Optimization of Reactor Startup and Nitrogen Removal of Aerobic Granular Sludge Systems

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Abstract

Aerobic granular sludge (AGS) in sequencing batch reactors (SBR) has recently made its proofs as full-scale technology for the biological treatment of urban wastewater. In contrast to conventional flocculent activated sludge, microorganisms aggregate to dense granular biofilm due to shear stress from aeration. Hence, AGS has much better settling characteristics and allows the construction of more compact wastewater treatment plants (WWTP) without secondary clarifiers. Due to diffusion limitations of oxygen through the biofilm, aerobic and anaerobic/anoxic zones coexist in AGS. Hence, AGS-SBR has the potential to treat organic matter (COD), nitrogen (N), and phosphorous (P) simultaneously in one single reactor.

The focus of the present PhD thesis was on the startup of AGS-SBR and the optimization of biological nutrient removal (BNR). For the investigation of the optimal startup conditions, a study testing seven parameters in parallel was conducted. The main conditions identified for a rapid startup of AGS-SBRs with good nutrient removal performances were (i) the alternation of high and low dissolved oxygen (DO) phases during aeration, (ii) a settling strategy avoiding too high biomass washout, (iii) the adaptation of the pollution load in the early stage of the startup in order to ensure that all soluble COD was consumed before the aeration phase, (iv) higher temperature (20°C), and (v) a neutral pH. Under such conditions it took less than 30 days to produce granular sludge with high removal performances for COD, N and P. A control run of the best startup strategy led to very similar results, proving the reproducibility of the experimental approach. This control run was operated for 80 days without any problems concerning the stability of the granular sludge or the nutrient removal performances.

Concerning the bacterial community composition during the startup phase, a general shift of the predominant populations from *Intrasporangiaceae* and *Sphingobacteriales* to *Dechloromonas* and *Zoogloea* was observed. This shift was mainly due to general conditions with lab-scale AGS-SBR, rather than the specific operation parameters tested in the different experimental runs. However, it has been observed that polyphosphate-accumulating organisms (PAO) and glycogen-accumulating organisms (GAO) related populations were favored by the adaptation of the pollution load in the early stage of the startup in order to ensure that all soluble COD was consumed before the aeration phase. Besides the pollution load, the temperature and the pH had a significant impact on the global bacterial community structure. The predominant PAO were presumably *Dechloromonas*.

In order to optimize BNR by AGS-SBR, different aeration strategies were tested. It has been concluded that the N-removal efficiency of COD-limited systems can be considerably enhanced with improved aeration strategies. Strategies promoting alternating nitrification and denitrification (AND) were significantly more efficient than simultaneous nitrification and denitrification (SND) strategies. The introduction of low DO phases or even anoxic phases in an early stage of the total aeration period probably enhanced denitrifying P-removal and led to COD savings. Intermittent aeration, which is a realistic AND strategy for full scale applications, led to the highest N-removal efficiency. The short mixing times implemented with this strategy were not problematic for the stability of the granules.

Finally, to further optimize N-removal in COD-limited systems, the potential of aeration control for the achievement of N-removal over nitrite was investigated. It was shown that aeration phase length control combined with intermittent aeration or alternating high-low DO, is an efficient way to achieve N-removal over nitrite. N-removal efficiencies of up to 95% were achieved with this way of reactor operation. At 20°C, N-removal over nitrite was achieved within 20 – 60 days and it was possible to switch from N-removal over nitrite to N-removal over nitrate and back again. At 15°C, the nitrite-oxidizing bacteria population could be reduced, but nitrite oxidation could not be completely repressed. However, the combination of aeration phase length control and high-low DO was successful to maintain the nitrite pathway at 15°C where the maximum growth rate of nitrite-oxidizing bacteria is clearly higher than the one of ammonium-oxidizing bacteria.

In conclusion, this thesis showed the potential of the optimization of operation conditions for the startup of AGS-SBR and BNR. An efficient strategy for the startup with flocculent inoculum sludge has been developed, maintaining high BNR. Moreover, it has been shown that N-removal under COD-limited conditions can be improved by aeration control, either by enhancing denitrifying P-removal or achieving N-removal over nitrite.

Keywords: wastewater treatment; aerobic granular sludge; biological nutrient removal; sequencing batch reactor; aeration strategies.

Zusammenfassung

Aerober, granulärer Schlamm (AGS) in Sequencing-Batch-Reaktoren (SBR) wird seit kurzem erfolgreich zur biologischen Abwasserreinigung eingesetzt. Im Gegensatz zu herkömmlichem, flockigem Belebtschlamm, aggregieren die Mikroorganismen zu einem dichten, granulären Biofilm. Dies geschieht aufgrund der Durchmischung durch die Belüftung und daher hat AGS viel bessere Absetzeigenschaften, was erlaubt kompaktere Abwasserreinigunsanlagen ohne Nachklärbecken zu bauen. Aufgrund der beschränkten Diffusion von Sauerstoff im Biofilm existieren im AGS gleichzeitig aerobe und anaerobe/anoxische Zonen. Daher bietet AGS-SBR die Möglichkeit organische Schmutzstoffe (gemessen als chemischer Sauerstoffbedarf; CSB), Stickstoff (N) und Phosphor (P) gleichzeitig in ein und demselben Reaktor abzubauen.

Das Hauptaugenmerk dieser Dissertation lag auf dem Anfahren (Inbetriebnahme) von AGS-SBR, sowie der Optimierung des biologischen Nährstoffabbaus. Zur Untersuchung der optimalen Anfahrbedingungen wurde eine Studie durchgeführt, welche den Einfluss von sieben Parametern gleichzeitig testete. Die wichtigsten Bedingungen für ein rasches Anfahren mit guten Leistungen bezüglich Nährstoffabbaus waren: (i) das Alternieren von Phasen mit hohem und niedrigem Sauerstoffgehalt während der Belüftung, (ii) eine Absetzstrategie welche ein zu hohes Auswaschen von Biomasse vermeidet, (iii) die Anpassung der Schadstoffbelastung im frühen Stadium der Inbetriebnahme, um sicherzustellen, dass der gesamte gelöste CSB vor der Belüftungsphase konsumiert wird, (iv) höhere Temperatur (20°C) und (v) einen neutralen pH-Wert. Unter solchen Bedingungen dauerte es weniger als 30 Tage um granulären Schlamm mit hohen Nährstoffabbauleistungen (CSB, N und P) zu produzieren. Diese optimale Anfahrstrategie wurde ein zweites Mal mit ähnlichen Resultaten durchgeführt, was die Reproduzierbarkeit der Strategie bestätigte. Dieser zweite Versuch wurde während 80 Tage durchgeführt ohne dass irgendwelche Probleme bezüglich der Stabilität des granulären Schlammes oder des Nährstoffabbaus auftraten.

Bezüglich der bakterielle Gemeinschaften wurde während der Startphase eine allgemeine Verschiebung der vorherrschenden Populationen von *Intrasporangiaceae* und *Sphingobacteriales* zu *Dechloromonas* und *Zoogloea* beobachtet. Diese Verschiebung wurde vor allem den allgemeinen Bedingungen mit AGS-SBR unter Laborbedingungen zugeschrieben und weniger den spezifischen Betriebsparametern in den verschiedenen Versuchsreihen. Jedoch wurde beobachtet, dass Polyphosphat-akkumulierende Organismen (PAO) und Glykogen-akkumulierenden Organismen (GAO) durch die Anpassung der Schadstoffbelastung im frühen Stadium der Inbetriebnahme begünstigt wurden. Neben der Anpassung der Schadstoffbelastung, hatten auch die Temperatur und der pH-Wert einen signifikanten Einfluss auf die globale Struktur der bakteriellen Gemeinschaft. Der vorherrschende PAO war vermutlich *Dechloromonas*.

Um den biologischen Nährstoffabbau mit AGS-SBR zu optimieren, wurden verschiedene Belüftungsstrategien getestet. Es wurde festgestellt, dass der N-Abbau in CSB-limitierten Systemen mit verbesserten Belüftungsstrategien deutlich gesteigert werden konnte. Strategien zur Förderung abwechselnder Nitrifikation und Denitrifikation (AND) waren effizienter als Strategien mit simultaner Nitrifikation und Denitrifikation (SND). Der massgebliche Punkt war dabei das Einführen von anoxischen Phasen oder von Phasen mit tiefem Sauerstoffgehalt in einem frühen Stadium der Belüftungsphase. Dies begünstigte sehr wahrscheinlich die Denitrifikation durch PAO und führte daher zu CSB Einsparungen. Intermittierende Belüftung, was auch für den Grossmassstab eine realistische Belüftungsstrategie ist, führte zu den höchsten N-Abbauleistungen. Die kurzen Mischzeiten mit dieser Strategie waren unproblematisch für die Stabilität des granulären Schlammes.

Abschliessend wurde eine weitere Strategie zur Optimierung des N-Abbaus in CSB-limitierten Systemen untersucht: Die Stickstoffeliminierung über Nitrit. Im Speziellen wurde getestet, ob mit Hilfe von Belüftungsregulierung N-Abbau über Nitrit erreicht werden konnte. Es konnte aufgezeigt werden, dass die Kontrolle der Belüftungsphasenlänge verbunden mit intermittierender Belüftung oder alternierendem hohem und tiefem Sauferstoffgehalt, eine effiziente Möglichkeit ist, N-Abbau über Nitrit zu erreichen. Auf diese Weise wurde ein N-Abbau von bis zu 95% erreicht. Bei 20°C wurde der N-Abbau über Nitrit in 20 – 60 Tagen erreicht und es war möglich von N-Abbau über Nitrit zu N-Abbau über Nitrat zu wechseln und wieder zurück. Bei 15°C konnte die Anzahl an Nitrit-oxidierenden Bakterien reduziert werden, jedoch konnte die Nitritoxidation nicht vollständig unterdrückt werden. Die Kombination von automatischer Belüftungsphasenlängenkontrolle und alternierendem hohem und tiefem Sauerstoffgehalt war jedoch auch bei 15°C erfolgreich, um den N-Abbau über Nitrit zu halten. Dies ist bemerkenswert, da bei dieser Temperatur die maximale Wachstumsrate von Nitrit-oxidierenden Bakterien deutlich höher ist als die von Ammonium-oxidierenden Bakterien, was den N-Abbau über Nitrit schwierig macht.

Zusammenfassend kann gesagt werden, dass diese Dissertation das Potenzial von optimierten Betriebsbedingungen für das Anfahren und den biologischen Nährstoffabbau mit AGS-SBR aufgezeigt hat. Eine effiziente Strategie zum Anfahren von AGS-SBR mit flockigem Belebtschlamm wurde entwickelt. Darüber hinaus hat sich gezeigt, dass der N-Abbau unter CSB-limitierten Bedingungen durch Belüftungsregulierung wesentlich verbessert werden kann. Eine Lösung ist dabei eine erhöhte Denitrifizierung durch PAO, eine andere der N-Abbau über Nitrit.

Stichwörter: Abwasserreinigung; Aerober, granulärer Schlamm; biologischer Nährstoffabbau; Sequencing-Batch-Reaktor; Belüftungsstrategien.

Résumé

La technologie des boues aérobies granulaires (AGS) dans des réacteurs à alimentation séquentielle (SBR) a récemment fait ses preuves comme alternative efficace pour le traitement biologique des eaux usées urbaines. Contrairement aux boues activées floculant classique, les micro-organismes s'agrègent dû à l'aération pour former un biofilm granulaire dense. Par conséquent, les AGS ont des meilleures caractéristiques de décantation et permettent la construction de station d'épuration (STEP) plus compactes et sans clarificateurs secondaires. En raison des limitations de diffusion de l'oxygène dans le biofilm, les zones aérobie et anaérobie / anoxique coexistent dans les AGS. Par conséquent, la technologie AGS-SBR a le potentiel de traiter la matière organique (DCO), l'azote (N) et le phosphore (P) simultanément dans un seul réacteur.

Le présent travail de thèse avait comme but d'étudier le démarrage de l'AGS-SBR et d'optimiser l'élimination biologique des nutriments (BNR). Pour étudier les conditions de démarrage optimales, une étude testant sept paramètres en parallèle a été menée. Les principales conditions identifiées pour un démarrage rapide et l'obtention de bonnes performances d'élimination des nutriments ont été (i) l'alternance de forte et faible teneur en oxygène dissous (OD) au cours de l'aération, (ii) une stratégie de décantation qui évite un lessivage trop élevée de biomasse, (iii) l'adaptation de la charge polluante dans un premier stade du démarrage, afin d'assurer que toute la DCO soluble est consommé avant la phase d'aération, (iv) une température plus élevée (20°C), et (v) un pH neutre. Dans ces conditions, des boues granulaires avec des bonnes performances d'élimination de DCO, N et P ont pu être produites en moins de 30 jours. La reproductibilité de cette stratégie ainsi que la stabilité des granules et des performances BNR a été démontré dans un essaie sur 80 jours.

En ce qui concerne la composition des communautés bactériennes pendant la phase de démarrage, un changement général des populations prédominantes d'*Intrasporangiaceae* et *Sphingobacteriales* à *Dechloromonas* et *Zoogloea* a été observé. Cette évolution s'observait dans tous les essaies indépendamment des paramètres étudiés et s'expliquait donc principalement par les conditions générales de cette étude à l'échelle du laboratoire. Cependant, il a été observé que les organismes accumulateurs de polyphosphate (PAO) et les organismes accumulateurs de glycogène (GAO) été favorisées par l'adaptation de la charge polluante dans la première phase du démarrage. Outre la charge polluante, la température et le pH ont également eu un impact significatif sur la structure globale de la communauté bactérienne. Le PAO prédominant était vraisemblablement *Dechloromonas.*

Afin d'optimiser l'élimination biologique des nutriments par AGS-SBR, différentes stratégies d'aération ont été testées. Il a été observé que des stratégies d'aération optimisées pouvaient considérablement améliorer l'efficacité de l'élimination du N dans les systèmes limités en DCO. Les stratégies favorisant une alternance de la nitrification et la dénitrification (AND) étaient significativement plus efficaces que les stratégies à nitrification et dénitrification simultané (SND). L'introduction de phases à faible DO (ou même anoxiques) à un stade précoce de l'aération favorise très probablement la dénitrification par PAO. Cela permet d'économiser de la DCO. La stratégie la plus efficace concernant l'élimination du N était l'aération intermittente.

Finalement, pour encore améliorer le BNR dans des systèmes limités en DCO, l'élimination du N par le nitrite a été étudiée. Il a été montré qu'un contrôle automatique de la durée de la phase d'aération combiné avec une aération intermittente, était un moyen efficace pour atteindre une élimination par le nitrite et des taux d'élimination du N allant jusqu'à 95% ont été obtenues. A 20 ° C, il était possible d'installer une élimination par le nitrite en 20 - 60 jours. A 15 ° C, il était possible de maintenir l'élimination par le nitrite. Cependant, il n'était pas possible de supprimer complètement l'oxydation des nitrites, même si la population de bactéries oxydant le nitrite (NOB) pouvait être fortement réduite.

En conclusion, cette thèse a montré le potentiel des conditions d'opération pour optimiser le démarrage de l'AGS-SBR et l'élimination biologique des nutriments. Une stratégie de démarrage efficace à partir de boues d'inoculation floculant a été développée. De plus, il a été démontré que l'élimination du N dans des systèmes limités en DCO peut être améliorée grâce au contrôle de l'aération, soit en favorisant la dénitrification par des PAO soit par l'élimination du N par le nitrite.

Mots-clés: traitement des eaux usées; boues granulaires aérobies; élimination biologique des nutriments; réacteur à alimentation séquentielle; stratégies d'aération.

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Table of abbreviations

AGS	Aerobic granular sludge
AND	Alternating nitrification and denitrification
ANOVA	Analysis of variance
AOB	Ammonium-oxidizing bacteria
AS	Activated sludge
ATU	Allylthiourea
BCS	Bacterial community structure
BNR	Biological nutrient removal
bp	base pairs
CFR	Continuous flow reactor
CH_4	Methane
CO_2	Carbon dioxide
COD	Chemical oxygen demand
DGAO	Denitrifying glycogen-accumulating organism
DGGE	Denaturing gradient gel electrophoresis
DO	Dissolved oxygen
DPAO	Denitrifying polyphosphate-accumulating organism
EBPR	Enhanced biological phosphorus removal
EPS	Exopolysaccharides
FA	Free ammonia
FISH	Fluorescence *in-situ* hybridization
FNA	Free nitrous acid
GAO	Glycogen-accumulating organism
HPLC	High performance liquid chromatography
HRT	Hydraulic retention time
MFA	Multiple factor analysis
MFC	Mass flow controller
N_2	Nitrogen gas
N_2O	Nitrous oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
NOB	Nitrite-oxidizing bacteria

NH_4^+	Ammonium
OHO	Ordinary heterotrophic organisms
OLR	Organic load rate
ORP	Oxidation–reduction potential
OUR	Oxygen uptake rate
PAO	Polyphosphate-accumulating organism
PCR	Polymerase chain reaction
PHA	Polyhydroxyalkanoates
PID	Proportional-Integral-Derivative
PO_4^{3-}	Phosphate
qPCR	Quantitative polymerase chain reaction
SBR	Sequencing batch reactor
SHARON	Single reactor high ammonia removal over nitrite
SND	Simultaneous nitrification and denitrification
SRT	Sludge retention time
SVI	Sludge volume index
TSS	Total suspended solids
T-RF	Terminal-restriction fragment
T-RFLP	Terminal-restriction fragment length polymorphism
VFA	Volatile fatty acids
VSS	Volatile suspended solids
WWTP	Wastewater treatment plant

General Introduction

1

1. General Introduction

During decades the standard for biological wastewater treatment has been conventional continuous activated sludge (AS) technology. Nowadays a promising new technology is coming up: aerobic granular sludge (AGS) in sequencing batch reactors (SBR). This technology is classified by experts as the most important process break-through in decades (Inocencio et al. 2013). First reported in the late nineties (Morgenroth et al. 1997; Beun et al. 1999; Peng et al. 1999), AGS-SBR is now in the stage of application. The first municipal full-scale plant with Nereda® aerobic granular sludge technology was put into operation two years ago and the results exceed all expectations. High biological removal performances for organic carbon (hereafter referred to as chemical oxygen demand, COD), nitrogen (N) and phosphorus (P) have been observed and the process has demonstrated remarkable robustness and stability under varying influent load conditions and extreme pH fluctuations (Inocencio et al. 2013). Furthermore, the two years of operation of this first full-scale plant proofed that aerobic granular sludge (AGS) technology has enormous advantages over classical continuous activated sludge technology, such as space savings (footprint just up to 25% of conventional technologies), investment and operational cost savings >25%, and lower energy consumption. Therefore, aerobic granular sludge technology is regarded as the upcoming new standard for biological treatment of domestic and industrial wastewater (Inocencio et al. 2013).

1.1 Background of wastewater treatment

The beginning of modern wastewater treatment is generally stated to be the 19th century. With the industrialization, urban areas grew considerably and the fast increase of population naturally caused an important rise in wastewater. At the same time, several epidemics like cholera and typhoid appeared in the cities and soon the bad sanitary conditions were identified to be at the source of these diseases. Moreover, the discharge of untreated wastewater to rivers and lakes was recognized of having a harmful effect on aquatic life. In a first time, the main problems were the accumulation of sludge and the decrease of the dissolved oxygen (DO) concentration, due to the discharge of oxygen-demanding pollutants such as organic compounds and ammonium. Thus, early wastewater treatment systems were mainly designed to retain suspended solids, to remove organic matter and sometimes to oxidize ammonium. With continuing industrialization and population growth, the increased discharge of nutrients such as nitrogen and phosphorus to rivers and lakes led to a new problem: eutrophication. The high availability of nutrients led to excessive growth of plants and

algae. Hence, in addition to the removal of suspended solids and carbonaceous organic matter, the removal of N and P became an important issue in wastewater treatment.

Including industries, the average water use per capita in Switzerland in 2010 was 325 L per day (http://www.trinkwasser.ch/dt/frameset.htm?html/wasserversorgung/wvs_wasserabgabe_03.htm~mainFrame, Accessed 2013-04-13). Hence, huge volumes of wastewater are produced by our society and have to be treated by wastewater treatment plants (WWTP). The main N and P pollutants present in this wastewater are ammonium (NH_4^+) and orthophosphate (PO_4^{3-}), respectively. The concentration of carbonaceous organic matter is usually expressed as chemical oxygen demand (COD) equivalents and the particulate matter as total suspended solids (TSS). Typical pollutant concentrations in Switzerland for urban wastewater are 200 gTSS m^{-3}, 350 gCOD m^{-3}, 20 gN-NH_4^+ m^{-3} and 6 gP-PO_4^{3-} m^{-3}. Another 10 gN m^{-3} is present as organic nitrogen.

1.1.1 Wastewater treatment plant

A classical WWTP includes pre-, primary, secondary and sometimes tertiary treatment (Figure 1-1). The pretreatment consists mainly of the screening of the wastewater to remove large objects such as trash, tree limbs, etc. and the removal of grits. The primary treatment is used to settle part of the suspended solids and often also to skim off grease and oil swimming on top of the wastewater. It is done in so-called primary clarifiers. In the secondary treatment, the biological treatment of the wastewater takes place. Microorganisms degrade, accumulate or transform pollutants to remove them from the water. Depending on the design of the biological treatment, only organic matter or also N and P are removed biologically. The biological treatment is followed by a secondary clarification. Part of the settled sludge from these secondary clarifiers is recirculated to maintain the concentration of microorganism in the biological treatment section constant. Sometimes a tertiary treatment is done, such as for example chemical removal of phosphorus.

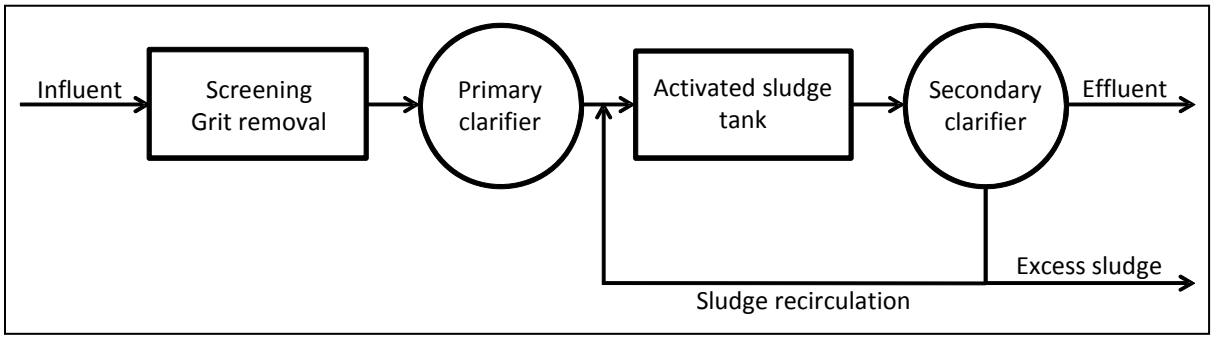

Figure 1-1: Schematic and simplified overview of WWTP.

The biological treatment is a key part of the WWTP and has a strong impact on the effluent water quality. Flocculent activated sludge in continuous flow reactors (CFR) is still the most widespread biological wastewater treatment process nowadays in industrialized countries. The basic principles of the activated sludge technology have first been published almost 100 years ago by Arden and Lockett (1914). They observed aerobic degradation of carbonaceous and nitrogenous pollutants by microorganisms in a fill-and-draw experiment with a glass bottle. In the following years, the technology was transformed into a continuous process with an aerated tank, a settling tank, and sludge recirculation. The first full-scale activated sludge plants were put into operation in the 1920's. Nowadays part of the tank is often unaerated, to allow anaerobic processes to take place. Despite the continuous improvement of conventional activated sludge technology over time, there is a clear trend towards new technologies for biological wastewater treatment nowadays. In view of more stringent water quality standards and to reduce costs, more compact solutions are required.

1.1.2 Biological nutrient removal

1.1.2.1 N-removal

N-removal is a two-step process with first nitrification under aerobic conditions followed by denitrification under anaerobic conditions and requiring COD (Figure 1-2). In nitrification, ammonium is first oxidized to nitrite (NO_2^-) and then to nitrate (NO_3^-). The first nitrification step is done by ammonium-oxidizing bacteria (AOB) such as *Nitrosomonas*, the second nitrification step by nitrite-oxidizing bacteria (NOB) such as *Nitrospira* or *Nitrobacter*. Nitrifiers are autotrophs growing with CO_2 as carbon source. Denitrification takes place in absence of oxygen (O_2) using nitrate or nitrite (NO_x^-) as electron acceptor. Absence of O_2 but presence of NO_x^- are called anoxic conditions. In denitrification, NO_x^- is reduced to inoffensive nitrogen gas (N_2) by heterotrophic denitrifying bacteria. The reduction steps of denitrification are $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$ (Figure 1-2). The denitrification process requires important amounts of COD.

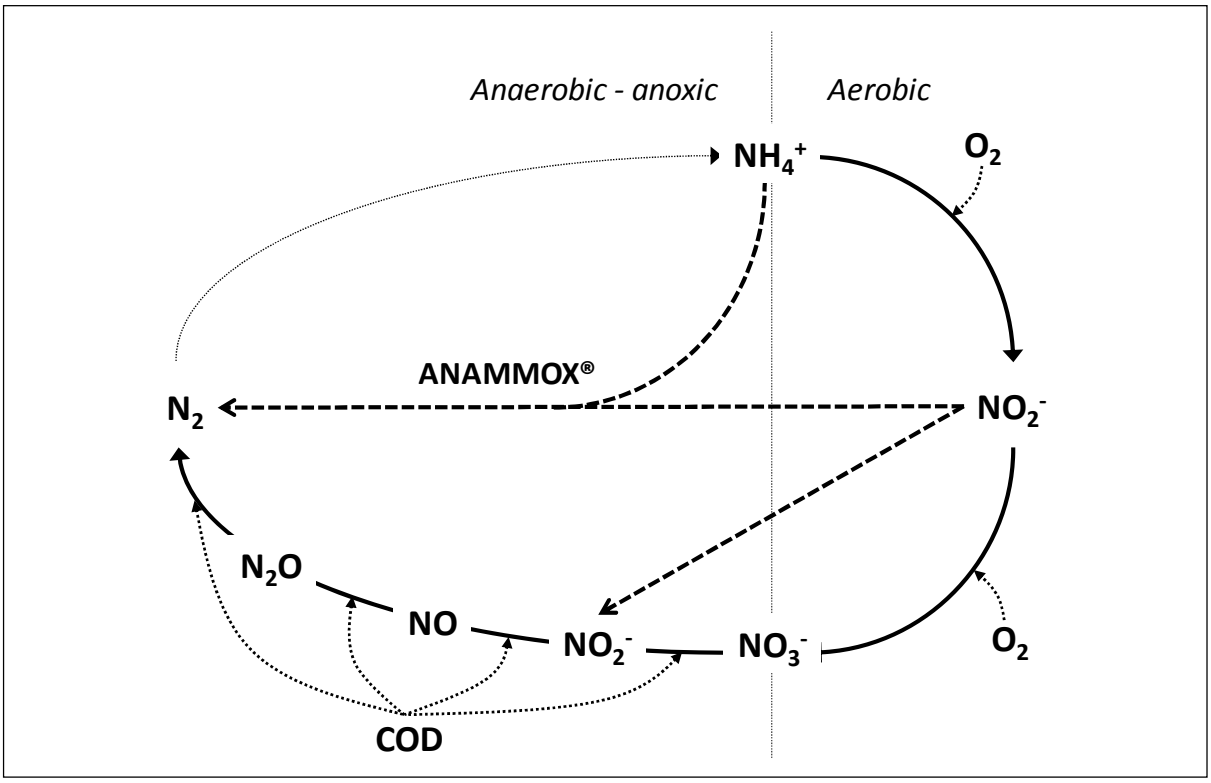

Figure 1-2: Simplified nitrogen cycle showing nitrification and denitrification. The dashed lines indicate the nitrite pathway and the ANAMMOX process.

As nitrite is an intermediate in nitrification and denitrification, there is the possibility to shortcut the N cycle. Nitrification can be stopped after the oxidation of ammonium to nitrite, which is subsequently denitrified without being oxidized to nitrate (Figure 1-2). This process shortcut is called nitrite pathway. The oxidation of ammonium to only nitrite is called nitritation or partial nitrification, in contrast to total nitrification. Complete or incomplete nitrification indicates if all ammonium is oxidized or not. The nitrite pathway has the advantage that 25% less oxygen is needed (Equation 1-1; Equation 1-2) and about 40% less COD (Equation 1-3; Equation 1-4).

Nitrification Step 1: $NH_4^+ + 1.5O_2 \rightarrow NO_2^- + H_2O + 2H^+$ **Equation 1-1**

Nitrification Step 2: $NO_2^- + 0.5O_2 \rightarrow NO_3^-$ **Equation 1-2**

Nitrate denitrification: $6NO_3^- + 5CH_3OH + CO_2 \rightarrow 3N_2 + 6HCO_3^- + 7H_2$ **Equation 1-3**

Nitrite denitrification: $6NO_2^- + 3CH_3OH + 3CO_2 \rightarrow 3N_2 + 6HCO_3^- + 3H_2$ **Equation 1-4**

Other advantages of N-removal over nitrite are a lower biomass production and a higher denitrification rate (Turk and Mavinic 1987). To achieve the nitrite pathway, conditions have to be

created such that the second nitrification step from nitrite to nitrate is not occurring which means that conditions are unfavorable for nitrite-oxidizing bacteria, but favorable for ammonium-oxidizing bacteria. A further possibility of N-removal is anaerobic ammonium oxidation ANAMMOX® process (Figure 1-2). In this process, ammonium and nitrite are converted directly into N_2, and the savings in aeration and COD are even higher than with N-removal over nitrite.

Efficient N-removal is a main challenge in wastewater treatment because of the fact, that denitrification takes place after nitrification, but requires COD. Nitrification is achieved under aerobic conditions; hence COD is consumed by fast-growing aerobic heterotrophic organisms. An often used solution is the so-called pre-denitrification, where the required COD for denitrification is provided by the influent wastewater. In CFR an anoxic zone is added in front of the aeration tank and water from the end of the aeration tank is recirculated to this anoxic zone (Ludzack and Ettinger 1962), where denitrification can take place. However, complete denitrification is not possible with pre-denitrification, since a fraction of the NO_x^- produced in the aerobic zone is discharged without being recirculated. To achieve very low NO_x^- concentrations in the effluent, more complex WWTP designs are required, like for example the Bardenpho® system (Barnard 1975). Another solution is the supply of an external carbon source, which is costly.

With the rising number of WWTP doing biological N-removal, the undesired emission of nitrous oxide (N_2O) is gaining more and more attention. N_2O is present in the atmosphere in a relatively low concentration of 325 ppb, but it has a life time of 319 years, which makes it the fourth most important greenhouse gas after water vapor, carbon dioxide (CO_2) and methane (CH_4) (Forster et al. 2007). Even more worrying than the role of N_2O as greenhouse gas is its role concerning the ozone hole, hence it is the dominating ozone-depleting gas nowadays (Ravishankara et al. 2009). In wastewater treatment, N_2O can be produced during nitrification and denitrification (Goreau et al. 1980; Lipschultz et al. 1981; Freitag and Bock 1990). Known trigger factors for N_2O production are low DO, low COD/N ratio, and the presence of nitrite (Kampschreur et al. 2009).

1.1.2.2 P-removal

Enhanced biological phosphorus removal (EBPR) has the advantage, compared to chemical P-removal, that no chemical precipitation agents have to be added and less excess sludge is produced. The microorganisms involved in this process are called polyphosphate-accumulating organisms (PAO). Cultivated under alternated anaerobic-aerobic conditions, these bacteria are able to store large amounts of phosphate intracellularly (Comeau et al. 1986). PAO can take up dissolved organic matter, such as volatile fatty acids (VFA) under anaerobic conditions and store intracellularly as

polyhydroxyalkanoates (PHA) (Figure 1-3A). The energy for this metabolism is provided by the hydrolysis of intracellularly stored polyphosphate, hence orthophosphate is released. Under aerobic conditions, the stored PHA is used for growth and provides the energy to replenish the poly-P stocks (Figure 1-3B). Therefore, under aerobic conditions orthophosphate is taken up. The accumulated poly-P is finally removed by purging sludge.

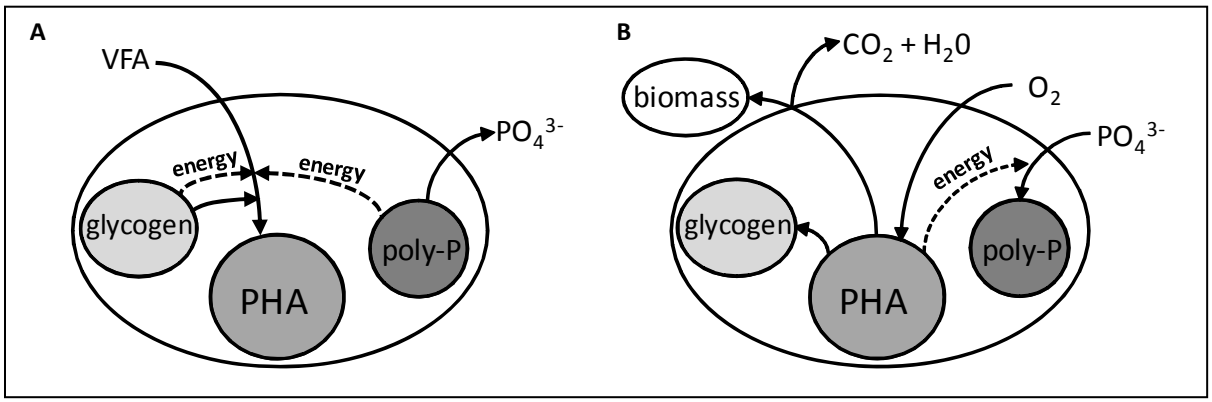

Figure 1-3: Schematic metabolism of PAO under **(A)** anaerobic and **(B)** aerobic conditions.

