Development and fundamental investigations of innovative technologies for biological nutrient removal from abattoir wastewater

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ETUDES FONDAMENTALES ET DEVELOPPEMENT DE PROCEDES INNOVANTS POUR L'ELIMINATION BIOLOGIQUE DE L'AZOTE ET DU PHOSPHORE DANS DES EFFLUENTS D'ABATTOIR.

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DEVELOPMENT AND FUNDAMENTAL INVESTIGATIONS OF INNOVATIVE TECHNOLOGIES FOR BIOLOGICAL NUTRIENT REMOVAL FROM ABATTOIR WASTEWATER.

by

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Declaration

I declare that the work presented in this thesis is, to the best of my knowledge and believe, original and my own work, except as acknowledged in the text, and that the material has not been submitted, in whole or in part, for a degree at this or any other institution.

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I remembered coming across a PhD student when I was still an undergraduate student in France. I was asking him some questions about the way his PhD was going and he told me: "don't worry too much about the subject of your PhD, what you really want is supervisors that are easy to talk to, timely with their comments and interested in your work". I did not fully understand his advice at the time but I can ensure you that I do now. While I had the chance to work on a subject I was passionate about, my excellent relation with both my principal supervisors, Prof. Zhiguo Yuan and Dr. Nicolas Bernet, their strong commitments, their availability and their insightful comments were instrumental in directing my research and helping me achieve my goal. I would also like to thank my associate supervisor Prof. Jürg Keller for his contribution and his valuable expertise in the field. I should also mention that, as the director of AWMC, Jürg welcomed me in his centre for a year when I was still an undergraduate student 6 years ago. I have not left since then as AWMC has become a vibrant and renowned research centre a credit to Jürg's effort and passion.

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- Dr. Gregory Crocetti of AWMC performed the FISH quantification and analysis reported in Appendix D. He also contributed to the development of a novel microbial technique used to determine the distribution of different microbial population inside aerobic granules (described in Appendix E).
- Dr. Rikke Meyer, formerly of AWMC, currently at the Department of Microbiology at Aarhus University, Denmark, constructed the N_2O microsensors used in this thesis and assisted with the experimental work reported in Appendix D.
- Ms Annelies Taske, formerly a student with the Department of Microbiology and Molecular Sciences, assisted with the N_2O microsensor measurements reported in Appendix D.
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List of Publications and Presentations

Journal publications forming chapters of the thesis, with author's contribution:

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- Yilmaz G., **Lemaire R.**, Keller J. and Yuan Z. (2007). Effectiveness of an Alternating Aerobic, Anoxic/Anaerobic Strategy for Maintaining Biomass Activity of BNR Sludge during Long-term Starvation. *Water Research,* **41**(12) 2590-2598.

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- **Lemaire R.**, Meyer R., Taske A., Crocetti G., Keller J. and Yuan Z. (2006). Identifying causes for N_2O accumulation in a lab-scale sequencing batch reactor performing simultaneous nitrification, denitrification and phosphorus removal. *Journal of Biotechnology*, **122**(1) 62-72.

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- **Lemaire R.**, Yuan Z., Blackall L. L. and Crocetti G. *in press*. Microbial Distribution of *Accumulibacter* spp. and *Competibacter* spp. in Aerobic Granules from a Lab-Scale Biological Nutrient Removal System. *Environmental Microbiology*.

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- **Lemaire R.**, Yilmaz G., Keller J. and Yuan Z. (2007). Aerobic granular sludge: breaking the sludge barrier. *Ozwater 2007 AWA conference, 4-8 March, Sydney, Australia*.
- **Lemaire R.**, Blackall L. L., Yuan Z. and Crocetti G. (2006). Microscale structure of the microbial community within simultaneous nitrification denitrification and phosphorus removal granules. *11th International Symposium on Microbial Ecology ISME-11, 20-25 August, Vienna, Austria*.
- **Lemaire R.**, Meyer R., Crocetti G., Zeng R. J., Yuan Z., Blackall L. L. and Keller, J. (2005). The microbial community dynamics in a simultaneous nitrification, denitrification and phosphorus removal bioreactor using different carbon sources. *4th Activated Sludge Population Dynamic IWA Specialist Conference, 17-20 July, Gold Coast, Australia*.

Patent:

- **Lemaire R.**, Yuan Z. and Keller J. "Biological process for at least partial removal of nitrogen, phosphorus and BOD from wastewaters which have very high levels of nitrogen as well as significant phosphorus levels, such as abattoir wastewaters." PCT *filed on 16th October 2007, IP Australia.*

Abstract

The meat processing industry requires large quantities of water, much of which is discharged as wastewater containing high levels of COD and nutrients such as nitrogen (N) and phosphorus (P). These nutrients must be removed to very low levels before any discharge into local waterways to avoid causing eutrophication in receiving aquatic systems. Reliable biological COD and nitrogen removal systems have been developed and applied for abattoir wastewater treatment using continuous activated sludge systems. However, P removal is achieved primarily through chemical precipitation with the addition of large amounts of iron or alum salts. Biological P removal is often consider as being a cheaper and more sustainable option as no chemical dosing is required and the quality of the excess sludge is usually suitable for land fertilisation purposes. Compare to well established continuous wastewater treatment systems, sequencing batch reactor (SBR) technology offers a great deal of operational flexibility and appears to be a promising vehicle for achieving high levels of N and P removal from abattoir wastewater.

The overall objective of this thesis is to develop a biological process that achieves high level of COD, N and P removal from abattoir wastewater, producing effluent suitable for being discharged into river systems (i.e. over 95% of the N and P contents removed). Achieving stable and reliable biological P removal from a wastewater containing high level of nitrogen is the main obstacle to overcome to achieve this objective. This study also investigate the feasibility of using two innovative technologies, namely the simultaneous nitrification, denitrification and phosphorus removal (SNDPR) process and the aerobic granular sludge technology, to further enhance the performance of the SBR system designed.

In this thesis, three different lab-scale SBRs were operated to demonstrate the feasibility to achieve high-levels of biological COD, N and P removal from abattoir wastewater. The first SBR was employed to experiment novel strategies, which can easily be implemented to current water treatment facilities used by the meat industry, to produce an effluent suitable for river discharge. The second SBR was operated to provide a platform for an in-depth investigation of the previously proposed SNDPR process, which has the potential for application to the treatment of abattoir wastewater. The third SBR was operated for the treatment of abattoir wastewater by combining together the aerobic granular sludge technology and the SNDPR process. A wide range of techniques was employed in this thesis which includes reactor process studies, microbial investigations (fluorescent *in-situ* hybridisation combined with confocal laser scanning microscopy) and several micro-scale techniques (e.g. microsensors, electron microscopy and light microscopy). The combination of these multi-disciplinary techniques has helped deliver significant insightful information.

The main contributions of this thesis are as follow. An SBR system was demonstrated to effectively remove 95%, 97% and 98% of the total COD, total N and total P present in abattoir wastewater. It could provide a real alternative to chemical P removal, which is the common practice in the meat industry. A multi-step feeding strategy was employed to prevent the accumulation of nitrate or nitrite in the SBR providing the right condition for the development of a stable biological P removal. The incorporation of a high-rate pre-fermentor as an integrated component of the nutrient removal system was found to be important. This stream, which contains a high-level of volatile fatty acids, provides supplementary carbon sources critical for both P and N removal. Further, an aeration control strategy consisting of stopping the aeration in the SBR immediately after ammonia is fully oxidised was effective in

achieving stable N removal via the nitrite pathway. It benefited the nutrient removal performance of the SBR by saving some valuable amount of COD.

The investigation of the SNDPR process revealed that the production of N_2O, often observed in lab-scale SNDPR bioreactors and source of concern due to the high global warming potential of N_2O, was found to be linked to the loss of diversity amongst the denitrifying microbial community due to the use of synthetic wastewater containing only a single carbon source. N_2O accumulation is unlikely to be an issue in a SNDPR bioreactor treating real wastewater which contains a large variety of different carbon sources.

The SNDPR process and the aerobic granular sludge technology were successfully combined in a single SBR process. The size and the dense structure of aerobic granules positively contributed to the oxygen mass transfer limitation required to achieve reliable SNDPR. It was also demonstrated that the minimum hydraulic retention time (HRT) for a granular sludge system is not governed by the sludge settleability, as is the case in a system with floccular sludge. Mass transfer limitations in granules are an important factor to be considered in the design of the HRT and the COD and nutrient loading rate in a granular sludge system.

The in-depth study of the structure and function of these aerobic granules fed with abattoir wastewater revealed interesting structural features that have not been reported in synthetic-fed granules. These observations initiated new hypotheses regarding the microbial structure and the fate of mature granules, the effect of pH on the granule structure stability and the role played by protozoa in the overall system performance. It also provided some new directions and recommendations for further experimental studies on aerobic granules in relation to their structure and behaviour in real systems.

Résumé en français

L'industrie de la viande utilise de larges quantités d'eau lors de l'abattage, du découpage et du nettoyage des équipements. Les effluents produits sont très chargés en DCO, azote et phosphore. Afin d'éviter toute pollution des milieux aquatiques environnants, ces effluents doivent subir des traitements poussés. Le but principal de cette thèse était de développer un procédé de traitement par boues activées qui puisse éliminer plus de 95% de la DCO, de l'azote et du phosphore dans les effluents d'abattoir permettant alors un rejet direct de l'effluent traité en rivière. La forte teneur en azote des effluents d'abattoir est l'obstacle majeur empêchant d'établir une élimination biologique du phosphore stable et efficace.

Durant cette thèse, un procédé biologique capable d'éliminer 98% du phosphore tout en abattant 95% de la DCO et 97% de l'azote a été développé dans un réacteur batch à alimentation séquentielle (SBR). Par rapport aux procédés chimiques classiques d'élimination du phosphore, ce nouveau procédé biologique offre une vraie alternative financière et environnementale pour l'industrie de la viande. Une stratégie d'alimentation séquentielle a permis de réduire l'accumulation des nitrates dans le SBR rendant ainsi possible l'élimination biologique du phosphore.

Cette thèse aborde aussi l'étude et l'utilisation de technologies innovantes pour améliorer les performances du procédé SBR. Le procédé de nitrification, dénitrification et déphosphatation simultanées (SNDPR) a été incorporé au procédé à boues granulaires aérobies. La taille, la densité et l'activité microbienne des granules aérobies génèrent de forts gradients d'oxygène à l'intérieur des granules, permettant alors d'obtenir un procédé SNDPR plus efficace. Le volume du réacteur et la demande en DCO nécessaire pour éliminer l'azote et le phosphore dans les effluents d'abattoir ont ainsi pu être fortement réduits. La structure interne et la composition microbienne de ces granules ont également été étudiées.

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List of Abbreviations

AOB	Ammonia oxidising bacteria
AS	Activated sludge
BNR	Biological nutrient removal
BOD5	Five-day biochemical oxygen demand
CLSM	Confocal laser scanning microscope
COD	Chemical oxygen demand
DAF	Dissolved air flotation
DO	Dissolved oxygen
EBPR	Enhanced biological phosphorus removal
EPS	Extracellular polymeric substances
FISH	Fluorescent in situ hybridisation
FOG	Fat, oil and grease
GAO	Glycogen accumulating organisms
HRT	Hydraulic retention time
MLSS	Mixed liquor suspended solids
MLVSS	Mixed liquor volatile suspended solids
NOB	Nitrite oxidising bacteria
OCT	Optimum cutting temperature
OLR	Organic loading rate
ORP	Oxido-reduction potential
OUR	Oxygen uptake rate
PAO	Polyphosphate accumulating organisms
PCA	Perchloric acid
PHA	Polyhydroxylalkanoates
SBOD5	Soluble five-day biochemical oxygen demand
SBR	Sequencing batch reactor
SCOD	Soluble COD
SEM	Scanning electron microscope
SKN	Soluble Kjeldahl nitrogen
SND	Simultaneous nitrification and denitrification
SNDPR	Simultaneous nitrification, denitrification and phosphorus removal
SRT	Sludge retention time
SVI	Sludge volume index
TCOD	Total COD
TDN	Total dissolved nitrogen
TDP	Total dissolved phosphorus
TEM	Transmission electron microscope
TKN	Total Kjeldahl nitrogen
TN	Total nitrogen
TP	Total phosphorus
UASB	Upflow anaerobic sludge-blanket
VSS	Volatile suspended solids
VFA	Volatile fatty acid
WWTP	Wastewater treatment plant

1.0 Introduction

The meat processing industry requires large quantities of water, much of which is discharged as wastewater containing high levels of COD and nutrients such as nitrogen (N) and phosphorus (P). These nutrients must be removed from the wastewater to very low levels before any discharge into local waterways to avoid causing eutrophication in receiving aquatic systems. Over the past two decades, biological COD and N removal from abattoir wastewater has received much greater attention than has the biological P removal. Reliable biological COD and nitrogen removal systems have been developed and applied for abattoir wastewater treatment using continuous activated sludge systems. Currently, P removal is achieved primarily through chemical precipitation with the addition of large amounts of iron or alum salts. Biological P removal is often consider as being a cheaper and more sustainable option as no chemical dosing is required and the quality of the excess sludge is usually suitable for land fertilisation purposes due to its low salt content and high phosphate recovery potential. The high level of nitrogen in abattoir wastewater has proved to be an obstacle to the development of a stable and reliable biological P removal process. Compare to well established continuous wastewater treatment systems, sequencing batch reactor (SBR) technology offers a great deal of operational flexibility and appears to be a promising vehicle for achieving high levels of N and P removal from abattoir wastewater. The meat industry in Australia is indeed starting to employ the SBR technology to treat their wastewater on site.

The overall objective of this thesis is to develop a biological process that achieves high level of COD, N and P removal from abattoir wastewater, producing effluent suitable for being discharged into river systems (i.e. over 95% of the nitrogen and phosphorus contents removed). Achieving stable and reliable biological P removal from a wastewater containing high level of nitrogen is the main obstacle to overcome to achieve this objective. This novel SBR process will have to be developed in a way that its future application to current abattoir wastewater treatment facilities can be achieved without large additional infrastructure and operating costs making it a real alternative option for the meat industry.

This study will also investigate the feasibility of using two innovative technologies to further enhance the performance of the SBR system designed. These technologies are still in their conceptual stage and knowledge gaps have to be filled before any possible industrial application. Firstly, the recently developed and demonstrated simultaneous nitrification, denitrification and phosphorus removal (SNDPR) process will be applied to the treatment of abattoir wastewater. This process promises savings in aeration costs and would also reduce demand for volatile fatty acids, which is crucial for biological P removal but often limiting in abattoir wastewater. Secondly, the feasibility of achieving high-levels of COD, N and P removal from abattoir wastewater using aerobic granular sludge technology will be investigated in this thesis. The excellent settleability of aerobic granular sludge allows for more biomass to be maintained in a relatively small reactor volume, enhancing the ability of the reactor to withstand high loading rates. This is of great interest for the treatment of high nutrient containing industrial wastewater such as abattoir wastewater compared to conventional floccular sludge systems. It is also believed that SNDPR could be more easily implemented in a granular system than in a floccular system.

2.0 Literature Review

This chapter explains the principles of the biological nitrogen and phosphorus removal processes, examines the characteristics of abattoir wastewater and identifies the main challenges for its treatment. The advantages and knowledge gaps related to two novel attractive technologies are also presented. A description of the achievements and limitations of previous studies is provided and based on these limitations, the focus of this research is established.

2.1. Introduction to biological nutrient removal processes

The general deterioration of water quality in rivers and streams in urban areas has resulted in an effort to prevent eutrophication by reducing the nutrient levels in wastewater - such as nitrogen (N) and phosphorus (P) - being discharged into local waterways (Mainstone and Parr, 2002). In order to achieve this reduction the wastewater needs to receive tertiary treatment, usually through biological treatment which is considered to be the easiest and most cost-effective way to remove nutrients from wastewater streams. Biological nutrient removal (BNR) relies on the activity of a diverse microbial community that transfers the nutrients from the wastewater (liquid phase) to the atmosphere (gas phase) and/or into biosolids (solid phase).

2.1.1. Nitrogen removal process

A conventional BNR process achieves nitrogen removal through a continuous two-stage treatment process (Figure 1): aerobic nitrification and anoxic denitrification (Metcalf & Eddy, 1991). During the nitrification stage, ammonia oxidising bacteria (AOB) aerobically oxidise ammonium, the major form of nitrogen in wastewater, to nitrite (i.e. nitritation process) which is subsequently oxidised to nitrate (i.e. nitratation process) by nitrite oxidising bacteria (NOB). Both AOB and NOB are autotrophic bacteria using oxygen as electron acceptor. During the denitrification stage, heterotrophic denitrifiers reduce nitrate to nitrite and then finally to dinitrogen gas under oxygen deficiency, using external organic substrates (chemical oxygen demand - COD) as electron donor. Typically the COD present in the incoming wastewater is used for denitrification in which case the denitrification reactor is located prior to the nitrification reactor (pre-denitrification) (Figure 1). This design is necessary to ensure sufficient amount of COD for complete denitrification as most of the COD present in the wastewater would be oxidised under aerobic conditions if the nitrification stage was located first. Nitrate produced by nitrification is then returned to the denitrification reactor via a recycle flow.

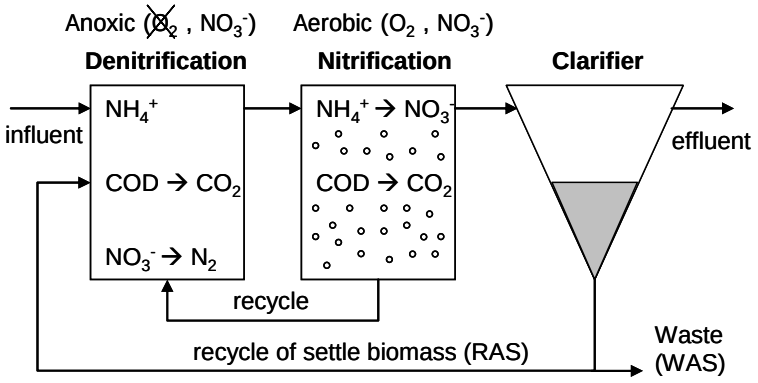


Figure 1. Conceptual design of a continuous nitrification and denitrification plant.

However, it has been observed that these two processes can occur concurrently in a single-sludge, single-stage process under low dissolved oxygen (DO) conditions called simultaneous nitrification and denitrification (SND) (Munch et al., 1996; Bertanza, 1997; Keller et al., 1997; Fuerhacker et al., 2000). SND relies on the formation of anoxic zones in the central part of the microbial aggregates caused by the mass transfer limitation of oxygen (Figure 2). In the aerobic zone on the edge of the aggregate, autotrophic bacteria can nitrify using oxygen, whereas in the anoxic zone at the centre of the aggregate, heterotrophic bacteria can denitrify. Therefore, factors that affect oxygen mass transfer limitation such as bulk liquid oxygen concentration, the aggregate size, and the specific activity of the microbial aggregates (oxygen uptake rate per volume of biomass) (Pochana and Keller, 1999; Meyer et al., 2005) also affect SND. Performing N removal via SND in full-scale plants has several potential benefits. Firstly, it will reduce the capital and operating cost through the elimination of the separate denitrification tank and recycle flow depicted in Figure 1. Secondly, the application of low DO for SND will save aeration costs.

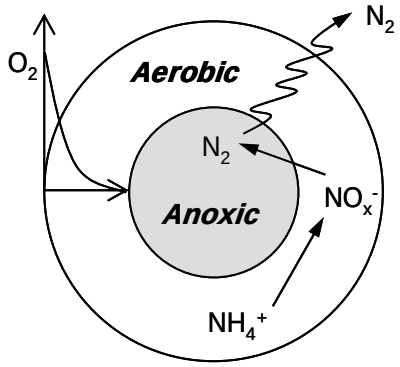


Figure 2. Illustration of oxygen profile inside a microbial aggregate under low oxygen concentration.

Additionally, nitrogen removal can be achieved through nitrification and denitrification via nitrite, by-passing the nitrate stage (Figure 3). This can be achieved by inhibiting or eliminating the NOB population from the system as NOBs are responsible for the second step of the nitrification (from NO_2^- to NO_3^-). This nitrification and denitrification via nitrite (also called "nitrite pathway") offers many advantages: (i) 40% lower requirement of COD in the denitrification stage, (ii) 25% lower consumption of oxygen in the nitrification stage, (iii)

higher denitrification rates and smaller sludge production (Turk and Mavinic, 1986). Implementing the nitrite pathway could have significant benefits for the operation of large-scale wastewater treatment facilities both for domestic and industrial effluents.

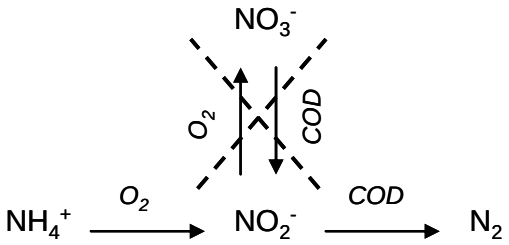


Figure 3. Illustration of nitrification and denitrification via nitrite (i.e. nitrite pathway).

2.1.2. Biological phosphorus removal process

The removal of phosphorus from wastewater is typically achieved by either chemical precipitation with the addition of iron and alum salts or through biological accumulation. However, the biological alternative has a number of significant advantages such as lower operating costs due to the non-requirement of chemicals addition, lower waste sludge production and higher reuse potential of the sludge as soil fertiliser due to its high phosphate recovery potential and its low salt content (Gaterell et al., 2000).

Wastewater treatment plants (WWTPs) performing biological P removal recirculate the sludge through an anaerobic and an aerobic zone as shown in Figure 4. This process is called enhanced biological phosphorus removal (EBPR) and is based on the ability of polyphosphate-accumulating organisms (PAOs) to take up P and accumulate it intracellularly as polyphosphate (poly-P) when exposed to alternating anaerobic (O_2 and nitrite/nitrate (NO_x^-) absent) and aerobic conditions (Comeau et al., 1986; Wentzel et al., 1988). PAOs are capable of taking up volatile fatty acids (VFAs) anaerobically which gives them a selective advantage over ordinary heterotrophic organisms, which are unable to take up VFAs anaerobically. VFAs are therefore the essential carbon substrates for EBPR. However they are not generally present to a sufficient concentration in wastewater. Many EBPR full-scale plants thus use prefermentors to generate additional VFAs from the incoming wastewater that can be added to the anaerobic zone.

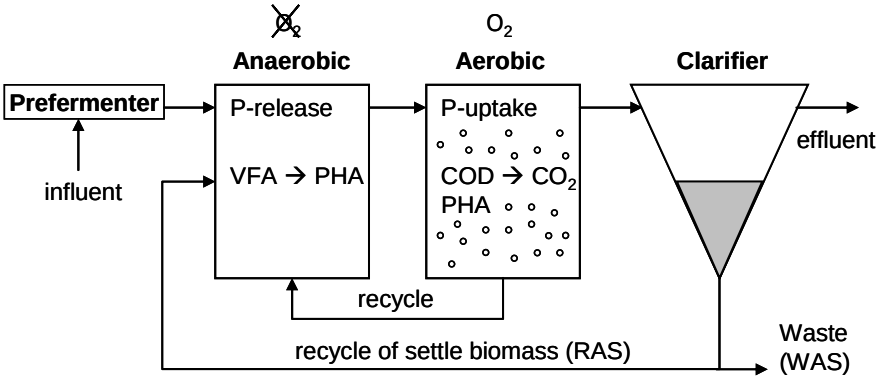


Figure 4. Conceptual design of an EBPR plant.

In the anaerobic zone, PAOs store VFAs as intracellular polyhydroxylalkanoates (PHAs) using energy gained from hydrolysing intracellular poly-P and releasing it to the liquid phase in the form of phosphate. In the subsequent aerobic zone, PAOs take up an amount of phosphate that is in excess of what was released under the proceeding anaerobic conditions, using their internally stored carbon (i.e. PHA) to provide the energy required. The accumulated P is then removed from the system together with the excess sludge that is produced in the process.

EBPR process has been successfully implemented in WWTPs and good P removal is regularly achieved (van Loosdrecht et al., 1997). However the control of the process can be difficult and EBPR failure has been frequently observed. Several reasons have been identified including excessive aeration (Brdjanovic et al., 1998), high nitrate concentration in the anaerobic zone (Pitman et al., 1983; Chang and Hao, 1996; Furumai et al., 1999) and the proliferation of microorganisms known as glycogen-accumulating organisms (GAOs) able to compete with PAOs for the carbon substrates in the anaerobic stage without contributing to any P removal (Mino et al., 1995). To store PHA from VFAs in anaerobic phase, GAOs gain energy exclusively from the glycolysis of intracellular glycogen, not from poly-P hydrolysing. Under aerobic condition, GAOs oxidise PHA for cell growth and glycogen replenishment. Therefore they can survive under EBPR conditions, but do not release/accumulate any phosphorus. It has been demonstrated in a recent survey of full-scale EBPR plants that carbon (i.e. VFA) requirements are increased considerably by the presence of GAOs (Saunders et al., 2003).

2.1.3. Advanced biological nutrient removal processes

In the case of very sensitive receiving environment, wastewater streams can be subjected to advanced tertiary treatment before being discharged to the local waterways. It usually consists of a combination between a conventional two-stage treatment process for N removal and the EBPR process for P removal. The conceptual design of this advanced BNR process is detailed in Figure 5. Such continuous BNR systems can vary slightly in terms of design but they generally include at least 3 separate mixing tanks, several recycling lines and a clarifier unit which make them relatively complex to operate and quite expensive to build. For example, the 5-stages Bardenpho® process, often referred to as the "Rolls-Royce" of all BNR systems because it can achieve really high-levels nutrient removal, consists in 5 separate tanks (i.e. a post-anoxic and second aerobic tanks are added to the process described in Figure 5 as polishing steps before the clarifier). In addition, an anaerobic prefermeter is often required at the head of the plant to provide sufficient VFAs for stable bio-P removal.

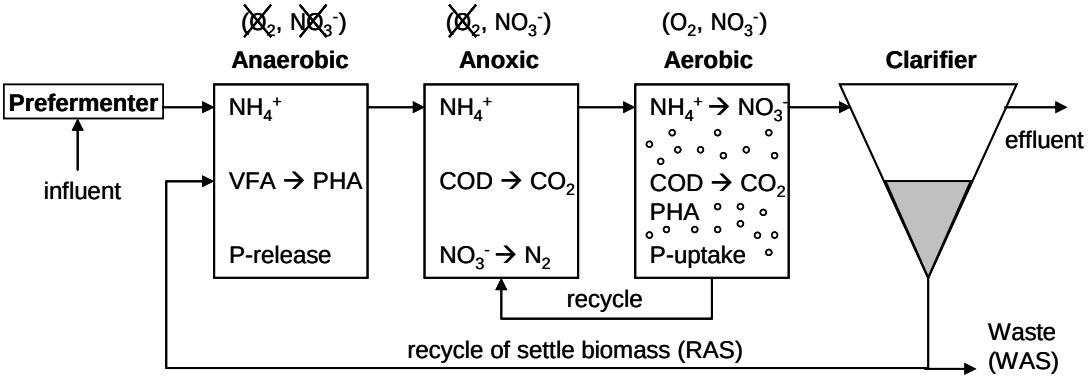


Figure 5. Conceptual design of a continuous BNR plant achieving both N and P removal.

2.1.4. Achieving BNR using sequencing batch reactor technology

Sequencing batch reactor (SBR) activated sludge processes are known to have several advantages over conventional continuous flow systems (Irvine and Busch, 1979; Wilderer et al., 2001). In recent years, the use of single tank SBRs for the biological treatment of wastewater has been widely extended from lab-scale studies to full-scale WWTPs (Tilche et al., 1999; Artan et al., 2001; Keller et al., 2001; Puig et al., 2004) since it offers a great deal of operational flexibility by easy adjustment of aerobic, anoxic and anaerobic periods through temporal control of aeration and filling in a cycle with no need for separate basins, recycling lines or clarifiers. SBRs have been utilised extensively for the removal of COD and, in many cases, nitrogen, from wastewater. It can be particularly useful in treating industrial effluent often presenting a large composition and flow variability. A comprehensive review of the SBR technology can be found in Wilderer et al. (2001).

More recently, a number of phosphorus removal processes using the SBR technology have also been developed which involves the use of a number of innovative concepts. Keller et al. (2001) demonstrated a novel filling process called "UniFed". The unique feature of this process is the uniform introduction of the influent into the settled sludge during the settling and decant periods of the SBR operation. This creates true anaerobic conditions under the sludge blanket by removing any remaining nitrate or nitrite using some of the freshly introduced influent COD. As a result, PAOs can release large amount of phosphate anaerobically which is critical to successful biological phosphorus removal. It also achieves a "selector" effect, which helps in generating a compact, well settling biomass in the reactor. Another innovative strategy is the use of a step-feeding scheme, characterised by several aerobic and anoxic phases in a SBR cycle (Anderottola et al., 2000; Lin and Jing, 2001; Puig et al., 2004). This strategy aims at improving the nitrification and denitrification performance of the SBR. The feeding is carried out in several anoxic periods so that easily biodegradable substrate would enhance the denitrification efficiencies and low nitrate levels may be achieved even from wastewater highly concentrated in nitrogen (Puig et al., 2004).

2.1.5. SBR control strategy

SBRs usually operate with fixed lengths for the different phases of filling, mixing (anaerobic, aerobic or anoxic), settling and decanting. Due to influent fluctuation and system state variations, it is beneficial to operate a SBR process with varying phase lengths. Therefore, higher levels of process control and automation are necessary to optimise the SBR operation. Many researchers have suggested that for a nitrogen removal system, on-line measurements of ORP, DO and pH contain some characteristic patterns that indicate the end of the biodegradation processes (Al-Ghusain et al., 1994; Wareham et al., 1994; Al-Ghusain and Hao, 1995; Hao and Huang, 1996). The control systems designed are inferential, due to the fact that ORP and pH are indirect measures of the nitrification and denitrification processes. Figure 6 shows typical ORP, pH and DO profiles in an alternating aerobic-anoxic nitrogen removal bioreactor with excess aerobic and anoxic periods. NH_4^+, and NO_3^- profiles are also shown in Figure 6. It is clear that the bending points detected on the ORP and pH curves correspond to the ends of the nitrification and denitrification, which enable the design of a real time control system for the process based on ORP, pH and DO signals. For example, Peng et al. (2004) recently demonstrated that stable nitrification and denitrification via nitrite could be obtained through the control of the aeration time in a sequencing batch reactor (SBR) treating domestic wastewater. DO and pH signals were used to detect the end of the nitritation process (i.e. complete oxidation of NH_4^+). Aeration was stopped as soon as nitritation finished, as indicated by a bending point on the pH profile and a sharp increase of the DO level. An

external carbon source (glucose) was then added to enable denitrification in the following anoxic period.

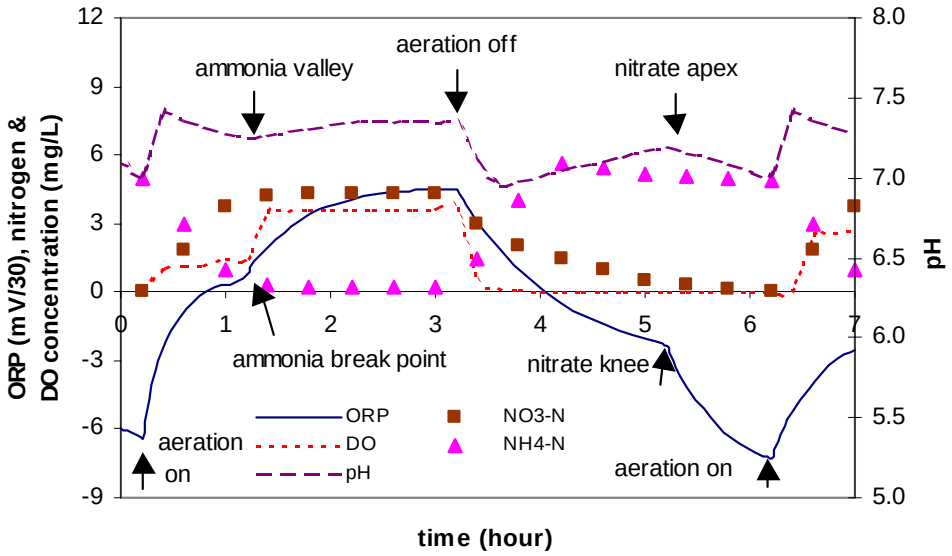


Figure 6. ORP, pH, DO, NH_4^+ and NO_3^- profiles in a nitrogen removal bioreactor (illustrative from (Yuan et al., 2003)).

In recent years, aeration control using nutrient sensors has been studied by many researchers. The continuous improvement of reliability, accuracy, ease of maintenance and *in-situ* location of ammonia and nitrate sensors (Londong and Wachtl, 1996; Lynggaard-Jensen et al., 1996; Ingildsen and Wendelboe, 2003) has resulted in some full-scale applications (Ingildsen and Olsson, 2002). Compared to other types of sensors, nutrient sensors support the direct control of the ammonia and nitrate nitrogen concentrations in the system. The control systems designed based on these sensors therefore exhibit more flexibility between nitrification and denitrification (Yuan et al., 2003).

2.2. Abattoir wastewater treatment

2.2.1. Abattoir wastewater characteristics

The meat processing industry requires large quantities of water, much of which is discharged as wastewater containing high levels of COD and nutrients such as nitrogen and phosphorus. In a recent review of the wastewater treatment in the meat industry, Mittal (2006) reported an average water usage of 300-700 L per pig slaughtered and 1,000-2,500 per cattle. The daily water usage for large plants processing up to 3,000 animals a day can therefore be quite consequent. Bhamidimarri (1991) indicated that a typical New-Zealand meat processing plant produces 10,000 $m^3.d^{-1}$ of wastewater with a pollution load equivalent to a city of 60,000-100,000 inhabitants. However, the water consumption varies considerably depending on the type of abattoir (e.g. integrated slaughterhouse, processing plant, rendering plant), the type of animals slaughtered (e.g. pig, sheep, cattle), the local practices employed (e.g. number of shifts per day and number of working days per week), the climate and also between private and public abattoirs (Koenig and Yiu, 1999).

Abattoir wastewater is composed of a mixture of grease, fat, protein, blood, intestinal content, manure, cleaning products as well as about 80% fresh water (Johns et al., 1995). The main characteristics of primarily treated abattoir wastewater reported in different studies are presented for comparison in Table 1. The strength of the wastewater varies among abattoirs depending upon operating practices. It also varies from day to day depending on the number and type of animals being processed. The main differences concern the COD and FOG concentrations whereas nitrogen and phosphorus content are less variable. According to Johns (1995), the typical concentrations of TKN and TP in slaughterhouse wastewater are 120-200 $mg.l^{-1}$ and 15-40 $mg.l^{-1}$, respectively, which are considerably higher than in domestic wastewater. Primarily treated wastewater also contains a high level of organic N that usually breaks down to ammonia during the subsequent treatment steps. The potentially high TCOD:TKN and TCOD:TP ratios in abattoir wastewater should be cautiously interpreted as 40 to 70% of the total COD is slowly biodegradable and not directly usable for N or P removal.

Table 1. Some characteristics of primarily treated abattoir wastewater. Adapted from Mittal (2004). Values in bracket are average value.

Reference	Country	TCOD ($mg.l^{-1}$)	SCOD ($mg.l^{-1}$)	FOG ($mg.l^{-1}$)	TKN ($mgN.l^{-1}$)	N-NH_4 ($mgN.l^{-1}$)	TP ($mgP.l^{-1}$)
Borja et al. (1994)	Spain	(5,100)	--	--	(310)	(95)	(30)
Caixeta et al. (2002)	Brazil	2,000-6,200	--	40-600	--	20-30	15-40
Li et al. (1986)	China	628-1,437	--	97-452	44-126	25-105	10-16
Manjunath et al. (2000)	India	1,100-7,250	--	125-400	90-150	--	8-15
Martinez et al. (1995)	Spain	(6,700)	(2,400)	(1,200)	(268)	--	(17)
Nunez and Martinez (1999)	Spain	1,440-4,200	720-2,100	45-280	--	--	--
Russell et al. (1993)	New-Zeal.	(1,900)	--	--	(115)	(30)	(15)
Sachon (1984)	France	(5,113)	--	(897)	(248)	--	(22)
Sayed et al. (1987)	Holland	1,500-2,200	--	--	120-180	--	12-20
Sayed et al. (1988)	Holland	1,925-11,118	780-10,090	--	110-240	--	13-22
Stebor at al. (1990)	US	4,200-8,500	1,100-1,600	100-200	114-148	65-87	20-30
Thayalakumaran (2003b)	New-Zeal.	490-2,050	400-1,010	250-990	105-170	26-116	25-47

2.2.2. Principal wastewater treatment processes used in the meat processing industry

Similar primary treatment processes (settling, screening, flocculation, dissolved air flotation – DAF) are used worldwide to partially remove the suspended solids and the FOG from the raw abattoir wastewater. The main process variation concerns secondary treatments and depends mostly on the location of the processing plant. Johns (1995) and more recently Mittal (2006) highlighted the differences existing between abattoirs located in Europe, North and South America, New-Zealand and Australia which are the main meat producing regions in the world (Figure 7). In Europe, primarily treated wastewater is usually directly discharged into municipal WWTPs for further treatment necessiting the payment of a surcharge. The treated effluent is then discharged into local waterways. In contrast, countries where land availability is less of an issue (North and South America, Australia and New-Zealand), large anaerobic and aerobic ponds systems are often employed to remove COD and achieve some partial nitrification before the effluent is used for land irrigation. These large pond systems achieve good COD removal but limited N removal and almost no P removal (Keller et al., 1997). Although this effluent contains the required nutrient for plant growth, the high nitrogen (ammonium and nitrate) infiltration rate in the soil may result in groundwater pollution during intensive irrigation (Russell et al., 1993). Activated-sludge systems are also employed in

North America to remove the COD and some of the nutrients before land application. Recently, advanced tertiary treatments using biological and physicochemical methods have been employed in the US and in Australia to achieve complete nitrification and partial or even complete denitrification together with chemical phosphorus removal. However, their use is very limited due to high-cost involved (Mittal, 2006).

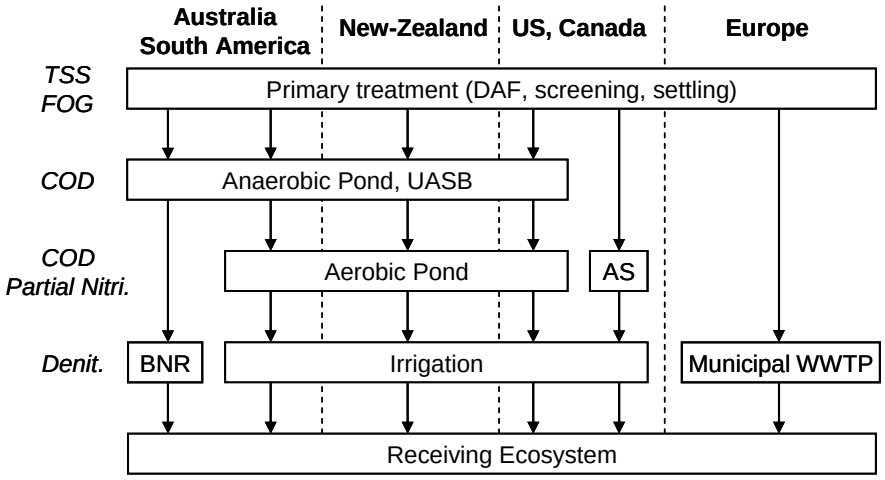


Figure 7. Principal wastewater treatment processes used in the meat industry worldwide. The targeted pollutant of each level of treatment is indicated on the left margin. Adapted from Johns (1995) and Mittal (2006).

2.2.3. SBR technology for abattoir wastewater treatment

Recently, several studies using the SBR technology to simultaneously remove COD, N and P from piggery wastewater have been very successful (Tilche et al., 1999; Obaja et al., 2003; Obaja et al., 2005). However, the characteristics of the piggery wastewater differ greatly from typical abattoir wastewater. TKN and TP are typically 3 to 4 times higher in piggery wastewater than in abattoir wastewater while the amount of TCOD is relatively similar. This has a direct effect on the overall TCOD:TKN and TCOD:TP ratios which are much lower in the case of piggery wastewater than in typical abattoir effluent. Therefore, an external source of easy biodegradable COD (acetate, methanol, non-digested pig manure) has to be added to achieve good N and P removal. In addition, the large amount of inorganic salts, minerals and metal ions present in the piggery wastewater promote chemical P removal by precipitation as only scarce P release occurred during the anaerobic stage of the process (Bortone et al., 1994). The absence of P release combined with the lack of microbial analysis of the sludge undermines the simultaneous "biological" removal of COD, N and P claimed in these studies.

Studies on nutrient removal have also been carried out with abattoir wastewater. Subramaniam et al. (1994) and Keller et al. (1997) firstly reported that simultaneous biological nutrient and COD removal was possible in SBR from anaerobic-treated abattoir wastewater under anoxic/anaerobic and aerobic conditions. In both studies, N removal was partially achieved through SND leaving enough readily biodegradable COD to remove 90% of the total N and P present in the anaerobic-treated abattoir wastewater without any external carbon addition. However, nutrient removal performance depended greatly on the degree of anaerobic treatment of the sludge - the longer the treatment, the less readily biodegradable COD is left for nutrient removal. The P removal was quite unstable due to some intermittently

high levels of NO_x^- being recycled to the anaerobic period reducing the amount of VFAs available for PAOs. More recently, Thayalakumaran (2003a) fed primary treated abattoir wastewater directly into a SBR under anoxic/anaerobic and aerobic conditions with low DO ($0.5\ mgO_2.l^{-1}$) to promote SND. As a result, 93% of the total N and 96% of the total P in the influent were biologically removed. It should be noted that the wastewater used in that study contained a particularly high fraction of soluble COD and readily biodegradable COD, representing 60% and 17% of the total COD, respectively. Most primary treated abattoir effluents have much lower soluble and readily biodegradable COD fractions which could not sustain high level of biological nutrient removal. The high variability of primary treated abattoir wastewater is also an obstacle to achieve stable N and P removal. Instead, using anaerobic-treated abattoir wastewater as influent for the SBR process offers more stability in term of influent composition due to the relatively long hydraulic retention time (HRT) of common anaerobic treatment processes. For example, the average HRT of large anaerobic pond systems widely used in the meat industry is around 10 days which is long enough to level out most of the daily fluctuations of flow and composition of the raw abattoir wastewater.

2.2.4. Challenges facing abattoir wastewater treatment

Effect of high nitrate level on Bio-P removal

The deterioration of Bio-P removal due to the presence of nitrate in the designated anaerobic period has been the topic of several studies. Comeau et al. (1986) reported that, when the nitrate concentration in the return sludge was less than 5 $mg.l^{-1}$, good P-release was easily achieved. Pitman et al. (1983) found that a nitrate concentration higher than 10 $mg.l^{-1}$ inhibited P-release resulting in a failed EBPR. Chang and Hao (1996) observed that when nitrate levels in effluent were reduced from 10.9 to 5.6 $mg.l^{-1}$, P removal efficiency increases from 80 to 98%. In SBR performing both N and P removal, nitrate concentration and the end of cycle must be low enough to provide a substantial true anaerobic period at the start of the next cycle, which is fundamental for Bio-P removal (Furumai et al., 1999).

In general, the remaining level of nitrate in SBR treating domestic wastewater varies from 2 to 5 $mg.l^{-1}$, which should not greatly affect the P removal. However, when dealing with abattoir wastewater containing a high level of nitrogen, the remaining nitrate levels are generally much higher and P removal can be severely affected due to the increased competition for organic substrate between denitrifiers and PAOs (Subramaniam et al., 1994; Keller et al., 1997).

Limitation of readily biodegradable COD

Sufficient COD, in particular readily biodegradable COD (primarily VFA), is required to achieve good biological nutrient removal. Therefore, a challenge for the abattoir wastewater primary treatment is to reduce the carbon content through anaerobic pond systems and, at the same time, have sufficient and suitable COD remaining to perform complete N and P removal in the BNR system (Keller et al., 1997). In some cases, COD required for denitrification can be provided by by-passing a fraction of the raw wastewater stream directly into the BNR system (Metzner and Temper, 1990). However, the direct addition of raw wastewater increases the fat content of the BNR mixed liquor and could lead to poor settling properties (see next paragraph). Alternatively, the high level of VFA necessary for P removal, mainly in the form of acetate or propionate (Pijuan et al., 2004; Oehmen et al., 2006), could be supplied from a well-controlled pre-fermenter.

Settling and bulking problems caused by the high level of fat/oil/grease

The high concentration of FOG and particulate matter (i.e. colloidal) of the abattoir wastewater (Table 1) is a concern as it generally produces a light, poor settling sludge (Hopwood, 1977). Travers and Lovett (1984) found that at low DO (<0.5 $mg.l^{-1}$), fat degradation was inhibited, leading to poor settling properties (sludge volume index – SVI – of 250 $ml.gMLSS^{-1}$) and excessive numbers of filamentous organisms. In a recent study of the edible oil industry, Reddy et al. (2003) observed significantly high TSS in the treated effluent as a result of sludge oil aggregation, pin point floc formation and high numbers of free swimming bacteria. However, microscopic analysis showed low abundance of filamentous bacteria. The high oil content of the effluent resulted in sludge bulking with SVI values as high as 770 $ml.gMLSS^{-1}$. Interestingly, Thayalakumaran et al. (2003a) had no major settling problem in their SBR (SVI between 100-200 $ml.gMLSS^{-1}$, effluent MLSS of 33 $mg.l^{-1}$) when directly using primary treated abattoir wastewater containing higher fat levels than anaerobically treated wastewater.

Fluctuations in abattoir wastewater composition and flow

One of the challenges for abattoir WWTPs is to cope with the large fluctuations of the wastewater flow and composition inherent to most industrial activities. Koenig and Yiu (1999) cited the type of animal slaughtered, the methods used, the type of equipment available, as well as the local mode of production as the main factors affecting the amount and composition of abattoir wastewater. In some cases, low activity periods (e.g. annual maintenance or seasonal production variations) would even result in complete interruptions of wastewater flows to the WWTPs for weeks and even months affecting the stability of the overall BNR process. It is crucially important to maintain the viability of biomass (activity and integrity) during the long idle or starvation periods to ensure a rapid return to previous level of treatment when normal operational condition are resumed.

2.3. New possible BNR technologies

The following technologies may potentially address the challenges facing the treatment of abattoir wastewater. The main advantages, drawbacks and knowledge gaps are presented.

2.3.1. Simultaneous Nitrification, Denitrification and Phosphorus Removal

Simultaneous nitrate/nitrite and phosphorus removal can be achieved in anaerobic-anoxic EBPR systems using ability of PAOs to simultaneously reduce NO_x^- and take up P (Kuba et al., 1993; Kerrn-Jespersen et al., 1994; Meinhold et al., 1999). This can be highly beneficial to lower the COD demand (same carbon source used for N and P removal) and reduce the aeration costs. In addition, PAOs exposed to such anaerobic-anoxic conditions are 40% less efficient in generating energy compare to in normal anaerobic-aerobic EBPR systems and thus have a 20-30% lower cell yield resulting in less sludge production (Kuba et al., 1994). Combining this anaerobic-anoxic EBPR system with SND could deliver even more substantial savings of COD especially if nitrification and denitrification via nitrite pathway is achieved. Indeed, the process of simultaneous nitrification and denitrification and phosphorus removal (SNDPR) has already been demonstrated in lab-scale SBRs treating mainly synthetic wastewater under alternating anaerobic and low DO aerobic stages (Zeng et al., 2003). In this process, nitrogen and phosphorus are removed simultaneously to very low levels. During the

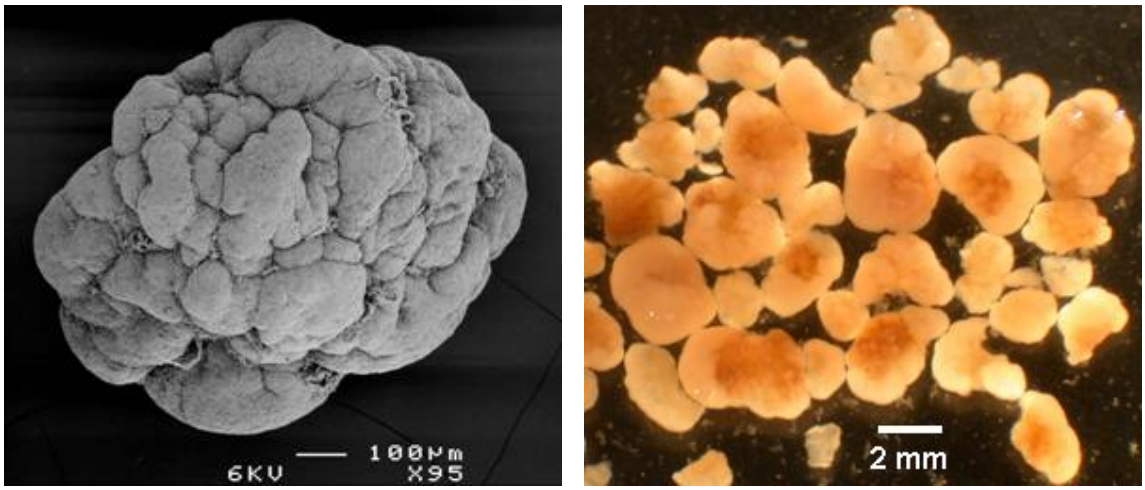
anaerobic stage, COD (i.e. acetate) is taken up accompanied by phosphorus release. During the subsequent aerobic stage, the conversion of ammonia to gaseous nitrogen products via nitrate or nitrite and P uptake are achieved concomitantly with almost no nitrite or nitrate accumulating in the bulk liquid.

However, it was observed that nitrous oxide (N_2O) rather than N_2 was the major denitrification end-product. This is a significant environmental concern due to the high greenhouse gas potential of N_2O (Zeng et al., 2003; Meyer et al., 2005). Meyer et al. (2005) identified the PHA-driven denitrification as a potential cause for this N_2O accumulation in lab-scale SNDPR processes. Furthermore, unstable nitrate/nitrite removal has been reported in such SNDPR bioreactors using floccular biomass. Meyer et al. (2005) showed that incomplete coupling between nitrification and denitrification would occur if the aerobic/anoxic zones in the microbial aggregates were not formed as illustrated in Figure 2, leading to the accumulation of NO_x^- in the bulk liquid. Based on the process data of these bioreactors, it was also reported that GAOs, known to compete with PAOs for carbon sources but without contributing to P removal, appear to be primarily responsible for the denitrification process (Zeng et al., 2003; Lemaire et al., 2006). Without denitrification by PAOs, there is no true link between SND and EBPR, and the two processes merely occur in the same sludge at the same time compromising the carbon savings proposed to be obtained by SNDPR. These findings undermine the stability and robustness of the SNDPR process which are crucial for any possible industrial application of this novel BNR technology.

2.3.2. Aerobic Granular Sludge technology

What is a granule?

Microbial granules can be described as compact and dense microbial aggregates of different bacterial species with an approximately spherical external appearance (Figure 8). The growth of such granules is sometimes regarded as a special case of biofilm development (Grotenhuis et al., 1991; El-Mamouni et al., 1998). While granules were first reported in an upflow anaerobic sludge blanket (UASB) bioreactor two decades ago (Lettinga et al., 1980), recent research efforts have been dedicated to the study of aerobic granules in SBR systems (Morgenroth et al., 1997; Beun et al., 1999; Peng et al., 1999; Etterer and Wilderer, 2001; Tay et al., 2001). The large microbial diversity found in both anaerobic and aerobic granules has led researchers to hypothesize that granulation is not a function of specific microbiological groups but of reactor operating conditions (Beun et al., 1999). Compared to conventional flocs, anaerobic or aerobic granules have a wide range of beneficial properties, including: a regular, dense and compact structure, high biomass retention in the bioreactor, good settleability, and the ability to withstand high flows rate and high organic loading rates. These properties explain why granular UASB process has been extensively applied to anaerobic wastewater treatment and why the recent aerobic granular technology has been the subject of extensive studies yielding more than 60 publications over the past 3 years (Liu and Tay, 2004).



(a) **(b)**

Figure 9. Aerobic granule(s) visualized **(a)** by scanning electron microscopy and **(b)** light microscopy. These granules were obtained in this thesis study for the treatment of abattoir wastewater. See appendix F and G for details of these granules and the conditions under which they were obtained.

Factors affecting aerobic granulation

The formation of aerobic granules is a gradual process from suspended sludge flocs to compact aggregates, to granular sludge and finally to mature aerobic granules (Tay et al., 2001). However, the exact mechanisms involved in the successive stages of the aerobic granulation process have not yet been fully explained. Recently, aerobic granules cultivated in synthetic wastewater in SBR have been reported to achieve COD and/or N removal (Tay et al., 2002; Liu et al., 2003; Yang et al., 2003) and, in some cases, bio-P removal (Dulekgurgen et al., 2003; Lin et al., 2003). Although nearly all studies on aerobic granulation have been carried out in lab-scale SBR with only a few using real wastewater (de Villiers and Pretorius, 2001; Arrojo et al., 2004; de Bruin et al., 2004), aerobic granulation technology is moving towards industrial use for biological wastewater treatment. Liu et al. (2005b) recently published some guidelines for up-scaling aerobic granular SBR from lab-scale to full-scale.

At laboratory scale, many factors influencing the properties of aerobic granules in SBR have been described (Liu and Tay, 2004). Four major selection pressures directly contributing to the formation mechanism of granules in SBR have been identified in the literature:

- Short settling and discharge time to select rapid settling granule (Arrojo et al., 2004; McSwain et al., 2004; Qin et al., 2004).
- High volume exchange ratio (defined as the volume of effluent discharged divided by the working volume of the SBR) (Liu et al., 2005a).
- Feast and famine regime to enhance the conversion of readily biodegradable substrates into slowly biodegradable stored substrate (Beun et al., 2001; de Kreuk and van Loosdrecht, 2004).
- High hydrodynamic shear force and high air upflow velocity to induce a compact, dense and round granule structure (Liu and Tay, 2004).

Benefits of aerobic granular sludge technology for industrial wastewater treatment

The potential of the aerobic granular sludge technology is very promising, in particular for the biological removal of high nutrient containing industrial wastewater such as abattoir effluents. First of all, the excellent settleability of granular sludge allows for more biomass to be maintained in a relatively small reactor volume, enhancing the ability of the reactor to withstand high loading rates and reducing the capital costs associated with the construction of large bioreactors. Secondly, the large size of granules and their dense and compact structure are expected to positively contribute to the oxygen mass transfer limitation required for stable and reliable SNDPR systems. Therefore, combining SNDPR and granular sludge technologies could indeed result in a very attractive novel BNR technology for industrial application.

3.0 Thesis Overview

This chapter offers an overview of the research work undertaken in this thesis. First, the research objectives are formulated based on the primary objectives of this study (Chapter 1), then the research methods employed to address theses objectives are briefly described and finally, the major outcomes of this research are outlined.

3.1. Research Objectives

i. Development of a process that achieves high levels of COD, nitrogen and phosphorus removal from abattoir wastewater.

As explained in the previous chapter, the biological removal of COD and nitrogen from abattoir wastewater has been widely studied resulting in successful full-scale applications whereas in comparison, biological phosphorus removal has not received great attention due to the general incompatibility between high level of nitrate/nitrite accumulation and stable EBPR processes. As a result, most full-scale WWTPs in the meat industry remove phosphorus through chemical precipitation by dosing iron or alumina ions. This method adds considerable costs to the overall abattoir effluent treatment and produces a sludge rich in metal ions limiting possible further application of this sludge as soil enrichment alternative. The first objective of this thesis is to investigate the feasibility of biologically removing COD, nitrogen and phosphorus to high-levels (>95%) from abattoir wastewater to produce an effluent of river discharge quality using an innovative SBR process aimed at limiting the nitrate/nitrite accumulation.

ii. Design of an automatic control system to consistently achieve nitrification and denitrification via nitrite.

The accumulation of nitrate/nitrite is not the only challenge faced by the meat industry to develop a reliable and cost effective biological phosphorus removal process. The limited amount of easily biodegradable COD contained in abattoir effluent is another source of concern for the removal of the high levels of N and P. It was established in the previous chapter that the use of the nitrite pathway to remove the high level of N present in abattoir wastewater could save significant amount of COD and improve the overall performance of the BNR system. Automatic control systems based on pH and DO signals have been used in several studies to promote nitrification and denitrification via nitrite in ammonia rich effluent. However, these studies relied upon the dosage of an external carbon source rather than the COD contained in the original wastewater to achieve a sufficient level of denitrification. The use of external carbon sources considerably undermines the overall benefits of the nitrite pathway. The second objective of this thesis is to implement an automatic control strategy to the SBR process developed under the first objective in order to achieve nitrite pathway without the need of external carbon source dosing. This automatic control strategy would improve COD usage efficiency and thus improve the reliability and robustness of biological phosphorus removal processes for the meat industry.

iii. Development of an operational strategy to maintain the biomass activity during long term starvation condition and during the subsequent resuscitation period.

Abattoir wastewater treatment plants (WWTPs) have to cope with large fluctuations of the wastewater flow and composition inherent to most industrial activities. In some cases, low activity periods for the industry (e.g. annual maintenance or seasonal production variations) would even result in complete interruptions of wastewater flows to the WWTPs for weeks and even months. It is crucially important to maintain the viability of biomass during the long idle or starvation periods and to ensure the process can fully recover when normal operations are resumed. Based on the limited literature available on the subject, it appears that keeping the sludge under alternating aerobic, anaerobic/anoxic conditions could be a suitable strategy for maintaining the biomass activities during an extended starvation period. However, most studies investigated the effect of different starvation conditions (aerobic, anoxic or anaerobic) on nitrifiers species only (i.e. AOB and NOB). This thesis aims to develop an integrative operational strategy easily applicable for the industry to (i) preserve the N and P removal capability of the biomass during long starvation periods and (ii) to resuscitate the biomass once the starvation period ceases.

iv. Identification of the causes of N_2O production in an SNDPR process enabling its application to the treatment of abattoir wastewater.

The possible benefits of implementing the SNDPR technology to the treatment of abattoir wastewater have been presented in the previous Chapter (i.e. lower COD demand, less accumulation of nitrate/nitrite, lower aeration costs and smaller sludge production). However, N_2O rather than N_2 has been observed in the SNDPR process as the end-product of denitrification, which poses a significant environmental problem. Before looking into the future application of SNDPR to abattoir wastewater treatment the causes of N_2O production/accumulation must be first identified and then eliminated. This forms another aim of this thesis.

v. Determination of the microscale microbial distribution of PAO and GAO in aerobic granules

As defined in Chapter 2, the aerobic granular sludge technology could be beneficial for the stability and reliability of the SNDPR process. Due to their large size and dense structure, aerobic granules are expected to positively contribute to the oxygen mass transfer limitation required for reliable SNDPR. As a result, different conditions (i.e. aerobic, anoxic or anaerobic) are likely to co-exist at different depths in granules creating different ecological micro-niches. It was also established in Chapter 2 that without denitrification by PAOs, there is no true link between SND and EBPR with the two processes merely occurring in the same sludge at the same time and thus compromising the carbon savings proposed to be obtained by SNDPR. If aerobic granules are to positively contribute to SNDPR, denitrification should be achieved by PAOs and not by GAOs. Therefore, PAOs should be the dominant population in the central part of the granules where no oxygen is present (anoxic zones) and denitrification occurs. To demonstrate ecological positions and roles for both populations in this complex system, a novel microbial method is developed in this thesis to establish the spatial distribution of the main PAO and GAO population in SNDPR granules, namely *Accumulibacter* spp. and *Competibacter* spp., respectively.

vi. Investigation of the feasibility of COD, N and P removal from abattoir wastewater using granular sludge technology.

As discussed in Chapter 2, the excellent settleability of aerobic granular sludge allows for more biomass to be maintained in a relatively small reactor volume, enhancing the ability of the reactor to withstand high loading rates. This is of great interest for the treatment of high nutrient containing industrial wastewater such as abattoir wastewater compared to conventional floccular sludge systems. To date, aerobic granular sludge technology has mainly been studied with synthetic wastewater, with the only real wastewater studies being performed using domestic wastewater. The feasibility of achieving high-levels of COD, N and P removal from abattoir wastewater using granular sludge technology is investigated in this thesis. In addition, the microscale structure of these aerobic granules is examined using a multi-disciplinary approach.

3.2. Research Methods

In this thesis, three different lab-scale SBRs were operated to demonstrate the feasibility to achieve high-levels of biological COD, N and P removal from abattoir wastewater. The first SBR, referred to as RivD-SBR, was employed to experiment novel strategies, which can easily be implemented to current water treatment facilities used by the meat industry, to produce an effluent suitable for river discharge (>95% TCOD, TN and TP removal). RivD-SBR was used to address research objectives 1, 2 and 3. The second SBR, referred to as SNDPR-SBR, was operated to provide a platform for an in-depth investigation of the previously proposed simultaneous nitrification, denitrification and phosphorus removal (SNDPR) process, which has the potential for application to the treatment of abattoir wastewater. SNDPR-SBR was first employed to identify the causes of N_2O emission in lab-scale SNDPR bioreactors fed with synthetic wastewater (research objective 4). The operation of this reactor was then modified to transform its floccular biomass into granules, which are believed to reinforce the SNDPR process. The microbial community structure in this new granular SNDPR-SBR was then examined via a newly developed method (research objective 5). The third SBR, referred to as Granular-SBR, was operated for the treatment of the high nutrient-containing abattoir effluent (research objective 6).

The operations of the three SBRs are briefly described below, which are followed by an overview of the key analytical methods employed in this thesis to address all of the above research objectives.

3.2.1. Operation of lab-scale SBRs used in this thesis

RivD-SBR

RivD-SBR had a working volume of 7 l (Figure 9) and was operated with a cycle time of 6 h in a temperature-controlled room (18-22°C). Each cycle, 1 l of abattoir wastewater collected weekly from a local abattoir (mixture of primarily treated and anaerobically treated wastewater) and stored in a cold room at 4°C was pumped into the reactor over 3 feeding periods. Each feeding period was followed by an anaerobic/anoxic period and an aerobic period (Table 2). During the aerobic periods, air was provided intermittently using an on/off control system to keep the DO level between 1.5 and 2 $mgO_2.l^{-1}$. The HRT and SRT in the SBR were kept constant at 42 h and 15 days, respectively. Full description of the SBR operation is given in Appendix A.

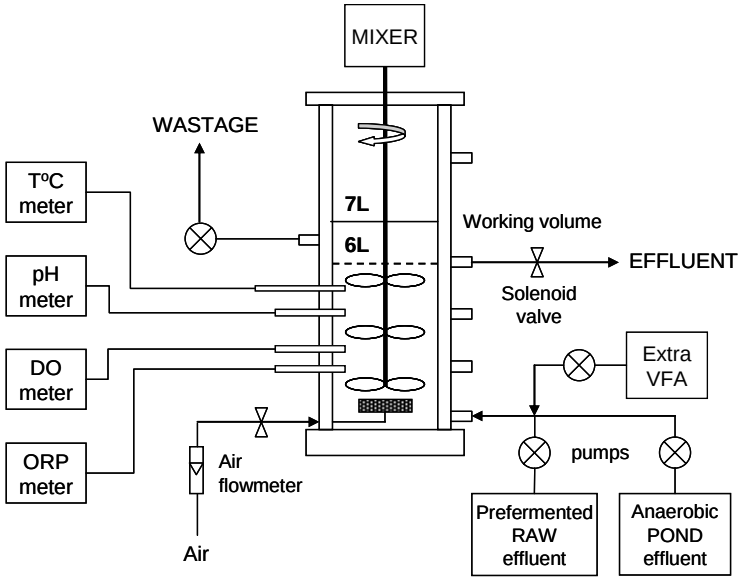


Figure 9. Design of RivD-SBR.

Table 2. Operating conditions of RivD-SBR (6 h cycle).

SBR sequences	**Duration** (min)
Fill no-mix 1	5
No-aerated mix 1*	30
Aerated mix 1	55
Fill mix 2	3
No-aerated mix 2*	70
Aerated mix 2	35
Fill mix 3	2
No-aerated mix 3*	60
Aerated mix 3	18
Wastage	2
Settle	70
Decant	10

* anoxic or anaerobic, depending when nitrate and nitrite are depleted.

SNDPR-SBR

A 5 l SBR performing SNDPR, which was previously designed and operated at the AWMC lab, was used in this thesis (Figure 10) to identify the causes for N_2O accumulation in lab-scale SNDPR processes (research objective 4). Initially, the SBR 6h cycle consisted of a 90 min anaerobic, 220 min aerobic, 40 min settling, and 10 min decanting period. In each cycle, 3 l of synthetic wastewater (230 mgCOD.l^{-1} as acetate, 23 mgN.l^{-1} as NH_4^+ and 18 mgP.l^{-1} as PO_4^{3-}) was pumped into the reactor resulting in an HRT of 10 h. The SRT was kept at 20 days. Aeration was provided intermittently using an on/off control system to keep the DO level relatively low between 0.35 and 0.5 mg.l^{-1}. Full description of the initial SBR operation is given in Appendix D.

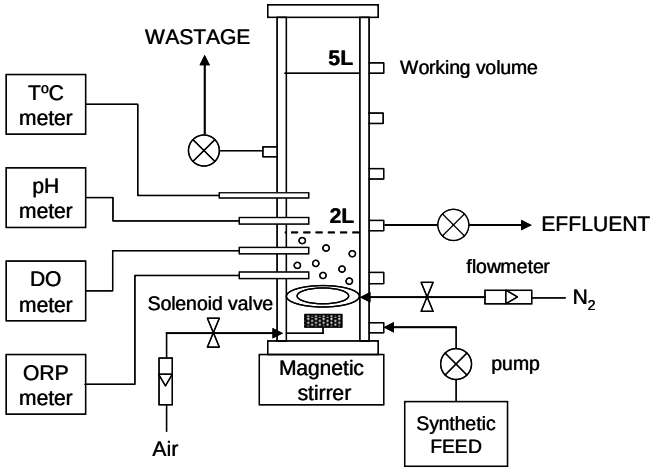


Figure 10. Design of SNDPR-SBR.

The SBR operation was then modified to promote the formation of aerobic granules. The settling time was gradually reduced from 40 min to only 5 min and the nutrient concentration in the synthetic wastewater was increased to 350 mgCOD.l^{-1} as acetate, 35 mgN.l^{-1} as NH_4^+ and 23 mgP.l^{-1} as PO_4^{3-}. The cycle time was reduced to 4 h and consisted in 55 min anaerobic period followed by 170 min aeration, 5 min settling, and 10 min decant. The DO was kept between 1.3-1.7 mgO_2.l^{-1}. Full description of the new SBR operation is given in Appendix E.

Granular-SBR

The design of granular-SBR was similar than SNDPR-SBR (Figure 10) with a working volume of 5 l. Granular-SBR was also operated in a temperature-controlled room (18-22°C). The reactor was seeded with the granular sludge obtained from the lab-scale SNDPR-SBR fed with synthetic wastewater. During the first 4 months of operation, the SBR was fed with a mixture of anaerobically treated abattoir effluent and synthetic wastewater and the cycle time varied between 4-8h. After the initial 4 month adaptation period, only anaerobically treated abattoir effluent was fed into the SBR. Once steady state was established, the cycle time was fixed at 8 h and consisted of an 18 min feeding, 60 min anaerobic/anoxic, 315 min aerobic, 80 min post-anoxic, 2 min settling and 5 min decanting period. During the aerobic period the DO level was kept between 3.0 and 3.5 mgO_2.l^{-1}. The HRT gradually increased from 6.7 h during the adaptation period to 13.3 h once stable operation was reached (3 l fed each cycle). Full description of the granular SBR operation is given in Appendix F.

3.2.2. Analytical Methods

A wide range of techniques was employed to address the research objectives defined in this thesis. It includes reactor process studies, microbial investigations (fluorescent in-situ hybridisation – FISH combined with confocal laser scanning microscopy – CLSM) and several micro-scale techniques (e.g. micro-sensors, electron microscopy and light microscopy). The combination of these multi-disciplinary techniques has helped deliver significant outcomes.

Chemical analysis

The ammonia ($NH_4^+ + NH_3$), nitrate (NO_3^-), nitrite (NO_2^-) and phosphate-P (PO_4^3-P) were analysed using a Lachat QuikChem8000 Flow Injection Analyser (Lachat Instrument, Milwaukee). Dissolved nitrous oxide (N_2O) was measured on-line with a N_2O microsensor constructed according to Andersen et al. (2001). Total and soluble COD (TCOD and SCOD), soluble BOD5, total and soluble Kjeldahl nitrogen (TKN and SKN), total phosphorus and total dissolved phosphorus (TP and TDP), mixed liquor suspended solid (MLSS) and volatile MLSS (MLVSS) were analysed according to the standard methods (APHA, 1995). The major ions present in the SBRs bulk liquid (Ca^{2+}, Fe^{2+}, K^+, Mg^{2+}, Na^+ and HS^-) were measured by Inductively Coupled Plasma - Atomic Emission Spectrometry (ICP-AES Varian Vista-PRO, Varian, Inc.). VFAs were measured by Perkin-Elmer gas chromatography with column DB-FFAP 15 m x 0.53 mm x 1.0 μm (length x ID x film) at 140°C, while the injector and FID detector were operated at 220°C and 250°C, respectively. High purity helium was used as carrier gas at a flow rate of 17 $ml.min^{-1}$. Polyhydroxylalkanoates (PHA=PHB+PHV+PH2MV) and glycogen were determined using the method described in Oehmen et al. (2005). The phosphorus fractionation in granules was determined using the cold perchloric acid (PCA) extraction procedure developed by De Haas et al. (2000) as detailed in Appendix F.