The capacity to take up VFA under anaerobic conditions gives PAO a selective advantage over ordinary heterotrophic organisms (OHO). However, glycogen-accumulating organisms (GAO) have the same capacity to take up VFA under aerobic conditions (Figure 1-4A) and are therefore competitors of PAO (Cech and Hartman 1990). As they do not accumulate P, they are undesired in biological P-removal systems. Several parameters have been reported to have an influence on the PAO-GAO competition. PAO are favored compared to GAO with high pH (Smolders et al. 1994; Lopez-Vazquez et al. 2009) and low temperature (Whang and Park 2002; Seviour et al. 2003; Lopez-Vazquez et al. 2007). Besides pH and temperature, the carbon source has also an influence on the PAO-GAO competition (Oehmen et al. 2004; Pijuan et al. 2004; Gonzalez-Gil and Holliger 2011).

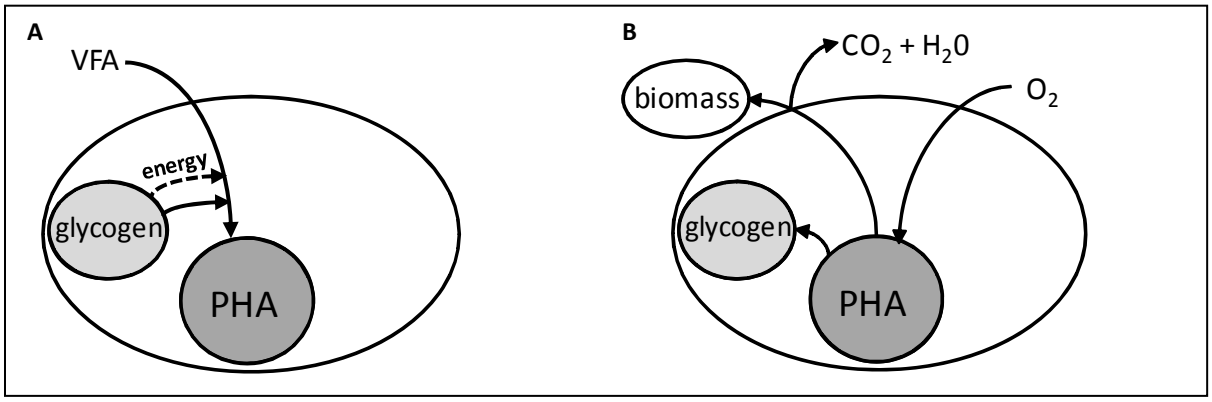

Figure 1-4: Schematic metabolism of GAO under **(A)** anaerobic and **(B)** aerobic conditions.

Some PAO are able to use NO_x^- as electron acceptor in absence of oxygen (Kuba et al. 1993; Kerrn-Jespersen et al. 1994; Meinhold et al. 1999) (Figure 1-5B). These organisms are called denitrifying PAO (DPAO). Denitrification by PAO can save COD, since the same PHA stock is used for P-removal and denitrification (Kuba et al. 1996). COD is often the limiting parameter in combined N- and P-removal systems (Kuba et al. 1996; Keller et al. 1997). However, COD is only saved, if P-removal and denitrification occur simultaneously with DPAO. In absence of orthophosphate, PAO can still act as denitrifiers, but there is no link to P-removal.

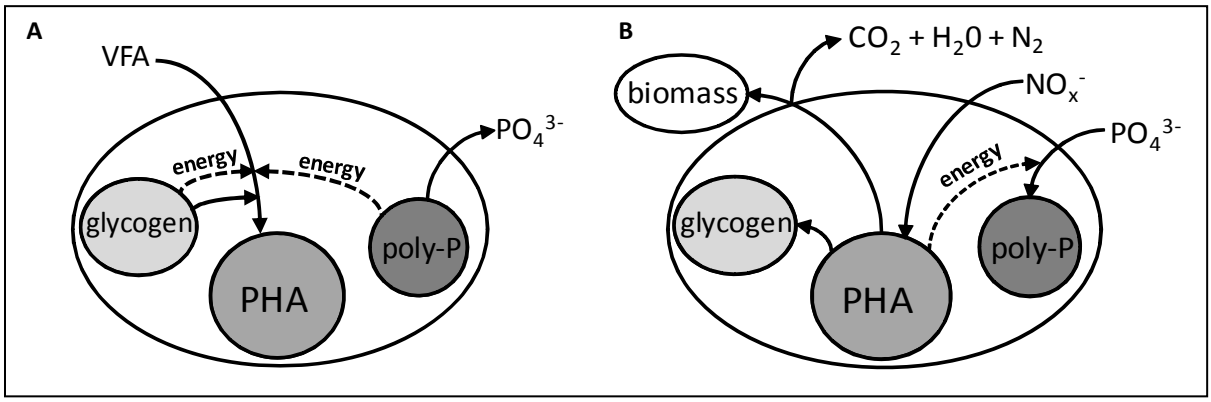

Figure 1-5: Schematic metabolism of DPAO under **(A)** anaerobic and **(B)** anoxic conditions.

1.1.3 Sequencing batch reactor

Nowadays more and more WWTP use SBR instead of CFR for the biological treatment. SBR is a fill-and-draw technology. A SBR cycle contains usually a fill phase, a reaction phase, a settling phase, a withdrawal phase and sometimes an idle phase. The separation of processes is time- rather than space-oriented like in CFR (Morgenroth and Wilderer 1998). In general, several SBR are run in parallel, so that always one reactor is in the fill phase and the wastewater does not have to be stored. The first activated sludge experiment by Ardern and Lockett (1914) was actually run in sequencing batch mode. For technical reasons this technology was given up soon after and only rediscovered in the 1970's (Irvine and Busch 1979). In the mentioned publication it has been concluded that the periodic nature of SBR expands the spectrum of treatment capabilities compared to CFR. Furthermore, microprocessors have been suggested as possible solution to handle the more complex operation control.

Nowadays, handling of operation control is not a problem anymore and an increasing number of WWTP use SBR instead of CFR as activated sludge tanks. SBR offer higher operation flexibility. In CFR the tank volume and design defines the hydraulic retention time (HRT) and the duration of the different process phases. With SBRs the operator has the control over the parameter “time” and the

duration of the process phases can be controlled by *in-situ* measurements. Moreover, with SBRs the settling phase is integrated and no supplementary clarifier is needed to treat the effluent. Besides the more complex operation, a drawback of SBR is the lower flexibility with respect to sludge recirculation (Morgenroth and Wilderer 1998).

1.2 Aerobic granular sludge in sequencing batch reactors

The idea to cultivate aerobic granular biofilm without carrier material came up in the late nineties, based on studies on biofilm structure and formation (van Loosdrecht et al. 1995; van Loosdrecht et al. 1997). In contrast to conventional activated sludge systems where bacteria form flocs, in aerobic granular systems, bacteria aggregate to compact granules due to the shear stress from aeration. Typical diameters of granules reported in literature are between 0.5-5 mm (Beun et al. 1999; Etterer and Wilderer 2001; de Kreuk and van Loosdrecht 2004; Yilmaz et al. 2007). Granular sludge has much better settling characteristics than conventional flocculent activated sludge. Hence, the separation of the biomass and the treated water is easier. Higher biomass concentrations can be achieved and higher volumetric loads applied (Beun et al. 2000; Etterer and Wilderer 2001), resulting in more compact WWTP. Moreover, no secondary clarification is needed (Beun et al. 1999). In contrast to moving bed biofilm reactors, no carrier material is applied, and compared to conventional attached biofilm technologies such as trickling filters or rotating biological contactors, AGS offers a higher specific contact surface between water and biofilm (Di Iaconi et al. 2005).

In the first aerobic granular sludge workshop 2004 in Munich, the following definition for aerobic granular sludge was proposed (de Kreuk et al. 2007a):

> Granules making up aerobic granular activated sludge are to be understood as aggregates of microbial origin, which do not coagulate under reduced hydrodynamic shear, and which settle significantly faster than activated sludge flocs.

In the second workshop on aerobic granular sludge in 2006, this definition has further been specified, resulting in the following statements (de Kreuk et al. 2007a):

(1) Aggregates of microbial origin: speaking of granular activated sludge in the statement implies that aerobic granules need to contain active microorganisms and cannot only consist of components of microbial origin (as proteins, exopolysaccharides (EPS), etc.). The microbial population in aerobic granular sludge are to be expected more or less similar to the ones in activated sludge and or biofilms, thus there is no need to describe specific groups of

microorganisms in the definition. Furthermore, this part implies that no carrier material is intentionally involved or added; the aggregate is formed without the dosage of such carrier material.

(2) No coagulation under reduced hydrodynamic shear: this part describes the difference in behavior between activated sludge and aerobic granular sludge. Activated sludge flocs tend to coagulate when they settle (when liquid-sludge mixture is not aerated or stirred), whilst granules do not coagulate and settle as separate units.

(3) Granules settle significantly faster than activated sludge flocs: this means that the sludge volume index after 10 min of settling (SVI_{10}) in combination with SVI_{30} (settling after 30 min) should be used for characterizing the settleability of granular activated sludge as was suggested by Schwarzenbeck et al. (2004). The difference between the SVI_{10} and SVI_{30} value gives an excellent indication about the granule formation and indicates the extent of thickening after settling.

(4) The minimum size of the granules should be such that the biomass still fulfills the previous condition. This minimum size was set to 0.2 mm, which was decided based on measurements in the past. This limit could be adjusted per case/granule type, as long as the other demands of the definition hold.

(5) Sieving is considered a proper method to harvest granules from activated sludge tanks or from aerobic granule reactors, which also determines certain strength of the required biomass matrix.

1.2.1 Formation of aerobic granular sludge

For the formation of AGS, different operating parameters have been identified to play a crucial role, such as a short settling time for the selection of fast settling particles (Beun et al. 1999; McSwain et al. 2004; Sheng et al. 2010), high shear stress from aeration (Liu and Tay 2002; Tay et al. 2004), and anaerobic feeding to favor the proliferation of slow growing microorganisms like PAO or GAO (de Kreuk and van Loosdrecht 2004). Under favorable conditions, the transformation of flocculent to granular sludge has been reported to take two to three weeks in lab-scale reactors fed with synthetic wastewater (Li and Li 2009; Ebrahimi et al. 2010), domestic sewage (de Kreuk and van Loosdrecht 2006), and industrial wastewater (Arrojo et al. 2004). However, in general, only COD removal was observed in a first time. High biomass washout during the first days of startup has been detected as the main reason for problems with nutrient removal in an early stage (Pijuan et al. 2011; Weissbrodt

et al. 2012), and strategies like the addition of divalent ions such as Ca^{2+} and Mg^{2+} to the wastewater (Jiang et al. 2003; Li et al. 2009; Liu et al. 2010) or crushed granules (Pijuan et al. 2011; Coma et al. 2012) have been proposed to reduce the sludge loss.

The precise formation mechanism of granular sludge is not yet completely elucidated. Several studies showed that EPS play a key role as "microbial glue" for the formation and stability of AGS (Tay et al. 2001; Liu et al. 2004; Wang et al. 2006; Seviour et al. 2009). A study combining confocal laser scanning microscopy and fluorescence *in-situ* hybridization (FISH) reported, that stalked ciliated protozoa act as backbone for bacterial growth in an early-stage of granulation (Weber et al. 2007). Moreover, based on the observation of physical differences between granules from the same reactor, it has been hypothesized that two different granulation mechanisms exist: microcolony outgrowth and microcolony aggregation (Barr et al. 2010). In recent years, a rising number of studies investigated the bacterial community composition in AGS and their evolution during the startup (Li et al. 2008; Ebrahimi et al. 2010; Sheng et al. 2010; Gonzalez-Gil and Holliger 2011; Weissbrodt et al. 2012).

1.2.2 Biological nutrient removal with AGS

Concerning the biological nutrient removal (BNR), different authors reported the feasibility of simultaneous COD-, N- and P-removal with AGS-SBR (Zeng et al. 2003; de Kreuk et al. 2005; Yilmaz et al. 2007). The proliferation of PAO in AGS has been achieved with anaerobic feed conditions (Zeng et al. 2003; de Kreuk and van Loosdrecht 2004). During this anaerobic feeding phase P is released while COD is consumed, whereas in the subsequent aeration phase P is taken up. For N-removal, simultaneous nitrification and denitrification (SND) has been observed with AGS (Beun et al. 2001). The penetration depth of oxygen in biofilm is limited due to the microbial activity (Figure 1-6). Hence, in biofilms of a certain thickness, anaerobic zones allowing denitrification are present deeper in the biofilm, while nitrification takes place closer to the surface of the biofilm (Münch et al. 1996; Keller et al. 1997). The main parameters governing SND in granular sludge are the concentration of dissolved oxygen (DO) in the bulk liquid (Beun et al. 2001; de Kreuk et al. 2005; Mosquera-Corral et al. 2005), the granule size (de Kreuk et al. 2005), and the microbial activity (Meyer et al. 2005; de Kreuk et al. 2007b; Yilmaz et al. 2007). The required COD for denitrification is present in PAO and GAO in form of PHA. It has been observed that PAO were more abundant in the outer layers, whereas GAO in the inner part of the AGS (Lemaire et al. 2008). Hence, denitrifying GAO (DGAO) are presumably the main denitrifiers with SND (Figure 1-6). The anaerobic storage of COD by PAO/GAO in the inner part of aerobic granules offers an elegant solution to the problem of COD availability for

denitrification after aeration. The COD which is stored in the inner anoxic part of granules is not available for strictly aerobic heterotrophs. Hence, it remains available for denitrification even during or after the aeration phase. Very recently the integration of anammox into the AGS process has been suggested to further optimize the N-removal (Winkler et al. 2012).

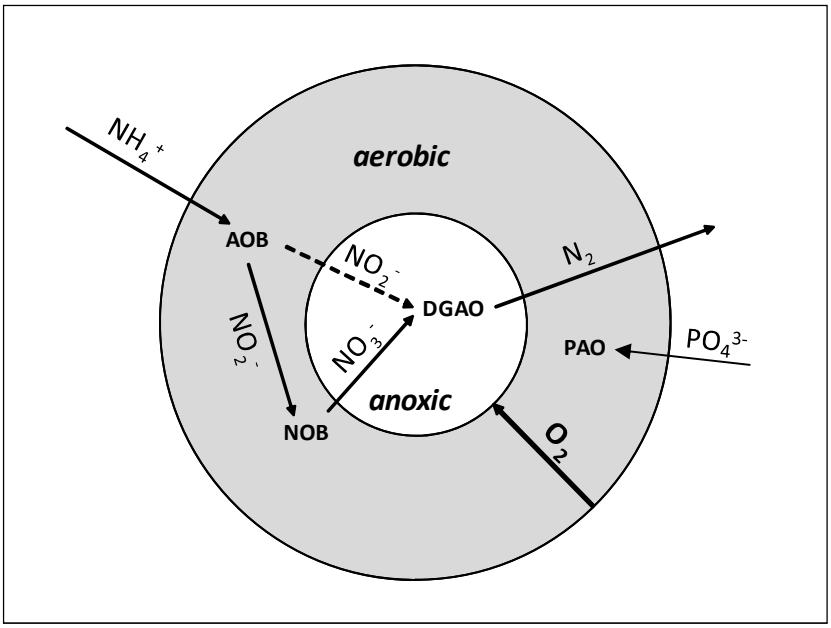

Figure 1-6: Scheme of nutrient removal in aerobic granules during the aeration phase.

1.3 Objectives and approach of the thesis

The startup of AGS-SBR with good nutrient removal efficiencies has often been reported to take a long time. Strategies are required to achieve rapid formation of granular sludge, with the capacity of N- and P-removal. After presenting in Chapter 2 the Materials and Methods common for the whole thesis, the subject of Chapter 3 is the impact of different operation conditions on the startup duration. The influence of different operation parameters was investigated in parallel, with a multifactorial experimental design. The aim was to screen the relevant parameters and based on that, optimize the startup strategy. In Chapter 4, the bacterial community compositions during the startup experiments were studied in order to understand the correlations between operation conditions and bacterial community dynamics.

In Chapter 5, the influence of different aeration strategies on N- and P-removal was investigated. With combined denitrification and P-removal, the BNR performances are often unsatisfying due to limiting COD availability. The study focused on the question if aeration strategies promoting alternating nitrification and denitrification (AND) offered advantages over simultaneous nitrification and denitrification (SND). Finally, in Chapter 6, a second possibility to overcome the problem of COD

limitations for BNR was studied, namely N-removal over nitrite. For this purpose, the possibility of the achievement and maintenance of the nitrite pathway by aeration control was investigated. Of special interest was the question, if such strategies were also applicable at temperatures lower than 20°C.

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Materials and Methods

2

2. Materials and Methods

2.1 Sequencing batch reactor set-up

Two sets of two slightly different lab scale bubble columns operated as sequencing batch reactors (SBR) have been used for the experiments of this thesis (Figure 2-1). The design of the two SBR sets is described in (Ebrahimi et al. 2010) and (Weissbrodt et al. 2012), respectively. The first set of reactors had an internal diameter of 5.2 cm and a working volume of 2.4 L, whereas the second set had an internal diameter of 6 cm and a working volume of 3.5 L. The effluent withdrawal point was positioned at a height of 54 and 61 cm, respectively, resulting in equal volumetric exchange ratios per cycle of 50%.

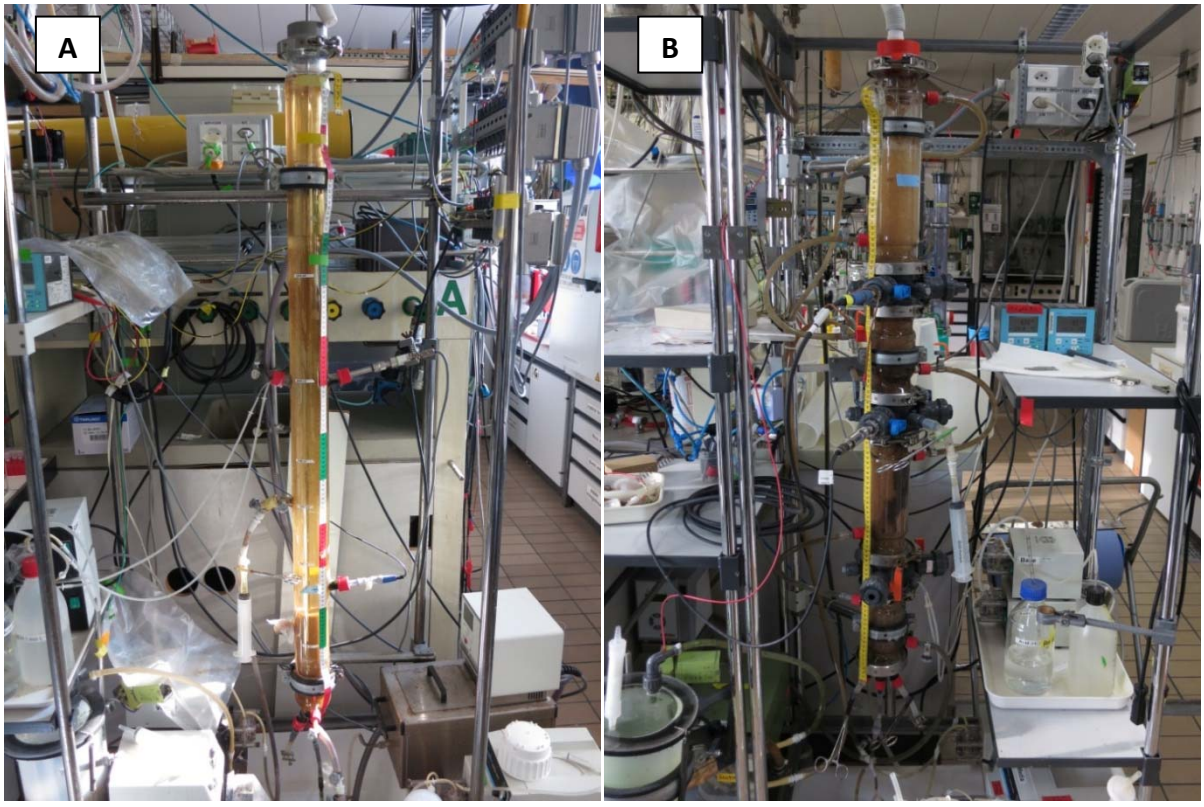

Figure 2-1: Pictures of the SBRs with a working volume of **(A)** 2.4 L and **(B)** 3.5 L.

The two reactors with a working volume of 2.4 L were made of a single glass column with three apertures for the installation of probes. The two reactors with a working volume of 3.5 L had a modular composition. They were made by glass tube sections of different lengths and PVC sections joining the glass pieces. Each PVC section had six apertures for the installation of probes or for sampling (Figure 2-2). The apertures were equipped with a ball stop valve allowing putting in or

removing probes, while the reactors were running. All glass parts of the SBRs had a double wall for temperature control. The water circulated in the double wall was heated up by thermostats (Haake, Julabo, Huber, all Germany) to the required temperatures.

Figure 2-2: PVC section with apertures for the installation of probes.

The SBRs were fed from the bottom in plug-flow mode with peristaltic pumps (Masterflex, Germany). The same type of pumps was used for the withdrawal of the effluent. The effluent from the reactor was discharged to a secondary clarifier (Figure 2-3), which allowed estimating the washout of biomass. The secondary clarifier had a low level volume of about 2 L, and the outlet was equipped with an electrical valve. A timer controlled the opening and closing of this valve. After the withdrawal of the effluent to the secondary clarifier, the valve remained closed during 2.5 hours for the settling of the suspended solids. Then, the valve was opened during 15 min for the discharge of the supernatant to the sewer.

Figure 2-3: Secondary clarifier with timer.

The SBRs were aerated with diaphragm gas pumps (KNF Neuberger, Switzerland) creating a constant gas up-flow rate of 3.6 L min^{-1} and porous diffusers making small bubbles. For an easier regulation of the dissolved oxygen (DO) concentration the headspace gas was recirculated as proposed by Mosquera-Corral et al. (2005). The DO was adjusted by the addition of air or N_2, controlled by mass flow controllers (MFC) (Figure 2-4). During the filling, the withdrawal and when air or N_2 was added, the excess gas valve was open in order to keep the pressure in the reactor constant. The pH was regulated during the mixed phases of the SBR cycle by the injection of 1 mol L^{-1} NaOH and HCl solutions.

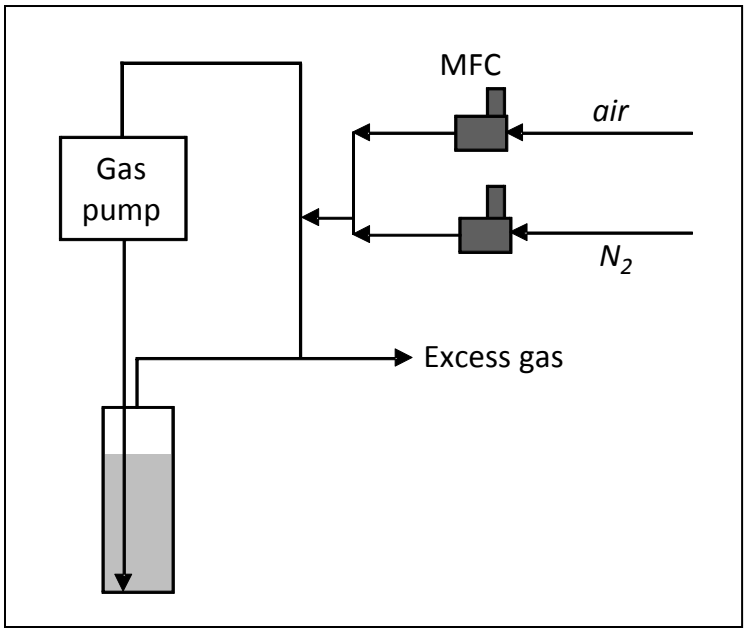

Figure 2-4: Scheme of aeration setup.

2.2 SBR operation

The SBR cycle was in general composed by four phases: (i) anaerobic feeding in plug-flow mode, (ii) aeration, (iii) settling, and (iv) withdrawal of the effluent. The cycle length varied slightly in the different studies but was usually around 3 hours, with 60 min of feeding, 112 - 120 min of aeration, 3-5 min of settling, and 5 min of withdrawal (Figure 2-5). It resulted in a hydraulic retention time (HRT) of around 6 hours. In some experiments, an aerobic mixing phase was added after the feeding phase. The detailed SBR operation schedule for each experiment is specified in the materials and methods section of the different chapters.

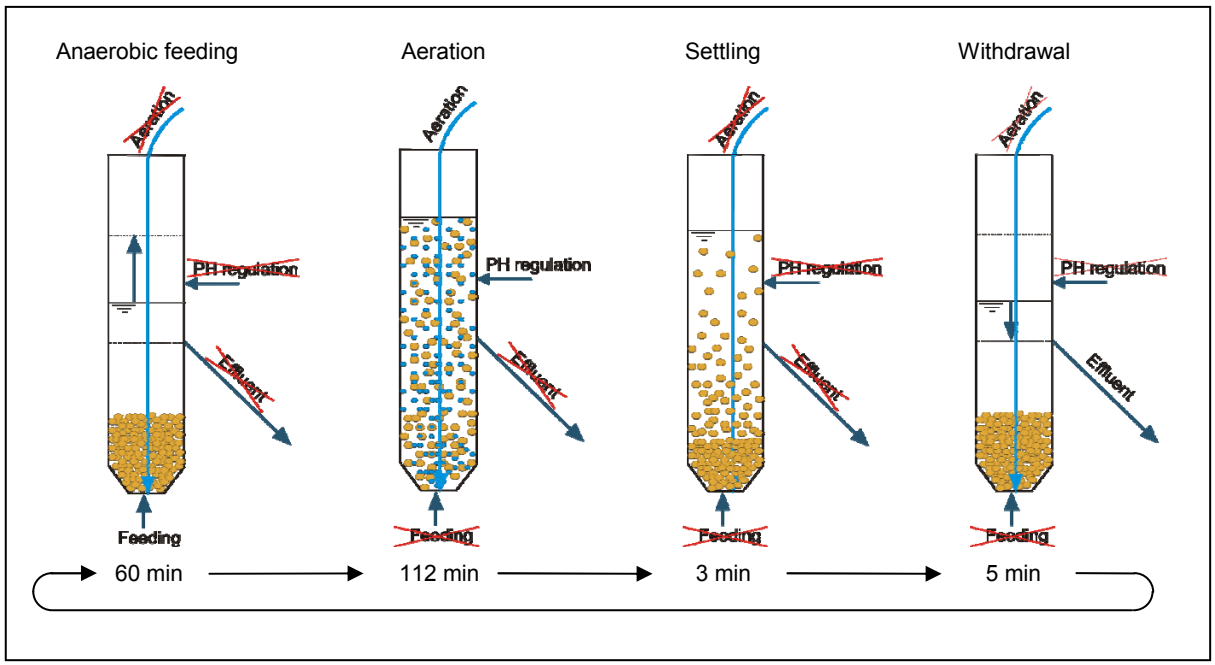

Figure 2-5: Typical SBR cycle.

2.3 SBR automation and data acquisition

The SBRs were connected to a computer, through an electrical control board (Siemens, Germany) and an output/input converter (Wago, Switzerland). DaqFactory software was used for system control and data acquisition (https://www.azeotech.com/daqfactory.php, Accessed 2013-04-26). This software steered the switch "on" and "off" of the pumps and the regulation of the DO. Moreover, it acquired the data collected by the different probes and the data relative to the supply of air or N_2 for the DO control. The data acquisition rate was one point per second for all parameters and the acquired data was stored in excel sheets. Matlab (http://www.mathworks.ch/products/matlab/, Accessed 2013-04-26) was used to post-analyze the large amount of acquired data, to assess the stability of the regulation, and to detect drifts in the measurements or technical problems.

2.4 DO and pH regulation

The DO was measured by an Ingold membrane electrodes (Mettler Toledo, Switzerland) connected to a signal amplifier (Endress + Hauser, Switzerland). The regulation of the DO was steered by DaqFactory software through proportional-integral-derivative (PID) controllers. If the measured DO was outside the range of ±3% to the setpoint, air or N_2 was injected. The parameters of the PID controller were adjusted by the Ziegler-Nichols method (Ziegler and Nichols 1942).

Similar to the DO, the pH was continuously measured by a glass electrode (Mettler Toledo, Switzerland) connected to a signal amplifiers (Endress + Hauser, Switzerland). During the mixed phases of the SBR cycle, the pH was controlled in a defined range by the injection of 1 mol L^{-1} NaOH and HCl solutions. The injection rate of these solutions was regulated by a PID control loop. Only the proportional term of the PID controller was used, in order to avoid signal accumulation during the unmixed phases. In contrast to the DO regulation, the pH regulation was directly done by the amplifier and not by DaqFactory. DaqFactory only acquired the pH.

2.5 Synthetic wastewater composition

The influent consisted of a mixture of two synthetic media and tap water adapted from de Kreuk et al. (2005). For the smaller SBR type 130 ml of each medium was diluted with 940 ml of tap water and for the bigger SBR type 190 ml of each medium with 1370 ml of tap water, resulting in the same influent concentrations. Medium A contained the carbon source, medium B the N and P sources and the trace elements. In most studies acetate and propionate was used as carbon source, in a ratio 1:1 according to COD equivalents. Medium A was composed of 4.28 g L^{-1} of sodium acetate trihydrate, 3.51 g L^{-1} of sodium propionate, 0.89 g L^{-1} of $MgSO_4 \cdot 7H_2O$, and 0.36 g L^{-1} of KCl, and medium B of 1.89 g L^{-1} of NH_4Cl, 0.73 g L^{-1} of K_2HPO_4, 0.23 g L^{-1} of KH_2PO_4, and 5 mL L^{-1} of a trace element solution as described by Vishniac and Santer (1957). This standard composition resulted in concentrations of about 400 mgCOD L^{-1}, 50 mgN-NH_4^+ L^{-1}, and 20 mgP-PO_4^{3-} L^{-1}. In some studies the media composition was adapted as described in the material and methods section of the concerned chapters. The tap water diluting the synthetic wastewater was flushed by N_2 in order to strip the oxygen.

2.6 Inoculation sludge

The conventional activated sludge used for the inoculation of the SBRs was taken from the aerated tank of the wastewater treatment plant in Uetendorf (ARA Thunersee, Switzerland). Besides COD- and N-removal, the wastewater treatment plant Thunersee does enhanced biological phosphorus removal (EBPR). Hence, all required organisms for COD-, N- and P-removal were present.

ARA Thunersee treats wastewater of 120'000 people, with average influent concentrations of COD, N and P are around 450 mgCOD L^{-1}, 50 mgN L^{-1} and 7 mgP L^{-1}. The biological tank volume is 6240m^3 and the average sludge retention times in the anaerobic, anoxic and aerobic tanks are around 4, 4 and 20 days, respectively.

2.7 Analytical methods

2.7.1 Analysis of soluble compounds and biomass

Volatile fatty acids (VFA), such as acetate and propionate, were measured by high performance liquid chromatography (HPLC). Inorganic cations and anions such as ammonium (NH_4^+), nitrate (NO_3^-), nitrite (NO_2^-), and orthophosphate (PO_4^{3-}) were measured by ion-chromatography. A detailed description of this analytical methods can be found in Gonzalez-Gil and Holliger (2011).

The method to measure total (TSS) and volatile suspended solids (VSS) was adapted from the standard methods (APHA 1998). An aliquot of 10 mL of well mixed sludge was taken from the middle of the SBR (effluent outlet) during the aeration, to have a sludge sample representative for the whole reactor. The filtration of the sludge sample as described in the standard methods was inapplicable due to the high biomass concentration, leading to rapid clogging of the filter. Hence, instead of filtering, the sludge sample was centrifuged and the supernatant removed. The remaining sludge pellet was put in a pre-weighted ceramic cup and dried at 105°C during at least 24 h as described in the standard methods (APHA 1998). For the calculation of the volatile suspended solids (VSS), the dried sludge sample was incinerated during 2 h at 550°C.

The sludge volume index (SVI) for granular sludge was calculated based on the TSS and the volume occupied by the sludge bed after 8 min. Schwarzenbeck et al. (2005) showed that with granular sludge the SVI_8 was comparable to the classical SVI_{30} used with flocculent sludge (Mohlman 1934).

The size and circularity of granules were measured by analyzing pictures of granular biomass with ImageJ software (http://rsb.info.nih.gov/ij/index.html, Accessed 2013-04-26). Diameters were calculated as circular equivalent diameters (Tijhuis et al. 1994) and only particles with a diameter > 0.2 mm were taken into account (de Kreuk et al. 2007). The biomass volume was calculated by the same principle, as sphere of same surface area.

2.7.2 Molecular analysis of bacterial communities

The bacterial community composition was assessed by terminal restriction fragment length polymorphism (T-RFLP) analysis targeting the v1-v3 hypervariable region of the *Eubacteria* 16S rRNA gene pool. The T-RFLP protocol was adapted from Rossi et al. (2009) with the modifications described in Weissbrodt et al. (2012).

2.7.3 Nitrous oxide emissions

Nitrous oxide (N_2O) emissions were measured by gas chromatography (HP 5890 series II, RTX-502.2 column, electron capture detector). The calibration curve was established by the injection of different volumes of a calibration gas (Messer, Switzerland) with a N_2O concentration of 200 ppm. To measure the N_2O emissions, discrete samples of the effluent gas were taken with a gas-tight syringe during the aeration phase and directly analyzed. Since the headspace gas was recirculated, the measured concentrations did not correspond to the real emissions. Experiments with tap water and a tracer gas were conducted under different aeration conditions, to determine the ratio between recirculated gas and gas leaving the system. Tests were conducted with air supplies of 0, 1, 2, and 3 L min^{-1} and the N_2O calibration gas was used as tracer gas (Figure 2-6A). Based on these results a correction model was established allowing calculating the actual N_2O emissions (Figure 2-6B). The detection limit of the gas chromatography measurement was approximately 1 ppm. However, due to the accumulation of gaseous compounds generated by gas recirculation loops, even much lower real gas concentrations could be calculated.

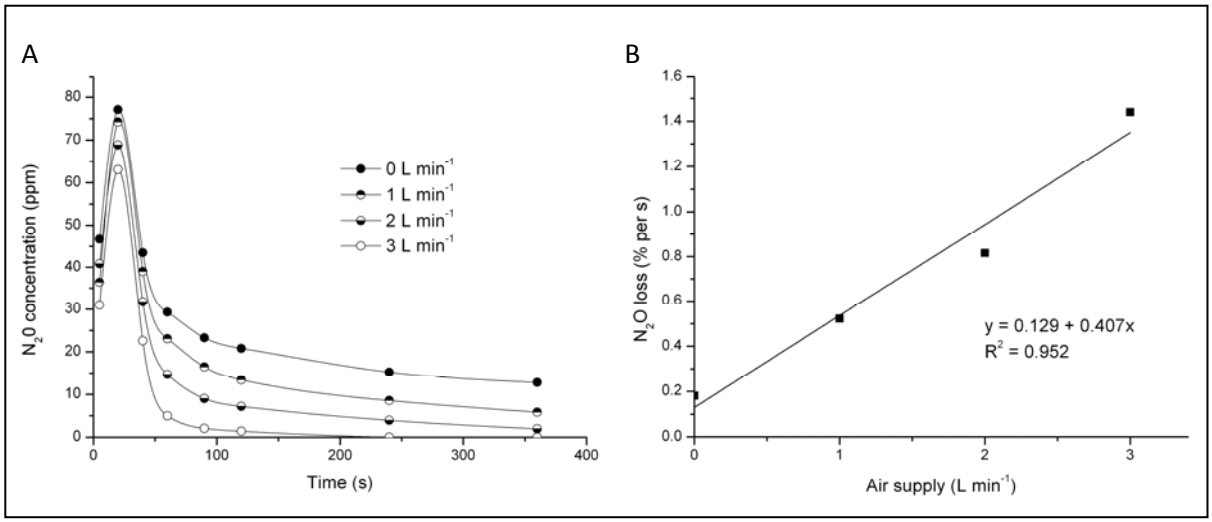

Figure 2-6: (A) Measured N_2O concentrations in the recirculation loop of the SBR in tests with different air supply rates. The SBR was filled with tab water and 300 mL of tracer gas at a concentration of 200 ppm N_2O was injected at time zero. **(B)** Correction curve for the calculation of N_2O emissions taking into account the effect of the gas recirculation. Relation between relative loss of N_2O per second and air supply.

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Optimized Startup Conditions

3

3. Optimization of operation conditions for the startup of aerobic granular sludge sequencing batch reactors

3.1 Introduction

The formation of aerobic granular sludge (AGS) in sequencing batch reactors (SBR) has first been reported in the late 1990s (Morgenroth et al. 1997; Beun et al. 1999; Dangcong et al. 1999). In a first time, most research on the startup of SBRs with AGS focused on parameters influencing the granulation process. A short settling time for the selection of fast settling particles (Beun et al. 1999; McSwain et al. 2004), high shear stress from aeration (Liu and Tay 2002; Tay et al. 2004), and anaerobic feeding to favor the proliferation of slow growing microorganisms like PAO or GAO (de Kreuk and van Loosdrecht 2004) have been pointed out as main conditions for the formation of dense and stable granular sludge. Under favorable conditions the transformation of flocculent into granular sludge has been reported to be in the order of two to three weeks in lab-scale reactors fed with synthetic wastewater (Li and Li 2009; Ebrahimi et al. 2010), domestic sewage (de Kreuk and van Loosdrecht 2006), and industrial wastewater (Arrojo et al. 2004). However, in all these studies only carbon removal occurred in an early stage. Much longer periods, in the order of 60-150 days, have been reported for the achievement of high N- and P-removal performances (de Kreuk et al. 2005; Xavier et al. 2007; Gonzalez-Gil and Holliger 2011). High biomass washout during the first days of startup has been detected as the main reason for problems with nutrient removal in an early stage (Pijuan et al. 2011; Weissbrodt et al. 2012). Hence, even if the seed sludge initially contained the required microorganisms for achieving N- and P-removal, this capacity got “lost” during the granulation process. The recovery of N- and P-removal is a lengthy process because nitrifyiers as well as PAO are slow growing organisms.

Different approaches have been investigated to avoid excessive biomass wash-out and maintain nutrient removal capacity during the startup. Cassidy and Belia (2005) have successfully started a SBR for the treatment of nutrient-rich abattoir wastewater with a high initial VSS concentration of 9.5 gVSS L^{-1} and a controlled stepwise reduction of the settling time in order to avoid excessive biomass washout. Other studies reported an improved granulation by adding divalent ions such as Ca^{2+} and Mg^{2+} to the wastewater (Jiang et al. 2003; Li et al. 2009; Liu et al. 2010). In two recent studies with abattoir (Pijuan et al. 2011) and domestic wastewater (Coma et al. 2012), the biomass loss during startup could be reduced by adding crushed granules to the flocculent seed sludge.

This study focused on the operation conditions for the transformation of conventional activated sludge to AGS. The objective was to determine the main parameters and conditions for a rapid formation of fast-settling granules with high removal capacity for COD, N and P. Seven parameters hypothesized to have an impact on startup of AGS-SBRs removing simultaneously COD, N, and P were tested with lab-scale reactors and synthetic wastewater with a multifactorial experimental design approach. The findings should help to tailor an optimal startup strategy in a second step.

3.2 Materials and Methods

3.2.1 Reactor setup

Two identical bubble column reactors with diameters of 5.2 cm and working volumes of 2.4 L were used in this study. A detailed description of these reactors and the gas recirculation system can be found in the general materials and methods chapter of this thesis (Chapter 2). For experimental runs with a reduced gas flow, a Hoffman's tubing clip was installed on the aeration tube. The membrane pumps (KNF Neuberger, Switzerland) used for aeration delivered a constant power. Hence, the increased resistance due to the tubing clip decreased the gas flow. This gas flow could easily be adjusted by tightening or loosening the screw of the Hoffman's clip and was measured by a volumetric gas flow meter (Brooks, USA).

3.2.2 Analytical methods

All analyses of dissolved chemical compounds as well as total suspended solids (TSS) and volatile suspended solids (VSS) measurements were made as described in the general materials and methods part (Chapter 2). For the sludge volume index (SVI), the classical index calculated after 30 min of settling was used (Mohlman 1934), in order to compare with the initial flocculent sludge.

3.2.3 Inoculation sludge

The inoculation sludge was taken at ARA Thunersee during the time period of 12.05.2011 to 25.07.2012. Since the inoculation sludge has been collected on different days over a period of about 15 months, the different inoculation samples had to be compared, in order to verify whether the start conditions were similar or not. For this purpose, the nutrient removal performances of the biological compartment in ARA Thunersee were compared for the five sampling periods. The 7-day average of the NH_4^+-, N- and P-removal centered on the sampling day was calculated, based on the measured concentrations in the primary and secondary clarifiers. The P-removal at ARA Thunersee is

done by combined biological accumulation and chemical precipitation. Hence, to compare the biological P-removal potential of the different sludge samples, the volumes of precipitating agent solution added have also been compared.

3.2.4 Experimental design

The influence of seven operation parameters was investigated in this study: (1) pollution load, (2) aeration strategy, (3) addition of allylthiourea, (4) airflow velocity, (5) strategy to reduce the settling time, (6) pH and (7) temperature. To test these parameters with a minimum of experiments, a Plackett-Burman design based on the Hadamard matrix was used. This design allows testing the influence of 7 parameters with only 8 runs. It is a screening approach with the aim to elucidate which parameters have a high impact on a process and which have not. Plackett-Burman is a two-level design. Hence, for each parameter two levels have been defined, one called negative (-1) and one positive (+1) (Table 3-1).

Table 3-1: Tested operation parameters during AGS-SBR startup and their negative and positive levels.

Parameters		Type	Negative (-1)	Positive (+1)
1	Pollution load	binomial	constant load	performance-adapted load
2	Aeration strategy	binomial	constant DO	alternate high-low DO
3	Allylthiourea	binomial	without	with
4	Airflow velocity	continuous	0.015 m/s	0.030 m/s
5	Settling time	binomial	strategy A	strategy B
6	pH	continuous	7.0-7.3	7.5-7.8
7	Temperature	continuous	15°C	20°C

The operation conditions of the 8 runs were defined according to the Hadamard matrix. Each parameter was either set to level (-1) or (+1) (Table 3-2). All columns and rows of the Hadamard matrix are orthogonal to each other. Hence, all results are interpreted as main effects and no interactions between parameters can be detected.

Table 3-2: Hadamard matrix for experimental design

	Parameter						
Run	Pollution load	Aeration strategy	Allylthiourea	Airflow velocity	Settling time	pH	Temperature
1	+	+	+	-	+	-	-
2	-	+	+	+	-	+	-
3	-	-	+	+	+	-	+
4	+	-	-	+	+	+	-
5	-	+	-	-	+	+	+
6	+	-	+	-	-	+	+
7	+	+	-	+	-	-	+
8	-	-	-	-	-	-	-

All runs were performed once except for run 7 which was performed twice. Runs 1 to 6 were tested during 45 days. Run 7a was stopped after 29 days, run 8 after 36 days and run 7b was performed during 80 days. The reasons for the different test durations for runs 7 and 8 are explained later.

3.2.4.1 Description of operation parameters and levels

The choice of the parameters and levels in a Plackett-Burman design is extremely important for two reasons. First, all results will be treated as main effects and no interactions between parameters can be detected, hence, parameters and levels must be chosen such that it can be assumed that there are no major interactions. Second, every single run has a high impact on the global result due to the minimal number of experiments. There is no extra data available allowing to detect, eliminate and treat statistically extreme events such as outliers or fails of experimental runs. Hence, all levels had to be chosen in realistic ranges for the formation of aerobic granular sludge. The selection of the parameters was made based on previous findings reported in literature and experience of our own laboratory. The seven parameters and their levels were the following:

(1) **Pollution load:** For level (-1) the influent wastewater had the same concentration in COD, N, and P during the whole run and the duration of the starvation phase was kept constant. It was defined as “constant pollution load”. For level (+1), the influent wastewater was diluted and an anaerobic mixing phase with N_2 of up to 3 hours was added after the non-mixed anaerobic feeding period. The wastewater concentration and the duration of the mixing time were continuously adapted to the anaerobic C-uptake capacity of the biomass, in order to avoid the presence of VFA during the aeration time. This level was called “performance-adapted pollution load”.

(2) **Aeration strategy:** For level (-1), the dissolved oxygen concentration (DO) was controlled at a constant value of 60% saturation and the level was called “constant DO”. For level (+1), high DO phases were alternated with low DO phases. Three high DO phases of 20 minutes with DOs of 50%, 40% and 30% saturation, respectively, were interrupted by three phases with a DO of 5%. This level was called “alternate high-low DO”.

(3) **Allylthiourea (ATU):** For level (-1), no ATU was added to the influent wastewater. For level (+1), 100 mg L^{-1} of ATU was added to the nutrient medium during the first 10 days of the run. After dilution with tap water, it resulted in a final concentration of about 10 mg L^{-1} in the influent. ATU is a nitrification inhibitor sometimes added in enhanced biological phosphorus removal systems.

(4) **Airflow velocity:** Superficial air velocities of 0.015 m s^{-1} and 0.030 m s^{-1} were used for level (-1) and level (+1), respectively.

(5) **Settling time strategy:** Settling time strategy A (level (-1)) consisted of a 20-days period with 30 min of settling time followed by a stepwise daily reduction to 25, 20, 15, 12, 9, 7, 5, 4, and finally 3 min at day 29. The latter was kept for the rest of the run (Figure 3-1). For strategy B (level (+1)), the settling time was 30 min for the first day, reduced to 20 min at day 2 and to 10 min at day 3. It was kept at 10 min until day 10 and then further reduced by 30 s per day to reach a final settling time of 5 min at day 20 which was kept until the end of the run (Figure 3-1).

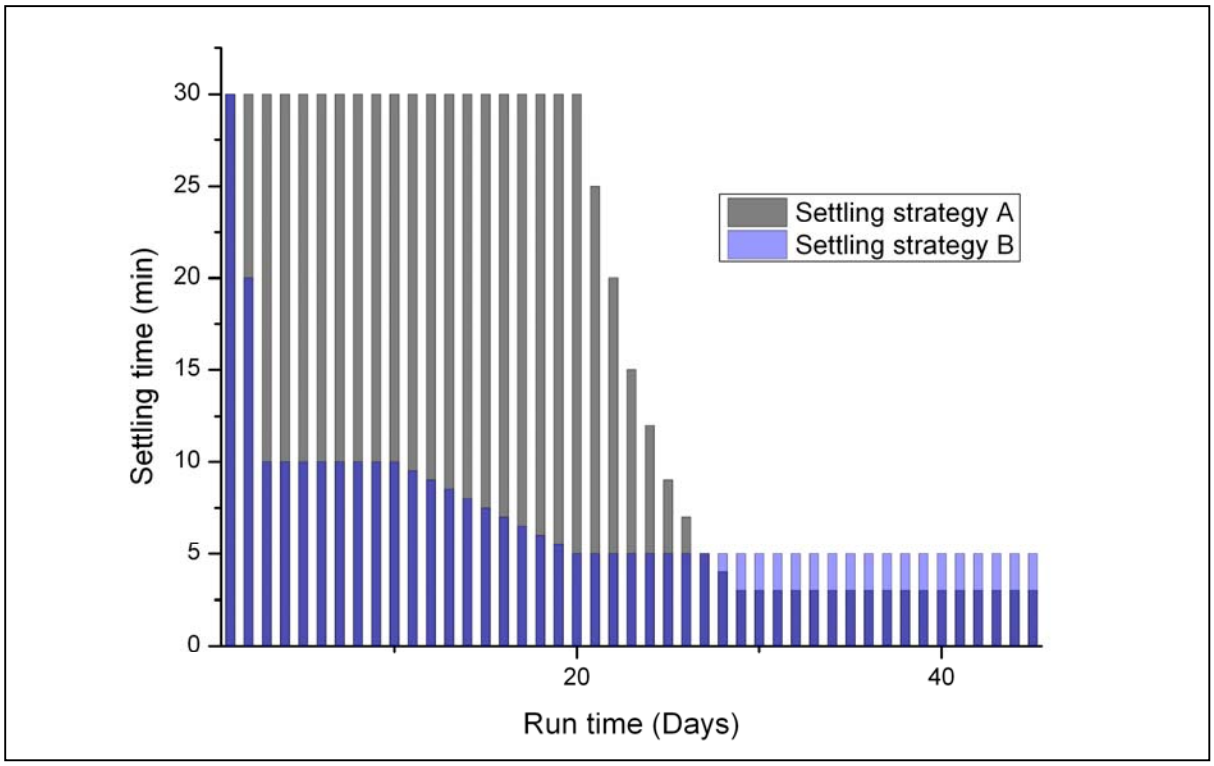

Figure 3-1: Evolution of the settling times with the two strategies.

(6) **pH:** For level (-1), the pH was controlled between 7 - 7.3, whereas for level (+1) the pH was kept between 7.5 - 7.8. During the non-mixed feeding phase, pH was not adjusted and therefore sometimes outside of these ranges.

(7) **Temperature:** Runs were operated at 15°C and at 20°C for level (-1) and level (+1), respectively.

3.2.4.2 Criteria for quantification of performances

The aim of the study was to elucidate the impact of the tested parameters on the startup duration. Hence, the success of the different runs was measured in number of days. Five different processes have been taken into account to decide if a run was successful or not: (i) anaerobic COD-removal, (ii) NH_4^+-removal, (iii) N-removal, (iv) P-removal, and (v) biomass growth and granulation. For each of the five processes one criterion has been defined, which had to be achieved (Table 3-3). It has been decided to choose reasonable values for the criteria to make sure that a majority of the runs would achieve most criteria. Stricter criteria, such as the allowed effluent concentrations for wastewater plants given by the Swiss Water Protection Ordinance (WPO, 1998), would require fine tuning of the operation parameters. However, in order to be scientifically rigorous it was not possible to modify or fine tune the operation conditions during the experimental runs.

Table 3-3: Criteria for the five processes defining the success of the experimental startup runs.

Process	Criterion	Approx. correspondence in %
Anaerobic COD-removal	> 1.4 g_{COD} L^{-1} d^{-1}	95
P-removal	> 60 mg_P L^{-1} d^{-1}	80
NH_4^+-removal	> 160 mg_N L^{-1} d^{-1}	85
N-removal	> 120 mg_N L^{-1} d^{-1}	65
Biomass growth and granulation	VSS > VSS_{start} and SVI < 50 mL g^{-1}	

A criterion was considered to be achieved when the 3-day average was higher as the limit value. A run was successful once all five criteria were achieved. Hence, the overall answer vector was the number of days for each run until all criteria were achieved. If a criterion was not achieved within the 45-day test period, a penalty relative to the difference to the limit value was added. The penalty was calculated by the following equation:

$$\text{penalty} = \frac{(\text{limit value-highest achieved value})}{\text{limit value}} * 100 \qquad \textbf{Equation 3-1}$$

So, for every percent difference to the limit value, one “penalty day” was added to the result. In order to avoid big differences in the quantification of similarly successful strategies, the highest achieved performance was taken into account for the calculation of the penalty which was not necessarily the performance at day 45.

3.2.4.3 Mathematical resolution of Plackett-Burman design

The impact of the different parameters on the processes was calculated solving the following linear equations:

$$
\begin{aligned}
&\textbf{Run 1}: \mathbf{a_0} + \mathbf{a_1}\, x_{11} + \mathbf{a_2}\, x_{12} + \mathbf{a_3}\, x_{13} + \mathbf{a_4}\, x_{14} + \mathbf{a_5}\, x_{15} + \mathbf{a_6}\, x_{16} + \mathbf{a_7}\, x_{17} = \text{answer run 1}\\
&\textbf{Run 2}: \mathbf{a_0} + \mathbf{a_1}\, x_{21} + \mathbf{a_2}\, x_{22} + \mathbf{a_3}\, x_{23} + \mathbf{a_4}\, x_{24} + \mathbf{a_5}\, x_{25} + \mathbf{a_6}\, x_{26} + \mathbf{a_7}\, x_{27} = \text{answer run 2}\\
&\textbf{Run 3}: \mathbf{a_0} + \mathbf{a_1}\, x_{31} + \mathbf{a_2}\, x_{32} + \mathbf{a_3}\, x_{33} + \mathbf{a_4}\, x_{34} + \mathbf{a_5}\, x_{35} + \mathbf{a_6}\, x_{36} + \mathbf{a_7}\, x_{37} = \text{answer run 3}\\
&\textbf{Run 4}: \mathbf{a_0} + \mathbf{a_1}\, x_{41} + \mathbf{a_2}\, x_{42} + \mathbf{a_3}\, x_{43} + \mathbf{a_4}\, x_{44} + \mathbf{a_5}\, x_{45} + \mathbf{a_6}\, x_{46} + \mathbf{a_7}\, x_{47} = \text{answer run 4}\\
&\textbf{Run 5}: \mathbf{a_0} + \mathbf{a_1}\, x_{51} + \mathbf{a_2}\, x_{52} + \mathbf{a_3}\, x_{53} + \mathbf{a_4}\, x_{54} + \mathbf{a_5}\, x_{55} + \mathbf{a_6}\, x_{56} + \mathbf{a_7}\, x_{57} = \text{answer run 5}\\
&\textbf{Run 6}: \mathbf{a_0} + \mathbf{a_1}\, x_{61} + \mathbf{a_2}\, x_{62} + \mathbf{a_3}\, x_{63} + \mathbf{a_4}\, x_{64} + \mathbf{a_5}\, x_{65} + \mathbf{a_6}\, x_{66} + \mathbf{a_7}\, x_{67} = \text{answer run 6}\\
&\textbf{Run 7}: \mathbf{a_0} + \mathbf{a_1}\, x_{71} + \mathbf{a_2}\, x_{72} + \mathbf{a_3}\, x_{73} + \mathbf{a_4}\, x_{74} + \mathbf{a_5}\, x_{75} + \mathbf{a_6}\, x_{76} + \mathbf{a_7}\, x_{77} = \text{answer run 7}\\
&\textbf{Run 8}: \mathbf{a_0} + \mathbf{a_1}\, x_{81} + \mathbf{a_2}\, x_{82} + \mathbf{a_3}\, x_{83} + \mathbf{a_4}\, x_{84} + \mathbf{a_5}\, x_{85} + \mathbf{a_6}\, x_{86} + \mathbf{a_7}\, x_{87} = \text{answer run 8}
\end{aligned}
$$

Equation 3-2

with x_{ij} = -1 or +1 according to the Hadamard matrix.

a_0 is the average duration for the process and a_1 to a_7 are the absolute half-effects of each parameter. The absolute half-effects are given in days and express the impact of the parameter on the duration. An effect can be negative or positive. Hence, the total effect of a parameter expresses the difference between levels (-1) and (+1) and corresponds to two times the value of the half-effect.

The half-effects in this study are given as relative half-effects in relation to the average duration. To avoid confusion in the interpretation of results, the signs of the half-effects were inversed, so that positive values express a positive impact of level (+1) on the result, which means a shorter duration. Whereas, a negative half-effect means that level (-1) reduces the startup duration. E.g. a relative half-effect of +10 means that level (+1) of the parameter reduces the startup duration by 10% compared to the average, whereas a value of -10 means that the startup duration is 10% longer than the average with level (+1).

3.2.4.4 Criteria for selection of relevant parameters

For the selection of the relevant parameters it is recommended to make an estimation of errors of the effects (Vander Heyden et al. 2001). An error estimate is only possible if more than *N*+1 runs are

available for *N* parameters. An often used strategy is to conduct a center point run, which means a run with all parameters set at the mean value between level (-1) and (+1). Since four of seven parameters were not numerical and continuous such a center point run was not applicable. In order to compensate the missing center point run, the run leading to the best performance was conducted a second time to get an estimation of the experimental error for each process. The error estimate allowed to calculate the critical effect ($E_{critical}$) for a significance level α = 0.05 and α = 0.1. The effect of a parameter *i* was considered significant at a given α, if $|E_i| > E_{critical}$.

Due the fact that only one run was duplicated, the power of the statistical approach had to be considered as rather low. Therefore, the parameter selection was rechecked by applying an analysis of variance (ANOVA) to the model obtained from the selected parameters.

3.2.4.5 Calculation of effect evolution over time

To obtain information on the evolution of the impact of the different parameters, the Plackett-Burman design was also analyzed according to the daily performances of the different runs. For the four nutrient removal processes (COD-, NH_4^+-, N- and P-removal) the absolute removal performances were taken into account. For the characterization of the biomass, the VSS and the SVI have been analyzed. The gaps due to missing measurements on some days have been filled by linear interpolation between the previous and the next available measurement. To reduce the effect of random noise from the measurements, an unweighted 3-day moving average filter has been applied to the different time series for nutrient removal and sludge characterization.

3.3 Results

3.3.1 Nutrient removal performances of inoculum sludge

NH_4^+-removal was between 99 - 100% around the day the five seed sludge samples were collected at ARA Thunersee, and N-removal amounted to 73 – 77.0%. The average P-removal was in three sampling periods around 92%, in the two others around 98%. P-removal performances were the result of biological P accumulation and chemical P precipitation. During the two periods with a P-removal around 98%, only about half the amount of precipitation agent was added, compared to the periods with 92% P-removal (Table 3-4), which emphasizes that the biological P-removal at ARA Thunersee was higher during these two sampling periods.

Table 3-4: Seven days average of the removal performances at ARA Thunersee around the inoculum sampling day

Sampling date	runs	NH_4^+-removal (%)	N-removal (%)	P-removal (%)	Fe coagulant added[2] ($L\ d^{-1}$)
12.05.2011	7a, 8	99.6	77.0	91.9	1214
17.06.2011	3, 5	99.6	73.0	91.8	1226
03.11.2011	6, 7b	99.9	74.3	98.4	630
14.02.2012	2	98.6	76.5	97.9	544
25.07.2012	1, 4	99.7	72.7	91.4	1184

[1]The removal performances were calculated by comparing the concentrations in the primary and secondary clarifier.

[2]Average volumes of iron coagulant solutions (FeCl, $FeClSO_4$, $FeSO_4$) added per day indicate the relative importance of chemical P-removal within overall P-removal.

3.3.2 Removal performances and biomass characteristics during different runs

Since the SBR cycle length and wastewater concentration were not equal in all runs and changed for some runs with time, the removal performances were expressed in relative and absolute values to be able to compare results of different runs. For strategies with constant influent wastewater concentrations, the cycle length was almost constant (runs 2, 3, 5, and 8). Hence, the relative and the absolute performance curves had very similar shapes, whereas for strategies with diluted influent wastewater and anoxic mixing phases after feeding (runs 1, 4, 6, and 7), the shapes of relative and absolute removal performances were very different (Figure 3-2). With diluted influent wastewater and longer cycle times, the absolute removal results were always low at the beginning even if the relative ones were high, because of the low pollution loads. Interestingly, only in four runs (run 1, 3, 4, and 5) the best removal performances were observed at the end of the 45 days test period. In run 2, 6, and 8, the best performances were achieved before day 25. Thereafter, first the P-removal performance decreased and around day 30 the nitrification.

Only in runs 1, 5, and 7 all four removal performance criteria were reached before day 45. In run 2, the P- and NH_4^+-removal criteria were not reached, in run 3 the COD- and P-removal criteria, in run 4 the N-removal criterion, in run 6 the N-removal criterion, and in run 8 the COD-, P- and N-removal criteria. Run 7a was stopped at day 30 because all criteria were reached.

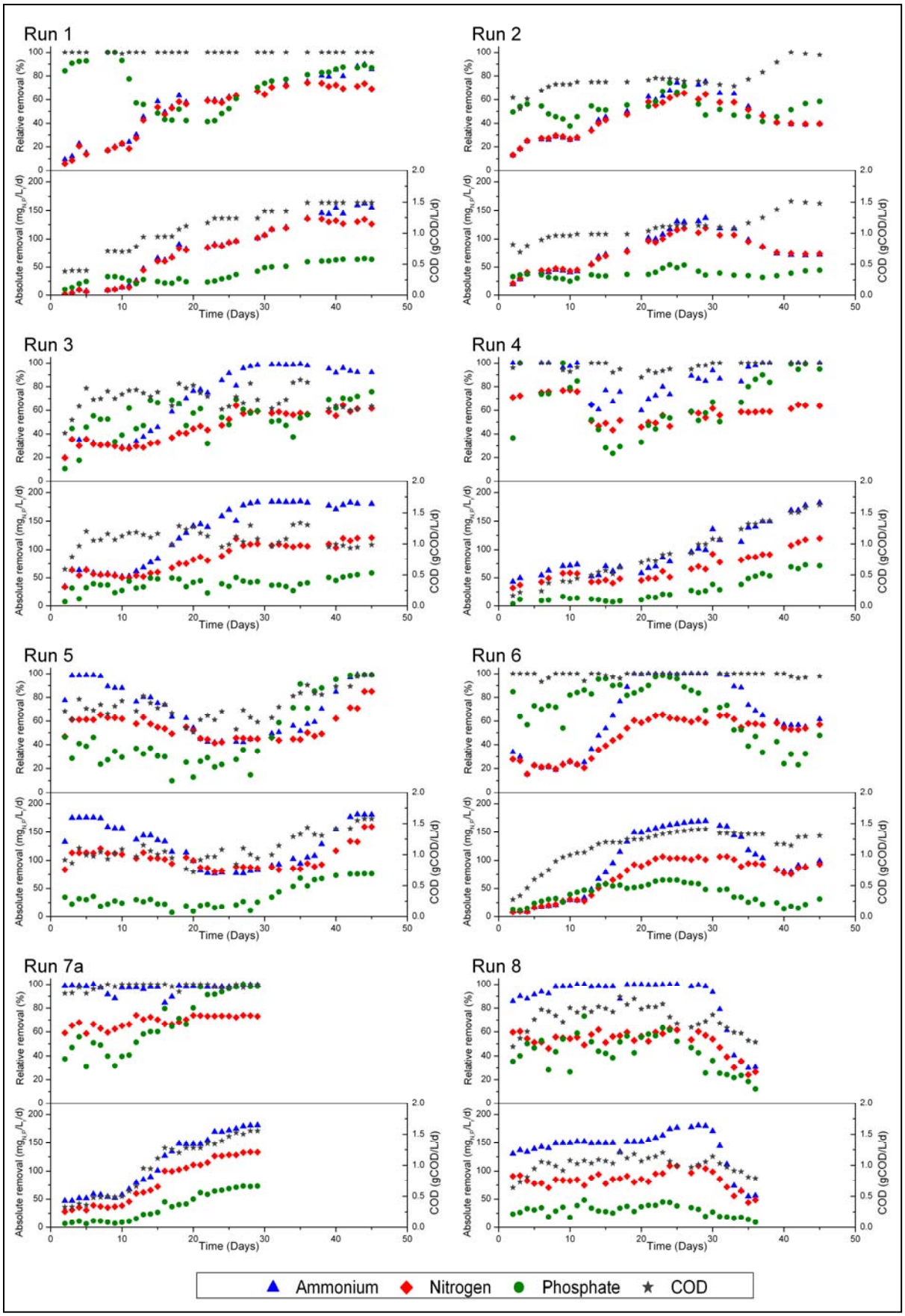

Figure 3-2: Absolute and relative removal performances for ammonium, nitrogen, phosphate and COD in the eight experimental runs.

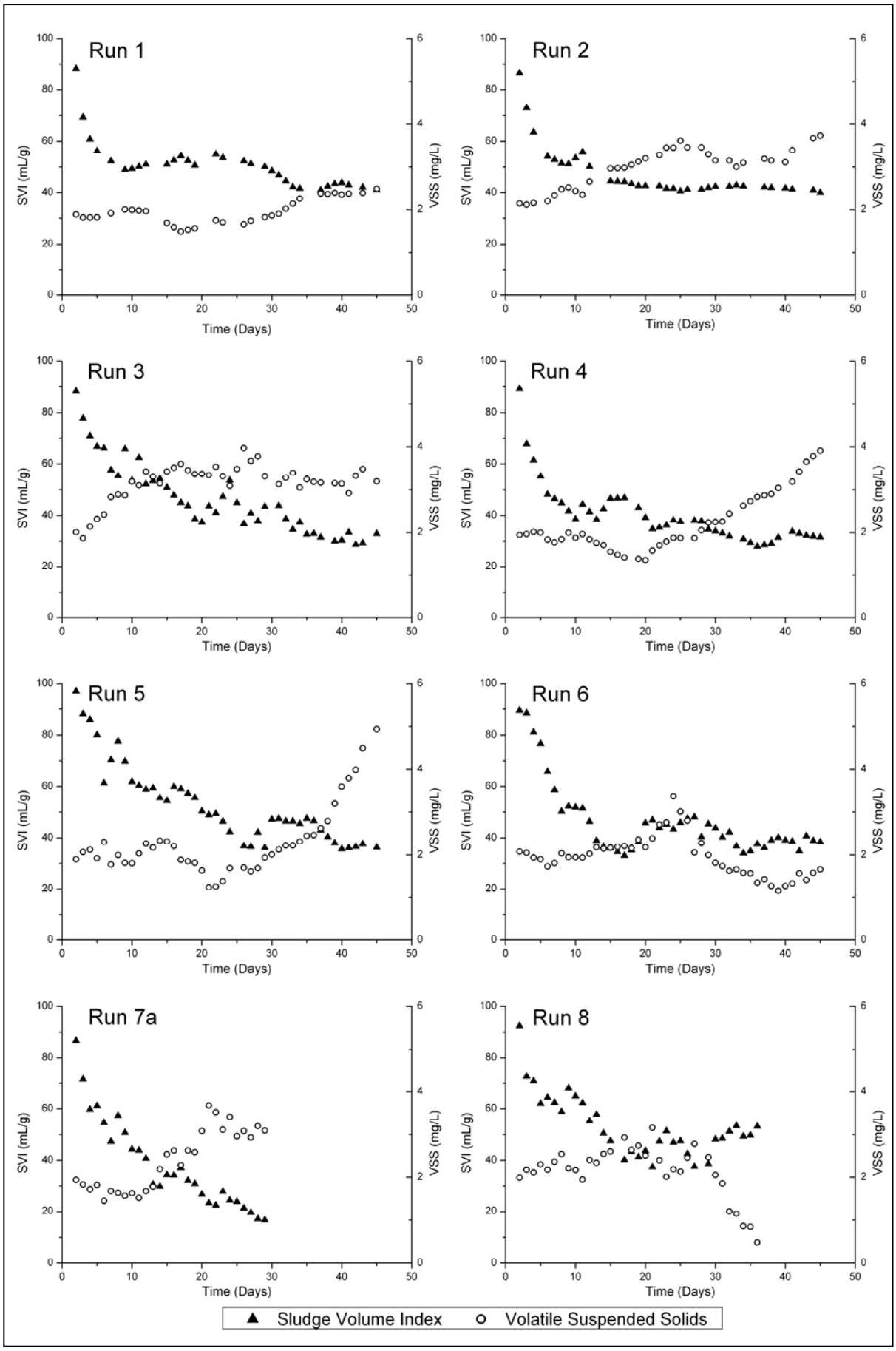

Figure 3-3: Sludge volume index (SVI) and volatile suspended solids (VSS) in the eight runs.

All runs were started with sludge concentrations between 2.6 – 2.9 gTSS L^{-1} and 1.9 – 2.2 gVSS L^{-1}. During the first 10 days, VSS remained almost stable, except for run 3 where an increase was observed (Figure 3-3). Between day 10 and 20, VSS decreased in runs 1, 4 and 5, but increased in runs 2 and 7a. At around day 25, VSS started to decrease in runs 2, 6 and 8. In run 2, the loss of biomass was moderate and stopped at around day 30 whereas in runs 6 and 8, biomass washout was massive, and run 8 was finally stopped at day 36 when VSS dropped below 0.5 gVSS L^{-1}. The highest biomass concentration after 45 days was measured in run 5 with 4.9 gVSS L^{-1} (Figure 3-3). The SVI was between 85 - 100 mL g^{-1} at the beginning of the runs and decreased rapidly during the first 10 to 15 days to values between 30 - 60 mL g^{-1}. The lowest values reached were around 20 mL g^{-1} in run 7a. In most runs, the SVI was around 40 mL g^{-1} at the end of the test period (Figure 3-3). All runs reached the criteria for biomass growth and granulation between day 13 (run 6) and day 32 (run 1).