Microbial analysis

Sludge samples were fixed and FISH probed as previously described (Amann, 1995). Oligonucleotide probes used in this thesis were the combination of EUB338 i-iii (EUBmix) for the detection of all bacteria (Daims et al., 1999), the combination of PAO462, PAO651 and PAO846 (PAOmix) for *Accumulibacter* spp. (Crocetti et al., 2000), the probe combination (GAOmix) of GAOQ989 (Crocetti et al., 2002) and GB_G2 (Kong et al., 2002) for *Competibacter* spp., NTSPA662 (Daims et al., 2001) for *Nitrospira* spp., NIT3 (Wagner et al., 1996) for *Nitrobacter* spp. and NSO1225 (Mobarry et al., 1996) for most of the ammonia oxidising bacteria (AOB) from the *Betaproteobacteria*. Additionally, probes for the proposed *Actinobacterial* PAOs (Kong et al., 2005) and *Defluviicoccus* spp.-related GAOs (Meyer et al., 2006) were also used. Fluorescently labelled oligonucleotides were purchased from Thermo (Ulm, Germany) with fluorescein isothiocyanate (FITC) or one of the sulfoindocyanine dyes indocarbocyanine (Cy3) or indodicarbocyanine (Cy5). FISH images were collected with a Zeiss LSM 510 (Carl Zeiss, Jena, Germany) CLSM using an argon laser (488 nm), a helium neon laser (543 nm) and a red diode laser (633 nm) fitted with 515-565 nm BP, 590 nm LP and 660-710 nm BP emission filters, respectively. Two different Zeiss oil immersion objectives were used, a Plan-Neofluar 40x/1.3 and a Plan-Apochromat 63x/1.4. FISH quantification was performed according to Crocetti et al. (2002) where the relative abundance of each group was determined as mean percentage of all bacteria based on pixel area counting.

Nile-Blue A staining (Ostle and Holt, 1982) was used to determine cells containing intracellular PHA and visualised on the confocal laser scanning microscope (see above).

Fixed granule samples for FISH and Nile Blue A staining were embedded in optimum cutting temperature (OCT) compound (TissueTek, Sakura, USA) for cryosectioning as previously described (Meyer et al., 2003). Embedded granules were then frozen and sectioned into 10 μm thick slices using a cryotome operated at -20°C (Kryo 1720, Leitz, Germany). The granule sections were collected on SuperFrost Plus microscope slides (Menzel-Glaser, Germany). Finally, the slides were dehydrated by sequential immersion for 3 min in 50%, 80% and 98% ethanol, followed by air-drying.

Physical analysis

To monitor the granule structure and characteristics, granule size distribution and density were measured. To determine the size distribution of the granules, 30 ml of well mixed granular sludge was sampled from the SBR at the end of the aeration period and pumped through a Malvern laser light scattering instrument, Mastersizer 2000 series (Malvern Instruments, Worcestershire, UK). The granule density, defined as the quantity of dry mass per biomass volume, was measured by the blue dextran method adapted from Di Iaconi et al. (2004) and further described in Appendix E.

The gradient of oxygen in granules was measured with oxygen microsensors (tip diameter <10 μm), which were constructed as described by Revsbech et al. (1989). Granules were transferred to a flow-cell with an upward flow where replicate oxygen profiles were then measured and averaged as described in Meyer et al. (2003). The pH gradient in granules was also measured with pH microsensors using the same experimental set-up.

Whole granules were photographed using an Olympus SZH10 stereo microscope mounted with a digital camera. To visualise the structure of aerobic granules at a microscale level, scanning and transmission electron microscopy (SEM and TEM, respectively) were employed. Prior to visualisation, granules were fixed in subsequent chemical solutions to minimise any structural artefacts arising from the dehydratation process (see Appendix G for more details). For TEM studies, dehydrated granules were embedded in Epon resin and ultrathin sections were cut and mounted on copper grid for visualisation with a JEOL 1010 transmission electron microscope operated at 80 kV. For SEM studies, dehydrated granules were mounted onto metal stubs and sputter coated with platinum to reduce charging. Viewing of samples was conducted using a JEOL 6300F scanning electron microscope operated at 5 or 10 kV. To observe the internal structure, some dehydrated granules were frozen in liquid nitrogen, fractured, sputter coated with platinium and visualised as described above.

3.3. Research Outcomes

i. Multi-step feeding strategy to prevent the accumulation of NO_x^-

Abattoir wastewater contains a high level of ammonia and organic nitrogen and the complete nitrification of these nitrogen compounds produces a high level of nitrate and/or nitrite, which has proved to be an obstacle to the development of a stable and reliable Bio-P removal process. The detrimental effect of nitrate/nitrite accumulation for biological P removal processes was discussed in Chapter 2. To address this challenge, a multi-step feeding strategy was implemented to keep the nitrate/nitrite concentration low during the entire SBR cycle providing suitable anaerobic conditions to perform reliable Bio-P removal. The SBR feeding strategy was split into 3 separate feeding stages each followed by a non-aerobic and aerobic periods. 50% of the total influent volume was fed during the first feeding, 30% during the second and 20% during the third. The "UniFed" filling process described in Chapter 2 was employed for the first feeding period. Fresh influent was introduced directly into the sludge blanket from the bottom of the SBR once the settling period was finished.

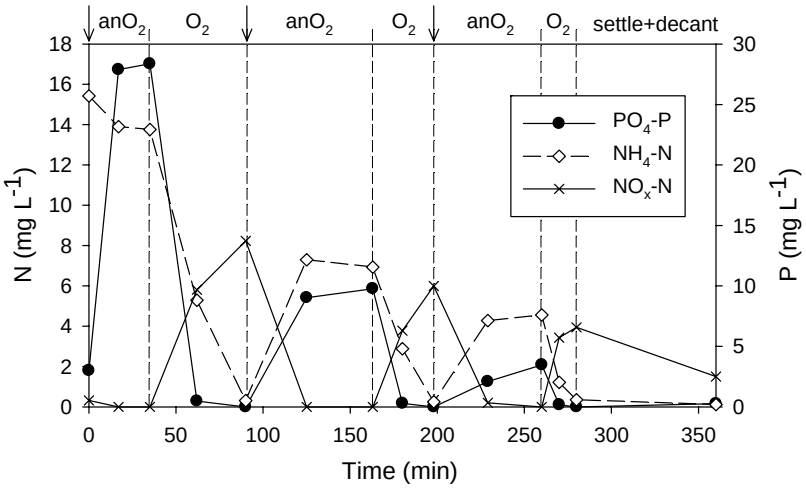


Figure 11. Nitrogen and phosphorus profiles during a cycle study of RivD-SBR. The vertical arrows indicate the 3 feeding periods.

This multi-feed strategy successfully limited the level of NO_x^- recycled to the anaerobic period. Figure 11 shows the nitrogen and phosphorus transformations in a typical SBR cycle study during the steady state period of the RivD-SBR. At the end of each aerobic period, NH_4^+ was fully oxidised, and the low level of NO_x^- accumulated was then removed in the following anoxic period. Very low level of NO_x^- was carried over to the next cycle, and was denitrified very quickly at the beginning of the first anaerobic period. Most of the anaerobic P release by PAOs occurred in the first non-aerated period. This indicates that the first non-aerated period was crucially important for P removal as PAOs could freely utilise all the VFA available without having to compete with heterotrophic denitrifiers. This multi-step strategy allowed RivD-SBR to consistently achieve 95%, 97% and 98% of TCOD, TN and TP removal, respectively. The full detail of this study is provided in Appendix A

ii. Strategies to meet the carbon demand for biological nutrient removal

The performance of a biological nutrient removal system depends greatly on the availability of easily biodegradable carbon sources in the wastewater, particularly VFAs. Therefore, it is required to optimise the use of the limited available COD to remove the high levels of nutrient in abattoir wastewater. Considering that it is difficult to control the VFAs content in large anaerobic ponds, a more controllable VFA source is necessary for reliable biological nutrient removal from abattoir wastewater. In this thesis, abattoir raw wastewater was subjected to a one-day high-rate pre-fermentation step before being mixed with anaerobic ponds effluent to increase the VFAs concentration of the wastewater fed into RivD-SBR. The pre-fermentation was performed in a 50 l tank continuously mixed with the temperature kept at 37˚C. No inoculum was introduced in the pre-fermentor, and hence the microbial population present in the raw abattoir wastewater was used to carry out the fermentation. The overall VFA concentration in the raw wastewater stream more than doubled as a direct result of this pre-fermentation. Acetate and propionate were the most abundant VFAs in the raw abattoir wastewater before and after pre-fermentation with propionate having a slightly higher production rate than acetate. However, it should be highlighted that the use of raw wastewater should be minimised due to its high FOG content which would likely deteriorate the sludge settleability.

An alternative approach to reduce the carbon demand is to achieve nitrogen removal via the nitrite pathway as presented in Chapter 2. To achieve this nitrite pathway in RivD-SBR, an on-line aeration phase length control system was integrated to the step-feed strategy described previously. The control strategy employed was based on the slope of the pH signal and on the oxygen uptake rate (OUR). The exact time of complete NH_4^+ oxidation (i.e. end of nitritation process) in each aerobic period could be detected through the pH bending point and the sharp OUR drop. The aeration could then be automatically switched off preventing the further oxidation of nitrite into nitrate. Instead of using an external carbon source, the organic carbon contained in wastewater was used for denitrification, thus delivering the true benefit of implementing the nitrite pathway. Figure 12 presents the level of nitrite pathway achieved in the SBR, measured as the average amount of NO_2^- produced per NO_x^- produced ($mgN.l^{-1}$) during the 3 aeration periods, and the relative abundance of NOBs in the SBR after implementation of the aeration control strategy. RivD-SBR had been already running for 5 months performing high level of COD, N and P removal (see Appendix A) before the aeration length control was implemented. During that time, no nitrite accumulation was observed during the aerobic periods. The control of the length of each aeration period was first implemented manually, which resulted in a rapid accumulation of nitrite reaching 95% of the total amount of NO_x^- produced and a sharp decrease of the NOB population (Figure 12). Then, the application of fixed aeration periods deteriorated the nitrite pathway previously established and NOB population slightly recovered. Finally, the implementation of the automatic aeration length control strategy re-established the nitrite pathway in the SBR. This strategy was therefore successful in controlling the level of the nitrite pathway through the elimination or the reduction of the NOB population in the system. Removing N via the nitrite pathway benefited the nutrient removal performance of the SBR by reducing the demand for COD. The full detail of this study is presented in Appendix B.

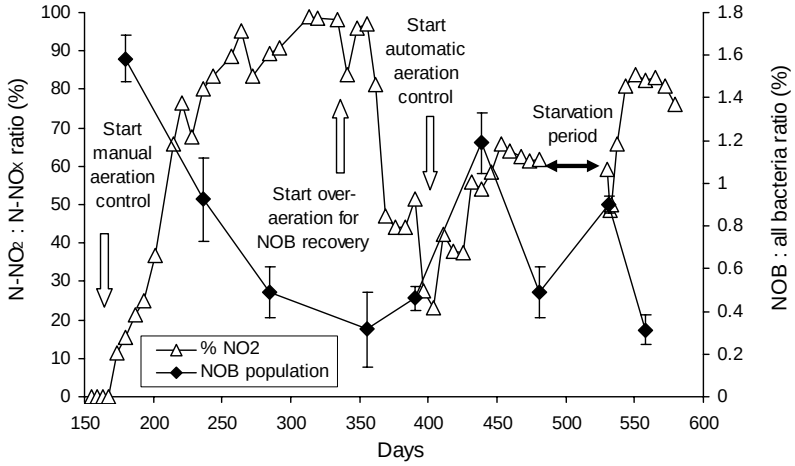


Figure 12. Degree of nitrite accumulation and abundance of NOB *Nitrospira* (FISH probe Nitspa-662) in RivD-SBR. The NOB quantification shown is an average (error bars=SE, n=3).

iii. Strategy to maintain the biomass activity during long term starvation condition.

RivD-SBR was put twice into a so-called "sleeping mode" for a period of 5-6 weeks when the abattoir from where the wastewater was sourced was closed for annual maintenance. The "sleeping mode" operation consisted of 15 min mixed aeration and 345 settling in a 6 h cycle.

During the first starvation period, the nitrifying activity was closely monitored through weekly pulse addition of ammonia and nitrite, while the activity of PAOs was only monitored by measuring the phosphate-P concentration in the liquid phase. During the second starvation period, the PAO activity was more comprehensively studied with weekly batch tests to monitor the anaerobic and aerobic activities of PAOs. The recovery processes of these organisms after the starvation were also investigated by applying a resuscitation strategy consisting of gradually increasing the wastewater load in the first few days after normal operation was resumed.

Batch tests monitoring the specific activity of nitrifiers and PAOs population in RivD-SBR over the course of each starvation period demonstrate a clear activity drop for both populations. However, after gradually resuming the normal reactor operation, the nitrification rate, denitrification rate and the amount of P-release and P-uptake quickly improve and reached their initial value within 4 days after both starvation periods (Table 3). Good nutrient removal performances were consistently achieved for several months after each starvation periods confirming that the strategies employed to maintain the biomass activity during long term starvation condition and to resuscitate that same biomass were very successful.

Table 3. Nitrification rate (rNH_4^+), denitrification rate (rNO_x^-) and amounts of P released and uptaken over a cycle, measured during cycle studies performed before the start of the starvation period, immediately after the starvation period (50% of normal load), 2 days after starvation (75% of normal load) and 4 days after the starvation (100% of normal load).

Parameter monitored	**rNH_4^+** ($mgN.l^{-1}.h^{-1}$)		**rNO_x^-** ($mgN.l^{-1}.h^{-1}$)		**P-release** ($mgP.l^{-1}$)		**P-uptake** ($mgP.l^{-1}$)	
"Sleeping mode" I or II	**I**	**II**	**I**	**II**	**I**	**II**	**I**	**II**
Before starvation	18.2	25.5	4.8	12.3	18.8	36.9	16.1	34.2
After 1st cycle (50%)	8.2	7.4	1.9	1.8	2.5	4.4	2.3	4.8
After 2 days (75%)	12.9	20.8	4.5	9.6	9.6	31	8.1	28.9
After 4 days (100%)	17.6	29.1	5.7	11.7	19.6	47	17.4	44.3

iv. Organisms responsible for denitrification in lab-scale floccular and granular SNDPR bioreactors

FISH analysis performed on the SNDPR-SBR floccular sludge has demonstrated a very high abundance of *Accumulibacter* spp. (PAOs) and *Competibacter* spp. (GAOs) accounting together for 70% of the total bacteria present in the reactor. Figure 13 shows that *Accumulibacter* spp. were always more abundant than *Competibacter* spp.. An interesting trend appears in Figure 13 when comparing the N removal efficiency to the percentage of GAOs in the system. Over the 5-months period, N removal efficiency decreased from 100% to 53% and the *Competibacter* spp. population in the reactor simultaneously decreased from 19% to 8% of all bacteria. At the same time, the *Accumulibacter* spp. population increased from 48% to 70%. These results strongly support the hypothesis that *Competibacter* spp. are the microorganisms primarily responsible for the denitrification occurring in the SNDPR reactor. It poses the question of whether there is a direct link between the enrichment of *Competibacter* spp. and N_2O accumulation from denitrification. More details of this study are given in Appendix D.

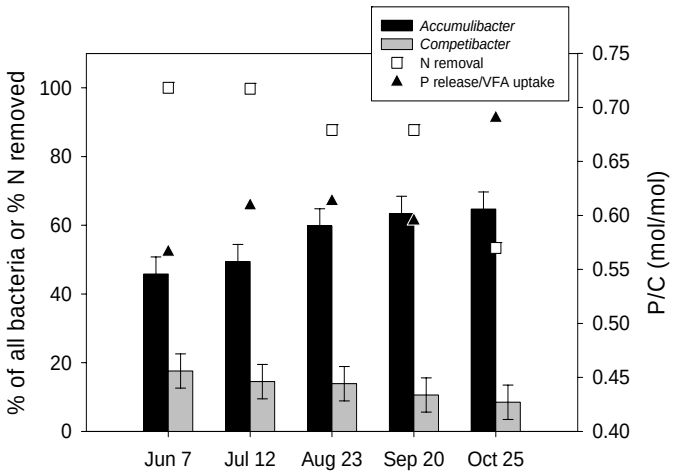


Figure 13. Abundance of *Accumulibacter* and *Competibacter* correlated with the N removal efficiency and the carbon-uptake to P-release ratio over the 5 month period.

In an attempt to demonstrate ecological positions and roles for each population in aerobic granules, the spatial distribution of PAOs and GAOs in this complex system was correlated with the dissolved oxygen profiles. The goal was to verify at a microscale level, which microbial community between PAO and GAO was more likely responsible for the denitrifcation in lab-scale granular SNDPR bioreactors fed with synthetic wastewater. A novel method using fluorescence *in situ* hybridisation (FISH) and confocal laser scanning microscopy (CLSM) was developed in this thesis to study the microscale distribution, organisation, and community composition of the bacterial community in aerobic granules. Oxygen profiles inside SNDPR granules were determined by microsensors. The exact method is described in depth in Appendix E. The population distribution of *Accumulibacter* spp. (the main PAO) and *Competibacter* spp. (the main GAO) within 24 different granules was expressed as the relative abundance at different depth of *Accumulibacter* spp. divided by that of *Competibacter* spp. (referred to as the PAO:GAO ratio). Using the ratio data, a mean distribution was calculated and is presented in Figure 14 alongside the in-situ dissolved oxygen profile. For each 50 µm zone of the granules, the PAO:GAO ratio strongly correlated (significant at the 0.01 level) with the dissolved oxygen concentration (Pearson correlation=0.86). *Accumulibacter* spp. was dominant (i.e. PAO:GAO ratio >1) in the aerobic zones (0-200 µm) while *Competibacter* spp. dominated (i.e. PAO:GAO ratio <1) in the central anoxic zones (200 µm inwards) of the studied granules. Therefore *Competibacter* spp. would be mostly responsible for denitrification in SNDPR granules which confirms the results obtained with floccular SNDPR systems also fed with synthetic wastewater. The low microbial diversity observed in both floccular and granular SNDPR bioreactors was suggested for this lack of denitrification by PAOs. The apparent role of GAOs in denitrification clearly compromises the carbon savings proposed to be obtained by SNDPR. More information is provided in Appendix E.

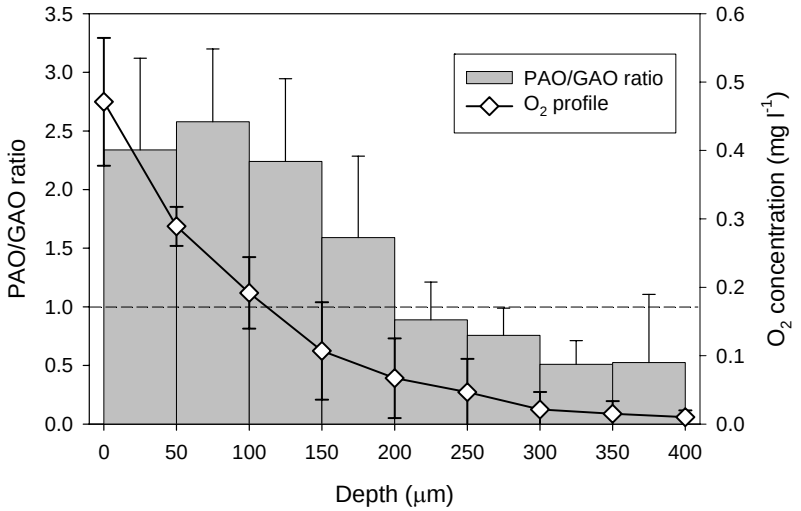


Figure 14. Average profile of the PAO:GAO ratio within 24 granules (error bar=95%CL) and mean O_2 profiles in granules at the end of the aerobic period (error bars=S.D., n=6).

v. Management of N_2O accumulation in SNDPR

To elucidate the factors responsible for N_2O accumulation in lab-scale SNDPR bioreactors operated with synthetic wastewater and test how it can be prevented, several anoxic batch tests were performed under different conditions. Sludge was sampled at the end of the anaerobic period from the parent reactor (SNDPR-SBR) and transferred to two 14.75 ml vials sealed with rubber stoppers to which a N_2O microsensor was inserted for on-line monitoring of the N_2O concentration. The mixed liquor was stirred using a magnetic stirrer, and the vials were filled completely to avoid any exchange of N_2O between liquid and gas phases. The two mini-reactors were operated in parallel with one acting as a negative control or a duplicate. Substrates could be added in small amounts at any time during experimentation with a syringe through the rubber stopper. Net N_2O production and consumption rates could then be measured with different electron acceptors (nitrate, nitrite and N_2O) using different carbon sources (intracellular PHA, acetate, propionate, methanol and abattoir wastewater). Both rates were corrected for MLVSS variations in different batch tests.

It was hypothesised that if denitrification is carried out simultaneously by GAOs and other denitrifiers in the SNDPR system, the N_2O accumulated by GAOs could be reduced by other denitrifiers, provided that carbon is available to these cells. To verify this hypothesis, anoxic batch tests were also performed with a mixture of sludge from the SNDPR reactor and a lab-scale nitrifying-denitrifying reactor treating domestic wastewater containing additional carbon in the form of methanol for denitrification. The addition of raw abattoir wastewater as carbon source after 35 min incubation with nitrate led to an immediate reduction and depletion of N_2O (Figure 15). At the same time, the nitrate reduction rate increased, underlining the capacity of non-PAO and non-GAO denitrifiers to reduce nitrate to N_2 while removing N_2O accumulated by the SNDPR sludge simultaneously.

The accumulation of N_2O in the SNDPR sludge is likely to be a result of the high enrichment of PAOs and GAOs because of the application of synthetic wastewater containing a single carbon source (i.e. acetate). If real wastewater is fed it is a realistic expectation that some carbon would be available for denitrification by non-PAO or non-GAO organisms during the low-DO aerobic period. The N_2O accumulation is unlikely to be an issue in future application

of the SNDPR process to remove nutrients from abattoir wastewater. This was indeed verified when abattoir wastewater was fed into Granular-SBR and SNDPR was achieved. Detailed explanation of this study is given in Appendix D.

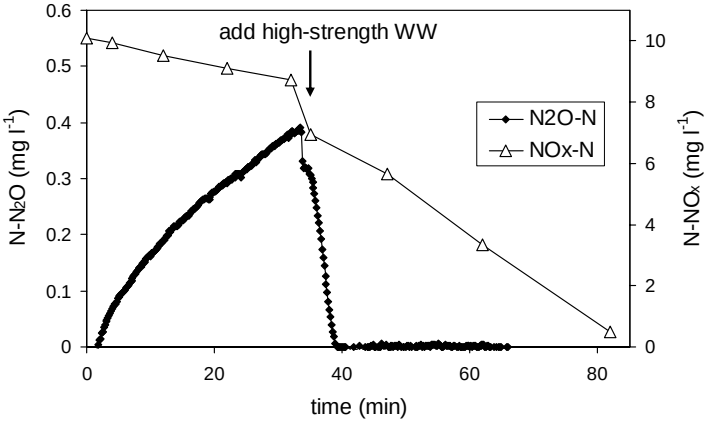


Figure 15. N_2O and NO_x^- concentration in the bulk liquid during an anoxic test in a 500 ml reactor. Raw high-strength wastewater was added at T=35 min.

vi. Nutrient removal from abattoir wastewater using aerobic granular sludge technology

Good nitrogen and phosphorus removal performance was achieved once stable operation was established. The organic, nitrogen and phosphorus loading rates applied were 2.7 $gCOD.l^{-1}.d^{-1}$, 0.43 $gN.l^{-1}.d^{-1}$ and 0.06 $gP.l^{-1}.d^{-1}$, respectively. The removal efficiency of soluble COD, total dissolved nitrogen and total dissolved phosphorus were 89%, 93% and 88%, respectively. The remaining soluble COD measured in the effluent (162 $mg.l^{-1}$) was non-biodegradable as indicated by the very low soluble BOD_5 value (<2 $mg.l^{-1}$). The oxidised nitrogen accumulating at the end of each cycle (i.e. around 10 $mgN.l^{-1}$) was almost exclusively nitrite (94.7%, SD=2.5%, n=63) suggesting that N was removed via the nitrite pathway in this granular SBR. However, the high suspended solids in the effluent (around 0.3 $g.l^{-1}$) limited the overall removal efficiency to 68%, 86% and 74% for total COD, total nitrogen and total phosphorus, respectively. Interestingly, *Competibacter* spp. was no longer detected in the granules via FISH analysis and *Accumulibacter* spp. were found to be involved in the denitrification indicating that true SNDPR occurred in this granular SBR fed with real wastewater. It was also found that the minimum hydraulic retention time in this aerobic granular sludge system was not governed by the sludge settleability, as is the case in a floccular sludge system, but likely by the limitations associated with the transfer of substrates in granules. More details of this study can be found in Appendix F. The structure of these aerobic granules fed with nutrient rich industrial wastewater was also studied at a microscale level. Observations were made using a wide range of techniques including light microscopy, scanning and transmission electron microscopy, fluorescent in-situ hybridisation (FISH) combined with confocal laser scanning microscopy (CLSM) and oxygen and pH microsensors. This multi-disciplinary approach allowed for interpretations to be made about the general structure and fate of mature granules, the microbial community structure, the effect of pH on the granule structure stability and the possible role played by protozoa in the overall system performance. Images of the granule structure and their interpretations are provided in Appendix G.

4.0 Conclusions and Recommendations for Future Work

4.1. Main Conclusions of the Thesis

A sequencing batch reactor system was demonstrated to effectively remove nitrogen, phosphorus and COD from abattoir wastewater. This provides a more cost-effective alternative to chemical phosphorus removal, the current practice in the meat industry which requires the addition of large amount of chemicals. The implementation of this novel SBR technology to the existing water treatment facilities employed in the meat industry is believed to be relatively simple and realistic. Indeed, this lab-scale RivD-SBR technology has been recently scaled up to a 10 m^3 on-site pilot plant for further trials. The following conclusions can be drawn:

- It is possible to achieve a high degree (>98%) of biological phosphorus removal from abattoir wastewater in the presence of high levels of nitrogen (200 – 300 $mgN.l^{-1}$) using a SBR process.
- The multi-step feeding strategy prevents high-level accumulation of nitrate or nitrite, and hence facilitates the creation of anaerobic conditions in the SBR. This strategy is strongly recommended for practical use in the biological treatment of abattoir wastewater.
- It is important to incorporate a high-rate pre-fermentor as an integrated component of the nutrient removal system in SBR. This stream, which contains a high-level of VFAs, is effective in providing supplementary carbon sources for both phosphorus and nitrogen removal.
- The aeration control strategy consisting of stopping the aeration in the SBR immediately after NH_4^+ is oxidised is effective in achieving stable N removal via the nitrite pathway. It benefits the nutrient removal performance of the SBR by saving some valuable amount of COD.
- The intermittent aeration of 15 minutes in every 6 hours is effective in maintaining the biomass activities of activated sludge performing biological nitrogen and phosphorus removal. Sludge can be stored under such conditions for at least six weeks with its nitrifying, denitrifying and phosphorus removal capabilities adequately maintained to allow for a quick recovery when wastewater feed resumes. The resuscitation strategy of gradually increasing the wastewater load in the first few days after a starvation period was also demonstrated to be successful.

Two novel technologies were also identified to have strong potentials to remove nutrient from abattoir wastewater, namely the simultaneous nitrification, denitrification and phosphorus removal (SNDPR) process and the aerobic granular sludge technology. The main conclusions drawn from the in-depth study of these two technologies are:

- GAOs and not PAOs are primarily responsible for the denitrification in lab-scale SNDPR bioreactor treating synthetic wastewater with floccular biomass. This compromises the carbon savings proposed to be obtained when using SNDPR process to remove nutrient from wastewater.

- The production of N_2O in lab-scale SNDPR bioreactors is likely linked to the loss of diversity amongst the denitrifying microbial community due to the use of synthetic wastewater containing only a single carbon source. However, N_2O accumulation is unlikely to be an issue in a SNDPR bioreactor treating real wastewater as such wastewater contains a combination of different carbon sources. It will enable denitrifiers other than PAO and GAO to participate to the denitrification and reduce the N_2O presumably produced by GAO.
- The size and the dense structure of aerobic granules positively contributed to the oxygen mass transfer limitation required to achieve reliable SNDPR process. Large and stable anoxic zones were created in the centre part of the granule which provided a better coupling between nitrification and denitrification. However, GAOs were still the main denitrifier in lab-scale granular SNDPR bioreactor treating synthetic wastewater.
- Granular sludge can be maintained in a bioreactor operated under alternating anaerobic and aerobic conditions using anaerobically pre-treated abattoir wastewater as feed. High-levels of COD, nitrogen and phosphorus removal can be achieved through true SNDPR process with PAOs carrying out the denitrification. However, the effluent produced requires post-treatment in order to remove the suspended solids before discharge into the receiving environment.
- The minimum HRT for a granular sludge system is not governed by the sludge settleability and retention, as is the case in a system with floccular sludge. Mass transfer limitations in granules are likely an important factor to be considered in the design of the HRT and the COD and nutrient loading rate in a granular sludge system.

4.2. Recommendation for Future Research

During the course of this PhD thesis, a number of other questions were raised that call for further investigations. Recommendations for future research in this field are listed below:

- The lab-scale SBR process developed in this PhD thesis for the treatment of abattoir wastewater still needs to be scaled up to a pilot and/or full scale treatment plant in order to validate the results obtain in this thesis and offer practical application for the industry. The specific feed mixture used in this study (80-90% anaerobic pond effluent supplemented with extra VFAs and 10-20% pre-fermented raw wastewater) may potentially limit the wider validity of results. Further investigations using different mixture ratio and better quality anaerobic pond effluent would therefore be beneficial.
- The operation and design of anaerobic pre-treatment processes currently employed in most abattoirs should be oriented towards maximising VFA production instead of solely focussing on removing COD. This will facilitate the downstream biological nutrient removal treatment of abattoir wastewater.
- The SBR cycle operation could be improved by the use of on-line NH_4^+, NO_3^-, NO_2^- and PO_4^{3-} sensors which are getting more and more reliable. Efficient control strategies could then be designed based on these on-line sensors as biological processes such as nitrification, denitrification and aerobic P-uptake would be directly monitored during the SBR cycle.

- The optimal aeration frequency and duration of the alternating anoxic/anaerobic and aerobic strategy to maintain the biomass activities of BNR process during long starvation period are yet to be identified through further experimental studies.
- Interesting structural properties of aerobic granules have been observed in this thesis using a multi-disciplinary approach. However, more specific studies are needed to fully understand the granule formation process and the exact role of EPS, mineral precipitation and protozoa in the overall behaviour of aerobic granules. The impact of pH fluctuations on the granule structure stability requires also further experimental work.
- In depth microsensors studies of aerobic granules could provide opportunities to model the complex diffusion processes occurring inside these granules and help to better understand and possibly predict the overall performance of granular sludge systems.
- Aerobic granular sludge technology seems to be particularly well suited for the treatment of industrial wastewater. The stability of aerobic granules under starvation condition needs to be investigated as industries are often subjected to temporal fluctuation of water usage which directly affects the flow and/or concentration of the effluent entering the treatment plant.

References

Al-Ghusain I. and Hao O.J. (1995). Use of pH as control parameter for aerobic/anoxic sludge-digestion. *Journal of Environmental Engineering-Asce*. **121**(3): 225-235.

Al-Ghusain I.A., Huang J., Hao O.J. and Lim B.S. (1994). Using pH as a real-time control parameter for waste-water treatment and sludge-digestion processes. *Water Science and Technology*. **30**(4): 159-168.

Amann R.I. (1995). In situ identification of microorganisms by whole cell hybridization with rRNA-targeted nucleic acid probes. In: Molecular Microbial Ecology Manual. Akkermans A.D.L., van Elsas J.D. and de Bruijn F.J. (eds). Dordrecht, Holland, *Kluwer Academic Publications*. **3.3.6**: 1-15.

Anderottola G., Canziani R., Foladori P., Ragazzi M. and Tatano F. (2000). Laboratory scale experimentation for RBCOD production from OFMSW for BNR systems: Results and kinetics. *Environmental Technology*. **21**(12): 1413-1419.

Andersen K., Kjaer T. and Revsbech N.P. (2001). An oxygen insensitive microsensor for nitrous oxide. *Sensors and Actuators B-Chemical*. **81**(1): 42-48.

APHA (1995). Standard methods for the examination of water and wastewater. Washington, DC, *American Public Health Association*.

Arrojo B., Mosquera-Corral A., Garrido J.M. and Mendez R. (2004). Aerobic granulation with industrial wastewater in sequencing batch reactors. *Water Research*. **38**(14-15): 3389-3399.

Artan N., Wilderer P., Orhon D., Morgenroth E. and Ozgur N. (2001). The mechanism and design of sequencing batch reactor systems for nutrient removal - the state of the art. *Water Science and Technology*. **43**(3): 53-60.

Bertanza G. (1997). Simultaneous nitrification-denitrification process in extended aeration plants: Pilot and real scale experiences. *Water Science and Technology*. **35**(6): 53.

Beun J.J., Heijnen J.J. and van Loosdrecht M.C.M. (2001). N-removal in a granular sludge sequencing batch airlift reactor. *Biotechnology and Bioengineering*. **75**(1): 82-92.

Beun J.J., Hendriks A., Van Loosdrecht M.C.M., Morgenroth E., Wilderer P.A. and Heijnen J.J. (1999). Aerobic granulation in a sequencing batch reactor. *Water Research*. **33**(10): 2283-2290.

Bhamidimarri S.M.R. (1991). Appropriate industrial-waste management technologies - the New-Zealand meat industry. *Water Science and Technology*. **24**(1): 89-95.

Borja R., Banks C.J. and Wang Z. (1994). Performance and Kinetics of an Upflow Anaerobic Sludge Blanket (Uasb) Reactor Treating Slaughterhouse Waste-Water. *Journal of Environmental Science and Health Part a-Environmental Science and Engineering & Toxic and Hazardous Substance Control*. **29**(10): 2063-2085.

Bortone G., Malaspina F., Stante L. and Tilche A. (1994). Biological nitrogen and phosphorus removal in an anaerobic/anoxic sequencing batch reactor with separated biofilm nitrification. *Water Science and Technology*. **30**(6): 303-313.

Brdjanovic D., Slamet A., van Loosdrecht M.C.M., Hooijmans C.M., Alaerts G.J. and Heijnen J.J. (1998). Impact of excessive aeration on biological phosphorus removal from wastewater. *Water Research*. **32**(1): 200-208.

Caixeta C.E.T., Cammarota M.C. and Xavier A.M.F. (2002). Slaughterhouse wastewater treatment: evaluation of a new three-phase separation system in a UASB reactor. *Bioresource Technology*. **81**(1): 61-69.

Chang C.H. and Hao O.J. (1996). Sequencing batch reactor system for nutrient removal: ORP and pH profiles. *Journal of Chemical Technology and Biotechnology*. **67**(1): 27-38.

Comeau Y., Hall K., Hancock R. and Oldham W. (1986). Biochemical model for enhanced biological phosphorus removal. *Water Research*. **20**: 1511-1521.

Crocetti G.R., Banfield J.F., Keller J., Bond P.L. and Blackall L.L. (2002). Glycogen accumulating organisms in laboratory-scale and full-scale activated sludge processes. *Microbiology*. **148**(11): 3353-3364.

Crocetti G.R., Hugenholtz P., Bond P.L., Schuler A., Keller J., Jenkins D. and Blackall L.L. (2000). Identification of polyphosphate-accumulating organisms and design of 16S rRNA-directed probes for their detection and quantitation. *Applied and Environmental Microbiology*. **66**(3): 1175-1182.

Daims H., Bruhl A., Amann R., Schleifer K.H. and Wagner M. (1999). The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: Development and evaluation of a more comprehensive probe set. *Systematic and Applied Microbiology*. **22**(3): 434-444.

Daims H., Nielsen J.L., Nielsen P.H., Schleifer K.H. and Wagner M. (2001). In situ characterization of Nitrospira-like nitrite oxidizing bacteria active in wastewater treatment plants. *Applied and Environmental Microbiology*. **67**(11): 5273-5284.

de Bruin L.M.M., de Kreuk M.K., van der Roest H.F.R., Uijterlinde C. and van Loosdrecht M.C.M. (2004). Aerobic granular sludge technology: an alternative to activated sludge? *Water Science and Technology*. **49**(11-12): 1-7.

de Haas D.W., Wentzel M.C. and Ekama G.A. (2000). The use of simultaneous chemical precipitation in modified activated sludge systems exhibiting biological excess phosphate removal Part 2: Method development for fractionation of phosphate compounds in activated sludge. *Water Sa*. **26**(4): 453-466.

de Kreuk M.K. and van Loosdrecht M.C.M. (2004). Selection of slow growing organisms as a means for improving aerobic granular sludge stability. *Water Science and Technology*. **49**(11-12): 9-17.

de Villiers G.H. and Pretorius W.A. (2001). Abattoir effluent treatment and protein production. *Water Science and Technology*. **43**(11): 243-250.

Di Iaconi C., Ramadori R., Lopez A. and Passino R. (2004). Preliminary biomass characterization in a sequencing batch biofilm reactor. *Annali di Chimica*. **94**(12): 889-898.

Dulekgurgen E., Ovez S., Artan N. and Orhon D. (2003). Enhanced biological phosphate removal by granular sludge in a sequencing batch reactor. *Biotechnology Letters*. **25**(9): 687-693.

El-Mamouni R., Leduc R. and Guiot S.R. (1998). Influence of synthetic and natural polymers on the anaerobic granulation process. *Water Science and Technology*. **38**(8-9): 341-347.

Etterer T. and Wilderer P.A. (2001). Generation and properties of aerobic granular sludge. *Water Science and Technology*. **43**(3): 19-26.

Fuerhacker M., Bauer H., Ellinger R., Sree U., Schmid H., Zibuschka F. and Puxbaum H. (2000). Approach for a novel control strategy for simultaneous nitrification/denitrification in activated sludge reactors. *Water Research*. **34**(9): 2499-2506.

Furumai H., Kazmi A.A., Fujita M., Furuya Y. and Sasaki K. (1999). Modeling long term nutrient removal in a sequencing batch reactor. *Water Research*. **33**(11): 2708-2714.

Gaterell M.R., Gay R., Wilson R., Gochin R.J. and Lester J.N. (2000). An economic and environmental evaluation of the opportunities for substituting phosphorus recovered from wastewater treatment works in existing UK fertiliser markets. *Environmental Technology*. **21**(9): 1067-1084.

Grotenhuis J.T.C., Vanlier J.B., Plugge C.M., Stams A.J.M. and Zehnder A.J.B. (1991). Effect of ethylene glycol-bis(beta-aminoethyl ether)-N,N-tetraacetic acid (egta) on stability and activity of methanogenic granular sludge. *Applied Microbiology and Biotechnology*. **36**(1): 109-114.

Hao O.J. and Huang J. (1996). Alternating aerobic-anoxic process for nitrogen removal: Process evaluation. *Water Environment Research*. **68**(1): 83-93.

Hopwood D. (1977). Effluent treatment in meat and poultry-processing Industries. *Process Biochemistry*. **12**(8): 5-&.

Ingildsen P. and Olsson G. (2002). Exploiting online in-situ ammonium, nitrate and phosphate sensors in full-scale wastewater plant operation. *Water Science and Technology*. **46**(4-5): 139-147.

Ingildsen P. and Wendelboe H. (2003). Improved nutrient removal using in situ continuous on-line sensors with short response time. *Water Science and Technology*. **48**(1): 95-102.

Irvine R.L. and Busch A.W. (1979). Sequencing batch biological reactors - overview. *Journal Water Pollution Control Federation*. **51**(2): 235-243.

Johns M.R. (1995). Developments in wastewater treatment in the meat processing industry: A review. *Bioresource Technology*. **54**(3): 203-216.

Johns M.R., Harrison M.L., Hutchinson P.H. and Beswick P. (1995). Sources of nutrients in wastewater from integrated cattle slaughterhouses. *Water Science and Technology*. **32**(12): 53-58.

Keller J., Subramaniam K., Gosswein J. and Greenfield P.F. (1997). Nutrient removal from industrial wastewater using single tank sequencing batch reactors. *Water Science and Technology*. **35**(6): 137-144.

Keller J., Watts S., Battye-Smith W. and Chong R. (2001). Full-scale demonstration of biological nutrient removal in a single tank SBR process. *Water Science and Technology*. **43**(3): 355-362.

Kerrn-Jespersen J.P., Henze M. and Strube R. (1994). Biological phosphorus release and uptake under alternating anaerobic and anoxic conditions in a fixed-film reactor. *Water Research*. **28**(5): 1253-1255.

Koenig A. and Yiu W.C. (1999). Waste management in Hong Kong abattoirs. *Water Science and Technology*. **40**(1): 379-387.

Kong Y., Ong S.L., Ng W.J. and Liu W.-T. (2002). Diversity and distribution of a deeply branched novel proteobacterial group found in anaerobic-aerobic activated sludge processes. *Environmental Microbiology*. **4**(11): 753-757.

Kong Y.H., Nielsen J.L. and Nielsen P.H. (2005). Identity and ecophysiology of uncultured actinobacterial polyphosphate-accumulating organisms in full-scale enhanced biological phosphorus removal plants. *Applied and Environmental Microbiology*. **71**(7): 4076-4085.

Kuba T., Smolders G.J.F., van Loosdrecht M.C.M. and and Heijnen J.J. (1993). Biological phosphorus removal from wastewater by anaerobic and anoxic sequencing batch reactor. *Water Science and Technology*. **27**(5-6): 241-252.

Kuba T., Wachtmeister A., Vanloosdrecht M.C.M. and Heijnen J.J. (1994). Effect of nitrate on phosphorus release in biological phosphorus removal systems. *Water Science and Technology*. **30**(6): 263-269.

Lemaire R., Meyer R., Taske A., Crocetti G.R., Keller J. and Yuan Z.G. (2006). Identifying causes for N_2O accumulation in a lab-scale sequencing batch reactor performing simultaneous nitrification, denitrification and phosphorus removal. *Journal of Biotechnology*. **122**(1): 62-72.

Lettinga G., Vanvelsen A.F.M., Hobma S.W., Dezeeuw W. and Klapwijk A. (1980). Use of the upflow sludge blanket (USB) reactor concept for biological wastewater-treatment, especially for anaerobic treatment. *Biotechnology and Bioengineering*. **22**(4): 699-734.

Li C.T., Shieh W.K., Wu C.S. and Huang J.S. (1986). Chemical Bio-Fluidized Bed Treatment of Slaughterhouse Waste-Water. *Journal of Environmental Engineering-Asce*. **112**(4): 718-728.

Lin Y.F. and Jing S.R. (2001). Characterization of denitrification and nitrification in a step-feed alternating anoxic-oxic sequencing batch reactor. *Water Environment Research*. **73**(5): 526-533.

Lin Y.M., Liu Y. and Tay J.H. (2003). Development and characteristics of phosphorus-accumulating microbial granules in sequencing batch reactors. *Applied Microbiology and Biotechnology*. **62**(4): 430-435.

Liu Y. and Tay J.-H. (2004). State of the art of biogranulation technology for wastewater treatment. *Biotechnology Advances*. **22**(7): 533-563.

Liu Y., Wang Z.W., Qin L., Liu Y.Q. and Tay J.H. (2005a). Selection pressure-driven aerobic granulation in a sequencing batch reactor. *Applied Microbiology and Biotechnology*. **67**(1): 26-32.

Liu Y., Wang Z.W. and Tay J.H. (2005b). A unified theory for upscaling aerobic granular sludge sequencing batch reactors. *Biotechnology Advances*. **23**(5): 335-344.

Liu Y., Yang S.F. and Tay J.H. (2003). Elemental compositions and characteristics of aerobic granules cultivated at different substrate N/C ratios. *Applied Microbiology and Biotechnology*. **61**(5-6): 556-561.

Londong J. and Wachtl P. (1996). Six years of practical experience with the operation of on-line analysers. *Water Science and Technology*. **33**(1): 159-164.

LynggaardJensen A., Eisum N.H., Rasmussen I., Jacobsen H.S. and Stenstrom T. (1996). Description and test of a new generation of nutrient sensors. *Water Science and Technology*. **33**(1): 25-35.

Mainstone C.P. and Parr W. (2002). Phosphorus in rivers - ecology and management. *Science of the Total Environment*. **282**: 25-47.

Manjunath N.T., Mehrotra I. and Mathur R.P. (2000). Treatment of wastewater from slaughterhouse by DAF-UASB system. *Water Research*. **34**(6): 1930-1936.

Martinez J., Borzacconi L., Mallo M., Galisteo M. and Vinas M. (1995). Treatment of slaughterhouse wastewater. *Water Science and Technology*. **32**(12): 99-104.

McSwain B.S., Irvine R.L. and Wilderer P.A. (2004). The influence of-settling time on the formation of aerobic granules. *Water Science and Technology*. **50**(10): 195-202.

Meinhold J., Filipe C.D.M., Daigger G.T. and Isaacs S. (1999). Characterization of the denitrifying fraction of phosphate accumulating organisms in biological phosphate removal. *Water Science and Technology*. **39**(1): 31-42.

Metcalf & Eddy (1991). Wastewater engineering: treatment, disposal and reuse. Singapore, *McGraw-Hill, Inc.*

Metzner G. and Temper U. (1990). Operation and optimization of a full-scale fixed-bed reactor for anaerobic-digestion of animal rendering waste-water. *Water Science and Technology*. **22**(1-2): 373-384.

Meyer R.L., Saunders A.M. and Blackall L.L. (2006). Putative glycogen-accumulating organisms belonging to the Alphaproteobacteria identified through rRNA-based stable isotope probing. *Microbiology-Sgm*. **152**: 419-429.

Meyer R.L., Saunders A.M., Zeng R.J.X., Keller J. and Blackall L.L. (2003). Microscale structure and function of anaerobic-aerobic granules containing glycogen accumulating organisms. *FEMS Microbiology Ecology*. **45**(3): 253-261.

Meyer R.L., Zeng R.J.X., Giugliano V. and Blackall L.L. (2005). Challenges for simultaneous nitrification, denitrification, and phosphorus removal in microbial aggregates: mass transfer limitation and nitrous oxide production. *FEMS Microbiology Ecology*. **52**(3): 329-338.

Mino T., Liu W.T., Kurisu F. and Matsuo T. (1995). Modeling glycogen-storage and denitrification capability of microorganisms in enhanced biological phosphate removal processes. *Water Science and Technology*. **31**(2): 25-34.

Mittal G.S. (2004). Characterization of the effluent wastewater from abattoirs for land application. *Food Reviews International*. **20**(3): 229-256.

Mittal G.S. (2006). Treatment of wastewater from abattoirs before land application - a review. *Bioresource Technology*. **97**(9): 1119-1135.

Mobarry B.K., Wagner M., Urbain V., Rittmann B.E. and Stahl D.A. (1996). Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. *Applied and Environmental Microbiology*. **62**(6): 2156-2162.

Morgenroth E., Sherden T., van Loosdrecht M.C.M., Heijnen J.J. and Wilderer P.A. (1997). Aerobic granular sludge in a sequencing batch reactor. *Water Research*. **31**(12): 3191-3194.

Munch E.V., Lant P. and Keller J. (1996). Simultaneous nitrification and denitrification in bench-scale sequencing batch reactors. *Water Research*. **30**(2): 277-284.

Nunez L.A. and Martinez B. (1999). Anaerobic treatment of slaughterhouse wastewater in an Expanded Granular Sludge Bed (EGSB) reactor. *Water Science and Technology*. **40**(8): 99-106.

Obaja D., Mace S., Costa J., Sans C. and Mata-Alvarez J. (2003). Nitrification, denitrification and biological phosphorus removal in piggery wastewater using a sequencing batch reactor. *Bioresource Technology*. **87**(1): 103-111.

Obaja D., Mace S. and Mata-Alvarez J. (2005). Biological nutrient removal by a sequencing batch reactor (SBR) using an internal organic carbon source in digested piggery wastewater. *Bioresource Technology*. **96**(1): 7-14.

Oehmen A., Keller-Lehmann B., Zeng R.J., Yuan Z.G. and Keller E. (2005). Optimisation of poly-beta-hydroxyalkanoate analysis using gas chromatography for enhanced biological phosphorus removal systems. *Journal of Chromatography A*. **1070**(1-2): 131-136.

Oehmen A., Saunders A.M., Vives M.T., Yuan Z.G. and Keller H. (2006). Competition between polyphosphate and glycogen accumulating organisms in enhanced biological phosphorus removal systems with acetate and propionate as carbon sources. *Journal of Biotechnology*. **123**(1): 22-32.

Ostle A.G. and Holt J.G. (1982). Nile blue-A as a fluorescent stain for poly-beta-hydroxybutyrate. *Applied and Environmental Microbiology*. **44**: 238-241.

Peng D.C., Bernet N., Delgenes J.P. and Moletta R. (1999). Aerobic granular sludge - A case report. *Water Research*. **33**(3): 890-893.

Peng Y.Z., Chen Y., Peng C.Y., Liu M., Wang S.Y., Song X.Q. and Cui Y.W. (2004). Nitrite accumulation by aeration controlled in sequencing batch reactors treating domestic wastewater. *Water Science and Technology*. **50**(10): 35-43.

Pijuan M., Saunders A.M., Guisasola A., Baeza J.A., Casas C. and Blackall L.L. (2004). Enhanced biological phosphorus removal in a sequencing batch reactor using propionate as the sole carbon source. *Biotechnology and Bioengineering*. **85**(1): 56-67.

Pitman A.R., Venter S.L.V. and Nicholls H.A. (1983). Practical experience with biological phosphorus removal plants in Johannesburg. *Water Science and Technology*. **15**(3-4): 233-259.

Pochana K. and Keller J. (1999). Study of factors affecting simultaneous nitrification and denitrification (SND). *Water Science and Technology*. **39**(6): 61-68.

Puig S., Vives M.T., Corominas L., Balaguer M.D. and Colprim J. (2004). Wastewater nitrogen removal in SBRs, applying a step-feed strategy: from lab-scale to pilot-plant operation. *Water Science and Technology*. **50**(10): 89-96.

Qin L., Liu Y. and Tay J.-H. (2004). Effect of settling time on aerobic granulation in sequencing batch reactor. *Biochemical Engineering Journal*. **21**(1): 47-52.

Reddy K., Drysdale G.D. and Bux F. (2003). Evaluation of activated sludge treatment and settleability in remediation of edible oil effluent. *Water Sa*. **29**(3): 245-250.

Revsbech N.P. (1989). An oxygen microsensor with a guard cathode. *Limnology and Oceanography*. **34**(2): 474-478.

Russell J.M., Cooper R.N. and Lindsey S.B. (1993). Soil denitrification rates at waste-water irrigation sites receiving primary-treated and anaerobically treated meat-processing effluent. *Bioresource Technology*. **43**(1): 41-46.

Sachon G. (1984). Traitment des eaux residuaires des slaughterhouses de betail. In: Food Industries and the Environment. Proceedings of the International Symposium, Budapest, Hungary, 9-11 Sep 1982. Hollo J. (eds). Amsterdam, Holland, *Elsevier*: 293-305. In French.

Saunders A.M., Oehmen A., Blackall L.L., Yuan Z. and Keller J. (2003). The effect of GAOs (glycogen accumulating organisms) on anaerobic carbon requirements in full-scale Australian EBPR (enhanced biological phosphorus removal) plants. *Water Science and Technology*. **47**(11): 37-43.

Sayed S. and Dezeeuw W. (1988). The performance of a continuously operated flocculant sludge UASB reactor with slaughterhouse waste-water. *Biological Wastes*. **24**(3): 199-212.

Sayed S., Vancampen L. and Lettinga G. (1987). Anaerobic treatment of slaughterhouse waste using a antigranulocytes sludge UASB reactor. *Biological Wastes*. **21**(1): 11-28.

Stebor T.W., Berndt C.L., Marman S. and Gabriel R. (1990). Operating experience: anaerobic treatment at packerland packing. Proceedings 44th Purdue Industrial Waste Conference, Chelsea, MI, *Lewis*.

Subramaniam K., Greenfield P.F., Ho K.M., Johns M.R. and Keller J. (1994). Efficient biological nutrient removal in high-strength waste-water using combined anaerobic-sequencing batch reactor treatment. *Water Science and Technology*. **30**(6): 315-321.

Tay J.H., Liu Q.S. and Liu Y. (2001). Microscopic observation of aerobic granulation in sequential aerobic sludge blanket reactor. *Journal of Applied Microbiology*. **91**(1): 168-175.

Tay J.H., Liu Q.S. and Liu Y. (2002). Characteristics of aerobic granules grown on glucose and acetate in sequential aerobic sludge blanket reactors. *Environmental Technology*. **23**(8): 931-936.

Thayalakumaran N., Bhamidimarri R. and Bickers P.O. (2003a). Biological nutrient removal from meat processing wastewater using a sequencing batch reactor. *Water Science and Technology*. **47**(10): 101-108.

Thayalakumaran N., Bhamidimarri R. and Bickers P.O. (2003b). Characterisation of aerobic bio treatment of meat plant effluent. *Water Science and Technology*. **48**(8): 53-60.

Tilche A., Bacilieri E., Bortone G., Malaspina F., Piccinini S. and Stante L. (1999). Biological phosphorus and nitrogen removal in a full scale sequencing batch reactor treating piggery wastewater. *Water Science and Technology*. **40**(1): 199-206.

Travers S.M. and Lovett D.A. (1984). Activated-sludge treatment of abattoir wastewater .2. Influence of dissolved-oxygen concentration. *Water Research*. **18**(4): 435-439.

Turk O. and Mavinic D. (1986). Preliminary assessment of a shortcut in nitrogen removal from wastewater. *Can Journal of Civil Engineering*. **13**: 600-605.

vanLoosdrecht M.C.M., Hooijmans C.M., Brdjanovic D. and Heijnen J.J. (1997). Biological phosphate removal processes. *Applied Microbiology and Biotechnology*. **48**(3): 289-296.

Wagner M., Rath G., Koops H.-P., Flood J. and Amann R. (1996). *In situ* analysis of nitrifying bacteria in sewage treatment plants. *Water Science and Technology*. **34**(1-2): 237-244.

Wareham D.G., Mavinic D.S. and Hall K.J. (1994). Sludge-digestion using ORP-regulated aerobic-nnoxic cycles. *Water Research*. **28**(2): 373-384.

Wentzel M.C., Loewenthal R.E., Ekama G.A. and Marais G.V. (1988). Enhanced polyphosphate organism cultures in activated-sludge systems .1. Enhanced culture development. *Water Sa*. **14**(2): 81-92.

Wilderer P., Irvine R.L. and Goronszy M.C. (2001). Sequencing batch reactor technology. London, *IWA*.

Yang S.F., Tay J.H. and Liu Y. (2003). A novel granular sludge sequencing batch reactor for removal of organic and nitrogen from wastewater. *Journal of Biotechnology*. **106**(1): 77-86.

Yuan Z., Keller J. and Lant P. (2003). Optimization and control of biological nitrogen removal in activated sludge processes: a review of recent developments. In: Biotechnology for the Environment. Agathos S.N. and Reineke W. (eds), *Kluwer Academic Publishers*. **3C**.

Zeng R.J., Lemaire R., Yuan Z. and Keller J. (2003). Simultaneous nitrification, denitrification, and phosphorus removal in a lab-scale sequencing batch reactor. *Biotechnology and Bioengineering*. **84**(2): 170-178.

Appendix A

A Sequencing Batch Reactor System for High-Level Biological Nitrogen and Phosphorus Removal from Abattoir Wastewater

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ABSTRACT

A sequencing batch reactor (SBR) system is demonstrated to biologically remove nitrogen, phosphorus and COD to very low levels from abattoir wastewater. Each 6h cycle contained three anoxic/anaerobic and aerobic sub-cycles with wastewater fed at the beginning of each anoxic/anaerobic period. The step-feed strategy was applied to avoid high-level build-up of nitrate or nitrite during nitrification, and therefore to facilitate the creation of anaerobic conditions required for biological phosphorus removal. A high degree removal of total phosphorus (>98%), total nitrogen (>97%) and total COD (>95%) was consistently and reliably achieved after a three-month start-up period. The concentrations of total phosphate and inorganic nitrogen in the effluent were consistently lower than 0.2 $mgP.l^{-1}$ and 8 $mgN.l^{-1}$ respectively. Fluorescence *in-situ* hybridization (FISH) revealed that the sludge was enriched in *Accumulibacter* spp. (20-40%), a known polyphosphate accumulating organism (PAO), whereas the known glycogen accumulating organisms (GAOs) were almost absent. The SBR received two streams of abattoir wastewater, namely the effluent from a full-scale anaerobic pond (75%) and the effluent from a lab-scale high-rate pre-fermenter (25%), both receiving raw abattoir wastewater as feed. The pond effluent contained approximately 250 $mgN.l^{-1}$ total nitrogen and 40 $mgP.l^{-1}$ of total phosphorus, but relatively low levels of soluble COD (around 500 $mg.l^{-1}$). The high-rate lab-scale pre-fermentor, operated at a temperature of 37˚C and a sludge retention time of 1 day, proved to be a cheap and effective method for providing supplementary VFAs allowing for high-degree of biological nutrient removal from abattoir wastewater.

Keywords: Abattoir wastewater, biological nutrient removal, pre-fermentation, SBR, step-feed, PAO.

INTRODUCTION

The meat processing industry requires large quantities of water, much of which is discharged as wastewater containing high levels of COD and nutrients such as nitrogen (N) and phosphorus (P). Over the past two decades, biological COD and N removal from abattoir wastewater has received much greater attention than has the biological P removal. Reliable biological COD and nitrogen removal systems have been successfully developed and applied for abattoir wastewater treatment using continuous activated sludge systems (Beccari et al., 1984; Frose and Kayser, 1985; Willers et al., 1993). However, P removal continues to be achieved primarily through chemical precipitation, despite biological P removal being a much cheaper and more environmentally sustainable option.

The main challenges for biological phosphorus removal from abattoir wastewater are:

- The wastewater contains a high level of ammonia and organic nitrogen. The complete nitrification of these nitrogen components produces a high level of nitrate, which has proved to be an obstacle to the development of a stable and reliable Bio-P removal process (Pitman et al., 1983; Comeau et al., 1986; Furumai et al., 1999). Phosphorus removal requires alternating anaerobic and aerobic/anoxic conditions. The high level of nitrate (due to the high influent nitrogen concentrations) makes the creation of true anaerobic conditions in the system difficult;
- Abattoir wastewater contains substantial amounts of fat, oil and grease (FOG), which would deteriorate the sludge settleability when directly fed to activate sludge systems. Primary treatment is typically required before abattoir wastewater is treated in biological nutrient removal systems. In Australia, the raw abattoir wastewater is typically pre-treated in anaerobic ponds with a hydraulic retention time ranging between 7 – 14 days. While reducing the FOG content, this anaerobic treatment process also removes a large fraction of the COD from the wastewater, resulting in COD limitations (particularly Volatile Fatty Acids – VFAs) for N and P removal (Keller et al., 1997).

In this paper, we demonstrate the use of a sequencing batch reactor (SBR) system for biological nitrogen and phosphorus removal from abattoir wastewater. In recent years, the use of SBRs for the biological treatment of wastewater has been widely extended from lab-scale studies to real WWTPs (Tilche et al., 1999; Artan et al., 2001; Keller et al., 2001; Puig et al., 2004). SBRs offer a great deal of operational flexibility as it allows for easy adjustment of aerobic, anoxic and anaerobic periods through temporal control of aeration and filling (Wilderer et al., 2001). To address the first challenge described above, a step-feed scheme, characterised by several aerobic and anoxic phases in a SBR cycle with the wastewater fed to the reactor during the anoxic phases is employed (Anderottola et al., 2000; Lin and Jing, 2001; Puig et al., 2004). This operational strategy allows timely removal of nitrate so that, when an adequate amount of COD is available, nitrate build-up is avoided (Puig et al., 2004).

To address the second challenge, the proposed system is equipped with a high-rate pre-fermentor, which is used to provide additional VFAs when the anaerobic pond effluent does not contain a sufficient amount for the biological phosphorus and nitrogen removal.

Details of the design, operation and performance of the proposed system are presented. Further potential optimisations are also highlighted.

MATERIAL AND METHODS

Reactor set-up and operation

A lab-scale SBR with a working volume of 7 l was used in this study. The SBR was seeded with non-EBPR (enhanced biological phosphorus removal) sludge from a full-scale SBR treating abattoir wastewater in Queensland, Australia. 1 l of EBPR sludge (MLSS around 4 $g.l^{-1}$) enriched in a lab reactor (Lemaire et al., 2006) was added on Day 60 to initiate the EBPR process in the reactor as there seemed to be no EBPR organisms present in the initial seed sludge used. The SBR was operated with a cycle time of 6 h in a temperature-controlled room (18-22˚C). In each cycle, 1 l of abattoir wastewater (more details given below) was pumped into the reactor over the three filling periods with a volume distribution of 0.5 l, 0.3 l and 0.2 l respectively. Each filling period was followed by non-aerated (either anoxic or anaerobic depending on when the oxidised nitrogen was completely consumed) and aerated periods (Table 1). During aerated periods, air was provided intermittently using an on/off control system to keep the dissolved oxygen (DO) level between 1.5 and 2 $mg.l^{-1}$. After the settling period, 1 l supernatant was removed from the reactor resulting in a HRT of 42 h. 115 ml of mixed liquor was wasted every cycle resulting in a SRT of 15 days. The pH in the system was recorded, ranging between 7.1-7.9, but not controlled. The ORP signal was also recorded to give indications of the nitrate levels in the reactor during the anoxic periods. The reactor was mixed with an overhead mixer except during the settling, decanting and first filling periods.

Table 1. Operating conditions of lab-scale SBR (6h cycle)

SBR sequences	**HRT = 42 h**	
	duration (min)	**DO** ($mgO_2.l^{-1}$)
Fill no-mix 1	5	~0
No-aerated mix 1 (anoxic or anaerobic*)	30	~0
Aerated mix 1 (no aeration in the last 5 min)	55	1.5-2
Fill mix 2	3	~0
No-aerated mix 2 (anoxic or anaerobic*)	70	~0
Aerated mix 2 (no aeration in the last 5 min)	35	1.5-2
Fill mix 3	2	~0
No-aerated mix 3 (anoxic or anaerobic*)	60	~0
Aerated mix 3 (sludge wasted at the end)	20	1.5-2
Settle	70	~0
Decant	10	~0

* when nitrate and nitrite depleted

Wastewater

The wastewater used in this study was from a local abattoir in Queensland, Australia. At this site, the raw effluent passes through four parallel anaerobic ponds before being treated in a SBR for biological nitrogen and COD removal. The anaerobic ponds serve to reduce FOG and COD, and also to produce easily biodegradable COD, particularly VFAs, to facilitate the down-stream biological nitrogen removal. The four anaerobic ponds produced VFAs at different concentrations due to different organic loading rates. Pond A, which was the only easily accessible pond for wastewater collection and hence had to be used in the study, was under-loaded leading to much lower COD and VFAs concentrations in comparison to other

ponds (see Table 2). Therefore, extra VFAs were added to pond A effluent to simulate the higher VFA levels present in other ponds, as will be detailed in Table 3.

Raw wastewater and anaerobic pond effluent from the abattoir were collected on a weekly basis and stored at 4˚C. The raw wastewater was subjected to one-day pre-fermentation before being pumped into the SBR. The pre-fermentation was performed in a 50 l tank continuously mixed with a submersible pump. No inoculum was introduced in the pre-fermentor, and hence the microbial population present in the raw abattoir wastewater was used to carry out the fermentation. The temperature inside the tank was kept at 37˚C by a heating probe and would not require any special heating system in a full-scale installation due to the temperature of the abattoir raw wastewater (typically around 40˚C). The aim of this pre-fermentation step was to increase the level of easy biodegradable COD, in particular VFAs, which is critical for bio-P removal. The characteristics of the pre-fermented raw wastewater and the anaerobic pond effluent are compared in Table 2.

Table 2. Characteristics of the different types of wastewater used in this study. The intervals represent the mid-95% range.

Parameter	Pre-fermented raw wastewater	Anaerobic pond A effluent [b]	Anaerobic pond B effluent
TCOD ($mg.l^{-1}$)	7460-9300	430-720	740-950
SCOD ($mg.l^{-1}$)	2360-2840	205-245	440-531
VFA[a] ($mgCOD.l^{-1}$)	703-869	24-32	272-358
TN ($mg.l^{-1}$)	271-317	218-262	240-262
NH_4-N ($mg.l^{-1}$)	139-160	207-224	220-226
TP ($mg.l^{-1}$)	44-53	33-37	37-40
PO_4-P ($mg.l^{-1}$)	38-43	32-34	33-36

[a] Acetate and propionate only

[b] Pond effluent used in this study; additional acetate and propionate was added (see Table 3) to simulate Pond B effluent, which was non-accessible for wastewater collection on site.

Table 3. Characteristics of the SBR influent during its nine-month operation.

Influent Parameters	Day 0-30	Day 30-80	After day 80
Ratio VFA:TP	3.7	12.2	15.1
Ratio TCOD:TN	5.5	8.7	12
% pre-fermented raw wastewater in influent	15%	15%	25%
VFAs added to Pond A to simulate other ponds	No	Yes[a]	Yes[a]

[a] 250 $mgCOD.l^{-1}$ acetate, and 100 $mgCOD.l^{-1}$ propionate

The abattoir closed down for a month over Christmas due to annual maintenance. During this period, the SBR cycle operation was modified in order to preserve the reactor biomass as no wastewater was available. The sludge was aerated and mixed for 15 min in each 6h cycle and was allowed to settle for the rest of the cycle. The performance during this starvation and recovery period is the focus of another paper (Yilmaz et al., 2007)

Analyses

The ammonia ($NH_3 + NH_4^+$), nitrate (NO_3^-), nitrite (NO_2^-) and total phosphate (PO_4^{3-}-P) were analysed using a Lachat QuikChem8000 Flow Injection Analyser (Lachat Instrument, Milwaukee). Total and soluble chemical oxygen demand (TCOD and SCOD, respectively), total Kjeldahl nitrogen (TKN), total phosphorus (TP), mixed liquor suspended solid (MLSS) and volatile MLSS (MLVSS) were analysed according to the standard methods (APHA, 1995). VFAs were measured by Perkin-Elmer gas chromatography with column DB-FFAP 15m x 0.53mm x 1.0µm (length x ID x film) at 140˚C, while the injector and FID detector were operated at 220˚C and 250˚C, respectively. High purity helium was used as carrier gas at a flow rate of 17 $ml.min^{-1}$. 0.9 ml of the filtered sample was transferred into a GC vial to which 0.1 ml of formic acid was added.

Fluorescence *in situ* hybridisation (FISH) was performed as specified in Amann (1995). Oligonucleotide probes used in this study were the combination of EUB338 i-iii (EUBmix) for the detection of all bacteria (Daims et al., 1999), the combination of PAO462, PAO651 and PAO846 (PAOmix) for *Accumulibacter* spp. (Crocetti et al., 2000), the probe combination (GAOmix) of GAOQ989 (Crocetti et al., 2002) and GB_G2 (Kong et al., 2002) for *Competibacter* spp., the probe combination (DF1mix) of TFO_DF218 and TFO_DF618 for Cluster 1 *Defluviicoccus vanus*-related spp. (Wong et al., 2004) and the probe combination (DF2mix) of DF988, DF1020 and helper probes H966 and H1038 for Cluster 2 *Defluviicoccus vanus*-related spp. (Meyer et al., 2006). FISH quantification was performed as described in Crocetti et al. (Crocetti et al., 2002).

RESULTS

Figure 1 presents the influent and effluent COD, N and P concentrations, along with the MLSS concentration in the reactor and its volatile fraction, during the nine months operation of the SBR. Also presented in Figure 1 is the fraction of *Accumulibacter* spp. (i.e. PAOs) in the system. GAOs, namely *Competibacter* spp. and the putative *Defluviicoccus vanus*-related spp. (Cluster 1 and 2), were negligible in this reactor (<1% of the total microbial population at all time). According to the effluent and MLSS data (Figure 1c and 1d), the SBR reached a steady state around day 170. The study can be divided into two periods: the start-up period from day 0 to 170 and the steady state period from day 170 to 275.

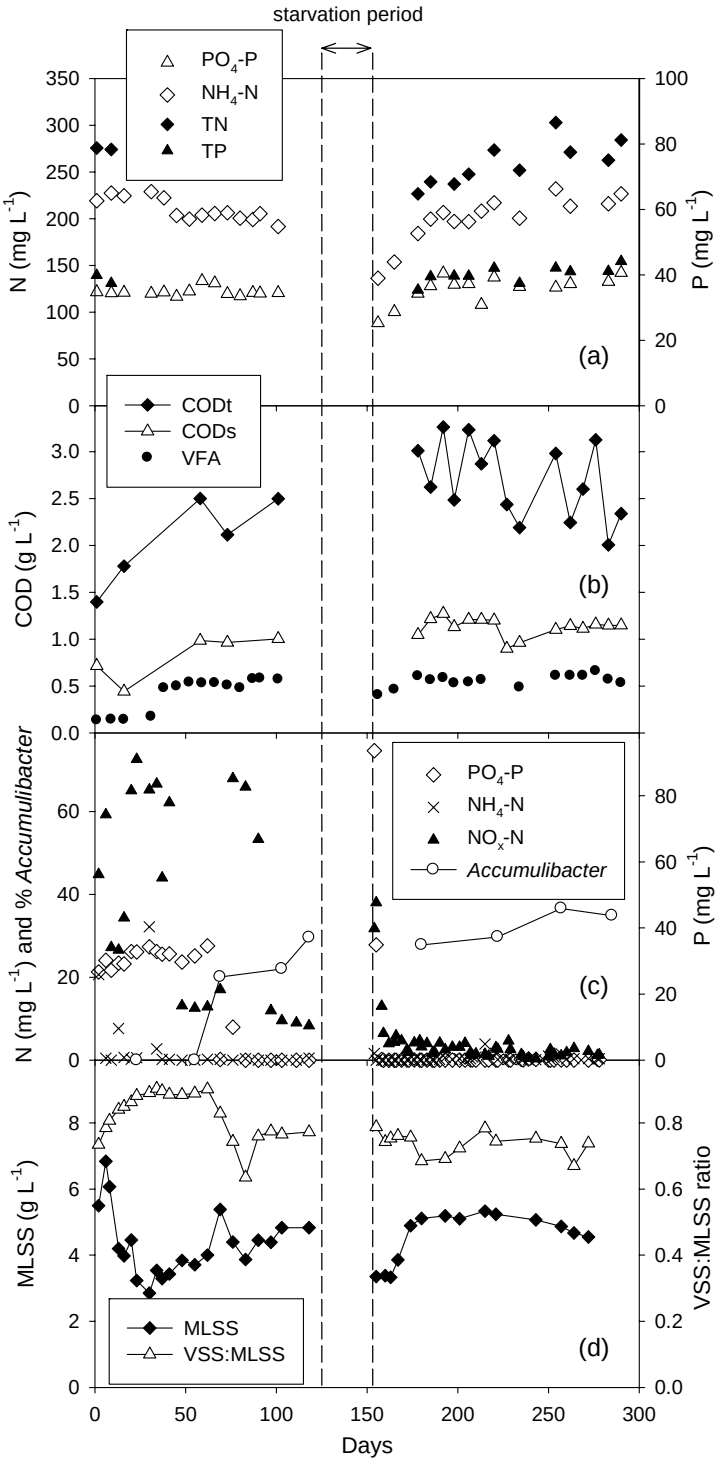

Figure 1. Characteristics of the influent **(a)** and **(b)**, effluent nutrient levels and the *Accumulibacter* spp. population **(c)**, and MLSS and the VSS:MLSS ratio in the reactor **(d)**. No wastewater was fed during the 33 days of starvation period.

Start up period (day 0 to 170)

Complete nitrification was achieved in the SBR after less than one week of operation as shown by the absence of NH_4^+ in the effluent (Figure 1c). However, denitrification was incomplete and NO_x^- (nitrate + nitrite) accumulated in the reactor reaching 60 mgN.l^{-1} in the effluent during the first 30 days of operation (Figure 1c). Clearly, In order to improve the denitrification, more COD was needed during anoxic periods. Therefore, extra VFAs (i.e. acetate and propionate) were added to pond A effluent on day 30 in order to simulate the concentration in the other ponds (typically 250 mgCOD.l^{-1} acetate and 100 mgCOD.l^{-1} propionate). These additional VFAs improved the denitrification and the level of NO_x^- in the effluent dropped to 15 mgN.l^{-1} (Figure 1c). The similar levels of PO_4^{3-} measured in the influent (Figure 1a) and effluent (Figure 1c) clearly indicate that phosphorus removal was negligible during the first 60 days. P removal was likely limited by the slow development of PAOs, which were possibly inhibited by the level of nitrate present during most of the time over a cycle as speculated by Keller et al. (1997). The fact that non-EBPR sludge was used to seed the reactor could have also contributed to the slow development of PAOs.

After the introduction of 1 l lab-scale EBPR sludge enriched in *Accumulibacter* spp. (details of the culture can be found in Lemaire et al., (2006)) on day 60, P removal improved dramatically, and consistent high-level of P removal was achieved and maintained thereafter. The process data clearly suggests that the enriched *Accumulibacter* spp. culture managed to survive and develop in a very different environmental setting. This is confirmed by the FISH quantitation results (Figure 1c) and the decrease of the organic fraction in the biomass due to intracellular poly-P storage by *Accumulibacter* spp. (Figure 2d).

However, Figure 1c also shows that while P removal was improving, NO_x^- started to accumulate again in the system. It was believed that a shortage of easily biodegradable COD in the reactor trigger this NO_x^- accumulation as PAOs and denitrifiers were competing for the same carbon sources. In order to further increase the amount of VFA available for P and N removal, the amount of pre-fermented raw wastewater in the influent was increased from 15% to 25% on day 80 resulting in a higher VFA:TP ratio and TCOD:TN ratio in the influent (Table 3). Denitrification improved immediately and from day 100 onwards, less than 10 mgN.l^{-1} remained in the effluent. Good COD, nitrogen and phosphorus removal was achieved after day 100. There was one interruption to the reactor operation between day 125 and 160, when the abattoir closed down and no wastewater could be supplied to the SBR (Figure 1). A detailed report of the reactor performance in this period can be found in Yilmaz et al. (2007). The reactor biomass concentration decreased by 30% during this long starvation period.

Steady state period (from day 170 to 275)

Following the long starvation period, the reactor performance quickly recovered (within four days) as clearly indicated by the low nutrient level in the effluent shortly after the normal SBR operation was resumed (Figure 1c). The biomass concentration returned to its previous level after 2 weeks and remained relatively constant around 5 g.l^{-1} with an organic fraction fluctuating between 0.7 and 0.75 (Figure 1d). Table 4 details the SBR effluent quality after the starvation period, between day 170 and 275. For comparison, the COD and nutrient levels in the influent are also presented. The SBR process consistently achieved 95, 97 and 98% of TCOD, TN and TP removal, respectively. The remaining COD in the effluent could be regarded as non-biodegradable and represented about 5% of the total COD initially present in

the influent. It was observed that the sludge volume index (SVI) was relatively high throughout the study period, between 180 and 250 ml.gMLSS^{-1}. This could have partially been caused by the remaining high fat/oil/grease content of the pre-fermented raw wastewater as suggested by Johns (1995). However, the suspended solids concentration in the effluent was lower than 25 mg.l^{-1} at all times (data not shown).

Table 4. Influent and effluent characteristics between day 170 and 275. (N represents the number of samples analysed between day 170 and 275)

Parameter (mg.l^{-1})	Influent (N=13)		Effluent (N=32)		Removal of TCOD, TN and TP
	mid-95% range	mean	mid-95% range	mean	
TCOD	2600-3120	2870	129-151	140	95 %
SCOD	1150-1320	1240	114-127	121	
TKN	236-277	256	5.3-7.7	6.5	
N-NH$_4$	196-215	206	0.2-0.8	0.5	97 %
N-NO$_x$	not detected		1.9-2.8	2.3	
TP	38-41	40	0.7-1.4	1.0	98 %
P-PO$_4$	35-38	37	0.04-0.09	0.06	

Figure 2 shows the nitrogen and phosphorus transformations in a typical SBR cycle study during the steady state period. At the end of each aerobic period, NH_4^+ was fully oxidised, and the low level of NO_x^- accumulated was then removed in the following anoxic period. It can be seen that a very low level of NO_x^- was carried over to the next cycle, and was denitrified very quickly at the beginning of the first anaerobic period. PO_4^{3-} level increased during each anaerobic period due to both anaerobic P release by PAOs and wastewater feeding (containing approximately 35 mgP.l^{-1}), but most P release occurred in the first non-aerated period. PO_4^{3-} was then fully taken up during the subsequent aerobic periods.

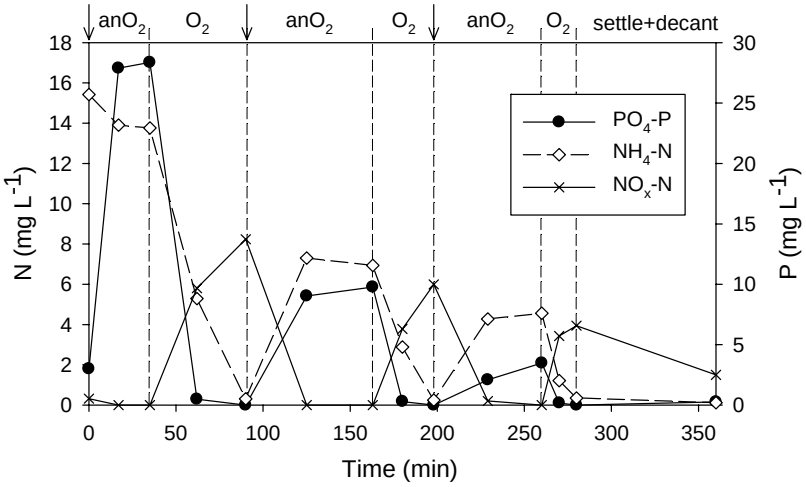

Figure 2. Nitrogen and phosphorus profiles during a SBR cycle study performed on day 243. The vertical arrows indicate the 3 feeding periods.

Performance of the pre-fermentor

The impact of the one-day pre-fermentation performed on raw wastewater is depicted in Figure 3. The overall VFA concentration more than doubled as a direct result of this pre-fermentation. Acetate and propionate were the most abundant VFAs in the raw abattoir wastewater before and after pre-fermentation with propionate having a slightly higher production rate than acetate. Also shown in Figure 3 is the impact of pre-fermentation on the NH_4^+ and PO_4^{3-} concentrations. While PO_4^{3-} concentration stayed constant, NH_4^+ concentration doubled due to partial mineralisation of the organic nitrogen which represents around 75% of the raw wastewater total nitrogen. The one week storage of the pre-fermented raw wastewater in the cold room at a temperature of 4˚C affected VFAs levels more than nutrient levels with a 20 % reduction of acetate and propionate concentration.

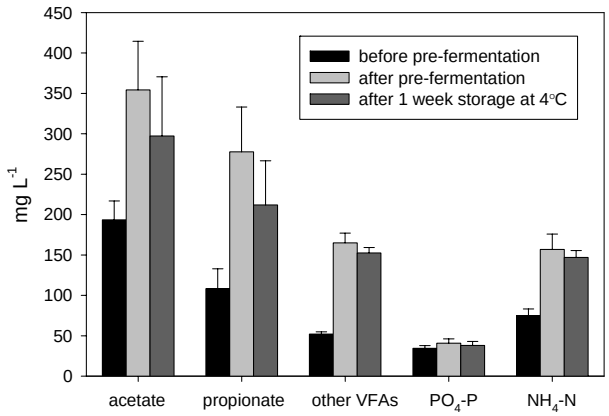

Figure 3. Concentration of the main VFAs, PO_4^{3-} and NH_4^+ in the raw wastewater before and after pre-fermentation, and after one week storage at 4°C. Other VFAs include iso-butyric, butyric and iso-valeric acids. (Error bars=SD, n=11)

DISCUSSION

Multi-feed strategy to promote biological P removal

Biological phosphorus removal from wastewaters containing a high level of nitrogen, such as abattoir wastewater, is challenging. Large accumulation of nitrate or nitrite must be avoided in order to secure anaerobic conditions required by PAOs. Several studies using the SBR technology to simultaneously remove COD, N and P from piggery wastewater have been reported (Tilche et al., 1999; Obaja et al., 2003; Obaja et al., 2005). However, the characteristics of piggery wastewater differ greatly from those of abattoir wastewater. The large amount of inorganic salts, minerals and metal ions present in the piggery wastewater promote chemical P removal by precipitation, as evidenced by the scarce P release observed during the anaerobic stage of the process (Bortone et al., 1994). Subramaniam et al. (1994) and Keller et al. (1997) attempted to achieve simultaneous COD, N and P removal biologically from abattoir wastewater using SBR systems. However, P removal was quite unstable due to intermittent recycling of high levels of NO_x^- to the anaerobic period.

The use of a multi-feed strategy in this study aimed to limit the level of NO_x^- recycled to the anaerobic period. Figure 2 shows that the strategy was very successful. The NO_x^- level was less than 8 $mgN.l^{-1}$ throughout the cycle, despite of the high level of NH_4^+ and organic nitrogen in the wastewater (over 250 $mgN.l^{-1}$, see Table 4). Based on the amount of

wastewater fed over the three feeding periods in the SBR cycle (i.e. 0.5 l, 0.3 l and 0.2 l, respectively) and the PO_4^{3-} concentration in the influent (i.e. 35 mgP.l^{-1}), the amounts of PO_4^{3-} introduced in the SBR bulk liquid during each feeding steps were estimated to be 2.7, 1.5 and 1.0 mgP.l^{-1}, respectively. The true anaerobic P release by PAOs following those three feeding periods was thus estimated to be 25.3, 4.5 and 1.0 mgP.l^{-1}, respectively. The very large discrepancy between the first anaerobic P release and the second and third is mostly due to the very low level of NO_x^- in the bulk liquid prior to the first feeding (< 0.5 mgN.l^{-1}) compared to the second (8 mgN.l^{-1}) and third (6 mgN.l^{-1}) feeding (Figure 2). This indicates that the first non-aerated period was crucially important for P removal as PAOs could freely utilise all the VFA available without having to compete with heterotrophic denitrifiers. Without the multi-feed strategy employed in this study, high level of NO_x^- is likely to have accumulated in the SBR at the end of the cycle and to have been recycled into the first anaerobic period preventing the stable high level of P removal reported.

Pre-fermentation of raw wastewater

The performance of a biological nutrient removal system depends greatly on the availability of easily biodegradable carbon sources in the wastewater, particularly VFAs. Considering the fact that it is difficult to control the VFAs content in large anaerobic ponds, a more controllable VFA source is necessary for reliable biological nutrient removal from abattoir wastewater. In this study, a high-rate pre-fermentation step was demonstrated to be a cheap and effective option. Table 3 shows that the VFA:TP ratio increased from 12.2 to 15.1 when the pre-fermented wastewater fraction in the SBR influent increased from 15% to 25% on day 80. This caused an immediate reduction in the nitrate level, with a drastic improvement to the reliability of P removal (Figure 1b and 1c). The results show that it is both necessary and practically feasible to include a high-rate pre-fermentor to generate VFAs that may be supplemented to the nutrient removal SBR when an inadequate amount of VFAs is present in the pond effluent.

However, it should be highlighted that the use of raw wastewater should be minimised. There is evidence suggesting that a high fraction of raw feed deteriorates the sludge settleability (data not shown) likely due to its higher FOG content compared to pond effluent. An over supply of carbon sources through this stream would also increase aeration costs and sludge production in the SBR system. Controlled addition of this stream using an on-line control system would be highly beneficial. However, the control of VFAs supplement to biological phosphorus removal systems in accordance to the actual demand for VFAs (varying with time) is still unresolved (Olsson et al., 2005).

An alternative solution that is being investigated is to reduce the demand for carbon sources by achieving nitrogen removal via nitrite instead of nitrate. This strategy, if successful, would reduce the carbon demand for denitrification by 40% (Turk and Mavinic, 1986). This would therefore reduce the amount of additional carbon supply, which in turn will also reduce the overall oxygen requirement. Such an improvement would have significant benefits for the operation of large-scale wastewater treatment facilities. Peng et al. (2004) demonstrated that stable nitrite accumulation during the nitrification process could be obtained through an aeration control system based on pH and DO signals. On-line control systems based on simple pH and DO signals are being developed to achieve this nitrite pathway in our lab-scale SBR.

A further opportunity to reduce the demand for carbon sources is to enhance the denitrification by PAOs. It has been found that *Accumulibacter* spp. are capable of taking up phosphorus under anoxic conditions (Kuba et al., 1993; Meinhold et al., 1999; Zeng et al., 2003a). This is particularly attractive as the same carbon could be used for both denitrification

and P removal. However, the exact conditions necessary to promote this type of denitrification are still unclear and further investigations are needed (Zeng et al., 2003a).

The low abundance of GAOs in the sludge

Competibacter spp. have been widely reported to be abundant in both lab-scale EBPR reactors (Mino et al., 1995; Crocetti et al., 2002; Kong et al., 2002; Zeng et al., 2003b) and full-scale EBPR plants (Crocetti et al., 2002; Saunders et al., 2003; Kong et al., 2006). Surprisingly, in this study, *Competibacter* spp. were scarcely present in the reactor representing always less than 1% of the total microbial population. *Defluviicoccus vanus*-related *Alphaproteobacteria* organisms, a new putative GAO recently reported in literature (Wong et al., 2004; Meyer et al., 2006), was also found to be in very low abundance in the reactor.

Several factors have been suggested in the literature that may influence on the competition between PAOs and GAOs. Filipe (2001) and Oehmen et al, (2005) found that pH has a significant impact on the PAO and GAO competition with *Accumulibacter* spp. possessing advantages over *Competibacter* spp. for anaerobic carbon uptake at relatively high pH (>8). The pH in the study fluctuated between 7.1 and 7.9 during a cycle (uncontrolled), which should have unlikely provided any selective advantages to PAOs over GAOs. Saito (2004) reported that the presence of nitrite in the anaerobic or aerobic period inhibited the PAO activity and could therefore enhance the presence of GAOs in the system. The presence of nitrite in the reactor during all three aerobic periods and during the second and third anaerobic periods apparently did not promote the growth of GAOs, as suggested by Saito (2004). Some studies also showed that better EBPR performance was achieved at relatively low temperature (5-15˚C) due to a shift in the microbial community from GAOs to PAOs (Whang and Park, 2002; Erdal et al., 2003). The temperature used in this study, controlled between 18-22˚C, is very similar to many reactor studies where GAOs appeared to be a problem, and is therefore not believed to be a significant contributor to the low abundance of GAOs. A more likely reason for the limited growth of GAOs in this reactor could be the large fraction of propionate present in the influent (propionate to acetate COD ratio was 0.8). (Pijuan et al., 2004; Oehmen et al., 2006) revealed that propionate as a carbon source may provide selective advantage to PAOs. The pre-fermentor used in this study largely contributed to the increase of the propionate fraction. If this hypothesis is true, the operation of the pre-fermentor should be optimised to not only maximise the total amount of VFAs produced but also to control the VFAs composition and particularly the acetate to propionate ratio.

CONCLUSION

A sequencing batch reactor system was demonstrated to effectively remove nitrogen, phosphorus and COD from abattoir wastewater. This provides a more cost-effective and environmentally friendly alternative to chemical phosphorus removal, which is a common practice at present. The following conclusions are drawn:

- It is possible to achieve a high degree (>98%) of biological phosphorus removal from abattoir wastewater in the presence of high levels of nitrogen (200 – 300 $mgN.l^{-1}$).
- The multi-step feeding strategy prevents high-level accumulation of nitrate or nitrite, and hence facilitates the creation of anaerobic conditions. The strategy is strongly recommended for practical use in the biological treatment of abattoir wastewater.
- It is important to incorporate a high-rate pre-fermentor as an integrated component of the nutrient removal system. This stream, which contains a high-level of VFAs, is effective in providing supplementary carbon sources for both phosphorus and nitrogen removal.

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REFERENCES

Amann R.I. (1995). In situ identification of microorganisms by whole cell hybridization with rRNA-targeted nucleic acid probes. In: Molecular Microbial Ecology Manual. Akkermans A.D.L., van Elsas J.D. and de Bruijn F.J. (eds). Dordrecht, Holland, *Kluwer Academic Publications*. **3.3.6:** 1-15.

Anderottola G., Canziani R., Foladori P., Ragazzi M. and Tatano F. (2000). Laboratory scale experimentation for RBCOD production from OFMSW for BNR systems: Results and kinetics. *Environmental Technology*. **21**(12): 1413-1419.

APHA (1995). Standard methods for the examination of water and wastewater. Washington, DC, *American Public Health Association*.

Artan N., Wilderer P., Orhon D., Morgenroth E. and Ozgur N. (2001). The mechanism and design of sequencing batch reactor systems for nutrient removal - the state of the art. *Water Science and Technology*. **43**(3): 53-60.

Beccari M., Carrieri C., Misiti A. and Ramadori R. (1984). Design of single sludge systems for the treatment of wastewaters with high ammonia content. *La Tribune du Cebedeau*. **37**(491): 387-394.

Bortone G., Malaspina F., Stante L. and Tilche A. (1994). Biological nitrogen and phosphorus removal in an anaerobic/anoxic sequencing batch reactor with separated biofilm nitrification. *Water Science and Technology*. **30**(6): 303-313.

Comeau Y., Hall K., Hancock R. and Oldham W. (1986). Biochemical model for enhanced biological phosphorus removal. *Water Research*. **20**: 1511-1521.

Crocetti G.R., Banfield J.F., Keller J., Bond P.L. and Blackall L.L. (2002). Glycogen accumulating organisms in laboratory-scale and full-scale activated sludge processes. *Microbiology*. **148**(11): 3353-3364.

Crocetti G.R., Hugenholtz P., Bond P.L., Schuler A., Keller J., Jenkins D. and Blackall L.L. (2000). Identification of polyphosphate-accumulating organisms and design of 16S rRNA-directed probes for their detection and quantitation. *Applied and Environmental Microbiology*. **66**(3): 1175-1182.

Daims H., Bruhl A., Amann R., Schleifer K.H. and Wagner M. (1999). The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: Development and evaluation of a more comprehensive probe set. *Systematic and Applied Microbiology*. **22**(3): 434-444.

Erdal U.G., Erdal Z.K. and Randall C.W. (2003). The competition between PAOs (phosphorus accumulating organisms) and GAOs (glycogen accumulating organisms) in EBPR (enhanced biological phosphorus removal) systems at different temperatures and the effects on system performance. *Water Science and Technology*. **47**(11): 1-8.

Filipe C.D.M., Daigger G.T. and Grady C.P.L. (2001). pH as a key factor in the competition between glycogen-accumulating organisms and phosphorus-accumulating organisms. *Water Environment Research*. **73**(2): 223-232.

Frose G. and Kayser R. (1985). Effective treatment of wastewater from rendering plants. Proceedings 40th Annual Purdue Industrial Waste Conference, Indiana, *Butterworths*.

Furumai H., Kazmi A.A., Fujita M., Furuya Y. and Sasaki K. (1999). Modeling long term nutrient removal in a sequencing batch reactor. *Water Research*. **33**(11): 2708-2714.

Johns M.R. (1995). Developments in wastewater treatment in the meat processing industry: A review. *Bioresource Technology*. **54**(3): 203-216.

Keller J., Subramaniam K., Gosswein J. and Greenfield P.F. (1997). Nutrient removal from industrial wastewater using single tank sequencing batch reactors. *Water Science and Technology*. **35**(6): 137-144.

Keller J., Watts S., Battye-Smith W. and Chong R. (2001). Full-scale demonstration of biological nutrient removal in a single tank SBR process. *Water Science and Technology*. **43**(3): 355-362.

Kong Y., Ong S.L., Ng W.J. and Liu W.-T. (2002). Diversity and distribution of a deeply branched novel proteobacterial group found in anaerobic-aerobic activated sludge processes. *Environmental Microbiology*. **4**(11): 753-757.

Kong Y.H., Xia Y., Nielsen J.L. and Nielsen P.H. (2006). Ecophysiology of a group of uncultured Gammaproteobacterial glycogen-accumulating organisms in full-scale enhanced biological phosphorus removal wastewater treatment plants. *Environmental Microbiology*. **8**(3): 479-489.

Kuba T., Smolders G.J.F., van Loosdrecht M.C.M. and and Heijnen J.J. (1993). Biological phosphorus removal from wastewater by anaerobic and anoxic sequencing batch reactor. *Water Science and Technology*. **27**(4-5): 241-252.

Lemaire R., Meyer R., Taske A., Crocetti G.R., Keller J. and Yuan Z.G. (2006). Identifying causes for N_2O accumulation in a lab-scale sequencing batch reactor performing simultaneous nitrification, denitrification and phosphorus removal. *Journal of Biotechnology*. **122**(1): 62-72.

Lin Y.F. and Jing S.R. (2001). Characterization of denitrification and nitrification in a step-feed alternating anoxic-oxic sequencing batch reactor. *Water Environment Research*. **73**(5): 526-533.

Meinhold J., Filipe C.D.M., Daigger G.T. and Isaacs S. (1999). Characterization of the denitrifying fraction of phosphate accumulating organisms in biological phosphate removal. *Water Science and Technology*. **39**(1): 31-42.

Meyer R.L., Saunders A.M. and Blackall L.L. (2006). Putative glycogen-accumulating organisms belonging to the Alphaproteobacteria identified through rRNA-based stable isotope probing. *Microbiology-Sgm*. **152**: 419-429.

Mino T., Liu W.T., Kurisu F. and Matsuo T. (1995). Modeling glycogen-storage and denitrification capability of microorganisms in enhanced biological phosphate removal processes. *Water Science and Technology*. **31**(2): 25-34.

Obaja D., Mace S., Costa J., Sans C. and Mata-Alvarez J. (2003). Nitrification, denitrification and biological phosphorus removal in piggery wastewater using a sequencing batch reactor. *Bioresource Technology*. **87**(1): 103-111.

Obaja D., Mace S. and Mata-Alvarez J. (2005). Biological nutrient removal by a sequencing batch reactor (SBR) using an internal organic carbon source in digested piggery wastewater. *Bioresource Technology*. **96**(1): 7-14.

Oehmen A., Saunders A.M., Vives M.T., Yuan Z.G. and Keller H. (2006). Competition between polyphosphate and glycogen accumulating organisms in enhanced biological phosphorus removal systems with acetate and propionate as carbon sources. *Journal of Biotechnology*. **123**(1): 22-32.

Oehmen A., Vives M.T., Lu H.B., Yuan Z.G. and Keller J. (2005). The effect of pH on the competition between polyphosphate-accumulating organisms and glycogen-accumulating organisms. *Water Research*. **39**(15): 3727-3737.

Olsson G., Nielsen M., Yuan Z., Jensen A. and Steyer J.-P. (2005). Instrumentation, control and automation in wastewater systems, *IWA Publishing*.

Peng Y.Z., Chen Y., Peng C.Y., Liu M., Wang S.Y., Song X.Q. and Cui Y.W. (2004). Nitrite accumulation by aeration controlled in sequencing batch reactors treating domestic wastewater. *Water Science and Technology*. **50**(10): 35-43.

Pijuan M., Saunders A.M., Guisasola A., Baeza J.A., Casas C. and Blackall L.L. (2004). Enhanced biological phosphorus removal in a sequencing batch reactor using propionate as the sole carbon source. *Biotechnology and Bioengineering*. **85**(1): 56-67.

Pitman A.R., Venter S.L.V. and Nicholls H.A. (1983). Practical experience with biological phosphorus removal plants in Johannesburg. *Water Science and Technology*. **15**(3-4): 233-259.

Puig S., Vives M.T., Corominas L., Balaguer M.D. and Colprim J. (2004). Wastewater nitrogen removal in SBRs, applying a step-feed strategy: from lab-scale to pilot-plant operation. *Water Science and Technology*. **50**(10): 89-96.

Saito T., Brdjanovic D. and van Loosdrecht M.C.M. (2004). Effect of nitrite on phosphate uptake by phosphate accumulating organisms. *Water Research*. **38**(17): 3760-3768.

Saunders A.M., Oehmen A., Blackall L.L., Yuan Z. and Keller J. (2003). The effect of GAOs (glycogen accumulating organisms) on anaerobic carbon requirements in full-scale Australian EBPR (enhanced biological phosphorus removal) plants. *Water Science and Technology*. **47**(11): 37-43.

Subramaniam K., Greenfield P.F., Ho K.M., Johns M.R. and Keller J. (1994). Efficient biological nutrient removal in high-strength waste-water using combined anaerobic-sequencing batch reactor treatment. *Water Science and Technology*. **30**(6): 315-321.

Tilche A., Bacilieri E., Bortone G., Malaspina F., Piccinini S. and Stante L. (1999). Biological phosphorus and nitrogen removal in a full scale sequencing batch reactor treating piggery wastewater. *Water Science and Technology*. **40**(1): 199-206.

Turk O. and Mavinic D. (1986). Preliminary assessment of a shortcut in nitrogen removal from wastewater. *Can Journal of Civil Engineering*. **13**: 600-605.

Whang L.M. and Park J.K. (2002). Competition between polyphosphate- and glycogen-accumulating organisms in biological phosphorus removal systems - effect of temperature. *Water Science and Technology*. **46**(1-2): 191-194.

Wilderer P., Irvine R.L. and Goronszy M.C. (2001). Sequencing batch reactor technology. London, *IWA*.

Willers H.C., Tenhave P.J.W., Derikx P.J.L. and Arts M.W. (1993). Temperature-dependency of nitrification and required anoxic volume for denitrification in the biological treatment of veal calf manure. *Bioresource Technology*. **43**(1): 47-52.

Wong M.T., Tan F.M., Ng W.J. and Liu W.T. (2004). Identification and occurrence of tetrad-forming Alphaproteobacteria in anaerobic-aerobic activated sludge processes. *Microbiology-Sgm*. **150**: 3741-3748.

Yilmaz G., Lemaire R., Keller J. and Yuan Z. (2007). Effectiveness of an alternating aerobic, anoxic/anaerobic strategy for maintaining biomass activity of BNR sludge during long-term starvation. *Water Research*. **41**(12): 2590-2598.

Zeng R.J., Saunders A.M., Yuan Z.G., Blackall L.L. and Keller J. (2003a). Identification and comparison of aerobic and denitrifying polyphosphate-accumulating organisms. *Biotechnology and Bioengineering*. **83**: 140-148.

Zeng R.J., van Loosdrecht M.C.M., Yuan Z.G. and Keller J. (2003b). Metabolic model for glycogen-accumulating organisms in anaerobic/aerobic activated sludge systems. *Biotechnology and Bioengineering*. **81**(1): 92-105.

Appendix B

Achieving the Nitrite Pathway Using Aeration Phase Length Control and Step-feed in a SBR Removing Nutrients from Abattoir Wastewater

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ABSTRACT

Aeration phase length control and step-feed of wastewater are used to achieve nitrogen removal from wastewater via nitrite in sequencing batch reactors (SBR). Aeration is switched off as soon as ammonia oxidation is completed, which is followed by the addition of a fraction of the wastewater that the SBR receives over a cycle to facilitate denitrification. The end-point of ammonia oxidation is detected from the on-line measured pH and oxygen uptake rate (OUR). The method was implemented in a SBR achieving biological nitrogen and phosphorus removal from anaerobically pre-treated abattoir wastewater. The degree of nitrite accumulation during the aeration period was monitored along with the variation in the nitrite oxidising bacteria (NOB) population using fluorescence *in situ* hybridisation (FISH) techniques. It is demonstrated that the nitrite pathway could be repeatedly and reliably achieved, which significantly reduced the carbon requirement for nutrient removal. Model-based studies show that the establishment of the nitrite pathway was primarily the result of a gradual reduction of the amount of nitrite that is available to provide energy for the growth of NOB, eventually leading to the elimination of NOB from the system.

Keywords: aeration control, denitritation, industrial wastewater, nitritation, NOB, multi-step feeding.

INTRODUCTION

The meat processing industry requires large quantities of water, much of which is discharged as wastewater containing high levels of COD and nutrients such as nitrogen (N) and phosphorus (P) with relatively high C/N and C/P ratios. Primary treatment is usually required before abattoir wastewater can be treated in biological nutrient removal (BNR) systems. In Australia, the raw abattoir wastewater is typically pre-treated in anaerobic ponds with a hydraulic retention time ranging between 7–14 days. While reducing the amounts of fat, oil and grease (FOG), this anaerobic treatment process also removes a large fraction of the COD from the wastewater, resulting in COD limitations (particularly easily biodegradable COD such as volatile fatty acids – VFAs) for subsequent N and P removal (Keller et al., 1997).

It has been widely reported that N removal through nitrification and denitrification via nitrite (i.e. nitrite pathway) not only reduces the oxygen consumption in the nitrification stage by

25% but also reduces the COD requirement in the denitrification stage by 40% (Turk and Mavinic, 1986). The use of this nitrite pathway to remove the high level of N present in abattoir wastewater could therefore save significant amount of COD and improve the overall performance of the BNR system.

Several factors have been identified to promote the nitrite pathway by selectively inhibiting or limiting the growth of nitrite oxidising bacteria (NOB) over ammonia oxidising bacteria (AOB). High pH (Villaverde et al., 1997; Pambrun et al., 2004), high concentrations of free ammonia (Balmelle et al., 1992; Villaverde et al., 2000) and free nitrous acid (Anthonisen et al., 1976; Vadivelu et al., 2006), low dissolved oxygen (DO) (Munch et al., 1996), and high temperature combined with short sludge retention time (SRT) (in the so-called SHARON – Single reactor High activity Ammonium Removal Over Nitrite – process detailed in Hellinga (1998)) all likely contribute, to various degrees, to the inhibition or elimination of NOB and the accumulation of nitrite. These factors have mainly been applied to wastewaters containing a high concentration of ammonium with low COD:N ratios (e.g. anaerobic digester supernatant, landfill leachate), whereas denitrification was achieved in a post anoxic period with the dosage of external carbon sources (Hellinga et al., 1998; Lai et al., 2004; Peng et al., 2004a; Mace et al., 2006).

These strategies may not be directly applicable to other types of wastewaters requiring simultaneous biological COD, nitrogen and phosphorus removal, due to the possible inhibitory effect that these factors could have on other bacterial populations involved in a more complex BNR system. For example, it has been reported recently that nitrite could severely inhibit the aerobic/anoxic P-uptake and the anaerobic P-release by the polyphosphate accumulating organisms (PAOs) in enhanced biological phosphorus removal (EBPR) processes (Saito et al., 2004; Yoshida et al., 2006; Zhou et al., 2007). The accumulation of nitrite has also been shown to trigger the emission of nitrous oxide (N_2O) during the denitrification step (Itokawa et al., 2001; Zeng et al., 2003).

Peng et al. (2004) demonstrated that stable N removal via nitrite could be obtained through the control of the aeration time in a sequencing batch reactor (SBR) treating domestic wastewater. Aeration was stopped as soon as nitritation finished (i.e. complete oxidation of NH_4^+), as indicated by a bending point on the pH profile. An external carbon source (glucose) was added to enable denitrification in the following anoxic period. Clearly, the use of an external carbon source rather than the COD contained in wastewater for denitrification considerably undermines the overall benefits of the nitrite pathway.

This study investigates the possibility of achieving nitrogen removal from abattoir wastewater via the nitrite pathway by integrating aeration phase length control with step-feed of wastewater. Instead of using an external carbon source, the organic carbon contained in wastewater is used for denitrification, thus delivering the true benefit of implementing the nitrite pathway. A lab-scale SBR, operated with three non-aerated and aerated sub-cycles with wastewater added to the three non-aerated periods, was employed to remove COD, N and P from abattoir wastewater. An on-line aeration phase length control system was implemented based on pH and DO signals to switch between aerobic and anoxic periods. The reactor performance in terms of the N and P removal efficiency and the degree of nitrite accumulation was monitored, along with the variation in the NOB population. The mechanisms responsible for the achievement of the nitrite pathway are explained using model-based analysis.

MATERIAL AND METHODS

Reactor set-up and operation

A lab-scale SBR with a working volume of 7 l was used in this study. The SBR was operated with a cycle time of 6 h in a temperature-controlled room (18-22˚C). In each cycle, 1 l of abattoir wastewater (more details given below) was pumped into the reactor over the three filling periods with a volume distribution of 0.5 l, 0.3 l and 0.2 l respectively. Each filling period was followed by non-aerated (either anoxic or anaerobic depending on when the oxidised nitrogen was completely consumed) and aerated periods (more details can be found in Lemaire et al. (submitted)). During aerated periods, air was provided intermittently using an on/off control system to keep the DO level between 1.5 and 2 $mgO_2.l^{-1}$. After the settling period, 1 l supernatant was removed from the reactor resulting in a HRT of 42 h. 115 ml of mixed liquor was wasted every cycle to keep a constant SRT of 15 days. The pH in the system was recorded, ranging between 7.1 and 7.9, but not controlled. The ORP signal was also recorded to give indications of the nitrate levels in the reactor during the anoxic periods. The reactor was mixed with an overhead mixer except during the settling, decanting and first filling periods. The SBR cycle operation was controlled by a programmable logic controller (PLC – Opto Control).

Wastewater

The wastewater used in this study was from a local abattoir in Queensland, Australia. At this site, the raw effluent passes through four parallel anaerobic ponds before being treated in a SBR for biological N and COD removal. The anaerobic ponds serve to reduce FOG and COD, and also to produce some easily biodegradable COD, particularly VFAs, to facilitate the down-stream biological nitrogen removal.

Raw wastewater and anaerobic pond effluent from the abattoir were collected on a weekly basis and stored at 4˚C. The raw wastewater was subjected to one-day pre-fermentation before being pumped into the SBR (more details can be found in Lemaire et al. (submitted)). The aim of this pre-fermentation step was to further increase the level of easily biodegradable COD, in particular VFAs, which is critical for bio-P removal. The characteristics of the pre-fermented raw wastewater and the anaerobic pond effluent are compared in Table 1. The wastewater fed to the lab-scale SBR consisted of a mixture of anaerobic pond effluent and pre-fermented raw wastewater as later described in Table 2. The modification of the fraction of raw pre-fermented wastewater used in the influent did not modify the overall N and P content of the influent due to the identical levels of N and P present in both type of wastewater.

Table 1. Characteristics of the different types of wastewater used in this study. The intervals represent the mid-95% range.

Parameter (mid-95% range)	Pre-fermented raw wastewater	Anaerobic pond effluent [b]
TCOD ($mg.l^{-1}$)	6,400-8,320	1,090-1,270
SCOD ($mg.l^{-1}$)	2,110-2,550	769-909
VFA[a] ($mgCOD.l^{-1}$)	699-797	502-626
TN ($mg.l^{-1}$)	260-306	235-254
NH_4-N ($mg.l^{-1}$)	141-157	223-229
TP ($mg.l^{-1}$)	44-50	36-39
PO_4-P ($mg.l^{-1}$)	37-42	34-35

[a] Acetate and propionate only

[b] Additional acetate and propionate were added to the anaerobic pond effluent to mimic the effluent of better operated, but physically inaccessible pond

Aerobic phase length control to promote nitrite pathway

The SBR was operated for approximately 18 months. Aerobic phase length control for achieving N removal via nitrite was trialled in the last 13 months. The control strategy employed was based on the slope of the pH signal and on the oxygen uptake rate (OUR). Figure 1 shows that the exact time of complete NH_4^+ oxidation in each aeration periods could be detected through the pH bending point and the sharp OUR drop. During each aeration period, the pH slope was calculated once the maximum pH has been reached and started to decrease. The slope of the pH was determined based on pH values in a 2 minute moving window. Due to the on-off control system of the DO in the reactor, the OUR was calculated during the time the oxygen valve was in an "off" state. Aeration is switched off when all the following three criteria are met:

(i) The pH slope is lower than a pre-specified value entered by the operator (typically between 0.005 – 0.01 pH unit.min^{-1}). This was used as the main criterion.

(ii) The OUR is lower than a pre-specified value entered by the operator (typically 1.2 $mgO_2.l^{-1}.min^{-1}$). This was only a safety condition to reduce the risk of switching aeration too early should the pH algorithm incorrectly detected a bending point. As such the set-point was not conservative giving more weight to the pH criterion.

(iii) The aeration period length is greater than a minimum aeration time entered by the operator (typically 15 min). The role of this criterion was to ensure a minimum aeration period in the case that the reactor was seriously under-loaded due to for example a very low nitrogen concentration in the feed, in which case both the pH and OUR algorithms could detect a bending point soon after aeration is started.

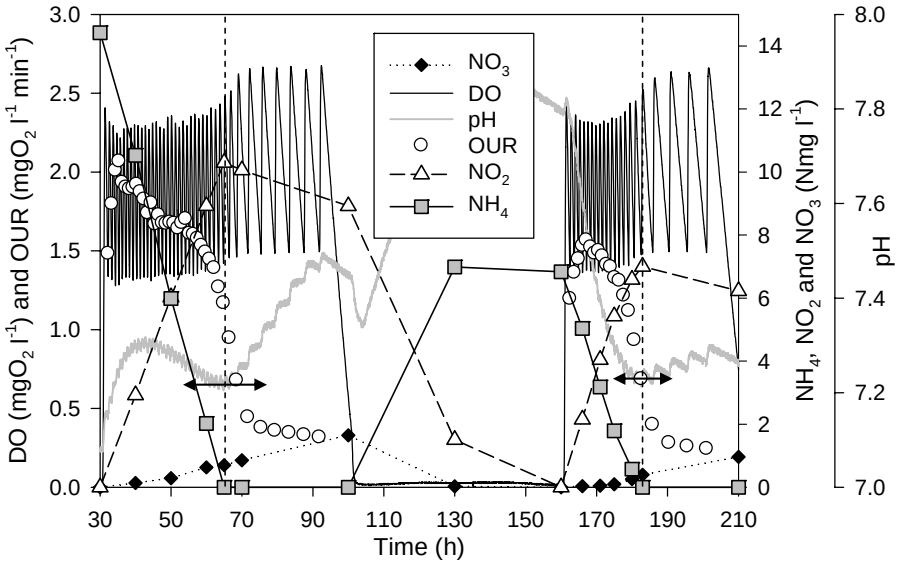

Figure 1. Typical pH, DO, OUR and ammonium profiles during the first two aeration periods of a SBR cycle, showing the correlation between the complete NH_4^+ oxidation (vertical dot line), the pH bending point (horizontal arrows) and the OUR drop. The nitrate and nitrite profiles are also presented.

The implementation and demonstration of the aeration length control consisted of three stages. In the first stage, from day 160 to 340, the aeration control was performed manually by the operator. Based on the pH slope and OUR calculated on-line, the length of each of the three aeration period was adjusted on a daily basis to ensure that the aeration was stopped immediately after complete oxidation of NH_4^+ in the SBR. Then, from day 340 to 410, the manual aeration control was ceased and fixed aeration lengths of 60 min, 36 min and 24 min for the three aeration periods, respectively, were applied, which were longer than the time required for complete NH_4^+ oxidation. The purpose of Stage II was to demonstrate that the nitrite pathway previously established under Stage I could be deteriorated by extending aeration. In Stage III (from day 410 onwards), the automatic aeration length control was implemented to re-establish the nitrite pathway. It should be mentioned that the abattoir closed down from day 480 to 525 due to annual maintenance. During this period, the SBR cycle operation was modified in order to preserve the reactor biomass as no wastewater was available. The sludge was aerated and mixed for 15 min in each 6h cycle and was allowed to settle for the rest of the cycle, more details of this operation can be found in Yilmaz et al. (2007).

Analyses

The ammonia ($NH_3 + NH_4^+$), nitrate (NO_3^-), nitrite (NO_2^-) and total phosphate (PO_4^{3-}-P) were analysed using a Lachat QuikChem8000 Flow Injection Analyser (Lachat Instrument, Milwaukee). Total and soluble chemical oxygen demand (TCOD and SCOD, respectively), total Kjeldahl nitrogen (TKN), total phosphorus, mixed liquor suspended solid (MLSS) and volatile MLSS (MLVSS) were analysed according to the standard methods (APHA, 1995). VFAs were measured by Perkin-Elmer gas chromatography with column DB-FFAP 15m x 0.53mm x 1.0µm (length x ID x film) at 140°C, while the injector and FID detector were

operated at 220°C and 250°C, respectively. High purity helium was used as carrier gas at a flow rate of 17 $ml.min^{-1}$.

Fluorescence *in situ* hybridisation (FISH) was performed as specified in Amann (1995). Oligonucleotide probes used in this study were EUBmix (Daims et al., 1999) for the detection of all Bacteria, NTSPA662 (Daims et al., 2001) for *Nitrospira* and NIT3 (Wagner et al., 1996) for *Nitrobacter*. FISH images were collected using a Zeiss LSM 510 confocal laser scanning microscope with a 63x Plan-Apochromat oil immersion lens. FISH quantification was performed according to Crocetti et al. (2002) where the relative abundance of each group was determined in triplicate as mean percentage of all bacteria.

Model based simulations

To investigate the mechanisms responsible for the decrease of the NOB population in the SBR with aeration phase length control and step-feed of wastewater, a model was devised based on the IWA ASM2d (Henze et al., 1999). The main change to ASM2d involved describing nitrification and denitrification as two-step processes, namely ammonia oxidation followed by nitrite oxidation, and nitrate reduction followed by nitrite reduction. Consequently, the nitrifier population in the original ASM2d model was replaced with two populations namely AOB and NOB. However, the two-step denitrification process was assumed to be catalysed by the same organisms as described in ASM2d, namely ordinary heterotrophic bacteria and PAOs. Both groups of bacteria were assumed to be able to reduce both nitrate and nitrite. The resulting model is presented in Table S1 – S6 in the Supplementary Materials.

In the simulation studies described below, default parameter values recommended in Henze et al. (1999) were used for the ASM2d model parameters. For the new kinetic parameters related to the two distinct nitrifier populations, identical values were selected for AOB and NOB. Rigorous model calibration was not considered necessary given the purpose of the simulation studies was purely theoretical. The model parameters along with the wastewater composition used in the simulations are summarised in Table S1 – S6 in the Supplementary Materials.

Two different scenarios were tested, with and without aeration length control. In the case with aeration length control, aeration was switched off as soon as the ammonium concentration in the SBR reached 1 $mgN.l^{-1}$. The lengths of the three aeration periods used in the second case were 60, 36 and 24 min, respectively, identical to those used in the SBR without aeration length control. These lengths are in average 10 to 15 min longer than those used in the first case.

RESULTS AND DISCUSSION

Effect of the aeration control strategy on the nitrite pathway

Figure 2a presents the level of nitrite pathway achieved in the SBR, measured as the amount of NO_2^- produced ($mgN.l^{-1}$) per NO_x^- produced ($mgN.l^{-1}$) during the 3 aeration periods, and the relative abundance of NOBs in the SBR throughout Stage I, Stage II and Stage III. Tests to identify the main NOB species present in the SBR using common FISH probes (i.e. NIT3 for *Nitrobacter* and NTSPA662 for *Nitrospira*) showed that only *Nitrospira* was present in the system. Therefore, only the *Nitrospira* population was quantified. The reactor used in this study had been already running for 5 months performing high level of COD, N and P removal (Lemaire et al., submitted) before the aeration length control was implemented. In that time, no nitrite accumulation was observed during the aerobic periods as depicted in Figure 2a.

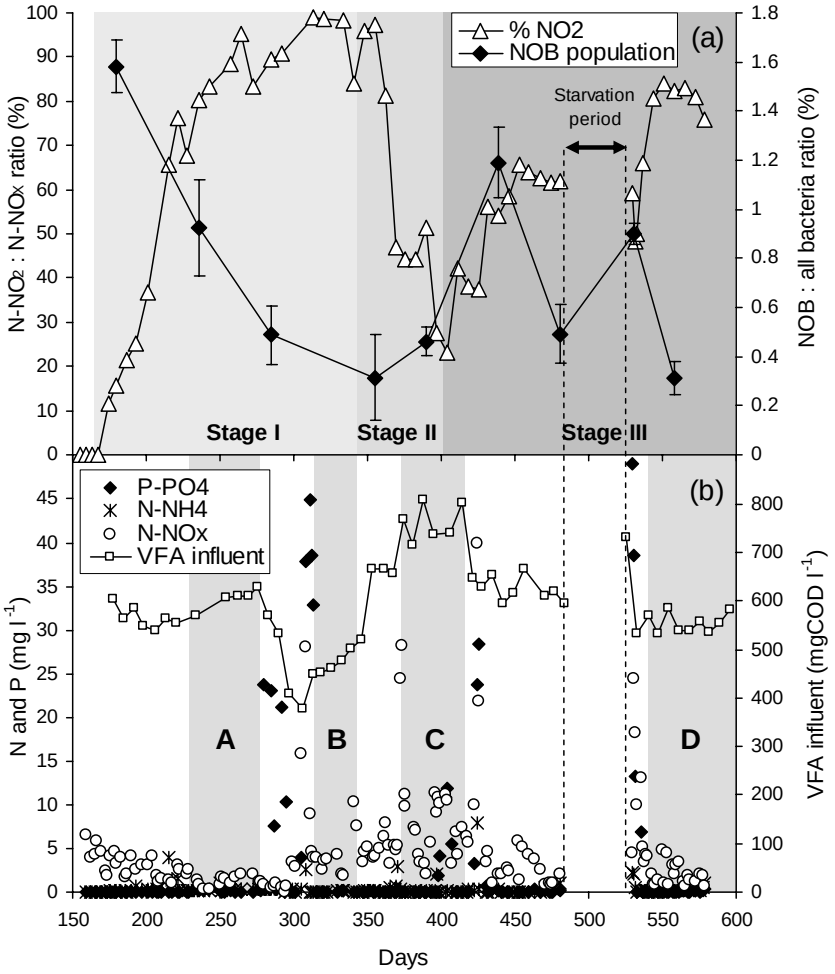

Figure 2. (a) Degree of nitrite accumulation in the three stages and the abundance of *Nitrospira* as the dominant NOB (FISH probe NTSPA662). The NOB quantification shown is an average (error bars=SE, n=3). **(b)** Ammonium, oxidised nitrogen and phosphate in the effluent and VFAs in the influent.

During Stage I, the manual control of the length of each aeration period resulted in a gradual accumulation of nitrite reaching 95% of the total amount of NO_x^- produced on day 280 (Figure 2a). This high level of nitrite pathway was maintained until the start of Stage II. During that second stage, the implementation of fixed aeration periods rapidly deteriorated the nitrite pathway previously established, resulting in only 20% of NO_2^- accumulation 50 days after the start of Stage II (Figure 2a). The implementation of the automatic aeration length control strategy during Stage III resulted in the recovery of the nitrite pathway in the SBR. A nitrite accumulation of 85% was reached 150 days (including 50 days of starvation period) after the application of the automatic controller. The aeration phase length control and step-feed strategy were therefore successful in establishing the nitrite pathway.

When comparing the level of nitrite pathway in the SBR and the *Nitrospira* population dynamics it clearly appears that the nitrite pathway was achieved through the elimination or the reduction of the NOB population. However, some delay was observed between the level of nitrite pathway measured and the abundance of NOB. While the NO_2^- accumulation decreased from 98% to 20% during Stage II, the *Nitrospira* population only increased from 0.3% to 0.5% but later increased to 1.2% of the total bacterial population 40 days into

Stage III (Figure 2a). The presence of this lag phase could be due to the complex dynamics involved in the NOB growth processes when the availability of their main energy source (i.e. NO_2^-) is modified. It should be said that in Stage III, no wastewater was fed to the SBR between day 480 and 530 due to the annual closure of the abattoir and the reactor cycle was modified in order to preserve the SBR biomass population (more detail given in Yilmaz et al. (2007)). The slight increase of the *Nitrospira* population observed during this starvation period was likely cause by the reduction of the total amount of bacteria present in the reactor (MLVSS in the SBR decreased by 20% over that period, data not shown) which had a direct impact on the relative amount of *Nitrospira* present in the reactor.

Effect of nitrite pathway on the overall SBR performance

In order to demonstrate the benefit of the nitrite pathway in COD savings, the COD concentration in the SBR influent was adjusted several times during the experimental period through changing the fraction of fermented raw feed and/or the VFA content in the pond effluent. The resulting VFA concentration profile in the SBR influent along with nutrient levels in the effluent are shown in Figure 2b.

From day 160 to 250, the level of nitrite pathway increased from 0 to 95% after the length of the aeration periods was manually controlled. High levels of COD, N and P removal (95%, 97% and 98%, respectively) were consistently achieved. Figure b shows that as the level of nitrite pathway increased the amount of NO_x^- in the effluent decreased, likely due to the reduced COD requirement for N removal via the nitrite pathway. The implementation of the aeration length control increased the anoxic time to aerobic time ratio for the SBR cycle which might have also improved the denitrification performance. The period between day 250 and 280 with stable high level of nitrite pathway is referred to as "Period A" in Figure 2b and the reactor performance in this period is summarised in Table 2.

From day 280 to 310, the VFA concentration in the SBR feed was gradually decreased from 600 to 400 $mgCOD.l^{-1}$ by first reducing the fraction of pre-fermented raw wastewater in the influent from 25% to 15%, and then gradually reducing the VFA concentration in pond effluent by 40% (mimicking a poorly performing anaerobic pond). The bio-P removal was immediately affected due to this sudden VFA shortage but soon recovered (Figure 2b). With the further reduction of VFA, NO_x^- started to accumulate in the effluent on day 300 due to incomplete denitrification apparently caused by the inadequate COD supply. The accumulation of NO_x^- was very detrimental to bio-P removal as it deteriorated anaerobic P release (data not shown), resulting in the loss of P removal (the effluent PO_4^{3-} concentration was similar to that in the influent). The amount of VFA had to be slightly increased to 400 $mgCOD.l^{-1}$, which led to the recovery of both N and P removal (Figure 2b). The stable period from day 310 and 340 is referred to as "Period B" in Figure 2b and the reactor performance is also summarised in Table 2.

With the implementation of fixed length aeration on day 340, the effluent NO_x^- and P levels deteriorated considerably, likely due to the increased COD requirement for denitrification via the gradually recovered nitrate pathway. Therefore, more VFAs (710 $mgCOD.l^{-1}$) had to be supplied to stabilise the N and P removal performance (Figure 2b). The stable period from day 380 to 420 is referred to as "Period C" in Figure 2b and Table 2.

Following the implementation of the automatic aeration control strategy, the VFA content in the SBR feed was reduced on day 420 by decreasing both the fraction of pre-fermented raw wastewater in the influent from 15% to 10% and the VFA concentration in pond effluent by 30%. Once again, NO_x^- and PO_4^{3-} immediately accumulated in the effluent due to the sudden VFA and COD shortage but promptly recovered (Figure 2b). Day 540 to 600, after the normal

wastewater load was resumed in the SBR following the long starvation period, is defined as "Period D" in Figure 2b and Table 2.

Table 2. Summary of the SBR performance and the VFA requirements over four stable periods, correlated with the degrees of nitrite pathway achieved.

Parameter (mid-95% range)	**Period A day 230-280**	**Period B day 310-340**	**Period C day 380-420**	**Period D day 540-600**
NO_2^- accumulation (%)	81-92	87-99	29-47	78-84
NO_x^- effluent (mgN l^{-1})	0.9-1.6	2.9-6.5	5.4-8.3	1.5-2.6
PO_4^{3-} effluent (mgP l^{-1})	0.06-0.20	0.01-0.07	0.1-3.6	0.05-0.13
% pre-fermented raw in SBR influent	25%	15%	15%	10%
Total VFAs in SBR influent (mgCOD l^{-1})	579-632	450-531	710-813	540-571

Table 2 clearly shows that during "Period C", when the level of nitrite pathway was the lowest, the worst N and P removal were achieved, despite the amount of VFA in the influent being considerably higher than in other periods. This clearly demonstrates the benefits of the nitrite pathway in saving COD and also in enhancing the nutrient removal performance.

Comparing Period B with Periods A and D, during all of which over 80% nitrite pathway was achieved, the P removal performance was almost identical and the N removal deteriorated only slightly, despite of a significantly lower VFA content in the feed (10-20%). Noting the immediate deterioration in the reactor performance each time the VFA content in the feed was reduced (Figure 2b), it appears that the biomass can slowly adapt to the feed variations, provided that a critical amount of VFA is supplied. However, abrupt changes in the feed should be avoided in reactor operation. The process data also showed that it is important to consider both N and P removal when assessing the possible COD and/or VFA savings via the nitrite pathway, as P removal depends strongly on the level of N removal.

The aeration control strategy was successful in achieving stable N removal via the nitrite pathway with reduced requirement for COD and VFAs. As a direct result, the fraction of pre-fermented raw wastewater in the influent was reduced from an initial 25% to 10% without affecting the performance of the SBR. The reduction of the raw wastewater fraction lowered the amount of FOG and colloidal matters, and is expected to improve the sludge settleability (Hopwood, 1977; Travers and Lovett, 1984) although this aspect was not investigated in this study.

The steep-feed strategy employed in this SBR ensured that no external carbon addition was needed to carry out the post denitrification or denitritation making the overall BNR process more attractive. However, compared to external carbon sources (e.g. methanol, ethanol, acetate and glucose), which contain no nitrogen, wastewater contains nitrogenous compounds and therefore the step-feed of wastewater increases the concentration of ammonium and organic nitrogen while reducing that of oxidised nitrogen compounds. This problem was resolved in this study by applying three feeding periods, with a gradually reduced amount of wastewater fed in each period (50%, 30% and 20%, respectively). The overall high-level removal of N, P and COD (>95%) demonstrates that this multiple step-feed strategy was successful.

Integration of aeration length control with step-feed of wastewater

In recent years, the use of SBR to remove nutrients from domestic or industrial wastewater has been extended from lab-scale studies to full-scale applications (Tilche et al., 1999; Artan et al., 2001; Keller et al., 2001; Puig et al., 2004). SBR usually operates with fixed lengths for the different phases of operation including filling, mixing (anaerobic, aerobic or anoxic), settling and decanting. Due to influent fluctuations and system state variations, it is beneficial to operate a SBR process with varying phase lengths. Therefore, higher levels of process control and automation are necessary to optimise the SBR operation. Many researchers have suggested that for a nitrogen removal system, on-line measurements of ORP, DO and pH are a cheap and easy way to detect the end of the nitrification and denitrification processes (Al-Ghusain et al., 1994; Wareham et al., 1994; Al-Ghusain and Hao, 1995; Hao and Huang, 1996).

More recently, Peng et al. (2004) used a control strategy based on the pH bending point to initiate the anoxic phase (by stopping aeration) and on the addition of an external carbon source (glucose) to achieve denitritation. In this study, we applied the control strategy to a more complex system achieving COD, N and P removal, and achieved denitritation through a step-feed strategy suppressing the need of external carbon dosage.

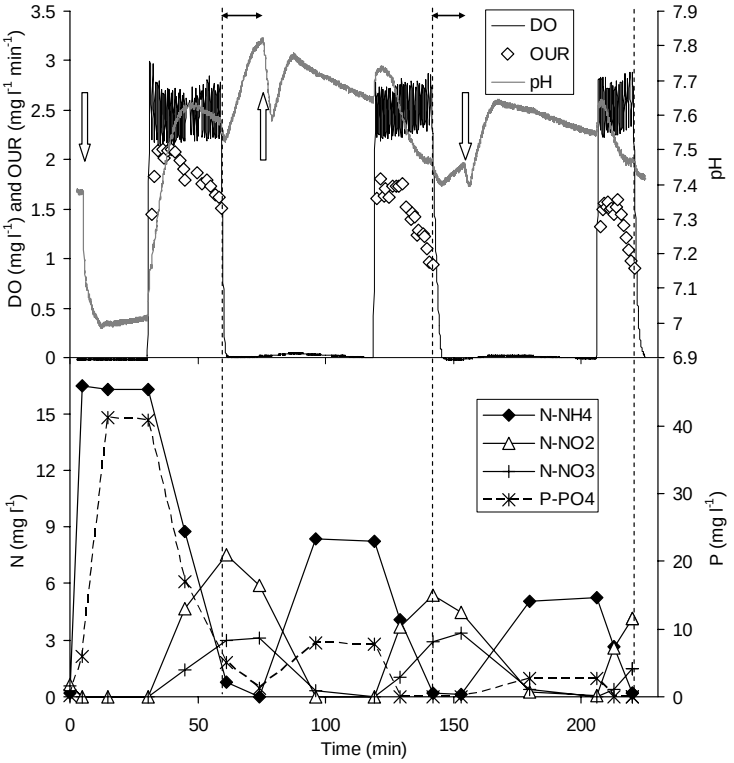

Figure 3. Example of pH, DO, OUR, nitrogen and phosphorous profiles during a SBR cycle performed on day 550 after the automatic aeration control was implemented. Vertical dot lines indicate when the aeration was automatically stopped and black arrows represent the additional anoxic time gained by aeration control. White arrows indicate feeding time.

Figure 1 already demonstrated the simultaneity between the depletion of NH_4^+, the bending points on pH and OUR drop during a SBR cycle where the aeration lengths were not controlled. The aeration length control strategy previously described could therefore be implemented in this complex BNR system. Figure 3 presents the pH, DO, OUR, nitrogen and phosphorus profiles in a SBR cycle after this automatic aeration length control strategy was implemented. This strategy was reliable in detecting the end of the nitritation process and stopping the aeration as indicated by the dot lines on Figure 3. The success of this control strategy was further confirmed by the good long-term performances of the SBR presented previously.

However, some technical issues and possible improvement of the control algorithm were identified. The pH and OUR profiles in each of the three aeration periods in a cycle were quite different (Figure 3) making it difficult to select universally applicable threshold values for the control algorithms. The pH profile even changed over time as shown by the difference between the profiles depicted in Figure 1 and in Figure 3. This was mainly due to the large difference of the initial pH value observed at the start of each aeration period but also to the different PAO activities in each aeration period, with most of the activity occurring in the first aeration period as indicated by the highest P uptake in Figure 3. The pH and OUR profiles evolution over time of in the SBR suggests that it would have been preferable to design an algorithm where the aeration was controlled based on relative rather than absolute threshold values. For example, the aeration could be turned off when a certain percentage (e.g. 5-10%) of the maximum pH slope and OUR value obtained in the on-going aerobic period is reached. Such an algorithm would have been easy to implement but the PLC software used in our study did not provide enough flexibility to enable the implementation of this algorithm.

Mechanisms leading to NOB elimination: model-based investigation

Many factors have been identified in literature to selectively inhibit or limit the growth of NOB, leading to their elimination from BNR systems. These included high pH, high ammonium/ammonia concentration and high nitrite/free nitrous acid concentration (Anthonisen et al., 1976; Balmelle et al., 1992; Villaverde et al., 1997; Villaverde et al., 2000; Pambrun et al., 2004; Vadivelu et al., 2006). However, these factors unlikely played a key role in the elimination/reduction of the NOB population in our system. Comparing the cycle study data with and without aeration length control (Figure 3 vs. Figure 1), both pH and ammonium varied within a similar range. While the nitrite profiles were different in the two cycles, nitrite accumulation could not have been initiated by itself. The lower affinity of NOB with oxygen in comparison to AOB has also been found to facilitate the washout of NOB (Blackburne et al., in press) under low DO conditions. However, the DO concentration applied in our study was relatively high, and was not lowered during the implementation of the nitrite pathway.

In the model used this study, none of the above factors were considered. As shown in the Supplementary Materials, the growth parameters including the maximum specific growth rate, the decay rate and the affinity constants with respect to oxygen and with respect to nitrogen sources were assumed identical for AOB and NOB. Yet, the simulation results successfully predicted the onset and full establishment of the nitrite pathway with aeration length control and step-feed, which was not the case when the aeration lengths were fixed (Figure 4). The results demonstrate that the nitrite pathway could be established without assuming that NOB have poorer growth kinetics than AOB.

Figure 4 shows a quick onset of nitrite accumulation after the implementation of the aeration length control. This is not unexpected. Nitrite, the energy source for NOB, is the product of ammonia oxidation. This implies that nitrite oxidation should always lag behind ammonia

oxidation. In other words, certain levels of nitrite must still be present in the liquid phase at the time when ammonia oxidation is completed. If the aeration is continued, the remaining nitrite would be converted to nitrate by NOB (Figure 1). However, if aeration is switched off at the exact time that ammonia oxidation finishes, as implemented in both the experimental and simulation studies, the residual nitrite would be carried into the following anoxic phase and reduced by denitrifiers and is thus no longer available for nitrite oxidisers (Figure 3). This means that NOB gained less energy for growth in a cycle with aeration length control in comparison to a cycle without aeration length control, resulting in comparatively less NOB growth. The reduced NOB growth would lead to a slightly lower nitrite oxidation rate in the following cycle, which is proportional to the NOB population size. As a result, the nitrite accumulation when ammonia oxidation finishes would be slightly higher than that in the previous cycle albeit the difference could be very small, further reducing the growth of NOB. Over many cycles the NOB population would decrease gradually, which should be accompanied by increased nitrite accumulation, a situation confirmed by both the experimental and simulation results.

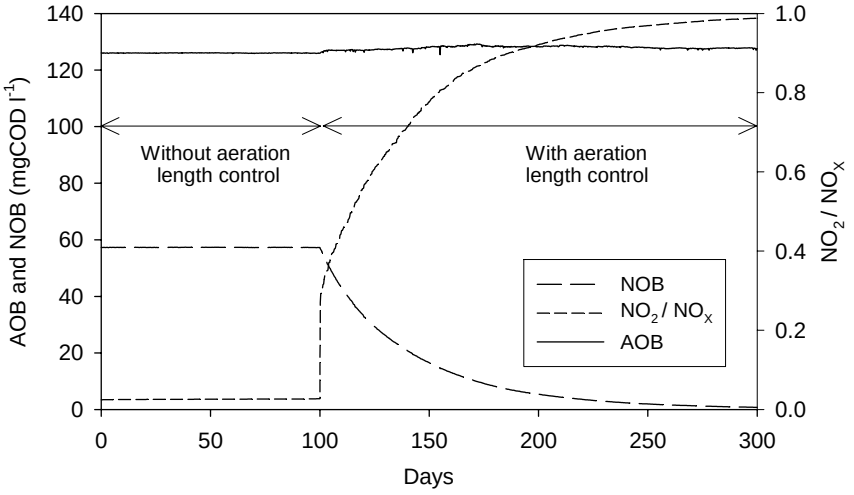

Figure 2. Simulated variation of the NOB and AOB population sizes and NO_2:NO_x ratio at the end of the aerobic periods without aeration length control (fixed aeration lengths) and with aeration length control.

Both the experimental and modelling results demonstrate that the elimination of NOB is a relatively slow process. This is likely because of the similar growth kinetics possessed by these groups of nitrifiers, as assumed in the model. Factors such as high free nitrous acid concentration that lower the NOB growth rate would help to speed up the elimination process. However, from the discussions above, we hypothesise that the primary reason for NOB elimination using aeration length control and the step-feed of wastewater, is the gradual reduction in energy supply to NOB. Indeed, further simulation studies showed that the nitrite pathway could be established even if NOB possess faster growth kinetics than AOB (data not shown). However, the time required for achieving the nitrite pathway becomes considerably longer in the latter case. In practice, it could be very difficult to establish the nitrite pathway in such conditions as there is typically a delay between the actual depletion of ammonium and the time of its detection. Such a delay could be sufficient for NOB to convert all the residual nitrite to nitrate and therefore not losing energy source for growth.

CONCLUSION

Nitrogen removal via the nitrite pathway can be achieved by integrating step-feed of wastewater with an on-line aeration phase length control system that switches off aeration as soon as ammonium oxidation is completed. The implementation of the nitrite pathway significantly reduces the carbon demand for biological nutrient removal.

In such systems, nitrite oxidising bacteria are eliminated likely due to the gradual reduction of their energy source through the use of denitrification, rather than due to the inhibition of their growth kinetics. However, any reduction of the NOB growth kinetics would help to speed-up the onset and establishment of the nitrite pathway.

The end-point of ammonium oxidation can be reliably detected from the on-line pH and DO signals. Therefore, the nitrite pathway can be implemented with relatively simple and cheap on-line sensors.

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REFERENCES

Al-Ghusain I. and Hao O.J. (1995). Use of pH as control parameter for aerobic/anoxic sludge-digestion. *Journal of Environmental Engineering-Asce*. **121**(3): 225-235.

Al-Ghusain I.A., Huang J., Hao O.J. and Lim B.S. (1994). Using pH as a real-time control parameter for waste-water treatment and sludge-digestion processes. *Water Science and Technology*. **30**(4): 159-168.

Amann R.I. (1995). In situ identification of microorganisms by whole cell hybridization with rRNA-targeted nucleic acid probes. In: Molecular Microbial Ecology Manual. Akkermans A.D.L., van Elsas J.D. and de Bruijn F.J. (eds). Dordrecht, Holland, *Kluwer Academic Publications*. **3.3.6:** 1-15.

Anthonisen A.C., Loehr R.C., Prakasam T.B.S. and Srinath E.G. (1976). Inhibition of nitrification by ammonia and nitrous-acid. *Journal Water Pollution Control Federation*. **48**(5): 835-852.

APHA (1995). Standard methods for the examination of water and wastewater. Washington, DC, *American Public Health Association*.

Artan N., Wilderer P., Orhon D., Morgenroth E. and Ozgur N. (2001). The mechanism and design of sequencing batch reactor systems for nutrient removal - the state of the art. *Water Science and Technology*. **43**(3): 53-60.

Balmelle B., Nguyen K.M., Capdeville B., Cornier J.C. and Deguin A. (1992). Study of factors controlling nitrite buildup in biological processes for water nitrification. *Water Science and Technology*. **26**(5-6): 1017-1025.

Crocetti G.R., Banfield J.F., Keller J., Bond P.L. and Blackall L.L. (2002). Glycogen accumulating organisms in laboratory-scale and full-scale activated sludge processes. *Microbiology*. **148**(11): 3353-3364.

Daims H., Bruhl A., Amann R., Schleifer K.H. and Wagner M. (1999). The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: Development and evaluation of a more comprehensive probe set. *Systematic and Applied Microbiology*. **22**(3): 434-444.

Daims H., Nielsen J.L., Nielsen P.H., Schleifer K.H. and Wagner M. (2001). In situ characterization of Nitrospira-like nitrite oxidizing bacteria active in wastewater treatment plants. *Applied and Environmental Microbiology*. **67**(11): 5273-5284.

Hao O.J. and Huang J. (1996). Alternating aerobic-anoxic process for nitrogen removal: Process evaluation. *Water Environment Research*. **68**(1): 83-93.

Hellinga C., Schellen A., Mulder J.W., van Loosdrecht M.C.M. and Heijnen J.J. (1998). The SHARON process: An innovative method for nitrogen removal from ammonium-rich waste water. *Water Science and Technology*. **37**(9): 135-142.

Henze M., Gujer W., Mino T., Matsuo T., Wentzel M.C., Marais G.V.R. and Van Loosdrecht M.C.M. (1999). Activated Sludge Model No.2d, ASM2d. *Water Science and Technology*. **39**(1): 165-182.

Hopwood D. (1977). Effluent treatment in meat and poultry-processing Industries. *Process Biochemistry*. **12**(8): 5-&.

Itokawa H., Hanaki K. and Matsuo T. (2001). Nitrous oxide production in high-loading biological nitrogen removal process under low COD/N ratio condition. *Water Research*. **35**(3): 657-664.

Keller J., Subramaniam K., Gosswein J. and Greenfield P.F. (1997). Nutrient removal from industrial wastewater using single tank sequencing batch reactors. *Water Science and Technology*. **35**(6): 137-144.

Keller J., Watts S., Battye-Smith W. and Chong R. (2001). Full-scale demonstration of biological nutrient removal in a single tank SBR process. *Water Science and Technology*. **43**(3): 355-362.

Lai E., Senkpiel S., Solley D. and Keller J. (2004). Nitrogen removal of high strength wastewater via nitritation/denitritation using a sequencing batch reactor. *Water Science and Technology*. **50**(10): 27-33.

Lemaire R., Yuan Z.G., Yilmaz G. and Keller J. (submitted). A sequencing batch reactor system for high-level biological nitrogen and phosphorus removal from abattoir wastewater. *Chemosphere*.

Mace S., Dosta J., Gali A. and Mata-Alvarez J. (2006). Optimization of biological nitrogen removal via nitrite in a SBR treating supernatant from the anaerobic digestion of municipal solid wastes. *Industrial & Engineering Chemistry Research*. **45**(8): 2787-2792.

Munch E.V., Lant P. and Keller J. (1996). Simultaneous nitrification and denitrification in bench-scale sequencing batch reactors. *Water Research*. **30**(2): 277-284.

Pambrun V., Paul E. and Sperandio M. (2004). Treatment of nitrogen and phosphorus in highly concentrated effluent in SBR and SBBR processes. *Water Science and Technology*. **50**(6): 269-276.

Peng Y.Z., Chen Y., Peng C.Y., Liu M., Wang S.Y., Song X.Q. and Cui Y.W. (2004). Nitrite accumulation by aeration controlled in sequencing batch reactors treating domestic wastewater. *Water Science and Technology*. **50**(10): 35-43.