The startup was considered successful once all five criteria were reached. This was the case during the experimental period of 45 days for runs 1, 5, and 7, whereas for runs 2, 3, 4, 6, and 8 at least on criterion was not reached (Table 3-5). In this case, a penalty time proportional to the difference with the criterion was added. The highest achieved performance has been taken into account for the calculation of the penalty. Hence, even if a criterion was not achieved within the 45 days, the result taken into account for the statistical analysis could reach values below 45 days. Run 2 for example achieved relatively high P-removal at around day 25, before suffering from sludge wash-out in the following days. For the statistical analysis, the penalty was calculated based on the P-removal at around day 25, resulting in a total time of 38 days (Table 3-5).

Table 3-5: Days of operation needed to reach the requested criteria for the different processes and total startup time of the eight experimental runs.

	Anaerobic COD-removal	**P-removal**	**NH_4^+-removal**	**N-removal**	**Biomass growth and granulation**	**Overall startup time**
run 1	37	37	44	33	32	**44**
run 2	40	(38)[1]	(45)[1]	27	15	**45**
run 3	(41)[1]	(50)[1]	25	44	16	**50**
run 4	36	40	41	(46)[1]	24	**46**
run 5	40	38	41	41	27	**41**
run 6	27	22	22	(38)[1]	13	**38**
run 7	24	22	23.5	26.5	15	**26.5**
run 8	(40)[1]	(52)[1]	23	(40)[1]	15	**52**

[1] In case the criterion was not reached at the end of the 45 days of operation, the number of days are put in brackets. In this case, a penalty time proportional to the difference with the criterion was added. The highest achieved performance was taken into account for the calculation of the penalty, resulting sometimes in times below 45 days, even if the criterion was in reality not achieved.

Startup of run 7 was the most successful since all five criteria were reached within 26.5 days (Table 3-5). For the statistical analysis the average of runs 7a and 7b was used. The limiting criterion in run 7 was N-removal. N-removal was also the limiting process in runs 4 and 6, whereas in runs 3 and 8 P-removal was the limiting process, and in runs 1 and 2 NH_4^+-removal. In run 5, both NH_4^+- and N-removal were limiting processes (Table 3-5).

3.3.3 Reproducibility of experimental runs

Run 7 that reached the five criteria after less than 30 days and therefore had the shortest startup period, was conducted a second time in order to get information on the reproducibility and variability. The inoculation sludge used for run 7b had a higher biological P-removal potential than the inoculation sludge of run 7a (Table 3-4). A difference concerning the anaerobic COD-removal was observed during the initial phase of the rune when comparing runs 7a and 7b. The general shape of COD-removal performances was similar, but run 7a was shifted by 5 to 8 days (Figure SI 3-1). However, after day 15 anaerobic COD uptake became very similar. Since in run 7 the pollution loads were adapted according to the anaerobic COD consumption, all other absolute nutrient removal performances were also lower during this initial startup phase (Figure SI 3-2). After this initial startup phase with quite big differences between removal performances of the two runs 7a and 7b, the removal performances became more and more similar and the criteria for the five processes were reached with a difference of 2 - 4 days (Table 3-6).

Table 3-6: Days of operation needed to reach the requested criteria in the two runs 7a and 7b.

	Anaerobic COD-removal	P-removal	NH_4^+-removal	N-removal	Biomass growth and granulation	Overall startup time
run 7a	23	24	25	25	14	25
run 7b	25	20	22	28	16	28

The evolution of VSS in the two runs was similar. During the first 10 days VSS was quite stable, between day 10 and 25 it increased to around 4 and 5 gVSS L^{-1} for run 7a and 7b, respectively. In run 7a, VSS remained between 3 - 4 gVSS L^{-1} until the run was stopped at day 29. In run 7b, high biomass washout was observed after day 25, resulting in a biomass concentration of about 2 gVSS L^{-1} at day 45. The SVI evolved differently in the two runs. In run 7a, SVI decreased rapidly, reaching 20 mL g^{-1} at day 29. In run 7b, SVI did not go < 40 mL g^{-1} in the first 30 days (Figure SI 3-3).

3.3.4 Statistical analysis of the Plackett-Burman design

Runs 7a and 7b were used to make an estimation of the error (Table 3-7). This error estimate was used to elucidate which half-effects of the parameters were relevant for the length of the startup process.

Table 3-7: Relative error estimates for the five processes based on runs 7a and 7b.

	Anaerobic COD-removal	**P-removal**	**NH_4^+-removal**	**N-removal**	**Biomass growth and granulation**
critical limit for α = 0.05	6.3 %	12.1 %	10.3 %	8.9 %	11.4 %
critical limit for α = 0.1	3.1 %	6.0 %	5.1 %	4.4 %	5.7 %

The statistical analysis of the answer vectors of the five processes resulted in the average durations and relative half-effects given in Table 3-8. Relative half-effects with values higher than the critical values were considered as significant at a level α=0.05 or α=0.1, respectively (Table 3-8).

Table 3-8: Average durations until reaching the different criteria, half-effects of the seven parameters, and indication of the relevance of the parameters for each process. The process durations under optimal conditions are calculated based on the models built with the relevant parameters.

Parameter		**Anaerobic COD-removal**	**P-removal**	**NH_4^+-removal**	**N-removal**	**Biomass growth and granulation**
a_0	average (days)	35.6	37.4	33.1	36.9	19.6
1	Pollution load	+13.0 % **	+19.1 % **	+1.3 %	+2.9 %	-7.0 % *
2	Aeration strategy	+1.1 %	+9.7 % *	-16.1 % **	+13.7 % **	-13.4 % **
3	Allylthiourea (ATU)	-1.8 %	+1.7 %	-2.8 %	+3.9 %	-3.2 %
4	Airflow	+1.1 %	-0.3 %	-1.7 %	+2.9 %	+10.8 % *
5	Settling time	-8.1 % **	-10.4 % *	-14.7 % **	-11.0 % **	-26.1 % **
6	pH	-0.4 %	+7.7 %	-12.7 % **	-2.9 %	-0.6 %
7	Temperature	+7.4 % *	+11.7 % *	+15.7 % **	-1.2 %	+9.6 % *
Process duration under optimal conditions (days)		25.5	21.3	24.3	27.8	14.5

* Relevant parameter at a significance level α=0.1

** Relevant parameter at a significance level α=0.05

The relevance of this parameter selection was checked by an ANOVA between significant and not significant parameters. For all five processes, p-values < 0.01 were obtained confirming that the parameter selection was valid from a statistical point of view. The significant parameters were used to build process models. A comparison between the models and the experimental results can be found in the supplementary material (Figure SI 3-4). Based on these five models, the optimal tested level of the seven parameters were determined. The optimal levels were not necessarily optimal for

all five processes, but they led overall to the shortest startup period. For parameters 1, 2, and 7, level (+1) was optimal, for parameters 5 and 6, level (-1). Parameters 3 and 4 had no significant impact on the overall startup duration. With these optimal levels, a startup duration time of 27.8 days was calculated. The limiting process appeared to be N-removal (Table 3-8).

In a next step, still based on the models taking into account only the significant parameters, an estimation of the impacts of each parameter on the overall startup duration was carried out. For this analysis, all parameters were set to their optimal level, except the one for which the impact was tested. The durations obtained by these suboptimal models were then compared to the shortest possible startup duration of 27.8 days calculated before. The difference between the two values was considered as the impact of the different parameters on the startup period length (Table 3-9).

Table 3-9: Impact of each parameter on the startup process compared to the optimal startup model and the limiting processes for the suboptimal models.

Parameter		duration (days)	impact (days)	limiting process
1	Pollution load	35.5	7.7	P-removal
2	Aeration strategy	37.9	10.1	N-removal
3	Allylthiourea (ATU)	27.8	0	N-removal
4	Airflow	27.8	0	N-removal
5	Settling time	35.9	8.1	N-removal
6	pH	32.7	4.9	NH_4^+-removal
7	Temperature	34.7	6.9	NH_4^+-removal

As mentioned previously, five parameters had an impact on the startup duration and two had not. The aeration strategy had the highest impact. A startup with optimal conditions for all parameters except the aeration strategy, would take about 10 days longer than the optimal startup. The other relevant parameters had an impact between 5 - 8 days (Table 3-9). According to the model, N-removal was the limiting process in most cases, with a lower temperature or a higher pH nitrification was limiting, and with a constant pollution load P-removal was the limiting process (Table 3-9).

3.3.5 Evolution of effects over time

The three parameters pollution load, use of ATU, and settling time contained dynamic changes during the 45-day test periods, which led to an evolution of the effects over time. For the calculation of the evolution of the half-effects, only run 7b has been taken into account for run 7, as run 7a was stopped before day 45. For run 8, which was stopped at day 36 because of massive biomass washout, the values of day 36 were used for days 37 to 45. That is a fair assumption as no rapid

recovery could be expected, taking into account the very low remaining biomass concentration and the short settling time at this period. For the interpretation of the effects, the significance limit was set arbitrarily at 5%, and below this limit, the effects were considered as experimental noise. It corresponded approximately to the average error estimate over the five processes at a significance level of α=0.1 (Table 3-7).

With level (+1) the pollution load was continuously adapted according to the anaerobic COD consumption, whereas with level (-1) the loads were kept constant. Only at the very end of the test period the pollution loads became about the same (Figure 3-4, black line). Half-effects above the black line mean that the relative removal performances were higher with performance-adapted pollution loads. Half-effects above the zero line mean that even the absolute removal performances were higher with performance-adapted pollution loads.

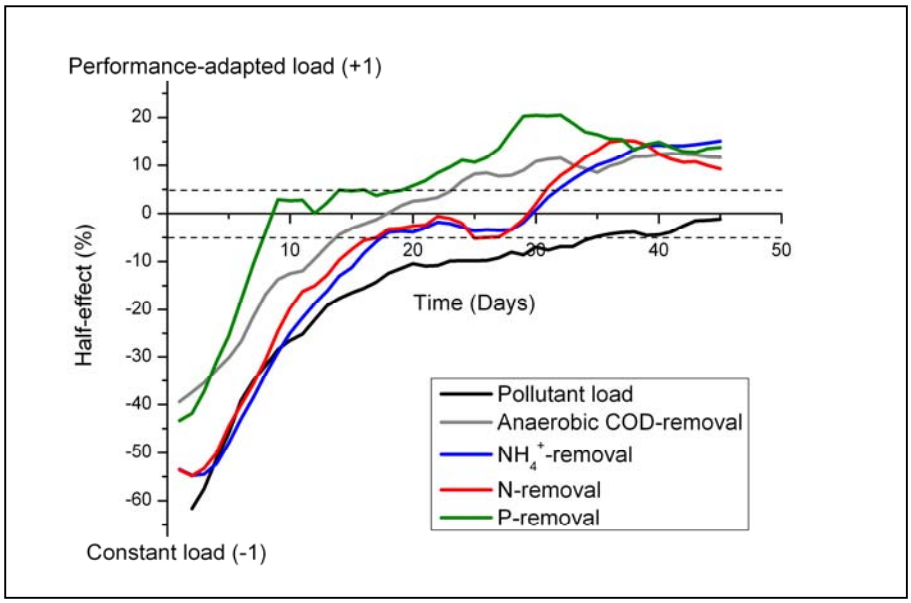

Figure 3-4: Evolution of the relative half-effects of the pollution load on the four nutrient removal processes. In black the relative half-effect of the pollution load parameter on the measured pollution load.

In a first time, the performance-adapted load strategy had a negative impact on the absolute nutrient removal. Over time the impact became positive for all 4 processes. The relative performances for anaerobic COD- and P-removal were from the beginning better under performance-adapted load conditions (Figure 3-4).

The addition of ATU had a very negative effect on NH_4^+- and N- removal in an early-stage which was expected as ATU is known to be a nitrification inhibitor. At day 10, the supply of ATU was stopped and another 10 days later ATU had no effect anymore on NH_4^+- or N-removal. During the first 7 days,

the addition of ATU seemed to have a positive effect on P-removal. However, this trend was not confirmed in the subsequent days of the study (Figure 3-5).

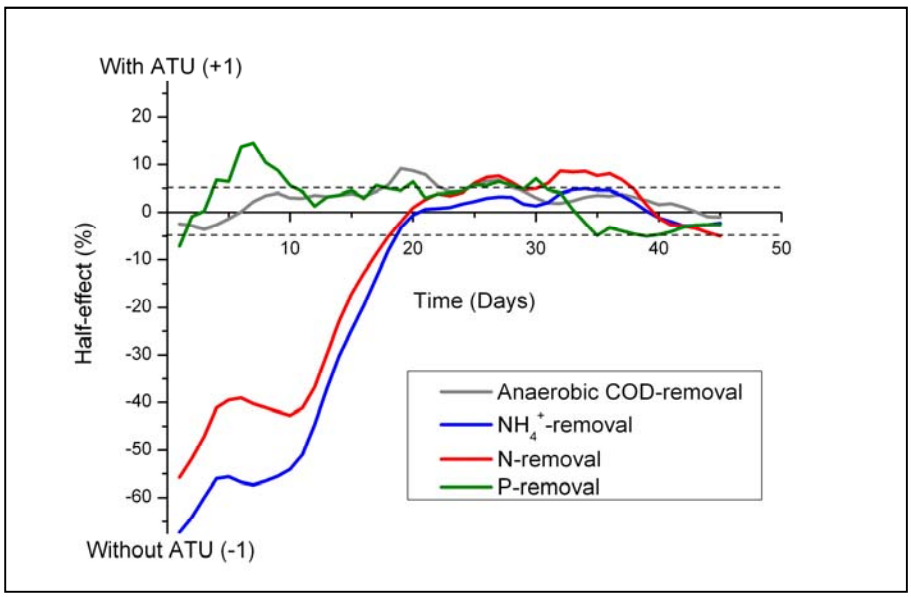

Figure 3-5: Evolution of the relative half-effects of ATU on the four nutrient removal processes.

Settling strategy A consisting in keeping settling time at 30 min during the first 20 days and then reducing it stepwise to 3 min until day 29, led initially to higher VSS and better nutrient removal. However, once the settling time reached 3 min in strategy A, strategy B where settling time was reduced to 10 min within the first 3 days, and then further to 5 min between day 10 and 20, became more favorable (Figure 3-6).

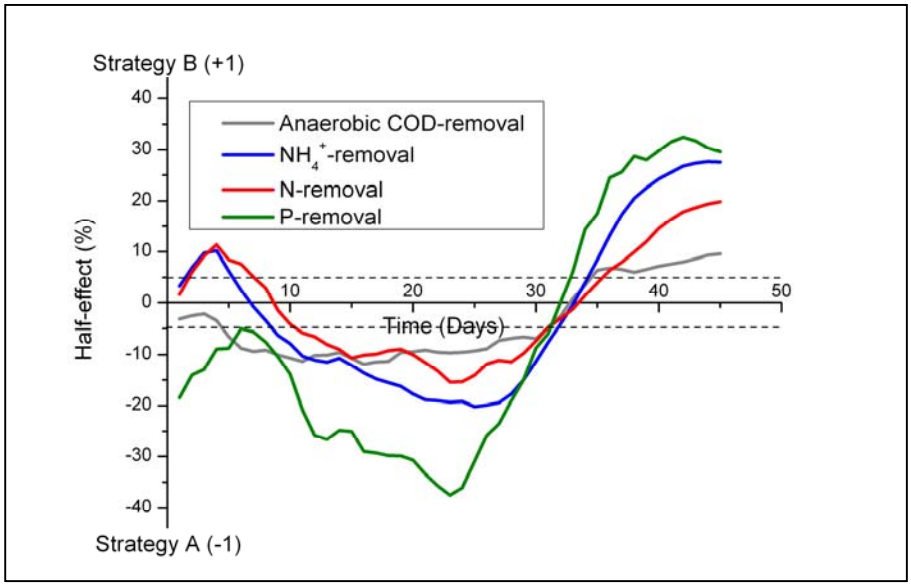

Figure 3-6: Evolution of the relative half-effect of the settling time strategies on the four nutrient removal processes and on VSS.

In relation to biomass concentration, not only the settling strategy had a major impact, but also airflow velocity. A higher airflow velocity had a positive impact on VSS starting at around day 13 and remained high until the end of the 45-day study period (Figure 3-7). Moreover, there was a trend to a lower and thus better SVI with higher airflow. However, this effect of higher airflow velocity on SVI seemed only to become significant towards the end of the runs (Figure 3-7).

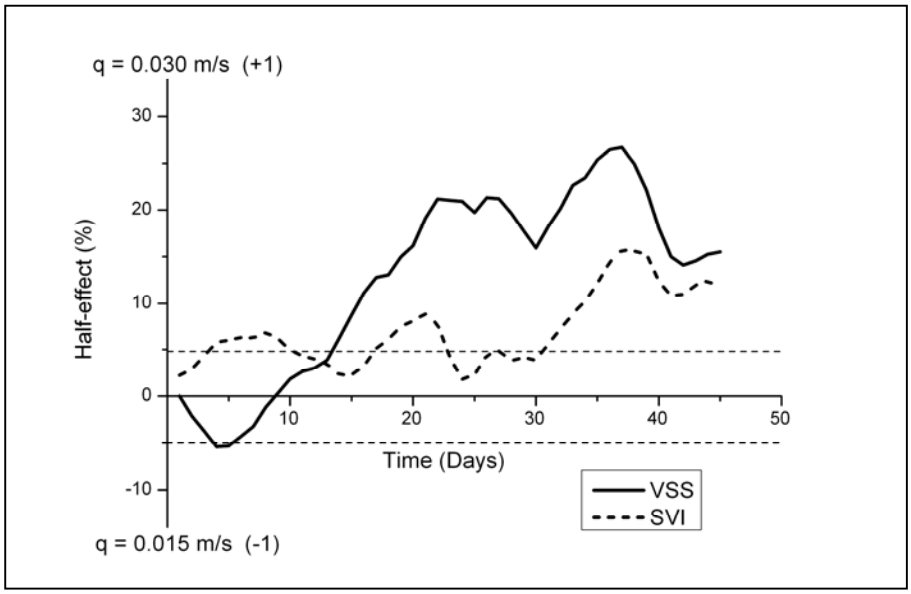

Figure 3-7: Evolution of the relative half-effect of the airflow velocity on VSS and SVI.

3.3.6 Long-term operation under optimal conditions

The conditions of our optimal startup model coincided with the conditions of run 7. Given the fact, that we had five significant parameters, the chance that one of the eight runs would perfectly correspond was $8/2^5$ = 25%. Due to this coincidence, run 7b was not only used to test the reproducibility, but also to test the long-term stability of this optimal run. The reactor was operated during 80 days without any changes in the operation conditions.

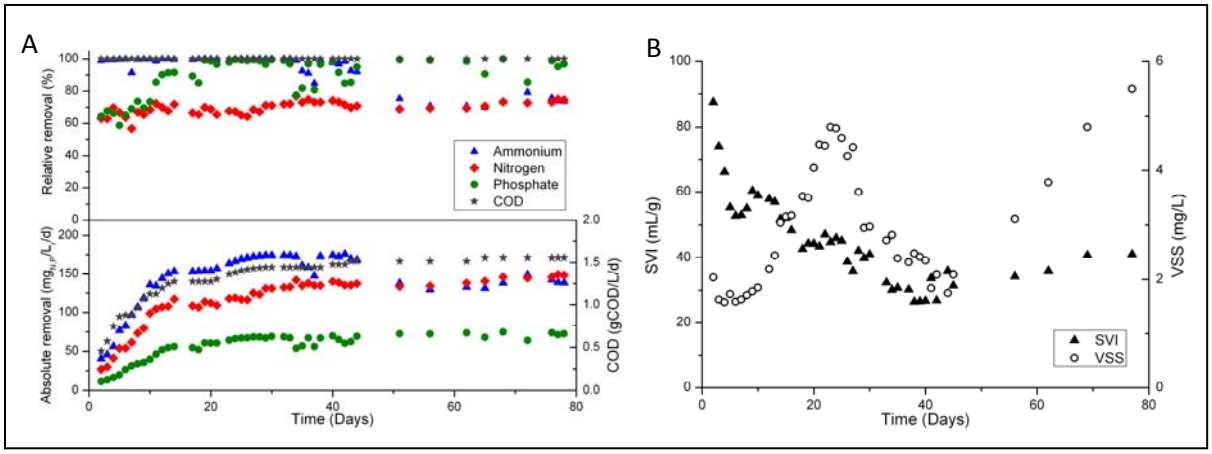

Figure 3-8: Relative and absolute nutrient removal performances **(A)** and biomass-relevant parameters **(B)** during the 80 days of operation of run 7b.

After day 30, P-removal and nitrification slightly decreased (Figure 3-8A). At the same time, an important biomass washout was observed (Figure 3-8B). VSS decreased by about 60% from 4.8 g L^{-1} to 1.9 g L^{-1} between day 24 and 41. The SVI improved from 45 to about 30 mL g^{-1}. Around day 50, the biomass concentration increased again, and P-removal was again close to 100% and rather stable. Nitrification remained between 70 and 80%, as well as the N-removal (Figure 3-8A). The SVI stabilized between 30 and 40 mL g^{-1} (Figure 3-8B). Approaches to recover improved nitrification and N-removal performances are presented in Chapter 6.

3.4 Discussion

3.4.1 Impact of the operation parameters on startup duration

The main parameters influencing the startup duration under the tested conditions were the aeration strategy, the settling time strategy, the pollution load, temperature and pH. The supply of ATU and the tested airflow velocities had no significant impact on the total startup duration. However, most parameters had only an impact on certain processes and not on others, and some parameters even had a positive effect on some processes and a negative on others.

The aeration strategy had the highest impact on the startup. The alternation of high and low DO phases had a positive impact on N- and P-removal, whereas a constant high DO had a positive impact on nitrification and on SVI. Several authors reported that a high DO favored nitrification but limited denitrification, whereas a lower DO enhanced denitrification (Beun et al. 2001; de Kreuk et al. 2005; Mosquera-Corral et al. 2005). Moreover, aeration strategies promoting alternating nitrification and denitrification (AND) such as alternating high and low DO phases, appeared to be more efficient for

N-removal than strategies leading to simultaneous nitrification and denitrification (SND) (Chapter 5). The positive impact on P-removal was presumably linked to the competition for COD between denitrifyiers and PAO in systems with combined denitrification and dephosphatation (Kuba et al. 1996; Keller et al. 1997). In runs with constant high DO (run 3, 4, 6 and 8), an accumulation of NO_x^- was observed as soon as nitrification got above 60% efficiency. The accumulation of NO_x^- can deteriorate P-removal in reactors operated in sequencing mode, because it leads to anoxic conditions during the non-aerated feeding phase (Furumai et al. 1999; Peng et al. 2010). Hence, denitrifyiers and PAO compete for carbon. In the present study, N-removal was the limiting process for the achievement of a rapid startup. Hence, the optimization of this process had a high impact on the global startup time. If for any reasons nitrification or granulation would be the limiting process, a constant high DO could be advantageous.

Settling strategy A had a positive impact on the startup process as well as on all five processes separately. For the biomass development, the positive impact was mainly related to VSS and not to SVI. Nevertheless, looking at the evolution of the half-effect of the settling strategy, it was observed that after day 30, strategy B became favorable for all processes. With strategy B more biomass was washed out between day 10 and 20, whereas with strategy A an enhanced biomass washout was observed after day 25. However, not all runs were equally concerned by the biomass washout. For example, in run 8 more than 80% of the biomass was washed out between day 25 and 36, whereas in run 2 with the same settling strategy, VSS remained quite stable. Overall a direct link between the biomass washout and nutrient removal performances was observed with both strategies. This results agree with findings from Pijuan et al. (2011), and show how important it is to control washout during the startup.

Both strategies tested to reduce the settling time and select for granular sludge were not really optimal. In strategy B, the reduction to 5 min within the first 20 days occurred too early whereas in strategy A the final settling time of 3 min was too short and led to important biomass washout for some runs. An option to avoid a too high loss of biomass during startup would be to link the settling time directly to the observed washout and to recirculate sludge from a secondary clarifier in case of a massive washout. However, granulation occurred in all runs independent of the settling strategy, and the SVI was not correlated to the applied settling times. Hence, based on this study one could recommend, as a measure of precaution, to reduce the settling time only until 10 min since no enhanced washout was observed with this settling time. The additional time required for the longer settling time represents less than 5% of the total cycle time.

The so-called performance-adapted pollution load appeared to be a handicap during the first days of startup, but became beneficial over time for all processes. The aim was to favor the growth of slow growing organisms such as PAO, which are able to take up and store VFA under anaerobic conditions. The relative P- and COD-removal were in fact right from the beginning higher with a performance-adapted pollution load and after day 9 for P-removal and day 17 for the COD-removal the performances became also better in absolute terms. The anaerobic COD uptake in aerobic granular sludge systems has been reported to be a key parameter for the long-term stability and P-removal (de Kreuk and van Loosdrecht 2004). The importance to favor the growth of slow-growing organisms rather than ordinary heterotrophic organisms (OHO) for the formation of dense and stable granules has also been reported in other studies (Liu et al. 2004; Wan et al. 2009). In case of COD availability during the aeration phase, enhanced growth of filamentous organisms has been observed, leading to fluffy granules (McSwain et al. 2004). In the present study, only in run 8 towards the end of the run, some fluffy granules were discovered. However, wall growth was observed in all runs with constant pollution loads (run 2, 3, 5 and 8) with the consequence that the reactor had to be cleaned about once per week. In runs where no VFA reached the aerobic phase, no significant wall growth was observed and reactors were not cleaned during the 45 days of operation.

A temperature of 20°C had a positive impact on the VSS as well as on all removal processes, except for N-removal. The effect of temperature on nitrification is largely known, whereas for P-removal the question is more complex. On the one hand, all metabolic processes of PAO have higher rates at 20°C than at 15°C (Brdjanovic et al. 1998). On the other hand higher temperatures have been shown to favor GAO in competition with PAO (Panswad et al. 2003; Lopez-Vazquez et al. 2007; Ebrahimi et al. 2010). However, a recent modeling study by Lopez-Vazquez et al. (2009) on the influence of the carbon source, pH, and temperature on the PAO-GAO competition has shown that with equal acetate and propionate fractions and pHs of 7 or 7.5, *Candidatus* Accumulibacter phosphatis PAO were the dominant species between 10 and 30°C, independent of the temperature, and only a very small temperature effect was reported in the range between 15 and 20°C. Hence, this supports our findings that in an overall perspective a temperature of 20°C had a positive impact on the P-removal and the anaerobic COD-uptake.

The startup process was faster with a pH of 7.0 - 7.3 compared to 7.5 – 7.8. However, a positive effect of a lower pH was only observed for nitrification, whereas a higher pH was favorable for P-removal. All other processes were not affected by pH. The high positive effect of a pH between 7.0 – 7.3 on nitrification is rather surprising. Normally, quite stable nitrification values are reported between pH 7 - 8.5 with an optimum for nitrification at around pH 8 (Painter and Loveless 1983; Antoniou et al. 1990). The positive effect of alkaline conditions on the P-removal however is well

known. The anaerobic VFA uptake and P-release as well as the aerobic P-uptake are higher at pH values of 7.5 - 8.0 compared to values of 6.5 - 7.0. (Oehmen et al. 2005).

The two airflows tested in this study did not have a relevant impact on the overall startup duration. However, a higher airflow had a positive effect on both biomass criteria, VSS and SVI. Interestingly, the higher airflow started to have a positive impact on the VSS between day 10 and 20. It coincided with the period when the settling time was decreased below 10 min in runs with strategy B, and enhanced biomass washout was observed in some runs. Although the experimental design did not allow quantifying the interaction between parameters, the effect evolution analysis indicated that there was an interaction between settling time and airflow. Nevertheless, in contrast to Tay et al. (2004) we did not observe a direct correlation between airflow and SVI. None of the seven tested parameters could be clearly identified as parameter governing SVI and granulation took place in all runs. Hence, a low airflow is presumably unproblematic as long as the settling time is high enough. Only if the airflow and the settling time are low, enhanced biomass washout will take place.

3.4.2 Further optimization of the startup

The main objective of this study was to determine the most relevant parameters for a startup process that includes rapid granulation and maintenance of nutrient removal capacities of the flocculent inoculation sludge. The optimization of the startup based on the information gained by this study was intended to occur as a separate step. However, the optimal conditions according to the statistical analysis coincided with run 7 of the experimental runs. Hence, this run was at the same time part of the experimental design and also served as test of an optimal startup strategy according to the parameters and levels we defined. Nevertheless, the study also indicated that some of the most relevant parameters could be further optimized. This mainly concerns the settling strategy since none of the two tested was optimal, but also the pollution loads and the aeration strategy could presumably be further optimized. With level (+1) the pollution loads have been constantly adapted according to the anaerobic COD consumption potential. The anaerobic COD consumption potential was supposed to be similar for all inoculation sludges. Hence, with a perfect process control, e.g. with *in-situ* measurements and automated switch from anaerobic mixing to aeration, the anaerobic COD uptake would have been similar with both tested levels at day one. However, in the present study the anaerobic COD uptake was 40% lower with level (+1) at day one, because the pollution load was operator controlled, giving priority to ensure that no dissolved COD entered the aerobic phase.

Concerning the aeration, the DO setpoints were kept unchanged during the whole study, even though the influent pollution concentrations as well as the biomass concentrations in the reactor changed over time. Hence, there would still be some potential to further optimize the startup.

3.5 Conclusion

The main conditions identified in this study leading to a rapid startup of AGS SBRs with good nutrient removal performances were (i) the alternation of high and low DO phases during aeration, (ii) a settling strategy avoiding too high biomass washout, (iii) the adaptation of the pollution load in the early stage of the startup in order to ensure that all soluble COD was consumed before the aeration phase, (iv) higher temperature (20°C), and (v) a neutral pH. Under such conditions it took less than 30 days to produce granular sludge with high removal performances for COD, N and P. A control run of the best startup strategy led to very similar results, proving the reproducibility of the experimental approach. This control run was in addition operated for 80 days under the conditions reached when all criteria were fulfilled (day 28) without any problems concerning the stability of the granular sludge or the nutrient performances.

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Supplementary information

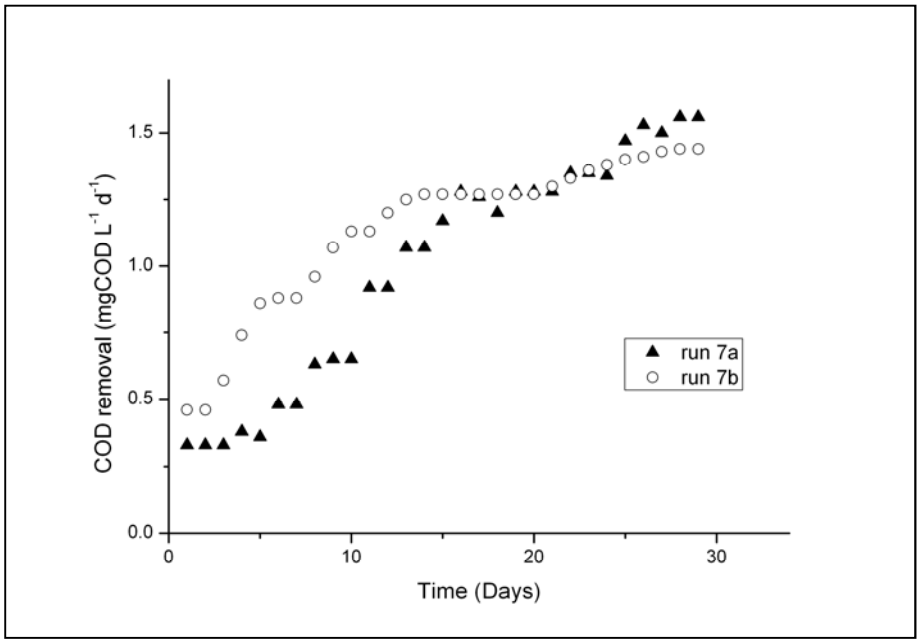

Figure SI 3-1: Comparison of anaerobic COD-removal in run 7a and 7b.

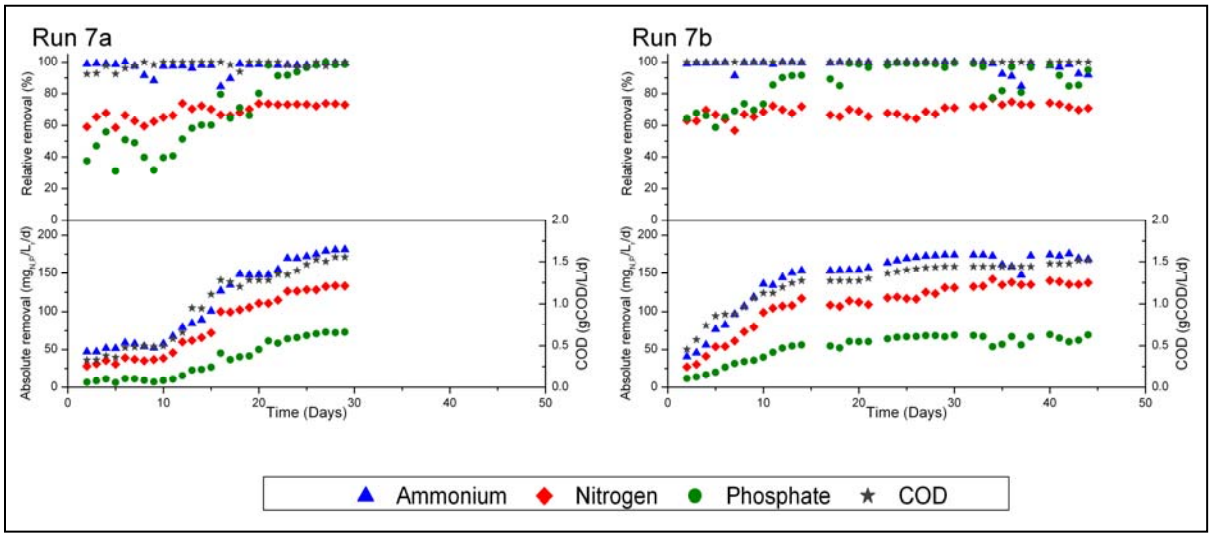

Figure SI 3-2: Comparison of removal performances in run 7a and 7b.

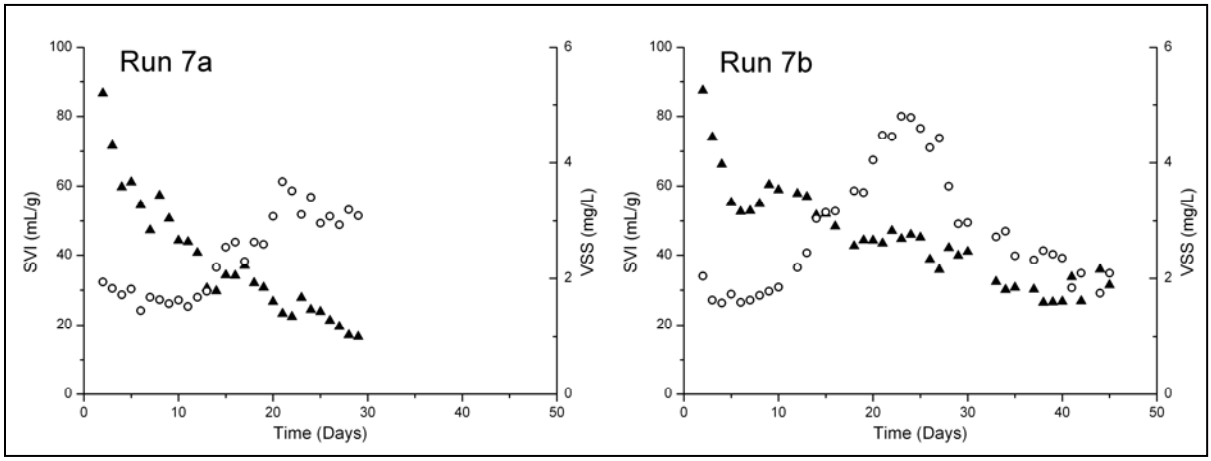

Figure SI 3-3: Comparison of SVI and VSS between run 7a and 7b.

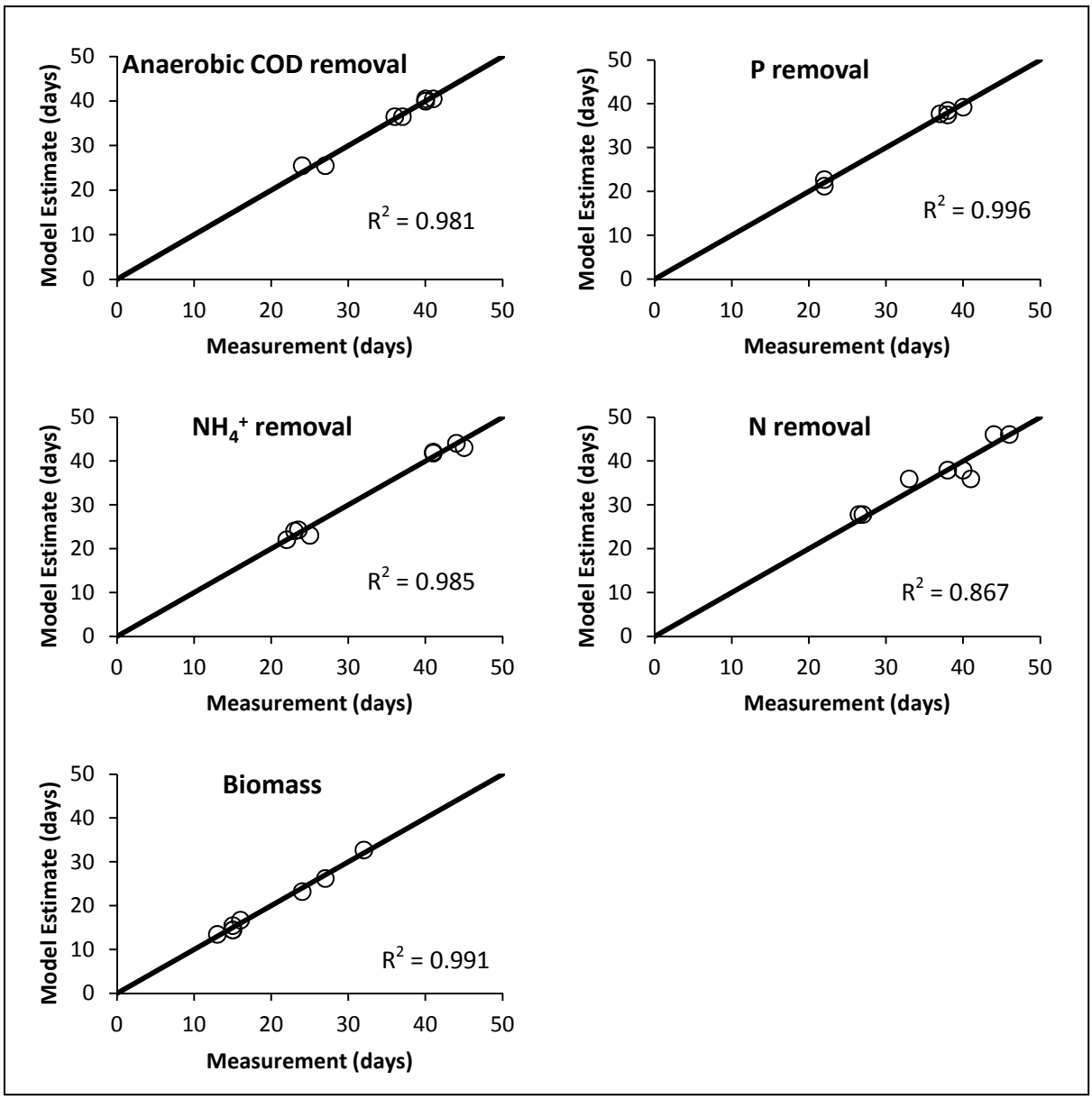

Figure SI 3-4: Comparison between model and experimental data for the five processes.

Bacterial Communities during Startup

4

4. Bacterial community dynamics during startup of aerobic granular sludge sequencing batch reactors

4.1 Introduction

Aerobic granular sludge (AGS) is formed by self-aggregation of microorganisms. The precise formation mechanism is not yet elucidated. Exopolysaccharides (EPS) are known to have an important influence on the structure of biofilms acting as 'microbial glue' (Sutherland 2001) and several studies showed that EPS play a key role for the formation and stability of AGS (Tay et al. 2001; Liu et al. 2004; Wang et al. 2006; Seviour et al. 2009). Moreover, differences between EPS in AGS and flocculent activated sludge have been shown (McSwain et al. 2005; Seviour et al. 2009; Lin et al. 2013). Moreover, based on the observation of physical differences between granules from the same reactor, it has been hypothesized that two different granulation mechanisms exist: microcolony outgrowth and microcolony aggregation (Barr et al. 2010).

In recent years, a rising number of studies investigated the bacterial community composition and evolution during the startup of AGS. In a startup study using *tert*-Butyl alcohol as carbon source, a low bacterial diversity with a predominance of α- and β-proteobacteria was observed (Tay et al. 2005). With acetate as sole carbon source *Thiothrix* was found to be predominant in early-stage granules and with propionate *Zoogloea* (Gonzalez-Gil and Holliger 2011). A predominance of *Zoogloea* has also been observed with selective discharge of small and slow-settling sludge flocs (Sheng et al. 2010) and under wash-out conditions (Weissbrodt et al. 2012b). It has been hypothesized that *Zoogloea* mainly proliferate when not enough biomass is present to consume the influent COD under anaerobic conditions due to washout. A study investigating the effect of different temperatures on the microbial community composition during startup reported also a predominance of *Zoogloea* in early-stage granules, for both 20°C and 30°C (Ebrahimi et al. 2010). After several weeks of operation the proliferation of *Rhodocyclaceae*-affiliated PAO was observed. However, after increasing the temperature to 35°C, the PAO population disappeared and putative GAO appeared instead. Cultivated under different organic loads *Zoogloea*, *Flavobacterium* and *Pseudomonas* were found to be predominant after granulation (Li et al. 2008). With COD loads up to 3 gCOD L^{-1} d^{-1} also *Comamonadaceae* and *Thauera* were observed to be predominant in the granular sludge.

For the assessment of the bacterial community in AGS different genetic fingerprint methods such as terminal-restriction fragment length polymorphism (T-RFLP) (Liu et al. 2008; Rossi et al. 2009),

denaturing gradient gel electrophoresis (DGGE) (Jiang et al. 2004; Tay et al. 2005), and more recently pyrosequencing (Weissbrodt et al. 2012a) have been applied.

The present study focused on the evolution of the bacterial community composition during the startup under different operation conditions described in Chapter 3. Eight experimental startup runs were conducted testing the influence of seven operation parameters in parallel. The bacterial community composition was assessed with T-RFLP combined with pyrosequencing of selected samples. The correlations between bacterial communities, operation conditions, and nutrient removal performances was investigated by multivariate analyses.

4.2 Materials and Methods

4.2.1 Setup of startup experiments

The influence of seven parameters on the startup of AGS with sequencing batch reactors (SBR) was investigated by a Plackett-Burman experimental design. Eight experimental runs were conducted over a period of 45 days. The tested parameters were: (1) pollution load, (2) aeration strategy, (3) addition of allylthiourea (ATU), (4) airflow velocity, (5) strategy to reduce the settling time, (6) pH, and (7) temperature (Table 4-1). For each parameter two levels were tested, according to the experimental matrix (Table SI 4-2). The N-, NH_4^+-, P-, and anaerobic COD-removal performances were measured, as well as the sludge volume index (SVI) and the volatile suspended solids (VSS). These startup experiments including detailed information on the parameters are described in Chapter 3. For experimental run 7, which was conducted twice, only run 7b was considered for this study.

Table 4-1: Tested operation parameters during AGS-SBR startup and the two tested levels of each parameter.

Parameters		Type	Negative (-1)	Positive (+1)
1	Pollution load	binomial	constant load	performance-adapted load
2	Aeration strategy	binomial	constant DO	alternate high-low DO
3	Allylthiourea	binomial	without	with
4	Airflow velocity	continuous	0.015 m/s	0.030 m/s
5	Settling time	binomial	strategy A	strategy B
6	pH	continuous	7.0-7.3	7.5-7.8
7	Temperature	continuous	15°C	20°C

4.2.2 Microbial analysis of bacterial community composition

A combination of T-RFLP and pyrosequencing was used to investigate the bacterial community composition. Mixed aliquots of biomass were taken three times per week and stored at -20°C. After each experimental run, the bacterial community composition of 7-9 samples was analyzed by T-RFLP as described in Chapter 2. Once all startup runs finished, two samples per run were analyzed in a common T-RFLP series to control for possible biases in the different T-RFLP series analyzed previously.

Seventeen samples were selected for in-depth analysis of the 16S rRNA gene pools by pyrosequencing as described in Weissbrodt et al. (2012b), two representative samples per experimental run plus one sample of inoculum sludge. The data from pyrosequencing was post-treated by PyroTRF-ID bioinformatics procedure (Weissbrodt et al. 2012a), which includes sequence annotation with the Greengenes database (McDonald et al. 2012). The denoised digital T-RFLP (dT-RFLP) profiles obtained were compared with the experimental T-RFLP (eT-RFLP) profiles, and corrected for base pair (bp) shifts if required. The corrected dT-RFLP results of the seventeen samples were summed by number of reads per phylotype contributing to the same terminal-restriction fragment (T-RF), to establish a phylogenetic annotation table representative for the whole eT-RFLP dataset.

4.2.3 Multivariate statistical analyses based on T-RFLP profiles

For the multivariate analyses to determine correlations between T-RFLP profiles and environmental variables (experimental conditions and measured nutrient removal and granule formation performances) R software with the additional package Vegan was used. The bacterial community dataset was transformed using a Hellinger transformation (Legendre and Gallagher 2001), and the removal performances were calculated as specific removal performances per g of VSS.

Multiple factor analysis (MFA) based on principal component analysis was chosen (Escofier and Pagès 1994; Bécue-Bertaut and Pagès 2008) to analyze the data. In a first time, RV multivariate correlation coefficients and p-values (Robert and Escoufier 1976) were calculated, to get numerical correlations between the environmental variables and the bacterial community structure (BCS). T-RFs and environmental variables were projected on a two-dimensional MFA ordination plot, to observe groups of related variables. Finally, the Spearman's rank correlation coefficient was calculated to further investigate correlations between single T-RFs and environmental variables. Based on the Spearman's rank correlation, a hierarchical clustering using the Ward's minimum variance algorithm was performed.

4.3 Results

4.3.1 Affiliation of T-RFs

Based on the results from eT-RFLP, seventeen T-RFs have been retained based on their frequency of detection in different samples and their relative abundance in the communities. Only T-RFs which were present in >10% of the samples or with an abundance >3% in at least one sample were taken into consideration for the T-RFLP profiles. PyroTRF-ID of seventeen samples led to the phylogenetic annotations presented in Table 4-2.

Table 4-2: Phylogenic annotation of selected eT-RFs.

T-RF[1] (bp)	Counts[2] (-)	Relative contribution to T-RF[3] (%)	Affiliation[4]
32	3411	42.6	F: *Rhodobacteraceae*
	1948	24.3	F: *Xanthomonadaceae*
	1497	18.7	F: *Saprospiraceae*
	678	8.5	F: *Flavobacteriaceae*
62	267	66.8	P: candidate phylum TM7
	26	6.5	G: *Tessaracoccus*
72	547	78.1	G: *Thauera*
185	871	39.1	O: *Rhizobiales*
	636	28.5	F: *Hyphomonodaceae*
	421	18.9	C: *Gammaproteobacteria*
193	7174	93.6	F: *Comamonadaceae*
195	11039	95.2	G: *Zoogloea*
208	1521	82	G: *Sphaerotilus*
214	4302	84.8	G: *Rhodocyclus*
	341	6.7	F: *Methyloversatilis*
215	28481	96.5	G: *Dechloromonas*
224	3371	80.1	F: *Intrasporangiaceae*
233	318	61.4	P: candidate phylum TM7
248	332	68.2	F: *Burkholderiaceae*
250	248	74.6	G: *Acinetobacter*
260	1571	84.0	O: *Sphingobacteriales*
	145	7.8	G: *Nitrospira*
264	1295	82.7	G: *Thiothrix*
285	167	74.5	F: *Sphingomonadaceae*
305	858	79.2	C: *Gammaproteobacteria*

[1] T-RFs according to eT-RFLP. Peaks from dT-RFLP were shifted to correspond to eT-RF.

[2] Total counts per phylotype for the corresponding T-RF over the 17 samples of the shifted and denoised dT-RFLP.

[3] Counts per phylotype divided by the total counts for the corresponding T-RF.

[4] Phylogenetic affiliation (P:phylum, C: class, O:order, F:family, G:genus). Only the last identified phylogenetic branch is presented here.

Only phylotypes which contributed for at least 5% to the T-RF were taken into account. For fifteen out of seventeen T-RFs one phylotype was clearly dominant. Only the two T-RFs 32 and 185 could not be clearly affiliated to one phylotype. For T-RF 32 *Rhodobacteraceae* was the most abundant (42.6%), followed by *Xanthomonadaceae* (24.3%) and *Saprospiraceae* (18.7%), for T-RF 185, *Rhizobiales* (39.1%), *Hyphomonodaceae* (28.5%), and *Gammaproteobacteria* (18.9%) were the most abundant phylotypes. Candidate phylum TM7 was affiliated to two T-RFs, namely T-RF 62 and 233. For T-RFs with a high numbers of counts, the relative contributions were very high. For example, *Dechloromonas* with >28'000 counts over the 17 samples represented 96.5% of T-RF 215. With lower number of counts the relative contribution decreased.

4.3.2 Comparison of bacterial communities in inoculation sludge

The T-RFLP profiles of the five inoculation sludges used for the startup of the different runs were very similar (Figure 4-1). The dominant T-RFs were in all five samples 224 (*Intrasporangiaceae*) with 28.1 - 35.8%, 32 (*Rhodobacteraceae, Xanthomonadaceae, Saprospiraceae, Flavobacteriaceae*) with 13.4 – 17.1%, and 260 (*Sphingobacteriales*) with 8.1 - 10.3% relative abundance. Other highly abundant T-RFs were 215 (*Dechloromonas*; 4.6 – 7.6%), 250 (*Acinetobacter*; 2.3 – 7.7%), and 248 (*Burkholderiaceae*; 1.8 – 7.1%). T-RFs 62 (candidate phylum TM7; 1.8 – 4.9%), 214 (*Rhodocyclus*; 1.8 – 4.8%), and 193 (*Comamonadaceae*; 1.4 – 2.6%) were also present in all inoculum samples. Correlation coefficients between the five inoculum samples were >0.92 (Table SI 4-2), confirming their high similarity and hence, comparable start conditions in the eight runs.

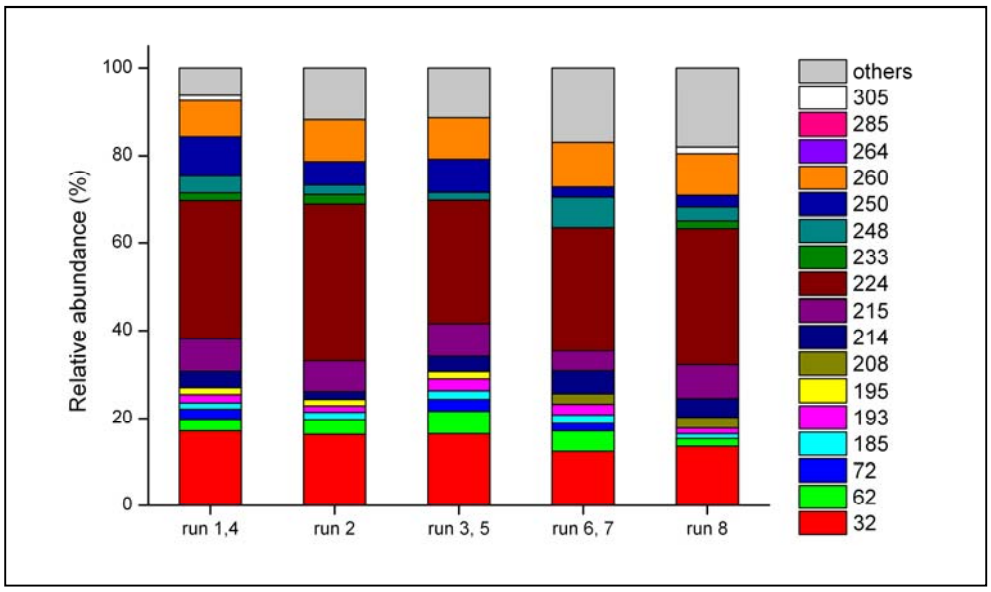

Figure 4-1: T-RFLP profiles of the inoculation sludge used in the different runs.

4.3.3 Evolution of bacterial communities

The seventeen retained T-RFs represented between 80-98% of the relative abundance of the T-RFLP profiles (Figure 4-2). The two samples per run analyzed in a common T-RFLP run at the end of the study showed high similarities with the surrounding samples concerning the dominant bacterial populations (Figure 4-2). In all eight runs, the initially dominant T-RF 224 (*Intrasporangiaceae*) decreased over time, and in most runs (1, 2, 3, 7b and 8), T-RF 215 (*Dechloromonas*) became dominant. T-RFs 195 (*Zoogloea*) and 305 (*Gammaproteobacteria*), hardly present in the inoculation sludge, proliferated in runs 1, 3, 5, 6, 7b and runs 1, 4, 6, 7b, respectively. However, the bacterial diversity slightly decreased in general during the startup, and T-RFs 62, 233 (both candidate phylum TM7), 248 (*Burkholderiaceae*), and 250 (*Acinetobacter*) typically disappeared during the first 10-15 days (Figure 4-2). On average, 13 of the 17 selected T-RFs were present in the inoculation sludge and only 9.5 in samples after day 30. The numerical calculation of the correlation coefficients between the cumulated bacterial abundance over the eight runs and the experiment duration confirmed a shift in the bacterial community composition over time: T-RFs 62, 224, 233, 248, 250 and 260 were strongly negatively correlated with the experiment duration, whereas T-RFs 72, 185, 195, 214, 215, 264, 285 and 305 were positively correlated (Table SI 4-3).

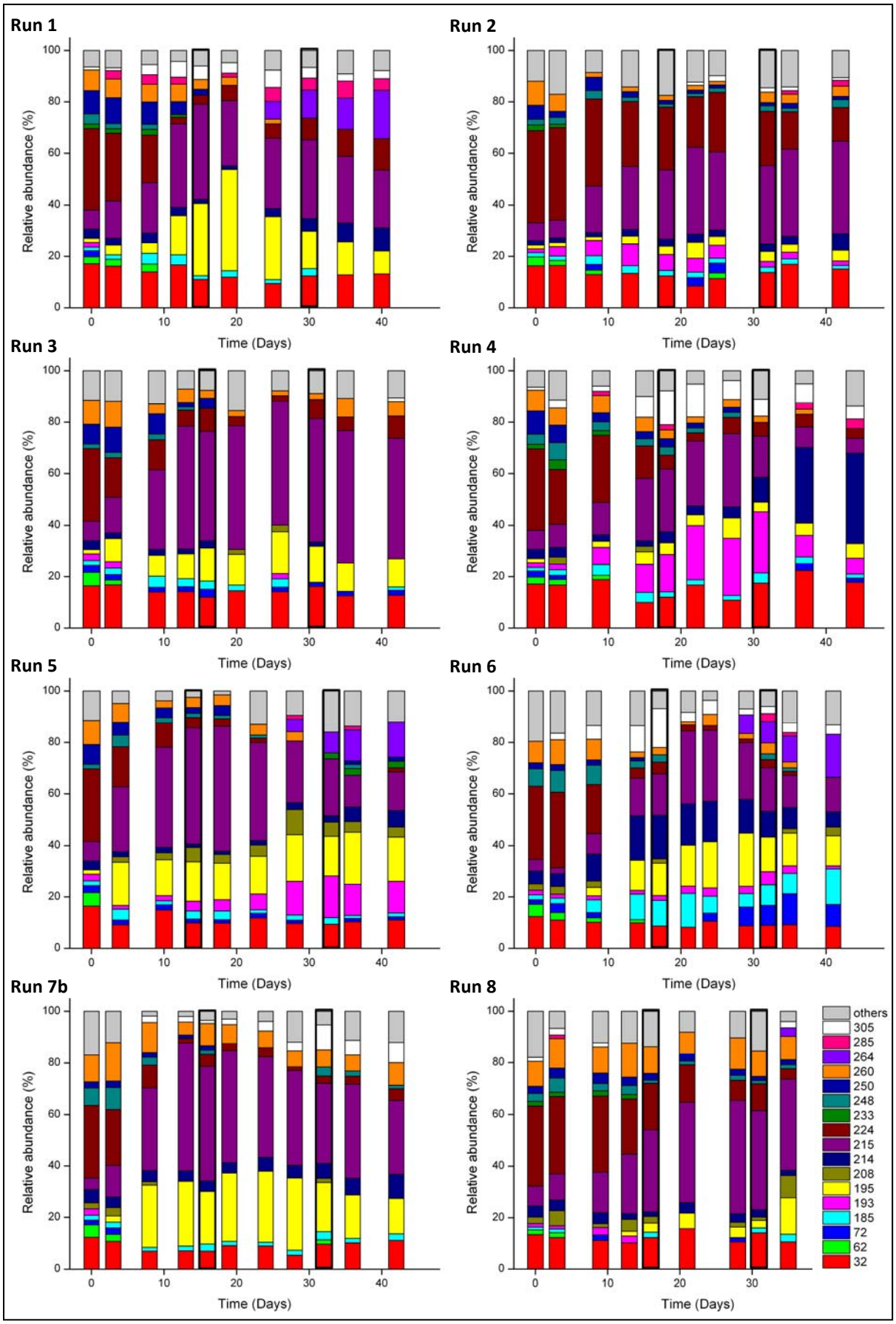

Figure 4-2: T-RFLP profiles of the different experimental runs. The bold frames indicate the samples that were analyzed at the end of all runs as control for biases of T-RFLP analysis.

4.3.4 Relationship between bacterial community structure and environmental variables

RV correlation coefficients between the BCS and environmental variables were calculated (Table 4-3). It was observed that the performances (i-v) had in general a higher correlation with BCS than the operation conditions (1-7). All correlations between performances and BCS were highly significant. The highest correlation was observed for the SVI. In contrast, for the operation conditions, only the parameters pollution load, pH and temperature had a highly significant correlation with BCS (Table 4-3).

Table 4-3: RV correlation coefficients calculated between the bacterial community dataset and each environmental variable. The p-values were calculated using a permutation test with 1000 permutations.

Environmental variables		RV coefficients	p-value	Significance[1]
i	Anaerobic COD-removal	0.33	<0.001	***
ii	NH_4^+-removal	0.22	<0.001	***
iii	N-removal	0.27	<0.001	***
iv	P-removal	0.28	<0.001	***
v	SVI	0.47	<0.001	***
1	Pollution load	0.18	<0.001	***
2	Aeration strategy	0.06	0.059	ns
3	Allylthiourea	0.06	0.051	ns
4	Airflow velocity	0.07	0.025	*
5	Settling time	0.07	0.031	*
6	pH	0.20	<0.001	***
7	Temperature	0.18	<0.001	***

[1] Degree of significance: $p < 0.001$: *** (highly significant); $p < 0.01$: ** (significant); $p < 0.05$: * (moderately significant); $p > 0.05$: ns (not significant).

4.3.5 Correlation between T-RFs and environmental variables

To observe correlations between T-RFs and environmental variables, a MFA ordination plot was created (Figure 4-3). The correlation between MFA and bacterial community, operation parameter and performances dataset was 0.71, 0.85 and 0.54, respectively. Hence, the operation parameters and the bacterial communities were better represented by the two dimensional MFA plot, than the performances. An important number of T-RFs (250, 224, 32, 62, 233, 248 and unaffiliated T-RFs)

were negatively correlated to the performances. T-RFs 214, 185 and 305 were positively correlated to the parameter pollution load, T-RF 193 to pH, and T-RF 195 to temperature.

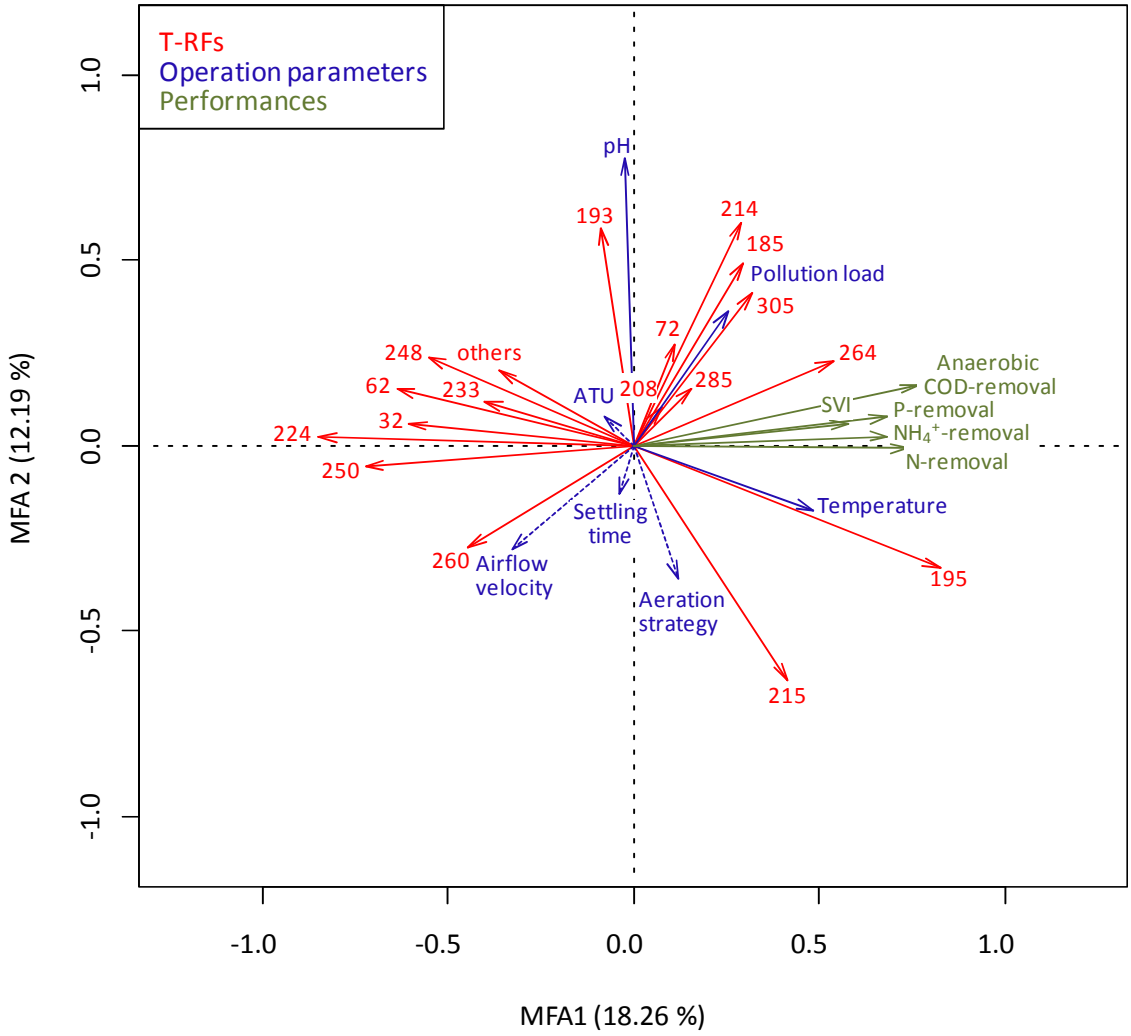

Figure 4-3: Multiple Factor Analysis (MFA) carried out on T-RFs and the environmental variables, representing the first two principal components (MFA1 and MFA2). The percentages indicated on the axes express the variance related to the axes. Operation conditions which were only moderately significant or not significant are represented with dashed lines.

For a deeper analyze of the correlations between T-RFs and environmental variables the Spearman's rank correlation was computed. Figure 4-4 depicts the correlation heatmap and the corresponding dendrogram. The bacterial communities could be separated in three clusters: Cluster I (T-RFs 195, 214, 215, 264, 285 and 305), cluster II (T-RFs 72, 185, 193, 208 and unaffiliated bacteria) and cluster III (32, 62, 224, 233, 248, 250 and 260) (Figure 4-4). Cluster I correlated positively with good removal performances, a low SVI and pollution load. Cluster III correlated negatively with all the mentioned variables, as well as with temperature. Cluster II was in general less correlated with the environmental variables. Looking at single T-RFs, the strongest positive correlations were observed

between T-RF 193 (*Comamonadaceae*) and pH, as well as between T-RFs 214 (*Rhodocyclus*), 305 (*Gammaproteobacteria*) and pollution load. TR-Fs 224 (*Intrasporangiaceae*) and 250 (*Acinetobacter*) were strongly negatively correlated with SVI, and T-RF 264 (*Thiothrix*) with the airflow velocity (Figure 4-4).

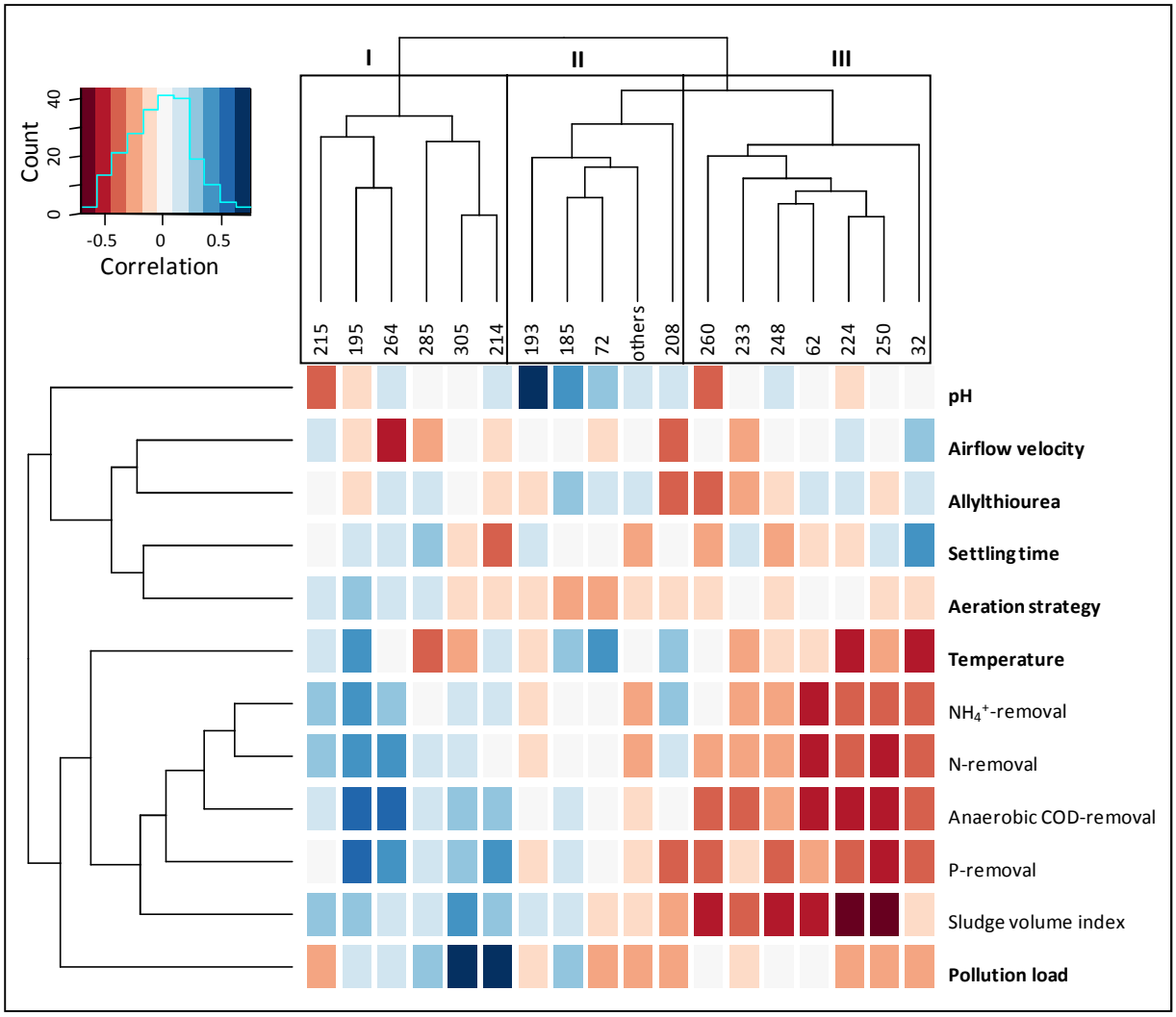

Figure 4-4: Heatmap of the pairwise correlations between T-RFs and environmental variables, based on the Spearman rank correlation. The operation conditions are in bold.