Peng Y.Z., Li Y.Z., Peng C.Y. and Wang S.Y. (2004a). Nitrogen removal from pharmaceutical manufacturing wastewater with high concentration of ammonia and free ammonia via partial nitrification and denitrification. *Water Science and Technology*. **50**(6): 31-36.

Puig S., Vives M.T., Corominas L., Balaguer M.D. and Colprim J. (2004). Wastewater nitrogen removal in SBRs, applying a step-feed strategy: from lab-scale to pilot-plant operation. *Water Science and Technology*. **50**(10): 89-96.

Saito T., Brdjanovic D. and van Loosdrecht M.C.M. (2004). Effect of nitrite on phosphate uptake by phosphate accumulating organisms. *Water Research*. **38**(17): 3760-3768.

Tilche A., Bacilieri E., Bortone G., Malaspina F., Piccinini S. and Stante L. (1999). Biological phosphorus and nitrogen removal in a full scale sequencing batch reactor treating piggery wastewater. *Water Science and Technology*. **40**(1): 199-206.

Travers S.M. and Lovett D.A. (1984). Activated-sludge treatment of abattoir wastewater .2. Influence of dissolved-oxygen concentration. *Water Research*. **18**(4): 435-439.

Turk O. and Mavinic D. (1986). Preliminary assessment of a shortcut in nitrogen removal from wastewater. *Can Journal of Civil Engineering*. **13**: 600-605.

Vadivelu V.M., Yuan Z.G., Fux C. and Keller J. (2006). The inhibitory effects of free nitrous acid on the energy generation and growth processes of an enriched Nitrobacter culture. *Environmental Science & Technology*. **40**(14): 4442-4448.

Villaverde S., Fdz-Polanco F. and Garcia P.A. (2000). Nitrifying biofilm acclimation to free ammonia in submerged biofilters. Start-up influence. *Water Research*. **34**(2): 602-610.

Villaverde S., GarciaEncina P.A. and FdzPolanco F. (1997). Influence of pH over nitrifying biofilm activity in submerged biofilters. *Water Research*. **31**(5): 1180-1186.

Wagner M., Rath G., Koops H.-P., Flood J. and Amann R. (1996). *In situ* analysis of nitrifying bacteria in sewage treatment plants. *Water Science and Technology*. **34**(1-2): 237-244.

Wareham D.G., Mavinic D.S. and Hall K.J. (1994). Sludge-digestion using ORP-regulated aerobic-nnoxic cycles. *Water Research*. **28**(2): 373-384.

Yilmaz G., Lemaire R., Keller J. and Yuan Z. (2007). Effectiveness of an alternating aerobic, anoxic/anaerobic strategy for maintaining biomass activity of BNR sludge during long-term starvation. *Water Research*. **41**(12): 2590-2598.

Yoshida Y., Takahashi K., Saito T. and Tanaka K. (2006). The effect of nitrite on aerobic phosphate uptake and denitrifying activity of phosphate-accumulating organisms. *Water Science and Technology*. **53**(6): 21-27.

Zeng R., Yuan Z. and Keller J. (2003). Enrichment of denitrifying glycogen-accumulating organisms in anaerobic/anoxic activated sludge systems. *Biotechnology and Bioengineering*. **81**(4): 397-404.

Zhou Y., Pijuan M. and Yuan Z. (in press). Free nitrous acid inhibition on anoxic phosphorus uptake and denitrification by poly-phosphate accumulating organisms. *Biotechnology and Bioengineering*.

Table S1. Enhanced ASM2d - reaction stoichiometry for soluble components

Process	S_{O2} $mgO_2\ l^{-1}$	S_F mgCOD l^{-1}	S_A mgCOD l^{-1}	S_{NH4} mg COD l^{-1}	S_{NO3} mgN l^{-1}	S_{NO2} mgN l^{-1}	S_{N2} mgN l^{-1}	S_P mgP l^{-1}	S_I mgCOD l^{-1}	S_{ALK} mmol l^{-1}
concentration of	oxygen	Fermentable COD	VFA COD	Ammonium + ammonia nitrogen	Nitrate nitrogen	Nitrite nitrogen	Dissolved N_2	Phosphate	Inert soluble COD	Alkalinity
1. Aerobic hydrolysis		$1-f_{SI}$		ν_{1,NH_4}				ν_{1,PO_4}	f_{SI}	ν_{1,ALK_4}
2. Anoxic hydrolysis		$1-f_{SI}$		ν_{2,NH_4}				ν_{2,PO_4}	f_{SI}	ν_{1,ALK_4}
3. Anaerobic hydrolysis		$1-f_{SI}$		ν_{3,NH_4}				ν_{3,PO_4}	f_{SI}	ν_{1,ALK_4}
4. Aerobic growth on S_F (X_H)	$1-1/Y_H$	$-1/Y_H$		ν_{4,NH_4}				ν_{4,PO_4}		ν_{1,ALK_4}
5. Aerobic growth on S_A (X_H)	$1-1/Y_H$		$-1/Y_H$	$-i_{NBM}$				$-i_{PBM}$		ν_{1,ALK_4}
6. Anoxic growth on S_F (X_H) $NO_3 \rightarrow NO_2$		$-1/Y_H$		ν_{6,NH_4}	$-\frac{(1-Y_H)}{(8/7\cdot Y_H)}$	$\frac{(1-Y_H)}{(8/7\cdot Y_H)}$		ν_{6,PO_4}		ν_{1,ALK_4}
7. Anoxic growth on S_F (X_H) $NO_2 \rightarrow N_2$		$-1/Y_H$		ν_{7,NH_4}		$-\frac{(1-Y_H)}{(1.72\cdot Y_H)}$	$\frac{(1-Y_H)}{(1.72\cdot Y_H)}$	ν_{7,PO_4}		ν_{1,ALK_4}
8. Anoxic growth on S_A (X_H) $NO_3 \rightarrow NO_2$			$-1/Y_H$	$-i_{NBM}$	$-\frac{(1-Y_H)}{(8/7\cdot Y_H)}$	$\frac{(1-Y_H)}{(1.72\cdot Y_H)}$		$-i_{PBM}$		ν_{1,ALK_4}
9. Anoxic growth on S_A (X_H) $NO_2 \rightarrow N_2$			$-1/Y_H$	$-i_{NBM}$		$-\frac{(1-Y_H)}{(1.72\cdot Y_H)}$	$\frac{(1-Y_H)}{(1.72\cdot Y_H)}$	$-i_{PBM}$		ν_{1,ALK_4}
10. Fermentation		-1	1	i_{NSF}				i_{PSF}		ν_{1,ALK_4}
11. Lysis				ν_{11,NH_4}				ν_{11,PO_4}		ν_{1,ALK_4}
12. Storage of X_{PHA}			-1					Y_{PO4}		ν_{1,ALK_4}
13. Aerobic storage of X_{PP}	$-Y_{PHA}$							-1		ν_{1,ALK_4}
14. Anoxic storage of X_{PP} $NO_3 \rightarrow NO_2$					$-\frac{Y_{PHA}}{(8/7)}$	$\frac{Y_{PHA}}{(8/7)}$		-1		ν_{1,ALK_4}
15. Anoxic storage of X_{PP} $NO_2 \rightarrow N_2$						$-\frac{Y_{PHA}}{1.72}$	$\frac{Y_{PHA}}{1.72}$	-1		ν_{1,ALK_4}
16. Aerobic growth of X_{PAO}	$1-1/Y_{PAO}$			$-i_{NBM}$				$-i_{PBM}$		ν_{1,ALK_4}
17. Anoxic growth of X_{PAO} $NO_3 \rightarrow NO_2$				$-i_{NBM}$	$\frac{(1-1/Y_{PAO})}{(8/7)}$	$-\frac{(1-1/Y_{PAO})}{(8/7)}$		$-i_{PBM}$		ν_{1,ALK_4}
18. Anoxic growth of X_{PAO} $NO_2 \rightarrow N_2$				$-i_{NBM}$		$\frac{(1-1/Y_{PAO})}{(1.72)}$	$-\frac{(1-1/Y_{PAO})}{(1.72)}$	$-i_{PBM}$		ν_{1,ALK_4}
19. Lysis of X_{PAO}				ν_{19,NH_4}				ν_{19,PO_4}		ν_{1,ALK_4}
20. Lysis of X_{PP}								1		ν_{1,ALK_4}
21. Lysis of X_{PHA}			1							ν_{1,ALK_4}
22. Aerobic growth of X_{AOB}	$\frac{-(3.43-Y_{AOB})}{Y_{AOB}}$			$-i_{NBM}-1/Y_{AOB}$		$1/Y_{AOB}$		$-i_{PBM}$		ν_{1,ALK_4}
23. Aerobic growth of X_{NOB}	$\frac{-(1.14-Y_{NOB})}{Y_{NOB}}$			$-i_{NBM}$	$1/Y_{NOB}$	$-1/Y_{NOB}$		$-i_{PBM}$		ν_{1,ALK_4}
24. Lysis of X_{AOB}				ν_{24,NH_4}				ν_{24,PO_4}		ν_{1,ALK_4}
25. Lysis of X_{NOB}				ν_{25,NH_4}				ν_{25,PO_4}		ν_{1,ALK_4}
26. Precipitation								-1		ν_{1,ALK_4}
27. Redissolution								1		ν_{1,ALK_4}

Table S2. Enhanced ASM2d - reaction stoichiometry for particulate components

Process	X_I mgCOD l^{-1}	X_S mgCOD l^{-1}	X_H mgCOD l^{-1}	X_{PAO} mgCOD l^{-1}	X_{PP} mgP l^{-1}	X_{PHA} mgCOD l^{-1}	X_{AOB} mgCOD l^{-1}	X_{NOB} mgCOD l^{-1}	X_{TSS} mgTSS l^{-1}	X_{MeOH} mgTSS l^{-1}	X_{MeP} mgTSS l^{-1}
concentration of	Inert particulate COD	Slowly biodegradable particulate COD	Ordinary heterotrophs	Poly-P accumulating organisms	Poly-P	PHA	Ammonia oxidising bacteria	Nitrite oxidising bacteria	Total suspended solids	Metal hydroxide	Metal phosphate
1. Aerobic hydrolysis		-1							i_{TSSBM}		
2. Anoxic hydrolysis		-1							i_{TSSBM}		
3. Anaerobic hydrolysis		-1							i_{TSSBM}		
4. Aerobic growth on S_F (X_H)			1						i_{TSSBM}		
5. Aerobic growth on S_A (X_H)			1						i_{TSSBM}		
6. Anoxic growth on S_F (X_H) $NO_3 \rightarrow NO_2$			1						i_{TSSBM}		
7. Anoxic growth on S_F (X_H) $NO_2 \rightarrow N_2$			1						i_{TSSBM}		
8. Anoxic growth on S_A (X_H) $NO_3 \rightarrow NO_2$			1						i_{TSSBM}		
9. Anoxic growth on S_A (X_H) $NO_2 \rightarrow N_2$			1								
10. Fermentation									$v_{10,TSS}$		
11. Lysis	f_{XIBM}	1- f_{XIBM}	-1						$v_{11,TSS}$		
12. Storage of X_{PHA}					-Y_{PO4}	1			$v_{12,TSS}$		
13. Aerobic storage of X_{PP}					1	-Y_{PHA}			$v_{13,TSS}$		
14. Anoxic storage of X_{PP} $NO_3 \rightarrow NO_2$					1	-Y_{PHA}			$v_{14,TSS}$		
15. Anoxic storage of X_{PP} $NO_2 \rightarrow N_2$					1	-Y_{PHA}			$v_{15,TSS}$		
16. Aerobic growth of X_{PAO}				1		-1/Y_{PAO}			$v_{16,TSS}$		
17. Anoxic growth of X_{PAO} $NO_3 \rightarrow NO_2$				1		-1/Y_{PAO}			i_{TSSBM}		
18. Anoxic growth of X_{PAO} $NO_2 \rightarrow N_2$				1		-1/Y_{PAO}			i_{TSSBM}		
19. Lysis of X_{PAO}	f_{XIBM}	1- f_{XIBM}		-1					$v_{19,TSS}$		
20. Lysis of X_{PP}					-1				-3.23		
21. Lysis of X_{PHA}						-1			-0.60		
22. Aerobic growth of X_{AOB}							1		i_{TSSBM}		
23. Aerobic growth of X_{NOB}								1	i_{TSSBM}		
24. Lysis of X_{AOB}	f_{XIBM}	1- f_{XIBM}					-1		$v_{24,TSS}$		
25. Lysis of X_{NOB}	f_{XIBM}	1- f_{XIBM}						-1	$v_{25,TSS}$		
26. Precipitation									1.42	-3.45	4.87
27. Redissolution									-1.42	3.45	-4.87

Table S3. Enhanced ASM2d - reaction kinetics.

Process	Kinetic rate
1. Aerobic hydrolysis	$k_H \cdot \frac{S_O}{K_O + S_O} \cdot \frac{X_S / X_H}{K_X + X_S / X_H} \cdot X_H$
2. Anoxic hydrolysis	$\eta_{FE} \cdot k_H \cdot \frac{K_O}{K_O + S_O} \cdot \frac{S_{NO_x}}{K_{NO_x} + S_{NO_x}} \cdot \frac{X_S / X_H}{K_X + X_S / X_H} \cdot X_H$
3. Anaerobic hydrolysis	$\eta_{FE} \cdot k_H \cdot \frac{K_O}{K_O + S_O} \cdot \frac{K_{NO_x}}{K_{NO_x} + S_{NO_x}} \cdot \frac{X_S / X_H}{K_X + X_S / X_H} \cdot X_H$
4. Aerobic growth on S_F (X_H)	$\mu_H \cdot \frac{S_O}{K_O + S_O} \cdot \frac{S_F}{K_F + S_F} \cdot \frac{S_F}{S_A + S_F} \cdot \frac{S_{NH_4}}{K_{NH_4} + S_{NH_4}} \cdot \frac{S_{PO_4}}{K_{PO_4} + S_{PO_4}} \cdot \frac{S_{ALK}}{K_{ALK} + S_{ALK}} \cdot X_H$
5. Aerobic growth on S_A (X_H)	$\mu_H \cdot \frac{S_O}{K_O + S_O} \cdot \frac{S_A}{K_A + S_A} \cdot \frac{S_A}{S_A + S_F} \cdot \frac{S_{NH_4}}{K_{NH_4} + S_{NH_4}} \cdot \frac{S_{PO_4}}{K_{PO_4} + S_{PO_4}} \cdot \frac{S_{ALK}}{K_{ALK} + S_{ALK}} \cdot X_H$
6. Anoxic growth on S_F (X_H) $NO_3 \rightarrow NO_2$	$\mu_H \cdot \eta_{NO_3} \cdot \frac{K_O}{K_O + S_O} \cdot \frac{S_{NO_x}}{K_{NO_x} + S_{NO_x}} \cdot \frac{S_{NO_3}}{S_{NO_2} + S_{NO_3}} \cdot \frac{S_F}{K_F + S_F} \cdot \frac{S_F}{S_A + S_F} \cdot \frac{S_{NH_4}}{K_{NH_4} + S_{NH_4}} \cdot \frac{S_{PO_4}}{K_{PO_4} + S_{PO_4}} \cdot \frac{S_{ALK}}{K_{ALK} + S_{ALK}} \cdot X_H$
7. Anoxic growth on S_F (X_H) $NO_2 \rightarrow N_2$	$\mu_H \cdot \eta_{NO_2} \cdot \frac{S_{NO_x}}{K_{NO_x} + S_{NO_x}} \cdot \frac{S_{NO_2}}{S_{NO_2} + S_{NO_3}} \cdot \frac{S_F}{K_F + S_F} \cdot \frac{S_F}{S_A + S_F} \cdot \frac{S_{NH_4}}{K_{NH_4} + S_{NH_4}} \cdot \frac{S_{PO_4}}{K_{PO_4} + S_{PO_4}} \cdot \frac{S_{ALK}}{K_{ALK} + S_{ALK}} \cdot X_H$
8. Anoxic growth on S_A (X_H) $NO_3 \rightarrow NO_2$	$\mu_H \cdot \eta_{NO_3} \cdot \frac{S_{NO_x}}{K_{NO_x} + S_{NO_x}} \cdot \frac{S_{NO_3}}{S_{NO_2} + S_{NO_3}} \cdot \frac{S_A}{K_A + S_A} \cdot \frac{S_A}{S_A + S_F} \cdot \frac{S_{NH_4}}{K_{NH_4} + S_{NH_4}} \cdot \frac{S_{PO_4}}{K_{PO_4} + S_{PO_4}} \cdot \frac{S_{ALK}}{K_{ALK} + S_{ALK}} \cdot X_H$
9. Anoxic growth on S_A (X_H) $NO_2 \rightarrow N_2$	$\mu_H \cdot \eta_{NO_2} \cdot \frac{K_O}{K_O + S_O} \cdot \frac{S_{NO_2}}{K_{NO_2} + S_{NO_2}} \cdot \frac{S_A}{K_A + S_A} \cdot \frac{S_A}{S_A + S_F} \cdot \frac{S_{NH_4}}{K_{NH_4} + S_{NH_4}} \cdot \frac{S_{PO_4}}{K_{PO_4} + S_{PO_4}} \cdot \frac{S_{ALK}}{K_{ALK} + S_{ALK}} \cdot X_H$
10. Fermentation	$q_{HFe} \cdot \frac{K_O}{K_O + S_O} \cdot \frac{K_{NO_x}}{K_{NO_x} + S_{NO_x}} \cdot \frac{S_F}{K_F + S_F} \cdot \frac{S_{ALK}}{K_{ALK} + S_{ALK}} \cdot X_H$
11. Lysis	$\left(b_{AerH} \cdot \frac{S_O}{K_O + S_O} + b_{AnoxH} \cdot \frac{K_O}{K_O + S_O} \cdot \frac{S_{NO_x}}{K_{NO_x} + S_{NO_x}} \right) \cdot X_H$
12. Storage of X_{PHA}	$q_{PHA} \cdot \frac{S_A}{K_A + S_A} \cdot \frac{S_{ALK}}{K_{ALK} + S_{ALK}} \cdot \frac{X_{PP} / X_{PAO}}{K_{PP} + X_{PP} / X_{PAO}} \cdot X_{PAO}$
13. Aerobic storage of X_{PP}	$q_{PP} \cdot \frac{S_O}{K_O + S_O} \cdot \frac{S_{PO_4}}{K_{PS} + S_{PO_4}} \cdot \frac{S_{ALK}}{K_{ALK} + S_{ALK}} \cdot \frac{X_{PHA} / X_{PAO}}{K_{PHA} + X_{PHA} / X_{PAO}} \cdot \frac{K_{MAX} \cdot X_{PP} / X_{PAO}}{K_{iPP} + K_{MAX} - X_{PP} / X_{PAO}} \cdot X_{PAO}$
14. Anoxic storage of X_{PP} $NO_3 \rightarrow NO_2$	$\rho_{13} \cdot \eta_{NO_3} \cdot \frac{K_O}{K_O + S_O} \cdot \frac{S_{NO_x}}{K_{NO_x} + S_{NO_x}} \cdot \frac{S_{NO_3}}{S_{NO_3} + S_{NO_2}}$
15. Anoxic storage of X_{PP} $NO_2 \rightarrow N_2$	$\rho_{13} \cdot \eta_{NO_2} \cdot \frac{K_O}{K_O + S_O} \cdot \frac{S_{NO_x}}{K_{NO_x} + S_{NO_x}} \cdot \frac{S_{NO_2}}{S_{NO_3} + S_{NO_2}}$
16. Aerobic growth of X_{PAO}	$\mu_{PAO} \cdot \frac{S_O}{K_O + S_O} \cdot \frac{S_{NH_4}}{K_{NH_4} + S_{NH_4}} \cdot \frac{S_{PO_4}}{K_{PO_4} + S_{PO_4}} \cdot \frac{S_{ALK}}{K_{ALK} + S_{ALK}} \cdot \frac{X_{PHA} / X_{PAO}}{K_{PHA} + X_{PHA} / X_{PAO}} \cdot X_{PAO}$
17. Anoxic growth of X_{PAO} $NO_3 \rightarrow NO_2$	$\rho_{16} \cdot \eta_{NO_3} \cdot \frac{K_O}{K_O + S_O} \cdot \frac{S_{NO_x}}{K_{NO_x} + S_{NO_x}} \cdot \frac{S_{NO_3}}{S_{NO_3} + S_{NO_2}}$
18. Anoxic growth of X_{PAO} $NO_2 \rightarrow N_2$	$\rho_{16} \cdot \eta_{NO_2} \cdot \frac{K_O}{K_O + S_O} \cdot \frac{S_{NO_x}}{K_{NO_x} + S_{NO_x}} \cdot \frac{S_{NO_2}}{S_{NO_3} + S_{NO_2}}$
19. Lysis of X_{PAO}	$\left(b_{AerPAO} \cdot \frac{S_O}{K_O + S_O} + b_{AnoxPAO} \cdot \frac{K_O}{K_O + S_O} \cdot \frac{S_{NO_x}}{K_{NO_x} + S_{NO_x}} \right) \cdot S_{ALK} / (K_{ALK} + S_{ALK}) \cdot X_{PAO}$
20. Lysis of X_{PP}	$\left(b_{AerPP} \cdot \frac{S_O}{K_O + S_O} + b_{AnoxPP} \cdot \frac{K_O}{K_O + S_O} \cdot \frac{S_{NO_x}}{K_{NO_x} + S_{NO_x}} \right) \cdot S_{ALK} / (K_{ALK} + S_{ALK}) \cdot X_{PAO}$
21. Lysis of X_{PHA}	$\left(b_{AerPHA} \cdot \frac{S_O}{K_O + S_O} + b_{AnoxPHA} \cdot \frac{K_O}{K_O + S_O} \cdot \frac{S_{NO_x}}{K_{NO_x} + S_{NO_x}} \right) \cdot S_{ALK} / (K_{ALK} + S_{ALK}) \cdot X_{PAO}$
22. Aerobic growth of X_{AOB}	$\mu_{AOB} \cdot \frac{S_O}{K_O + S_O} \cdot \frac{S_{NH_4}}{K_{NH_4} + S_{NH_4}} \cdot \frac{S_{PO_4}}{K_{PO_4} + S_{PO_4}} \cdot \frac{S_{ALK}}{K_{ALK} + S_{ALK}} \cdot X_{AOB}$
23. Aerobic growth of X_{NOB}	$\mu_{NOB} \cdot \frac{S_O}{K_O + S_O} \cdot \frac{S_{NO_2}}{K_{NO_2} + S_{NO_2}} \cdot \frac{S_{PO_4}}{K_{PO_4} + S_{PO_4}} \cdot \frac{S_{ALK}}{K_{ALK} + S_{ALK}} \cdot X_{NOB}$
24. Lysis of X_{AOB}	$b_{AerAOB} \cdot \frac{S_O}{K_O + S_O} + b_{AnoxAOB} \cdot \frac{K_O}{K_O + S_O} \cdot \frac{S_{NO_x}}{K_{NO_x} + S_{NO_x}} \cdot X_{AOB}$
25. Lysis of X_{NOB}	$\left(b_{AerNOB} \cdot \frac{S_O}{K_O + S_O} + b_{AnoxNOB} \cdot \frac{K_O}{K_O + S_O} \cdot \frac{S_{NO_x}}{K_{NO_x} + S_{NO_x}} \right) \cdot X_{NOB}$
26. Precipitation	$K_{PRE} \cdot S_{PO_4} \cdot X_{MeOH}$
27. Redissolution	$K_{RED} \cdot X_{MeP} \cdot S_{ALK} / (K_{ALK} + S_{ALK})$

Table S4: Definition of the stoichiometric coefficients and their ASM2d default values or selected values for the new coefficients (in bold).

Coefficient	Definition	Value	Unit
i_{NSI}	N content of inert soluble COD S_I	0.01	gN $gCOD^{-1}$
i_{NSF}	N content of fermentable soluble substrates S_F	0.03	gN $gCOD^{-1}$
i_{NXI}	N content of inert particulate COD X_I	0.02	gN $gCOD^{-1}$
i_{NXS}	N content of slowly biodegradable particulate substrate X_I	0.04	gN $gCOD^{-1}$
i_{NBM}	N content of biomass X_H, X_{PAO}, X_{AOB}, X_{NOB}	0.07	gN $gCOD^{-1}$
i_{PSI}	P content of inert soluble COD S_I	0	gP $gCOD^{-1}$
i_{PSF}	P content of fermentable soluble substrates S_F	0.01	gP $gCOD^{-1}$
i_{PXI}	P content of inert particulate COD X_I	0.01	gP $gCOD^{-1}$
i_{PXS}	P content of slowly biodegradable particulate substrate X_I	0.01	gP $gCOD^{-1}$
i_{PBM}	P content of biomass X_H, X_{PAO}, X_{AOB}, X_{NOB}	0.02	gP $gCOD^{-1}$
i_{TSSXI}	TSS to COD ratio for X_I	0.75	gTSS $gCOD^{-1}$
i_{TSSXS}	TSS to COD ratio for X_S	0.75	gTSS $gCOD^{-1}$
i_{TSSBM}	TSS to COD ratio for biomass X_H, X_{PAO}, X_{AOB}, X_{NOB}	0.90	gTSS $gCOD^{-1}$
f_{SI}	Production of S_I in hydrolysis	0	gCOD $gCOD^{-1}$
Y_H	Yield coefficient heterotrophs	0.625	gCOD $gCOD^{-1}$
Y_{PAO}	Yield coefficient PAO (biomass/PHA)	0.625	gCOD $gCOD^{-1}$
Y_{PO4}	Poly-P requirement (PO_4 release) per PHA stored	0.40	gP $gCOD^{-1}$
Y_{PHA}	PHA requirement for Poly-P storage	0.20	gCOD gP^{-1}
Y_{AOB}	**Yield coefficient AOB per NO_2^-**	**0.17**	**gCOD gN^{-1}**
Y_{NOB}	**Yield coefficient NOB per NO_3^-**	**0.07**	**gCOD gN^{-1}**
f_{XI}	Fraction of inert COD generated in biomass lysis X_H, X_{PAO}, X_{AOB}, X_{NOB}	0.10	gCOD $gCOD^{-1}$

Table S5: Definition of the kinetic parameters and their ASM2d default values at 20°C or selected values for the new parameters (in bold).

Parameters	Description	Default (20°C) or selected (in bold)	Units
k_H	Hydrolysis rate constant	3.00	d^{-1}
η_{NO3}	Anoxic hydrolysis reduction factor	0.60	
η_{Fe}	Anaerobic hydrolysis reduction factor	0.40	
K_{O2}	Saturation/inhibition coefficient for oxygen	0.20	$gO_2\ m^{-3}$
K_{NO3}	Saturation/inhibition coefficient for nitrate	0.50	$gN\ m^{-3}$
K_X	Saturation coefficient for particulate COD	0.10	$gXs\ gX_H^{-1}$
μ_H	Maximum growth rate on substrate	6.00	d^{-1}
q_{Fe}	Maximum rate for fermentation	3.00	$gS_F\ gX_H^{-1}\ d^{-1}$
b_H	Rate constant for lysis and decay	0.40	d^{-1}
K_{O2}	Saturation/inhibition coefficient for oxygen	0.20	$gO_2\ m^{-3}$
K_F	Saturation coefficient for growth on S_F	4.00	$gCOD\ m^{-3}$
Kfe	Saturation coefficient for fermentation of S_F	4.00	$gCOD\ m^{-3}$
K_A	Saturation coefficient for growth on acetate/propionic S_A	4.00	$gCOD\ m^{-3}$
K_{NO3}	Saturation/inhibition coefficient for nitrate	0.50	$gN\ m^{-3}$
$\mathbf{K_{NO2}}$	**Saturation/inhibition coefficient for nitrite**	**0.50**	**$gN\ m^{-3}$**
K_{NH4}	Saturation/inhibition coefficient for ammonium (Nutrient)	0.05	$gN\ m^{-3}$
K_P	Saturation/inhibition coefficient for phosphate (Nutrient)	0.01	$gP\ m^{-3}$
K_{ALK}	Saturation/inhibition coefficient for alkalinity (HCO_3^-)	0.10	$gHCO_3^-\ m^{-3}$
$\boldsymbol{\eta_{NO2}}$	**Anoxic(NO_2^-) reduction factor for denitrification**	**0.80**	
η_{NO3}	Anoxic(NO_3^-) reduction factor for denitrification	0.80	
$\boldsymbol{\mu_{AOB}}$	**Maximum growth rate of AOB**	**1.0**	**d^{-1}**
$\boldsymbol{\mu_{NOB}}$	**Maximum growth rate of NOB**	**1.0**	**d^{-1}**
$\boldsymbol{b_{AOB}}$	**Rate constant for lysis of X_{AOB}**	**0.15**	**d^{-1}**
$\boldsymbol{b_{NOB}}$	**Rate constant for lysis of X_{NOB}**	**0.15**	**d^{-1}**
K_{O2}	Saturation/inhibition coefficient for oxygen	0.20	$gO_2\ m^{-3}$
K_{AOBNH4}	Saturation coefficient for ammonium (AOB activity)	1.0	$gN\ m^{-3}$
$\boldsymbol{K_{NOBNO2}}$	**Saturation coefficient for nitrite (NOB activity)**	**1.0**	**$gN\ m^{-3}$**
K_{ALK}	Saturation/inhibition coefficient for alkalinity (HCO_3^-)	0.10	$gHCO_3^-\ m^{-3}$
K_P	Saturation/inhibition coefficient for phosphate (Nutrient)	0.01	$gP\ m^{-3}$
μ_{PAO}	Maximum growth rate of PAO	1.00	d^{-1}
q_{PP}	Rate constant for storage of X_{PP}	1.50	$gX_{PP}\ gX_{PAO}^{-1}\ d^{-1}$
q_{PHA}	Rate constant for storage of X_{PHA} (base X_{PP})	3.00	$gX_{PHA}\ gX_{PAO}^{-1}\ d^{-1}$
b_{PAO}	Rate constant for lysis of X_{PAO}	0.20	d^{-1}
b_{PP}	Rate constant for lysis of X_{PP}	0.20	d^{-1}
b_{PHA}	Rate constant for lysis of X_{PP}	0.20	d^{-1}
K_{O2}	Saturation/inhibition coefficient for oxygen	0.20	$gO_2\ m^{-3}$
K_{NO3}	Saturation/inhibition coefficient for nitrate	0.50	$gN\ m^{-3}$
$\mathbf{K_{NO2}}$	**Saturation/inhibition coefficient for nitrite**	**0.50**	**$gN\ m^{-3}$**
K_A	Saturation coefficient for growth on acetate/propionic S_A	4.00	$gCOD\ m^{-3}$
K_{NH4}	Saturation/inhibition coefficient for ammonium (Nutrient)	0.05	$gN\ m^{-3}$
K_{PS}	Saturation coefficient for phosphorous in storage of PP	0.20	$gP\ m^{-3}$
K_P	Saturation/inhibition coefficient for phosphate (Nutrient)	0.01	$gP\ m^{-3}$
K_{ALK}	Saturation/inhibition coefficient for alkalinity (HCO_3^-)	0.10	$gHCO_3^-\ m^{-3}$
K_{MAX}	Maximum ratio of X_{PP}/X_{PAO}	0.34	$gX_{PP}\ gX_{PAO}$
K_{IPP}	Inhibition coefficient for PP storage	0.02	$gX_{PP}\ gX_{PAO}$
K_{PHA}	Saturation coefficient for PHA	0.01	$gX_{PP}\ gX_{PAO}$
$\boldsymbol{\eta_{NO2}}$	**Anoxic(NO_2^-) reduction factor for denitrification**	**0.60**	
η_{NO3}	Anoxic(NO_3^-)reduction factor for denitrification	0.60	
k_{PRE}	Rate constant for P precipitation	1.00	$m^3(gFe(OH)_3)^{-1}\ d^{-1}$
K_{RED}	Rate constant for redissolution	0.60	d^{-1}
K_{ALK}	Saturation coefficient for alkalinity	0.50	$mole\ HCO_3^-m^{-3}$

Table S6: Wastewater composition used in simulation.

Parameter	Value	Unit
S_{O2}	0	mg l^{-1}
S_A	625	mgCOD l^{-1}
S_F	450	mgCOD l^{-1}
S_{NO3}	0	mgN l^{-1}
S_{NO2}	0	mgN l^{-1}
S_{N2}	15	mgN l^{-1}
S_{NH4}	160	mgN l^{-1}
S_P	40	mgP l^{-1}
S_{HCO}	73	mmol l^{-1}
S_I	225	mgCOD l^{-1}
X_I	100	mgCOD l^{-1}
X_S	200	mgCOD l^{-1}
X_H	10	mgCOD l^{-1}
X_{PAO}	0	mgCOD l^{-1}
X_{PP}	0	mgP l^{-1}
X_{PHA}	0	mgCOD l^{-1}
X_A	0	mgCOD l^{-1}
X_{MeOH}	0	mgSS l^{-1}
X_{MeP}	0	mgSS l^{-1}
X_{ash}	10	mgSS l^{-1}
X_{AOB}	0	mgCOD l^{-1}
X_{NOB}	0	mgCOD l^{-1}

Appendix C

Effectiveness of an Alternating Aerobic, Anoxic/Anaerobic Strategy for Maintaining Biomass Activity of BNR Sludge during Long-term Starvation

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ABSTRACT

The effectiveness of an aerobic, anoxic/anaerobic strategy for maintaining the activity of activated sludge performing biological nitrogen and phosphorus removal during long-term starvation is investigated. A lab-scale sequencing batch reactor (SBR) treating abattoir wastewater and achieving high-levels (>95%) of nitrogen, phosphorus and COD removal was used. The reactor was put twice into a so-called "sleeping mode" for a period of 5-6 weeks when the abattoir, where the wastewater was sourced, was closed down for annual maintenance. The "sleeping mode" operation consisted of 15 minutes aeration in a 6 hour SBR cycle. The sludge was allowed to settle in the remaining time of the cycle. The decay rates for ammonia oxidising bacteria (AOB) and nitrite oxidising bacteria (NOB) were determined to be 0.017 d^{-1} and 0.004 d^{-1}, respectively. These decay rates correlated well with AOB and NOB population quantified using molecular techniques (FISH). There was negligible phosphate accumulation in the reactor during the first 1-2 weeks of starvation, which was followed by a linear net release of phosphate in the remaining 4-5 weeks at a very slow rate of 1-2 $mgP.gVSS^{-1}.d^{-1}$. A sudden decrease in the aerobic activities of polyphosphate accumulating organisms (PAOs), observed via anaerobic/aerobic batch tests, occurred after two weeks of starvation. This correlated with a dramatic increase of several metal ions in the liquid phase. The underlying reasons are not clear. A resuscitation period with a gradual increase of the wastewater load was applied during the re-startup of the reactor after both "sleeping mode" periods. Each time, the performance of the reactor in terms of nitrogen and phosphorus removal fully recovered in four days.

Keywords: Activated sludge, decay, EBPR, nitrifier, PAO, recovery, starvation

INTRODUCTION

Several industry sectors such as the dairy and food processing industries, paper mills and abattoirs are known to be great consumers of water. As a result, large volumes of wastewater are produced and need to be treated before being released into the municipal sewage or the natural ecosystems. One of the challenges for such wastewater treatment plants (WWTPs) is to cope with the large fluctuations of the wastewater flow and composition inherent to the industrial activities. In some cases, low activity periods for the industry (e.g. annual maintenance or seasonal production variations) would even result in complete interruptions of wastewater flows to the WWTPs for weeks and even months. It is crucially important to maintain the viability of biomass during the long idle or starvation periods.

Many researchers have investigated the effects of starvation on the bacterial population and activities. The main discoveries to date are summarized below:

The availability of electron acceptor has a major impact on the bacterial decay rates. This has been well-demonstrated for nitrifiers (Nowak et al., 1994; Roslev and King, 1995; Siegrist et al., 1999; Morgenroth et al., 2000; Lee and Oleszkiewicz, 2003; Salem et al., 2006). It is now well established that nitrifiers decay at a higher rate under aerobic conditions than under anoxic or anaerobic conditions.

There is no or little loss (<5%) of nitrifying activities when activated sludge is starved under alternating aerobic, anoxic/anaerobic conditions for a period of up to one week (Morgenroth et al., 2000; Yuan et al., 2000; Lee and Oleszkiewicz, 2003). The growth of nitrifiers during aerobic period utilising the ammonia released through the decay of heterotrophic bacteria can largely compensate for the reduction of nitrifying activity caused by the decay of nitrifiers (Morgenroth et al., 2000). This may explain, at least partially, the observation made in Lee and Oleszkiewicz (2003) that nitrifiers decayed under alternating aerobic, anoxic conditions at a rate that is 40% lower than under purely anoxic conditions.

Lopez et al. (2006) investigated the activity of polyphosphate accumulating organisms (PAOs) during long term aerobic or anaerobic storage (up to 28 days). It was found that, similar to nitrifiers, PAOs also displayed a considerably lower decay rate under anaerobic condition than under aerobic condition. It confirmed the results reported in an earlier study (Brdjanovic et al., 1998) that excessive aeration of enhanced biological phosphorus removal (EBPR) processes under starvation condition was detrimental to PAOs.

These findings suggest that keeping the sludge under alternating aerobic, anaerobic/anoxic conditions would be a feasible strategy for maintaining the biomass activities during an extended idle period.

This paper evaluates the effectiveness of a specific operating strategy, which creates alternating anaerobic, anoxic and aerobic conditions, in maintaining the nutrient removal capacity of activated sludge. The simple, easily implementable strategy proposed and evaluated consists of providing 15 minutes aeration in every six hours with biomass allowed to settle in the remaining period. The activities of ammonia-oxidising bacteria, nitrite-oxidising bacteria as well as PAOs were closely monitored through weekly batch tests over two five-week starvation periods. The recovery processes of these organisms after the starvation were also investigated.

MATERIAL AND METHODS

Reactor set-up and operation

A lab-scale sequencing batch reactor (SBR) treating abattoir wastewater with consistently high level (>95%) removal of COD, nitrogen and phosphorus was used in this study. The SBR with a working volume of 7 l was seeded with sludge from a full-scale SBR treating abattoir wastewater. The SBR was operated with a cycle time of 6 h in a temperature-controlled room (18-22°C). In each cycle, 1 l of abattoir wastewater (composition summarised in Table 1) was pumped into the reactor under anoxic or anaerobic (when oxidised nitrogen was completely consumed) conditions, allowing for denitrification and phosphorus release. During the subsequent aerobic periods, air was provided intermittently using an on/off control system to keep the dissolved oxygen (DO) level between 1.5 and 2 $mgO_2.l^{-1}$. In these periods, nitrification and phosphorus uptake occurred. The hydraulic retention time (HRT) and sludge retention time (SRT) applied to the reactor were 42 h, and 15 days, respectively. The pH in the system was recorded but not controlled and fluctuated between 7.0 and 7.9 during a typical cycle.

Table 1. Characteristics of the SBR influent.

Parameter	**Mid-95% range** (n=67)
TCOD ($mg.l^{-1}$)	1,792-3,126
SCOD ($mg.l^{-1}$)	992-1,288
VFA ($mgCOD.l^{-1}$)	552-712
TN ($mg.l^{-1}$)	238-267
NH_4-N ($mg.l^{-1}$)	193-220
TP ($mg.l^{-1}$)	37-40
PO_4-P ($mg.l^{-1}$)	33-36

Due to annual maintenance, the abattoir closes down for up to six consecutive weeks each year. During that time, the supply of wastewater to the SBR was interrupted and the SBR cycle operation was modified according to the alternating aerobic, anoxic (or anaerobic when nitrate depleted) strategy. The new 6h cycle consisted of 15 min mixed aeration and 345 min settling periods. This operation is referred to as the “sleeping mode” operation in the sequel with distinction made between the first and the second “sleeping mode” operations (i.e. “sleeping mode I” and “sleeping mode II”, respectively) which were applied to the SBR a year apart. This starvation operation lasted for 30 consecutive days during “sleeping mode I” and for 44 days during “sleeping mode II”. During the first starvation period, the nitrifying activity was closely monitored through weekly pulse addition of ammonia and nitrite, while the activity of PAOs was only monitored by measuring the ortho-phosphate concentration in the liquid phase. During the second starvation period, the PAO activity was more comprehensively studied with weekly batch tests to monitor the anaerobic and aerobic activities of PAOs.

Experiments performed

Before each "sleeping mode"

The performance of the reactor was monitored through measuring the NH_4^+, NO_3^-, NO_2^-, PO_4^{3-}, COD and TSS concentrations in the effluent, and the MLSS and MLVSS concentrations in the reactor 3 times a week. The detailed performance was further monitored by weekly cycle studies, during which mixed liquor samples were taken from the reactor every 10 – 35 minutes over the 6h cycle, and filtered for NH_4^+, NO_3^-, NO_2^- and PO_4^{3-}, analysis.

During "sleeping mode I"

Short experiments (2h) were carried out once a week directly into the parent SBR to monitor the nitrification activity of the biomass by measuring the ammonium and nitrite oxidation rates after a pulse addition of ammonia or nitrite. During each of these experiments, the SBR was continuously aerated. 15 $mgN.l^{-1}$ of ammonium and 10 $mgN.l^{-1}$ of nitrite were added at the start and after 1h of aeration, respectively. Mixed liquor samples were taken and filtered every 10 min for analysis of NH_4^+, NO_3^- and NO_2^-. The pH was manually controlled in the range 7.4-7.6. The ammonia oxidation rate and nitrite oxidation rate were determined as the slopes of the ammonia and nitrite profiles, respectively. The decay rates of ammonia oxidising bacteria (AOB) and nitrite oxidising bacteria (NOB) were determined using Excel through fitting an exponential function to the measured profiles of ammonia and nitrite oxidation rates based on the least square optimisation algorithm. Between each nitrification experiment, liquid samples were regularly taken from the parent reactor to monitor the evolution of NH_4^+, NO_x^- and PO_4^{3-} concentrations.

During "sleeping mode II"

During the second starvation period, only the PAO activity was monitored through weekly anaerobic/aerobic batch tests carried out in a 200 ml reactor. At the start of the experiment, 200 ml of sludge was taken out from the parent SBR and acetate was added to reach a concentration of 100 $mgCOD.l^{-1}$. After 1h of anaerobic period, the 200 ml reactor was sparged with air for 1h. Samples were taken every 10 min for analysis of acetate and PO_4^{3-}. The pH was manually controlled in the range 7.4-7.6. The sludge was then returned to the parent SBR at the completion of each batch test. Liquid samples were regularly taken from the parent reactor to monitor PO_4^{3-} and cations concentrations.

After each "sleeping mode"

After each starvation period, the SBR normal operation was gradually resumed. On the first day, the reactor received only 50% of the normal wastewater feed, which was further increased to 75% on the second day and 100% on the fourth day. Cycle studies were performed during the first cycle on Day 1 (50% feed), the second cycle on Day 2 (75%) and the second cycle on Day 4 (100% feed).

Physico-chemical analyses

The ammonia ($NH_3 + NH_4^+$), nitrate (NO_3^-), nitrite (NO_2^-) and total phosphate (PO_4^{3-}-P) were analysed using a Lachat QuikChem8000 Flow Injection Analyser (Lachat Instrument, Milwaukee). Total and soluble chemical oxygen demand (TCOD and SCOD, respectively), total Kjeldahl nitrogen (TKN), total phosphorus (TP), mixed liquor suspended solid (MLSS) and volatile MLSS (MLVSS) were analysed according to the standard methods (APHA, 1995). VFAs were measured by Perkin-Elmer gas chromatography with column DB-FFAP

15m x 0.53mm x 1.0µm (length x ID x film) at 140°C, while the injector and FID detector were operated at 220°C and 250°C, respectively. High purity helium was used as carrier gas at a flow rate of 17 $ml.min^{-1}$. 0.9 ml of the filtered sample was transferred into a GC vial to which 0.1 ml of formic acid was added. Calcium, potassium, magnesium and sodium cations were measured by Inductively Coupled Plasma - Atomic Emission Spectrometry (ICP-AES Varian Vista-PRO, Varian, Inc.).

Microbial analyses

Sludge samples were fixed and FISH probed as previously described (Amann, 1995). Oligonucleotide probes used in this study were EUBmix (Daims et al., 1999) for all Bacteria, PAOmix (Crocetti et al., 2000) for *Accumulibacter* spp., NTSPA662 (Daims et al., 2001) for *Nitrospira* spp., NIT3 (Wagner et al., 1996) for *Nitrobacter* spp. and NSO1225 (Mobarry et al., 1996) for most of the β-proteobacterial ammonia oxidising bacteria (AOB). FISH images were collected using a Zeiss LSM 510 Meta Confocal microscope with a 63x Plan-Apochromat oil immersion lens. FISH quantification was performed according to (Crocetti et al., 2002) where the relative abundance of each group was determined as mean percentage of all bacteria.

RESULTS

Steady state operation before both starvation periods

The SBR was operated at steady state conditions for over one month before the first starvation period started and for more than a year before the second started. This SBR consistently achieved over 95% COD and N removal and 98% of P removal after reaching steady state conditions. Table 2 shows the concentration of NH_4^+, NO_x^- (= NO_2^- + NO_3^-) and PO_4^{3-} in the effluent during the month preceding each starvation period. NH_4^+ and PO_4^{3-} in the effluent were almost undetected, indicating that nitrification and phosphorus removal were complete. The average MLSS concentration and MLVSS/MLSS ratio were 4.5 $g.l^{-1}$ and 0.75, respectively before the "sleeping mode I" and 4.8 $g.l^{-1}$ and 0.73 before "sleeping mode II".

Table 2. Nutrient concentration in the effluent during the month of steady state operation before and after each "sleeping mode" study.

Nutrient	**"sleeping mode" I** (Mid-95% range)		**"sleeping mode" II** (Mid-95% range)	
	Before	**After**	**Before**	**After**
PO_4^{3-} ($mgP.l^{-1}$)	0.00-0.04	0.01-0.04	0.00-0.17	0.04-0.08
NH_4^+ ($mgN.l^{-1}$)	0.0-0.5	0.02-0.08	0.07-0.43	0.04-0.30
NO_x^- ($mgN.l^{-1}$)	7.2-12.2	2.9-4.6	1.6-3.3	1.8-3.7

Nitrifiers monitoring during "sleeping mode I"

Figure 1 shows the profiles of NH_4^+ and NO_2^- oxidation rates measured weekly during the first starvation period. These rates were calculated from the NH_4^+ and NO_2^- profiles measured during batch tests as explained previously. Initially, the NH_4^+ oxidation rate was slightly higher than the NO_2^- oxidation rate (19.3 $mgN.l^{-1}.h^{-1}$ compare to 17.8 $mgN.l^{-1}.h^{-1}$). However, after 33 days of "sleeping mode I" operation, the NH_4^+ oxidation rate decreased by 40% to 11.9 $mgN.l^{-1}.h^{-1}$ while the NO_2^- oxidation rate barely changed (16.1 $mgN.l^{-1}.h^{-1}$). The NH_4^+ oxidation rate therefore decreased 4 times quicker than the NO_2^- oxidation rate. The decay rates calculated from the NH_4^+ and NO_2^- oxidation rates (Figure 1) were 0.017 d^{-1} for AOBs and 0.004 d^{-1} for NOBs. Using FISH quantification techniques, the NOB population (*Nitrospira* spp. and *Nitrobacter* spp.) was estimated to be 3.1% of the total microbial population before the starvation period and 2.7% after that. The AOB population (most of the β-proteobacterial AOB) was estimated at 5.8% and 3.8% before and after the starvation period, respectively.

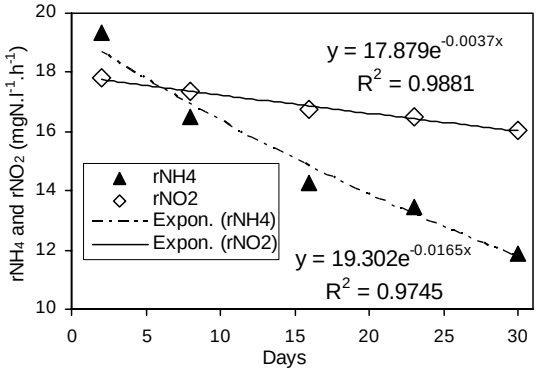

Figure 1. Variation of NH_4^+ and NO_2^- oxidation rates over the first starvation period and the best fits produced by the first-order decay model.

The measured NO_3^- concentration in the reactor during "sleeping mode I" is shown in Figure 2. NH_4^+ and NO_2^- concentrations were also monitored but almost undetectable in periods other than during the nitrification experiments and hence were not given in Figure 2. NO_3^- was present for most of the time so that the condition in the reactor was predominantly anoxic with short aerobic periods. The accumulation of NO_3^- seemed to have been primarily caused by the weekly nitrification experiments carried out with the addition of NH_4^+ and NO_2^-. It should be noted, however, that the total amount of NO_x^- produced during the 2 h oxidation experiment was 3.5 to 7 $mgN.l^{-1}$ higher than the total amount of NH_4^+ and NO_2^- added (i.e. 25 $mgN.l^{-1}$ in total, data not shown). It was likely caused by the oxidation of extra NH_4^+ coming from bacterial decay and from the slow breakdown of organic nitrogen under anoxic conditions. The denitrification rates over the period are also presented in Figure 2. The denitrification rate in the first week was significantly higher than that in the following weeks, suggesting that the biomass quickly ran out of carbon sources for denitrification.

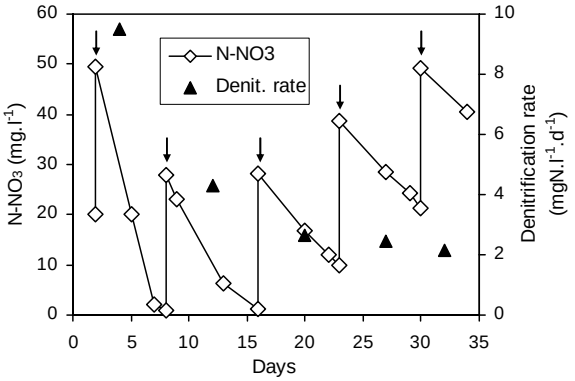

Figure 2. Nitrate profile during the starvation period and denitrification rates in periods between each nitrification experiment (shown by black arrows).

PAO activities during "sleeping mode II"

Figure 3 presents the anaerobic P-release rate, the aerobic P-uptake rate and the P-release:C-uptake ratio measured during each batch test throughout the starvation period. The values depicted are relative to their initial value at the start of the starvation on Day 0. Over the 43 days starvation period, the anaerobic P-release rate and aerobic P-uptake rate decreased by 70% and 60% respectively while the P-release:C-uptake ratio decreased by only 15%. In addition, Figure 3 shows the amounts of P released anaerobically and taken up aerobically during each anaerobic/aerobic batch test. It appears that the amount of P released in each batch test remained relatively constant (some slight increase after week 4). In contrast, the total amount of P taken up aerobically started to decrease sharply after 15 days of starvation and by the end of the starvation period, the amount of P taken up aerobically was only 30% of that released anaerobically.

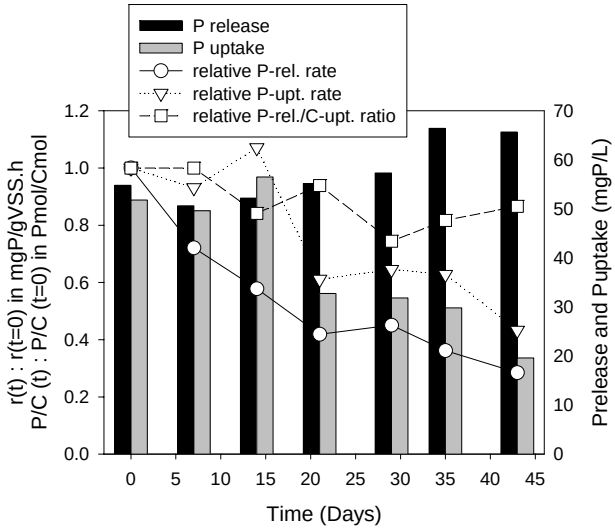

Figure 3. Anaerobic P-release rate, aerobic P-uptake rate and P-release/C-uptake ratio during anaerobic/aerobic batch tests, expressed relatively to their initial values at the start of the starvation. The total amounts of P released anaerobically and taken up aerobically during the batch tests are also depicted (black and grey bars).

The concentration of several cations (Ca^{2+}, K^{+}, Mg^{2+} and Na^{+}) in the liquid phase during the entire starvation period is presented in Figure 4. The very sharp release of those cations observed between Day 17 and Day 20 correlates well with the sharp decrease in the aerobic activities of PAOs reported in Figure 3. This release of cations occurred at the time PO_4^{3-} started to be released in the reactor (later depicted in Figure 5).

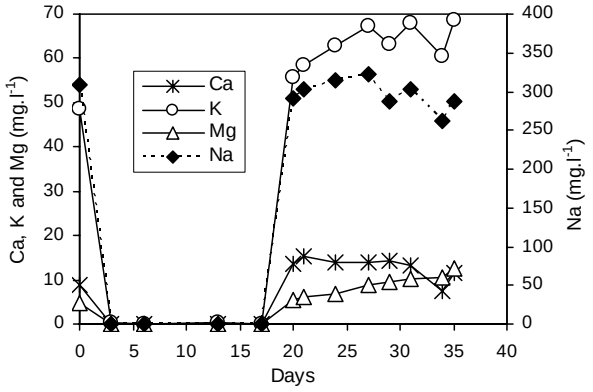

Figure 4. Profiles of some common cations (Ca^{++}, K^{+}, Mg^{++} and Na^{+}) in the SBR during the second starvation period.

Figure 5 compares the evolution of the MLSS, the volatile fraction of the biomass (i.e. VSS:MLSS ratio) and the PO_4^{3-} concentration in the SBR during both "sleeping mode" periods. The volatile fraction of the biomass was relatively constant during "sleeping mode I" and increased slightly during "sleeping mode II" by about 10% (Figure 5a and 5b). The evolution of the MLSS in the SBR was similar in the two "sleeping mode" periods with a final decrease of approximately 20% of their initial values (Figure 5a and 5b). In "sleeping mode I", the MLSS decreased sharply in the first week while in "sleeping mode II" it stayed relatively constant for the first 2 weeks and then started to decrease. This difference could be due to the different starvation conditions applied in the two periods (i.e. anoxic/aerobic for "sleeping mode I" and "anaerobic/aerobic for sleeping mode II"). The PO_4^{3-} profiles in the SBR during the two "sleeping mode" periods present similar trend, with an initial period of 7 days for "sleeping mode I" and 15 days for "sleeping mode II" with no accumulation of PO_4^{3-} followed by a period with a relatively linear accumulation of PO_4^{3-} (Figure 5c and 5d).

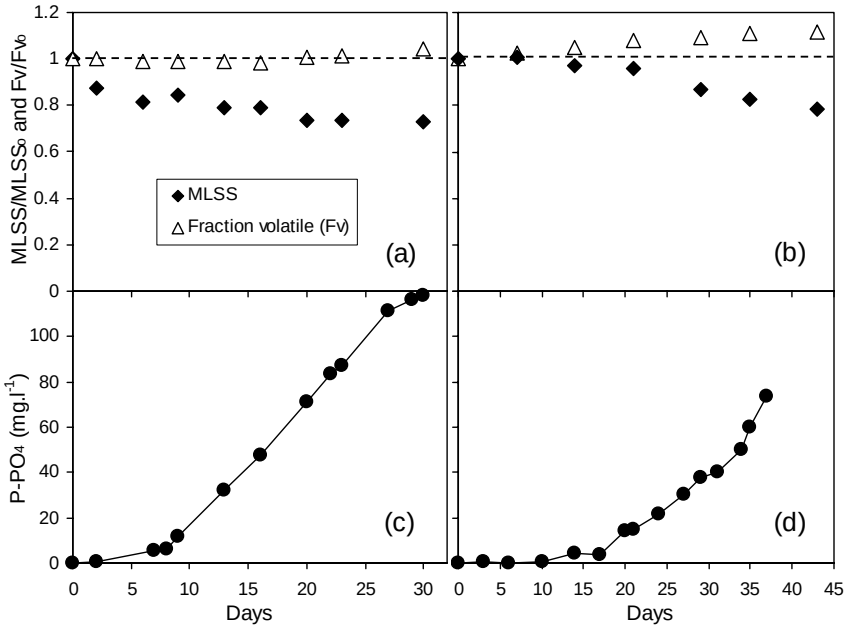

Figure 5. MLSS and volatile fraction (Fv) profiles relative to their initial values during **(a)** "sleeping mode I" and **(b)** "sleeping mode II". PO_4^{3-} profile in the SBR during **(c)** "sleeping mode I" and **(d)** "sleeping mode II".

Recovery after both "sleeping mode"

Table 3 compares the nitrification rate, the denitrification rate and the amount of P released and P uptake measured during SBR cycles shortly before the starvation period started, immediately after the completion of the starvation period (with 50% of normal wastewater load), two days after (with 75% of normal wastewater load) and four days after resuming wastewater feeding (with 100% of normal wastewater load) for both "sleeping mode" studies. The steady-state operations of the SBR prior to the two starvation experiments were quite different as a number of operating parameters had been changed for other research purposes. This resulted in a considerably higher nutrient removal performance of the process prior to "sleeping mode II" experiments. This is of no further relevance to this investigation as the behaviour in each starvation test is only related to the performance before the start of the test. After gradually resuming the normal reactor operation, the nitrification rate and denitrification rate quickly improved and reached their initial value within four days for both starvation periods (Table 3). The recovery of the P removal activity followed the same trend with the amount of P-release and P-uptake over a cycle returning to their initial level within four days after resuming wastewater feeding. The P removal activity was negligible in the first cycle after both starvation periods.

The good nutrient removal performances were consistently achieved for several months after both starvation periods. Table 2 summarizes the nutrient levels in the effluent during the months immediately after each starvation period. Notwithstanding the quick recovery of the reactor performance, it took around 30 days (2 sludge ages) for the MLSS to return to the original level in both starvation studies (data not shown).

Table 3. Nitrification rate (rNH_4^+), denitrification rate (rNO_x^-) and amounts of P released and uptaken over a cycle, measured during cycle studies performed before the start of the starvation period, immediately after the starvation period (50% of normal load), 2 days after starvation (75% of normal load) and 4 days after the starvation (100% of normal load).

Parameter monitored	rNH_4^+ ($mgN.l^{-1}.h^{-1}$)		rNO_x^- ($mgN.l^{-1}.h^{-1}$)		P-release ($mgP.l^{-1}$)		P-uptake ($mgP.l^{-1}$)	
"Sleeping mode" I or II	**I**	**II**	**I**	**II**	**I**	**II**	**I**	**II**
Before starvation	18.2	25.5	4.8	12.3	18.8	36.9	16.1	34.2
After 1st cycle (50%)	8.2	7.4	1.9	1.8	2.5	4.4	2.3	4.8
After 2 days (75%)	12.9	20.8	4.5	9.6	9.6	31	8.1	28.9
After 4 days (100%)	17.6	29.1	5.7	11.7	19.6	47	17.4	44.3

DISCUSSION

Nitrifiers decay rate under anoxic conditions with intermittent aeration

During the five batch tests, a sum of 75 mg NH_4-N and 50 mg NO_2-N were added to each litre of the mixed liquor. The AOB and NOB growth as a result of these additions is believed to be insignificant relative to the size of their populations, considering the fact that the SBR had a volumetric N load of approximately 150 $mgN.l^{-1}.d^{-1}$ during its normal operation. On the other hand, these ammonia and nitrite additions could have affected the maintenance metabolism of the nitrifiers as these bacteria were not truly maintained under starvation conditions, which could have impacted on the cell decay rate. However, one aim of aerating for 15 min in a 6 h cycle was to provide nitrifiers the opportunity to oxidise the nitrogen released from cell lysis and therefore avoid extended starvation conditions. The most significant impact caused by these additions was a change of the electron acceptor condition in the reactor. As shown in Figure 2, NO_3^- accumulated in the reactor during most of the time of the starvation period, which was caused by the addition of ammonia and nitrite during batch tests. It should be noted, however, that the NO_3^- measurements were carried out during periods when the reactor was mixed (15 min every 6h). Therefore, the measured NO_3^- concentration may not be representative to its level under the sludge blanket during settling periods, which constituted over 95% of the time over a cycle. It is likely that denitrification under the sludge blanket caused depletion of nitrate, resulting in localised anaerobic conditions. Unfortunately, the nutrient level under the sludge blanket was not measured directly. The decay rates determined during "sleeping mode I", 0.017 d^{-1} for ammonia oxidisers and 0.004 d^{-1} for nitrite oxidisers (Figure 1), were likely those of AOB and NOB under mainly alternating anoxic and anaerobic conditions with short intermittent aeration periods.

These decay rates are significantly lower than the decay rates generally reported in literature, including those obtained under anoxic or anaerobic conditions. Salem et al. (2006) presented a detailed comparison of nitrifier decay rates reported in various studies. Most studies only determined the AOB decay rate, which ranged between 0.15-0.21 d^{-1}, 0.025-0.06 d^{-1} and 0.05-0.2 d^{-1}, respectively, for activated sludge systems at 20°C (similar to the temperature used in this study) under aerobic, anaerobic and anoxic conditions. Salem et al. (2006) found that the NOB decay rates in an activated sludge system at 20°C were 0.21 d^{-1}, 0.06 d^{-1} and 0.12 d^{-1} under aerobic, anaerobic and anoxic conditions respectively.

The low decay rates found in this study, which were supported by the very quick recovery of nitrifying activity after the normal operation of the reactor was resumed (Table 3), were likely a result of the intermittent aeration strategy employed. Lee and Oleszkiewicz (2003) found

that the nitrifier decay rate under alternating aerobic and anoxic conditions was 62% and 40% lower than those under pure aerobic and anoxic conditions, respectively. Yuan et al. (2000) reported that the nitrifier decay rate was reduced from 0.08 d^{-1} to 0.03 d^{-1} in a storage tank when the aeration frequency was changed from 15 min in every 30 min to 15 min in every 45 min. The importance of short aeration periods during sludge storage was further described by Morgenroth et al. (2000). They suggested that intermittent aeration during anoxic storage of biomass would result in the removal of the potential toxic compounds such as H_2S that may be produced under anaerobic or anoxic conditions. However, Salem et al. (2006) recently studied the effect of H_2S on the nitrification activity of AOB and NOB and found that H_2S did not inhibit their activity and therefore was not considered to be toxic for nitrifiers. More recently, Mansar et al. (2006) reported an AOB and NOB decay rate of 0.015 d^{-1} and less than 0.001 d^{-1}, respectively, in a conventional activated sludge system under anoxic conditions for a period of 7 days. However, the starvation conditions used by Manser et al. (2006) were not completely anoxic as the reactor was aerated once a day for 5 min with the hypothesis that a complete lack of ATP for the nitrifiers would be prevented. The starvation conditions were indeed mainly anoxic with only limited aeration and were therefore relatively similar to that employed in this study, which could explain the similar low decay rates determined.

The relatively faster decay rate of AOB in comparison to NOB revealed by the process data in this study is not in agreement with Salem et al. (2006), who observed that the AOB and NOB decay rate is the same at the same corresponding conditions. Nevertheless, the results found in our study are supported by the FISH quantification results. Over the starvation period, the AOB population targeted by FISH probe NSO1225 decreased by some 40% which correlates well with the 40% decrease in the measured AOB activity. The NOB population targeted by probes NTSPA662 and NIT3 decreased by 13% only which also agrees well with the observed 10% decrease in the measured NOB activity. It should be noted, however, that the FISH quantification results should be interpreted qualitatively, as they are given as percentages of the size of the total bacteria population (targeted by the EUBmix probe) which is also subject to reduction during starvation. Also, FISH probes target the ribosome RNA, and consequently the signal intensity is proportional to the quantity of ribosome RNA present in cells rather than the number of cells.

PAOs activity under long term starvation condition

The behaviour of PAOs under long term starvation conditions has received limited attention to date. Lopez et al. (2006) investigated the decay rate and the activity of PAOs under maintained aerobic and anaerobic conditions for 4 weeks. The decay rate and activity of PAOs were both found to be considerably smaller under anaerobic conditions as compared to aerobic starvation conditions.

In this study, the monitoring of the PAO activity during “sleeping mode II” through anaerobic/aerobic batch tests revealed a sudden activity drop after about two weeks of mainly anaerobic starvation conditions with only brief aerobic periods. This sudden drop was simultaneously observed for (i) the P-uptake rate and amount of P taken-up (Figure 3), (ii) the release of cations in the parent SBR (Figure 4) and (iii) the beginning of PO_4^{3-} accumulation and decrease of biomass concentration in the SBR (Figure 5d and 5b). The exact reasons behind this sudden loss of activity are not clear.

The PO_4^{3-} profiles measured during the two starvation periods (Figure 5c and 5d) are very different from those reported in Lopez et al. (2006) under either aerobic or anaerobic conditions. During a four weeks anaerobic storage of an EBPR sludge, Lopez et al. (2006) observed that all the intracellular poly-phosphate was released in the first few days, and then P

release ceased for the remaining period of the study. In contrast, under aerobic condition, P was released at almost a constant rate over a three weeks period. The primary difference between the storage conditions used in this study and those used in Lopez et al. (2006) is that alternating aerobic, anoxic and anaerobic conditions rather than pure aerobic or anaerobic conditions were applied. An advantage of this strategy is that PAOs were provided conditions to perform their normal anaerobic and aerobic (or anoxic) metabolism. During anaerobic periods, which likely happened under the sludge blanket during settling periods, PAOs should be able to take up the VFAs produced by sludge hydrolysis and fermentation through the release of internal poly-phosphate. The formation of intracellular polyhydroxyalkanoates (PHA) should allow them to take up some of the phosphate released during the subsequent aerobic or anoxic period.

Two different rates of P release were observed during each "sleeping mode" (Figure 5c and 5d). The specific P release rate in the first week of "sleeping mode I" and in the first two weeks of "sleeping mode II" were calculated as 0.4 $mgP.gVSS^{-1}.d^{-1}$ and less than 0.1 $mgP.gVSS^{-1}.d^{-1}$, respectively. If we consider that *Accumulibacter* spp. (i.e. the only established organisms performing the PAO phenotype detected in the reactor using FISH techniques) constituted approximately 30% of the bacterial population prior to each starvation period (data not shown), the P release rates specific to the PAO biomass are roughly estimated to be 1.3 $mgP.gVSS^{-1}.d^{-1}$ and less than 0.3 $mgP.gVSS^{-1}.d^{-1}$ for "sleeping mode I" and "sleeping mode II", respectively. These estimations are considerably lower than the anaerobic P release rate required for maintenance reported in the literature (45 to 70 $mgP.gVSS^{-1}.d^{-1}$, see e.g. (Smolders et al., 1995; Filipe et al., 2001; Oehmen et al., 2005; Lopez et al., 2006). As discussed above, these low P release rates were likely due to the anaerobic and aerobic/anoxic recycling of phosphorus. Figure 5a shows that, during "sleeping mode I", the MLSS concentration decreased by 20% in the first week, implying that a considerable amount of COD was released in this period. This availability of COD supported denitrification at a relatively high rate as depicted in Figure 2, creating anaerobic conditions particularly under the sludge blanket during settling periods. Part of the COD released was likely recycled by PAOs, enabling these organisms to effectively keep their pools of poly-phosphate.

The relatively constant P release rates observed after one week of "sleeping mode I" and two weeks of "sleeping mode II" (2.1 $mgP.gVSS^{-1}.d^{-1}$ and 1 $mgP.gVSS^{-1}.d^{-1}$, respectively) were probably a consequence of maintenance processes and the lysis of PAO cells, both leading to release of PO_4^{3-}. Unfortunately, there are to our knowledge, no techniques at present to distinguish between the two processes. The relatively constant VSS:MLSS ratio suggests that the lysis of PAO cells could be the dominant process as it should lead to a proportional decrease of MLSS and VSS. When maintenance was the primary cause for P release, Lopez et al. (2006) reported that the volatile fraction of the biomass increased by 40% in two days of starvation. However, it is not possible to be conclusive here due to the complex nature of the VSS in the sludge used in this study. In addition to PAO biomass, it contains ordinary heterotrophs, nitrifiers, as well as other biodegradable or non-biodegradable organics originating from the wastewater or cell lysis.

Recovery of the process performance after long term starvation

When studying the impact of starvation on biomass activities, the most important questions to be answered from a practical point of view are (i) can the process fully recover when the starvation period ceases? (ii) how quickly can a full recovery occur?

The transient phase from a starved to a fully functional system is critical, and needs to be managed carefully. Even when an adequate amount of microorganisms is maintained in the

system through applying appropriate storage strategies, the level of intracellular materials such as enzymes and RNAs are likely to be very low, if not exhausted, due to maintenance mechanisms and stressful conditions, and need to be re-synthesised. Some cells may need to be 'waken up' through resuscitation strategies (Van Loosdrecht and Henze, 1999). Tappe et al. (1999) reported that five-day resuscitation allowed a *Nitrobacter* culture starved for 35 days to resume 50% of its original activity. A resuscitation period is believed to be even more important for PAOs, as their metabolism requires the cycling of phosphorus and carbon compounds under alternating anaerobic, aerobic or anoxic conditions. After an extended period of starvation, the pools of polyphosphate, PHA and glycogen, which are essential for the PAO metabolism (Smolders et al., 1994) are likely to be very low and need to be re-developed slowly.

The stepwise strategy adopted in this study was very successful as shown by the different activity rates depicted in Table 3. The nitrification rates in the first cycle after "sleeping mode I" and "sleeping mode II" were 50-70% lower than that obtained before each starvation started, but increased quickly to reach their previous level in just 4 days (Table 3). The complete nitrification observed throughout the whole month following the end of both starvation periods (Table 2) confirms that the recovery was permanent. The denitrification performance even improved after "sleeping mode I", as indicated by the lower level of NO_x^- in the effluent (Table 2). The reasons for this N removal improvement are not known.

The quick recovery of P removal after a long starvation period has not been widely reported in literature. The very low amount of P release and P uptake in the first cycle after the starvation (Table 3) is not unexpected. It was most likely due to the low level of polyphosphate and PHA in the PAO cells. The high level of NO_x^- in the reactor after "sleeping mode I" would have also inhibited the anaerobic VFA uptake by PAOs (Furumai et al., 1999). However, the amount of P-release and P-uptake measured 4 days after both long-term starvations are almost identical to those measured before starvations started (Table 3), suggesting PAOs had a full recovery. This recovery is, like for the nitrifiers, a genuine long term recovery as indicated by the excellent long term P removal performance observed after each starvation (Table 2).

CONCLUSION

Alternating anoxic/anaerobic and aerobic strategy is effective in maintaining the biomass activities of activated sludge performing biological nitrogen and phosphorus removal. Sludge can be stored under such conditions for at least six weeks with its nitrifying, denitrifying and phosphorus removal capabilities adequately maintained to allow for a quick recovery when wastewater feed resumes. While an intermittent aeration of 15 minutes in every 6 hours was used in this study and proved to be effective, the optimal aeration frequency and duration are yet to be identified through further experimental studies.

The resuscitation strategy of gradually increasing the wastewater load in the first few days after a starvation period was also demonstrated to be successful.

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REFERENCES

Amann R.I. (1995). In situ identification of microorganisms by whole cell hybridization with rRNA-targeted nucleic acid probes. In: Molecular Microbial Ecology Manual. Akkermans A.D.L., van Elsas J.D. and de Bruijn F.J. (eds). Dordrecht, Holland, *Kluwer Academic Publications*. **3.3.6:** 1-15.

APHA (1995). Standard methods for the examination of water and wastewater. Washington, DC, *American Public Health Association*.

Brdjanovic D., Slamet A., van Loosdrecht M.C.M., Hooijmans C.M., Alaerts G.J. and Heijnen J.J. (1998). Impact of excessive aeration on biological phosphorus removal from wastewater. *Water Research*. **32**(1): 200-208.

Crocetti G.R., Banfield J.F., Keller J., Bond P.L. and Blackall L.L. (2002). Glycogen accumulating organisms in laboratory-scale and full-scale activated sludge processes. *Microbiology*. **148**(11): 3353-3364.

Crocetti G.R., Hugenholtz P., Bond P.L., Schuler A., Keller J., Jenkins D. and Blackall L.L. (2000). Identification of polyphosphate-accumulating organisms and design of 16S rRNA-directed probes for their detection and quantitation. *Applied and Environmental Microbiology*. **66**(3): 1175-1182.

Daims H., Bruhl A., Amann R., Schleifer K.H. and Wagner M. (1999). The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: Development and evaluation of a more comprehensive probe set. *Systematic and Applied Microbiology*. **22**(3): 434-444.

Daims H., Nielsen J.L., Nielsen P.H., Schleifer K.H. and Wagner M. (2001). In situ characterization of Nitrospira-like nitrite oxidizing bacteria active in wastewater treatment plants. *Applied and Environmental Microbiology*. **67**(11): 5273-5284.

Filipe C.D.M., Daigger G.T. and Grady C.P.L. (2001). pH as a key factor in the competition between glycogen-accumulating organisms and phosphorus-accumulating organisms. *Water Environment Research*. **73**(2): 223-232.

Furumai H., Kazmi A.A., Fujita M., Furuya Y. and Sasaki K. (1999). Modeling long term nutrient removal in a sequencing batch reactor. *Water Research*. **33**(11): 2708-2714.

Lee Y. and Oleszkiewicz J.A. (2003). Effects of predation and ORP conditions on the performance of nitrifiers in activated sludge systems. *Water Research*. **37**(17): 4202-4210.

Lopez C., Pons M.N. and Morgenroth E. (2006). Endogenous processes during long-term starvation in activated sludge performing enhanced biological phosphorus removal. *Water Research*. **40**(8): 1519-1530.

Manser R., Gujer W. and Siegrist H. (2006). Decay processes of nitrifying bacteria in biological wastewater treatment systems. *Water Research*. **40**(12): 2416-2426.

Mobarry B.K., Wagner M., Urbain V., Rittmann B.E. and Stahl D.A. (1996). Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. *Applied and Environmental Microbiology*. **62**(6): 2156-2162.

Morgenroth E., Obermayer A., Arnold E., Bruhl A., Wagner M. and Wilderer P.A. (2000). Effect of long-term idle periods on the performance of sequencing batch reactors. *Water Science and Technology*. **41**(1): 105-113.

Nowak O., Schweighofer P. and Svardal K. (1994). Nitrification inhibition - a method for the estimation of actual maximum autotrophic growth-rates in activated-sludge systems. *Water Science and Technology*. **30**(6): 9-19.

Oehmen A., Zeng R.J., Yuan Z.G. and Keller J. (2005). Anaerobic metabolism of propionate by polyphosphate-accumulating organisms in enhanced biological phosphorus removal systems. *Biotechnology and Bioengineering*. **91**(1): 43-53.

Roslev P. and King G.M. (1995). Aerobic and anaerobic starvation metabolism in methanotrophic bacteria. *Applied and Environmental Microbiology*. **61**(4): 1563-1570.

Salem S., Moussa M.S. and van Loosdrecht M.C.M. (2006). Determination of the decay rate of nitrifying bacteria. *Biotechnology and Bioengineering*. **94**(2): 252-262.

Siegrist H., Brunner I., Koch G., Phan L.C. and Le V.C. (1999). Reduction of biomass decay rate under anoxic and anaerobic conditions. *Water Science and Technology*. **39**(1): 129-137.

Smolders G., Vandermeij J., Vanloosdrecht M. and Heijnen J. (1994). Model of the anaerobic metabolism of the biological phosphorus removal process - stoichiometry and pH influence. *Biotechnology and Bioengineering*. **43**(6): 461-470.

Smolders G.J.F., Klop J.M., van Loosdrecht M.C.M. and Heijnen J.J. (1995). A metabolic model of the biological phosphorus removal process I. Effect of sludge retention time. *Biotechnology and Bioengineering*. **48**(3): 222-233.

Tappe W., Laverman A., Bohland M., Braster M., Rittershaus S., Groeneweg J. and van Verseveld H.W. (1999). Maintenance energy demand and starvation recovery dynamics of Nitrosomonas europaea and Nitrobacter winogradskyi cultivated in a retentostat with complete biomass retention. *Applied and Environmental Microbiology*. **65**(6): 2471-2477.

Van Loosdrecht M.C.M. and Henze M. (1999). Maintenance, endogeneous respiration, lysis, decay and predation. *Water Science and Technology*. **39**(1): 107-117.

Wagner M., Rath G., Koops H.-P., Flood J. and Amann R. (1996). *In situ* analysis of nitrifying bacteria in sewage treatment plants. *Water Science and Technology*. **34**(1-2): 237-244.

Yuan Z.G., Bogaert H., Leten J. and Verstraete W. (2000). Reducing the size of a nitrogen removal activated sludge plant by shortening the retention time of inert solids via sludge storage. *Water Research*. **34**(2): 539-549.

Appendix D

Identifying Causes for N_2O Accumulation in a Lab-scale Sequencing Batch Reactor performing Simultaneous Nitrification, Denitrification and Phosphorus Removal

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ABSTRACT

The recently described process of simultaneous nitrification, denitrification and phosphorus removal (SNDPR) has a great potential to save capital and operating costs for wastewater treatment plants. However, the presence of glycogen accumulating organisms (GAOs) and the accumulation of nitrous oxide (N_2O) can severely compromise the advantages of this process. In this study, these two issues were investigated using a lab-scale sequencing batch reactor performing SNDPR over a five month period. The reactor was highly enriched in polyphosphate-accumulating organisms (PAOs) and GAOs representing around 70% of the total microbial community. PAOs were the dominant population at all times and their abundance increased while GAOs population decreased over the study period. Anoxic batch tests demonstrated that GAOs rather than denitrifying PAOs, were responsible for denitrification. N_2O accumulated from denitrification and more than half of the nitrogen supplied in a reactor cycle was released into the atmosphere as N_2O. After mixing SNDPR sludge with other denitrifying sludge, N_2O present in the bulk liquid was reduced immediately if external carbon was added. We therefore suggest that the N_2O accumulation observed in the SNDPR reactor is an artefact of the low microbial diversity facilitated by the use of synthetic wastewater with only a single carbon source.

Keywords: GAO, microbial diversity, N_2O, PAO, SBR, SNDPR.

INTRODUCTION

The general degradation of water quality in rivers and streams in both rural and urban areas has resulted in stronger actions taken towards preventing eutrophication by reducing nutrient levels in wastewater discharged into local waterways. On a global scale, pollution with greenhouse gases contributing to global warming is the focus of a large field of research, and the link between eutrophication and greenhouse gas (N_2O, CO_2, CH_4) emissions from natural systems (Brink et al., 2001) reinforces the motivation for removing nutrients from wastewater in an efficient and environmentally friendly way. However, biological wastewater treatment plants contribute to the emission of greenhouse gas. While CO_2 is an inevitable product from the breakdown of organic carbon, the much stronger greenhouse gas nitrous oxide (N_2O), an intermediate product of both nitrification and denitrification (Beline et al., 2001), may also be emitted from wastewater treatment processes. In the present study, we investigate and discuss the cause and prevention of N_2O emission from a novel biological nutrient removal process, from which the accumulation of N_2O had previously been identified as a major concern.

In conventional biological nutrient removal systems, ammonium (NH_4^+) is first oxidised to nitrite or nitrate (NO_x^-), which is then reduced to di-nitrogen gas in a two-stage aerobic and anoxic (O_2 absent, NO_x^- present) process (Metcalf & Eddy, 1991), while phosphorus (P) can be removed through enhanced biological phosphorus removal (EBPR). EBPR is based on the ability of polyphosphate-accumulating organisms (PAOs) to take up P and accumulate it intracellularly as polyphosphate when exposed to alternating anaerobic (O_2 and NO_x^- absent) and aerobic conditions (Comeau et al., 1986; Wentzel et al., 1988). An essential requirement for successful EBPR is to only provide carbon under anaerobic conditions in order to provide PAOs with a selective advantage, as other heterotrophic organisms cannot take up carbon in the absence of an electron acceptor. Unfortunately, these conditions are not compatible with the conditions required for N removal, and EBPR failure is regularly observed when NO_x^- is present in the designated anaerobic zone due to competition for carbon between denitrifying organisms and PAOs (Pitman et al., 1983).

It has been found that PAOs capable of denitrification (DPAOs) can perform P uptake with NO_x^- as electron acceptor (Kuba et al., 1993; Meinhold et al., 1999). This finding offers a possibility for removing N and P from wastewater, even with low COD:N and COD:P ratios, as the same carbon could be used for both denitrification and P removal. More recently, Zeng et al. (2003b) successfully demonstrated a process in an anaerobic-aerobic reactor where nitrification and denitrification occurs simultaneously with P uptake under low-oxygen concentration. This novel integrated process was called simultaneous nitrification, denitrification and phosphorus removal (SNDPR). In SNDPR, acetate was taken up under anaerobic conditions, accompanied by phosphorus release. During the following aerobic period, phosphorus was fully taken up, while NH_4^+ was converted through simultaneous nitrification and denitrification to gaseous nitrogen without accumulation of nitrite or nitrate intermediates. However, detailed off-gas analysis in several studies of the SNDPR process have shown that N_2O rather than N_2 was the major denitrification end-product (Zeng et al., 2003b; Meyer et al., 2005), which is of significant environmental concern due to the high global warming potential of N_2O. This finding significantly diminishes the overall benefits of the SNDPR process and limits the prospect of implementing this process in wastewater treatment plants.

This study seeks to characterise the SNDPR process with the aim to elucidate factors responsible for N_2O accumulation, and test how it can be eliminated. Emission of N_2O from SNDPR is the main obstacle for pursuing the full potential of this process, and it is therefore our goal to address how this issue can be managed.

MATERIAL AND METHODS

Reactor setup and operation

The biomass was enriched in a 5 l sequencing batch reactor (SBR) seeded with sludge from the Caboolture Sewage Treatment Plant, Queensland, Australia. The SBR was operated at room temperature (20-22°C) with a cycle time of 6 h, consisting of a 90 min anaerobic period, followed by 220 min aeration, 40 min settling, and 10 min decant. Three litres of synthetic wastewater was pumped into the reactor in the first 7 min of the anaerobic period, and 3 l supernatant was removed after settling, resulting in a hydraulic retention time (HRT) of 10 h. Before settling, 62.5 ml mixed liquor was wasted to keep the solids retention time (SRT) at approximately 20 days. Aeration was provided at a flow of 0.5 $l.min^{-1}$, using an on/off control system to keep the dissolved oxygen level at between 0.35-0.5 $mg.l^{-1}$. The pH in the system was recorded but not controlled, and fluctuated between 7.0 and 7.5. The synthetic wastewater fed in each cycle was prepared as described in Zeng et al. (2003b) with the adjustment of carbon, nitrogen and phosphorus to the level of 230 $mg.l^{-1}$ COD as acetate, 23 $mg.l^{-1}$ NH_4-N and 18 $mg.l^{-1}$ PO_4-P. The performance of the reactor was monitored by weekly cycle studies, during which samples for NH_4^+, PO_4^{3-}, and NO_x^- were taken regularly during the cycle and N_2O was measured on-line with a N_2O microsensor (see below). The N_2O concentration was measured with high temporal resolution, and these data were used for calculating the amount of N_2O stripped from the sludge during the cycle. As described above, the aeration of the reactor was controlled in an on/off manner. Assuming the N_2O transfer from the liquid to the gas phase is negligible during aeration off periods (due to the limited surface area in this reactor and the relatively high solubility of N_2O, 26.2 $mmol.l^{-1}$ at 22°C), the slopes of the N_2O data in these periods represent the net biological production (when slopes are positive) or consumption (when slopes are negative) rates of N_2O. Further assuming that these rates remain constant during each aeration on/off cycle as each cycle is of a period of 1-3 min only, the integration of the net biological production/consumption rates of N_2O over the entire aerobic period of the SBR operation yields a rough estimate of the amount of N_2O that was stripped during the entire aerobic period.

Physico-chemical analyses

Ammonia nitrogen (NH_4-N), nitrate nitrogen (NO_3-N), nitrite nitrogen (NO_2-N) and orthophosphate (PO_4-P) were analysed using a Lachat QuikChem8000 Flow Injection Analyser (Lachat Instrument, Milwaukee). Mixed liquor suspended solid (MLSS) and volatile MLSS (MLVSS) were analysed according to the standard methods of APHA (APHA, 1995). Acetate was measured by Perkin-Elmer gas chromatography with column DB-FFAP 15 m x 0.53 mm x 1.0 μm (length x ID x film) at 140°C while the injector and FID detector were operated at 220°C and 250°C respectively. Polyhydroxybutyrate (PHB), polyhydroxyvalerate (PHV) and glycogen were determined using the method described in Zeng et al. (2003b).

Oxygen mass transfer limitation

The gradient of oxygen in granules present in the reactor was measured with oxygen microsensors (tip diameter <10 μm), which were constructed as described by Revsbech et al. (1989). Granules were sampled from the parent reactor 1h into the aerobic period, at which time NH_4^+ was usually depleted, and transferred to flow-cell with an upward flow as described in Meyer et al. (2003). Replicate oxygen profiles were then measured over the

following hour. Profile measurements were analysed in the absence of NH_4^+ to simulate the conditions in the parent reactor after NH_4^+ depletion when oxygen was more likely to fully penetrate the flocs or granules.

The granules analysed with oxygen microsensors were larger than 500 μm in diameter, as the floccular biomass was not amenable to microsensor analysis. The sensor was moved stepwise into the granules from above, and movement of the sensor and data acquisition was obtained with the software Profix (Unisense A/S, Aarhus, Denmark). The composition of medium in the flowcell was identical to that in the parent reactor at the end of the anaerobic period except for NH_4^+. The oxygen concentration was adjusted within the nutrient medium by controlling the air/nitrogen ratio in the gas inlet.

Microbial analyses

Sample fixation and FISH experiments were performed as previously described in Amann (1995). Oligonucleotide probes used in this study were EUBmix for the detection of all bacteria (Daims et al., 1999), PAOmix for "*Candidatus* Accumulibacter phosphatis*"* (referred to as *Accumulibacter* in the following) (Crocetti et al., 2000) and GAOQ989 (Crocetti et al., 2002) and GB_G2 (Kong et al., 2002) for "*Candidatus* Competibacter phosphatis" (referred to as *Competibacter* in the following). FISH quantification was performed using previously published method in Crocetti et al. (2002).

Batch tests

Measurement of net N_2O production and consumption by denitrification in SNDPR sludge with nitrate or nitrite as electron acceptors

Sludge was sampled at the end of the anaerobic period from the parent reactor and amended with KNO_3 or $NaNO_2$ to reach a concentration of 7.5 $mgN.l^{-1}$ of NO_3^- or NO_2^- before being transferred to two 14.75 ml vials sealed with rubber stoppers to which a N_2O microsensor was inserted for on-line monitoring of the N_2O concentration. The mixed liquor was stirred using a magnetic stirrer, and the vials were filled completely to avoid any exchange of N_2O between liquid and gas phases. The two mini-reactors were operated in parallel with one acting as a negative control or a duplicate. A sample was taken at the end of the experiment for NO_x^- analysis to confirm the depletion of nitrite or nitrate from denitrification. Substrates could be added in small amounts at any time during experimentation with a syringe through the rubber stopper. The N_2O microsensors used in these experiments were constructed according to Andersen et al. (2001). The maximum N_2O net production rate was calculated from the very first part of the graph when N_2O accumulated almost linearly, whereas the gross consumption rate was calculated from the last part of the graph when nitrate or nitrite was depleted and the N_2O concentration started to decrease. Both rates were corrected for MLVSS variations in different batch tests.

Measurement of net N_2O production and consumption by denitrification in mixed SNDPR and denitrifying sludges in presence of external carbon sources.

To investigate how the presence of denitrifying bacteria other than those found in the SNDPR sludge affect N_2O accumulation, batch tests were performed in duplicate with a mixture of sludge from the SNDPR reactor and a lab-scale nitrifying-denitrifying reactor treating domestic wastewater containing additional carbon in the form of methanol for denitrification.

The SNDPR sludge was sampled at the end of the anaerobic period, mixed with the denitrifying sludge in a 1:2 ratio, and amended with KNO_3 to a concentration of 7.5 $mgN.l^{-1}$

of NO_3^- at time=zero, before being transferred to the two sealed vials for monitoring of the N_2O concentration. After 10 min, methanol was injected into one of the vials to a concentration of 30 mgCOD.l^{-1}. The addition of external carbon was delayed to allow observation of N_2O accumulation when only bacteria with intracellular PHA were active, before studying the effect of other denitrifying bacteria on N_2O accumulation after external carbon was added. As a negative control, this experiment was repeated with SNDPR sludge only.

It could be argued that adding methanol to a methanol-adapted sludge would bias the results from the experiment detailed above, and therefore the effect of adding real wastewater to SNDPR sludge mixed with denitrifying sludge that had not been adapted to a particular carbon source was also tested. This was done in a 500 ml reactor from which NO_x^- samples could be taken during the experiment. SNDPR sludge was sampled at the end of the anaerobic period, mixed in a 1:2 ratio with sludge from a denitrifying SBR treating domestic wastewater, and transferred to the 500 ml reactor. The reactor was sealed at the top and a N_2O microsensor was mounted through the lid for on-line measurements, but it should be noted that the headspace in the reactor would cause some stripping of N_2O to the gas phase during the experiment. Prior to the experiment, helium was sparged for 10 min to obtain anaerobic conditions before nitrate was added to a concentration of 7.5 mgN.l^{-1} of NO_3^- at Time=zero. After 35 min, 50 ml of high-strength wastewater from meat processing industry (approx. 6000 mgCOD.l^{-1}) was added to the mixed liquor.

Denitrification test

To determine whether PAOs were contributing to denitrification in the reactor, batch tests were performed to measure if denitrification was accompanied by P uptake under strictly anoxic conditions. Sludge (500 ml) was taken from the SNDPR reactor at the end of the anaerobic period and placed in the same 500 ml reactor described before mounted with a N_2O microsensor. After 10 min of helium sparging, 5 ml of $NaNO_3$ or $NaNO_2$ solution was added to obtain an initial concentration of 8 mgN.l^{-1} of NO_3^- or NO_2^- in the reactor. Liquid samples were taken at regular intervals to monitor the PO_4^{3-} , NH_4^+ and NO_x^- concentration.

RESULTS and DISCUSSION

Process and microbial characterisation

Over the 5 months operation period, MLSS varied between 3.94 and 4.64 g.l^{-1} with a standard deviation (S.D) of the mean of 0.23, and MLVSS varied between 2.18 and 2.7 g.l^{-1} with a S.D. of 0.21. The microbial community consisted mostly of *Accumulibacter* PAOs and *Competibacter* GAOs, where the sum of these two populations accounted for 63 to 78.5% of the biomass. *Accumulibacter* increased in abundance from 45 to 70% over the 5 months operational period while the abundance of *Competibacter* decreased from 17.6% to only 8.6% of the total population.

Weekly cycle studies showed little variation in the performance of the reactor in June- July (the first 2 months), but the performance gradually changed in the months of August to October towards increasing NO_x^- level present at the end of the reactor cycle. Figure 1 shows two cycle studies representing the stable period from June to July (Figure 1a), and the period after a gradual increase in the effluent NO_x^- concentration from August to October (Figure 1b). During the anaerobic period of the reactor cycle, acetate was rapidly consumed and stored as PHA (PHB and PHV), which was accompanied by release of phosphorus and

the consumption of glycogen (see Figure 1b). During the aerobic period, phosphorus was completely taken up, PHA was oxidised and the glycogen pool was replenished (Figure 2b). Ammonium was removed without accumulation of NO_x^- in the June-July period (Figure 1a), but in the August-October period, NO_x^- accumulated in the liquid (Figure 1b).

A transient accumulation of N_2O was observed in the aerobic period throughout the study (Figure 1a and b). The N_2O concentration peaked at the same time as NH_4^+ was depleted in the June-July test (Figure1a) and slightly earlier in the August-October test (Figure 1b). The accumulated N_2O was eventually removed before the end of the cycle for both periods. Stripping of N_2O occurred during the aerobic period was estimated to be 27.5 mgN, corresponding to 51% of the N removed in each cycle. Although SNDPR has been proposed as an economically feasible process (Zeng et al., 2003b), the apparent emission of N_2O poses an environmental problem which hinders its implementation in wastewater treatment.

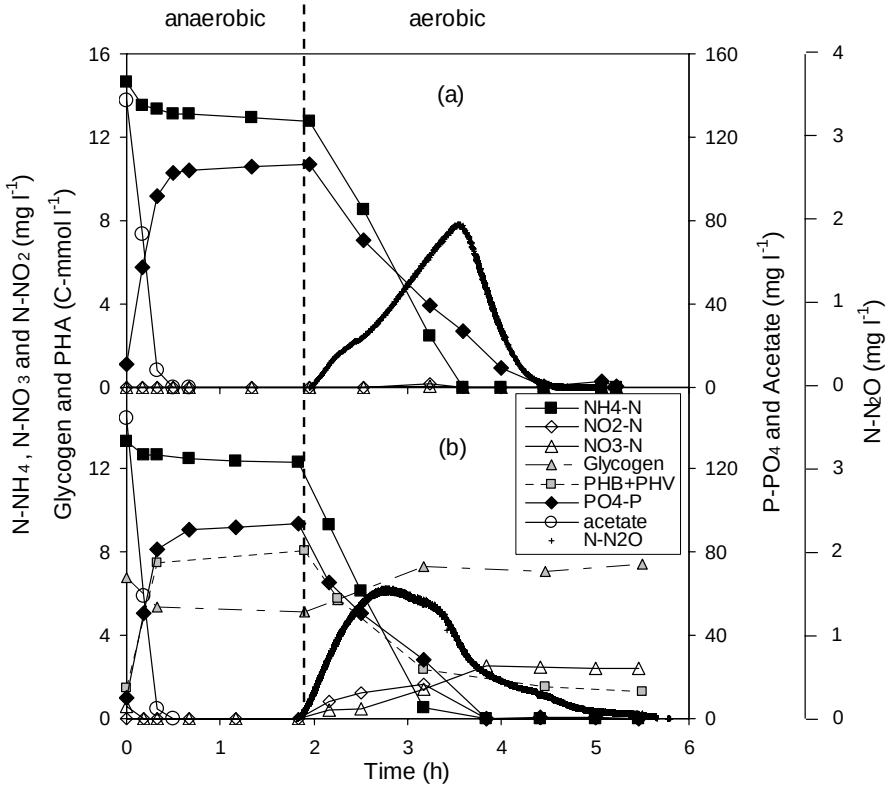

Figure 1. Typical weekly cycle study of the parent SBR **(a)** for the first 2 months (June-July) when complete N removal was achieved and **(b)** in September when the reactor showed only partial removal of N. The dashed line indicates the transition from the anaerobic to the aerobic period.

Factors affecting N_2O accumulation

Nitrous oxide can potentially accumulate from both nitrification and denitrification (Beline et al., 2001), however, accumulation of N_2O under strictly anoxic conditions (Figure 2) indicates that denitrification was the main source of N_2O in the present study. Di-nitrogen gas is most often the predominant product of denitrification and N_2O is rarely monitored in wastewater treatment systems.

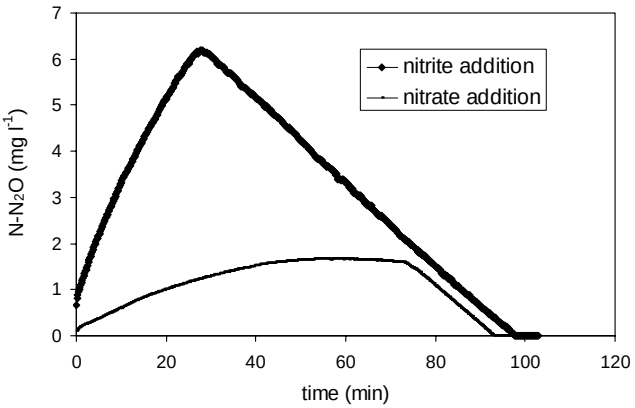

Figure 2. N_2O concentration during anoxic batch tests (14.75 ml sealed reactors) amended with either nitrate or nitrite (7.5 $mgN.l^{-1}$) at T=0.

A number of factors have been suggested to cause N_2O accumulation from denitrification in activated sludge. Hanaki et al. (1992) reported N_2O production during denitrification in the presence of low COD:N ratio (3.5), low pH (6.5), and short solids retention time (<1 day). Kishida et al. (2004) also reported N_2O production under low COD:N ratio (2.6). The SNDPR reactor in our study was operated at a COD:N ratio of 10, pH of 7-7.5, and a solids retention time of 20 days. As such, these factors are therefore not believed to affect the N_2O production in our study.

The N_2O reductase is very sensitive to oxidative stress (Otte et al., 1996; Noda et al., 2003), and denitrification occuring at low oxygen concentrations may therefore lead to N_2O accumulation, as it has been shown in pure culture studies (Otte et al., 1996). As SND relies on an oxygen gradient into the biomass, it is possible that denitrification takes place under microaerobic conditions. However, the N_2O accumulation observed under completely anoxic conditions (Figure 2) excludes oxygen as the single causing factor.

Another possible cause for N_2O production is the presence of nitrite. Zeng et al. (2003b) suggested that nitrite accumulation at even at very low levels triggered the N_2O production observed in their study. Itokawa et al. (2001) also reported nitrite accumulation as a possible cause of N_2O production in a denitrifying sludge, yet hypothesised that the accumulation may be in combination with denitrification based on intracellular carbon. The potential rates of net N_2O production and consumption from denitrification investigated in anoxic, sealed batch reactors with on-line monitoring of N_2O, showed that the maximum net production rates of N_2O varied greatly with the electron acceptor provided (Table 1). Addition of nitrite to the sludge led to a 5 times higher net N_2O production rate, as compared to nitrate addition. Figure 2 shows, as an example, one of 5 replicate batch tests. The peak concentration of N_2O corresponded to 77% of the N added in the incubations with nitrite, whereas only 26% of the N added as nitrate accumulated as N_2O. When accumulation of N_2O stopped, there was no significant difference between the N_2O reduction rates measured in the different incubations (Table 1), and these rates were similar to N_2O reduction rates obtained when only N_2O was added as electron donor (data not shown). These results could suggest an effect of nitrite on N_2O production. However, a difference in the rate of nitrate and nitrite reduction could also affect the accumulation of N_2O if nitrate reduction is the rate limiting step. The two anoxic batch tests performed with nitrate or nitrite as electron acceptors in the non-sealed 500 ml reactor allowed NO_x^- samples to be taken simultaneously to N_2O measurements. Figure 3a

and 3b show that the nitrite reduction rate was twice as high as the nitrate reduction rate and nitrite did not accumulate in the batch test amended with nitrate. Nitrate reduction being the rate limiting step may therefore explain the differences in N_2O accumulation rate observed in Figure 2. The profile showing the sum of nitrogen oxides (NO_x^- plus N_2O) in Figure 3b suggests that high concentrations of nitrite (> approx. 5 $mgN.l^{-1}$) may inhibit N_2O reduction as the concentration of nitrogen oxides did not decrease in the first 10 min, indicating that nitrite was only reduced to N_2O in this period. After the 10 min, an inflection point in the N_2O concentration curve was observed, indicating a lower net N_2O production rate. At the same time, the nitrogen oxide concentration began to decrease, and the inflection point therefore reflected the onset of N_2O reduction. It should be noted that N_2O accumulated even in the absence of nitrite (Figure 3a), and the presence of nitrite cannot be the sole factor responsible for the accumulation of N_2O.

Table 1. Production and consumption rates of N_2O ($\mu mol.h^{-1}.gVSS^{-1}$) by the SNDPR sludge.

Electron acceptor	**Mean N_2O net production rate** ± S.E. (n=5)	**Mean N_2O gross consumption rate** ± S.E. (n=5)
NO_2^-	201.5 ± 14.2	90.8 ± 4.7
NO_3^-	37.7 ± 4.2	86.7 ± 5.5

In addition to the physico-chemical factors that may trigger N_2O accumulation, the faster reduction of nitrite as compared to N_2O may simply be related to the enzyme kinetics of the organism(s) carrying out denitrification in the reactor. Nitrous oxide accumulation has previously been observed in SNDPR reactors (Zeng et al., 2003b; Meyer et al., 2005) and in reactors enriched in denitrifying glycogen-accumulating organisms (DGAO) (Zeng et al., 2003a). These reactors share the feature of performing denitrification using intracellular carbon, which was taken up under anaerobic conditions where NO_x^- was absent. The DGAO and the SNDPR reactors could therefore harbour similar denitrifying organisms, and the observed N_2O accumulation could be linked either to the phenotype of denitrification from stored carbon, or to the particular denitrifying organism enriched in these systems. Several attempts were made in the present study to investigate N_2O accumulation from denitrification in the presence of an external carbon source by adding acetate or propionate to one of two parallel anoxic batch tests that were otherwise identical to the tests shown in Figure 2. Addition of carbon led to a substantial increase in N_2O accumulation, and it was found that acetate and propionate was first accumulated intracellularly as PHA before being used as electron donor in denitrification (data not shown). A true test of denitrification using extracellular versus intracellular carbon could therefore not be performed.

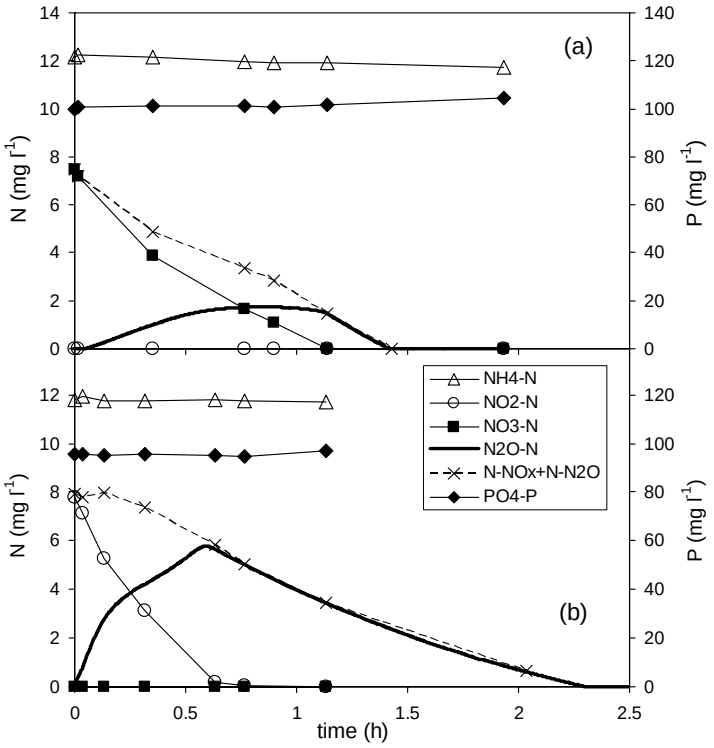

Figure 3. Measurements of NH_4^+, NO_3^-, NO_2^-, N_2O, and PO_4^{3-} during anoxic batch tests (500 ml reactor) with either nitrate **(a)** or nitrite **(b)** addition. It should be noted that some stripping of N_2O occurred during these batch tests.

Organisms responsible for denitrification

The role of PAOs versus GAOs in the denitrification process was specifically addressed by investigating the ability of the SNDPR sludge to simultaneously reduce NO_x^- and take up PO_4^{3-} under strictly anoxic conditions. Batch tests showed that PO_4^{3-} was not taken up by the biomass under conditions where NO_x^- reduction occurred, using both nitrite and nitrate as electron acceptors (Figure 3a and 3b). Although PAOs have the potential to denitrify (Kuba et al., 1993; Meinhold et al., 1999), they did not appear to play a role in denitrification in this reactor. As carbon was only available to organisms capable of anaerobic acetate uptake, GAOs were therefore assumed to be responsible for denitrification.

The role of GAOs in denitrification was further supported by the developments in denitrification performance and the abundance of PAOs and GAOs over the study period. Figure 4 shows the abundance of *Accumulibacter* and *Competibacter* at five sampling times during the 5 months period, the N removal efficiency and the P release to acetate uptake ratio over the same period. While *Accumulibacter* increased from 46 to 70% of the total biomass, *Competibacter* decreased from 18 to 9%. It is evident that *Accumulibacter* became more abundant and more active, as the P release to acetate uptake ratio increased over the period to reach 0.69 Pmol.Cmol^{-1} on day 140. This ratio reflects the fraction of carbon being taken by PAOs relative to GAOs, and it was in this case higher than what was reported in previous studies (0.5 Pmol.Cmol^{-1}) of lab-scale reactors enriched in PAOs using acetate as the sole carbon source (Smolders et al., 1994; Oehmen et al., 2004). The increase in PAO abundance and PAO activity was accompanied by a decrease in the abundance of *Competibacter,* which

halved its population over the 5 months period. If *Competibacter* was primarily responsible for denitrification, the decline in their population can explain the deterioration of the N removal efficiency from 100% to 53% over the same period.

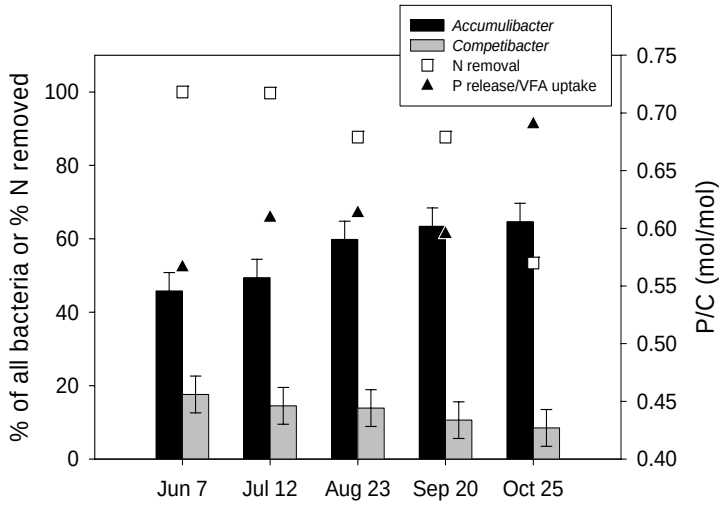

Figure 4. Abundance of *Accumulibacter* and *Competibacter* correlated with the N removal efficiency and the carbon-uptake to P-release ratio over the 5 month period.

It should be noted that denitrification in the SNDPR process relies on the formation of anoxic zones in the central part of the microbial aggregates caused by the mass transfer limitation of oxygen. Lack of anoxic zones caused by a change in floc size or oxygen uptake rate of the aggregates could therefore also cause the observed effect on denitrification. Measurement of the oxygen penetration into aggregates during the June-July and the August-October periods did, however, confirm that anoxic microzones were present in the aggregates throughout the study (Figure 5).

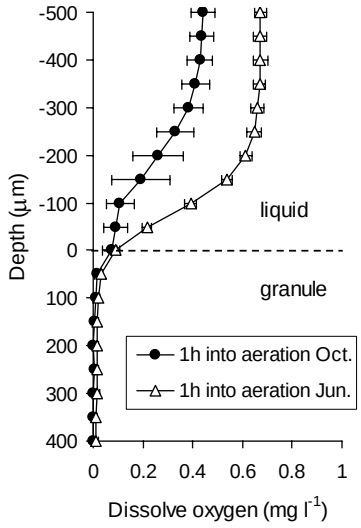

Figure 5. O_2 profiles measured in aggregates at 1 h into the aeration period (i.e. after nitrification activity ceased) in June and October. Profiles shown are averages (error bars=S.E., n=3).

The apparent role of GAOs in denitrification has two implications to the SNDPR process. Firstly, it compromises the carbon savings proposed to be obtained by SNDPR. Without denitrification by PAOs, there is no true link between SND and EBPR, and the two processes merely occur in the same sludge at the same time. Secondly, it poses the question of whether there is a direct link between the enrichment of *Competibacter* and N_2O accumulation from denitrification. The enrichment of *Competibacter* in previous SNDPR and DGAO reactors where N_2O accumulation was observed (Zeng et al., 2003a; Zeng et al., 2003b) suggests this, however, the question cannot be addressed fully without studying *Competibacter* in pure culture.

Management of N_2O accumulation in SNDPR

Whether N_2O accumulation is linked to the particular organisms performing denitrification in the SNDPR reactor or to the phenotype of PHA-driven denitrification, the high enrichment of particular organisms in lab-scale reactors fed with synthetic wastewater containing a single carbon source may exacerbate the effect of that organism or phenotype on N_2O accumulation. The abundance of *Accumulibacter* and *Competibacter* in full-scale treatment plants is usually below 15% (Saunders et al., 2003), and the diversity of denitrifying bacteria in sludge treating real wastewater may impact greatly on the potential for accumulating denitrification intermediates. We therefore hypothesise that if denitrification is carried out by GAOs and other denitrifiers simultaneously, the N_2O accumulated by GAOs could be reduced by other denitrifiers, provided that carbon is available to these cells.

The hypothesis was first tested by adding methanol during an anoxic batch test containing a mixture of sludge from the SNDPR reactor, and a reactor treating domestic wastewater amended with methanol (Figure 6a). Methanol was added to one of two parallel batch reactors after 10 min of incubation with nitrate, and while the control reactor continued to accumulate N_2O, the N_2O concentration immediately decreased in the test reactor after methanol addition. As a negative control, the same experiment was repeated with SNDPR sludge alone, demonstrating that methanol had no effect on N_2O accumulation in this sludge (Figure 6b). Hence, the denitrifying bacteria in the sludge mixed with the SNDPR sludge did not only denitrify without accumulating N_2O, they also acted as scavengers for the N_2O accumulated by GAOs in the SNDPR sludge.

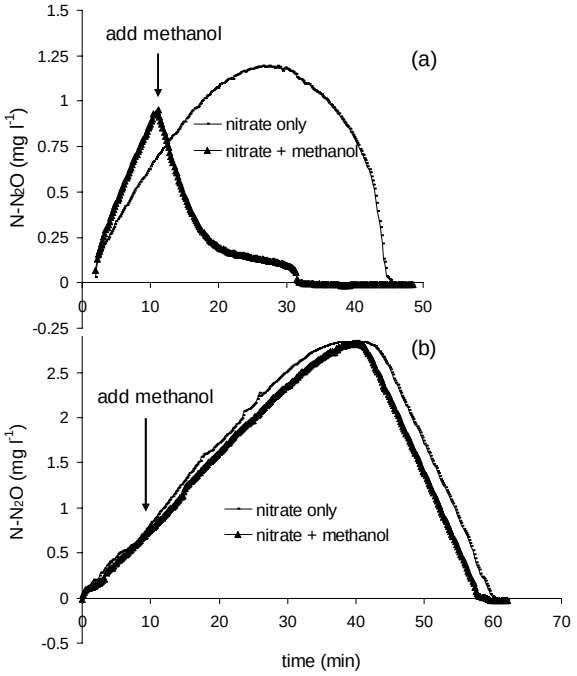

Figure 6. N_2O profiles in the bulk liquid during anoxic batch tests in 14.75-ml sealed reactors with and without methanol addition as carbon source using **(a)** a 2:1 mixture of SNDPR sludge and denitrifying sludge, or **(b)** SNDPR sludge alone as a negative control. The initial nitrate concentration at T=0 was 7.5 $mgN.l^{-1}$ of NO_3^-.

The denitrifying sludge used in the experiment above was adapted to methanol, which may have biased the results. The experiment was therefore repeated using raw, high-strength wastewater as the carbon source and mixing the SNDPR sludge with sludge from a denitrifying reactor which had not been adapted to a particular carbon compound. This batch test was carried out in a 500 ml reactor, allowing sampling of NO_x^- during the incubation. Addition of raw wastewater after 35 min of incubation with nitrate lead to an immediate reduction and depletion of N_2O (Figure 7). At the same time, the nitrate reduction rate increased, underlining the capacity of the non-GAO denitrifiers to reduce nitrate to N_2 while simultaneously removing N_2O accumulated by GAOs. The somewhat abrupt decrease in the nitrate and N_2O concentration upon addition of the raw wastewater was caused by the dilution effect imposed by the added volume of liquid.

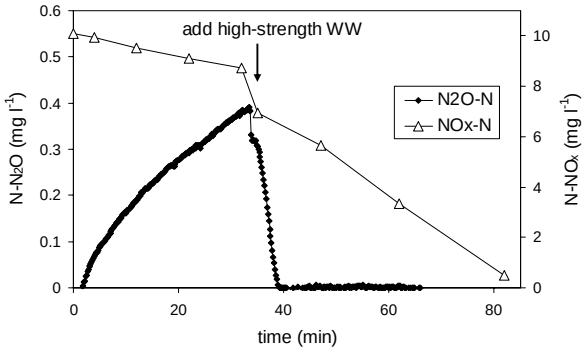

Figure 7. N_2O and NO_x^- concentration in the bulk liquid during an anoxic test in a 500 ml reactor. Raw high-strength wastewater was added at T=35 min.

The net production of N_2O from denitrification is most likely linked to the organisms (*Competibacter* and possibly other GAOs) responsible for denitrification in the present study, while the enrichment of these organisms and loss of diversity amongst the denitrifying microbial community enhanced the effect of these organisms on N_2O accumulation in the SNDPR reactor. This loss of diversity was primarily a result of feeding the reactor synthetic wastewater containing only a single carbon source. Real wastewater contains a combination of different carbon sources with different degradability, and because GAOs take up mainly volatile fatty acids, many carbon compounds are likely to still be present when the aerobic period sets in. It is therefore a realistic expectation that some carbon would be available for denitrification by non-GAO organisms during the aerobic period. We therefore conclude that N_2O accumulation is unlikely to be an issue in a SNDPR reactor treating real wastewater.

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REFERENCES

Amann R.I. (1995). In situ identification of microorganisms by whole cell hybridization with rRNA-targeted nucleic acid probes. In: Molecular Microbial Ecology Manual. Akkermans A.D.L., van Elsas J.D. and de Bruijn F.J. (eds). Dordrecht, Holland, *Kluwer Academic Publications*. **3.3.6:** 1-15.

Andersen K., Kjaer T. and Revsbech N.P. (2001). An oxygen insensitive microsensor for nitrous oxide. *Sensors and Actuators B-Chemical*. **81**(1): 42-48.

APHA (1995). Standard methods for the examination of water and wastewater. Washington, DC, *American Public Health Association*.

Beline F., Martinez J., Marol C. and Guiraud G. (2001). Application of the N-15 technique to determine the contributions of nitrification and denitrification to the flux of nitrous oxide from aerated pig slurry. *Water Research*. **35**(11): 2774-2778.

Brink C., Kroeze C. and Klimont Z. (2001). Ammonia abatement and its impact on emissions of nitrous oxide and methane in Europeb - Part 1: method. *Atmospheric Environment*. **35**(36): 6299-6312.

Comeau Y., Hall K., Hancock R. and Oldham W. (1986). Biochemical model for enhanced biological phosphorus removal. *Water Research*. **20**: 1511-1521.

Crocetti G.R., Banfield J.F., Keller J., Bond P.L. and Blackall L.L. (2002). Glycogen accumulating organisms in laboratory-scale and full-scale activated sludge processes. *Microbiology*. **148**(11): 3353-3364.

Crocetti G.R., Hugenholtz P., Bond P.L., Schuler A., Keller J., Jenkins D. and Blackall L.L. (2000). Identification of polyphosphate-accumulating organisms and design of 16S rRNA-directed probes for their detection and quantitation. *Applied and Environmental Microbiology*. **66**(3): 1175-1182.

Daims H., Bruhl A., Amann R., Schleifer K.H. and Wagner M. (1999). The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: Development and

evaluation of a more comprehensive probe set. *Systematic and Applied Microbiology*. **22**(3): 434-444.

Hanaki K., Hong Z. and Matsuo T. (1992). Production of nitrous oxide gas during denitrification of wastewater. *Water Science and Technology*. **26**(5-6): 1027-1036.

Itokawa H., Hanaki K. and Matsuo T. (2001). Nitrous oxide production in high-loading biological nitrogen removal process under low COD/N ratio condition. *Water Research*. **35**(3): 657-664.

Kishida N., Kim J.H., Kimochi Y., Nishimura O., Sasaki H. and Sudo R. (2004). Effect of C/N ratio on nitrous oxide emission from swine wastewater treatment process. *Water Science and Technology*. **49**(5-6): 359-365.

Kong Y., Ong S.L., Ng W.J. and Liu W.-T. (2002). Diversity and distribution of a deeply branched novel proteobacterial group found in anaerobic-aerobic activated sludge processes. *Environmental Microbiology*. **4**(11): 753-757.

Kuba T., Smolders G.J.F., van Loosdrecht M.C.M. and and Heijnen J.J. (1993). Biological phosphorus removal from wastewater by anaerobic and anoxic sequencing batch reactor. *Water Science and Technology*. **27**: 241-252.

Meinhold J., Filipe C.D.M., Daigger G.T. and Isaacs S. (1999). Characterization of the denitrifying fraction of phosphate accumulating organisms in biological phosphate removal. *Water Science and Technology*. **39**(1): 31-42.

Metcalf & Eddy (1991). Wastewater engineering: treatment, disposal and reuse. Singapore, *McGraw-Hill, Inc.*

Meyer R.L., Saunders A.M., Zeng R.J.X., Keller J. and Blackall L.L. (2003). Microscale structure and function of anaerobic-aerobic granules containing glycogen accumulating organisms. *FEMS Microbiology Ecology*. **45**(3): 253-261.

Meyer R.L., Zeng R.J.X., Giugliano V. and Blackall L.L. (2005). Challenges for simultaneous nitrification, denitrification, and phosphorus removal in microbial aggregates: mass transfer limitation and nitrous oxide production. *FEMS Microbiology Ecology*. **52**(3): 329-338.

Noda N., Kaneko N., Milkami M., Kimochi Y., Tsuneda S., Hirata A., Mizuochi M. and Inamori Y. (2003). Effects of SRT and DO on N2O reductase activity in an anoxic-oxic activated sludge system. *Water Science and Technology*. **48**(11-12): 363-370.

Oehmen A. (2004). The competition between polyphosphate accumulating organisms and glycogen accumulating organisms in the enhanced biological phosphorus removal process. PhD Thesis. *University of Queensland*, Brisbane, Australia.

Otte S., Grobben N.G., Robertson L.A., Jetten M.S.M. and Kuenen J.G. (1996). Nitrous oxide production by *Alcaligenes faecalis* under transient and dynamic aerobic and anaerobic conditions. *Applied and Environmental Microbiology*. **62**(7): 2421-2426.

Pitman A.R., Venter S.L.V. and Nicholls H.A. (1983). Practical experience with biological phosphorus removal plants in Johannesburg. *Water Science and Technology*. **15**(3-4): 233-259.

Revsbech N.P. (1989). An oxygen microsensor with a guard cathode. *Limnology and Oceanography*. **34**(2): 474-478.

Saunders A.M., Oehmen A., Blackall L.L., Yuan Z. and Keller J. (2003). The effect of GAOs (glycogen accumulating organisms) on anaerobic carbon requirements in full-scale Australian EBPR (enhanced biological phosphorus removal) plants. *Water Science and Technology*. **47**(11): 37-43.

Smolders G., Vandermeij J., Vanloosdrecht M. and Heijnen J. (1994). Model of the anaerobic metabolism of the biological phosphorus removal process - stoichiometry and pH influence. *Biotechnology and Bioengineering*. **43**(6): 461-470.

Wentzel M.C., Loewenthal R.E., Ekama G.A. and Marais G.V. (1988). Enhanced polyphosphate organism cultures in activated-sludge systems .1. Enhanced culture development. *Water SA*. **14**(2): 81-92.

Zeng R., Yuan Z. and Keller J. (2003a). Enrichment of denitrifying glycogen-accumulating organisms in anaerobic/anoxic activated sludge systems. *Biotechnology and Bioengineering*. **81**(4): 397-404.

Zeng R.J., Lemaire R., Yuan Z. and Keller J. (2003b). Simultaneous nitrification, denitrification, and phosphorus removal in a lab-scale sequencing batch reactor. *Biotechnology and Bioengineering*. **84**(2): 170-178.

Appendix E

Microbial Distribution of *Accumulibacter* spp. and *Competibacter* spp. in Aerobic Granules from a Lab-Scale Biological Nutrient Removal System

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ABSTRACT

Granular sludge for simultaneous nitrification, denitrification and phosphorus removal (SNDPR) was generated and studied in a laboratory-scale sequencing batch reactor (SBR). The SBR was monitored for 450 days during which the biomass was transformed from flocs to granules, which persisted for the last 130 days of operation. Short sludge settling time was employed to successfully generate the granules, with the 10^{th} and 90^{th} percentiles of diameter being 0.7 and 1.6 mm, respectively. Good phosphorus removal and nitrification occurred throughout the SBR operation but only when granules were generated was denitrification and full nutrient removal complete. Fluorescence *in situ* hybridisation and oxygen microsensors were used to study the granules at a microscale. *Accumulibacter* spp. (a polyphosphate accumulating organism, PAO) and *Competibacter* spp. (a glycogen non-polyphosphate accumulating organism, GAO) were the most abundant microbial community members (together 74% of all *Bacteria*) and both are capable of denitrification. In the aerobic period of the SBR operation, the oxygen penetrated 250 μm into the granules leaving large anoxic zones in the centre part where denitrification can occur. In granules > 500 μm in diameter, *Accumulibacter* spp. was dominant in the outermost 200 μm region of the granule while *Competibacter* spp. dominated in the granule central zone. The stratification of these two populations between the outer aerobic and inner anoxic part of the granule was highly significant ($P<0.003$). We concluded that GAO *Competibacter* spp., and not the PAO *Accumulibacter* spp., was responsible for denitrification in this SBR. This is undesirable for SNDPR as savings in carbon demand cannot be fulfilled with P removal and denitrification being achieved by different groups of bacteria.

Keywords: Aerobic granule, biological nutrient removal, GAO, microbial communities, PAO, SNDPR.

INTRODUCTION

The removal of nutrients, mainly nitrogen (N) and phosphorus (P), from domestic and industrial wastewater is required in order to prevent eutrophication of receiving water systems. Biological nutrient removal (BNR) systems are the most cost effective way to reduce the nutrient load from large volumes of wastewater. In conventional BNR systems, N is removed via a two-stage process (Metcalf & Eddy, 1991) featuring aerobic nitrification and anoxic denitrification. However, it has been observed that these two processes can occur concurrently in a single-sludge, single-stage process under low dissolved oxygen conditions called simultaneous nitrification and denitrification (SND) (Munch et al., 1996; Bertanza, 1997; Keller et al., 1997; Fuerhacker et al., 2000). SND relies on the formation of anoxic zones in the central part of microbial aggregates caused by the mass transfer limitation of oxygen. In the aerobic zone on the edge of the aggregate, autotrophic bacteria can nitrify using oxygen, whereas in the anoxic zone in the centre of the aggregate, heterotrophic bacteria can denitrify. Factors that affect oxygen mass transfer limitation such as bulk liquid oxygen concentration, the aggregate size, and the specific activity of the microbial aggregates (oxygen uptake rate per volume of biomass) (Pochana and Keller, 1999; Meyer et al., 2005) also affect SND.

The removal of P from wastewater is typically achieved by either chemical precipitation or through the biological process called enhanced biological phosphorus removal (EBPR). EBPR is based on the ability of polyphosphate-accumulating organisms (PAOs) to take up P and accumulate it intracellularly as polyphosphate when exposed to alternating anaerobic (O_2 and nitrite/nitrate (NO_x^-) absent) and aerobic conditions (Comeau et al., 1986; Wentzel et al., 1988). Simultaneous NO_x^- and P removal was achieved in anaerobic-anoxic EBPR systems using denitrifying PAOs (Kuba et al., 1993; Kerrn-Jespersen et al., 1994; Meinhold et al., 1999). The use of denitrifying PAOs in BNR systems is highly beneficial in terms of lower COD requirement (i.e. same carbon source used for both N and P removal) and reduced aeration cost. EBPR and SND were amalgamated into one process called simultaneous nitrification, denitrification and phosphorus removal (SNDPR) by Zeng et al. (2003). In the anaerobic period of SNDPR, COD was taken up and P was released. During the following aerobic period, there were concomitant P uptake and NH_4^+ removal through SND. No accumulation of nitrite or nitrate was observed. However, incomplete nitrate removal has been reported in lab-scale SNDPR bioreactors. Meyer et al. (2005) showed that if the aerobic/anoxic zones in the microbial aggregates were not formed, incomplete coupling between nitrification and denitrification could occur. Based on the process data of these bioreactors, it was also reported that glycogen-accumulating organisms (GAOs), known to compete with PAOs for carbon sources but without contributing to P removal, appear to be primarily responsible for the denitrification process in many lab-scale SNDPR systems (Zeng et al., 2003; Lemaire et al., 2006). The presence of GAOs in such systems reduces the potential for SNDPR to be used by the wastewater treatment industry.

Recent research efforts exploring the formation and use of aerobic granular biomass for nutrient removal in sequencing batch reactor (SBR) systems (Tay et al., 2002; Lin et al., 2003; Liu et al., 2003a; Yang et al., 2003; Cassidy and Belia, 2005; de Kreuk et al., 2005), suggest that granules could be beneficial for SNDPR. Compared to conventional flocs, granules are relatively large, compact, dense microbial aggregates of different bacterial species with an approximately spherical external appearance. The granular structure might positively contribute to the oxygen mass transfer limitation that is important in facilitating aerobic/anoxic zones required for SNDPR. Several studies investigated the structure and distribution of bacteria in nitrifying biofilms (Schramm et al., 2000; Gieseke et al., 2002;

Gieseke et al., 2003; Lydmark et al., 2006) and anaerobic granules (Sekiguchi et al., 1999; Liu et al., 2003b; Diaz et al., 2006) using molecular and microsensor techniques, but few attempted to comprehensively describe the relationship between the oxygen mass transfer limitation and the spatial organisation of the microbial community in aerobic granules (Ivanov et al., 2005; Kishida et al., 2006).

This study reports on a lab-scale SBR operated for SNDPR with selection for granular biomass. The microscale distribution, organization, and community composition of the bacterial community in granules were studied using fluorescence *in situ* hybridisation (FISH) and confocal laser scanning microscopy (CLSM). Oxygen profiles inside SNDPR granules were determined by microsensors. The spatial distribution of PAOs and GAOs in granules was correlated with the dissolved oxygen profiles in an attempt to demonstrate "ecological positions" and roles for each population in this complex system.

RESULTS

Reactor performance

Figure 1 shows various process data collected from the SBR from day 600 (i.e. 9 months before initiation of granule selection) until day 1000. The SBR effluent quality is depicted in Figure 1a. The low concentration of PO_4^{3-} and NH_4^+ in the effluent indicates that good P removal and nitrification were consistently achieved in the SBR. However, incomplete denitrification occurred occasionally as shown by the accumulation of NO_x^- (mostly nitrate) in the effluent between day 700 and day 900. Before granulation started, the mixed liquor suspended solid (MLSS) and volatile suspended solid (VSS) concentration in the SBR were relatively stable with means of 4.2 $g.l^{-1}$ (SD=0.53, n=32) and 2.4 $g.l^{-1}$ (SD=0.36, n=25), respectively, and a mean VSS:MLSS ratio of 0.59 (SD=0.04, n=25) (Figure 1b). During the same period, the aggregates in the SBR were relatively small as indicated by the 10^{th}, 50^{th} and 90^{th} percentile of the aggregates size distribution illustrated in Figure 1c.

The reduction of the SBR settling time after day 870 resulted in an immediate increase in the suspended solids concentration in the effluent due to the washout of slowly settling aggregates from the SBR (Figure 1b). After a slight decrease of the biomass concentration in the SBR, the MLSS and VSS concentrations reached 9 $g.l^{-1}$ and 6 $g.l^{-1}$ respectively while the size of the microbial aggregates (now called granules) increased sharply to stabilise between 0.7 and 1.6 mm (10^{th} and 90^{th} percentile respectively) (Figure 1b and 1c). During the granule selection process, the nutrient levels in the SBR effluent stayed relatively low with only a slight increase of the PO_4^{3-} concentration. The denitrification improved rapidly and was complete by day 900. Figure 2 shows the SBR cycle study carried out on day 970. The profiles of PO_4^{3-}, NH_4^+ and NO_x^- in the SBR bulk liquid depicted in Figure 2 illustrated typical SNDPR patterns with simultaneous removal of N and P during the aerobic stage without significant accumulation of NO_x^- in the bulk liquid.

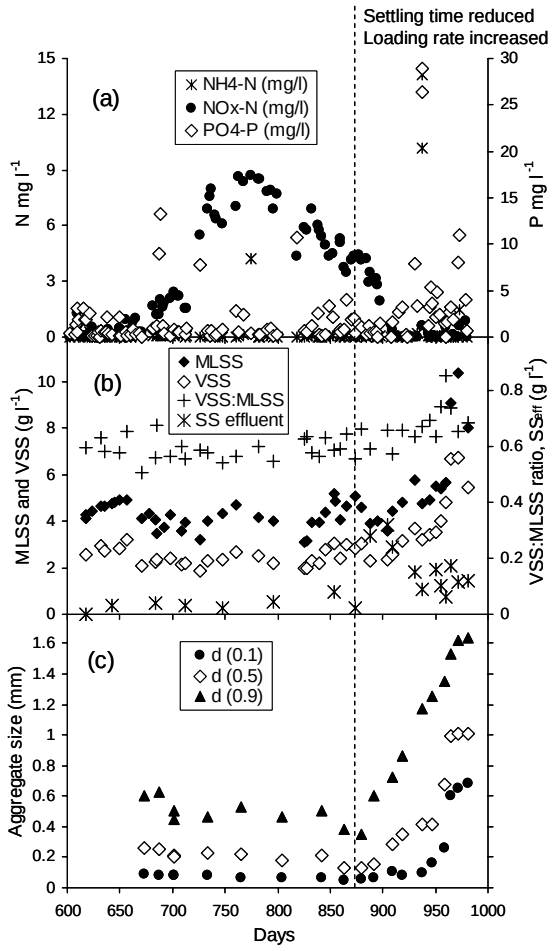

Figure 1. Process data since SBR was started. **(a)** Nutrient concentration in the SBR effluent. **(b)** MLSS and VSS concentration and their ratio in the SBR and the effluent suspended solids concentration. **(c)** 10^{th}, 50^{th} and 90^{th} percentile of the volumetric size distribution of the microbial aggregates in the SBR. The vertical dotted-line indicates when the SBR settling time was reduced to promote the formation of granules.

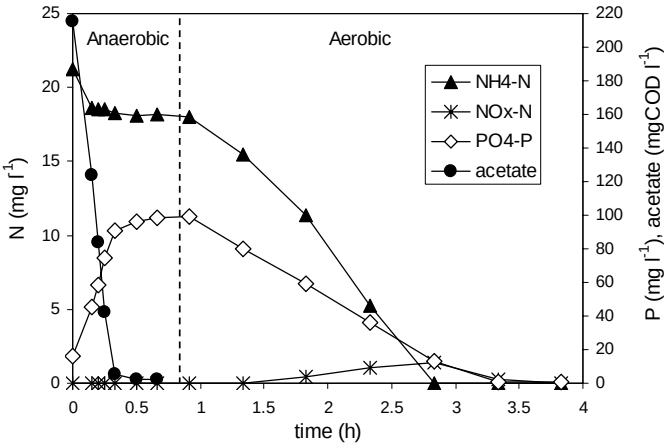

Figure 2. N, P and Acetate profile during a cycle study performed on day 970 when granule samples were taken to visualize microbial population distribution within the granule structure.

Oxygen profiles

After 100 days of implementing operational conditions to promote the formation of granules (i.e. reducing the settling time and increasing the organic and nutrient loading rates, see Experimental Procedures), the SBR biomass was mainly constituted of large (diameter > 500 μm) and dense granules (biomass density = 125 $gSS.l^{-1}$ biomass, SD=9, n=5) with only few loose floccular aggregates observed. To determine the oxygen penetration boundary into the >500 μm granules, oxygen gradient measurements were determined on day 970. Figure 3 shows that the oxygen profiles at the start and at the end of the aeration period were very similar with a boundary between the aerobic and anoxic part of the granule occurring at approximately 250 μm from the granule surface.

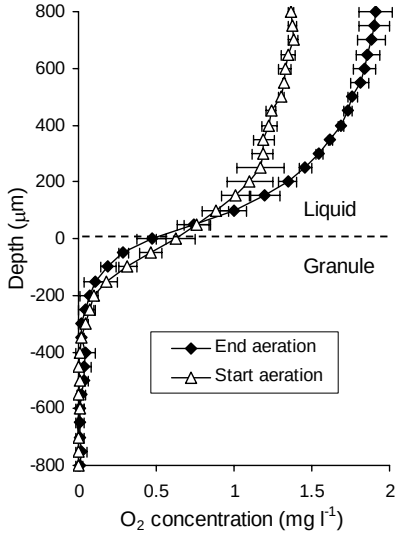

Figure 3. Oxygen profiles in granules at the start and the end of the aerobic period performed on day 970. Profile shown are average (error bars=S.D., n=6).

Microbial diversity and distribution of PAO and GAO in granules

A preliminary investigation of the diversity of PAO and GAO populations present in homogenised granular sludge samples was carried out by FISH with probes targeting organisms previously shown to exhibit the PAO phenotype (*Accumulibacter* spp., *Actinobacter*-related spp.) or GAO phenotype (*Competibacter* spp. and *Defluviicoccus* spp.). *Accumulibacter* spp. (48% of all bacteria, SD=18, n=24) and *Competibacter* spp. (26% of all bacteria, SD=8, n=24) were the only PAO and GAO (together 74% of all bacteria, SD=16, n=24) detected in these granules. The abundance of the nitrifier populations was also estimated using FISH with probes targeting most ammonia oxidising bacteria (AOB) from the *Betaproteobacteria* as well as some of the known nitrite oxidising bacteria (NOB) such as *Nitrospira* spp. and *Nitrobacter* spp. These are the most common AOB and NOB in activated sludge. *Nitrobacter* spp. was not detected while few *Nitrospira* spp. clusters were observed (under the detection limit of the quantification method employed). AOB comprised 1.9% of all bacteria (SD=0.3%, 3 replicates) in homogenised granular sludge samples. Given the fact that nitrate rather than nitrite accumulated at the end of aerobic periods during days 700 – 900, NOB were likely present in the sludge in that period, but declined during the development of the granular sludge.

The mean distribution of *Accumulibacter* spp. and *Competibacter* spp. obtained from 24 granules (15 granules of >500 µm diameter and 9 granules of <500 µm diameter) and expressed as the PAO (*Accumulibacter spp.*) to GAO (*Competibacter spp.*) ratio calculated for each concentric 50 µm zone (see description in Experimental Procedures) is depicted in Figure 4 along with the mean dissolved oxygen concentration at each zone measured in several granules at the end of the aerobic period. For each 50 µm zone of the granules, the PAO:GAO ratio correlated strongly (significant at the 0.01 level) with the dissolved oxygen concentration (Pearson correlation=0.86). *Accumulibacter* spp. was dominant from 0-200 µm from the granule surface (i.e. PAO:GAO ratio > 1) while *Competibacter* spp. dominated from 200 µm inwards (i.e. PAO:GAO ratio < 1). The statistical validity of this observation was tested by comparing the difference of the mean PAO:GAO ratio in the outer part of the granule (0-100 µm, likely aerobic) and in an inner part (250-350 µm, likely anoxic). The stratification of the two populations between the aerobic and anoxic part of the granules was highly significant (P<0.003). The FISH micrographs of median sections of granules shown in Figure 5a and 5e illustrate this stratification. However, this trend was not always observed in smaller granules with diameters less than 500 µm as shown in the FISH micrograph depicted in Figure 5c.

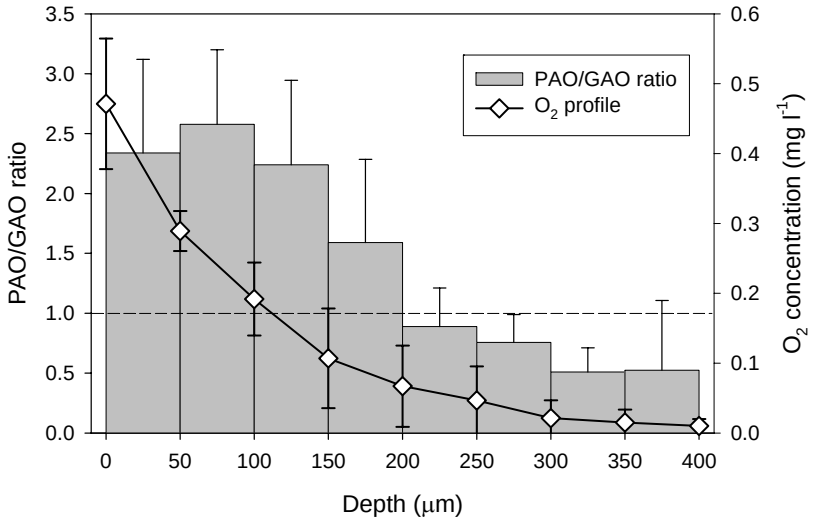

Figure 4. Average profile of the PAO:GAO ratio within 24 granules (error bar=95%CL) and mean O_2 profiles in granules at the end of the aerobic period performed on day 970 (error bars=S.D., n=6).

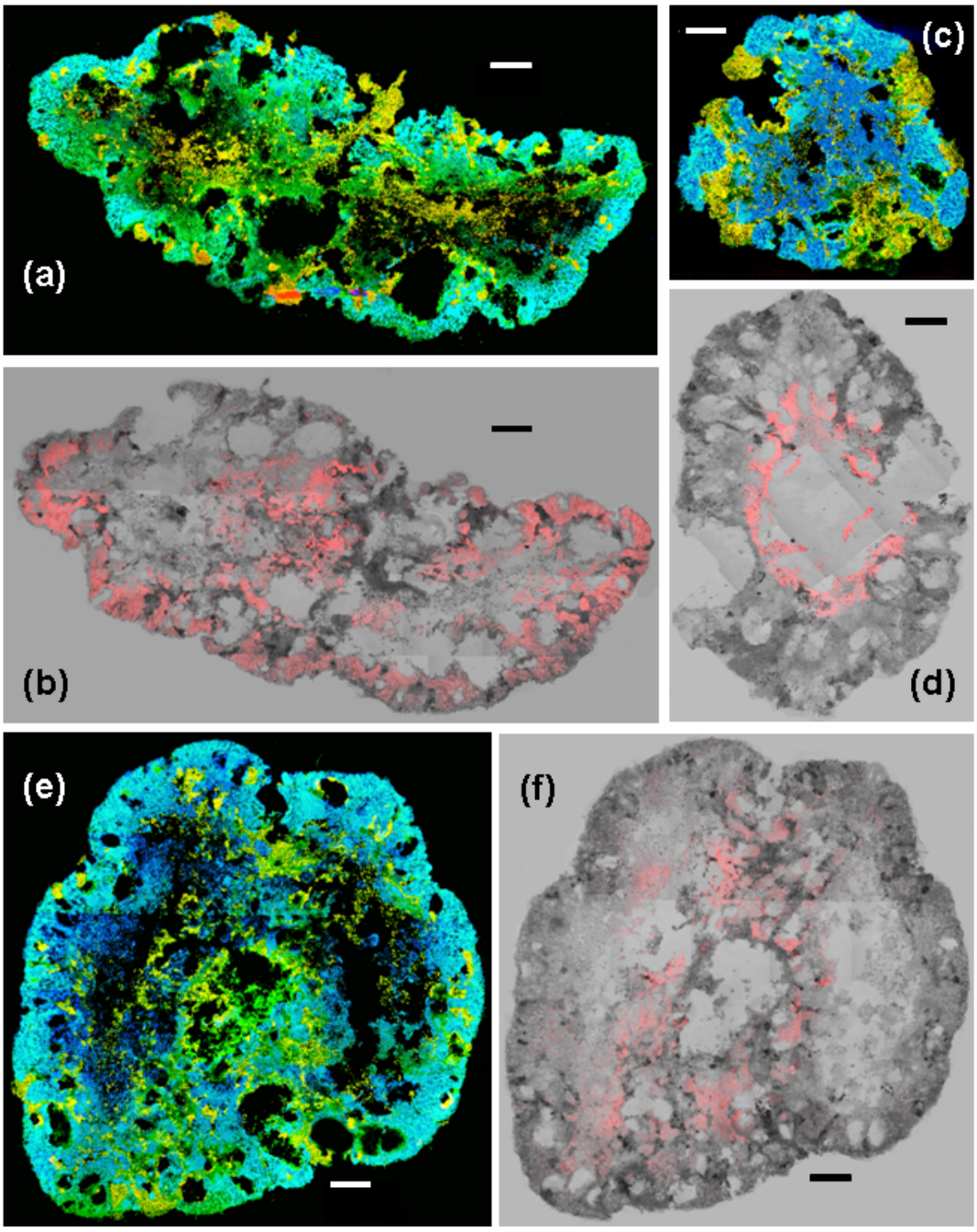

Figure 5. Reconstructed CLSM images of FISH micrographs (a, c and e) and Nile-Blue-stain micrographs (b, d and f) of entire granule sections. In **(a)**, **(c)** and **(e)** *Accumulibacter* spp. cells are cyan (overlay of blue PAOmix and green EUBmix), *Competibacter* spp. cells are yellow (overlay of red GAOmix and green EUBmix) and other *Bacteria* are green (green EUBmix). In **(b)**, **(d)** and **(f)**, overlays were of transmitted light images (black and white) and Nile-Blue-stained PHAs in red. Subsequent granule sections (7 µm apart) of two different granules are presented in (a)-(b) and (e)-(f). (c) and (d) are not images of the same granules. The granules were sampled at the end of the anaerobic (a-b and c) and at the end of the aerobic (d and e-f) periods. Scale bar = 100 µm.