4.4 Discussion

The combination of T-RFLP with pyrosequencing of selected samples was successful to establish bacterial community profiles including the main T-RFs and to create the related affiliation table. Minor shifts of the measured length of eT-RFs are common in T-RFLP analyis, especially if different T-RFLP runs are performed over a long time period. If T-RFs with similar length contribute significantly to the biomass, as it is the case in this study with T-RF 214 (*Rhodocyclus*) and 215 (*Dechloromonas*), shifts of even <1bp can be problematic. The analysis of key samples by pyrosequencing was a

sensible solution to this problem. The affiliation table representing the whole T-RFLP dataset was created based on the results from pyrosequencing. Fifteen out of seventeen T-RFs could be affiliated to a dominant phylotype. The key was to cumulate the information of diverse pyrosequencing samples. For low-abundance populations there is in general no correspondence between eT-RFs and dT-RFs with the bioinformatics PyroTRF-ID approach (Weissbrodt et al. 2012a). The more pyrosequencing samples available, the higher are the chances to have a clear match between all selected eT-RFs and dT-RFs at least in one sample and hence, to be able to affiliate a phylotype to the T-RF.

However, the assessment of low-abundance population remains limited with the PyroTRF-ID approach, even with a high number of well selected pyrosequencing samples. T-RFLP does not allow to assess populations with very low abundances (<1% of community) (Dunbar et al. 2000; Bent et al. 2007). Slow-growing bacteria like nitrifiers typically playing an important role in AGS, were observed in pyrosequencing samples, but could not be clearly affiliated to an eT-RF. *Nitrosomonas* counted for 0.1% of the total reads in pyrosequencing samples, but no correspondent eT-RF was found with T-RFLP. *Nitrospira* (0.2%) could be affiliated to eT-RF 260, however the dominant phylotype for T-RF 260 was *Sphingobacteriales.* Hence, the nitrifying populations could not be integrated in the statistical analysis.

In relation to the environmental variables, the bacterial community could be regrouped in three clusters. The performances and not the operation conditions dominated this ordination. Cluster I was composed by bacteria positively correlated to good performances, cluster II by bacteria only weakly correlated to the performances, and cluster III by bacteria negatively correlated to the performances. T-RFs from cluster I were all positively correlated to the experiment duration, T-RFs from cluster III all negatively. Hence, the main shift in the bacterial community composition was not due to the specific operation conditions applied in the different runs, but it was a general shift from a more diverse flocculent sludge cultivated in a continuous flow full scale reactor towards a lab-scale AGS-SBR. This shift was characterized not only by a decrease in diversity, but also by a change of the predominant populations. The initially predominant *Intrasporangiaceae* and *Sphingobacteriales* decreased and a proliferation of *Dechloromonas* and in some runs of *Zoogloea* was observed. *Dechloromonas* has been observed to be the predominant population in enhanced biological phosphorus removal (EBPR) systems under certain conditions (Goel et al. 2005), and has been proposed as putative PAO (Kong et al. 2007). The proliferation of *Zoogloea* during the startup of AGS has been reported in several other studies (Ebrahimi et al. 2010; Sheng et al. 2010; Weissbrodt et al. 2012b). It has been hypothesized that the presence of volatile fatty acids (VFA) under aerobic

conditions was the main reason for the proliferation of *Zoogloea* (Weissbrodt et al. 2012b). The results from the present study did not support this hypothesis. Even in experimental runs with performance-adapted pollution loads (runs 1, 4, 6 and 7b) (Table SI 4-1), where all COD was consumed anaerobically, *Dechloromonas* and/or *Zoogloea* proliferated. Looking at the operation conditions, *Dechloromonas* was slightly correlated to a lower pH and *Zoogloea* to a higher temperature, but not to the parameter pollution load (Figure 4-4). Other studies on AGS reported a predominance of *Thiothrix* with acetate and *Zoogloea* with propionate as carbon source (Gonzalez-Gil and Holliger 2011), *Comamonadaceae* in denitrifying granules (Adav et al. 2010) and *Sphingomonas* at 35°C (Ebrahimi et al. 2010). Whether this indicates new physiological properties of members of the genera *Dechloromonas* and *Zoogloea,* remains to be elucidated.

Despite of this general shift in populations, independent of the operation conditions, some significant correlations between T-RFs and operation conditions were observed. T-RFs 214 (*Rhodocyclus*) and 305 (*Gammaproteobacteria*) were highly correlated to the pollution load parameter. Other T-RFs that positively correlated to the pollution load were 185 (Rhizobiales / *Hyphomonodaceae / Gammaproteobacteria*) and 285 (*Sphingomonadaceae*). With performance-adapted pollution loads and hence absence of COD in the aerobic phase, these populations were more abundant. Certain *Rhodocyclus*-affiliated bacteria are known to be a polyphosphate-accumulating organisms (PAO) (Hesselmann et al. 1999), and certain *Gammaproteobacteria* to be putative glycogen-accumulating organisms (GAO) (Nielsen et al. 1999; Crocetti et al. 2002; Kong et al. 2002), as well as some *Sphingomonadaceae* (Beer et al. 2004). Hence, these populations are probably able to take up VFA under anaerobic conditions which would explain their correlation with the pollution load parameter. Another high correlation was observed between T-RF 193 (*Comamonadaceae*) and pH. So far, no information on the influence of pH on *Comamonadaceae* is available in literature. Members of the family *Comamonadaceae* can be involved in denitrification (Khan et al. 2002). Hence, it can be hypothesized that they prefer slightly alkaline conditions, since in a study with activated sludge higher denitrification rates have been reported under alkaline conditions (Glass and Silverstein 1998). Negative correlations were found between temperature and T-RFs 32 and 224 (*Intrasporangiaceae*). *Tetrasphaera* spp. that are belonging to the *Intrasporangiaceae* family are putative PAO (Hanada et al. 2002; Kong et al. 2005). *Intrasporangiaceae* was the predominant population in the inoculation sludge but decreased in most runs drastically over time. Based on the results from this study it can be hypothesized that *Intrasporangiaceae* are more competitive at lower wastewater temperatures. Finally, T-RF 264 (*Thiothrix*), a genus of filamentous bacteria, was strongly negatively correlated to the airflow velocity. High shear stress from aeration has been reported to be important for the formation of

dense and smooth granules (Tay et al. 2004). Other filamentous populations such as T-RF 233 (candidate phylum TM7) and T-RF 208 (*Sphaerotilus*) are also negatively correlated to the airflow velocity.

Since no single T-RF highly correlated with the nutrient removal performances, the question remains which populations had key roles in biological nutrient removal in the experimental runs. The predominant PAO in the inoculation sludge provided by a wastewater treatment plant with EBPR were presumably *Intrasporangiaceae* affiliates. *Tetrasphaera* spp. affiliated to *Intrasporangiaceae* have been reported to be a predominant PAO in full-scale EBPR plants (Mielczarek et al. 2013). Since *Intrasporangiaceae* affiliates decreased over time, other population had to be involved in P-removal in the AGS-SBRs. *Candidatus* Accumulibacter phosphatis that is affiliated to *Rhodocyclus* was in most runs not predominant, hence, most likely *Dechloromonas*-like populations acted as PAO. Nitrifying bacteria were not abundant enough to be assessed by T-RFLP. However, from pyrosequencing samples it could be concluded that *Nitrosomonas* and *Nitrospira* were present. Besides of denitrifying PAO and GAO, *Thauera* (Liu et al. 2006), *Zoogloea* (Strand et al. 1988), and *Comamonadaceae* (Khan et al. 2002) were potential denitrifying populations in the present study.

4.5 Conclusion

The general shift of the predominant populations from *Intrasporangiaceae* and *Sphingobacteriales* to *Dechloromonas* and *Zoogloea* was mainly due to general conditions with lab-scale AGS-SBR, rather than the specific operation conditions in the different runs. However, it has been observed that relative abundance of PAO- and GAO-related populations were enhanced by the performance-adapted pollution load strategy as applied in this study. Besides pollution load, temperature and pH were the parameters with a significant impact on the global bacterial community structure. The predominant PAO population was presumably *Dechloromonas* in most runs.

The combination of T-RFLP with selected pyrosequencing samples was successful to establish bacterial community profiles including the main populations. However, nitrifying populations could not be assessed with due to their low abundance.

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Supplementary information

Table SI 4-1: Matrix of experimental design.

	Parameter						
Run	Pollution load	Aeration strategy	Allylthiourea	Airflow velocity	Settling time	pH	Temperature
1	+	+	+	-	+	-	-
2	-	+	+	+	-	+	-
3	-	-	+	+	+	-	+
4	+	-	-	+	+	+	-
5	-	+	-	-	+	+	+
6	+	-	+	-	-	+	+
7	+	+	-	+	-	-	+
8	-	-	-	-	-	-	-

Table SI 4-2: Correlation coefficients between the bacterial communities of the inoculation sludge samples of the different runs.

	run 1, 4	run 2	run 3, 5	run 6, 7b	run 8
run 1, 4	1				
run 2	0.987	1			
run 3, 5	0.981	0.981	1		
run 6, 7b	0.926	0.950	0.942	1	
run 8	0.945	0.971	0.953	0.977	1

Table SI 4-3: Correlation coefficients between T-RFs and duration of run.

T-RF[1] (bp)	Correlation to experiment duration[2]	Affiliation
32	-0.55	F: *Rhodobacteraceae* (42.6%), F: *Xanthomonadaceae* (24.3%), F: *Saprospiraceae* (18.7%), F: *Flavobacteriaceae* (8.5%)
62	-0.74	P: candidate phylum TM7 (66.7%), G: *Tessaracoccus* (6.5%)
72	0.59	G: *Thauera*
185	0.43	O: *Rhizobiales* (39.1%), F: *Hyphomonodaceae* (28.5%), C: *Gammaproteobacteria* (18.9%)
193	0.27	F: *Comamonadaceae*
195	0.71	G: *Zoogloea*
208	-0.05	G: *Sphaerotilus*
214	0.85	S: *Rhodocyclus tenuis* (84.8%), F: *Methyloversatilis* (6.7%)
215	0.45	G: *Dechloromonas*
224	-0.89	F: *Intrasporangiaceae*
233	-0.70	P: candidate phylum TM7
248	-0.84	F: *Burkholderiaceae*
250	-0.93	F: *Acinetobacter*
260	-0.87	O: *Sphingobacteriales* (84.0%), G: *Nitrospira* (7.8%)
264	0.88	F: *Thiothrix*
285	0.46	F: *Sphingomonadaceae*
305	0.69	C: *Gammaproteobacteria*

[1] T-RFs according to eT-RFLP.

[2] Correlation coefficients between T-RFs and duration of the experimental runs.

Aeration Strategies for N-removal

5

5. Optimized aeration strategies for nitrogen and phosphorus removal with aerobic granular sludge

5.1 Introduction

The aerobic granular sludge process is a promising technology for biological wastewater treatment. It offers the possibility to remove organic carbon (hereafter referred to as chemical oxygen demand, COD), nitrogen (N) and phosphorus (P) in a single reactor (Zeng et al. 2003; de Kreuk et al. 2005). One of the major challenges of this technology is the efficient N-removal, a two-step process with first, nitrification under aerobic conditons followed by denitrification under anoxic conditions and requiring COD. The association of these two processes in a single stage system is delicate and can result in considerable accumulation of nitrate (NO_3^-) or nitrite (NO_2^-) in the effluent. Residual effluent NO_x^- concentrations should be minimized to protect the aquatic ecosystem, and to prevent deterioration of the P-removal in reactors operated in sequencing batch mode (Furumai et al. 1999; Peng et al. 2010).

In biofilms of a certain thickness, nitrification and denitrification can occur simultaneously (SND) during aeration (Münch et al. 1996; Keller et al. 1997) since the penetration depth of oxygen is limited due to microbial activity. Hence, the outer layer of aerobic granules is aerobic allowing nitrification and the inner layer anoxic allowing denitrification. The main parameters governing SND in granular sludge are the concentration of dissolved oxygen (DO) in the bulk liquid, the granule size, and the microbial activity. Several studies have shown that a higher DO favors nitrification but limits denitrification, whereas a lower DO enhances denitrification but limits nitrification (Beun et al. 2001; de Kreuk et al. 2005; Mosquera-Corral et al. 2005). For the granule size, a maximal N removal was observed with average diameters of 1.3 mm (de Kreuk et al. 2005). However, no strategy to control the granule size is yet known. Parameters influencing the granule size are the food-to-microorganism ratio (Li et al. 2011) and the hydrodynamics during the mixing phase (Tay et al. 2004).

Besides the spatial dimension, there is also a temporal dimension influencing the oxygen penetration depth in granular biofilm. Modeling studies and micro-sensor measurements have shown that oxygen penetrated much deeper into the granules at the end of the aeration phase than at the beginning because of decreased aerobic microbial activity towards the end of aeration (de Kreuk et al. 2007; Yilmaz et al. 2007). Once COD oxidized and ammonium completely nitrified, oxygen fully

penetrates the biofilm (Meyer et al. 2005) which also stops denitrification because of inhibition by oxygen.

In addition to too high DO, the COD to N and P ratio in the influent wastewater can be a reason for impaired denitrification. In combined N- and enhanced biological P-removal (EBPR) systems, the COD concentration in the influent is often the limiting parameter (Kuba et al. 1996; Keller et al. 1997). During anaerobic feeding, polyphosphate-accumulating organisms (PAO) and glycogen-accumulating organisms (GAO) store carbon as polyhydroxyalkanoates. These intracellular stocks of COD allow denitrification during the aeration phase in the anoxic parts of the biofilm. Denitrification by PAO with concurrent ortho-phosphate uptake is called denitrifying P-removal and allows saving COD.

From an operating perspective, lowering the DO has been proposed as solution to improve SND in aerobic granular sludge (Beun et al. 2001). This has been confirmed by lab-scale tests (de Kreuk et al. 2005), however it has also been reported that a lower DO can lead to granule disintegration (Mosquera-Corral et al. 2005). Besides a lower DO, the addition of an anoxic phase after aeration has been tested to remove accumulated NO_x^- (Yilmaz et al. 2007). With a relatively high COD to N and P ratio (COD:N:P = 20:2.5:1) and mainly N-removal over nitrite, this study reported removal efficiencies for soluble COD, N and P of over 80%. The introduction of an anoxic phase leads to a temporal separation of nitrification and denitrification. Instead of SND, the main N-removal occurs via alternating nitrification and denitrification (AND). A modeling study has come to the conclusion that AND would have a higher N-removal potential than SND (Xavier et al. 2007).

The present study investigated the potential of different aeration strategies to reach maximum efficiency of both N- and P-removal. We tested two strategies for optimized SND and two for AND. In addition, we also varied the influent COD concentrations to detect limitations due to lack of organic substrate.

5.2 Materials and Methods

5.2.1 Reactor setup

Two similar bubble-column SBRs were used, with designs described in (Ebrahimi et al. 2010) and (Weissbrodt et al. 2012), respectively. The first had an internal diameter of 5.2 cm and a working volume of 2.4 L, whereas the second had an internal diameter of 6 cm and a working volume of 3.5 L. The effluent withdrawal point was positioned at a height of 54 and 61 cm, respectively, resulting in equal volumetric exchange ratios per cycle of 50%. The SBR cycle length was 3 hours with 60 min of

anaerobic plug-flow feeding, 112 min of aeration, 3 min of settling and 5 min of withdrawal. The hydraulic retention time was for both reactors 6 hours. The temperature was controlled at 20°C and the pH was regulated between 7.0-7.3 by adding 1 mol L^{-1} NaOH and HCl solutions.

The up-flow gas flow rate was maintained constant at 3.6 L min^{-1} with headspace gas recirculation pumps (KNF Laboport). DO setpoints were varied according to the defined oxygenation strategies. To control DO, an off-gas recirculation system was installed with the possibility to add air or nitrogen gas, as described by Mosquera-Corral *et al*. (2005).

5.2.2 Synthetic wastewater composition

The influent consisted of a mixture of two synthetic media and tap water adapted from de Kreuk *et al.* (2005). For the smaller reactor 130 ml of each medium was diluted with 940 ml of tap water and for the bigger reactor 190 ml of each medium with 1370 ml of tap water, resulting in the same influent concentrations. Medium A was composed of 3.51 g L^{-1} of sodium propionate, 0.89 g L^{-1} of $MgSO_4 \cdot 7H_2O$, and 0.36 g L^{-1} of KCl, and medium B of 1.89 g L^{-1} of NH_4Cl, 0.73 g L^{-1} of K_2HPO_4, 0.23 g L^{-1} of KH_2PO_4, and 5 mL L^{-1} of a trace element solution as described by Vishniac and Santer (1957). For the tests conducted with higher COD loading rates, the concentration of sodium propionate was increased to 4.61 g L^{-1} and 5.27 g L^{-1} in Medium A.

5.2.3 Analytical methods

Concentrations of propionate and of inorganic cations and anions such as ammonium (NH_4^+), nitrate (NO_3^-), nitrite (NO_2^-), and orthophosphate (PO_4^{3-}) were measured by high performance liquid chromatograpgy and ion chromatography, respectively, as described previously (Gonzalez-Gil and Holliger 2011). DO and pH were measured online with electrodes and controlled by control loops.

Nitrous oxide (N_2O) in the off-gas was measured by gas chromatography (HP 5890 series II, RTX-502.2 column, electrical conductivity detector). Discrete samples of the effluent gas were taken with a gas-tight syringe during the aeration phase and directly analyzed. Since we recirculated the headspace gas, these measured concentrations did not correspond to the real emissions. Experiments with tap water and N_2O as a tracer gas were conducted under different aeration conditions, to determine the ratio between recirculated gas and gas leaving the system. The tracer gas N_2O was at a concentration of 200 ppm, and the tests were conducted with air supplies of 0, 1, 2 and 3 L min^{-1}. Based on these results a model was establishing allowing calculating the actual N_2O emissions. The detection limit of the gas chromatography measurement was approximately 1 ppm.

However due to the accumulation of gaseous compounds generated by gas recirculation loops, even much lower real gas concentrations could be calculated.

The ImageJ software (http://rsb.info.nih.gov/ij/index.html, Accessed 2012-11-19) was used to analyze the size and circularity of the granules. The diameters were calculated as circular equivalent diameters (Tijhuis et al. 1994) by only taking particles with a diameter > 0.2 mm into account (de Kreuk et al. 2007). The biomass volume was calculated by the same principle, as sphere of same surface area.

5.2.4 Denitrification tests

Denitrification tests under anoxic conditions were carried out *in situ* in the reactor and in off-line batch tests. *In situ* tests were conducted between day 285 and day 332 before investigating the different aeration strategies. After the anaerobic feeding phase, the reactor was mixed with N_2 gas instead of air. Anoxic conditions were generated by injecting a concentrated solution of $NaNO_3$ or $NaNO_2$. The starting NO_x^- concentration was in all tests between 20 and 60 mgN L^{-1}, similar to the concentrations measured during normal aeration phases.

Five denitrification batch tests were performed over a period of 12 months with NO_2^- and NO_3^- as terminal electron acceptors. Aliquots of 200 ml of granular sludge under mixed conditions (about 2.5 gVSS) were taken from the reactors after the anaerobic feeding phase. The sludge sample was placed in an Erlenmeyer flask, where 600 ml of a $NaNO_3$ or $NaNO_2$ solution were added. The starting concentration was again between 20 and 60 mgN L^{-1}. To have strict anoxic conditions, the sludge was mixed with N_2 gas, similar as in the *in situ* tests.

Denitrification efficiencies were determined by collecting liquid phase samples every 20 minutes over a period of at least 2 hours. The samples were analyzed by ion chromatography to observe the evolution of NO_3^-, NO_2^-, NH_4^+ and PO_4^{3-}.

5.2.5 Aeration strategies

Four different aeration strategies were tested: (i) optimized constant DO, (ii) headspace gas recirculation, (iii) alternating high and low DO, and (iv) intermittent aeration. The strategies (i) and (ii) were tested for SND. The strategies (iii) and (iv) were used to promote AND. The optimal constant set-point for strategy (i) was obtained by lowering DO such as to allow SND during the whole aeration phase, hence such that ammonium was still present at the end of the aeration phase, although at very low concentrations. The SND strategy (ii) consisted of an aeration phase at a DO of

50% saturation for a certain period of time followed by an aeration phase with a hermetically closed headspace recirculation until the start of the settling phase. A lower limit of 5% DO was defined to allow a minimum of nitrification until the end of the aeration phase. The AND strategy (iii) consisted of three 20-minute periods with high DO that were interrupted by periods with only 5% DO (2 periods of 20 min and a final period of 12 min). The initial values of the high DO phases were 50%, 40% and 30% saturation, respectively. N_2 gas was added into the aeration gas flow to reach rapidly low DO and keep the gas flow constant. The AND strategy (iv) consisted of intermittent aeration that included six aeration pulses of 6-10 min that were interrupted by periods of 7-12 min where the sludge was neither aerated nor mixed. Before the start of the settling phase, a 2-min aeration phase mixed the sludge once again before settling. Before comparing the different strategies, they have been optimized to reach maximal N-removal efficiencies under the corresponding aeration regime. This included adaptation of the duration of the aeration phases and the DO levels.

5.2.6 Experimental schedule

The granular sludge used in the present work was taken from a reactor that has been operated for more than 18 months (Gonzalez-Gil and Holliger 2011). During the start-up period of this reactor, DO was not controlled and no excess sludge was purged. At day 553, the granular sludge was partitioned into two reactors.

From day 554 to day 669, Reactor 1 was operated with alternating high and low DO, and Reactor 2 with headspace gas recirculation. During this period no sludge was purged in order to increase the biomass concentration. Moreover, several tests were undertaken for a better understanding of the system control, as well as for optimizing the aeration strategies.

At day 670, the systematic tests of the different aeration strategies were started (Table 5-1). The biomass from the two reactors were mixed and re-partitioned between the two reactors, to start experiments with identical sludge composition. During the rest of the study, excess sludge was purged to maintain the concentration of total suspended solids (TSS) between 15-20 g L^{-1}, resulting in a sludge retention time (SRT) of approximately 30 days. Sludge was purged from the bottom of the reactor at the very end of the SBR cycle just before restarting the new cycle. Depending on biomass increase in the reactor, sludge was purged up to two times per week.

Table 5-1: Experimental conditions during operation of parent and experimental reactors.

Reactor	Control of DO/SRT[1]	COD loading rate (gCOD L_r^{-1} d^{-1})	Aeration strategy	Period (days)
Parent reactor	no/no	1.6	uncontrolled high DO	1-341
			uncontrolled head-space gas recirculation	342-481
			uncontrolled high DO	482-553
Reactor 1	yes/no	1.6	alternating high/low DO	554-669
	yes/yes	1.6	alternating high/low DO	670-854
		2.1	alternating high/low DO	855-978
		2.4	alternating high/low DO	979-1016
		2.1	optimized constant DO	1017-1036
		1.6	intermittent aeration	1037-1095
Reactor 2	yes/no	1.6	headspace gas recirculation	554-669
	yes/yes	1.6	headspace gas recirculation	670-877
		2.1	headspace gas recirculation	878-978
		2.4	headspace gas recirculation	979-1021
		1.6	optimized constant DO	1022-1095

[1] DO was controlled by the supply of N_2 gas or air. The SRT was controlled by purging excess sludge at the bottom of the reactor and was kept at about 30 days.

5.3 Results

5.3.1 Denitrification activity of the aerobic granular sludge

The denitrification capacity of the granular sludge was tested to evaluate whether the potential for full N-removal was present and whether denitrifying PAO and GAO could be involved in N-removal. *In situ* denitrification tests conducted in the SBRs with nitrate amendment displayed N-removal of 31.3 ±9.5 mgN L_r^{-1} during the aeration phase of 112 minutes. This resulted in an apparent daily denitrification potential of 240 mgN L_r^{-1} d^{-1}, hence 20% more than the daily nitrogen load. The denitrification rate over nitrite with 55.7 ±13.5 mgN L_r^{-1} removed within 112 minutes was about twice the rate over nitrate. By taking the biomass concentrations of 12-15 gVSS L^{-1} into account, the average specific denitrification rate was 1.24 ±0.15 mgN h^{-1} $gVSS^{-1}$ and 2.50 ±0.08 mgN h^{-1} $gVSS^{-1}$ in tests conducted with nitrate and nitrite, respectively (Table 5-2).

Table 5-2: Specific denitrification rates of aerobic granular sludge biomass determined with *in situ* and batch tests.

Test type	Specific denitrification rate with	
	Nitrate ($mgN\ h^{-1}\ gVSS^{-1}$)	Nitrite ($mgN\ h^{-1}\ gVSS^{-1}$)
In situ tests [1]	1.24 ± 0.15	2.50 ± 0.08
Batch tests [2]	1.46 ± 0.48	2.46 ± 0.56

[1] *In situ* tests were carried out on days 285, 305, 316, and 332.

[2] *Ex situ* batch tests were carried out on days 706, 785, 891, 955, and 1061.

In addition to *in situ* denitrification tests, also *ex situ* batch tests were conducted on a regular basis over the whole period of the aeration strategy experiments which confirmed the results of the *in situ* tests. The specific nitrate removal rate in batch tests was 1.46 ±0.48 $mgN\ h^{-1}\ gVSS^{-1}$ whereas the average specific nitrite removal rate was 2.46 ±0.56 $mgN\ h^{-1}\ gVSS^{-1}$. In all tests, the specific denitrification rate was higher for nitrite than for nitrate. This difference between nitrite and nitrate removal was significant in both *in situ* and batch test with p-values < 0.001 and <0.01, respectively. Furthermore, in all denitrification tests a decrease in orthophosphate concentration could be observed, indicating that denitrifying P-removal occurred.

5.3.2 Optimization approach of the different aeration strategies

The objective of the study was to compare the different aeration strategies according to their maximal potential for total N-removal. Hence, the strategies had first to be optimized to make them comparable. According to the period where the SBRs where operated with full aeration and without DO control, we observed that the optimum in N-removal was reached when still some NH_4^+ as well as NO_x^- was present in the effluent indicating an optimal balance between nitrification and denitrification. In this optimal zone, minor adaptations of the oxygen supply changed the ratio between NH_4^+ and NO_x^- in the effluent, but the total N-removal remained stable as depicted in Figure 5-1 for the SND aeration strategy (ii). For this aeration strategy, the supply of oxygen was controlled by the duration of the initial aeration phase before switching to a closed headspace gas recirculation. With a shorter initial aeration phase, NH_4^+ was the dominant N pollutant in the effluent, whereas with a slightly longer initial aeration time, NO_x^- became dominant. In the following, all aeration strategies tested were first optimized to obtain an effluent containing both NH_4^+ and NO_x^- and then results were taken for comparison of the different strategies.

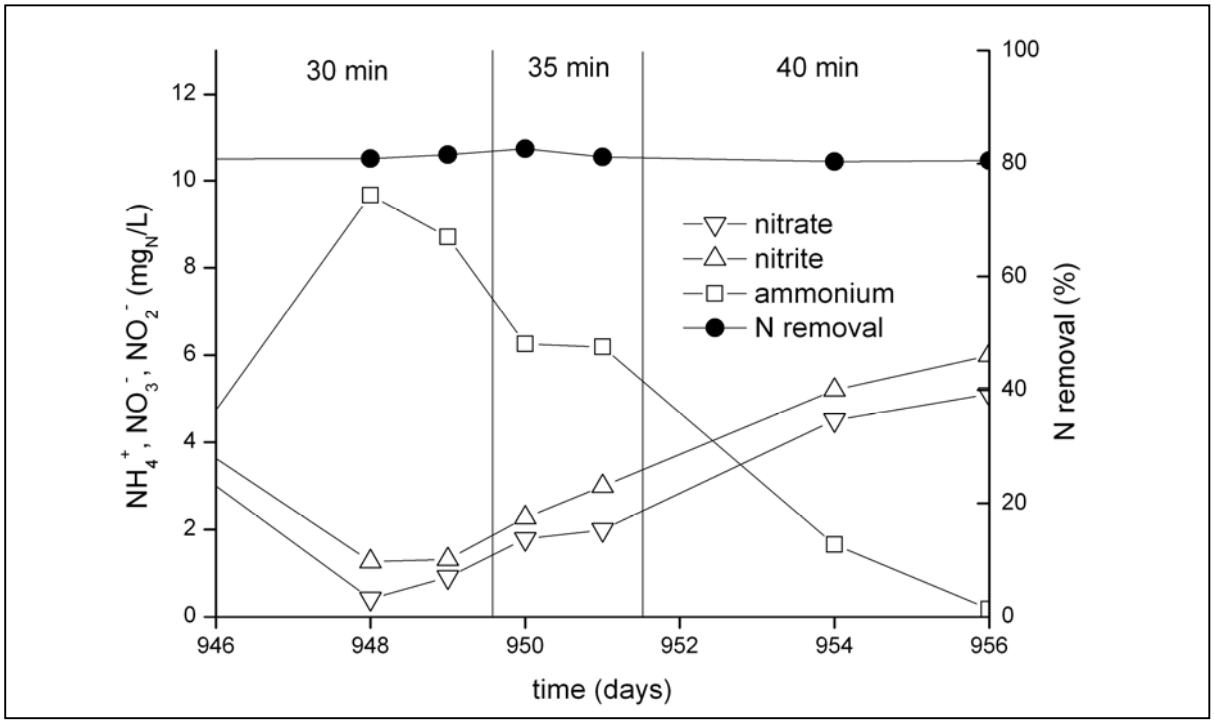

Figure 5-1: Effect of the duration of the initial aeration phase in the headspace gas recirculation strategy on the residual concentrations of N pollutants in the effluent.

5.3.3 Dissolved oxygen profiles of the different aeration strategies

During the start-up period, the parent reactor was run with a constant up-flow aeration of 3.6 L of air min^{-1}. No excess sludge was purged, which resulted in a sludge concentration of 20 - 25 gTSS L^{-1} and a SRT of about 40 days between days 195 and 313. During this period of uncontrolled aeration, DO reached 80% of saturation at the beginning and approximately 95% at the end of the aeration phase (Figure 5-2A). After 16 months, the sludge concentration was around 30 - 35 gTSS L^{-1} and the SRT was more than 50 days. DO reached only 50 - 60% at the beginning of the aeration phase with the same constant aeration of 3.6 L min^{-1}. However, at the end of the aeration phase, the DO was again as high as 95% (Figure 5-2B).

The optimal constant DO for SND strategy (i), the set-point where some ammonium was still present at the end of the aeration phase, fluctuated between 25 and 40%. A typical profile of an SBR cycle operated with SND strategy (i) is depicted in Figure 5-2C where DO was at 28% of saturation. To further optimize SND, the aeration strategy (ii) with headspace gas recirculation in a closed system was tested. After a certain period of aeration at a DO of 50% saturation, a switch to a hermetic headspace recirculation was conducted that led to a decrease in DO due to microbial activity (Figure 5-2D). The optimum according to effluent ammonium concentration was reached at durations of the initial aeration phase at 50% DO of 30-40 min (Figure 5-1).

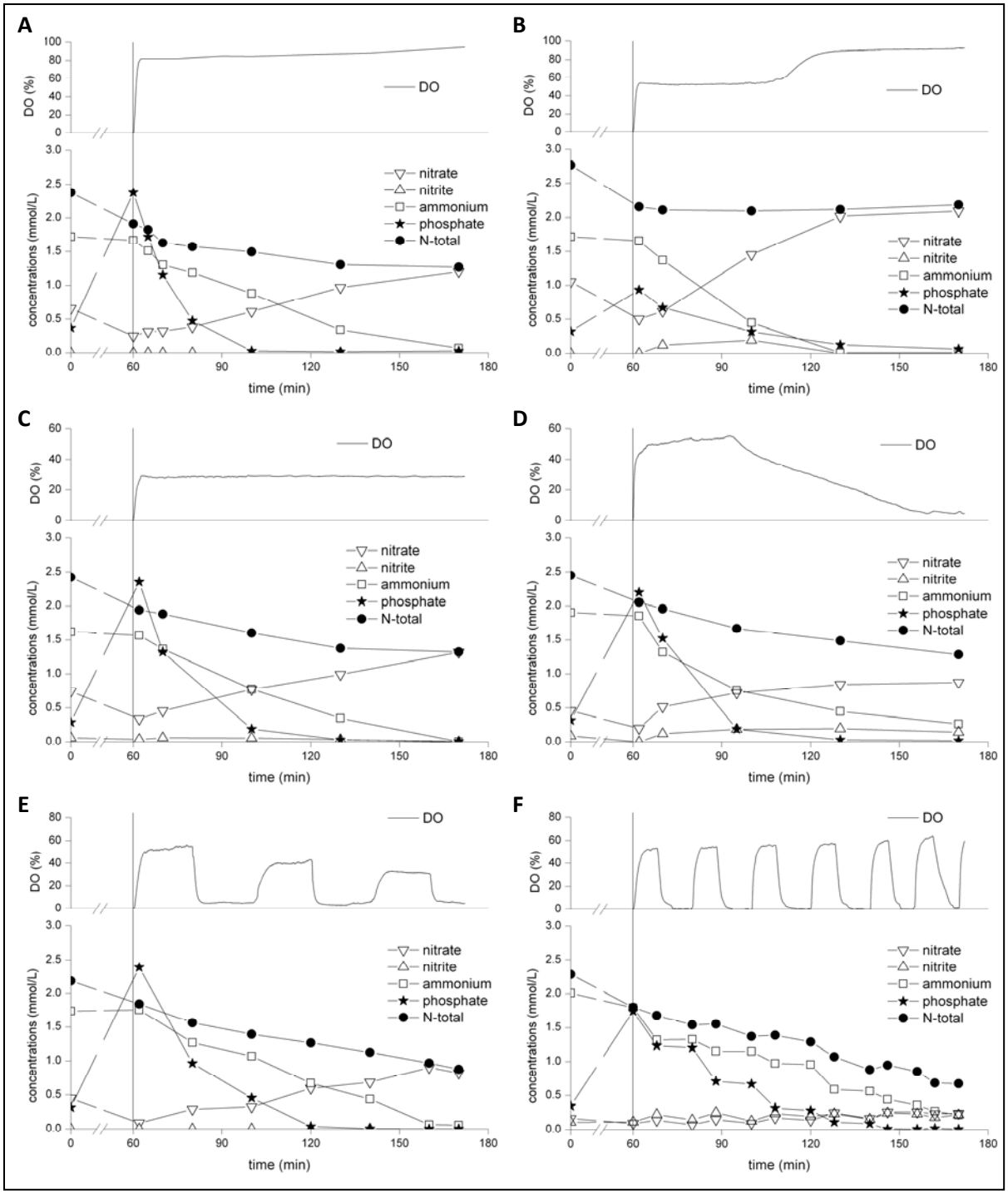

Figure 5-2: Evolution of oxygen and nutrient concentrations during a cycle of the AGS-SBRs operated with different aeration startegies: **(A)** uncontrolled high DO on day 267, **(B)** uncontrolled high DO on day 503, **(C)** optimized DO, **(D)** headspace gas recirculation, **(E)** alternating high/low DO, and **(F)** intermittent aeration. The COD loading rate was 1.6 gCOD L_r^{-1} day^{-1} for all cycles depicted. The initial concentrations shown at t = 0 are the theoretical concentrations if the influent wastewater would be fed all at once and not in plug-flow mode over 60 min. They are calculated based on the measured influent and effluent concentrations.

AND aeration strategy (iii) with alternating phases with high and low DO (Figure 5-2E) was optimized by slightly increasing the set-points of the high DO phases. The optimum was reached with set-points of 50-55%, 40-45%, and 30-35% for the three high DO phases, respectively. For AND strategy (iv) involving intermittent aeration which is a realistic strategy for full-scale applications, DO reached values of approximately 60% saturation during the aerated phases and resulted in completely anoxic phases during the non-aerated phases (Figure 5-2F). Initially, intermittent aeration was run with aerated periods of 4-times 10 min, 2-times 8 min, and a final period of 2 min, resulting in a total aerated time of 58 min. These aerated phases were interrupted by 4 phases of 10 min and 2 of 7 min where the aeration was switched off. To optimize the strategy, the aerated phases were successively shortened. Optimal N-removal was reached with 4 periods of 8 min, 2 periods of 6 min and one period of 2 min. All non-aerated periods were extended by 2 min in order to keep the total time at 112 min. Under these optimized conditions, the sludge was only aerated during 46 min, a bit less than half the time of the normal aeration phase.

5.3.4 Nutrient removal performances with the different aeration strategies

5.3.4.1 Constant high aeration

During the operation of the parent reactor, the nutrient removal performances became stable after approximately 6 months. More than 90% of P and around 60% of N were removed from the liquid phase (Table 5-3). Nitrification was complete and the remaining dissolved N was present in form of NO_3^-. However, ammonium was present until almost the end of the aeration phase. In contrast, most orthophosphate was removed during the first 40 min of aeration (Figure 5-2A). The yield of P-release per propionate uptake during the anaerobic phase was 0.39 ± 0.14 P-mol C-mol^{-1}.

In a later stage (day 488 to 525) with still constant high aeration, N-removal decreased to 40.0 ± 3.4%. Ammonium was completely nitrified after 50 to 70 min of aeration and massive NO_3^- accumulation was observed (Figure 5-2B). SND was absent and denitrification took only place during the feeding phase. P-removal was 88.3 ± 7.4% and the yield of P-release per propionate uptake during the anaerobic phase decreased to 0.18 ± 0.09 P-mol C-mol^{-1}.

5.3.4.2 Optimized simultaneous nitrification-denitrification (SND)

With optimized low constant DO, the total N-removal increased to 61.2 ± 5.2%. The air supplied to maintain a constant DO progressively decreased during the aeration phase. At the beginning of the aeration phase, a specific oxygen uptake rate (SOUR) of 0.224 ± 0.022 mgO_2 min^{-1} gVSS^{-1} was measured. At the end of the aeration phase, the SOUR was 0.100 ± 0.037 mgO_2 min^{-1} gVSS^{-1} in the

case where some NH_4^+ was still present and only 0.017 ± 0.002 mgO_2 min^{-1} $gVSS^{-1}$ in the case where all NH_4^+ was nitrified. This lower aerobic activity probably led to a deeper oxygen penetration into the granules and therefore to a smaller inner anoxic zone.

With headspace gas recirculation, DO decreased gradually during the aeration phase. The aim was to have a more stable equilibrium over the whole aeration phase between aerobic and anoxic zones which could have led to a further improvement of SND. N-removal with this aeration strategy was very similar compared with optimized constant DO (Table 5-3). To test whether denitrification was limited due to lack of COD, COD loading rate was increased from 1.6 to 2.1 gCOD L_r^{-1} day^{-1} which improved N-removal to 77.4 ± 3.5% and 80.5 ± 3.6% for constant DO and headspace gas recirculation, respectively. For the headspace gas recirculation, a further increase to 2.4 gCOD L_r^{-1} day^{-1} resulted in an increase in N-removal of another 4%. P-removal was almost 100% for optimized constant low DO and between 90-95% for the headspace gas recirculation strategy (Table 5-3). The yield of P-release per propionate uptake during the anaerobic phase was for all optimized SND strategies between 0.34 - 0.38 P-mol C-mol^{-1}.

5.3.4.3 Alternating nitrification-denitrification (AND)

Aeration by alternating high and low DO periods markedly improved denitrification. N-removal was about 10% higher compared with headspace gas recirculation for all three tested influent COD concentrations (Table 5-3). The intermittent aeration further increased N-removal to 78.3 ± 2.9% with the initial low COD loading rate of 1.6 gCOD L_r^{-1} day^{-1}. Hence, N-removal was 7% higher than for alternating high and low DO and even 17% than for headspace gas recirculation. P-removal was around 95% for both tested AND aeration strategies. No sludge stability problems occurred with intermittent aeration, even if the total mixing time was reduced to less than one third of the total cycle time.

Analyzing separately the high and low DO phases showed that 66% of nitrification and 40% of denitrification occurred during the high DO phases, and the other 34% and 60%, respectively, during the low DO phases. An uptake of 76% of orthophosphate was observed during the high DO phases and only 24% during the low DO phases. With intermittent aeration, the differences were even higher. More than 90% of nitrification and P-removal took place during the aerated phases, and only about 10% of NH_4^+ and 8.5% of orthophosphate was removed during the non-aerated phases. Denitrification occurred for about 65% during the non-aerated phases and 35% during the aerated phases. Taking the whole SBR cycle into account, up to 25% of denitrification took place during the anaerobic feeding phase, depending on how much NO_x^- was present at the end of the SBR cycle.

Table 5-3: Average N- and P-removal efficiencies obtained in granular sludge SBRs run with different aeration strategies after optimization for N-removal.

Aeration strategy	COD load	nitrification	N-removal	P-removal	Yield of anaerobic P–release per propionate uptake	duration
	(gCOD L_r^{-1} d^{-1})	(%)	(%)	(%)	(P-mol C-mol^{-1})	(d)
Uncontrolled high DO (day 179 - 313)	1.6	99.6 ± 0.7	62.3 ± 3.4	92.6 ± 7.5	0.39 ± 0.14	134
Uncontrolled high DO (day 488 - 525)	1.6	98.1 ± 1.6	40.0 ± 3.4	88.3 ± 7.4	0.18 ± 0.09	37
Optimized constant low DO	1.6	99.4 ± 0.7	61.2 ± 5.2	96.3 ± 3.8	0.38 ± 0.07	27
Optimized constant low DO	2.1	99.3 ± 0.8	77.7 ± 2.8	98.3 ± 0.2	0.34 ± 0.05	14
Head-space gas recirculation	1.6	89.3 ± 6.9	61.4 ± 2.8	90.2 ± 9.6	0.35 ± 0.06	52
Head-space gas recirculation	2.1	94.5 ± 6.0	80.5 ± 3.6	97.7 ± 1.4	0.38 ± 0.02	39
Head-space gas recirculation	2.4	95.3 ± 5.4	85.7 ± 3.8	94.8 ± 4.3	0.34 ± 0.10	26
Alternating high/low DO	1.6	96.3 ± 5.3	71.2 ± 5.6	94.1 ± 6.1	0.38 ± 0.04	178
Alternating high/low DO	2.1	97.5 ± 4.1	87.1 ± 2.6	97.5 ± 2.2	0.34 ± 0.03	36
Alternating high/low DO	2.4	97.5 ± 1.9	94.7 ± 1.7	98.1 ± 1.1	0.54 ± 0.12	35
Intermittent aeration	1.6	93.9 ± 4.5	78.3 ± 2.9	94.9 ± 8.6	0.27 ± 0.05	29

5.3.5 Granule size distribution and settling capacity

The average size of granules was fairly stable over time, varying from 0.85 to 1.13 mm during the whole study. However, with regard to the removal efficiency, the granule size distribution related to the biomass volume is of much more interest than the average size of the granules. At day 310 after reactor start, with uncontrolled high DO, a volume fraction of 63% of the granular biomass was related to diameters of 2 mm and above. In all later tests (day 488 to 1095), a minimum of 70% of the biomass was related to diameters of 2 mm and above, and 93-97% of the biomass was composed by granules with diameters above 1 mm (Figure 5-3).

The sludge volume index after 8 min of sedimentation (SVI_8) was very low during the whole experimental period (SVI_8 = 21.0 ± 5.0 ml g_{TSS}^{-1}) showing excellent settling characteristics of the granular sludge. During the phase with intermittent aeration where the reactor was only mixed during one third of the cycle time, the settling capacity of the sludge remained in the same range (SVI_8 = 19.3 ± 2.8 ml g_{TSS}^{-1}).

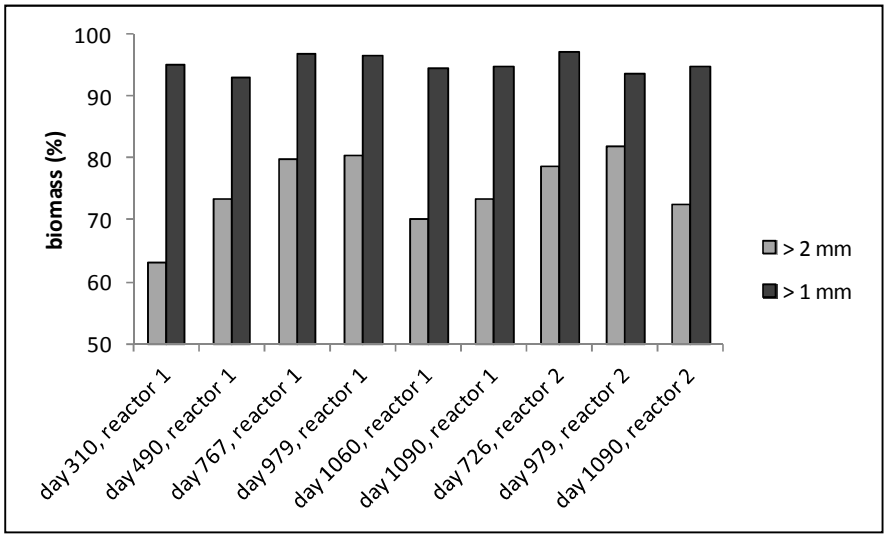

Figure 5-3: Volume fraction of biomass with granular diameters above 1 and 2 mm, respectively.

5.3.6 N_2O emissions

N_2O emissions were measured during testing headspace gas recirculation and alternating high/low DO as aeration strategies. The emissions appeared to be very different for the different aeration strategies and the different COD loads. The highest emissions were measured with the lowest COD loading rates of 1.6 gCOD L_r^{-1} day^{-1} and with alternating high/low DO where 9% of the nitrogen load left the reactor in form of N_2O (Table 5-4). However, with higher COD loading rates of 2.1 and 2.4 gCOD L_r^{-1} day^{-1}, the N_2O emissions decreased to 2.1 and 1%, respectively. With headspace gas

recirculation, no clear trend was observed concerning the impact of COD loading rates. The N_2O emissions represented 7.3, 3.0 and 4.8% of the nitrogen load for 1.6, 2.1 and 2.4 gCOD L_r^{-1} day^{-1}, respectively (Table 5-4). With both aeration strategies, the N_2O emissions were generally higher during the low DO phases.

Table 5-4: Nitrous oxide emissions with alternating high/low DO and head-space gas recirculation and different influent COD concentrations.

	COD load	Average N_2O emission		
	(gCOD L_r^{-1} d^{-1})	(ppm)[1]	(mgN L_{ww}^{-1})[2]	(% of N load)[3]
Alternating high/low DO	1.6	11.8	5.0	9.0
	2.1	2.8	1.2	2.1
	2.4	1.2	0.5	1.0
Head-space gas recirculation	1.6	12.8	4.0	7.3
	2.1	5.2	1.6	3.0
	2.4	8.4	2.6	4.8

[1] Emissions in ppm based on volume ratio

[2] Milligrams of nitrogen emitted as N_2O per liter of wastewater

[3] Part of nitrogen load leaving the reactor as N_2O gas

5.4 Discussion

The removal performances obtained under COD-limited conditions showed that AND aeration strategies led to markedly better N-removal efficiency than strategies promoting SND. This can be explained by the possible involvement of denitrifying P-removal. The introduction of low DO phases early in the aeration created favorable conditions for combined denitrification and P-removal. Low DO enlarged the inner anoxic part of granular biofilms while dissolved orthophosphate was still present at this moment of the cycle (Figure 5-2). Denitrifying P-removal allows saving COD (Kuba et al. 1996), and more COD remains available for the denitrification, improving the N-removal. With intermittent aeration, the N-removal efficiency further increased. During the non-aerated phases, granules settled forming a compact sludge bed and oxygen was rapidly consumed leading to completely anoxic conditions. The formation of a compact sludge bed reduced the contact between biomass and orthophosphate in solution. As a result, the P-uptake was lower during the non-aerated phases and the presence of orthophosphate in the liquid phase was extended (Figure 5-2). Moreover, the P-uptake rates are reported to be lower under anoxic conditions than under aerobic conditions (Kerrn-Jespersen and Henze 1993). Hence, denitrifying dephosphatation could take place during the non-aerated phases almost until the end of the aeration phase.

In contrast, orthophosphate was consumed within the first 40 to 60 minutes of the aeration phase with SND, excluding denitrifying dephosphatation during the rest of the aeration phase (Figure 5-2). Lemaire *et al.* (2008) have reported higher abundance of PAO in the outer layer, concluding that mainly GAO present in the inner part of the granules were responsible for denitrification. Confocal laser-scanning microscope images of granules used in this study and stained by FISH showed the same trend (results not shown). A modeling study looking at the distribution of different microbial populations inside the granules also suggested that PAO are mainly situated in the outer layer of granules (de Kreuk et al. 2007). Hence, it is quite probable that even with moderate DO and presence of orthophosphate the potential for denitrifying dephosphatation remained rather limited with SND.

In activated sludge systems with separate nitrification and denitrification tanks it has been demonstrated that denitrifying P-removal can save up to 50% of COD (Kuba et al. 1996). In single reactor systems like the aerobic granular biofilm technology, such high COD savings are unrealistic. The reactor has to be aerated during a certain time for nitrification. During that time, orthophosphate is taken up aerobically and denitrification is presumably conducted by GAO, as described above. This can reduce the potential for denitrifying P-removal and could explain why even with intermittent aeration which performed the best, only about 9% of orthophosphate was removed during the non-aerated phases.

Comparing the cycle measurements of the different aeration strategies, we conclude that best nutrient removal performances were achieved when the three main processes of nitrification, denitrification, and P-removal were active during the whole aeration phase. The alternation of aerated and non-aerated phases has the advantage that the rates of the different processes are very different during the two phases. It allows to slow down the more rapid heterotrophic process (i.e. aerobic dephosphatation) and to speed up the slower one (i.e. denitrification).

In contrast, with SND strategies the separation between aerobic and anoxic processes is mainly spatial within the granular biofilm and not temporal. Hence, the aim of these strategies was to find the optimal balance between aerated and unaerated zones of the granules. With constant DO strategies, it has earlier been shown that the oxygen penetrates deeper in the biofilm towards the end of the aeration phase, because of lower aerobic microbial activity in the outer layer due to depletion of ammonium and orthophosphate (Yilmaz et al. 2007). A better balance was therefore expected with headspace gas recirculation, where the DO decreases over time. However, the N-removal performances of the two SND strategies were very similar indicating that headspace gas recirculation did not improve the balance between aerobic and anoxic zones within the granules. Although there were indications that COD availability rather than oxygen inhibition was the main

limiting parameter, increasing the COD loading rate resulted in only slightly better N-removal performances with headspace gas recirculation than with constant low DO. However, the differences between performances were too small to conclude that with higher COD availability the optimal balance between aerated and unaerated zones becomes more relevant to reach higher removal efficiencies. The optimal constant DO range for SND was between 25-40% of saturation which is in the same range as in other studies with similar operating conditions (de Kreuk et al. 2005).

A too small granule size as limiting parameter for denitrification could be excluded. During the whole study the biomass was composed by a high fraction of large granules with diameters of 2 mm and above. Hence, as long as the metabolic activity in the outer layer was high, inner anoxic zones were presumably present, allowing SND. With alternating high/low DO, and even more with intermittent aeration, the size of the granules is of less importance. The separation between nitrification and denitrification is mainly temporal and not spatial as with SND. Nevertheless, the granular stability is important for the settling capacity of the sludge. It has been suspected that a switch-off of aeration could be problematic, as the aeration has not only the function to provide oxygen to the system but also to mix the sludge and to create shear to maintain smooth granules (Xavier et al. 2007). In our study, we did not observe increased sludge disruption during the 58 days of intermittent aeration, even if only during one third of the cycle time the sludge was mixed by aeration. The low SVI_8 of around 20 ml g_{TSS}^{-1} confirmed that the settling capacity was excellent and was not affected by the reduced mixing time. Another study with aerobic granular sludge reported very similar SVI_8 values (Cassidy and Belia 2005).

When comparing the P- and N-removal under the different test conditions, we observed negative correlations between NO_x^- accumulation and P-removal ($r = -0.74$, $p<0.01$) and also between NO_x^- accumulation and anaerobic P-release ($r = -0.60$, $p<0.05$). The lowest P-removal efficiency, and also the lowest P-release, was observed with uncontrolled high DO between day 488 and 525. With that aeration strategy, about 60% of the N load was present as NO_3^- at the end of the cycle. This led to anoxic conditions in the subsequent feeding phase and to competition for COD between denitrifying bacteria, PAO, and GAO. The highest yield of P-release to propionate uptake under anaerobic conditions of 0.54 ± 5.0 P-mol C-mol^{-1} was observed with alternating high/low DO and a high COD load. Under such conditions, NO_x^- accumulation was insignificant. Similar ratios have been reported in EBPR studies with biomass composed by over 50% by PAO affiliating with *Accumulibacter* (Pijuan et al. 2004; Oehmen et al. 2006). Such results on the relation between P-removal and NO_x^- accumulation agree with former studies on dephosphatation (Furumai et al. 1999; Peng et al. 2010)

and demonstrate the crucial importance of efficient N-removal in reactors with combined N- and P-removal.

The off-gas analysis showed that under certain conditions an important part of N left the reactor as undesired N_2O. With alternating high/low DO and head-space gas recirculation the highest emissions were measured with low COD loads. Moreover, higher emissions were observed during the phases with low DO. These results agree with studies on N_2O emissions in wastewater treatment processes that have identified COD and oxygen limitations as possible reasons for N_2O production (Kampschreur et al. 2009). Interestingly, with headspace gas recirculation the N_2O emissions remained quite important even with higher COD loads. With headspace gas recirculation we observed during the whole aeration phase NO_2^- concentrations between 1 - 4 mg L^{-1} independently from the tested COD loads. NO_2^- is known to be a trigger for N_2O emissions. Recent studies have shown that under SND conditions the concurrent presence of NH_4^+ and NO_2^- leads to enhanced N_2O emissions (Kim et al. 2010; Wunderlin et al. 2012). In our study, only punctual control measurements of N_2O were made. We observed big variations in the different samples, highlighting the complexity of N_2O production in aerobic granules. It is known that N_2O production can take place during nitrification, denitrification and also by the reaction of nitrite with hydroxylamine (Kampschreur et al. 2009). With aerobic granular sludge and SND conditions all three pathways are possible. Therefore, continuous measurements would be needed to have a deeper insight of the impact of the aeration strategies on N_2O production.

5.5 Conclusions

The N-removal efficiency of COD-limited aerobic granular sludge wastewater systems can be considerably enhanced with improved aeration strategies. Strategies promoting AND were significantly more efficient concerning N-removal than SND strategies. The introduction of low DO phases or even anoxic phases in an early stage of the total aeration period probably enhanced denitrifying P-removal and led to COD savings. Intermittent aeration, which is a realistic AND strategy for full scale applications, led to the highest N-removal efficiency. The short mixing times implemented with this strategy were not problematic for the stability of the granules.

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Nitrogen removal over Nitrite

6

6. Nitrogen removal over nitrite by aeration control in AGS-SBRs

6.1 Introduction

Biological wastewater treatment by aerobic granular sludge technology offers the possibility to remove carbon (hereafter referred to as chemical oxygen demand, COD), nitrogen and phosphorus in one single reactor (Zeng et al. 2003; de Kreuk et al. 2005; Yilmaz et al. 2007). Nevertheless, the simultaneous removal of nitrogen and phosphorus is not always successful. Denitrifying bacteria and polyphosphate accumulating organisms (PAO) are both heterotrophic, and PAO as well as some of the former group are able to take up carbon under anaerobic conditions and store it intracellularly until a suitable electron acceptor is present in order to use the stored carbon for growth. In combined denitrification and enhanced biological phosphorus removal (EBPR) systems, COD availability is often the limiting parameter (Kuba et al. 1996; Keller et al. 1997) and PAO and denitrifiers are in direct competition for the available COD. Both processes have been reported to be potentially disturbed by this competition. In lab-scale experiments with different aeration strategies, it has been observed that nitrate accumulated due to a lack of COD whereas P-removal efficiency remained quite high (Chapter 5; Cassidy and Belia 2005; Yilmaz et al. 2007). On the other hand, it has also been reported that the presence of nitrate (NO_3^-) or nitrite (NO_2^-) can disturb the P-removal in sequencing batch mode (Furumai et al. 1999; Peng et al. 2010). Presence of nitrate/nitrite (NO_x^-) at the end of the cycle leads to anoxic conditions in the subsequent feeding phase. Under such conditions, part of the COD is consumed by denitrifying bacteria, leaving less for PAO to accumulate during the anaerobic phase resulting in lower P-removal overall (Peng et al. 2010).

In full scale installations unsatisfying denitrification is sometimes improved by the addition of an external carbon source, for example ethanol, whereas P-removal can be complemented by a chemical post-treatment. Nevertheless, both solutions are costly and lead to a higher excess sludge production. Hence, solutions intending to reduce COD requirements are needed. An interesting approach to reduce COD requirements is N-removal over nitrite. From stoichiometric equations with methanol as carbon source, it can be derived that 25% less oxygen and 40% less COD is required to transform ammonium (NH_4) into nitrogen gas (N_2) over nitrite compared to conventional N-removal over nitrate. Other advantages are a lower biomass production and a higher denitrification rate (Turk and Mavinic 1987). To achieve the nitrite pathway, conditions have to created such that the second nitrification step from nitrite to nitrate it is not occurring which means that conditions are

unfavorable for nitrite-oxidizing bacteria (NOB) to be active but not for ammonium-oxidizing bacteria (AOB).

One of the first applied examples of a selective elimination of NOB has been the so-called SHARON process (Single reactor High activity Ammonia Removal Over Nitrite; Hellinga et al. (1998)). At temperatures >25°C, AOB have a higher maximum specific growth rate than NOB. Hence, by reducing the sludge retention time (SRT), NOB can be washed out. This strategy works only for elevated temperatures, and although large variations of growth rates for nitrifiers have been reported in literature (Munz et al. 2011), it is widely accepted that at temperatures <20°C, the maximum specific growth rate of NOB is higher than the growth rate of AOB. Hence, for temperatures <20°C strategies acting directly on the growth rate of and suitable growth conditions for NOB are required. Several chemical substances such as free ammonia (FA) and free nitrous acid (FNA)(Anthonisen et al. 1976; Vadivelu et al. 2007), chlorate (Xu et al. 2011) or formic acid (Philips et al. 2002) have been shown to be able to selectively inhibit NOB. Besides of chemical inhibitors, the competition for oxygen has been used as a parameter to achieve N-removal over nitrite. AOB have a lower oxygen half-saturation coefficient than NOB (Guisasola et al. 2005; Ciudad et al. 2006), which means that NOB are more sensitive to low oxygen concentrations. Several studies confirmed the possibility to achieve the nitrite pathway with low dissolved oxygen (DO) concentrations (Bernet et al. 2001; Ruiz et al. 2003; Tokutomi 2004). Beside low DO, aeration phase length control (Blackburne et al. 2008; Lemaire et al. 2008) and intermittent aeration (Fux et al. 2006; Li et al. 2011) have been investigated with sequencing batch reactors (SBR) for the achievement or maintenance of the nitrite pathway. With aeration phase length control, the aeration has been stopped upon completion of ammonium oxidation. Lemaire et al. (2008) have advanced the following mechanism to explain the elimination of NOB by this strategy. Nitrite oxidation finishes naturally after ammonium oxidation. If the aeration is stopped prior to or right at the moment of complete ammonium oxidation, no extra time is left for the completion of the nitrite oxidation by NOB. It gradually reduces the energy supply for NOB and therefore its growth. Over the cycles it leads to a stepwise decrease of NOB. In the study of Lemaire et al. (2008), wastewater has been fed after stop of aeration to supply COD for post-denitrification. In Blackburne et al. (2008) formic acid has been used to reduce the NOB population at the beginning of the study, and the main mechanism of nitrite elimination has been pre-denitrification during the feeding phase of the subsequent cycle. The temperature was in both studies around 20°C. Fux et al. (2006) have implemented an automated intermittent aeration strategy based on the oxidation–reduction potential (ORP). They achieved the nitrite pathway in a SBR operated at 30-32°C and with ethanol supply during the unaerated phases to enhance denitrification. Li et al. (2011) have maintained partial nitrification with intermittent aeration in a

SBR operated at 20°C in a long-term study. NOB have been eliminated by free ammonia inhibition and high temperature. Very limited research has been done so far on achieving or maintaining the nitrite pathway at temperatures below 20°C. Yang et al. (2007) maintained N-removal over nitrite with automated aeration control and step-feeding, while gradually decreasing the temperature from 22 to 12°C.

The aim of this study was to achieve and maintain the nitrite pathway at temperatures of 20°C and 15°C by aeration control in a SBR with aerobic granular sludge (AGS) for biological nutrient removal. A combination of aeration phase length control and DO variation was tested to achieve N-removal over nitrite. No chemical inhibitors were used and no additional carbon was supplied after aeration stop. The COD for denitrification came from the carbon stored intracellularly by certain bacteria during the anaerobic feeding phase. A matter of particular interest was the time necessary to achieve the nitrite pathway, because the switch from the nitrate to nitrite pathway with aeration control has been reported to be a very slow process of 100-300 days (Blackburne et al. 2008; Lemaire et al. 2008).

6.2 Materials and Methods

6.2.1 Technical setup

Two identical bubble column reactors with diameters of 5.2 cm and working volumes of 2.4 L were used in this study. A detailed description of these reactors and the gas recirculation system can be found in the general materials and methods chapter of this thesis (Chapter 2). The SBR cycle was composed of four steps: an anaerobic feeding phase of 60 min, a starvation phase of maximum 150 min, 3 - 5 min of settling, and 5 min of withdrawal. The pH was controlled between 7 - 7.3. The inoculation sludge was provided by the wastewater treatment plant Thunersee. The carbon source was acetate and propionate in a ratio of 1:1 according to COD. The detailed composition of the wastewater is described in Chapter 2.

6.2.2 Experimental schedule and aeration strategies

The study was divided in five phases. Phase I from day 1 – 96: the granular sludge was produced from activated sludge in a parent reactor operated with alternate high - low DO phases (Figure 6-1). The two hours of aeration were split into three phases with high and three with low DO of 20 min each. From day 1 - 80, the setpoints of the high DO phases were at 50%, 40% and 30%, respectively and the low DO setpoints at 5%. At day 81 (Phase II), the setpoints of the high DO phases were

increased by 10% to 60%, 50% and 40%, respectively. In Phase II from day 97 – 155, the reactor was aerated with 3.6 L min^{-1} of air during the high DO phases, without defined setpoints. However, the last high DO phase was stopped when all ammonium was consumed. The temperature was maintained at 20°C during Phases I and II.

For Phase III that lasted from day 156-186, the sludge was distributed over two reactors, one operated at 20°C and the other at 15°C (Figure 6-1). The aeration strategy was the same as at in Phase II, alternating high-low DO with uncontrolled full aeration during the high DO phases and with aeration phase length control (Figure 6-1). In Phase IV from 187-219, the reactors were fully aerated during 2 hours without low DO phases (Figure 6-1). Finally, in Phase V from day 220-261, the reactors were operated with intermittent aeration and aeration phase length controlled (Figure 6-1). Aeration pulses of 12.5 min were followed by idle phases of 12.5 min without aeration or mixing. A maximum of six aeration pulses were carried out. If all ammonium was consumed before the end of the sixth aeration pulse, the aeration was stopped earlier. After the aeration stop, another period of 10.5 min without aeration was added, followed by a short aeration pulse of 2 min. The 2 min aeration pulse served to mix the sludge once again before final settling.

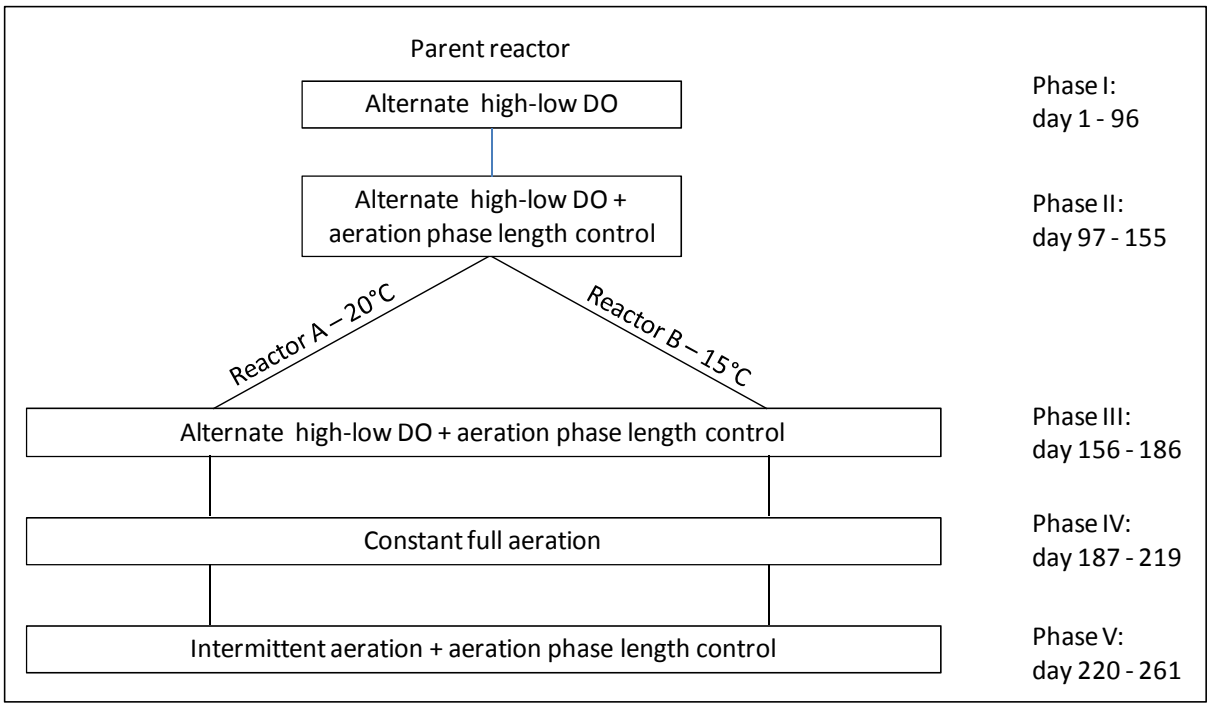

Figure 6-1: Aeration strategies during the five experimental phases.