Intracellular poly-hydroxyalkanoates (PHA) distribution in granules section

PAOs and GAOs take up acetate and convert it to PHA under anaerobic conditions and then oxidise their stored PHA in either aerobic or anoxic (NO_x^- as electron acceptor) conditions. Nile Blue staining of PHA was carried out to observe the location in granules of cells containing PHA. Figure 5b shows that at the end of the anaerobic period, cells containing PHA could be detected from the edge of the granule to the centre. This was consistently observed in all the granules sampled at the end of the anaerobic period. At the end of the aerobic period, PHA was still present, however only in the centre of the granule as shown in Figure 5d and 5f. In all the granules sampled at the end of the aerobic period, the depletion boundary of PHA correlated relatively well with the penetration boundary of oxygen, around 250 μm inside the granule.

DISCUSSION

Role of granules for SNDPR process reliability

The denitrification performance of the SBR operating for SNDPR was occasionally less efficient leading to the accumulation of NO_3^- in the effluent (Figure 1a). Previous research showed the causes to be a lack of anoxic zones in the inner part of the microbial aggregates (flocs or granules) (Meyer et al., 2005) and a decrease in the denitrifier population (Lemaire et al., 2006). We hypothesised that biomass comprised of a high proportion of larger granules would be better at sustaining stable gradations of dissolved oxygen which are required for the establishment of a robust SNDPR process. The strategy employed on day 870 proved successful in generating biomass in mostly large and dense granules, which rapidly achieved complete denitrification (Figure 1a) due to good coupling between nitrification and denitrification. Additionally, NO_x^- was barely detectable in the bulk liquid during the aeration period of the SBR cycle (Figure 2), indicating that SND was occurring. However, in order to benefit fully from the advantages of SNDPR, it is essential that PAOs carry out most of the denitrification and not GAOs as previously found (Zeng et al., 2003; Lemaire et al., 2006).

Microscale distribution of Accumulibacter spp. and Competibacter spp.

The 15 large granules (diameter > 500 μm) were comprised of *Accumulibacter* spp (the PAO in the SBR) mostly in the outer part of the granule, and *Competibacter* spp. (the GAO in the SBR) mostly in the inner zone ($P<0.003$). This PAO:GAO stratification was strongly correlated with the dissolved oxygen concentration gradient (Figure 3, Pearson coefficient = 0.86). In the 9 small granules (diameter < 500 μm) studied, the central anoxic zone was likely absent and there was no clear stratification of PAOs and GAOs. A totally random distribution was observed in 4 of the 9 small granules (data not shown). Overall, our results are in contrast to those of Kishida et al. (2006) who reported that *Accumulibacter* spp. were located in granules past the point where dissolved oxygen was not measurable, *Competibacter* spp. were mainly located in the aerobic parts of the granules. This discrepancy may be due to aerobic granules being relatively heterogeneous and having many different local microenvironments (Ivanov et al., 2005) including channels and voids which allow transport of nutrients from the bulk liquid to the granule interior. To address the heterogeneity, a sufficient number of granules should be studied to allow statistical analysis of the data. Therefore, true comparisons with Kishida et al. (2006) are difficult due to the few granules they studied. We

analysed 24 granules of various shapes and sizes so as to provide a representative sample of granules in order to establish a microscale distribution profile with statistical support.

The irregular spherical shape of the granules prevented the use of basic software tools to automatically generate the different zones of microorganism distribution, as have been used in biofilm studies (Schramm et al., 2000; Gieseke et al., 2003; Lydmark et al., 2006). Our developed methods were highly reproducible and the PAO:GAO ratio highlighted the population distribution. Errors that might have been introduced by drawing concentric zones following the specific contour of each granule were statistically evaluated by replication and the SD was less than 3% of the mean (data not shown).

Several biofilm studies have focused on the microscale distribution of nitrifiers in aerobic biofilms (Okabe et al., 1999; Schramm et al., 2000; Gieseke et al., 2003). Lydmark et al. (2006) suggested that the availability of different specific substrates (ammonium or nitrite) was the main factor responsible for the stratification of ammonia oxidisers and nitrite oxidisers. *Accumulibacter* spp. and *Competibacter* spp. use the same substrates (acetate) under anaerobic conditions. The presence of PHA in *Accumulibacter* spp. and *Competibacter* spp. cells located in the centre of large granules indicated that acetate diffused fully into granules (Figure 5b). Phosphorus transformations distinguish *Accumulibacter* spp. from *Competibacter* spp., but there are no *in situ* methods that suitably allow the study of inorganic phosphate (Pi) diffusion into granules. Through experimental and modelling results, Falkentoft et al. (2001) reported that Pi diffusion limitation could be significant in continuous-flow EBPR biofilm systems with constant phosphate concentration in the bulk liquid of 28 $mgP.l^{-1}$. The batch process used in our study produced a concentration of Pi in the bulk liquid of up to 100 $mgP.l^{-1}$ (Figure 2) at the start of the aerobic period which should provide sufficient concentration gradient for a full penetration of Pi inside the granules. In contrast, the relatively low concentration of DO (in comparison to acetate and Pi) in the bulk liquid phase has been found to create zones in granules devoid of oxygen or with low oxygen concentrations. We hypothesise that the DO gradient in granules was most likely responsible for the stratification of *Accumulibacter* spp. and *Competibacter* spp. in SNDPR granules. This could either be caused by different affinities of *Accumulibacter* spp. and *Competibacter* spp. with respect to oxygen, or directly by the availability of oxygen in different zones of the granules.

Role of microbial diversity in denitrification process

An important feature of SNDPR is that most of the carbon in the bulk liquid (i.e. acetate) is fully taken up and stored intracellularly during the anaerobic period by specific organisms (i.e. PAOs and GAOs). Thus, most microorganisms present in the biomass will have no access to an energy source which would allow them to carry out nitrate or nitrite reduction during the oxidative period (oxygen and/or NO_x^- present), with the exception of denitrifiers able to use exudates or extracellular polymeric substances as carbon sources. However, these alternate carbon sources are limited and also slowly biodegradable. Consequentially, they are unlikely to contribute in any substantial fashion to denitrification in SNDPR. The determination of the organisms (whether they are PAOs or GAOs) primarily responsible for denitrification in SNDPR is important, because carbon savings are a major advantage of SNDPR compared to conventional BNR systems. PAOs are desirable because they use the same carbon source for both N and P removal. Instead, if GAOs are the main denitrifiers, then there is no link between SND and EBPR and no saving of carbon.

Using the PAO:GAO ratio, *Accumulibacter* spp. dominated in the aerobic zones and *Competibacter* spp. in the central anoxic zones of the studied granules. Nile Blue staining on

several granule sections demonstrated intracellular PHA inclusions in cells throughout the granules when sampled at the end of the anaerobic period. After the aerobic period, only cells in the central anoxic zone (mostly *Competibacter* spp.) still contained PHA. We suggest that these cells only oxidised a portion of their stored PHA while denitrifying the NO_x^- produced. However, since Nile Blue staining is not quantitative, we could not confirm this hypothesis. We conclude that *Competibacter* spp. would be mostly responsible for denitrification in SNDPR granules. Previous research also found *Competibacter* spp. to be the main denitrifying population in lab-scale SNDPR systems (Zeng et al., 2003; Lemaire et al., 2006).

Nitrate rather than nitrite was the primary end product of nitrification in the process leading to the formation of granular sludge. The genome sequence of *Accumulibacter* spp. (Martin et al., 2006) does not contain respiratory nitrate reductase genes but does have the genes for enzymes in the denitrification pathway from nitrite reduction onwards. Several studies reported that *Accumulibacter* spp. could reduce nitrate to N_2 under anoxic conditions (Kuba et al., 1993; Meinhold et al., 1999; Shoji et al., 2003). It is possible that strains of *Accumulibacter* spp. other than those sequenced by Martin et al. (2006) may indeed be able to reduce nitrate to N_2. Since complex mixed cultures were studied, organisms other than *Accumulibacter* spp. might have carried out nitrate reduction to nitrite. *Accumulibacter* spp. then would have access to this formed nitrite to survive in anoxia. If *Competibacter* spp. has the capacity to reduce nitrate, this organism would have a competitive edge over *Accumulibacter* spp. in anoxic conditions which may explain the spatial organisation of the organisms in our granules. The abundance of *Competibacter* spp. in the community shows this organism has limited competitors for nitrate. A more complex community would provide this competition and likely limit the dominance of *Competibacter* spp. in the centre part of the granules. Therefore, maintaining a threshold of microbial biodiversity above that in the present SBR with a synthetic feed by using more complex wastewater might facilitate efficient SNDPR by allowing PAOs to carry out denitrification and P removal. Alternatively, the long term inhibition or elimination of the nitrite oxidising bacterial population in a SNDPR system would prevent nitrite being oxidised to nitrate allowing PAOs to perform denitrification of nitrite to N_2 without relying on GAOs or any other denitrifiers to carry out the first denitrification step. The process data and FISH quantification results indicate that NOB were indeed gradually eliminated during the granulation process. However, the impact of this change on the interactions between PAOs and GAOs and on their spatial locations in granules was not assessed in this study. Further investigations are warranted.

EXPERIMENTAL PROCEDURES

Reactor Setup and Operation

The biomass was enriched in a 5 l SBR operated at room temperature (20-22°C) and seeded with sludge from the Caboolture Sewage Treatment Plant, Queensland, Australia. The SBR was initially operated with a cycle time of 6 h, consisting of a 90 min anaerobic period, followed by 220 min aeration, 40 min settling, and 10 min decanting periods (Lemaire et al., 2006). After 870 days of operation, the SBR cycle was modified to promote the formation of granules. The cycle time was shortened to 4 h and the time allowed for settling was gradually reduced to reach 5 min by day 900. From day 900 onward, the new 4 h cycle operation consisted of 55 min anaerobic period, followed by 170 min aeration, 5 min settling, and 10 min decant. Every cycle, three litres of synthetic wastewater was pumped into the reactor in the first 7 min of the anaerobic period, and 3 l supernatant was removed after settling, resulting in a hydraulic retention time (HRT) of 6.7 h. The synthetic wastewater was prepared

as described in Zeng et al. (2003) with 350 mg.l^{-1} COD as acetate, 35 mg.l^{-1} NH_4-N and 23 mg.l^{-1} PO_4-P. To provide adequate shear force in the SBR during the aerobic period, air was provided at a flow of 5 l.min^{-1} throughout an air diffuser producing coarse bubbles resulting in an upflow air velocity of 0.9 cm.s^{-1}. Dissolved oxygen was kept between 1.3-1.7 mg.l^{-1} using an on/off control system. The pH in the system was recorded but not controlled, and fluctuated between 7.0 and 7.5.

To monitor the reactor performance, the SBR effluent was sampled daily for analysis of PO_4^{3-}, NH_4^+ and NO_x^- using a Lachat QuikChem8000 Flow Injection Analyser (Lachat Instrument, Milwaukee). Cycle studies were performed once a week during which liquid samples were taken and filtered every 20-30 min for analysis of acetate, PO_4^{3-}, NH_4^+ and NO_x^-. Acetate was measured on a Perkin-Elmer gas chromatograph with column DB-FFAP 15 m x 0.53 mm x 1.0 µm (length x ID x film) at 140°C while the injector and flame ionisation detector were operated at 220°C and 250°C, respectively. The reactor MLSS, VSS and effluent suspended solids concentrations were measured weekly according to standard methods (APHA, 1995).

Oxygen Microsensors

The gradient of oxygen in granules was measured with oxygen microsensors (tip diameter <10 µm), which were constructed as described by Revsbech et al. (1989). Granules were sampled from the SBR on day 970 at the start of the aerobic period when NH_4^+ and PO_4^{3-} were present at high concentrations and at the end of the aerobic period, at which time NH_4^+ and PO_4^{3-} were usually depleted. Granules with diameter > 500 µm were transferred to a flow-cell with an upward flow as described in Meyer et al. (2003). Six replicate oxygen profiles were then measured over the following hour and averaged. The sensor was moved stepwise into the granules from above, and movement of the sensor and data acquisition was obtained with the software Profix (Unisense A/S, Aarhus, Denmark). The composition of medium and the dissolved oxygen concentration in the flow-cell was identical to that in the SBR at either the start or the end of the aerobic period. The oxygen concentration was adjusted within the nutrient medium by controlling the air/nitrogen ratio in the gas inlet.

Biomass Composition and Structure

The granule size distribution was determined with a Malvern laser light scattering Mastersizer 2000 series instrument (Malvern Instruments, Worcestershire, UK) on granules in 30 ml of well mixed granular sludge from the SBR at the end of the aeration period. The granule density, defined as the quantity of dry mass per biomass volume, was determined using the procedure described below. The biomass volume was measured using the blue dextran method adapted from Di Iaconi et al. (2004). Dextran blue is not absorbed by biomass. Briefly, 5 ml granular sludge was gently mixed with 5 ml of dextran blue solution (1 g.l^{-1}), the mixture was centrifuged for 3 minutes at 10,000 rpm and the absorbance of the supernatant at 620 nm was measured. The same procedure was carried out with distilled water (blank).

Biovolume (ml) = 5 x (1 – Blank A_{620}/Sample A_{620})

The biomass density (mg.l^{-1}) was calculated by dividing the dry weight of the biomass in the 10 ml suspension by the volume that the granules occupied in the initial 5 ml sample determined above. The reported value was determined from the mean of five replicates.

Granule samples for FISH and chemical staining were fixed in 3% paraformaldehyde (Amann, 1995) on day 970. The fixed granules were embedded in optimum cutting temperature (OCT) compound (TissueTek, Sakura, USA) for cryosectioning as previously described (Meyer et al., 2003) or they were homogenised in 1.5 ml tube using a pestle (Astral

Scientific, Australia). Embedded granules were then frozen and sectioned into 10 µm thick slices using a cryotome operated at -20°C (Kryo 1720, Leitz, Germany). The granule sections were collected on SuperFrost Plus microscope slides (Menzel-Glaser, Germany). Finally, the slides were dehydrated by sequential immersion for 3 min in 50%, 80% and 98% ethanol, followed by air-drying.

FISH was then carried out on the granule sections (PAO and GAO) or on homogenised granule samples (nitrifiers) as described by Amann (1995). To ensure that only the median part of each cryosectioned granule was visualised by FISH, microscopic slides with the largest sections of subsequent granule sections (as determined by eye) were first selected for each cryosectioned granule. Oligonucleotide probes used in this study were the combination of EUB338 i-iii (EUBmix) for the detection of all bacteria (Daims et al., 1999), the combination of PAO462, PAO651 and PAO846 (PAOmix) for *Accumulibacter* spp. (Crocetti et al., 2000), the probe combination (GAOmix) of GAOQ989 (Crocetti et al., 2002) and GB_G2 (Kong et al., 2002) for *Competibacter* spp, NIT3 (Wagner et al., 1996) for *Nitrobacter* spp., NSPA662 (Daims et al., 2001) for *Nitrospira* spp. and NSO1225 (Mobarry et al., 1996) for most of the ammonia oxidizing bacteria (AOB) from the *Betaproteobacteria*. Additionally, probes for proposed *Actinobacteria* PAOs (Kong et al., 2005) and *Defluviicoccus* spp. GAOs (Meyer et al., 2006) were used. Fluorescently labelled oligonucleotides were purchased from Thermo (Ulm, Germany) with fluorescein isothiocyanate (FITC) or one of the sulfoindocyanine dyes indocarbocyanine (Cy3) or indodicarbocyanine (Cy5).

Hybridised granule sections were visualised with a Zeiss LSM 510 (Carl Zeiss, Jena, Germany) CLSM using an argon laser for FITC excitation (488 nm), a helium neon laser for Cy3 (543 nm) and a red diode laser for Cy5 (633 nm) fitted with 515-565 nm BP, 590 nm LP and 660-710 nm BP emission filters, respectively. To obtain images of entire granule sections, between 10 and 50 overlapping, consecutive images of 1024x1024 pixels were collected (depending on the size of the granule) using a Zeiss Neofluar 40x/1.3 oil objective. The final composite image of the granule section was then reconstructed from all the single images collected using Adobe Photoshop 7.0 (Adobe Systems, USA). For preliminary investigation of the microbial composition and for the quantification of nitrifier populations in homogenised granule samples, images were taken using a Zeiss Apochromat 63x/1.4 oil objective.

Consecutive granule sections following those used for FISH were stained with Nile Blue A (Ostle and Holt, 1982) and visualised on the CLSM using the Cy3 settings (above) to determine cells containing intracellular PHA. Images of sections of 15 granules sampled from the SBR at the end of the anaerobic period and 15 from the end of the aerobic period were reconstructed.

Image analysis and statistical analysis

Using the brush tool in Adobe Photoshop 7.0, sequential 50 µm wide concentric zones, following the external contour of each granule, were manually drawn. The creation of these zones was continued stepwise towards the centre of the granule, following the contour of the outer boundary from each previous zone, until the centre of the granule was reached. The relative abundance of each microbial population (*Accumulibacter* spp. (PAOmix-targeted) or *Competibacter* spp. (GAOmix-targeted)) was determined in each concentric 50 µm granule zone of 24 individual granules with diameters ranging from 400 to 1000 µm using the pixel Measure/Count tool in Image Pro 4.0 (Media Cybernetics, USA). Briefly, the area of pixels contributed by PAOmix or GAOmix probes above a manually determined threshold was divided by the area of pixels contributed by the EUBmix probes (also applying threshold) for

each granule zone. Individual pixels counted as 'positive' PAOmix or GAOmix were also required to have a 'positive' pixel signal from EUBmix, above given thresholds. The population distribution of *Accumulibacter* spp. and *Competibacter* spp. within each granule was expressed as the relative abundance of *Accumulibacter* spp. divided by that of *Competibacter* spp. (referred as the PAO:GAO ratio). Using the ratio data, a mean distribution was calculated. The statistical validity of the differences between the PAO:GAO ratio in the outer layer of the granule (0-100 μm, likely aerobic) and the PAO:GAO ratio in an inner layer of the granule (250-350 μm, likely anaerobic) was analysed using a Student's paired t-Test, with a two-tailed distribution. The correlation between the PAO:GAO ratio and the oxygen profile in the granule was established using the Pearson correlation coefficient.

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REFERENCES

Amann R.I. (1995). In situ identification of microorganisms by whole cell hybridization with rRNA-targeted nucleic acid probes. In: Molecular Microbial Ecology Manual. Akkermans A.D.L., van Elsas J.D. and de Bruijn F.J. (eds). Dordrecht, Holland, *Kluwer Academic Publications*. **3.3.6:** 1-15.

APHA (1995). Standard methods for the examination of water and wastewater. Washington, DC, *American Public Health Association*.

Bertanza G. (1997). Simultaneous nitrification-denitrification process in extended aeration plants: Pilot and real scale experiences. *Water Science and Technology*. **35**(6): 53.

Cassidy D.P. and Belia E. (2005). Nitrogen and phosphorus removal from an abattoir wastewater in a SBR with aerobic granular sludge. *Water Research*. **39**(19): 4817-4823.

Comeau Y., Hall K., Hancock R. and Oldham W. (1986). Biochemical model for enhanced biological phosphorus removal. *Water Research*. **20**: 1511-1521.

Crocetti G.R., Banfield J.F., Keller J., Bond P.L. and Blackall L.L. (2002). Glycogen accumulating organisms in laboratory-scale and full-scale activated sludge processes. *Microbiology*. **148**(11): 3353-3364.

Crocetti G.R., Hugenholtz P., Bond P.L., Schuler A., Keller J., Jenkins D. and Blackall L.L. (2000). Identification of polyphosphate-accumulating organisms and design of 16S rRNA-directed probes for their detection and quantitation. *Applied and Environmental Microbiology*. **66**(3): 1175-1182.

Daims H., Bruhl A., Amann R., Schleifer K.H. and Wagner M. (1999). The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: Development and evaluation of a more comprehensive probe set. *Systematic and Applied Microbiology*. **22**(3): 434-444.

Daims H., Nielsen J.L., Nielsen P.H., Schleifer K.H. and Wagner M. (2001). In situ characterization of Nitrospira-like nitrite oxidizing bacteria active in wastewater treatment plants. *Applied and Environmental Microbiology*. **67**(11): 5273-5284.

de Kreuk M., Heijnen J.J. and van Loosdrecht M.C.M. (2005). Simultaneous COD, nitrogen, and phosphate removal by aerobic granular sludge. *Biotechnology and Bioengineering*. **90**(6): 761-769.

Di Iaconi C., Ramadori R., Lopez A. and Passino R. (2004). Preliminary biomass characterization in a sequencing batch biofilm reactor. *Annali di Chimica*. **94**(12): 889-898.

Diaz E.E., Stams A.J.A., Amils R. and Sanz J.L. (2006). Phenotypic properties and microbial diversity of methanogenic granules from a full-scale upflow anaerobic sludge bed reactor treating brewery wastewater. *Applied and Environmental Microbiology*. **72**(7): 4942-4949.

Falkentoft C.M., Arnz P., Henze M., Mosbaek H., Muller E., Wilderer P.A. and Harremoes P. (2001). Possible complication regarding phosphorus removal with a continuous flow biofilm system: Diffusion limitation. *Biotechnology and Bioengineering*. **76**(1): 77-85.

Fuerhacker M., Bauer H., Ellinger R., Sree U., Schmid H., Zibuschka F. and Puxbaum H. (2000). Approach for a novel control strategy for simultaneous nitrification/denitrification in activated sludge reactors. *Water Research*. **34**(9): 2499-2506.

Gieseke A., Arnz P., Amann R. and Schramm A. (2002). Simultaneous P and N removal in a sequencing batch biofilm reactor: insights from reactor- and microscale investigations. *Water Research*. **36**(2): 501-509.

Gieseke A., Bjerrum L., Wagner M. and Amann R. (2003). Structure and activity of multiple nitrifying bacterial populations co-existing in a biofilm. *Environmental Microbiology*. **5**(5): 355-369.

Ivanov V., Tay S.T.L., Liu Q.S., Wang X.H., Wang Z.W. and Tay J.H. (2005). Formation and structure of granulated microbial aggregates used in aerobic wastewater treatment. *Water Science and Technology*. **52**(7): 13-19.

Keller J., Subramaniam K., Gosswein J. and Greenfield P.F. (1997). Nutrient removal from industrial wastewater using single tank sequencing batch reactors. *Water Science and Technology*. **35**(6): 137-144.

Kerrn-Jespersen J.P., Henze M. and Strube R. (1994). Biological phosphorus release and uptake under alternating anaerobic and anoxic conditions in a fixed-film reactor. *Water Research*. **28**(5): 1253-1255.

Kishida N., Kim J., Tsuneda S. and Sudo R. (2006). Anaerobic/oxic/anoxic granular sludge process as an effective nutrient removal process utilizing denitrifying polyphosphate-accumulating organisms. *Water Research*. **40**(12): 2303-2310.

Kong Y., Ong S.L., Ng W.J. and Liu W.-T. (2002). Diversity and distribution of a deeply branched novel proteobacterial group found in anaerobic-aerobic activated sludge processes. *Environmental Microbiology*. **4**(11): 753-757.

Kong Y.H., Nielsen J.L. and Nielsen P.H. (2005). Identity and ecophysiology of uncultured actinobacterial polyphosphate-accumulating organisms in full-scale enhanced biological phosphorus removal plants. *Applied and Environmental Microbiology*. **71**(7): 4076-4085.

Kuba T., Smolders G.J.F., van Loosdrecht M.C.M. and and Heijnen J.J. (1993). Biological phosphorus removal from wastewater by anaerobic and anoxic sequencing batch reactor. *Water Science and Technology*. **27**: 241-252.

Lemaire R., Meyer R., Taske A., Crocetti G.R., Keller J. and Yuan Z.G. (2006). Identifying causes for N_2O accumulation in a lab-scale sequencing batch reactor performing simultaneous nitrification, denitrification and phosphorus removal. *Journal of Biotechnology*. **122**(1): 62-72.

Lin Y.M., Liu Y. and Tay J.H. (2003). Development and characteristics of phosphorus-accumulating microbial granules in sequencing batch reactors. *Applied Microbiology and Biotechnology*. **62**(4): 430-435.

Liu Y., Xu H.L., Yang S.F. and Tay J.H. (2003a). Mechanisms and models for anaerobic granulation in upflow anaerobic sludge blanket reactor. *Water Research*. **37**(3): 661-673.

Liu Y., Yang S.F. and Tay J.H. (2003b). Elemental compositions and characteristics of aerobic granules cultivated at different substrate N/C ratios. *Applied Microbiology and Biotechnology*. **61**(5-6): 556-561.

Lydmark P., Lind M., Sorensson F. and Hermansson M. (2006). Vertical distribution of nitrifying populations in bacterial biofilms from a full-scale nitrifying trickling filter. *Environmental Microbiology*. **8**(11): 2036-2049.

Martin H.G., Ivanova N., Kunin V., Warnecke F., Barry K.W., McHardy A.C., Yeates C., He S.M., Salamov A.A., Szeto E., Dalin E., Putnam N.H., Shapiro H.J., Pangilinan J.L., Rigoutsos I., Kyrpides N.C., Blackall L.L., McMahon K.D. and Hugenholtz P. (2006). Metagenomic analysis of two enhanced biological phosphorus removal (EBPR) sludge communities. *Nature Biotechnology*. **24**(10): 1263-1269.

Meinhold J., Filipe C.D.M., Daigger G.T. and Isaacs S. (1999). Characterization of the denitrifying fraction of phosphate accumulating organisms in biological phosphate removal. *Water Science and Technology*. **39**(1): 31-42.

Metcalf & Eddy (1991). Wastewater engineering: treatment, disposal and reuse. Singapore, *McGraw-Hill, Inc.*

Meyer R.L., Saunders A.M. and Blackall L.L. (2006). Putative glycogen-accumulating organisms belonging to the Alphaproteobacteria identified through rRNA-based stable isotope probing. *Microbiology-Sgm*. **152**: 419-429.

Meyer R.L., Saunders A.M., Zeng R.J.X., Keller J. and Blackall L.L. (2003). Microscale structure and function of anaerobic-aerobic granules containing glycogen accumulating organisms. *FEMS Microbiology Ecology*. **45**(3): 253-261.

Meyer R.L., Zeng R.J.X., Giugliano V. and Blackall L.L. (2005). Challenges for simultaneous nitrification, denitrification, and phosphorus removal in microbial aggregates: mass transfer limitation and nitrous oxide production. *FEMS Microbiology Ecology*. **52**(3): 329-338.

Mobarry B.K., Wagner M., Urbain V., Rittmann B.E. and Stahl D.A. (1996). Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. *Applied and Environmental Microbiology*. **62**(6): 2156-2162.

Munch E.V., Lant P. and Keller J. (1996). Simultaneous nitrification and denitrification in bench-scale sequencing batch reactors. *Water Research*. **30**(2): 277-284.

Okabe S., Satoh H. and Watanabe Y. (1999). In situ analysis of nitrifying biofilms as determined by in situ hybridization and the use of microelectrodes. *Applied and Environmental Microbiology*. **65**(7): 3182-3191.

Ostle A.G. and Holt J.G. (1982). Nile blue-A as a fluorescent stain for poly-beta-hydroxybutyrate. *Applied and Environmental Microbiology*. **44**: 238-241.

Pochana K. and Keller J. (1999). Study of factors affecting simultaneous nitrification and denitrification (SND). *Water Science and Technology*. **39**(6): 61-68.

Revsbech N.P. (1989). An oxygen microsensor with a guard cathode. *Limnology and Oceanography*. **34**(2): 474-478.

Schramm A., De Beer D., Gieseke A. and Amann R. (2000). Microenvironments and distribution of nitrifying bacteria in a membrane-bound biofilm. *Environmental Microbiology*. **2**(6): 680-686.

Sekiguchi Y., Kamagata Y., Nakamura K., Ohashi A. and Harada H. (1999). Fluorescence in situ hybridization using 16S rRNA-targeted oligonucleotides reveals localization of methanogens and selected uncultured bacteria in mesophilic and thermophilic sludge granules. *Applied and Environmental Microbiology*. **65**(3): 1280-1288.

Shoji T., Satoh H. and Mino T. (2003). Quantitative estimation of the role of denitrifying phosphate accumulating organisms in nutrient removal. *Water Science and Technology*. **47**(11): 23-29.

Tay J.H., Liu Q.S. and Liu Y. (2002). Characteristics of aerobic granules grown on glucose and acetate in sequential aerobic sludge blanket reactors. *Environmental Technology*. **23**(8): 931-936.

Wagner M., Rath G., Koops H.-P., Flood J. and Amann R. (1996). *In situ* analysis of nitrifying bacteria in sewage treatment plants. *Water Science and Technology*. **34**(1-2): 237-244.

Wentzel M.C., Loewenthal R.E., Ekama G.A. and Marais G.V. (1988). Enhanced polyphosphate organism cultures in activated-sludge systems .1. Enhanced culture development. *Water SA*. **14**(2): 81-92.

Yang S.F., Tay J.H. and Liu Y. (2003). A novel granular sludge sequencing batch reactor for removal of organic and nitrogen from wastewater. *Journal of Biotechnology*. **106**(1): 77-86.

Zeng R.J., Lemaire R., Yuan Z. and Keller J. (2003). Simultaneous nitrification, denitrification, and phosphorus removal in a lab-scale sequencing batch reactor. *Biotechnology and Bioengineering*. **84**(2): 170-178.

Appendix F

Simultaneous Nitrification, Denitrification and Phosphorus Removal from High Nutrient Containing Industrial Wastewater using Granular Sludge

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ABSTRACT

The biological removal of nitrogen and phosphorus from nutrient-rich abattoir wastewater using granular sludge has been investigated. A lab-scale sequencing batch reactor, seeded with granular sludge developed using synthetic wastewater, was operated for 13 months under alternating anaerobic and aerobic conditions. It is demonstrated that the granules could be sustained and indeed further developed with the use of abattoir wastewater. The organic, nitrogen and phosphorus loading rates applied were 2.7 $gCOD.l^{-1}.d^{-1}$, 0.43 $gN.l^{-1}.d^{-1}$ and 0.06 $gP.l^{-1}.d^{-1}$, respectively. The removal efficiency of soluble COD, soluble nitrogen and soluble phosphorus were 85%, 93% and 89%, respectively. However, the high suspended solids in the effluent limited the overall removal efficiency to 68%, 86% and 74% for total COD, TN and TP, respectively. This good nutrient removal was achieved through the process known as simultaneous nitrification, denitrification and phosphorus removal, likely facilitated by the presence of large anoxic zones in the centre of the granules. The removal of nitrogen was likely via nitrite optimising the use of the limited COD available in the wastewater. *Accumulibacter* spp. was found to be responsible for most of the denitrification, further reducing the COD requirement for nitrogen and phosphorus removal. Mineral precipitation was evaluated and was not found to significantly contribute to the overall nutrient removal. It is also shown that the minimum HRT in a granular sludge system is not governed by the sludge settleability, as is the case with floccular sludge systems, but likely by the limitations associated with the transfer of substrates in granules.

Keywords: abattoir wastewater, BNR, granular sludge, HRT, SBR, SNDPR.

INTRODUCTION

The meat processing industry requires large quantities of water, much of which is discharged as wastewater containing high levels of COD and nutrients such as nitrogen (N) and phosphorus (P). Over the past two decades, biological COD and N removal from abattoir wastewater has received much greater attention than has the biological P removal. Reliable biological COD and nitrogen removal systems have been successfully developed and applied for abattoir wastewater treatment using continuous activated sludge systems (Beccari et al., 1984; Frose and Kayser, 1985; Willers et al., 1993). However, P removal continues to be

achieved primarily through chemical precipitation, despite biological P removal being a much cheaper and more environmentally sustainable option.

There are two main challenges for biological phosphorus removal from abattoir wastewater. First of all, the wastewater contains a high level of ammonia and organic nitrogen, the oxidation of which results in a high level of nitrate accumulation. Nitrate accumulation has proved to be an obstacle to the development of a stable and reliable enhanced biological phosphorus removal (EBPR) process (Comeau et al., 1986; Furumai et al., 1999; Pitman et al., 1983). Secondly, abattoir wastewater contains substantial amounts of fat, oil and grease (FOG) which negatively impact on sludge settleability. As a solution, the raw abattoir wastewater is often pre-treated in anaerobic ponds to reduce the FOG content, which also removes a large fraction of COD, resulting in COD limitations (particularly Volatile Fatty Acids – VFAs) for N and P removal (Keller et al., 1997).

Simultaneous nitrification and denitrification (SND) potentially offers a solution to the first problem as it prevents accumulation of NO_x^- (NO_3^- + NO_2^-) in the system. SND relies upon the formation of anoxic zones in the central part of microbial aggregates caused by the mass transfer limitation of oxygen (Bertanza, 1997; Fuerhacker et al., 2000; Keller et al., 1997; Munch et al., 1996). The NO_x^- formed in the outer aerobic layer of the aggregates due to nitrification can be reduced in the central anoxic zone. The accumulation of NO_x^- in the bulk liquid phase is therefore minimised.

To address the second challenge, optimised use of COD for N and P removal could be achieved by combining EBPR with SND. Polyphosphate-accumulating organisms (PAOs) have the ability to simultaneously reduce NO_x^- and take up P using the same carbon source (Kerrn-Jespersen et al., 1994; Kuba et al., 1993; Meinhold et al., 1999). Indeed, the process of simultaneous nitrification and denitrification and phosphorus removal (SNDPR) has already been demonstrated in lab-scale SBRs treating mainly synthetic wastewater (Zeng et al., 2003a). Such a process requires alternating anaerobic and aerobic conditions. In the aerobic period, the conversion of ammonia to gaseous nitrogen products and P uptake were achieved concomitantly. However, unstable nitrate/nitrite removal has been reported in lab-scale SNDPR bioreactors using floccular biomass. Meyer et al. (2005) showed that incomplete coupling between nitrification and denitrification would occur if the aerobic/anoxic zones in the microbial aggregates were not formed, leading to the accumulation of NO_x^-.

Recent research efforts exploring the formation and use of aerobic granular biomass in sequencing batch reactor (SBR) systems (de Kreuk et al., 2005; Mosquera-Corral et al., 2005), suggest that granules could indeed be beneficial for SNDPR. While granules were firstly reported in an up-flow anaerobic sludge blanket (UASB) bioreactor over two decades ago (Lettinga et al., 1980), they have been recently investigated in aerobic SBR systems for nutrient removal (Beun et al., 1999; Cassidy and Belia, 2005; de Kreuk et al., 2005; Lin et al., 2003; Peng et al., 1999; Yang et al., 2003). Compared to conventional flocs, granules are relatively large, compact, dense microbial aggregates of different bacterial species with an approximately spherical external appearance. The size and the dense, compact structure of granules are expected to positively contribute to the oxygen mass transfer limitation required for reliable SNDPR systems. In addition, the excellent settleability of granular sludge allows for more biomass to be maintained in a relatively small reactor volume, enhancing the ability of the reactor to withstand high loading rates. This is of great interest for the treatment of nutrient-rich industrial wastewater.

The work presented investigates the feasibility of using granular sludge to sustain a stable and robust SNDPR system allowing high-level of nutrient removal from nutrient-rich industrial wastewater such as abattoir effluents. A lab-scale SBR, seeded with granular sludge

developed with synthetic wastewater, was operated under alternating anaerobic and aerobic conditions. The feed was changed gradually from synthetic wastewater to real abattoir wastewater. The size distribution of the granules and the long term performance of the reactor in terms of COD, nitrogen and phosphorus removal were monitored. The effect of chemical precipitation on the overall nutrient removal performance and the impact of the SBR cycle time on the reactor nitrification capability were also investigated.

MATERIAL AND METHODS

Abattoir wastewater

The wastewater used in this study was from a local abattoir in Queensland, Australia. At this site, the raw effluent passes through four parallel anaerobic ponds before being treated in a SBR for biological COD and N removal. Anaerobic pond effluent from the abattoir was collected on a weekly basis and stored at 4˚C. The characteristics of the anaerobic pond effluent are detailed in Table 1. The change in the wastewater composition during the one week storage period is also shown in Table 1.

Table 1. Characteristics of the anaerobic pond effluent used in this study. The intervals represent the mid-95% range.

Parameter	Anaerobic pond effluent	Variation after one week storage at 4°C
TCOD ($mg.l^{-1}$)	600-783	-12%
SCOD ($mg.l^{-1}$)	265-384	+5%
VFA* ($mgCOD.l^{-1}$)	58-116	-15%
TN ($mg.l^{-1}$)	225-277	N.A.
NH_4-N ($mg.l^{-1}$)	215-240	+2%
TP ($mg.l^{-1}$)	35-42	N.A.
PO_4-P ($mg.l^{-1}$)	32-38	-5%
TSS ($mg.l^{-1}$)	185-217	N.A.

* Containing acetate and propionate only N.A.: Not Analysed

SBR operation

The SBR had a working volume of 5 l and was operated in a temperature-controlled room (18-22°C). The SBR had an height:diameter ratio of 5.5 and its mixing was carried out via a combination of magnetic stirring (200 rpm) and intermittent sparging of either nitrogen gas (anaerobic/anoxic periods) or oxygen (aerobic period). The reactor was seeded with aerobic granules obtained from another lab-scale SBR fed with synthetic wastewater (Lemaire et al., in press). During the first 40 days (i.e. start-up period), the SBR was fed only with synthetic wastewater. From day 40 to 133 (i.e. transition period), the SBR was fed with a mixture of abattoir and synthetic wastewater. The percentage of abattoir wastewater in the influent was gradually increased from 0 to 100% to allow the granular biomass to acclimate to the high levels of nutrients present in the anaerobically pre-treated abattoir wastewater. During this transition period, the average COD, ammonia and phosphate concentrations in the SBR feed increased from 350 $mgCOD.l^{-1}$, 40 $mgN.l^{-1}$ and 20 $mgP.l^{-1}$ to 1420 $mgCOD.l^{-1}$, 240 $mgN.l^{-1}$ and 40 $mgP.l^{-1}$, respectively. From day 133 onwards the reactor was fed only with

anaerobically pre-treated abattoir wastewater. Additional acetate had to be supplemented to the SBR as the amount of easily biodegradable COD (i.e. VFAs) available in the particular abattoir wastewater used in this study was insufficient (due to highly effective operation of the anaerobic pond) for the removal of the high levels of N and P present. The N, P and VFA concentrations in the SBR feed during the entire period of study is depicted in Figure 1a. Detailed operations of the SBR over the 13 months period are summarised in Table 2. In each cycle, 3 l of anaerobically pre-treated abattoir wastewater supplemented with additional acetate was pumped into the bottom of the reactor without mixing. The feeding period was followed by a mixed anaerobic/anoxic period (Table 2) with intermittent sparging of nitrogen gas (10 seconds every minute) at 2.5 $l.min^{-1}$ to ensure good mixing and shear force in the reactor. In the first two phases, the dissolved oxygen (DO) concentration during the subsequent aerobic period was not controlled, and constant aeration was provided. The DO level in the SBR was stable around 2.0-2.5 $mgO_2.l^{-1}$ during most of the aerobic period, reaching 6 $mgO_2.l^{-1}$ only when ammonia and phosphate were completely depleted, typically in the last 15 minutes of the aerobic period. After day 290, the DO level was controlled between 3.0-3.5 $mgO_2.l^{-1}$ during the entire aerobic period using an on/off aeration control system. To provide adequate shear force during the aerobic period an air diffuser producing coarse bubbles was used for aeration and the air flow rate was maintained at 4 $l.min^{-1}$ (resulting in an upflow superficial air velocity of 0.6 $cm.s^{-1}$). On day 333 (phase IV), a post-anoxic period was introduced after the aerobic period to further enhance denitrification. At the end of the cycle, sludge was rapidly settled before 3 l of supernatant was decanted. During the initial four months (phase I), a hydraulic retention time (HRT) of 6.7 h was applied. Once the percentage of abattoir wastewater in the SBR feed reached 100%, the aerobic period was gradually extended resulting in a HRT of 13.3 h. Wastage was manipulated to maintain MLSS at approximately 20 $g.l^{-1}$, resulting in an SRT of 15-20 days. The pH in the system, which was recorded but not controlled, typically fluctuated between 7.0 and 8.6 over the cycle.

Table 2. Cycle composition during four phases of the SBR operation over the 13 month period.

Cycle operation	Phase I Day 0-133	Phase II Day 133-290	Phase III Day 290-333	Phase IV Day 333-390
Settle (min)	2	2	2	2
Decant (min)	5	5	5	5
Feed (min)	18	18	18	18
Anaerobic (min)	50	60	60	60
Aerobic (min)	160	from 160 to 400	400	315
Post anoxic (min)	0	0	0	80
Total cycle (min)	240	from 240 to 480	480	480
HRT (h)	6.7	from 6.7 to 13.3	13.3	13.3
DO ($mgO_2.l^{-1}$)	no control	no control	control (3.0-3.5)	control (3.0-3.5)

Reactor and granules monitoring procedures

To monitor the reactor performance, the SBR effluent was sampled daily for analysis of phosphate-P (PO_4-P), ammonia (NH_4^+ + NH_3), nitrate and nitrite. Cycle studies were performed once a week during which liquid samples were taken and filtered every 20-30 minutes for analysis of phosphate-P, ammonia, nitrate and nitrite. The reactor mixed liquor suspended solids (MLSS) and volatile MLSS (MLVSS) concentrations were also measured weekly as well as the effluent total suspended solids (TSS).

To monitor the granule structure and characteristics, granule size distribution and density were measured once a week. To determine the size distribution of the granules, 30 ml of well mixed granular sludge was sampled from the SBR at the end of the aeration period and pumped through a Malvern laser light scattering instrument, Mastersizer 2000 series (Malvern Instruments, Worcestershire, UK). The granule density, defined as the quantity of dry mass (TSS) per biomass volume, was measured every week by the blue dextran method adapted from Di Iaconi et al. (2004). The reported value was determined from the mean of five replicates.

Chemical analyses

The ammonia (NH_4^+ + NH_3), nitrate (NO_3^-), nitrite (NO_2^-) and phosphate-P (PO_4-P) concentrations were measured using a Lachat QuikChem8000 Flow Injection Analyser (Lachat Instrument, Milwaukee). Total and soluble chemical oxygen demand (TCOD and SCOD, respectively), soluble five-day biological oxygen demand ($SBOD_5$), total and soluble Kjeldahl nitrogen (TKN and SKN), total phosphorus and total dissolved phosphorus (TP and TDP), MLSS and MLVSS were analysed according to the standard methods (APHA, 1995). The major ions present in the SBR (Ca^{2+}, K^+, Mg^{2+}, Na^+ and HS^-) were measured by Inductively Coupled Plasma - Atomic Emission Spectrometry (ICP-AES Varian Vista-PRO, Varian, Inc.). VFAs were measured by Perkin-Elmer gas chromatography with column DB-FFAP 15m x 0.53mm x 1.0μm (length x ID x film) at 140°C, while the injector and FID detector were operated at 220°C and 250°C, respectively.

The fractions of various types of phosphorus-containing compounds in granules were determined using the cold perchloric acid (PCA) extraction procedure developed by de Haas et al. (2000). The initial centrifugation steps were used to separate the supernatant (containing the phosphate species initially present in the sample bulk liquid (PO_4^{3-}, HPO_4^{2-}, $H_2PO_4^-$ and H_3PO_4) plus the "interstitial" loosely bound phosphates) from the solid fraction or "pellet". The subsequent steps were aimed at distinguishing the biologically and chemically stored phosphorus in the "pellet" (i.e. granules) by measuring the total P and ortho-P of cold PCA extracts. The slow hydrolysis of biologically stored phosphorus (poly-P + nucleic acids + phospholipids + minor organics P = "complex P") in cold PCA implies that, provided the ortho-P is measured immediately after the PCA extraction, the ortho-P content of the PCA extracts may be assumed to originate from chemically bound P (mineral complexes) while non-ortho-P (total P – ortho-P) is assumed to be of biological origin (i.e. "complex P") (de Haas et al., 2000). The residue of the cold PCA extraction can be analysed for total P and contains mostly poly-P associated in some manner with proteins, or other macromolecules which are not extractable in cold PCA. However, these non-PCA soluble phosphate compounds can be almost fully extracted with NaOH (de Haas et al., 2000). To determine the origin (i.e. chemical or biological) of the residue P content of the cold PCA extraction, this additional NaOH fractionation step was performed on 3 different residues.

Microbial analysis

Granule samples for FISH were fixed in 3% paraformaldehyde (Amann, 1995). The fixed granules were embedded in OCT (TissueTek, Sakura, USA) for cryosectioning as previously described (Meyer et al., 2003). FISH was then carried out on the granule sections as described by Amann (1995). Oligonucleotide probes used in this study were the combination of EUB338 i-iii (EUBmix) for the detection of all bacteria (Daims et al., 1999), the combination of PAO462, PAO651 and PAO846 (PAOmix) for *Accumulibacter* spp. (Crocetti et al., 2000), the probe combination (GAOmix) of GAOQ989 (Crocetti et al., 2002) and GB_G2 (Kong et

al., 2002) for *Competibacter* spp., Actino-658 (Kong et al., 2005) for proposed *Actinobacteria* PAO, NTSPA662 (Daims et al., 2001) for *Nitrospira* and NIT3 (Wagner et al., 1996) for *Nitrobacter*.

Hybridised granule sections were visualised with a confocal laser scanning microscope (CLSM) Zeiss LSM 510 (Carl Zeiss, Jena, Germany). To obtain images of entire granule sections, between 10 and 40 overlapping, consecutive images of 1024x1024 pixels were collected (depending on the size of the granule) using a Zeiss Neofluar 40x/1.3 oil objective. The final composite image of the granule section was then reconstructed from all the single images collected using Adobe Photoshop 7.0 (Adobe Systems, USA). FISH quantification was performed on reconstructed granule images as described in Crocetti et al. (2002).

Oxygen profiles

The gradient of oxygen in granules was measured with oxygen microsensors (tip diameter <10μm), which were constructed as described by Revsbech et al. (1989). Granules were sampled from the SBR at the start of the aerobic period when ammonia and phosphates were present at high concentrations and at the end of the aerobic period, at which time ammonia and phosphates were usually depleted. Profile measurements were performed as described in Lemaire et al. (2006). The composition of medium and the dissolved oxygen concentration in the measuring flowcell was identical to that in the SBR at the start or the end of the aerobic period.

Calculation of the degree of struvite saturation during a SBR cycle

To assess the contribution of struvite precipitation ($Mg\ NH_4\ PO_4 \cdot 6H_2O$) on nutrient removal in a SBR cycle, the degree of saturation of the relevant ions in the reactor bulk liquid was calculated over the course of a SBR cycle. Liquid samples were taken for phosphate-P, ammonia and Mg analysis and the pH was recorded on-line. The degree of struvite saturation was expressed by the critical supersaturation ratio (S_c), which relates to the conditional solubility product (P_s) and the product of the concentrations of phosphate-P, ammonia and Mg (P_{so}) as shown in Eq. (1)

$$S_c = (P_{so}/P_s)^{1/3} \quad \textbf{(1)}$$

P_s depends on the pH, the ionic strength and the ion activity and was calculated as described by Ohlinger et al. (1998). The chemical equilibria for phosphate species ($PO_4^{3-}/HPO_4^{2-}/H_2PO_4^{-}/H_3PO_4$), magnesium complexes ($Mg^{2+}/MgOH^{+}$; $Mg^{2+}/MgPO_4^{-}$; $Mg^{2+}/MgHPO_4$ and $Mg^{2+}/MgH_2PO_4^{+}$), ammonia (NH_3/NH_4^{+}) and water were solved to determine the speciation of the ions PO_4^{3-}, NH_4^{+} and Mg^{2+} from the measured concentration of phosphate-P, ammonia and Mg and measured pH value. For each equilibrium equation, the stability constant values applied by Ohlinger et al. (1998) were used. The ions activity coefficients were calculated using the Davies approximation of the Debye-Huckel equations at 20°C. The ionic strength of the SBR bulk liquid was estimated based on the measured concentrations of the major ions present. The solubility product of struvite ($K_{sp}=10^{-13.26}$) proposed by Ohlinger et al. (1998) was employed in this study as it includes the effect of ionic strength and magnesium phosphate complex formation.

Anoxic batch tests

To determine whether PAOs were contributing to denitrification in the reactor, anoxic batch tests were performed to measure if denitrification was accompanied by P uptake under strictly anoxic conditions. Granular mixed liquor (200 ml) was taken from the SBR at the end of the

anaerobic period and placed in two 100 ml vessels. After 10 min of helium sparging, 5 ml of $NaNO_3$ solution was added to one vessel while 5 ml of $NaNO_2$ solution was added to the other vessel resulting in an initial concentration of 55 $mgN.l^{-1}$ of $N\text{-}NO_3$ or $N\text{-}NO_2$ in the two vessels, respectively. Liquid samples were taken every 10 min to monitor the concentration of phosphate, ammonia, nitrate and nitrite.

RESULTS AND DISCUSSION

Granule structure and characteristics

Following the inoculation of the SBR, an initial start-up period of 40 days was used to gradually increase the COD and nutrient concentrations in the synthetic feed (Figure 1a). As shown in Figure 1b, the granule size increased from an initial diameter of 0.1-0.8 mm (10^{th}-90^{th} percentiles) to 0.9-1.6 mm over that period. The introduction of anaerobically pre-treated abattoir wastewater to the reactor influent on day 40 resulted in a temporary decrease of the granule size by about 25%, which fully recovered within a few weeks. From thereon, a relatively stable granule size of 0.7-1.6 mm was maintained in the reactor. The fluctuations of the granule size observed after day 133 are hypothesized to be a consequence of the dynamic processes regulating the size of the granules; big granules are expected to fracture into smaller aggregates that can then grow into bigger granules again.

Figure 1c shows the MLSS and MLVSS concentrations and also the granule density over the experimental period. MLSS linearly increased from 2.8 to 20.2 $g.l^{-1}$ in the first 160 days, largely as a result of the increased COD load but also partially due to the fact that no sludge was deliberately wasted from the reactor in this period. Sludge was regularly wasted after day 160 to maintain the biomass concentration at approximately 20 $g.l^{-1}$. The MLVSS concentration displayed a similar trend to that of MLSS, but the MLVSS:MLSS ratio decreased with time. The granule density gradually decreased from 290 $gTSS.l_{biomass}^{-1}$ with synthetic feed to around 150 $gTSS.l_{biomass}^{-1}$ with real wastewater feed, with reasons yet to be identified. The density measured in this study is considerably higher than the density values reported in literature. For example, de Kreuk et al. (2005) reported a granule density of 78-89 $gTSS.l_{biomass}^{-1}$, while Cassidy and Belia (2005) reported a density of 12-32 $gTSS.l_{biomass}^{-1}$ in a granular SBR using abattoir wastewater.

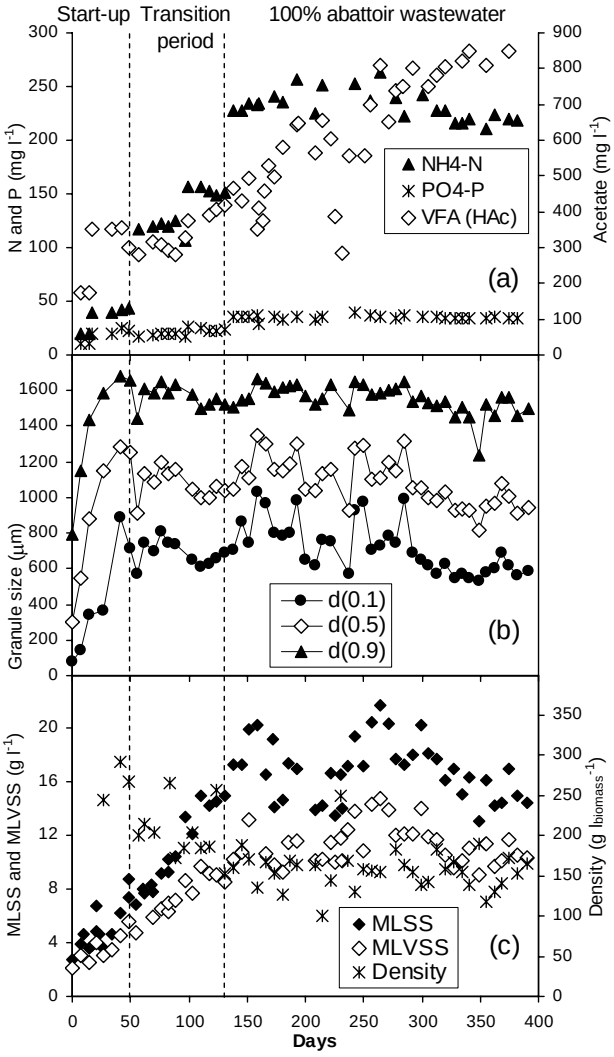


Figure 1. (a) N, P and VFA concentrations in the SBR feed. During the start-up phase only synthetic wastewater was used, which was then stepwise changed to real wastewater during the transition period and after day 133 only anaerobically pre-treated abattoir wastewater was fed to the reactor. **(b)** Volumetric distribution of granule size in the reactor during the entire study. d(0.1), d(0.5), d(0.9) are the 10^{th}, 50^{th} and 90^{th} percentiles of the distribution. **(c)** MLSS, MLVSS and granule density during the study.

Nitrogen and Phosphorus Removal Performance

The SBR performance in terms of nitrogen and phosphorus removal varied considerably during the process of gradually increasing the fraction of abattoir wastewater in the feed (data not shown). Indeed, the unstable performance continued for 3-4 months after 100% of abattoir wastewater was applied, as shown in Figure 2. As the nitrification gradually improved, more carbon was needed to denitrify the increasing amount of NO_x^- produced and therefore the extra acetate was gradually increased from 330 to 750 mgCOD.l^{-1} (Figure 1a). The acetate concentration in the influent was stable after day 280 (750 mgCOD.l^{-1}) which corresponds to the time when complete nitrification was achieved in the reactor.

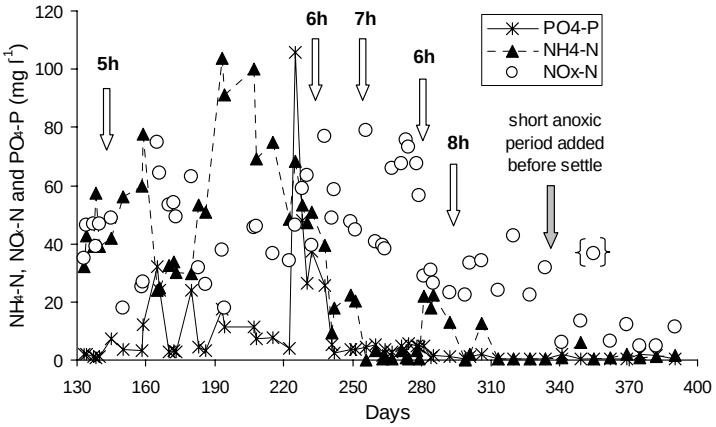


Figure 2. Long-term effluent NH_4-N, NO_x-N and PO_4-P concentrations showing the nitrogen and phosphorus removal performance of the SBR, and the impact of cycle time changes (indicated by vertical arrows) on the nitrification performance. The NO_x^- data point around day 360, enclosed with {}, was an outlier caused by a hardware failure of the control system.

Good nitrogen and phosphorus removal performance was achieved once stable operation was established. Table 3 shows the average reactor performance during the last 2 months of operation under an organic, nitrogen and phosphorus loading rate of 2.7 $gCOD.l^{-1}.d^{-1}$, 0.43 $gN.l^{-1}.d^{-1}$ and 0.06 $gP.l^{-1}.d^{-1}$, respectively. Both the ammonia and phosphate removal efficiencies were over 98%. The removal efficiency for the soluble COD was 85%. The remaining soluble COD measured in the effluent (162 $mg.l^{-1}$) was non-biodegradable as indicated by the very low soluble BOD_5 value (<2 $mg.l^{-1}$) (Table 3). It indicates that the abattoir pond wastewater used contained a relatively high fraction of non-biodegradable COD estimated to be around 40% of its soluble COD. During the last two months of stable operation, the biomass yield in the granular SBR was estimated to be 0.3 $gVSS.gCOD^{-1}$. Considering a biomass composition of $CH_{1.8}O_{0.55}N_{0.2}P_{0.015}$, the biomass growth accounted for approximately 16% and 22% of the total N and P removal in the SBR, respectively.

Table 3. Summary of reactor performance in the last 2 months (day 333 to day 390).

Parameter	Influent average (SD; NS)	Effluent average (SD; NS)	Nutrient removal efficiency
TKN ($mgN\ l^{-1}$)	237.3 (6.6; 8)	25.3 (5.2; 8)	85.7 % (TN)
SKN ($mgN\ l^{-1}$)	220.1 (4.1; 7)	7.6 (0.7; 7)	92.7 % (TDN)
NH_4 ($mgN\ l^{-1}$)	221.7 (6.7; 7)	0.8 (0.5; 10)	99.7 %
NO_3 ($mgN\ l^{-1}$)	N.D.	5.2 (1; 9)	---
NO_2 ($mgN\ l^{-1}$)	N.D.	3.3 (4.1; 9)	---
NO_x ($mgN\ l^{-1}$)	N.D.	8.5 (3.7; 9)	---
TP ($mgP\ l^{-1}$)	34.3 (1.2; 8)	9.0 (2.8; 7)	73.8 %
TDP ($mgP\ l^{-1}$)	32.4 (1.4; 7)	3.6 (1.2; 7)	89.5 %
PO_4 ($mgP\ l^{-1}$)	33.6 (0.8; 7)	0.6 (0.3; 10)	98.3 %
TCOD ($mg\ l^{-1}$)	1480* (138; 6)	467 (83; 5)	68.4 %
SCOD ($mg\ l^{-1}$)	1072* (53; 6)	162 (33; 7)	84.8 %
$SBOD_5$ ($mg\ l^{-1}$)	N.A.	<2 (--; 4)	---
TSS ($mg\ l^{-1}$)	205 (17; 8)	306 (88; 8)	---

* including 750 $mgCOD.l^{-1}$ of acetate added

SD: Standard Deviation; NS: Number of Samples; N.D.: Not Detectable, N.A.: Not Analysed

It is known that granular sludge systems produce effluent containing higher levels of suspended solids compared to floccular sludge systems due to their specific design (i.e. short settling time and sludge wasting with the effluent). In this study, the average effluent TSS concentration was 306 $mg.l^{-1}$, which is in the higher range of effluent TSS values reported in literature for granular systems (McSwain et al., 2004; Mosquera-Corral et al., 2005; Pan et al., 2004). However, the influent of those granular systems did not contain any solids (i.e. synthetic wastewater) whereas in this study, the average influent TSS of 205 $mg.l^{-1}$ would have certainly contributed to the high level of TSS in the effluent given the short HRT of the SBR. This high level of suspended solids in the effluent significantly reduced the TN, TP and TCOD removal efficiencies of the SBR (Table 3). Cassidy and Belia (2005) reported higher TN, TP and TCOD removal efficiencies in their granular SBR treating abattoir wastewater under very similar organic, nitrogen and phosphorus loading rates and similar SRT. The reason for this might be the much longer HRT they employed compared to this study (72 h vs. 13.3 h), resulting in only 8% of the SBR working volume being discharged in each cycle. Considering that the same settling time of 2 min was applied, the suspended solids in their effluent (55 $mg.l^{-1}$) were therefore much lower than in our study which could explain the higher nutrient removal efficiencies reported. More biomass was mechanically wasted in their SBR each day in order to keep the SRT constant around 20 days. In either case though, an additional filtration or floatation system would be necessary to remove the remaining solids from the SBR effluent to meet environmental discharge limits. The additional costs associated with such a solids removal step are likely limited at least for the meat industry since post-treatment using dissolved air flotation (DAF) is already required in many abattoirs to remove remaining FOG and solids from the activated sludge process effluent before release to the receiving waterways. On the other hand, the HRT applied in the last 2 months of the reactor operation (i.e. 13.3 h) was less than 20% of the HRT currently applied in the full-scale floccular SBR system used in the studied abattoir. This implies that the reactor volume could theoretically be reduced by more than 80% if a granular SBR system was used, which would result in considerable savings that could more than offset the extra costs for a post-treatment step. It may therefore be concluded that granular sludge is a very attractive option for the treatment of nutrient-rich industrial wastewater such as abattoir wastewater. However, the operation of the anaerobic pre-treatment processes of the raw abattoir wastewater might have to be modified (i.e. high rate prefermentor, anaerobic lagoons with shorter HRT) to ensure that sufficient amount of VFAs are available for the subsequent N and P removal in the granular sludge system.

Contribution of chemical precipitation in the overall nutrient removal efficiency

Nutrient-rich wastewaters often provide the right conditions for the formation of mineral complexes such as apatites ($Ca_2HPO_4(OH)_2$, hydroxydicalcium phosphate or HDP; $Ca_5(PO_4)_3OH$, hydroxyapatite or HAP; $Ca_3(PO_4)_2.xH_2O$, amorphous calcium phosphate or ACP), newberyite ($MgHPO4.3H_2O$) or struvite ($MgNH_4PO_4.6H_2O$) (Maurer et al., 1999; Musvoto et al., 2000). The precipitation of these minerals is controlled by pH, degree of supersaturation, temperature and the presence of other ions in the wastewater and can occur when the concentrations of Ca^{2+}, Mg^{2+}, NH_4^+ and PO_4^{3-} ions exceed the solubility products of the minerals (HDP: $K_{sp}=10^{-22.6}$, Maurer et al. (1999); newberyite: $K_{sp}=10^{-5.51}$, Abbona et al. (1982); struvite: $K_{sp}=10^{-13.26}$, Ohlinger et al. (1998)). It is well established that the large release of PO_4^{3-} and Mg^{2+} during the anaerobic phase of the EBPR process (ratio P:Mg of 3:1, mole based) favours the precipitation of phosphates into diverse minerals, which is referred to as “biologically induced precipitation” (Maurer et al., 1999).

In our granular SBR system, the combination of nutrient-rich industrial wastewater, high loading rate and strong EBPR activity could have induced chemical precipitation, contributing to the overall nutrient removal described previously. Figure 3 shows the concentrations of Ca, Mg, ammonia and phosphate-P in the bulk liquid during a cycle study performed on day 270 along with the pH recorded. The important decrease of ammonia during the last 30 minutes of the anaerobic period (around 30 $mgN.l^{-1}$) was consistently observed in all cycles and, to our knowledge, cannot be explained by any anaerobic biological processes. The relatively long lag phase (15 min) observed between the end of feeding and the start of the sharp ammonium decrease (Figure 3) indicates that the adsorption of ammonium to the granular sludge surface unlikely contributed to this phenomenon as biosorption is a relatively fast process reaching equilibrium within minutes (Nielsen, 1996). This means that chemical precipitation of ammonium-containing minerals such as struvite likely occurred in this granular SBR during the anaerobic phase, which is not surprising given the very high concentration of phosphate-P (250 $mgP.l^{-1}$), ammonia (120 $mgN.l^{-1}$) and Mg (70 $mg.l^{-1}$) at that time. To further demonstrate the possibility for struvite formation in this SBR, the degree of struvite saturation, or critical supersaturation ratio (S_c), is also depicted in Figure 3. When $S_c>1$, the SBR bulk liquid is supersaturated and struvite precipitation could likely occur (represented by the grey period). The initial formation of struvite (i.e. nucleation) can be facilitated by the presence of suitable nuclei such as solid impurities (Doyle and Parsons, 2002). In our granular SBR, the biopolymers matrix (i.e. external polymeric substances - EPS) of the granules may play this role. When $S_c<1$, the bulk liquid is undersaturated and struvite would dissolve releasing PO_4^{3-}, NH_4^+, and Mg^{2+} in the bulk liquid. However, no corresponding increase of their respective concentration was observed in the later part of the cycle (Figure 3). Any PO_4^{3-} released under aerobic condition would be immediately taken up by PAOs (alongside with Mg^{2+}) to form intracellular poly-P and any NH_4^+ released would be oxidised to nitrite/nitrate by nitrifiers, therefore masking the possible effect of struvite dissolution. Nevertheless, indirect evidence of this dissolution can be glanced when considering that 35 $mgN.l^{-1}$ of NO_x^- accumulated in the bulk liquid in the last 150 min of the cycle (data not shown) while only 20 $mgN.l^{-1}$ of NH_4^+ was oxidised (Figure 3). The production of NO_x^- in excess of NH_4^+ consumption was consistently observed in the last 1-2 h of the aerobic period (see also Figure 4) and was likely due to the instantaneous oxidation of NH_4^+ released through the dissolution of struvite. It is also interesting to notice that the calcium concentration in the bulk liquid more than doubled in the last 200 min of the aerobic period (Figure 3). It could be that apatites formed during the anaerobic period when the PO_4^{3-} concentration was very high, later re-dissolved during the aerobic period when PO_4^{3-} level is very low and pH is decreasing (from 8.3 to 7.8). Unlike PO_4^{3-}, Ca^{2+} released from apatites dissolution (mostly HDP and ACP) was not subsequently removed from the bulk liquid via biological processes and therefore accumulated. This agrees well with the dynamic model proposed by Maurer et al. (1999) to describe the pH-sensitive and partly reversible precipitation of calcium phosphates observed in EBPR systems.

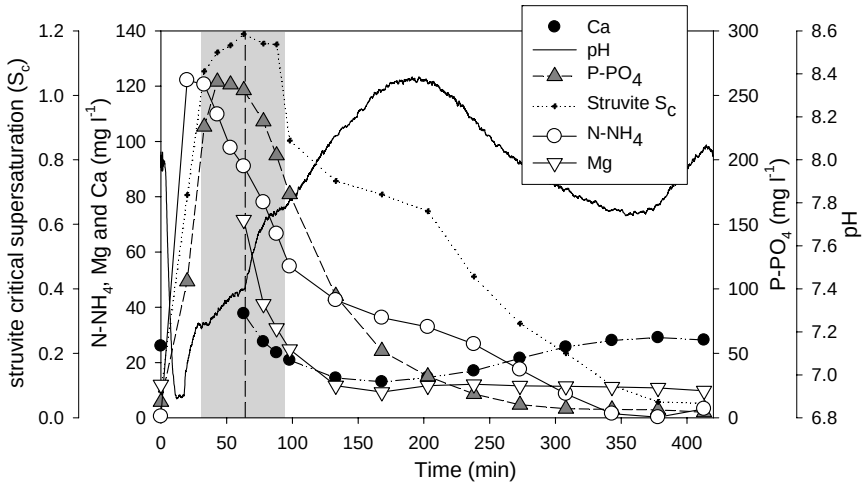


Figure 3. Cycle study performed on day 270 illustrating the role of chemical precipitation in the overall nutrient removal. The vertical dotted line delimits the anaerobic and aerobic periods. The profiles of total phosphate, ammonia, magnesium and calcium along with the pH and the ratio of struvite critical supersaturation (S_c) are depicted. In the calculation of S_c, the magnesium concentration during the anaerobic phase was estimated using the measured concentrations at the beginning and the end of the anaerobic period and the phosphate profile, assuming a Mg-released:P-released ratio of 0.33 mol.mol^{-1} (Smolders et al., 1994). The grey zone indicates when struvite is likely to precipitate (S_c>1) in the cycle based on the thermodynamic equilibrium. The rest of the time (S_c<1), struvite should dissolve.

To assess the exact contribution of chemical mineral precipitation to the overall nutrient removal, the possible interactions between the main mineral formed and the different ions species present in wastewater, as well as nucleation thermodynamics, crystal growth kinetics and dissolution rates would have to be considered, which can rapidly become very complex. A simpler method to evaluate the contribution of phosphate precipitation during the SBR cycle is to apply the phosphorus fractionation procedure based on cold perchloric acid (PCA) developed by de Haas et al. (2000) to mixed liquor samples taken at the end of the anaerobic period and at the end of the cycle. This PCA fractionation procedure is capable of distinguishing between biologically-stored forms of phosphate (mainly poly-P in EBPR systems) and chemically precipitated forms of phosphate (ortho-P) in activated sludge samples.

Table 4 presents the phosphorus fractionation of mixed liquor samples taken at the end of the anaerobic and aerobic periods in different SBR cycles. First of all, it should be noted that the typical EBPR pattern is well illustrated by the P fractionation results. By the end of the anaerobic period, 30.5% of the total amount of P contained in the sample was released into the bulk liquid (or supernatant) as ortho-P. This ortho-P was subsequently taken-up and stored intracellularly as poly-P (i.e. “complex P”) in the aerobic period as shown by the higher fraction of “complex P” in the granules at the end of the cycle compared to the end of the anaerobic period (76.8% and 35.7%, respectively - see Table 4). The important information given in Table 4 concerns the fraction of P held in mineral complexes formed in an SBR cycle, which is represented by the ortho-P fraction in the PCA extract. As previously suggested, this fraction was significant at the end of the anaerobic period (10.6%) but was very small at the end of the SBR cycle (1.9%). This confirms that most of the mineral complexes formed during the anaerobic period (due to higher bulk liquid phosphate-P

concentrations) were subsequently re-dissolved during the aerobic period. Therefore mineral complexes (or precipitates) did not appear to contribute significantly to the overall nutrient removal performance of the SBR. The P left over in the residue of the cold PCA extract was found to be at least 95% "complex P" (i.e. most likely of biological origin) when employing a NaOH based extraction procedure on samples taken at the end of a cycle and at the end of the anaerobic period; less than 2% was of chemical origin (i.e. ortho-P) and around 3% were unaccounted for (data not shown).

Table 4. Phosphorus fractionation determined by the cold PCA procedure in five mixed liquor samples taken at the end of the anaerobic and at the end of the aerobic period during five different cycles.

Sample/Extract	**Ortho P** (% of sample TP)	**Total P** (% of sample TP)	**"Complex P" (Total P – Ortho P)** (% of sample TP)
End of anaerobic period (n=5)			
Supernatant	30.5% ; SD=1.8	30% ; SD=4.0	(-0.5%) ; SD=2.1
PCA	10.6% ; SD=1.8	46.3% ; SD=5.8	35.7% ; SD=4.0
Residue (not extracted)	---	29.5% ; SD=3.9	---
Recovery	---	105.7% ; SD=2.8	---
End of cycle (n=5)			
Supernatant	0.2% ; SD=0.2	2.6% ; SD=0.9	2.4% ; SD=0.7
PCA	1.9% ; SD=0.5	78.7% ; SD=4.3	76.8% ; SD=3.8
Residue (not extracted)	---	18.9% ; SD=2.3	---
Recovery	---	99.4% ; SD=5.0	---

Simultaneous Nitrification, Denitrification and Phosphorus Removal (SNDPR) using Granular Sludge

SNDPR via nitrite was previously demonstrated using floccular sludge and synthetic wastewater (Zeng et al., 2003a). However, denitrification was not attributed to PAOs but to glycogen-accumulating organisms (GAOs), their known competitors. Without denitrification by PAOs, there is no true link between SND and EBPR, the two processes simply occurring in the same sludge at the same time. Figure 4 shows a cycle study on the SBR performed on day 369. During the first 3 h of the aerobic period, before phosphates were completed taken up, 70 $mgN.l^{-1}$ of NH_4^+ was removed with only the accumulation of less than 10 $mgN.l^{-1}$ of NO_2^-, demonstrating that simultaneous nitrification and denitrification (SND) occurred. The oxidised nitrogen present at the end of the aerobic period was almost exclusively nitrite (NO_2^- : NO_x^- ratio=0.95, SD=2.5%, n=63) as depicted in Figure 4, suggesting that N was removed via the nitrite pathway in this granular SBR. At a microbial level, common nitrite oxidising bacteria population (i.e. *Nitrobacter* and *Nitrospira*) were not detected in the SBR (data not shown) providing further supporting evidence that SND via nitrite occurred in this SBR.

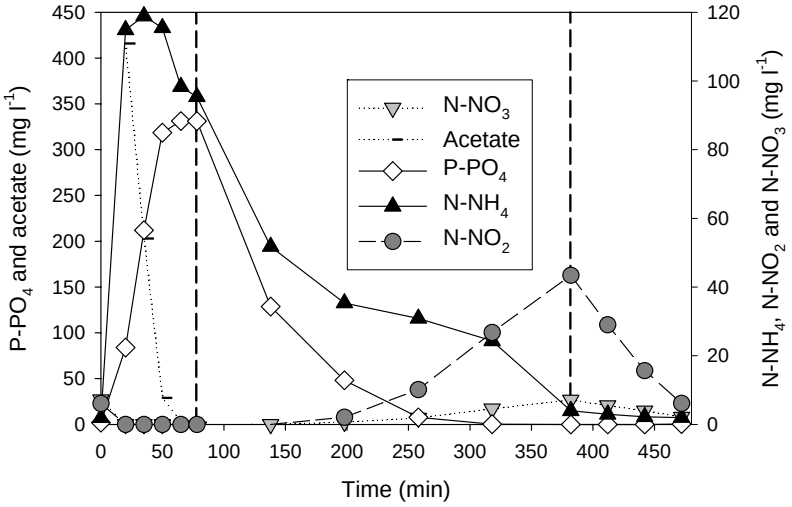


Figure 4. Cycle study performed on the SBR on day 369 demonstrating the occurrence of simultaneous nitrification, denitrification and phosphorus removal. The two dotted lines delimit the anaerobic period (0-78 min), the aerobic period (78-393 min) and the post anoxic period (380-470 min).

Most of the nitrite accumulation in the SBR (i.e. up to 45 mgN-NO_2.l^{-1}) occurred during the last 2 h of the aerobic period, when phosphates was completely taken up, before being later reduced to less than 5 mgN.l^{-1} in the post-anoxic period (Figure 4). The concomitance of the nitrite accumulation and the depletion of phosphate suggested that PAOs might be responsible for most of the denitrification and that SNDPR occurred in this granular SBR. This was further confirmed by the anoxic batch test presented in Figure 5a. Phosphate was taken up in the absence of oxygen while NO_2^- was being reduced. The P-uptake:N-denitrification ratio was 0.84 mgP.mgN^{-1} which is comparable to the value of 1.18 mgP.mgN^{-1} reported by Zeng et al. (2003b) for an enriched culture of denitrifying PAOs. When nitrate instead of nitrite was added to the granular sludge under otherwise identical anoxic condition (Figure 5b), the denitrification rate decreased by more than 80% (from 50.5 to 8.5 mgN.l^{-1}.h^{-1}) and no simultaneous P-uptake was observed.

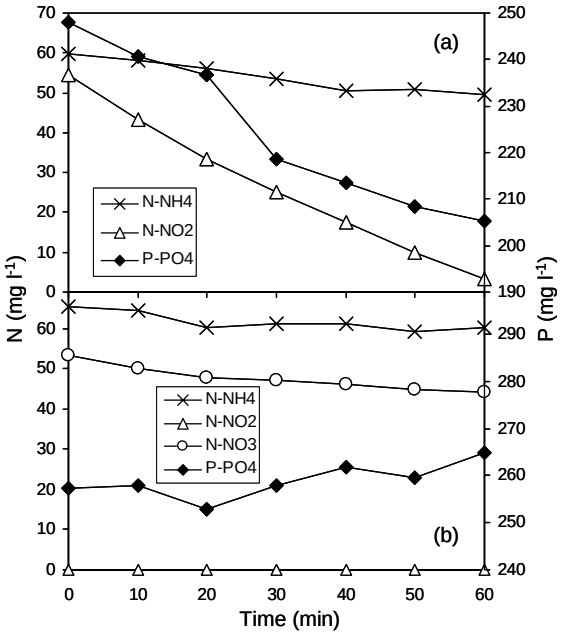


Figure 5. Measurement of phosphate, nitrate, nitrite and ammonia during anoxic batch tests with nitrite **(a)** and nitrate **(b)** addition.

This study shows that SNDPR via nitrite can be achieved with nutrient-rich abattoir wastewater using granular sludge and that SND and EBPR are truly linked, optimising the use of the limited available COD for nutrient removal.

The presence of granules probably facilitated the SNDPR process. As shown in Figure 6, oxygen was barely penetrating into the granules 1 h after the aerobic period started, when the granule biomass was highly active. At the end of the aerobic period (i.e. the last 15 min) when the microbial activity inside the granule was low and the DO level in the liquid phase reached 5-6 $mgO_2.l^{-1}$, oxygen was only penetrating the granules to a limited depth (i.e. 300-400 μm). Anoxic zones in the inner part of the granules were therefore present during most of the aerobic period.

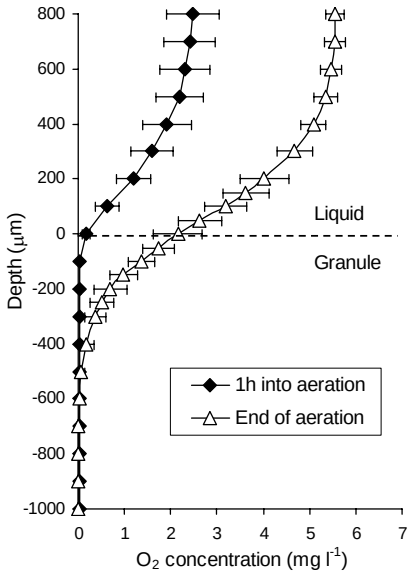
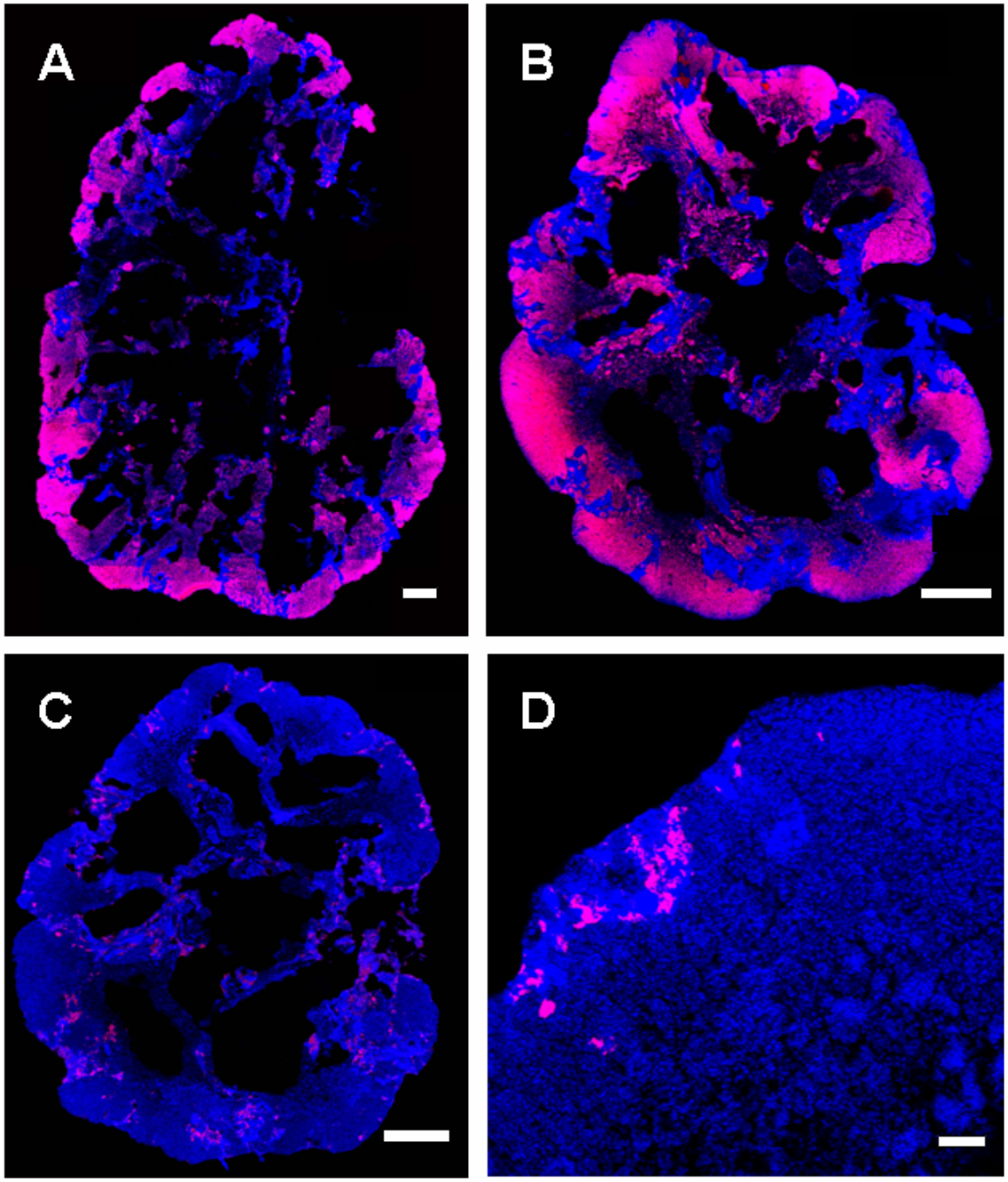


Figure 6. O_2 profiles in granules measured on day 240 at 1 h into the aeration period and at the end of the aeration period (i.e. after DO level reached 5-6 $mgO_2.l^{-1}$ due to low microbial activity in the granules). Profiles shown are averages (error bars=S.E., n=4).

Looking at a microbial community level, *Accumulibacter* spp. was the dominant PAO detected in 10 different cryosectioned granules by fluorescent in situ hybridisation (41% of all bacteria, SD=9, n=10, Supplementary Figures S1a and S1b). *Actinobacteria* PAOs were also present but not dominant (4.1% of all bacteria, SD=1.2, n=7, Supplementary Figures S1c and S1d). Very few clusters of *Competibacter* spp. cells, the only GAO detected in the system, could be observed in each granule. Their low abundance was under the detection limit of the FISH quantification method employed. Given the microbial composition of this granular SNDPR system, the above stated proposition that PAOs (mainly *Accumulibacter* spp.) are responsible for the denitrification in this reactor is strongly supported. The discrepancy between the nitrite and nitrate reduction ability may also be explained by the information contained in the genome sequence of *Accumulibacter* spp. recently published (Martin et al., 2006). This genome does not appear to contain the genetic information for the known respiratory nitrate reductase enzymes whereas genes for enzymes in the denitrification pathway from nitrite reduction onwards were present. Since granular sludge is a complex mixed cultures, organisms other than *Accumulibacter* spp. could have first reduced nitrate to nitrite, before *Accumulibacter* spp. could finish the denitrification from nitrite to nitrogen gas. It is very interesting to highlight that before being exposed to real wastewater conditions, the original granules cultured on synthetic wastewater with acetate as the sole carbon source, contained a high fraction of *Competibacter* spp. (around 25% of all bacteria) which was found to be responsible for denitrification in that SNDPR system (Lemaire et al., in press). In this study, the disappearance of *Competibacter* spp. from the SNDPR system and the denitrification likely by *Accumulibacter* spp. coincide with the use of real abattoir wastewater. In addition, the inhibition or elimination of the nitrite oxidiser bacteria population in this SNDPR system prevented nitrite to be oxidised to nitrate which might have allowed PAOs to perform full denitrification to N_2 without relying on GAOs or any other denitrifiers to carry out the first denitrification step.



Supplementary Figure S1. Reconstructed CLSM images of FISH micrographs of entire granule sections (a, b and c) and part of the section (d). In **(a)** and **(b)** *Accumulibacter* spp. cells are magenta (overlay of red PAOmix and blue EUBmix) and other *Bacteria* are blue (blue EUBmix). In **(c)** and **(d)** *Actinobacteria* PAO cells are magenta (overlay of red Actino-658 and blue EUBmix) and other *Bacteria* are blue (blue EUBmix). Scale bar = 100 μm for (a), (b) and (c) and 10 μm for (d).

Hydraulic retention time (HRT) determination

Several factors were identified to have contributed to the variation of reactor performance. These included the adaptation of the biomass to the increased N and P load caused by the stepwise increase of the fraction of abattoir wastewater in the feed (see Figure 1a), and also the variation of the VFA level in the feed. For example, the effluent P peak observed around day 230 (Figure 2) correlated well with the low VFA concentration in the feed in this period (Figure 1a). Of most significance to the reactor operation is the finding that the cycle time (or equivalently the HRT when the amount of wastewater fed in each cycle remained constant) has a major impact on the nitrification performance. Figure 2 shows that a cycle time of 8 h is required in order to achieve complete nitrification. Between day 133 and day 250, when a cycle time of 5 h or 6 h was applied, the degree of nitrification varied between 60 – 90%. This is in clear contrast to the results obtained in periods when a cycle time of 8 h was applied (day 250 to day 280 and from day 300 onwards), during which over 99% of the influent ammonia was removed (see also Table 3). It is interesting to observe the ammonia peak after day 280, which further confirms that a cycle time of 6 h was too short.

Theoretically, the degree of nitrification is primarily determined by the SRT, with the HRT playing little or no role. The fundamental reason for this prediction is that, in a steady state operation of a reactor, the nitrifier concentration in the reactor is proportional to 1/HRT (see Appendix). Consequently, the nitrification capability of the sludge is proportional to the nitrogen load resulting in an effluent quality that is independent of HRT (see Appendix). Based on the oxygen profiles inside the granules measured on day 240 (Figure 6), we hypothesize that the contradiction between the theoretical and experimental results in this study was caused by the mass transfer limitation of oxygen in granules. The limitation in oxygen transfer implies that only a fraction of nitrifiers are able to contribute to the oxidation of nitrogen. Consequently, the increase in the nitrifier population (X_A) as the result of a reduced HRT (Eq. (A3)) does not necessarily lead to a proportional increase in the nitrification rate of the sludge, leading to incomplete nitrification when a too short HRT (or cycle time) is applied. The shortening of the HRT leads to an increase in the COD and nutrient loading of the process, and it is likely that this increased loading rate is the performance-limiting factor in this case. However, no independent experimental study of the effect of the loading rate was undertaken in this case as actual abattoir wastewater with a given concentration range was used. The experimental results obtained in this study show that a minimum cycle time of 8h is required for the wastewater and operational conditions (SRT = 15–20 days, DO = 2.5–3.0 $mgO_2.l^{-1}$) used in this study.

In the design of a conventional wastewater treatment plant, the SRT is typically chosen to ensure complete nitrification, while the HRT is designed such that the resulting solids concentration is below a certain limit (e.g. 4 $g.l^{-1}$) determined by the sludge settleability. When granular sludge is used, a much higher solids concentration can be used (e.g. 20 $g.l^{-1}$ in this study). In this case, sludge settleability is no longer a limiting factor, and the minimum HRT (or maximum loading rate) applicable is likely determined by other factors, as indicated by the results obtained in this study. Mass transfer limitation could be one of these factors. The design criteria for the HRT and loading rates in granular sludge systems require further investigation.

CONCLUSION

Nitrogen and phosphorus removal using granular sludge from anaerobically pre-treated abattoir wastewater was investigated. The following conclusions are drawn:

- Good nitrogen and phosphorus removal from nutrient-rich industrial wastewater can be achieved biologically using granular sludge operated under alternating anaerobic and aerobic conditions. The use of granules likely facilitated the simultaneous nitrification, denitrification and phosphorus removal process observed;
- Unlike in granular SNDPR systems using synthetic wastewater, *Accumulibacter* spp. were found to be responsible for both denitrification and P removal resulting in lower carbon demand. Increased microbial diversity due to the use of more complex wastewater and utilisation of the nitrite pathway are believed to be the main reasons for this denitrification by PAOs.
- Mineral precipitation was observed in the reactor mostly during the anaerobic period. However, the overall contribution of this mineral precipitation in the nutrient removal was only minor due to subsequent dissolution in the aerobic period.
- The minimum HRT for a granular sludge system is not governed by the sludge settleability and retention, as is the case in a system with floccular sludge. Mass transfer limitations in granules are likely an important factor to be considered in the design of the HRT and the COD and nutrient loading rate in a granular sludge system.

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APPENDIX

The following mass balance equations can be made for an aerobic nitrifying system operated with hydraulic and sludge retention times of *HRT* and *SRT*, respectively:

$$\frac{dX_A}{dt} = \mu_{A,\max}\frac{S_{NH}}{K_{NH}+S_{NH}}X_A - b_A X_A - \frac{1}{SRT}X_A$$

$$\frac{dS_{NH}}{dt} = \mu_{A,\max}\frac{S_{NH}}{K_{NH}+S_{NH}}\frac{X_A}{Y_A} + \frac{1}{HRT}(S_{NN,\inf} - S_{NH}) \qquad \textbf{(A1)}$$

where X_A and S_{NH} are, respectively, the nitrifier and ammonium (including ammonia) concentrations in the system; $S_{NN,,inf}$ is the nitrifiable nitrogen concentration in the feed; $\mu_{A,max}$, b_A, K_{NH} and Y_A are the maximum specific growth rate (d^{-1}), the decay rate (d^{-1}), the affinity

constant with respect to ammonia ($mgN.l^{-1}$) of nitrifiers and the biomass yield ($mgCOD.mgN^{-1}$), respectively.

Solving Eq. (A1) in steady state for X_A and S_{NH} gives:

$$S_{NH} = \frac{K_{NH}(b_A + 1/SRT)}{\mu_{A,\max} - b_A - 1/SRT} \quad \textbf{(A2)}$$

$$X_A = \frac{Y_A(S_{NN,\inf} - S_{NH})}{HRT(b_A + 1/SRT)} \quad \textbf{(A3)}$$

The nitrification capacity of the sludge ($r_{N,max}$, $mgN.l^{-1}.d^{-1}$) is:

$$r_{N,\max} = \frac{\mu_{A,\max}}{Y_A} X_A = \frac{\mu_{A,\max}(S_{NN,\inf} - S_{NH})}{HRT(b_A + 1/SRT)} \quad \textbf{(A4)}$$

Eq. (A4) implies that, with a given HRT, the nitrifiers are able to oxidise the incoming nitrogen load to the same level (S_{NH} determined by Eq. (A2)), which is independent of the HRT (and indeed the influent nitrifiable nitrogen concentration as well).

While the above equations were derived for continuously fed reactors, the conclusion that the effluent ammonia concentration should be independent of HRT is applicable to a fed-batch reactor such as the SBR used in this study.

REFERENCES

Abbona F., Madsen H.E.L. and Boistelle R. (1982). Crystallization of 2 Magnesium Phosphates, Struvite and Newberyite - Effect of Ph and Concentration. *Journal of Crystal Growth*. **57**(1): 6-14.

Amann R.I. (1995). In situ identification of microorganisms by whole cell hybridization with rRNA-targeted nucleic acid probes. In: Molecular Microbial Ecology Manual. Akkermans A.D.L., van Elsas J.D. and de Bruijn F.J. (eds). Dordrecht, Holland, *Kluwer Academic Publications*. **3.3.6:** 1-15.

APHA (1995). Standard methods for the examination of water and wastewater. Washington, DC, *American Public Health Association*.

Beccari M., Carrieri C., Misiti A. and Ramadori R. (1984). Design of single sludge systems for the treatment of wastewaters with high ammonia content. *La Tribune du Cebedeau*. **37**(491): 387-394.

Bertanza G. (1997). Simultaneous nitrification-denitrification process in extended aeration plants: Pilot and real scale experiences. *Water Science and Technology*. **35**(6): 53.

Beun J.J., Hendriks A., Van Loosdrecht M.C.M., Morgenroth E., Wilderer P.A. and Heijnen J.J. (1999). Aerobic granulation in a sequencing batch reactor. *Water Research*. **33**(10): 2283-2290.

Cassidy D.P. and Belia E. (2005). Nitrogen and phosphorus removal from an abattoir wastewater in a SBR with aerobic granular sludge. *Water Research*. **39**(19): 4817-4823.

Comeau Y., Hall K., Hancock R. and Oldham W. (1986). Biochemical model for enhanced biological phosphorus removal. *Water Research*. **20**: 1511-1521.

Crocetti G.R., Banfield J.F., Keller J., Bond P.L. and Blackall L.L. (2002). Glycogen accumulating organisms in laboratory-scale and full-scale activated sludge processes. *Microbiology*. **148**(11): 3353-3364.

Crocetti G.R., Hugenholtz P., Bond P.L., Schuler A., Keller J., Jenkins D. and Blackall L.L. (2000). Identification of polyphosphate-accumulating organisms and design of 16S rRNA-directed probes for their detection and quantitation. *Applied and Environmental Microbiology*. **66**(3): 1175-1182.

Daims H., Bruhl A., Amann R., Schleifer K.H. and Wagner M. (1999). The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: Development and evaluation of a more comprehensive probe set. *Systematic and Applied Microbiology*. **22**(3): 434-444.

Daims H., Nielsen J.L., Nielsen P.H., Schleifer K.H. and Wagner M. (2001). In situ characterization of Nitrospira-like nitrite oxidizing bacteria active in wastewater treatment plants. *Applied and Environmental Microbiology*. **67**(11): 5273-5284.

de Haas D.W., Wentzel M.C. and Ekama G.A. (2000). The use of simultaneous chemical precipitation in modified activated sludge systems exhibiting biological excess phosphate removal Part 2: Method development for fractionation of phosphate compounds in activated sludge. *Water SA*. **26**(4): 453-466.

de Kreuk M., Heijnen J.J. and van Loosdrecht M.C.M. (2005). Simultaneous COD, nitrogen, and phosphate removal by aerobic granular sludge. *Biotechnology and Bioengineering*. **90**(6): 761-769.

Di Iaconi C., Ramadori R., Lopez A. and Passino R. (2004). Preliminary biomass characterization in a sequencing batch biofilm reactor. *Annali di Chimica*. **94**(12): 889-898.

Doyle J.D. and Parsons S.A. (2002). Struvite formation, control and recovery. *Water Research*. **36**(16): 3925-3940.

Frose G. and Kayser R. (1985). Effective treatment of wastewater from rendering plants. Proceedings 40th Annual Purdue Industrial Waste Conference, Indiana, *Butterworths*.

Fuerhacker M., Bauer H., Ellinger R., Sree U., Schmid H., Zibuschka F. and Puxbaum H. (2000). Approach for a novel control strategy for simultaneous nitrification/denitrification in activated sludge reactors. *Water Research*. **34**(9): 2499-2506.

Furumai H., Kazmi A.A., Fujita M., Furuya Y. and Sasaki K. (1999). Modeling long term nutrient removal in a sequencing batch reactor. *Water Research*. **33**(11): 2708-2714.

Keller J., Subramaniam K., Gosswein J. and Greenfield P.F. (1997). Nutrient removal from industrial wastewater using single tank sequencing batch reactors. *Water Science and Technology*. **35**(6): 137-144.

Kerrn-Jespersen J.P., Henze M. and Strube R. (1994). Biological phosphorus release and uptake under alternating anaerobic and anoxic conditions in a fixed-film reactor. *Water Research*. **28**(5): 1253-1255.

Kong Y., Ong S.L., Ng W.J. and Liu W.-T. (2002). Diversity and distribution of a deeply branched novel proteobacterial group found in anaerobic-aerobic activated sludge processes. *Environmental Microbiology*. **4**(11): 753-757.

Kong Y.H., Nielsen J.L. and Nielsen P.H. (2005). Identity and ecophysiology of uncultured actinobacterial polyphosphate-accumulating organisms in full-scale enhanced biological phosphorus removal plants. *Applied and Environmental Microbiology*. **71**(7): 4076-4085.

Kuba T., Smolders G.J.F., van Loosdrecht M.C.M. and and Heijnen J.J. (1993). Biological phosphorus removal from wastewater by anaerobic and anoxic sequencing batch reactor. *Water Science and Technology*. **27**: 241-252.

Lemaire R., Meyer R., Taske A., Crocetti G.R., Keller J. and Yuan Z.G. (2006). Identifying causes for N_2O accumulation in a lab-scale sequencing batch reactor performing

simultaneous nitrification, denitrification and phosphorus removal. *Journal of Biotechnology*. **122**(1): 62-72.

Lemaire R., Yuan Z.G., Blackall L.L. and Crocetti G.R. (in press). Microbial distribution of Accumulibacter spp. and Competibacter spp. in aerobic granules from a lab-scale biological nutrient removal system. *Environmental Microbiology*.

Lettinga G., Vanvelsen A.F.M., Hobma S.W., Dezeeuw W. and Klapwijk A. (1980). Use of the upflow sludge blanket (USB) reactor concept for biological wastewater-treatment, especially for anaerobic treatment. *Biotechnology and Bioengineering*. **22**(4): 699-734.

Lin Y.M., Liu Y. and Tay J.H. (2003). Development and characteristics of phosphorus-accumulating microbial granules in sequencing batch reactors. *Applied Microbiology and Biotechnology*. **62**(4): 430-435.

Martin H.G., Ivanova N., Kunin V., Warnecke F., Barry K.W., McHardy A.C., Yeates C., He S.M., Salamov A.A., Szeto E., Dalin E., Putnam N.H., Shapiro H.J., Pangilinan J.L., Rigoutsos I., Kyrpides N.C., Blackall L.L., McMahon K.D. and Hugenholtz P. (2006). Metagenomic analysis of two enhanced biological phosphorus removal (EBPR) sludge communities. *Nature Biotechnology*. **24**(10): 1263-1269.

Maurer M., Abramovich D., Siegrist H. and Gujer W. (1999). Kinetics of biologically induced phosphorus precipitation in waste-water treatment. *Water Research*. **33**(2): 484-493.

McSwain B.S., Irvine R.L. and Wilderer P.A. (2004). The influence of-settling time on the formation of aerobic granules. *Water Science and Technology*. **50**(10): 195-202.

Meinhold J., Filipe C.D.M., Daigger G.T. and Isaacs S. (1999). Characterization of the denitrifying fraction of phosphate accumulating organisms in biological phosphate removal. *Water Science and Technology*. **39**(1): 31-42.

Meyer R.L., Saunders A.M., Zeng R.J.X., Keller J. and Blackall L.L. (2003). Microscale structure and function of anaerobic-aerobic granules containing glycogen accumulating organisms. *FEMS Microbiology Ecology*. **45**(3): 253-261.

Meyer R.L., Zeng R.J.X., Giugliano V. and Blackall L.L. (2005). Challenges for simultaneous nitrification, denitrification, and phosphorus removal in microbial aggregates: mass transfer limitation and nitrous oxide production. *FEMS Microbiology Ecology*. **52**(3): 329-338.

Mosquera-Corral A., de Kreuk M.K., Heijnen J.J. and van Loosdrecht M.C.M. (2005). Effects of oxygen concentration on N-removal in an aerobic granular sludge reactor. *Water Research*. **39**(12): 2676-2686.

Munch E.V., Lant P. and Keller J. (1996). Simultaneous nitrification and denitrification in bench-scale sequencing batch reactors. *Water Research*. **30**(2): 277-284.

Musvoto E.V., Wentzel M.C. and Ekama G.A. (2000). Integrated chemical-physical processes modelling - II. Simulating aeration treatment of anaerobic digester supernatants. *Water Research*. **34**(6): 1868-1880.

Nielsen P.H. (1996). Adsorption of ammonium to activated sludge. *Water Research*. **30**(3): 762-764.

Ohlinger K.N., Young T.M. and Schroeder E.D. (1998). Predicting struvite formation in digestion. *Water Research*. **32**(12): 3607-3614.

Pan S., Tay J.H., He Y.X. and Tay S.T.L. (2004). The effect of hydraulic retention time on the stability of aerobically grown microbial granules. *Letters in Applied Microbiology*. **38**(2): 158-163.

Peng D.C., Bernet N., Delgenes J.P. and Moletta R. (1999). Aerobic granular sludge - A case report. *Water Research*. **33**(3): 890-893.

Pitman A.R., Venter S.L.V. and Nicholls H.A. (1983). Practical experience with biological phosphorus removal plants in Johannesburg. *Water Science and Technology*. **15**(3-4): 233-259.

Revsbech N.P. (1989). An oxygen microsensor with a guard cathode. *Limnology and Oceanography*. **34**(2): 474-478.

Smolders G.J.F., Vandermeij J., Vanloosdrecht M.C.M. and Heijnen J.J. (1994). Stoichiometric model of the aerobic metabolism of the biological phosphorus removal process. *Biotechnology and Bioengineering*. **44**(7): 837-848.

Wagner M., Rath G., Koops H.-P., Flood J. and Amann R. (1996). *In situ* analysis of nitrifying bacteria in sewage treatment plants. *Water Science and Technology*. **34**(1-2): 237-244.

Willers H.C., Tenhave P.J.W., Derikx P.J.L. and Arts M.W. (1993). Temperature-dependency of nitrification and required anoxic volume for denitrification in the biological treatment of veal calf manure. *Bioresource Technology*. **43**(1): 47-52.

Yang S.F., Tay J.H. and Liu Y. (2003). A novel granular sludge sequencing batch reactor for removal of organic and nitrogen from wastewater. *Journal of Biotechnology*. **106**(1): 77-86.

Zeng R.J., Lemaire R., Yuan Z. and Keller J. (2003a). Simultaneous nitrification, denitrification, and phosphorus removal in a lab-scale sequencing batch reactor. *Biotechnology and Bioengineering*. **84**(2): 170-178.

Zeng R.J., Saunders A.M., Yuan Z.G., Blackall L.L. and Keller J. (2003b). Identification and comparison of aerobic and denitrifying polyphosphate-accumulating organisms. *Biotechnology and Bioengineering*. **83**: 140-148.

Appendix G

Micro-scale Observations of the Structure of Aerobic Microbial Granules used for the Treatment of Nutrient-Rich Industrial Wastewater

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ABSTRACT

The structure and function of aerobic microbial granules from a lab-scale sequencing batch reactor (SBR) treating nutrient-rich abattoir wastewater were investigated. These wastewater-fed granules were examined using a wide range of micro-scale techniques including light microscopy, scanning and transmission electron microscopy, fluorescent *in situ* hybridisation (FISH) combined with confocal laser scanning microscopy (CLSM) and oxygen and pH microsensors, in conjunction with a range of measurements in the bulk liquid phase. Interesting structural features were observed in these granules that have not been reported in synthetic-fed granules. The complex nature of abattoir wastewater was suggested to be responsible for accelerating the breaking process of large mature granules due to a rapid clogging of the granules pores and channels and for the very diverse microbial community observed displaying specific spatial distribution throughout the granules. More importantly, the dissolution at lower pH of mineral complexes associated to the granule matrix of extracellular polymeric substances (EPS) might have caused the structural damages observed on the granules even though some pH buffer capacity were observed inside these granules. Ciliate protozoa were found to be very abundant on the surface of these wastewater-fed granules which could potentially assist with reducing the high levels of suspended solids usually present in aerobic granular sludge effluent. All these observations provide support to future studies on aerobic granular sludge treating real wastewater especially with regard to the granule structure and the mechanisms involved in their formation.

Keywords: aerobic granule, electron microscopy, EPS structure, FISH, industrial wastewater, micro-scale.

INTRODUCTION

The deterioration of water quality in rivers, lakes and other fresh water streams is often related to the depletion of dissolved oxygen. When the level of organic compounds and nutrient discharged into local waterways as a result of human activity is too high, the natural oxidation processes of organic compounds and the proliferation of aquatic plant such as algae in nutrient-rich waters (process known as eutrophication) will consume most of the dissolved oxygen available in the water system. To prevent happening, the level of organic compounds

(often measured as chemical oxygen demand – COD) and nutrient – such as nitrogen (N) and phosphorus (P) – in discharged wastewater must be significantly reduced by improving the treatment processes. Tertiary wastewater treatment, usually based on biological treatment known as the activated sludge system, is considered to be the easiest and most cost-effective way to remove nutrients from wastewater streams. Biological nutrient removal relies on the activity of a diverse microbial community that transfers organic matter and nutrients from the wastewater (liquid phase) to the atmosphere (gas phase) and/or into biosolids (solid phase).

In conventional activated floccular sludge systems, microorganisms and small particles in suspension in the wastewater shape into small aggregates or flocs (50 to 300 μm in size). Under special condition, these aggregates can become much bigger and compact forming granules (0.3 to 5 mm). While granules were first reported in an upflow anaerobic sludge blanket (UASB) bioreactor two decades ago (Lettinga et al., 1980), recent research efforts have been dedicated to the study of aerobic granules (Morgenroth et al., 1997; Beun et al., 1999; Etterer and Wilderer, 2001). Aerobic granules can be described as compact and dense aggregates of microbial origin with an approximately spherical external appearance which do not coagulate under reduced hydrodynamic shear and settle significantly faster than conventional activated sludge flocs. The growth of such granules is sometimes regarded as a special case of biofilm development without the presence of any substratum for attachment (Grotenhuis et al., 1991; El-Mamouni et al., 1998). It appears that aerobic granules do not form naturally and must be cultivated in bioreactors under specific operating conditions providing strong selective pressures (Liu and Tay, 2004). The large microbial diversity found in aerobic granules has also led researchers to hypothesize that granulation is not a function of specific microbiological groups (Beun et al., 1999). However, the exact mechanisms involved in the successive stages of the aerobic granulation process have not yet been fully explained.

More recently, aerobic granules cultivated in synthetic wastewater bioreactors have been reported to achieve COD and/or N removal (Tay et al., 2002; Liu et al., 2003; Yang et al., 2003) and, in some cases, P removal (Dulekgurgen et al., 2003; Lin et al., 2003). Sequencing batch reactors (SBR) have been identified as the most suitable bioreactor design to harbour this novel aerobic granular sludge technology. The main advantages of the aerobic granule technology are high biomass retention in the bioreactor, good settling properties, and the capacity to withstand high organic loading rates which all contribute to the very small footprint of this technology in comparison to conventional floccular activated sludge systems. It should be noted that all these advantages originate from the unique compact and dense structure of aerobic granules.

So far, most of the research have been primarily focussing on macro-scale characterisation of aerobic granular sludge systems whether there were designed for COD removal only or for both COD and nutrient removal. The effects of several key operating parameters (e.g. dissolved oxygen (DO) concentration, shear force, settling time, feast/famine regime and organic loading rate) on granule size and reactor performance were comprehensively investigated with several different carbon substrates (acetate, glucose and phenol) (Liu and Tay, 2004). Only few recent studies explored the micro-scale structure of aerobic granules (Ivanov et al., 2005; Chen et al., 2007a; Liu and Tay, 2007; Wang et al., 2007; Zheng and Yu, 2007). The majority of these studies focussed on extracellular polymeric substances (EPS) and its distribution within aerobic granules since non-biodegradable EPS appear to provide an architectural structure and mechanical stability for such granules (Wang et al., 2007). Unfortunately, to date, all these micro-scale studies were performed on granular systems fed with synthetic wastewater containing a single carbon source (i.e. mainly acetate) and operated for COD removal only. The ultimate goal of aerobic granular sludge technology is to treat real wastewater, either from domestic or industrial origin, which often contains diverse carbon

sources along with a multitude of organic and inorganic compounds and some particulate matters. This complex nature of real wastewater and the different substrates degradation rates are likely to have an effect on the structure of aerobic granules and their ability to remove COD and/or nutrients due to a change in the granule microbial communities affecting both the type of EPS produced and the mass transfer of substrate within the granule (Schwarzenbeck et al., 2005).

The aim of this study is to make micro-scale observations of the structure and function of aerobic granules fed with real wastewater, providing support to future studies on granule structure and the mechanisms involved in their formation. The granules from a lab-scale SBR fed with nutrient-rich industrial wastewater (Yilmaz et al., submitted) were employed in the study. The structure and function of granules were examined using a wide range of micro-scale techniques including light microscopy, scanning and transmission electron microscopy, fluorescent in-situ hybridisation (FISH) combined with confocal laser scanning microscopy (CLSM) and oxygen and pH microsensors, in conjunction with a range of measurements in the bulk liquid phase. The work is to our knowledge the most comprehensive micro-scale study on wastewater-fed aerobic granules using a variety of multi-disciplinary tools. Some of the structural features observed provided support to the hypotheses made previously by other researchers from aerobic granules obtained with synthetic feed. Others initiated new hypotheses regarding the general and microbial structure and the fate of mature granules, the effect of pH on the granule structure stability and the possible role played by protozoa in the overall system performance. The work also provided some new directions and recommendations for further experimental studies on aerobic granules in relation to their structure and behaviour in real systems.

MATERIAL AND METHODS

Nutrient-rich industrial wastewater

The wastewater used in this study was from a local abattoir in Queensland, Australia. At this site, the raw effluent passes through four parallel anaerobic ponds before being treated in a SBR for biological COD and N removal. Anaerobic pond effluent from the abattoir was collected on a weekly basis and stored at 4˚C. The average concentration of COD, ammonia and phosphate were 1420 $mgCOD.l^{-1}$, 240 $mgN.l^{-1}$ and 40 $mgP.l^{-1}$, respectively.

Reactor operation and sampling of granules

The aerobic granule SBR had a working volume of 5 l and was operated in a temperature-controlled room (18-22°C). It was operated on an 8 h cycle consisting of 18 min non-mixed feeding (3 l each cycle), 60 min mixed anaerobic/anoxic, 315 min mixed aerobic, 80 min mixed anoxic, 2 min settling and 5 min decanting periods. The DO level was controlled between 3.0-3.5 $mgO_2.l^{-1}$ during the entire aerobic period using an on/off aeration control system. The hydraulic retention time (HRT) was 13.3 h and the sludge retention time (SRT) was kept around 15-20 days through sludge wastage. The pH in the system, which was recorded but not controlled, typically fluctuated between 7.0 and 8.6 over the cycle.

All the granules analysed in this paper were sampled during steady state operation, after the SBR had been operated for more than a year achieving stable COD, N and P removal. Granules were either sampled at the end of the anaerobic period or at the end of the SBR cycle.

Due to presence of mineral complexes inside these wastewater-fed granules over the course of the SBR cycle (Yilmaz et al., submitted) and the dissolution of these complexes at lower pH, the effect of pH fluctuation on the granule structure stability was investigated in several anaerobic batch tests. For each batch test, 200 ml of a mixture of granule and liquor was sampled from the parent SBR at the end of the anaerobic period and transferred into a pH-controlled vessel kept under anaerobic condition. The vessel was consistently mixed and granules were sampled after 1 h for examination under light and electron microscopy to monitor their structure. Bulk liquid pH of 7.5 (pH in the parent SBR at the end of the anaerobic period) and 6.5 were tested.

Physico-chemical analysis

To monitor the granule structure and characteristics, granule size distribution and density were measured. To determine the volumetric size distribution of the granules, 30 ml of well mixed granular sludge was sampled from the SBR at the end of the aeration period and pumped through a Malvern laser light scattering instrument, Mastersizer 2000 series (Malvern Instruments, Worcestershire, UK). The granule density, defined as the quantity of dry mass per biomass volume, was measured by the blue dextran method described in Lemaire et al. (in press), which was adapted from Di Iaconi et al. (2004).

The gradient of oxygen in granules was measured with oxygen microsensors (tip diameter <10 μm), which were constructed as described by Revsbech et al. (1989). Granules were sampled from the SBR at the start of the aerobic period when ammonia and phosphates were present at high concentrations and at the end of the aerobic period, at which time ammonia and phosphates were usually depleted. They were then transferred to a flow-cell with an horizontal flow where replicate oxygen profiles were measured and averaged as described in Meyer et al. (2003). The composition of medium and the dissolved oxygen concentration in the measuring flow-cell was identical to that in the SBR at the start or the end of the aerobic period. The pH gradient in granules was measured with pH microsensors using the same experimental set-up than for oxygen profile measurement. Granules were sampled from the SBR at the end of the anaerobic period. The medium used in the flow-cell was sampled from the SBR (liquid phase) at the same time to keep the substrate concentrations identical.

Microbial analysis

Granule samples were fixed and FISH probed as previously described (Amann 1995). Prior to FISH probing, fixed granule samples were embedded in optimum cutting temperature (OCT) compound (TissueTek, Sakura, USA) for cryosectioning as previously described (Meyer et al. 2003). Embedded granules were then frozen and sectioned into 10 μm thick slices using a cryotome operated at -20°C (Kryo 1720, Leitz, Germany). The granule sections were collected on SuperFrost Plus microscope slides (Menzel-Glaser, Germany). Finally, the slides were dehydrated by sequential immersion for 3 min in 50%, 80% and 98% ethanol and air-dried.

Oligonucleotide probes applied on the granule sections were the combination of EUB338 i-iii (EUBmix) for the detection of all bacteria (Daims et al. 1999), the combination of PAO462, PAO651 and PAO846 (PAOmix) for *Accumulibacter* spp. (Crocetti et al. 2000), the probe combination (GAOmix) of GAOQ989 (Crocetti et al. 2002) and GB_G2 (Kong et al. 2002) for *Competibacter* spp., NTSPA662 for *Nitrospira* spp. (Daims et al. 2001), NIT3 for *Nitrobacter* spp. (Wagner et al. 1996) and NSO1225 for most of the ammonia oxidising bacteria (AOB) from the *Betaproteobacteria* (Mobarry et al. 1996). Additionally, probes for the newly proposed *Actinobacterial* PAOs (Kong et al. 2005) and *Defluviicoccus* spp.-related

GAOs (Meyer et al. 2006) were also used. Fluorescently labelled oligonucleotides were purchased from Thermo (Ulm, Germany) with fluorescein isothiocyanate (FITC) or one of the sulfoindocyanine dyes indocarbocyanine (Cy3) or indodicarbocyanine (Cy5).

Microscopy Images

Whole fresh granules were photographed using an Olympus SZH10 stereo microscope with a DP70 digital camera.

FISH images were collected with a confocal laser scanning microscope (CLSM) Zeiss 510 (Carl Zeiss, Jena, Germany) using an argon laser (488 nm), a helium neon laser (543 nm) and a red diode laser (633 nm) fitted with 515-565 nm BP, 590 nm LP and 660-710 nm BP emission filters, respectively. To obtain images of entire granule sections, between 10 and 50 overlapping, consecutive images of 1024x1024 pixels were collected (depending on the size of the granule) using a Zeiss Neofluar 40x/1.3 oil objective. The final composite image of the granule section was then reconstructed from all the single images collected using Adobe Photoshop 7.0 (Adobe Systems, USA). For single images of specific part of the granule section a Zeiss Apochromat 63x/1.4 oil objective was used. FISH quantification was performed according to (Crocetti et al., 2002) where the relative abundance of each group was determined as mean percentage of all bacteria based on pixel area counting.

To visualise the structure of aerobic granules at a micro-scale level, scanning and transmission electron microscopy (SEM and TEM, respectively) were employed. Prior to visualisation, granules EPS material was first stabilised using 2.5% glutaraldehyde and 75 mM lysine in 0.1 M cacodylate buffer for 10 minutes to minimise any structural damages arising from the dehydration procedure (Jacques and Graham, 1989). All subsequent processing was performed in a Pelco Biowave microwave oven. Granules were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer and after washing in 0.1 M cacodylate buffer, were postfixed in 1% osmium tetroxide.

For transmission electron microscopy (TEM) studies, granules were dehydrated in a graded acetone series and embedded in Epon resin. Semi-thin sections of 500 nm were stained with 1% Toluidine Blue and 1% borax and viewed with an Olympus BX61 stereo microscope. Ultra-thin sections of 60 nm thickness were cut using a Leica Ultracut UC6 ultramicrotome and mounted on the Formvar coated copper grids, stained with 5% uranyl acetate in 5% methanol and Reynolds lead citrate, and viewed using a JEOL 1010 transmission electron microscope operated at 80 kV. To further reduce any possible artefacts coming from the fixation steps, some fresh granules were also frozen in a Leica EMPACT 2 high pressure freezer and cryosubstituted at -85^0C over 2 days in 2% osmium tetroxide 0.5% uranyl acetate in acetone. Specimens were then warmed to room temperature over 13 hours, washed in acetone and embedded in Epon resin. Ultra-thin sections were cut and viewed by TEM as described above.

For scanning electron microscopy (SEM) studies, granules were dehydrated in a graded ethanol graded series and infiltrated with the drying agent hexamethyldisilazane and left overnight to dry before being sputter coated with platinum. Viewing of samples was conducted using a JEOL 6300F scanning electron microscope operated at 5-10 kV. To observe the internal structure, some dehydrated granules were taken from 100% ethanol, frozen in liquid nitrogen and fractured. These fractured granules were then thawed in ethanol, dried as described above and coated with platinum. The internal structure of granules were visualised by SEM as described above.

RESULTS AND DISCUSSION

General macro characteristics of mature granules

Over the course of this study, the granular sludge SBR was in steady state and consistently removed 85%, 93% and 89% of the soluble COD, soluble nitrogen and soluble phosphorus present in the abattoir wastewater, respectively. This good nutrient removal was achieved through the process known as simultaneous nitrification, denitrification and phosphorus removal (SNDPR), facilitated by the presence of large and stable anoxic zones in the inner part of the granules (Yilmaz et al., submitted).

Figure 1 presents an overview of the macro-characteristics of the mature granules developed in this SBR fed with industrial wastewater. The volumetric size distribution of the granules presented in Figure 1a indicates that more than 80% of the biomass volume is made of granules with a size larger than 600 μm while the volume percentage of biomass with a size smaller than 300 μm (likely flocs) is less than 5% which demonstrates that granules were the dominant form of bacterial aggregates in this SBR. The average density of these granules was measured at 150 $g.l_{biomass}^{-1}$, which is considerably higher than what is generally reported for synthetic wastewater-fed granules (30-80 $g.l_{biomass}^{-1}$). Figure 1b and 1c show that most granules have a round shape with a clear outline; however, smaller granules with concave shapes were also observed suggesting they might have been part of a bigger granule that disintegrated, which will be further discussed later in this paper.

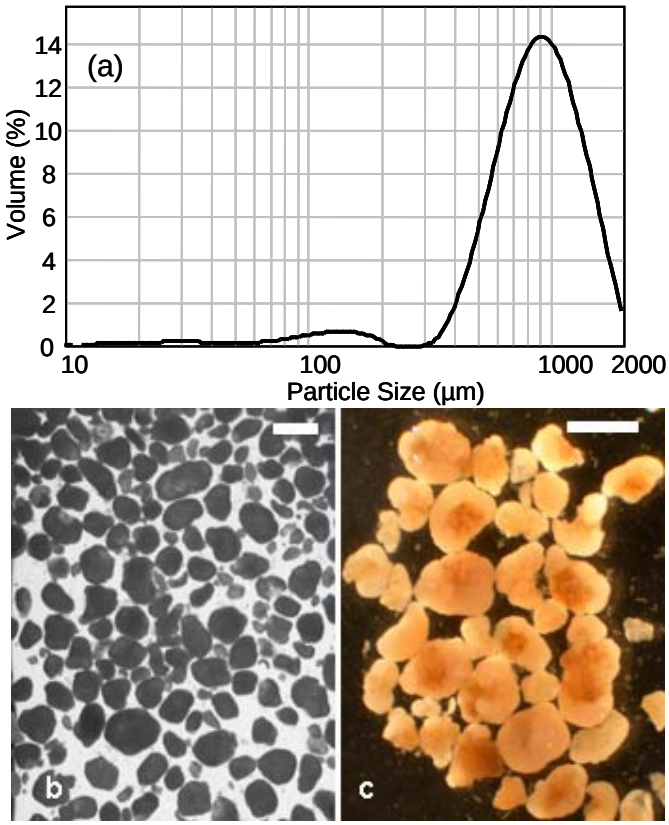

Figure 1. (a) Volumetric size distribution of the SBR mature granules. Pictures of granules obtained **(b)** with a scanner and **(c)** with a light microscope. Scale bars = 2 mm.

Micro-scale structure of granules and illustration of the role played by EPS

To explore the micro-scale structure of these granules especially in regards to the EPS structural matrix, electron microscopy (both SEM and TEM) was employed (Figure 2). The overall round shape of aerobic granules is actually made up of large cauliflower-like outgrowths (Figure 2a). These outgrowths have also been reported in acetate-fed aerobic granules (Tay et al., 2004; Liu and Tay, 2007), but were not as pronounced as observed in this study. On the surface of the granule (Figure 2c) some glue-like substances (i.e. EPS), provide the cohesive material to maintain the bacteria bound to each other. To demonstrate the importance of the lysine based pre-fixation step before visualising granules with SEM, a SEM image of a granule which has not been pre-fixed in lysine solution is shown in Figure 2e. The EPS matrix has obviously shrunk during the dehydration process revealing more rod-shaped bacteria underneath it. Extreme caution should be exercised when preparing biological samples for SEM or TEM observation to avoid any structural artefacts. The internal EPS structure was observed on granules fractured in liquid nitrogen to prevent any structural damages coming from the use of a cutting tool. The SEM picture of the inner part of a fractured granule is presented in Figure 2b. Figure 2d presents a close up image of the field delimited in black in Figure 2b, which is located near the surface of the granule. All bacteria cells visible on this image are embedded in the EPS matrix. The cellular origin of this EPS is clearly demonstrated in the high magnification SEM image of a fractured granule displayed in Figure 2f. Each individual cell is enclosed in a thick EPS capsule. An ostensible difference can be made between the EPS of this cell capsule and the main EPS matrix cementing the overall granule. The real distance between bacteria in granules is very difficult to assess due to the thickness of the sections generally observed by light microscopy or CLSM where lots of bacteria are piled up on the top of each other. The TEM image of a granule ultra-thin section depicted in Figure 2g reveals the exact distance between each cell in three different bacteria clusters defined by their size, shape, grey scale intensity and general texture at high magnification. The cell to cell distance varied substantially depending on the type of bacteria resulting in sparse or dense cell clusters. The space between cells is filled by some EPS material, which means that the cells from the sparse cluster might indeed produce more EPS than those from the dense cluster. The type of EPS produce by different clusters of bacteria is also likely to be different throughout the granule. To illustrate that point, Figure 2h shows a TEM image of two different clusters of bacteria, one cocci-shaped and one rod-shaped, embedded in what appear to be different types of EPS matrix based on the grey scale intensity and texture.

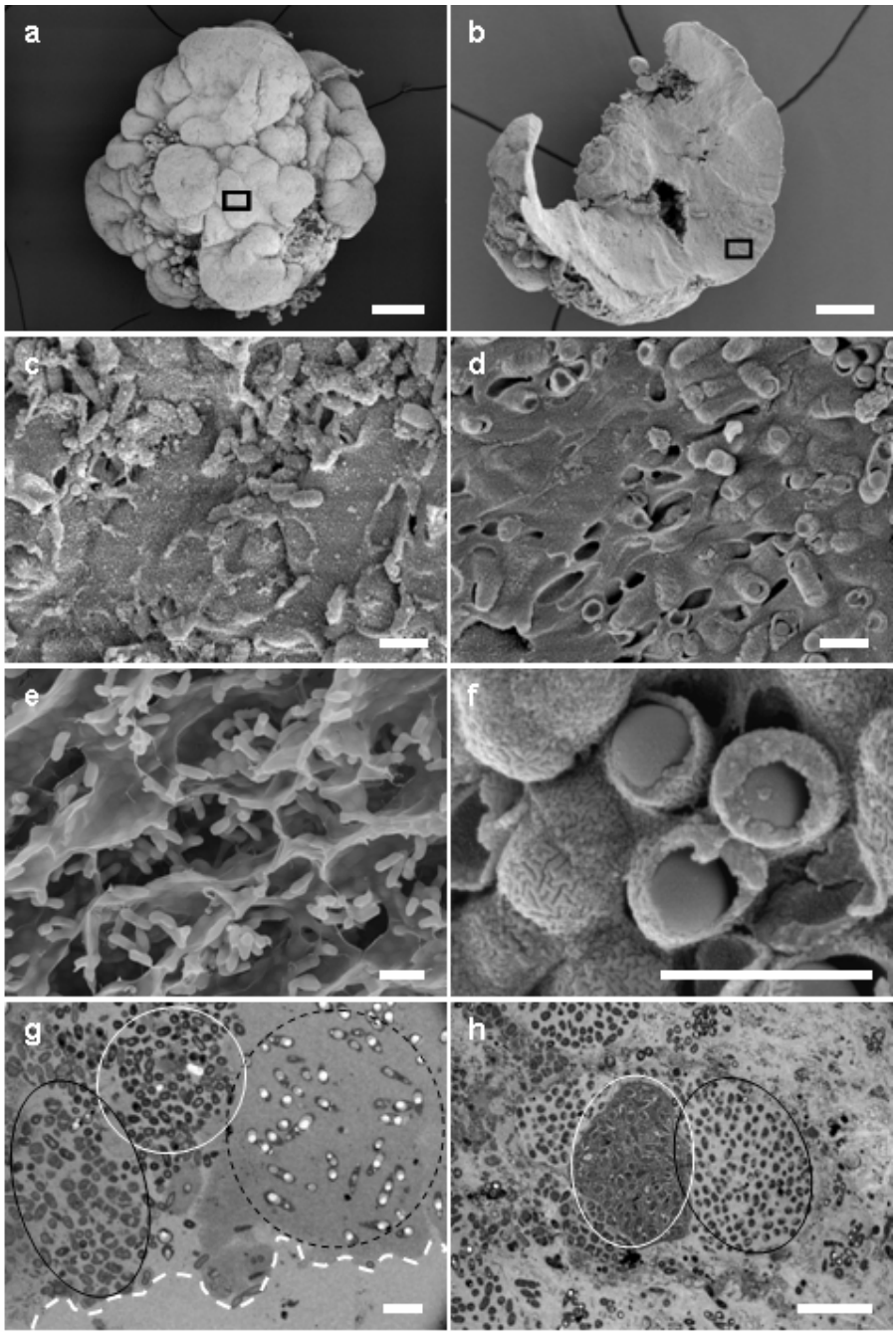

Figure 2. SEM images of **(a)** an entire granule, scale bar = 200 μm; **(b)** a fractured granule, scale bar =200 μm; **(c)** granule outer surface delimited in black in (a), scale bar = 1 μm; **(d)** granule inner surface delimited in black in (b) , scale bar = 1 μm; **(e)** outer surface of a granule without any pre-fixation step, scale bar =1 μm; **(f)** granule inner surface at high magnification, scale bar = 1 μm; TEM image of **(g)** three bacteria clusters in the outer part of a granule, scale bar = 5 μm; **(h)** two bacteria clusters showing different types of EPS, scale bar = 5 μm.

Voids, channels and fate of large mature granules

Voids and channels have been reported in some acetate-fed granules (Ivanov et al., 2005; Zheng and Yu, 2007) and were found to play a key role in the transport of substrate and metabolites in and out of the granules. Such voids or cavities were observed in almost every granule sections examined. One of these large voids can be seen in the centre of the granule section depicted in Figure 3a. These voids were most of the time connected to the outside of the granules via channels-like structures as illustrated in the light microscope images of Figure 3b and 3c and the TEM image of Figure 3d. The channels depicted in Figure 3c and 3d are filled with some material, again likely EPS, and could be compared to a ground water system where liquid can circulate in and out of the granules through some porous material. Very few clusters of bacteria are present in these channels probably due to the constant flow of liquid which prevent them from attaching firmly on to the granule and thus being washed away. In comparison, the channel presented in Figure 3b is not filled with any material and appears to be more like an open river system where liquid can circulate more freely. These types of channels or interstices are usually located on the boundary line between two cauliflower-like outgrowths. The two large blue shapes at the entrance of this channel are two protozoa (ciliates) and their presence will be discussed later in this paper.

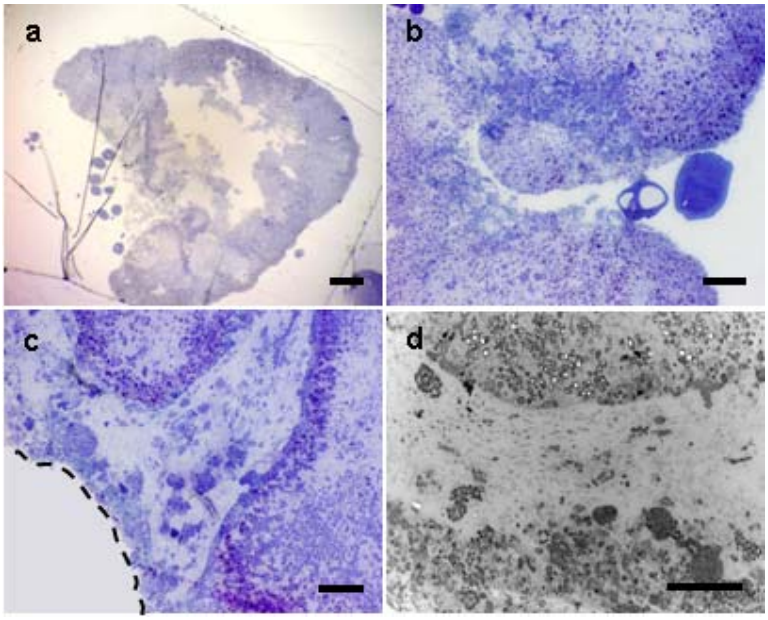

Figure 3. Light microscope images of semi-thin sections (500 μm) embedded in resin and stained with Toluidine Blue of **(a)** an entire granule, scale bar=100 μm; **(b)** and **(c)** channels on the granule surface, scale bar=10μm; **(d)** TEM image of an inner channel, scale bar=10μm.

As mentioned earlier, not all granules in our SBR were big and round shaped. A large number of granules were of concave shape with smooth, dense cauliflower-like outgrowths on one side and loose and fluffy organisation on the other. Some examples of such granules are presented in Figure 4a and 4b. A close up image of the smooth and dense side of such a granule is shown in the Figure 4c while the loose and fluffy side is shown in Figure 4d. These highly heterogenous granule structures seem to originate from the disintegration of bigger granules. The newly formed granules can then grow to become themself big mature granules that would break into smaller aggregates. However, the properties of these "recycled" mature granules would likely differ from that of the original large mature granules due to the

heterogeneity of the structure from which they had to re-develop. A recent research study by Zheng and Yu (2007) found a positive correlation between the bioactivity of acetate-fed granules and their porosity and also reported that the granule porosity decreased as the granule size increased. They suggested that the pores of large granules were more readily plugged by EPS leading to a decrease of the biological activity because of a lack of nutrient or metabolites transport. In our system, the particular and colloidal matter and the high level of fat, oil and grease (FOG) present in abattoir wastewater might have speeded up the clogging process of the granule channels and pores and increased the mass transfer limitation of nutrient and substrates. This would have weakened the inner structure of the granule and explain why our granules only grew to a maximum size of 1.5-2 mm before breaking up into smaller and less homogenous granules. Therefore, not only the well studied operating parameters such as settling time, shear force and organic loading rate can have an impact on the size of the granules but also the characteristics of the wastewater to be treated.

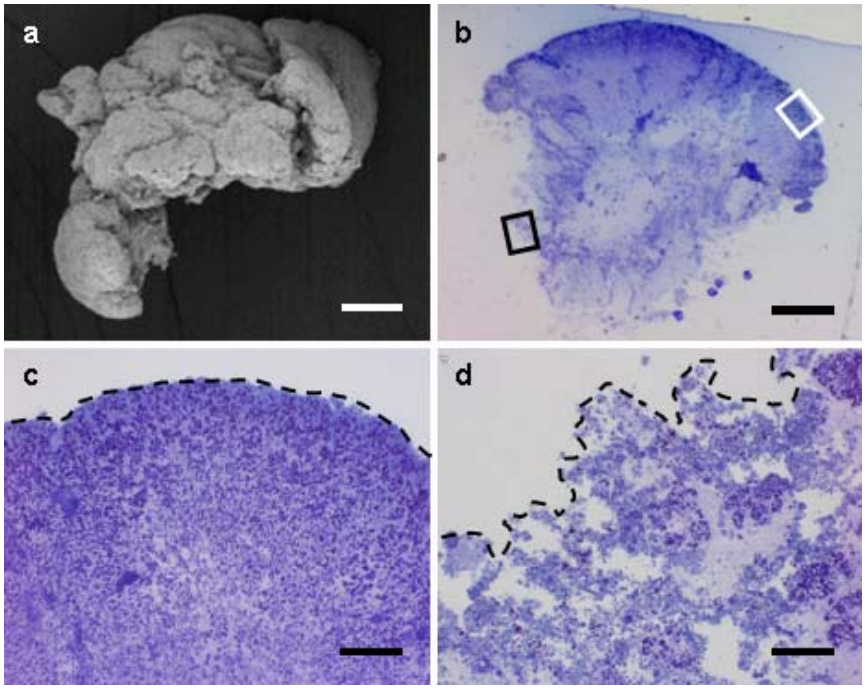

Figure 4. (a) SEM image of a broken granule, scale bar = 100 μm; light microscope images of semi-thin sections (500 μm) embedded in resin and stained with Toluidine Blue of **(b)** a broken granule, scale bar = 100 μm; **(c)** part of the granule edge delimited in white in (b), scale bar = 10 μm; **(d)** part of the granule edge delimited in black in (b), scale bar = 10 μm.

Microbial population and distribution in granules

The mass transfer limitation of nutrient, substrates and metabolites inside large granule was proposed to cause the breaking up of mature granules into smaller aggregates. This theory implies that the core part of mature granules must be deprived of any substantial microbial activity due to substrate diffusion limitation. Some researchers have used staining methods to establish the distribution profile of total (SYTO 63) and dead cells (SITOX Blue, *Bac*Light Live-Dead staining kit) in acetate-fed aerobic granules (McSwain et al., 2005; Chen et al., 2007b; Chiu et al., 2007). The problem with these methods is that no standard procedure is available in the literature for the staining of entire granules and different incubation times, ranging from 5 to 30 min have been employed by different groups. Due to the diffusion

limitation likely to occur in large granules, the time of incubation should probably be defined based on the size of the granules if anything. Due to the lack of consistency of these staining procedures, the cell distribution in our wastewater-fed granules was investigated qualitatively using SEM and TEM images. A SEM image of the central part of a fractured granule is illustrated in Figure 5a. This image can be compared to that of Figure 2f taken near the granule surface. The same kind of EPS capsule is observed in both images but the shape of the cells enclosed in these capsules are very different. Cells in the central part of the granules (Figure 5a) are mal-formed compared to the smooth round shaped cells of Figure 2f. It is very likely that these amorphous cells are indeed dead cells. To confirm that, several juxtaposing TEM images were taken from the edge to the centre of the granule. Figure 5b and 5c are two examples of what could be observed in the centre and on the edge of every granule, respectively. Mostly cell walls embedded in the EPS matrix are left in the central part of the granules whereas lots of compact cells are located on the edge of the granule. This cell distribution in wastewater-fed granules supports the nutrient and substrate diffusion limitation theory, which may be the main cause for the breaking up of large mature granules.

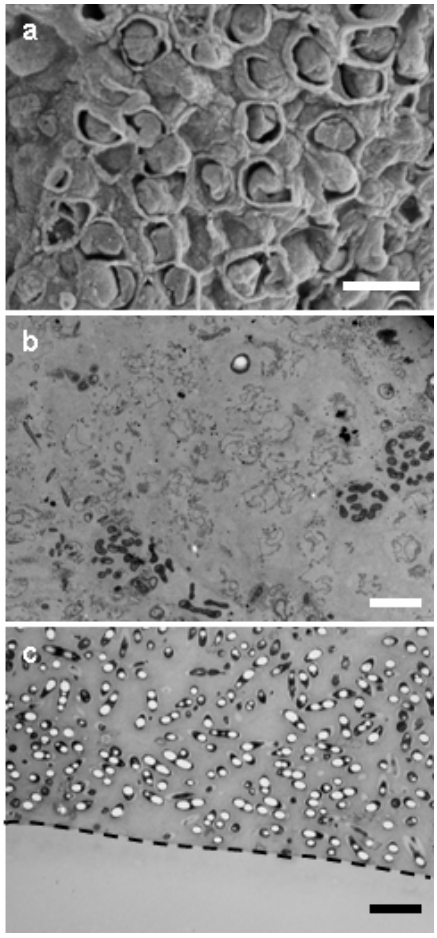

Figure 5. (a) SEM image of the central part of a fractured granule, scale bar = 1 μm; TEM images of **(b)** the central part of a granule section, scale bar = 5 μm; **(c)** the edge of the same granule section, scale bar = 5 μm.

The microbial diversity within the granule structure and spatial distributions of various groups of bacteria of importance to biological nutrient removal was investigated using a wide range of FISH probes designed to identify the most common microbial communities found in

activated sludge performing nutrient removal. *Accumulibacter* spp., the main polyphosphate accumulating organism (PAO) found in biological P removal system, were dominant in these wastewater-fed granules (41% of all bacteria). This domination is illustrated in Figure 6a and 6b where FISH images of entire granule sections are reconstructed (*Accumulibacter* spp. in magenta, ammonia oxidising bacteria (AOB) in cyan and other bacteria in blue). Oxygen micro-profiles were measured in these granules during the first hour of the aeration period when the microbial activity was the highest (i.e. substrates in excess) and at the end of the aeration period, 4 h later, when the microbial activity was lower (i.e. most substrates depleted). Oxygen was found to penetrate only as far as 50 μm inside the granules in the first hour of the aeration period and around 400 μm deep at the end of the aeration (Yilmaz et al., submitted). *Accumulibacter* spp. were located on the outer part of these wastewater-fed granules where the dissolved oxygen concentration was higher. This preferred location has been already reported and quantified in acetate-fed granules performing N and P removal (Lemaire et al., in press). Other P removal microorganisms recently speculated, *Actinobacteria*-PAO, were also present but in much lower abundance (4.1% of all bacteria) in comparison to *Accumulibacter* spp. as depicted in Figure 6c. Very few clusters of *Competibacter* spp. cells, the main glycogen accumulating organisms (GAO) usually found in large numbers in biological P removal processes, could be detected in the granules. This is considered desirable as GAOs compete with *Accumulibacter* spp. for the same carbon source but without performing any P removal. One of the few *Competibacter* spp. cluster present in these wastewater-fed granules is shown in Figure 6e in cyan.

The nitrifying organisms (i.e. ammonium and nitrite oxidising bacteria, AOB and NOB respectively) are also important microbial populations in nutrient removal processes due to their ability to oxidise ammonium/nitrite to nitrite/nitrate, which can then be reduced to di-nitrogen gas by other organisms. AOBs were present in these granules as expected from the good nitrification performance of this SBR. A typical dense AOB cluster is depicted in Figure 6d in magenta. According to their strict aerobic metabolism, they should also be located on the outer part of the granule where oxygen is always available. Fig 6a and 6b shows that most AOB clusters were indeed situated in the first 200 μm from the granule surface but rarely in the most outer part of the granule (i.e. 0-50 μm layer) where oxygen availability is high. Instead, AOBs appear to grow just behind the thick layer of *Accumulibacter* spp. surrounding the granule edge. Strangely, some AOB clusters were even found right in the centre of the granule (indicated by the white circles on Figure 6a and 6b) where, according to the oxygen micro-profiles, oxygen should not be usually present. However, these AOB clusters were always located along the edge of large internal voids where small “pockets” of oxygen might have been present after diffusing through some of the channels described earlier in this paper. This could explain why some oxygen micro-profiles presented small surges of oxygen concentration deep inside the granule. It clearly highlights the heterogenous nature of aerobic granules and the need to study a sufficient number of granules when investigating their micro-scale structure.

No NOB targeted by the FISH probes applied was found in these wastewater-fed granules. The oxidised nitrogen accumulating in the liquid phase of the SBR at the end of each cycle was almost exclusively nitrite (data not shown) confirming the absence of NOB in the system and that N was likely removed through the nitrite pathway. The limitation of oxygen transfer inside the granules could have advantaged the nitrite reducing organisms over NOB by providing large anoxic zones where denitrification could occur using the soluble COD present in the wastewater. In addition, the domination of *Accumulibacter* spp. on the outer part of the granules where oxygen was abundant might have prevented NOB to adequately perform their aerobic metabolism due to insufficient oxygen availability.

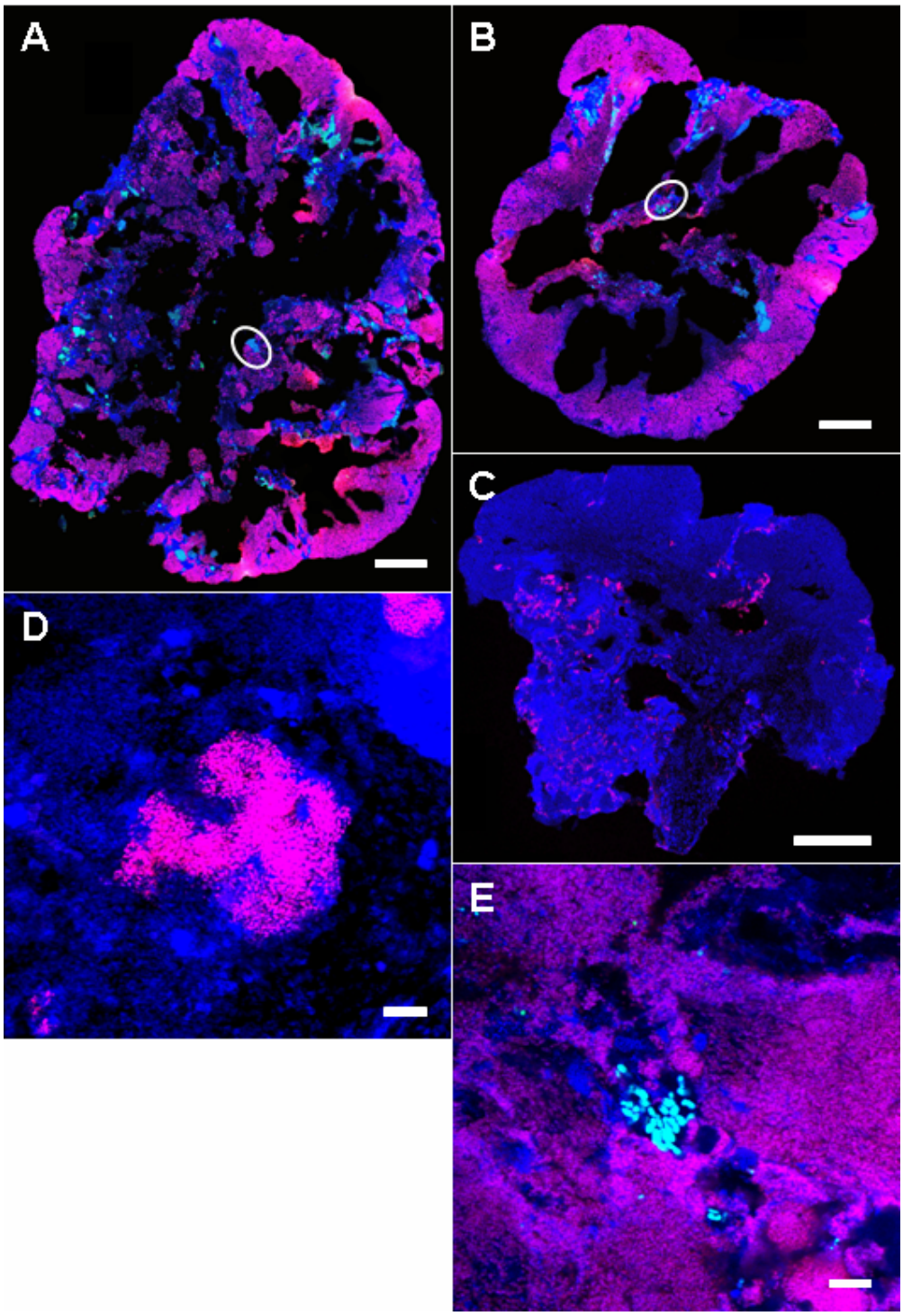

Figure 6. Reconstructed CLSM images of FISH micrographs of entire granule sections **(a, b and c)** and part of the section **(d and e)**. In (a) and (b) *Accumulibacter* spp. cells are magenta (overlay of red PAOmix and blue EUBmix) most of the ammonia oxidising bacteria (AOB) from the *Betaproteobacteria* are in cyan (overlay of green NSO1225 and blue EUBmix) and other *Bacteria* are blue (blue EUBmix). The white circle highlights the presence of AOB in the centre of the granule. In (c) *Actinobacteria* PAO cells are magenta (overlay of red Actino-658 and blue EUBmix) and other *Bacteria* are blue (blue EUBmix). In (d) AOB from the *Betaproteobacteria* are in magenta (overlay of red NSO1225 and blue EUBmix) and other *Bacteria* are blue (blue EUBmix). In (e) *Accumulibacter* spp. cells are magenta (overlay of red PAOmix and blue EUBmix) *Competibacter* spp. are in cyan (overlay of green GAOmix and blue EUBmix) and other *Bacteria* are blue (blue EUBmix). Scale bars = 100 μm for (a), (b) and (c) and 10 μm for (d) and (e).

Impact of bulk liquid pH on the granule structure

Aerobic granular sludge technology is particularly well suited to treat industrial wastewater due to its small footprint and capacity to withhold high loading rates. Most intensive water user industries are subject to inherent production variability often resulting in changes of the wastewater composition which could affect its pH. Yilmaz et al. (submitted) reported that mineral complexes such as struvite and apatites could precipitate in these wastewater-fed granules during the anaerobic phase of the SBR cycle when the concentration of Ca^{2+}, Mg^{2+}, NH_4^+ and PO_4^{3-} ions is the highest. Due to the dissolution of these complexes at lower pH and the possible effect that could have on the overall granule structure, the influence of pH variation on the macro-structure of wastewater-fed granules was investigated. Series of batch tests were performed in a small pH-controlled vessel to investigate the effect of pH on the granule structure. Figure 7a and 7c show light microscopy and SEM images of the granules after a 1h batch test at pH 7.5, simulating the bulk liquid pH of the parent SBR at the end of the anaerobic period, while example images obtained in the case of pH 6.5 are shown in Figure 7b and 7d. After 1h batch test at pH 6.5, granules started to lose their smooth and compact external appearance (Figure 7d) and most of the smaller granules even completely disintegrate (Figure 7b). This disintegration led to a significant decrease of the volumetric size distribution of granules with the 10^{th}, 50^{th} and 90^{th} percentiles dropping by an average of 80%, 50% and 20%, respectively.

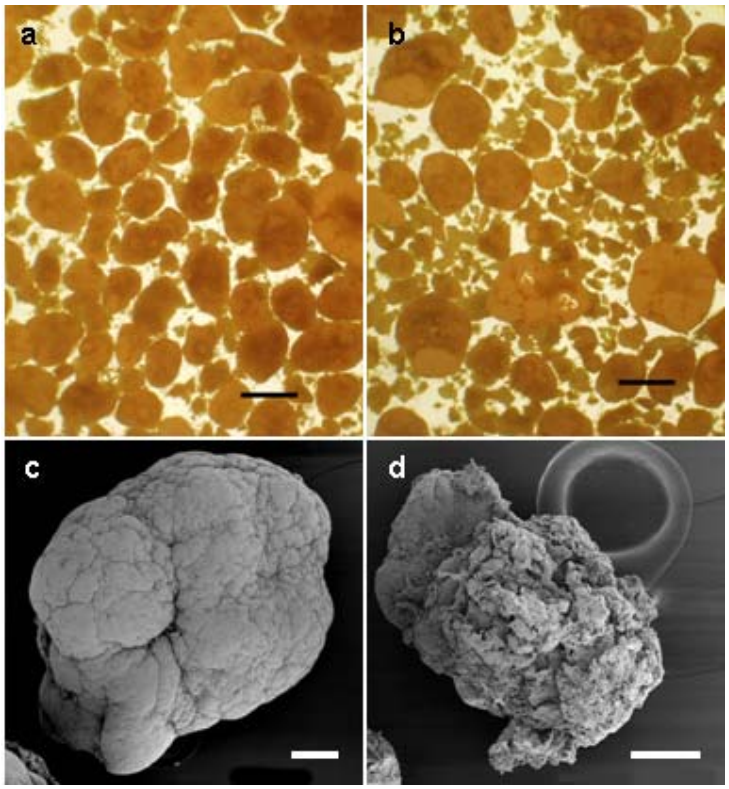

Figure 7. Light microscope images of granules after 1h anaerobic batch test performed **(a)** at pH 7.5 and **(b)** at pH 6.5, scale bars = 1 mm. SEM images of the same granules after batch test **(c)** at pH 7.5 and **(d)** at pH 6.5, scale bars = 200 μm.

To better understand the effects that lower bulk liquid pH can have on the granule structure, microsensors were employed to measure the *in situ* pH profiles in these wastewater-fed granules after immersion in a pH-controlled flow cell. One of several pH profile time series measured is presented in Figure 8. The first pH profile (t=0) was measured as soon as the granule was transferred from the parent SBR bulk liquid (pH=7.6) into the measuring flow-cell where the pH was controlled at 6.5. The subsequent profiles were measured on the same granule (different location) after 10, 20 and 60 minutes. The presence of a clear pH gradient indicates that these granules have a pH buffer capacity due to diffusion limitation and/or *in situ* biological activities. This buffering effect decreased overtime but a slight pH gradient remained visible even after 60 min. If the dissolution of mineral complexes likely associated with the granules biopolymers (i.e. EPS matrix) is indeed the main reason for the structural damage observed at lower pH, the presence of sharp pH gradient inside the granules would protect them from total disintegration. It could explain why during the batch tests at pH 6.5 small granules were more affected than large granules. The relatively limited pH buffer capacity of smaller granules would result in similar pH levels in the bulk liquid and in the granules and therefore increase their vulnerability under lower pH condition. Although these are only preliminary results, the high probability of having mineral complexes associated with the EPS matrix in granules treating nutrient-rich industrial wastewater calls for more extensive research to be done on the effect of dynamic pH fluctuations on the granule structural stability.

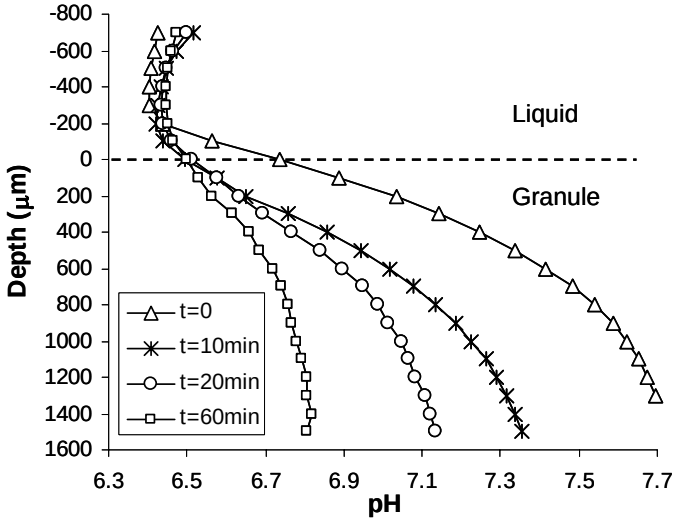

Figure 8. Typical pH profile time series measured in one granule using a microsensor in a flow-cell controlled at pH 6.5.

Presence and role of ciliates on real wastewater-fed granules

Protozoa from the ciliate group (likely from the *Vorticella* genus based on the observed morphology) were present in "bouquets" on the surface of almost every granule examined (Figure 9a). These ciliates are attached to the granule via their retractable stalk as depicted in Figure 9d. They are always located in the concave part of the granule (Figure 9b and Figure 3a) or in interstices between large cauliflower-like outgrowths (Figure 9c). Due to the high shear force applied in the SBR, the constant collision between granules would have prevented

ciliates to stay attached on exposed part of the granules explaining why they are mostly located in sheltered areas.

The presence of these ciliates on the surface of the granules raises the question of their specific role and possible impact on the overall granular process. During measurement of oxygen profiles in granules it was observed that when the microsensor tip had to progress through a ciliate bouquet before reaching the surface of the granule (Fig 9e), the measured oxygen concentration immediately dropped to zero. It indicates that ciliates were indeed interfering with the oxygen diffusion process in the granule by creating some localised oxygen depleted zones at the surface of the granule. The high abundance of ciliates on each granule could have had an impact on the overall oxygen diffusion limitation in the granules but also on the diffusion of other substrates.

However, the main role of these ciliates in the system was probably related to their predatory behaviour. Like most protozoa, ciliates feed on small organic particulates including bacterial cells. They can sweep into their mouth free floating bacteria by creating a vortex through the rotation of their oral cilia. The mouth of these ciliates can be observed in Figure 9f although their oral cilia are hidden inside. The TEM image presented in Figure 9g shows a cross section of a ciliate mouth with a group of free floating bacteria close by. These bacteria are then digested by the ciliate via the formation of internal food vacuoles that are depicted in Figure 9h (black arrows). High level of suspended solids in granular sludge effluent is a well known drawback of the technology and post-treatments for solids removal are often required (Schwarzenbeck et al., 2005). It is directly linked to the process operation (i.e. short settling time) where slowly settling biomass has to be washed out from the system continuously. The predation by these ciliates of the bacteria suspended in the SBR bulk liquid could help reduce the level of suspended solids discharged in the effluent at the end of each cycle and reduce the cost of post-treatments. More experimental work has to be done to estimate the fraction of suspended solids removed from the bulk liquid by these ciliates.

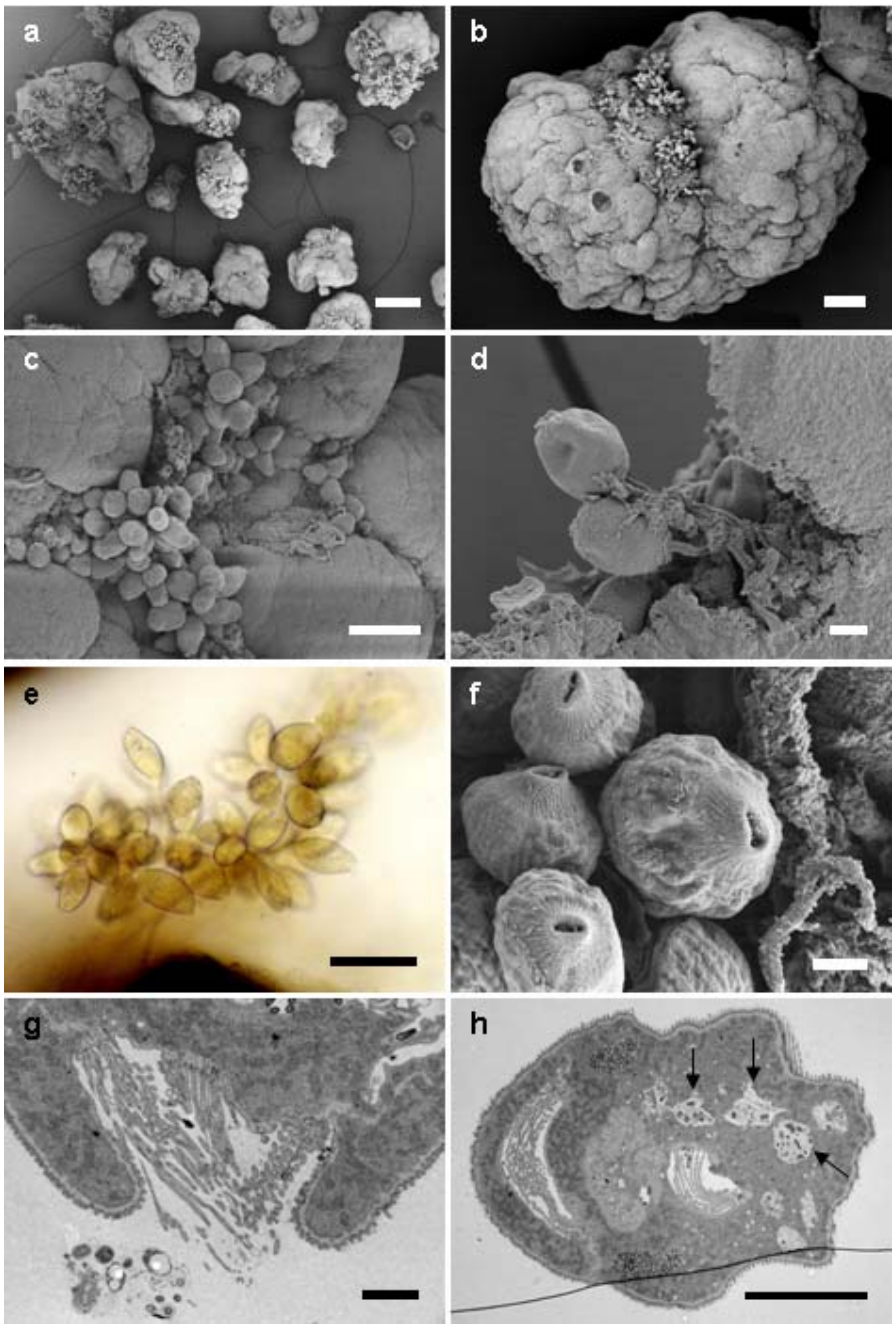

Figure 9. (a)-(d) and **(f)** SEM images of ciliates attached to the granule surface, scale bars = 1 mm (a), 200 μm (b), 100 μm (c) and 10 μm (d) and (f); **(e)** light microscope image of a bunch of ciliates, scale bar =100 μm; TEM images of **(g)** oral cilia attracting free floating bacteria in the ciliate mouth, scale bar =2 μm; **(h)** a ciliate cross section with internal food vacuole indicated by the black arrows, scale bar =10 μm.

CONCLUDING REMARKS

The structure of aerobic granules treating nutrient-rich wastewater in a SBR was investigated. Some interesting structural features were observed in these granules that have not been reported in synthetic-fed granules. The particulate and colloidal matter along with the fat, oil and grease present in abattoir wastewater appeared to have enhanced the breaking process of large mature granules due to a rapid clogging of the granules pores and channels. The various and complex types of substrates available also resulted in a very diverse microbial community with specific spatial distribution throughout the granules. This diverse community is likely to produce different sorts of EPS with different function. More importantly, the dissolution of mineral complexes associated to the granule EPS matrix at lower pH could indeed affect the structural stability of the granules. Further experimental studies are needed to understand the real impact of pH on the granule stability especially in regard to dynamic pH fluctuations as granules exhibit some *in-situ* pH buffer capacity. Finally the abundance of ciliates on the surface of these wastewater-fed granules raises the question of their real contribution in removing small particulate matter from the bulk liquid. More targeted studies on that aspect could be beneficial for the overall aerobic granular sludge technology which is known to produce an effluent with high levels of suspended solids.

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REFERENCES

Amann R.I. (1995). In situ identification of microorganisms by whole cell hybridization with rRNA-targeted nucleic acid probes. In: Molecular Microbial Ecology Manual. Akkermans A.D.L., van Elsas J.D. and de Bruijn F.J. (eds). Dordrecht, Holland, *Kluwer Academic Publications*. **3.3.6:** 1-15.

Beun J.J., Hendriks A., Van Loosdrecht M.C.M., Morgenroth E., Wilderer P.A. and Heijnen J.J. (1999). Aerobic granulation in a sequencing batch reactor. *Water Research*. **33**(10): 2283-2290.

Chen M.Y., Lee D.J. and Tay J.H. (2007a). Distribution of extracellular polymeric substances in aerobic granules. *Applied Microbiology and Biotechnology*. **73**(6): 1463-1469.

Chen M.Y., Lee D.J., Tay J.H. and Show K.Y. (2007b). Staining of extracellular polymeric substances and cells in bioaggregates. *Applied Microbiology and Biotechnology*. **75**(2): 467-474.

Chiu Z.C., Chen M.Y., Lee D.J., Wang C.H. and Lai J.Y. (2007). Oxygen diffusion in active layer of aerobic granule with step change in surrounding oxygen levels. *Water Research*. **41**(4): 884-892.

Crocetti G.R., Banfield J.F., Keller J., Bond P.L. and Blackall L.L. (2002). Glycogen accumulating organisms in laboratory-scale and full-scale activated sludge processes. *Microbiology*. **148**(11): 3353-3364.

Crocetti G.R., Hugenholtz P., Bond P.L., Schuler A., Keller J., Jenkins D. and Blackall L.L. (2000). Identification of polyphosphate-accumulating organisms and design of 16S

rRNA-directed probes for their detection and quantitation. *Applied and Environmental Microbiology*. **66**(3): 1175-1182.

Daims H., Bruhl A., Amann R., Schleifer K.H. and Wagner M. (1999). The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: Development and evaluation of a more comprehensive probe set. *Systematic and Applied Microbiology*. **22**(3): 434-444.

Daims H., Nielsen J.L., Nielsen P.H., Schleifer K.H. and Wagner M. (2001). In situ characterization of Nitrospira-like nitrite oxidizing bacteria active in wastewater treatment plants. *Applied and Environmental Microbiology*. **67**(11): 5273-5284.

Di Iaconi C., Ramadori R., Lopez A. and Passino R. (2004). Preliminary biomass characterization in a sequencing batch biofilm reactor. *Annali di Chimica*. **94**(12): 889-898.

Dulekgurgen E., Ovez S., Artan N. and Orhon D. (2003). Enhanced biological phosphate removal by granular sludge in a sequencing batch reactor. *Biotechnology Letters*. **25**(9): 687-693.

El-Mamouni R., Leduc R. and Guiot S.R. (1998). Influence of synthetic and natural polymers on the anaerobic granulation process. *Water Science and Technology*. **38**(8-9): 341-347.

Etterer T. and Wilderer P.A. (2001). Generation and properties of aerobic granular sludge. *Water Science and Technology*. **43**(3): 19-26.

Grotenhuis J.T.C., Vanlier J.B., Plugge C.M., Stams A.J.M. and Zehnder A.J.B. (1991). Effect of ethylene glycol-bis(beta-aminoethyl ether)-N,N-tetraacetic acid (egta) on stability and activity of methanogenic granular sludge. *Applied Microbiology and Biotechnology*. **36**(1): 109-114.

Ivanov V., Tay S.T.L., Liu Q.S., Wang X.H., Wang Z.W. and Tay J.H. (2005). Formation and structure of granulated microbial aggregates used in aerobic wastewater treatment. *Water Science and Technology*. **52**(7): 13-19.

Jacques M. and Graham L. (1989). Improved preservation of bacterial capsule for electron-microscopy. *Journal of Electron Microscopy Technique*. **11**(2): 167-169.

Kong Y., Ong S.L., Ng W.J. and Liu W.-T. (2002). Diversity and distribution of a deeply branched novel proteobacterial group found in anaerobic-aerobic activated sludge processes. *Environmental Microbiology*. **4**(11): 753-757.

Kong Y.H., Nielsen J.L. and Nielsen P.H. (2005). Identity and ecophysiology of uncultured actinobacterial polyphosphate-accumulating organisms in full-scale enhanced biological phosphorus removal plants. *Applied and Environmental Microbiology*. **71**(7): 4076-4085.

Lemaire R., Yuan Z.G., Blackall L.L. and Crocetti G.R. (in press). Microbial distribution of Accumulibacter spp. and Competibacter spp. in aerobic granules from a lab-scale biological nutrient removal system. *Environmental Microbiology*.

Lettinga G., Vanvelsen A.F.M., Hobma S.W., Dezeeuw W. and Klapwijk A. (1980). Use of the upflow sludge blanket (USB) reactor concept for biological wastewater-treatment, especially for anaerobic treatment. *Biotechnology and Bioengineering*. **22**(4): 699-734.

Lin Y.M., Liu Y. and Tay J.H. (2003). Development and characteristics of phosphorus-accumulating microbial granules in sequencing batch reactors. *Applied Microbiology and Biotechnology*. **62**(4): 430-435.

Liu Y. and Tay J.-H. (2004). State of the art of biogranulation technology for wastewater treatment. *Biotechnology Advances*. **22**(7): 533-563.

Liu Y., Yang S.F. and Tay J.H. (2003). Elemental compositions and characteristics of aerobic granules cultivated at different substrate N/C ratios. *Applied Microbiology and Biotechnology*. **61**(5-6): 556-561.

Liu Y.Q. and Tay J.H. (2007). Characteristics and stability of aerobic granules cultivated with different starvation time. *Applied Microbiology and Biotechnology*. **75**(1): 205-210.

McSwain B.S., Irvine R.L., Hausner M. and Wilderer P.A. (2005). Composition and distribution of extracellular polymeric substances in aerobic flocs and granular sludge. *Applied and Environmental Microbiology*. **71**(2): 1051-1057.

Meyer R.L., Saunders A.M. and Blackall L.L. (2006). Putative glycogen-accumulating organisms belonging to the Alphaproteobacteria identified through rRNA-based stable isotope probing. *Microbiology-Sgm*. **152**: 419-429.

Meyer R.L., Saunders A.M., Zeng R.J.X., Keller J. and Blackall L.L. (2003). Microscale structure and function of anaerobic-aerobic granules containing glycogen accumulating organisms. *FEMS Microbiology Ecology*. **45**(3): 253-261.

Mobarry B.K., Wagner M., Urbain V., Rittmann B.E. and Stahl D.A. (1996). Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. *Applied and Environmental Microbiology*. **62**(6): 2156-2162.

Morgenroth E., Sherden T., van Loosdrecht M.C.M., Heijnen J.J. and Wilderer P.A. (1997). Aerobic granular sludge in a sequencing batch reactor. *Water Research*. **31**(12): 3191-3194.

Revsbech N.P. (1989). An oxygen microsensor with a guard cathode. *Limnology and Oceanography*. **34**(2): 474-478.

Schwarzenbeck N., Borges J.M. and Wilderer P.A. (2005). Treatment of dairy effluents in an aerobic granular sludge sequencing batch reactor. *Applied Microbiology and Biotechnology*. **66**(6): 711-718.

Tay J.H., Liu Q.S. and Liu Y. (2002). Characteristics of aerobic granules grown on glucose and acetate in sequential aerobic sludge blanket reactors. *Environmental Technology*. **23**(8): 931-936.

Tay J.H., Liu Q.S. and Liu Y. (2004). The effect of upflow air velocity on the structure of aerobic granules cultivated in a sequencing batch reactor. *Water Science and Technology*. **49**(11-12): 35-40.

Wagner M., Rath G., Koops H.-P., Flood J. and Amann R. (1996). *In situ* analysis of nitrifying bacteria in sewage treatment plants. *Water Science and Technology*. **34**(1-2): 237-244.

Wang Z.W., Liu Y. and Tay J.H. (2007). Biodegradability of extracellular polymeric substances produced by aerobic granules. *Applied Microbiology and Biotechnology*. **74**(2): 462-466.

Yang S.F., Tay J.H. and Liu Y. (2003). A novel granular sludge sequencing batch reactor for removal of organic and nitrogen from wastewater. *Journal of Biotechnology*. **106**(1): 77-86.

Yilmaz G., Lemaire R., Keller J. and Yuan Z.G. (submitted). Simultaneous nitrification, denitrification and phosphorus removal from nutrient-rich industrial wastewater using granular sludge. *Biotechnology and Bioengineering*.

Zheng Y.M. and Yu H.Q. (2007). Determination of the pore size distribution and porosity of aerobic granules using size-exclusion chromatography. *Water Research*. **41**(1): 39-46.

Appendix H

Résumé détaillé en français

INTRODUCTION

L'industrie de la viande utilise de très grandes quantités d'eau lors de l'abattage et le découpage des bêtes ainsi que pour le nettoyage des équipements. Les effluents produits sont très chargés en DCO, azote et phosphore. Afin d'éviter toute pollution des cours d'eau environnants, les effluents doivent être traités pour diminuer considérablement leurs teneurs en DCO, azote et phosphore (> 95%) avant de pouvoir être rejetés dans le milieu naturel. Durant ces vingt dernières années, l'élimination biologique de la DCO et de l'azote dans les effluents d'abattoir a reçu beaucoup plus d'attention que l'élimination biologique du phosphore. Cela a abouti au développement de procédés à boues activées fiables pour le traitement en continu de la DCO et de l'azote dans ce type d'effluents. Cependant, le phosphore continue à être principalement éliminé par l'intermédiaire de procédés chimiques de précipitation, même si l'élimination biologique du phosphore est généralement moins coûteuse et meilleure pour l'environnement. La haute teneur en azote des effluents d'abattoir c'est avérée être un obstacle au développement d'un procédé fiable et résistant d'élimination biologique du phosphore. Contrairement aux procédés de traitements des effluents en continu, les procédés à réacteurs séquentiels discontinus, sequencing batch reactors en anglais (SBR) permettent d'avoir une plus grande flexibilité de fonctionnement, ce qui peut être un atout majeur pour le traitement des effluents d'abattoirs à fortes teneurs azotées et phosphorées. D'ailleurs, en Australie, de plus en plus d'abattoirs ont opté pour le procédé SBR afin de traiter leurs effluents sur site.

L'objectif principal de cette thèse est de développer un procédé à boues activées purement biologique qui parvienne à réduire fortement les teneurs en DCO, azote et phosphore des effluents d'abattoir (> 95%) afin de répondre aux contraintes de rejet en rivière de plus en plus sévères et financièrement pénalisantes pour l'industrie. Pour ce faire, le principal obstacle à surmonter est de parvenir à éliminer durablement le phosphore par suraccumulation biologique dans un milieu fortement azoté. Pour pouvoir être considéré comme une réelle alternative pour l'industrie de la viande, ce nouveau procédé SBR devra être facilement transférable aux installations de traitement des eaux déjà présentes sur site afin d'éviter des coûts de construction trop élevés et une opération trop complexe.

Ce projet de thèse s'intéressera aussi à l'utilisation de technologies innovantes pour améliorer les performances du procédé SBR. Ces nouvelles technologies ou concepts sont encore au stade de développement avec beaucoup de questions fondamentales à résoudre avant de pouvoir être utilisées à l'échelle industrielle. Tout d'abord, le procédé de nitrification, dénitrification et déphosphatation simultanée (SNDPR), récemment développé et validé pour le traitement d'effluents synthétiques, sera utilisé pour traiter des effluents d'abattoir. Ce procédé permet de réduire les coûts d'aération mais surtout de diminuer la demande en DCO (surtout en acides gras volatils – AGV) qui est un paramètre très important pour l'élimination du phosphore par suraccumulation biologique, souvent déficient dans les effluents d'abattoir (faible teneur en AGV). Dans un deuxième temps, la possibilité d'éliminer la DCO, l'azote et le phosphore en utilisant un procédé à boues activées granulaires sera étudiée. A cause de leur

structure dense et compacte, les boues granulaires décantent très rapidement, ce qui permet d'avoir une concentration de boues plus élevée dans le réacteur et par conséquent de réduire le volume du réacteur. Cette concentration élevée en boues granulaires permet au procédé de pouvoir répondre à de fortes charges, ce qui est un grand avantage par rapport aux procédés classiques à boues activées floculantes pour traiter des effluents industriels généralement très concentrés. Il est également reconnu que le procédé SNDPR pourrait être plus facilement mis en œuvre dans un système granulaire à cause d'un gradient en oxygène plus important dans les granules que dans les flocs.

OBJECTIFS de RECHERCHE

1. Développement d'un procédé pouvant éliminer les concentrations en DCO, azote et phosphore contenues dans les effluents d'abattoir.

L'élimination biologique de la DCO et de l'azote dans des effluents d'abattoir a déjà été largement étudiée, aboutissant au développement de procédés d'épuration de grande échelle. En revanche, l'élimination biologique du phosphore dans ces mêmes effluents n'a été que peu étudiée en raison de problèmes d'instabilité causés par une accumulation de nitrate trop élevée lors de l'étape de nitrification. En effet, l'élimination du phosphore par suraccumulation biologique nécessite la présence de périodes d'anaérobies (sans oxygène ni nitrate ou nitrite) pendant lesquelles les bactéries déphosphatantes (PAO) peuvent stocker des AGV pour s'en servir ensuite comme source d'énergie durant les périodes d'aérobies. Par conséquent, la plupart des stations d'épuration traitant des effluents d'abattoir éliminent le phosphore par précipitation chimique en ajoutant des ions métalliques (fer ou aluminium). Cette technique alourdit considérablement les coûts de traitement et produit des boues à fortes teneurs métalliques ce qui peut limiter leur valorisation agricole. Le premier objectif de cette thèse est de développer un nouveau procédé de traitement en SBR limitant l'accumulation des nitrates et/ou nitrites et permettant du même coup d'éliminer par voie biologique plus de 95% de la DCO, de l'azote et du phosphore des effluents d'abattoir.

2. Création d'un système de contrôle automatique permettant d'obtenir une nitrification et une dénitrification via les nitrites.

La trop forte accumulation de nitrates et/ou nitrites n'est pas le seul problème limitant la stabilité et l'efficacité du procédé d'élimination du phosphore par suraccumulation biologique dans les stations de traitement d'effluents d'abattoir. La faible teneur en DCO facilement biodégradable de ces effluents est aussi un problème pour arriver à éliminer les fortes concentrations en azote et en phosphore. Le fait d'arrêter le processus de nitrification au stade nitrite permet d'économiser de la DCO lors de la dénitrification (en théorie 40%) et d'améliorer les performances du procédé. Des systèmes de contrôle automatique du pH et de la concentration en oxygène dissous ont déjà été utilisés pour favoriser la nitrification et dénitrification via les nitrites pour des effluents très concentrés en ammonium. Cependant, l'ajout de composés carbonés facilement biodégradables est à chaque fois nécessaire durant l'étape de dénitrification car la teneur en DCO des effluents n'est généralement pas suffisante pour obtenir une dénitrification satisfaisante. L'utilisation de sources externes de carbone ne permet donc pas de bénéficier pleinement des avantages du shunt des nitrates. Le second objectif de cette thèse est de mettre en place sur le procédé SBR développé précédemment un système de contrôle automatique pour établir un shunt des nitrates sans avoir recours à l'ajout de composés carbonés facilement dégradables. Ce système aura pour but d'améliorer

l'utilisation de la DCO présente dans les effluents d'abattoir et rendra le procédé d'élimination du phosphore par suraccumulation biologique plus efficace et plus résistant.

3. Expérimentation d'une stratégie pour maintenir l'activité des boues durant de longues périodes de famine ainsi que durant la phase de redémarrage du procédé.

Les stations de traitement d'effluents d'abattoir doivent s'adapter aux larges fluctuations de débit et de composition de l'effluent d'entrée en fonction du niveau de production. Durant certaines périodes de faible production (production saisonnière, maintenance annuelle des équipements) aucun effluent n'arrivera en tête de station pendant des semaines voire même des mois. Il est donc très important de pouvoir maintenir l'intégrité des boues biologiques durant ces longues périodes d'interruption afin de s'assurer que le procédé de traitement soit opérationnel lors du retour à une production normale. D'après le peu de recherches faites sur ce sujet, il semble que la stratégie consistant à alterner les conditions aérobies et anaérobies soit la plus efficace pour maintenir l'activité des boues durant de longues périodes de famine. Cependant, ces travaux de recherche se sont seulement intéressés aux boues nitrifiantes. Cette thèse a donc pour but de développer une stratégie de stockage des boues facilement applicable par l'industrie qui puisse préserver les capacités nitrifiantes, dénitrifiantes et déphosphatantes des boues lors de longues périodes d'interruption de la production et qui permette leur rapide réactivation lors de la reprise d'une production normale.

4. Identification des causes de la production de N_2O dans le procédé SNDPR afin de pouvoir utiliser ce procédé pour le traitement d'effluents d'abattoir.

Le procédé SNDPR présente de nombreux avantages qui pourraient être très bénéfiques pour le traitement d'effluents d'abattoir comme une plus faible demande en DCO, moins d'accumulation de nitrates ou nitrites durant l'étape aérobie, des coûts d'aération plus faible et une production de boue limitée. Cependant, la production de N_2O au lieu de N_2 comme produit final de la dénitrification pose un problème environnemental à cause du fort pouvoir à effet de serre de ce gaz (300 fois plus élevé que celui du CO_2). Avant de pouvoir utiliser cette technologie pour le traitement d'effluents d'abattoir, les causes exactes de cette accumulation et cette émission de N_2O doivent être identifiées et si possible éliminées. Ceci constitue un autre objectif de cette thèse.

5. Etude de la distribution spatiale des populations microbiennes responsables de la dénitrification à l'intérieur de boues granulaires.

L'utilisation de boues granulaires a la capacité à améliorer la stabilité et l'efficacité du procédé SNDPR. La taille et la densité de ces granules aérobies sont supposées générer de plus forts gradients d'oxygène qui seront bénéfiques pour la stabilité du procédé SNDPR en créant des zones anaérobies au centre de chaque granule. La présence simultanée de zones aérobies et anaérobies à l'intérieur d'un même granule entraînera vraisemblablement une organisation des populations microbiennes en fonction du gradient d'oxygène existant dans cette structure granulaire. Par exemple, les populations dénitrifiantes seront sans doute localisées plutôt vers le centre du granule dépourvu d'oxygène. La réduction de la demande en DCO grâce au procédé SNDPR implique que la dénitrification soit principalement assurée par les populations déphosphatantes (PAO) qui utiliseront la même source de carbone pour éliminer l'azote et le phosphore de façon simultanée. Malheureusement, il a souvent été observé que d'autres populations ne participant pas à l'élimination du phosphore (glycogen accumulating organisms – GAO) soient en fait responsables de la dénitrification dans le procédé SNDPR rendant ce procédé moins attractif. Dans un procédé SNDPR à boues

granulaires idéal, les PAO seraient en charge de la dénitrification ce qui impliquerait qu'ils soient plutôt localisés au centre du granule là où l'oxygène est absent. Pour vérifier les rôles écologiques respectifs des populations de PAO et GAO dans le système SNDPR à boues granulaires, une méthode a été développée afin d'établir les positions de chaque population à l'intérieur des granules. Le développement de cette méthode ainsi que le rôle joué par ces deux importantes populations dans le procédé SNDPR est un autre objectif de cette thèse.

6. Possibilité d'éliminer la DCO, l'azote et le phosphore présents dans les effluents d'abattoir par l'intermédiaire d'un procédé SBR aérobie à boues granulaires.

L'excellente décantation des boues granulaires et leur densité élevée permettent d'avoir une forte concentration de boues dans un réacteur de petite taille. L'utilisation d'un tel procédé peut donc permettre de traiter des effluents très chargés en DCO, azote et phosphore plus efficacement par rapport aux procédés à boues floculantes conventionnels. Aujourd'hui, les procédés à boues granulaires ont été principalement étudiés avec des effluents synthétiques. Les quelques études utilisant des effluents réels se sont limitées à des eaux résiduaires urbaines. La possibilité d'utiliser cette technologie pour traiter des effluents d'abattoir très chargés en DCO, azote et phosphore est étudiée dans cette thèse. De plus, la structure des granules ainsi développés est examinée en utilisant une grande variété de méthodes microscopiques.

PRINCIPAUX RESULTATS

1. Utilisation d'une stratégie d'alimentation fractionnée pour limiter l'accumulation de nitrates et/ou nitrites dans le SBR

Pour réduire l'effet néfaste des nitrates sur la stabilité et l'efficacité du procédé d'élimination du phosphore par suraccumulation biologique, l'alimentation du SBR avec l'effluent d'abattoir a été répartie en 3 périodes pour chaque cycle. Après la décantation et la décharge de l'effluent traité, le premier remplissage ne consiste qu'en 50% du volume total d'effluent à traiter dans le cycle, et est suivi d'une période sans aération (anaérobie) puis avec aération (aérobie). Lors du deuxième remplissage, 30% du volume total d'effluent à traiter sont ajoutés, suivi là aussi d'une période anaérobie puis aérobie. Le reste du volume à traiter est ajouté lors du troisième remplissage, suivi également d'une période anaérobie et aérobie. Grâce à cette stratégie le niveau de nitrates à la fin de chaque période aérobie reste suffisamment faible pour permettre une suraccumulation biologique du phosphore efficace et stable. La figure 11 montre l'évolution des concentrations d'ammonium, de NO_x^- (nitrate + nitrite) et de phosphates durant un cycle SBR typique. A la fin de chaque période aérobie, l'ammonium était totalement nitrifié et les NO_x^- produits étaient rapidement consommés durant la période anoxique qui suivait. Le peu de NO_x^- restant dans le SBR à la fin du cycle était immédiatement consommé une fois le premier remplissage du cycle suivant achevé. Grâce au faible niveau de NO_x^- durant tout le cycle du SBR, les populations déphosphatantes (PAO) étaient très actives comme le montrent les forts relargages de phosphates durant les périodes anaérobies et leur rapide consommation pendant les périodes aérobies. Cette stratégie de remplissages successifs a permis de maintenir une élimination de plus de 95% de la DCO totale en entrée, 97% de l'azote total et 98% du phosphore total.

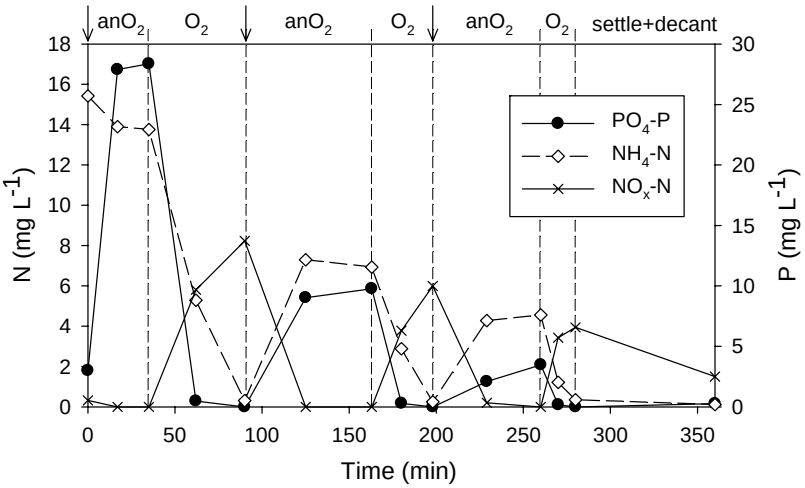

Figure 11. Concentrations en azote et phosphore pendant un cycle du SBR. Les flèches indiquent les 3 périodes d'alimentation.

2. Stratégies pour satisfaire la demande en DCO nécessaire pour éliminer les fortes concentrations d'azote et de phosphore des effluents d'abattoir.

Les performances d'un procédé de traitement biologique pour l'élimination de l'azote et du phosphore dépendent énormément de la DCO facilement biodégradable présente dans l'effluent à traiter, en particulier la teneur en AGV. Pour éliminer les fortes concentrations d'azote et de phosphore des effluents d'abattoir, il est donc primordial d'optimiser l'utilisation de la DCO disponible. En pratique, les effluents bruts d'abattoir sont envoyés dans des grandes lagunes anaérobies afin de réduire les teneurs en graisses ainsi que d'hydrolyser la DCO particulaire. Malheureusement, beaucoup de DCO est abattue durant ce prétraitement, ce qui rend l'élimination de l'azote et du phosphore difficile. La taille de ces lagunes ne permet pas de contrôler avec précision les quantités d'AGV produites à cause de la compétition entre les processus d'acidogénèse et de méthanogénèse. Afin de mieux contrôler les niveaux d'AGV présents dans l'effluent d'entrée du SBR, une fraction de l'effluent brut a été soumise à une pré-fermentation d'un jour à 37°C dans un réacteur batch de 50 L avant d'être mélangée avec l'effluent prétraité en sortie de lagune anaérobie et introduite dans le SBR. Aucun inoculum n'était utilisé dans notre pré-fermenteur, seules les bactéries présentes dans l'effluent brut d'abattoir effectuèrent cette pré-fermentation où la teneur en AGV a plus que doublé. Les acides acétique et propionique étaient les AGV les plus abondamment produits lors de cette pré-fermentation. Cependant, l'utilisation d'une trop grande fraction d'effluent pré-fermenté pendant 24h dans l'alimentation d'entrée du procédé SBR doit être évitée à cause de la forte teneur en graisses de cet effluent et du risque que cela pose pour avoir une bonne décantation des boues.

L'autre stratégie pour réduire la demande en DCO du procédé est d'utiliser le shunt des nitrates lors de l'étape de nitrification et de dénitrification. Pour ce faire, un système de contrôle automatique de la durée de chacune des 3 périodes aérobies du cycle SBR a été mis en place. Ce système de contrôle est basé sur la pente du signal pH ainsi que sur la vitesse de consommation d'oxygène (OUR). Durant chaque aération, l'instant exact où tout l'azote a été nitrifié était déterminé grâce à la rupture de pente du profil pH et à la soudaine diminution de l'OUR. L'aération dans le SBR pouvait donc être arrêtée automatiquement, évitant ainsi l'oxydation des nitrites en nitrates. Au lieu d'apporter une source de carbone externe, la DCO

présente dans l'effluent d'abattoir était utilisée pour accomplir la dénitrification, ce qui a permis de profiter pleinement des atouts du shunt des nitrates. La figure 12 montre la relation entre le niveau moyen d'accumulation des nitrites dans le SBR à la fin de chacune des 3 aérations (indicateur du niveau de shunt des nitrates réalisé) et l'abondance relative des populations microbiennes responsables de l'oxydation des nitrites en nitrates (NOB) dans les boues du SBR. Pendant les 5 premiers mois de fonctionnement du SBR, la durée de chaque phase d'aération n'était pas strictement contrôlée et tous les nitrites étaient oxydés en nitrates. Après avoir d'abord ajusté manuellement la durée de chacune des 3 périodes d'aération afin que l'aération s'arrête dès que tout l'azote était oxydé, la proportion de nitrites augmenta très rapidement de 0% à 95% des NO_x^- produits et les populations NOB diminuèrent en conséquence. Après avoir rétabli un régime d'aération plus longue que le temps nécessaire pour avoir une totale oxydation de l'azote, le pourcentage d'accumulation des nitrites chuta et les populations NOB augmentèrent très légèrement. Enfin, après 400 jours d'opération, le contrôle automatique de la durée d'aération fut mis en place ce qui entraîna un retour a un shunt des nitrates d'environ 70%. Après une période d'interruption de 6 semaines suite à la fermeture annuelle de l'abattoir, le shunt des nitrates atteignit 85% et les populations NOB disparurent presque complètement du SBR. Cette stratégie du contrôle de la durée d'aération a donc permis d'atteindre un fort shunt des nitrates ce qui a fortement réduit la demande en DCO du procédé durant l'étape de dénitrification.

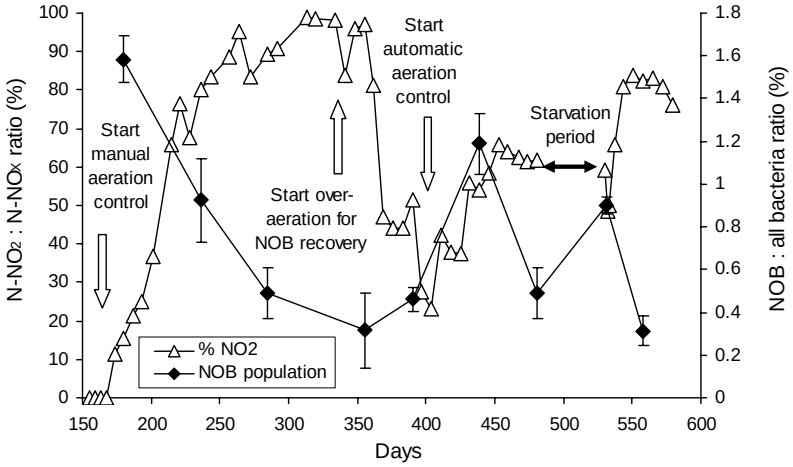

Figure 12. Accumulation de nitrite et abondance des bactéries nitratantes (NOB) du genre *Nitrospira* (FISH probe Nitspa-662) dans le SBR. La quantification des NOB indiquée est une moyenne (barres d'erreur=SE, n=3).

3. Stratégie pour maintenir l'activité biologique des boues de traitement d'effluents d'abattoir durant de longues périodes sans alimentation.

Le fonctionnement du SBR a du être modifié durant deux périodes de 5 à 6 semaines lorsque l'abattoir a interrompu sa production pour cause de maintenance annuelle de ses équipements, ce qui nous a obligé à arrêter l'alimentation du SBR. Le nouveau mode opératoire du SBR, appelé « mode veille », consistait en seulement 15 min d'aération pour chaque cycle de 6h (décantation le reste du temps). Durant le premier « mode veille » de 5 semaines, l'activité des populations nitrifiantes était mesurée chaque semaine en injectant de l'ammonium puis des nitrites et en suivant leur cinétique de consommation respective alors que le SBR était aéré pendant 2 h. L'activité générale des populations déphosphatantes était suivie en mesurant la

concentration des phosphates dans le SBR plusieurs fois par semaine. Durant le second « mode veille », de 6 semaines cette fois-ci, l'activité aérobie et anaérobie des PAO était plus rigoureusement suivie à l'aide de tests en batch réalisés chaque semaine avec 200 ml de boues prélevés dans le SBR. Après la réouverture de l'abattoir et le retour à une production d'effluent normale, une stratégie de réalimentation progressive du SBR étalée sur 4 jours a été expérimentée pour permettre aux boues de se ré-acclimater aux fortes concentrations de DCO, d'azote et de phosphore.

Pendant ces deux « mode veille », l'activité des populations nitrifiantes a diminué de 40% pour les AOB et de 10% pour les NOB, alors que pour les populations déphosphatantes (PAO) elle a chuté de plus de 60%. Malgré cela, après avoir progressivement réalimenté le SBR, les cinétiques de nitrification, de dénitrification, et de déphosphatation ont rapidement retrouvé leur valeur initiale (voir Tableau 3). La nouvelle stratégie opératoire du SBR pendant ces longues périodes d'interruption et lors de la réalimentation progressive, a donc permis de maintenir les capacités nitrifiantes, dénitrifiantes et déphosphatantes des boues ainsi que de rapidement retrouver les cinétiques d'élimination initiales.

Tableau 3. Vitesses de nitrification (rNH_4^+), dénitrification (rNO_x^-) et quantités de P relargué et accumulé par cycle, mesurées durant des cycles réalisés avant la période de famine, juste après le début de la période de famine (50% de la charge normale), 2 jours après (75% de la charge normale) et 4 jours après la famine (100% de la charge normale).

Paramètres	**rNH_4^+** ($mgN.l^{-1}.h^{-1}$)		**rNO_x^-** ($mgN.l^{-1}.h^{-1}$)		**Relargage de P** ($mgP.l^{-1}$)		**Accumulation de P** ($mgP.l^{-1}$)	
"mode veille" I or II	**I**	**II**	**I**	**II**	**I**	**II**	**I**	**II**
Avant famine	18.2	25.5	4.8	12.3	18.8	36.9	16.1	34.2
Après 1st cycle (50%)	8.2	7.4	1.9	1.8	2.5	4.4	2.3	4.8
Après 2 days (75%)	12.9	20.8	4.5	9.6	9.6	31	8.1	28.9
Après 4 days (100%)	17.6	29.1	5.7	11.7	19.6	47	17.4	44.3

4. Identification des principaux microorganismes dénitrifiants dans des bioréateurs SNDPR à boues floculantes et granulaires traitant des effluents synthétiques

Les analyses microbiennes (FISH) faites sur les boues floculantes du bioréacteur SNDPR alimenté avec un effluent synthétique ont montré une forte abondance de bactéries *Accumulibacter* (PAO) et *Competibacter* (GAO) représentant environ 70% de la totalité des bactéries dans les boues. La figure 13 indique que les PAO étaient toujours plus nombreux que les GAO dans le SBR. Il est intéressant de noter que le taux d'élimination de l'azote a diminué de 100% à 53% pendant que le pourcentage de GAO dans les boues est passé de 19% à 8%. Durant cette même période, le pourcentage de PAO a lui augmenté de 48% à 70%. Ces résultats semblent suggérer que les GAO, et non pas les PAO, étaient responsables de la dénitrification dans le SBR. Cela pose donc la question de savoir s'il y a un lien entre cette apparente dénitrification par les GAO et la production de N_2O souvent constatée dans les procédés SNDPR à boues floculantes traitant des effluents synthétiques.

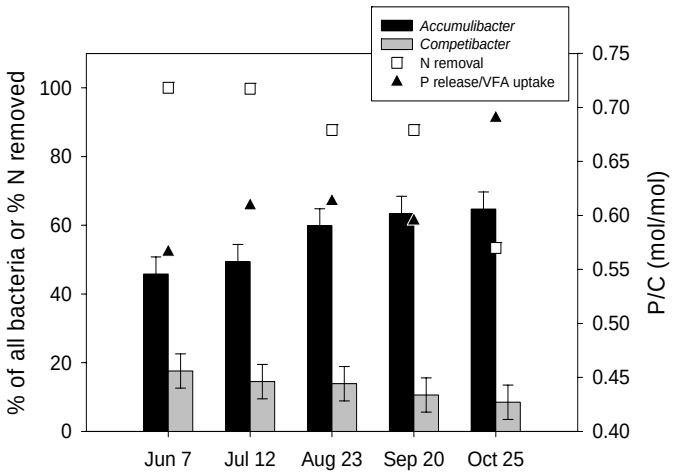

Figure 13. Abondance de *Accumulibacter* et *Competibacter* corrélée à l'élimination de l'azote et au rapport carbone accumulé:phosphate relargué pendant les 5 mois d'expérience.

Pour essayer de déterminer à l'échelle microbienne quelle était, entre les PAO et les GAO, la population la plus apte à dénitrifier dans un procédé SNDPR traitant des effluents synthétiques, on a comparé, mais cette fois à l'aide de boues granulaires, la distribution spatiale des PAO et GAO à l'intérieur des granules ainsi que le gradient d'oxygène existant dans ces mêmes granules. Pour pouvoir établir avec précision la distribution de ces deux populations dans la structure granulaire, une nouvelle méthode utilisant la technique FISH et la microscopie laser confocal (CLSM) a donc été développée durant cette thèse. Les gradients d'oxygène à l'intérieur des granules ont eux été mesurés à l'aide de microsondes à oxygène. La distribution des PAO et GAO a été déterminée en analysant une fine section médiane de 24 granules différents. Pour chaque granule étudié, le pourcentage relatif de PAO était divisé par celui des GAO pour chaque niveau de profondeur (tous les 50 µm) à partir de la surface du granule. En utilisant ce ratio PAO/GAO obtenu pour chaque zone, une distribution moyenne a été calculée et est présentée dans la figure 14 ainsi que la concentration en oxygène mesurée tous les 50 µm en partant de la surface du granule. Une forte corrélation a été établie entre le pourcentage de PAO et la concentration en oxygène. Les PAO étaient beaucoup plus nombreux que les GAO dans la zone aérobie des granules (0-200 µm) alors que les GAO étaient dominateurs dans la zone centrale anaérobie (à partir de 200 µm de profondeur). A cause de cette prédominance des GAO dans la partie du granule dépourvu d'oxygène, il apparaît donc comme très probable que les GAO soient les bactéries dénitrifiantes dans ce procédé SNDPR granulaire, ce qui va dans le même sens que les résultats obtenus avec des boues SNDPR floculantes. Alors que cette dénitrification par les GAO compromet les économies de DCO du procédé SNDPR, il est possible que l'utilisation d'un effluent réel à la place d'un effluent synthétique puisse atténuer le rôle dénitrifiant des GAO.

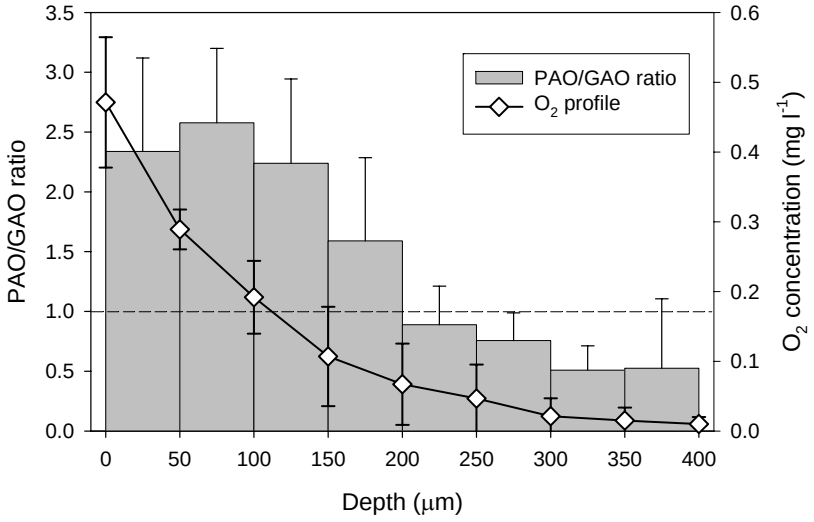

Figure 14. Profil moyen du rapport PAO:GAO dans 24 granules (bar d'erreur=95%CL) et profil moyen de la concentration en O_2 dans les granules à la fin de la période aérobie (barre d'erreur=S.D., n=6).

5. Gestion de l'accumulation de N_2O dans le procédé SNDPR

Afin d'identifier les raisons de l'accumulation de N_2O observée dans le procédé SNDPR traitant des effluents synthétiques et essayer d'y remédier, plusieurs expériences en batch ont été réalisées sous différentes conditions. Pour chaque expérience, des boues étaient prélevées du SBR et transvasées dans deux petits flacons de 15 ml fermés hermétiquement avec un bouchon en caoutchouc à travers lequel une microsonde à N_2O était introduite pour suivre la concentration en N_2O dissous en continu. Les flacons étaient mélangés continuellement avec un barreau magnétique et ne contenaient aucune poche d'air afin d'éviter tout transfert de N_2O de la phase liquide vers la phase gazeuse. Chaque expérience était faite en parallèle dans les deux flacons avec un des flacons utilisé comme témoin. Durant chaque expérience, de faibles volumes de substrat pouvaient être injectés dans chaque flacon par l'intermédiaire d'une seringue. Les vitesses de production et de consommation du N_2O lors de l'étape de dénitrification pouvaient ainsi être mesurées en utilisant différents accepteurs d'électrons (nitrate, nitrite et N_2O) et plusieurs sources de carbone (réserves de carbone intracellulaire, acétate, propionate, méthanol et des effluents d'abattoir).

Une des hypothèses proposées pour éviter l'accumulation de N_2O est que si la dénitrification dans le procédé SNDPR n'était pas uniquement supportée par les GAO comme vu précédemment mais aussi par d'autres populations dénitrifiantes, l'excès de N_2O produit par ces GAO lors de la dénitrification pourrait donc être consommé par d'autres organismes dénitrifiants, pourvu que suffisamment de DCO soit encore disponible pendant la période aérobie du procédé SNDPR. Pour vérifier cette hypothèse, des expériences batch supplémentaires ont été réalisées en mélangeant cette fois les boues du procédé SNDPR avec celles d'un autre procédé éliminant l'azote d'effluents urbains par nitrification et dénitrification avec ajout de méthanol. La figure 15 montre que l'addition d'effluent d'abattoir après 35 min d'incubation avec des nitrates a permis aux boues du réacteur traitant un effluent urbain de consommer immédiatement tout le N_2O accumulé par les boues provenant du procédé SNDPR.

L'accumulation de N_2O observée dans les boues du procédé SNDPR est sûrement le résultat de la très forte abondance des PAO et GAO dans le SBR due à l'utilisation d'un effluent synthétique contenant leur source de carbone préférée (acide acétique) comme unique composé organique. Si ce procédé SNDPR était alimenté avec un effluent d'origine domestique ou industrielle, il est très probable que des organismes dénitrifiants autres que les PAO ou GAO pourraient participer à la dénitrification en utilisant la multitude de composés organiques présents dans cet effluent. Il est donc vraisemblable que l'accumulation de N_2O observée lors du traitement d'effluents synthétiques à une seule source de carbone par le procédé SNDPR ne soit plus observée lors du traitement d'effluents réels contenant une large diversité de composés organiques.

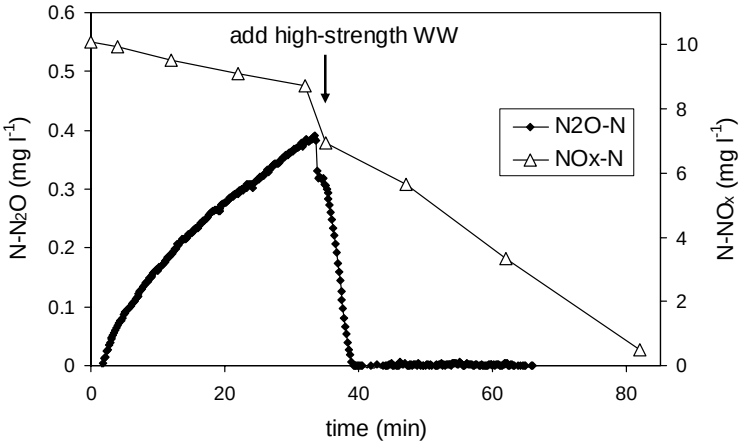

Figure 15. Concentrations en N_2O et NO_x^- dans la phase liquide pendant un test anoxique dans un réacteur de 500 ml. L'eau usée brute chargée a été ajoutée à T=35 min.

6. Elimination de la DCO, de l'azote et du phosphore des effluents d'abattoir à l'aide de boues granulaires

Les charges en DCO, azote et phosphore appliquées dans le SBR granulaire étaient de 2.7 $gDCO.l^{-1}.j^{-1}$, 0.43 $gN.l^{-1}.j^{-1}$ et 0.06 $gP.l^{-1}.j^{-1}$. Les rendements d'épuration obtenus après avoir atteint un régime stationnaire étaient de 89% pour la DCO soluble, 93% pour l'azote dissous total et 88% pour le phosphore dissous total. Le reste de la DCO soluble dans l'effluent de sortie (162 $mg.l^{-1}$) était considérée non biodégradable à cause de la très faible valeur de la DBO_5 soluble mesurée (<2 $mg.l^{-1}$). A la fin de chaque cycle SBR environ 10 $mgN.l^{-1}$ de NO_x^- restait dans l'effluent principalement sous la forme de nitrites (95%) signifiant que l'azote était vraisemblablement éliminé via le shunt des nitrates. Cependant, la forte concentration des matières en suspension de l'effluent en sortie du SBR (autour de 0.3 $g.l^{-1}$) limita les taux d'épuration de la DCO totale, de l'azote total et du phosphore total à 68%, 86% et 74% respectivement. Il est intéressant de noter que les GAO n'étaient pas présents dans ces boues granulaires et que les PAO étaient responsables de la dénitrification. Cela montre que le véritable procédé SNDPR a pu être établi dans ce SBR à boues granulaires traitant des effluents d'abattoir. La structure interne de ces boues granulaires a également été étudiée à l'aide de microscopes à balayage et à transmission électronique, de microsondes à oxygène et à pH et de techniques de microbiologie (FISH).

CONCLUSION

Un procédé SBR (réacteur discontinu à alimentation séquentielle) a été développé pour éliminer efficacement les fortes teneurs en DCO, azote et phosphore des effluents d'abattoir. Ce procédé biologique offre une vraie alternative par rapport aux procédés chimiques conventionnels utilisés pour éliminer le phosphore dans ces effluents, que ce soit au niveau du coût ou de l'impact sur l'environnement. L'utilisation de ce nouveau procédé par l'industrie de la viande est donc rapidement envisageable et relativement facile à mettre place. Ce procédé est d'ailleurs en train d'être testé à l'échelle pilote (10 m^3) dans un abattoir australien. Les principales conclusions de la recherche effectuée sur ce procédé durant cette thèse sont :

- Ce procédé SBR peut traiter de façon biologique des effluents d'abattoir à forte teneur en azote (200 – 300 $mgN.l^{-1}$) et d'obtenir des taux épuration supérieurs à 95% pour la DCO totale, l'azote total et le phosphore total.
- La stratégie d'alimentation fractionnée a permis de réduire l'accumulation des nitrates et nitrites dans le SBR rendant possible l'élimination du phosphore par suraccumulation biologique.
- Il est important de faire passer une fraction de l'effluent d'abattoir brute dans un pré-fermenteur en amont du procédé SBR afin d'augmenter sa teneur en AGV. Cela améliorera la stabilité du procédé SBR ainsi que ses taux d'épuration.
- L'arrêt automatique du système d'aération du SBR en fonction du pH dès que tout l'ammonium était oxydé a permis d'éviter la production de nitrates. Grâce à ce shunt des nitrates, l'utilisation de la DCO présente dans l'effluent d'abattoir a été optimisée pour obtenir une élimination de l'azote et du phosphore plus stable et plus poussée.
- Pour maintenir l'activité biologique des boues lors de longues périodes d'interruption et faciliter le redémarrage du procédé, la stratégie consistant à aérer les boues pendant 15 minutes toutes les 6h a été très efficace. Après avoir utilisé cette stratégie pendant plus de 5 semaines alors que l'alimentation du SBR avait été arrêtée, le retour aux cinétiques d'épuration initiales a pris seulement 4 jours.

Deux autres nouveaux procédés très prometteurs pour le traitement biologique des effluents industriels chargés ont été étudiés. Le procédé de nitrification, dénitrification et déphosphatation simultanées (SNDPR) et le procédé à boues granulaires aérobies. Les principales conclusions de ces deux études sont les suivantes :

- Les GAO sont principalement responsables de la dénitrification dans le procédé SNDPR à boues floculantes traitant des effluents synthétiques. L'absence de dénitrification par les PAO compromet sérieusement la réduction de la demande en DCO supposée être réalisée avec ce procédé lors de l'élimination de l'azote et du phosphore.
- La production de N_2O dans des SBR traitant des effluents synthétiques par le procédé SNDPR est très probablement liée à la présence d'une seule source de carbone (acétate) dans cet effluent. L'émission de gaz N_2O ne sera vraisemblablement plus un problème lors du traitement d'effluents d'abattoir à cause de la diversité des sources de carbone présentes.
- La taille, la densité et l'activité microbienne des granules aérobies contribuent à la présence d'un fort gradient d'oxygène à l'intérieur des granules. L'existence de larges zones anaérobies au centre de chaque granule permet d'obtenir un procédé SNDPR plus stable grâce à un meilleur couplage entre les zones aérobies pour la nitrification et celles

anaérobies pour la dénitrification. Cependant, les GAO étaient toujours responsables de la dénitrification dans ce procédé SNDPR à boues granulaires traitant des effluents synthétiques.

- Des taux d'épuration élevés peuvent être obtenus lorsque des effluents d'abattoir sont traités avec des boues granulaires. Le procédé SNDPR a pu être employé avec cette fois les PAO responsables de la dénitrification permettant enfin au procédé SNDPR de développer tout son potentiel.

RESUME

L'industrie de la viande utilise de larges quantités d'eau lors de l'abattage, du découpage et du nettoyage des équipements. Les effluents produits sont très chargés en DCO, azote et phosphore. Afin d'éviter toute pollution des milieux aquatiques environnants, ces effluents doivent subir des traitements poussés. Le but principal de cette thèse était de développer un procédé de traitement par boues activées qui puisse éliminer plus de 95% de la DCO, de l'azote et du phosphore dans les effluents d'abattoir permettant alors un rejet direct de l'effluent traité en rivière. La forte teneur en azote des effluents d'abattoir est l'obstacle majeur empêchant d'établir une élimination biologique du phosphore stable et efficace. Durant cette thèse, un procédé biologique capable d'éliminer 98% du phosphore tout en abattant 95% de la DCO et 97% de l'azote a été développé dans un réacteur batch à alimentation séquentielle (SBR). Par rapport aux procédés chimiques classiques d'élimination du phosphore, ce nouveau procédé biologique offre une vraie alternative financière et environnementale pour l'industrie de la viande. Une stratégie d'alimentation séquentielle a permis de réduire l'accumulation des nitrates dans le SBR rendant ainsi possible l'élimination biologique du phosphore. Cette thèse aborde aussi l'étude et l'utilisation de technologies innovantes pour améliorer les performances du procédé SBR. Le procédé de nitrification, dénitrification et déphosphatation simultanées (SNDPR) a été incorporé au procédé à boues granulaires aérobies. La taille, la densité et l'activité microbienne des granules aérobies génèrent de forts gradients d'oxygène à l'intérieur des granules, permettant alors d'obtenir un procédé SNDPR plus efficace. Le volume du réacteur et la demande en DCO nécessaire pour éliminer l'azote et le phosphore dans les effluents d'abattoir ont ainsi pu être fortement réduits. La structure interne et la composition microbienne de ces granules ont également été étudiées.

Development and Fundamental Investigations of Innovative Technologies for Biological Nutrient Removal from Abattoir Wastewater.

ABSTRACT

The meat processing industry requires large quantities of water, much of which is discharged as wastewater containing high levels of COD and nutrients such as nitrogen (N) and phosphorus (P). These nutrients must be removed to very low levels before the wastewater can be discharged into local waterways to avoid causing eutrophication. The aim of this thesis was to develop a biological process that could achieve more than 95% of COD, N and P removal from abattoir wastewater, producing an effluent suitable for direct discharge into river systems. The main challenge is to achieve stable and reliable biological P removal in nitrogen-rich wastewater. A sequencing batch reactor (SBR) system was demonstrated to effectively remove 95%, 97% and 98% of the total COD, total N and total P present in abattoir wastewater. It could provide a more cost-effective and environmentally friendly alternative to chemical P removal, which is the common practice in the meat industry at present. A multi-step feeding strategy was employed to prevent the accumulation of nitrate or nitrite in the SBR providing the right condition for the development of a stable biological P removal. An automatic aeration length control strategy was developed and demonstrated to remove N via the nitrite pathway which benefited the nutrient removal performance of the SBR by reducing the amount of readily biodegradable COD required. This study also investigated the feasibility of using two innovative technologies to further enhance the performance of the SBR system. The simultaneous nitrification, denitrification and phosphorus removal (SNDPR) process and the aerobic granular sludge technology were successfully combined in a single SBR process. The size and the dense structure of aerobic granules positively contributed to the oxygen mass transfer limitation required to achieve reliable SNDPR. The structure and function of these granules fed with nutrient-rich wastewater were closely investigated using a wide range of microbial and micro-scale techniques, which yielded insightful information about aerobic granules and provided support for future in-depth studies on the mechanisms involved in aerobic biogranulation.

DISCIPLINE: Génie des Procédés – Process Engineering

MOTS-CLES: Boues granulaires aérobies, Dénitrification, Effluent d'abattoir, Elimination biologique de l'azote et du phosphore, Nitrification, Populations microbiennes, Réacteur séquentiel discontinu, Shunt des nitrates.

KEYWORDS: Aerobic granular sludge, Denitrification, Abattoir wastewater, Biological nutrient removal, Nitrification, Microbial communities, Sequencing batch reactor, Nitrite pathway.

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