6.2.3 Analytical methods

Dissolved chemical compounds as well as the total (TSS) and volatile suspended solids (VSS) were analyzed as described in Chapter 2.

6.2.4 Nitrification batch tests

Nitrification batch tests were carried out in a 1 L glass beaker with 0.5 L working volume and about 2 gVSS granular sludge. The sludge was taken from the SBR at the end of the aeration phase. An aliquot of a NH_4Cl stock solution (10 mmol L^{-1}) resulting in a final concentration of 1.2 mmol L^{-1} was added. A porous diffuser was used for aeration, with an airflow high enough to completely mix the bulk liquid. The test was carried out during 2 hours with liquid sampling every 20 minutes for ammonium, nitrite, and nitrate analysis.

6.2.5 Quantification of nitrite-oxidizing bacteria

NOB were quantified by quantitatve PCR targeting specifically the 16S rRNA gene of *Nitrospira*. A reference plasmid named pNOB was used as standard. This plasmid was obtained by cloning a 151-bp fragment of the *Nitrospira* 16S rRNA gene amplified from total DNA obtained from granular sludge with the primers NTSPA-D16S-F (5'-CCTGCTTTCAGTTGCTACCG-3') and NTSPA-D16S-R (5'-GTTTGCAGCGCTTTGTACCG-3') taken from Dionisi et al (2002). The PCR reaction mix contained: 66.5 µl of ddH2O, 10 µl of 10x PCR buffer S (Peqlab), 3 µl of dNTPs at 2.5 mM, 5 µl of 10 µM primer and 0.5 µl of Taq polymerase at 5 U/µl (Peqlab). One µl of DNA at 1 ng/µl was added as template. The PCR program was designed as follows: 5 min of initial denaturation at 95°C, 30 cycles of amplification with each cycle including 45 s denaturation at 95°C, 45 s of primer annealing at 52°C, and 60 s of elongation at 72°C; a 5 min step of final elongation at 72°C was added at the end. The PCR product was purified with the PCR purification Kit (Qiagen) and ligated into the vector pGEM-T Easy (Promega) following the manufacturer's instructions. The resulting plasmid was transformed in $CaCl_2$-competent *E. coli* DH5α cells using standard heat shock protocol. Transformants were selected by colony PCR and verified by sequencing using in-house facility as described previously in Rupakula et al (2013). A positive transformant harboring pNOB was cultivated for plasmid extraction using the Qiaquick Miniprep Kit (Qiagen). One µg of plasmid DNA was linearized by digestion with the restriction enzyme *Sca*I (Promega), dephosphorylated by shrimp alkaline phosphatase (TaKaRa), and finally purified using the PCR purification kit (Qiagen). DNA concentration was measured with a NanoDrop ND-1000 apparatus. Standards for qPCR were prepared by serial dilution from $1.15 \cdot 10^7$ to 10^1 plasmid copies/µl. Alternatively, a second qPCR standard plasmid was prepared similarly as for

Nitrobacter spp. targeting a 323-bp fragment of the *nxrA* gene family using primers NTBCR-nxrA-F (5'-CAGACCGACGTGTGCGAAAG-3') and NTBCR-nxrA-R (5'-TCCACAAGGAACGGAAGGTC-3') taken from Wertz et al (2008).

Runs of qPCR consisted of the standard dilution series and samples in triplicates. The 10 µl qPCR reaction mixture was composed of 5 µl of KAPA SYBR® FAST Universal 2X qPCR Master Mix, 0.2 µl of primers NTSPA-D16S-F and -R (runs 1-5) (and NTBCR-F and -R, runs 6-7) at 10 µM, 2.1 µl of sterile water and 2.5 µl of DNA template. The program consisted of 15 min initial denaturation at 94°C, followed by 40 cycles of 30 s denaturation at 94°C, 20 s primer annealing at 60°C, 30 s elongation at 72°C, and 15 s fluorescence acquisition at 80°C. A melt curve ranging from 72 to 99°C was added at the end for quality assessment. The reaction was run in the RotorGene RG3000 machine (Corbett Research).

Data were analyzed using the RotorGene 6 software with a fluorescence threshold fixed at 0.25. The copy number (cn) of *Nitrospira* 16S rRNA genes (and alternatively *Nitrobacter nxrA* genes) was calculated for each sample using Equation 6-1 from the average cycle threshold (CT) value (with standard deviation kept below 3% of the average), and the parameters M and B calculated from the run-specific standards dilution series. These parameters along with reaction efficiency and R^2 value of the linear regression of the standards are given in Table 6-1.

$$cn = 10^{[(CT-B)/M]} \qquad \textbf{Equation 6-1}$$

Table 6-1: Parameters for individual qPCR runs. B and M are the parameters of the linear calibration curve of each run, B is the constant and M the slope.

Parameters	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Run 7
B	33.289	33.735	36.637	37.055	37.379	31.605	35.612
M	-3.537	-3.438	-3.446	-3.422	-3.542	-3.434	-3.514
Efficiency	0.917	0.954	0.951	0.960	0.916	0.955	0.926
R^2	0.999	0.998	0.998	0.994	0.997	0.997	0.998

6.2.6 Nitrous oxide tests

Dissolved concentrations and emissions of nitrous oxide (N_2O) were investigated in a separate study. Sludge with N-removal over nitrite and nitrate, respectively, was maintained in two SBRs. A liquid phase microsensor (Unisense, Denmark) was used for the continuous measurement of N_2O in the bulk liquid. The probe was calibrated with nitrogen gas and a N_2O calibration gas at a concentration of 200 ppm. For both sludge types, N_2O was measured with two different aeration strategies: constant DO of 30% and intermittent aeration, and with two different influent COD concentrations: 400 mgCOD L^{-1} and 600 mgCOD L^{-1} (Table 6-2).

Table 6-2: Tested conditions for dissolved N_2O concentrations and N_2O emissions.

Run No	N-removal over	Aeration strategy	COD concentration (mgCOD L^{-1})
1	NO_2^-	Constant DO 30%	400
2	NO_2^-	Constant DO 30%	600
3	NO_2^-	Intermittent aeration	400
4	NO_2^-	Intermittent aeration	600
5	NO_3^-	Constant DO 30%	400
6	NO_3^-	Constant DO 30%	600
7	NO_3^-	Intermittent aeration	400
8	NO_3^-	Intermittent aeration	600

The microsensor gave stable results also for N_2O concentrations in the gas phase. Eighteen grab samples were analyzed by gas chromatography (GC) (see Chapter 2 for details) to compare with the microsensor measurements (Figure SI 6-1). The same conditions were tested with the microsensor installed in the head-space zone of the SBR reactor to measure N_2O emissions. The head-space gas recirculation was disconnected during the N_2O study. All runs were tested over 1 day (8 cycles).

6.3 Results

6.3.1 Strategy for aeration phase length control

In Phases II, III, and IV the aeration was stopped once ammonium oxidation was upon completion. The control strategy implemented for the automatic aeration phase length control was based on the DO signal. The oxygen uptake rate (OUR) decreased when ammonium oxidation was about to finish which led to a rise of DO. The slope change of the DO signal was easily observable (Figure 6-2). The aeration was automatically stopped when over a period of one minute a higher slope was observed. If no DO slope change was observed, the aeration was stopped according to the normal aeration strategy. It was after three times 20 minutes of full aeration in Phase II and III, and after six aeration pulses of 12.5 min in Phase V with intermittent aeration.

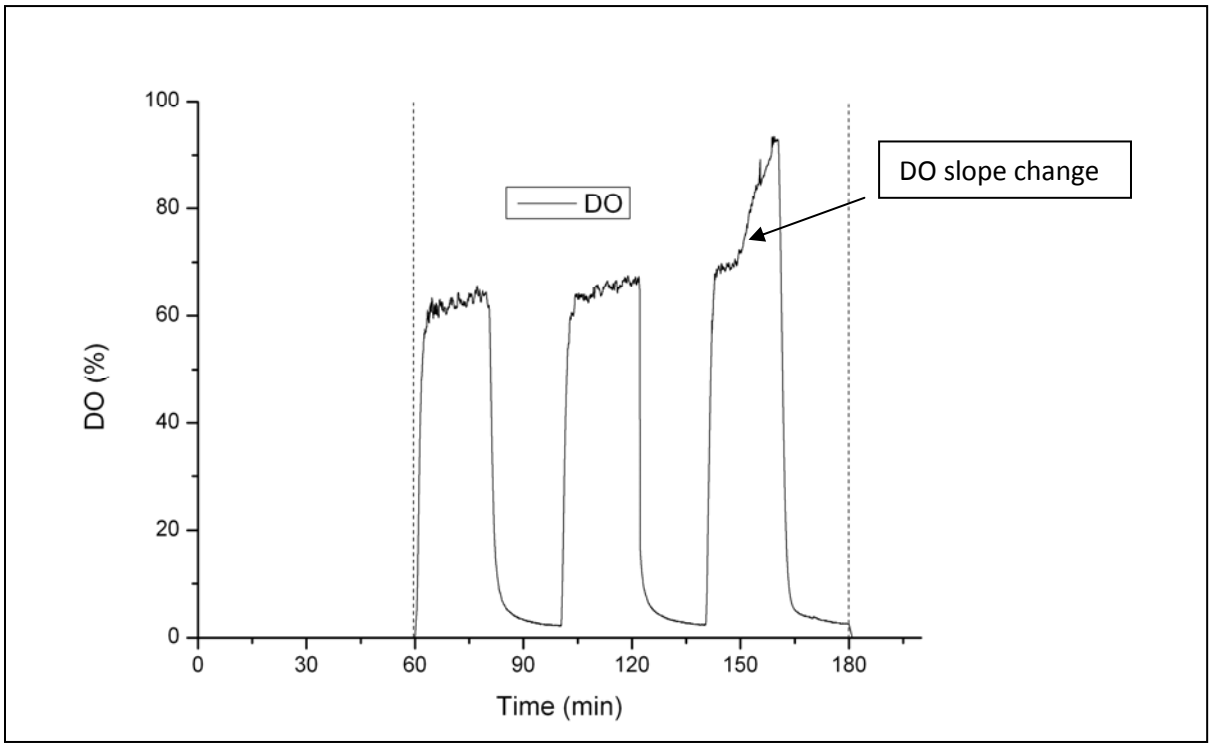

Figure 6-2: DO profile in a SBR cycle with alternate high-low DO phases with full aeration during the high DO phases. Slope change of DO towards end of nitrification during the third high DO phase.

6.3.2 N-removal and NOB abundance in the parent reactor

The nutrient removal performances during the start phase until day 80 are described in Chapter 3, run 7b. Before increasing the setpoints of the high DO phases on day 81, nitrification and N-removal performance were at 74%, and P-removal was at 97% (Figure 6-3A). The increase of the setpoints from 50%, 40% and 30% to 60%, 50% and 40%, respectively, resulted in an increase of NH_4^+-removal

to 79% without leading to significant NO_x^- accumulation. In Phase II, with full aeration during the high DO phases, nitrification further increased. Between days 100 – 155, NH_4^+-removal was 98%. The NO_x^- effluent concentrations remained very low (Figure 6-3B) leading to an average N-removal of over 95% for this period (Figure 6-3A). *Nitrospira* was the dominant NOB population in the granular sludge and a clear decrease of the NOB population was observed after day 100. In the inoculation sludge the concentration of *Nitrospira* 16S rRNA genes was about 1800 cn ng^{-1} of DNA (Figure 6-3C). At day 42 where clear accumulation of nitrate was occurring, this concentration was more than twice as high, but decreased to values below 1000 cn ng^{-1} of DNA, and finally, after day 100, the concentration was in the order of 100 cn ng^{-1} of DNA (Figure 6-3C).

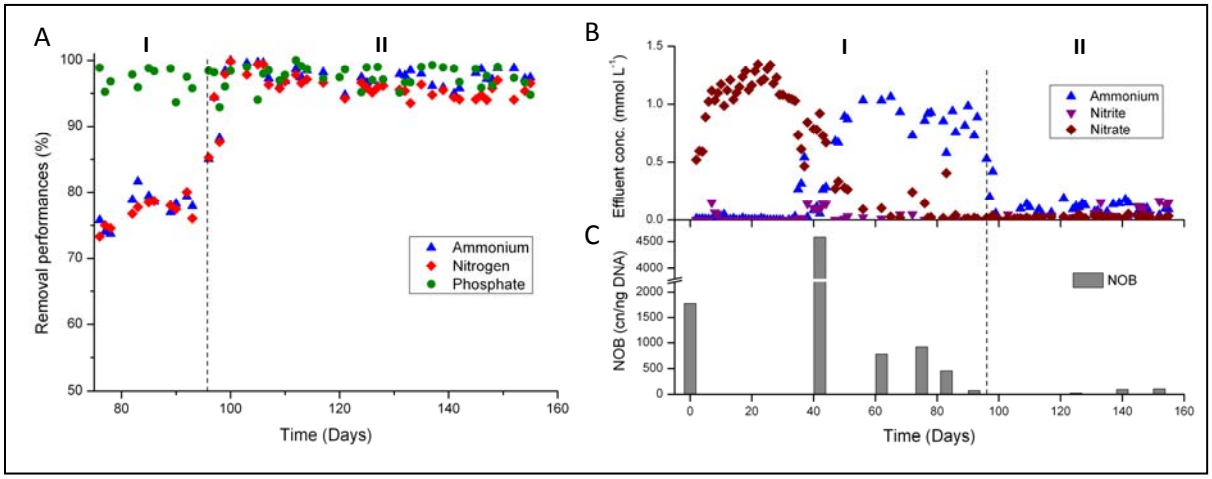

Figure 6-3: (A) Nutrient removal performances of parent reactor between days 75 – 155. **(B)** Concentrations of N compounds in the effluent and **(C)** *Nitrospira* 16S rRNA gene concentrations measured by qPCR until day 155. At day 96, the oxygen supply was increased, but automatically stopped upon completion of ammonium oxidation.

An analysis of the pollutant concentrations during an SBR cycle at day 98 revealed that almost no NO_x^- accumulated during the phases with high DO (Figure 6-4A). The same result was observed in a test cycle with uncontrolled full aeration. No nitrate and only traces of nitrite was detected (Figure 6-4B). All ammonium was removed by simultaneous nitrification and denitrification (SND).

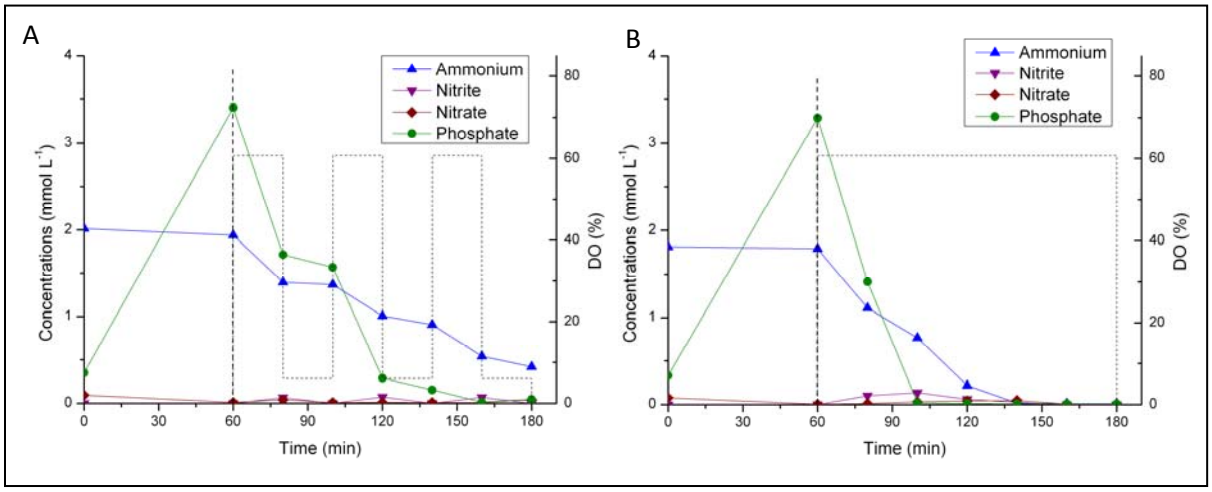

Figure 6-4: Concentrations of N and P compounds during a SBR cycle with **(A)** high-low DO and **(B)** in a test cycle with uncontrolled full aeration. Concentrations at time 0 were calculated based on the effluent concentrations of the previous cycle and the influent concentrations. Aeration started after 60 min of plug-flow feeding. The dashed lines show schematically the aeration strategy.

Finally, a nitrification batch test with unfed sludge was carried out at day 118 to elucidate whether nitrate or nitrite was the main product of ammonium oxidation under unfavorable conditions for denitrification. Between 90% and 94% of the accumulated NO_x^- in the liquid phase was in the form of nitrite while nitrate was hardly detected (Figure 6-5) which indicated that N-removal occurred mainly over nitrite at that stage of the study.

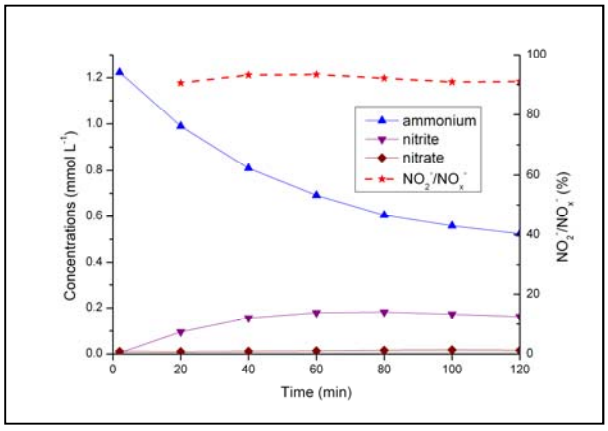

Figure 6-5: Nitrification batch test with sludge taken at the end of the starvation phase without COD supply.

6.3.3 N-removal and NOB abundance at 20°C

In the reactor at 20°C, 52% of NH_4^+-removal was observed on the first day after startup (Figure 6-6A). This sharp performance decrease compared with the parent reactor was obviously due to reduced biomass. Nitrification recovered rapidly during Phase III and after one week NH_4^+-removal was back

at 80%. Between day 180 and 186, NH_4^+-removal was on average over 96% and aeration was stopped by automatic aeration phase length control slightly before the end of the normal starvation time of 2 hours. The average N-removal during this last week of Phase II was 93%. P-removal was constantly over 97% (Figure 6-6A). The accumulated NO_x^- was in the form of nitrite (Figure 6-6B), and the *Nitrospira* abundance remained very low (Figure 6-6C), indicating N-removal over nitrite.

In Phase IV, after switching to two hours of full aeration, the N-removal performance gradually decreased from 93% to 74% (Figure 6-6A). In a first time, nitrite was the main ammonium oxidation product and it accumulated, but over time more and more nitrate was produced and was at the end of Phase IV the main NO_x in the effluent (Figure 6-6B). At the same time, a re-growth of *Nitrospira* was observed (Figure 6-6C).

Finally, in Phase V, the reactor was operated with intermittent aeration and aeration phase length control. N-removal improved immediately to >80%, and after 20 days even to >90%. Between day 241 – 261, the average N-removal was 95% and P-removal 98% (Figure 6-6A). The nitrate concentration in the effluent decreased to values below 0.1 mmol L^{-1} (Figure 6-6B) and the *Nitrospira* 16S rRNA gene concentrations were below 100 cn ng^{-1} of DNA at the end of Phase V (Figure 6-6C). However, no nitrite was detected in the effluent (Figure 6-6B).

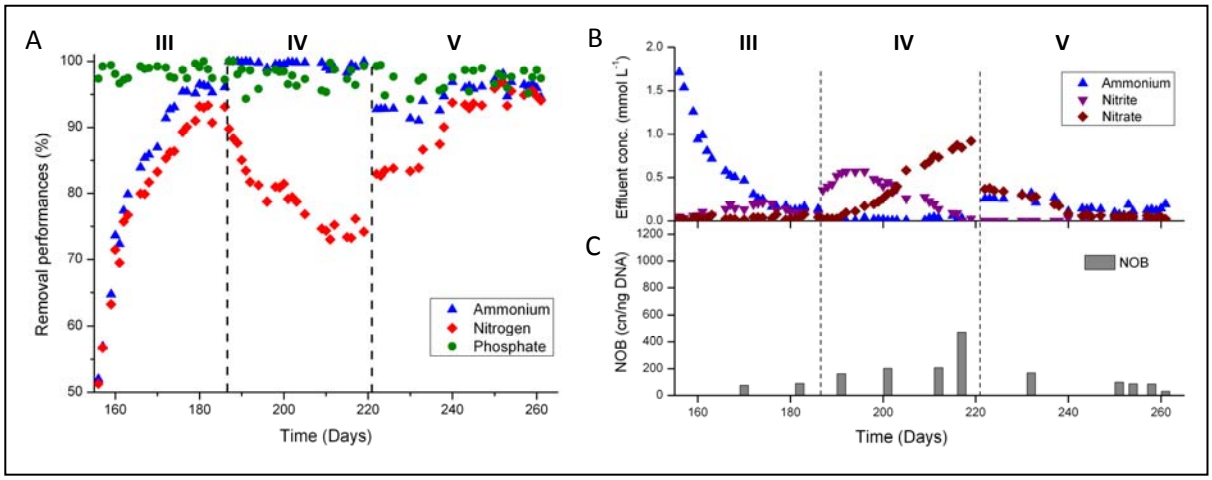

Figure 6-6: (A) Nutrient removal performances at 20°C from days 156 - 261. **(B)** Concentrations of N compounds in the effluent and **(C)** *Nitrospira* 16S rRNA gene concentrations measured by qPCR in the period from days 156 - 261. Until day 186, the reactor was operated with alternating high-low DO and aeration phase length control, from days 187 - 220 the reactor was fully aerated during two hours, and finally, from days 221 – 261, the reactor was operated with intermittent aeration and aeration phase length control.

6.3.4 N-removal and NOB abundance at 15°C

At 15°C, NH_4^+-removal also decreased to around 50% after startup, but recovered within two weeks (Figure 6-7A). N-removal stabilized at around 90% and nitrite was the major N compound in the effluent during Phase III (Figure 6-7B). In Phase IV with to 2 hours of full aeration, N-removal immediately decreased to 80% and within two weeks to 55 – 60% (Figure 6-7A). While during the first 10 days mainly nitrite was measured in the effluent, a switch towards total nitrification was observed. After 16 days, no more nitrite was present in the effluent, but over 1.4 mmol L^{-1} of nitrate (Figure 6-7B). The *Nitrospira* 16S rRNA gene concentration increased to almost 2000 cn ng^{-1} of DNA (Figure 6-7C). After switching to intermittent aeration and aeration phase length control in Phase V, N-removal increased to about 80% within 17 days (Figure 6-7A). Concentrations of 0.3 mmol L^{-1} of NH_4^+ and 0.4 mmol L^{-1} of nitrate were measured in the effluent, but no nitrite (Figure 6-7B). The concentration of *Nitrospira* 16S rRNA genes clearly decreased, but remained between 200 and 400 cn ng^{-1} of DNA (Figure 6-7C).

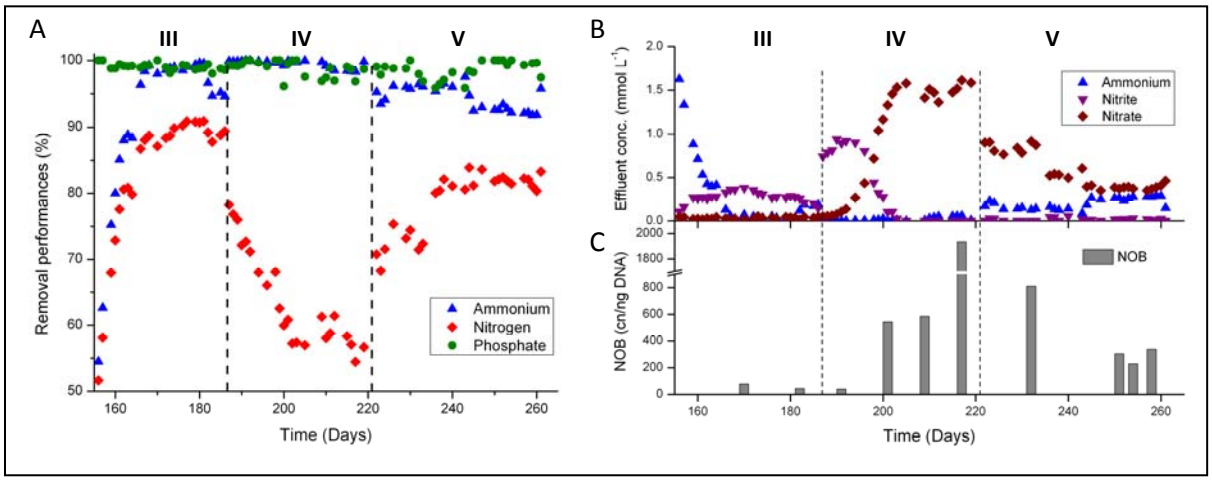

Figure 6-7: (A) Nutrient removal performances at 15°C from days 156 - 261. **(B)** Concentrations of N compounds in the effluent and **(C)** *Nitrospira* 16S rRNA gene concentrations measured by qPCR in the period from days 156 - 261. Until day 186, the reactor was operated with alternating high-low DO and aeration phase length control, from days 187 - 220 the reactor was fully aerated during two hours, and finally from days 221 - 261 the reactor was operated with intermittent aeration and aeration phase length control.

6.3.5 Nitrous oxide emissions and concentrations in the liquid phase

With N-removal over nitrite and intermittent aeration N_2O was only emitted during the aeration pulses (Figure 6-8A). However, in the bulk liquid the concentrations sharply increased right at the end of the aeration phase (Figure 6-8A). Except for the first aeration pulse, two peaks of N_2O emissions were measured during each aeration pulse, one at the beginning and one at the end (Figure 6-8A). Similar profiles for N_2O emissions and liquid concentrations were observed with N-

removal via nitrate (Figure 6-8B). However, the concentrations were lower than via nitrite. Moreover, a small peak of N_2O appeared in the liquid phase during the first minutes of the feeding phase and a N_2O emission peak was observed at the beginning of the first aeration pulse.

With a constant DO of 30% and N-removal via nitrite, N_2O concentrations in the gas and the liquid phase constantly increasing during the first minutes of the aeration, before reaching a steady state (Figure 6-8C). With N-removal via nitrate, the N_2O concentrations were again a bit lower (Figure 6-8D). Also with constant DO a peak of N_2O in the liquid phase was observed during the first minutes of the feeding phase and in the gas phase right after the aeration start (Figure 6-8D). With N-removal via nitrite, these peaks did not appear.

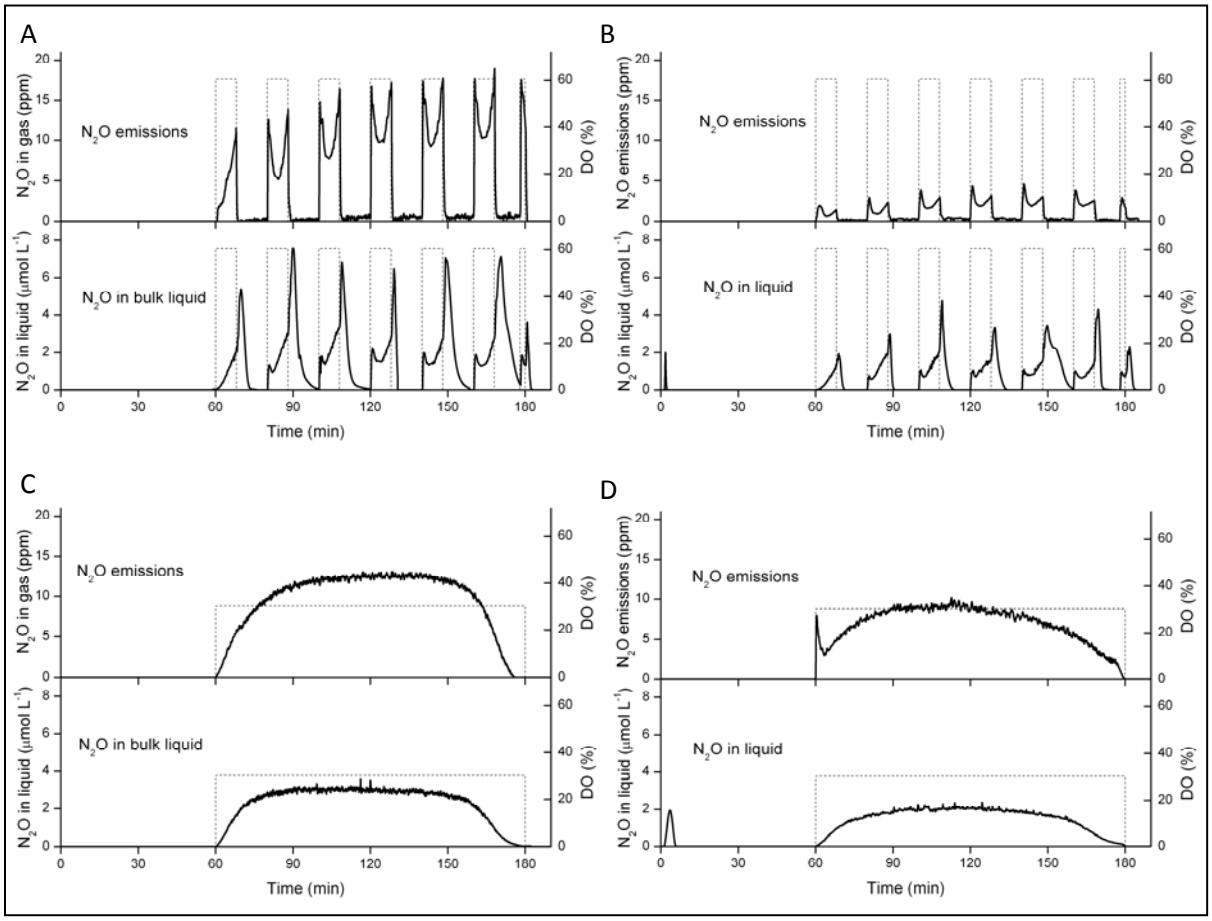

Figure 6-8: Typical profiles of nitrous oxide concentrations in the effluent gas and the bulk liquid during a SBR cycle. The dashed lines show schematically the aeration strategy: **(A)** N-removal over nitrite with intermittent aeration, **(B)** N-removal over nitrate with intermittent aeration, **(C)** N-removal over nitrite with constant DO of 30% and **(D)** N-removal over nitrate with constant DO of 30%. The shown profiles have been measured with 400mgCOD L^{-1} influent concentration.

The N_2O emissions varied between 0.7% - 12.9% of the influent N load under the different conditions tested (Table 6-3). The highest N_2O emission of 12.9% was observed with constant DO, N-removal over nitrite and influent COD concentrations of 400 mgCOD L^{-1}, whereas the lowest emission of 0.7% was found with N-removal over nitrate and intermittent aeration. The *Nitrospira* concentration was 22 ± 9 cell numbers per ng of DNA in the nitrite pathway sludge, and 445 ± 79 cn ng^{-1} of DNA in the nitrate pathway sludge. COD concentration had a decreasing effect on nitrous oxide emissions under almost all conditions tested.

Table 6-3: N_2O emissions with N-removal over nitrite and nitrate, respectively, with intermittent aeration and constant DO of 30%.

Aeration strategy	COD concentration (mgCOD L^{-1})	NO_2^- pathway (% of N load) [1]	NO_3^- pathway (% of N load) [1]
Intermittent aeration	400	5.2 ± 1.1	0.8 ± 0.2
Intermittent aeration	600	2.4 ± 0.8	0.7 ± 0.3
Constant aeration	400	12.9 ± 2.1	9.3 ± 2.4
Constant aeration	600	8.1 ± 1.7	5.9 ± 0.9

[1] Part of nitrogen load leaving the reactor as N_2O gas

6.4 Discussion

Aeration by alternating high-low DO and incomplete nitrification between days 40 – 96 let to a switch from N-removal over nitrate to N-removal over nitrite. Quantitative PCR showed a decrease of the abundance of *Nitrospira* in the granular biofilm by over 95% during this period. The subsequent increase of oxygen supply (Phase II) let to N-removal of over 95%. In a previous study with the same aeration strategy, the same influent wastewater concentrations and the same reactor set-up, N-removal was limited to 71% (Chapter 5). A lack of COD had been found to be the reason for incomplete denitrification, resulting in nitrate accumulation. In Phase II of the present study, with N-removal over nitrite, NO_x^- was hardly detected in the effluent, meaning that denitrification was no longer limited by available COD. These results agreed with the theory on important COD savings with the nitrite pathway (Turk and Mavinic 1987) and emphasize the great potential of N-removal over nitrite in COD-limited systems.

The incomplete nitrification between day 40 and 100 very likely played a major role in the switch to the nitrite pathway. The effect of an incomplete nitrification is comparable to an aeration phase

length control (Blackburne et al. 2008; Lemaire et al. 2008). In both studies, the transition from total to partial nitrification by aeration phase length control was described as a slow process taking 100 - 300 days at 20°C. In our study, the low DO phases of three times 20 minutes at around 0.5 mg O_2 L^{-1} presumably accelerated the transition. A DO < 1mg L^{-1} has been successfully tested to achieve N-removal over nitrite with granular sludge (Tokutomi 2004). Inhibition by free ammonia was very unlikely to play a major role. With 50 mgN-NH_4^+ L^{-1} in the influent and a pH controlled between 7 - 7.3, the free ammonia concentration remained below 0.4 mg L^{-1}. For the selective elimination of NOB, free ammonia concentrations of 1 - 20 mg L^{-1} have been reported to be necessary to inhibit NOB (Bae et al. 2001; Chung et al. 2006). Inhibition of NOB by free nitrous acid could also be excluded, since no nitrite accumulated during this phase of the study.

Alternation of high-low DO phases and aeration phase length control was successful in maintaining the nitrite pathway with both tested temperatures of 20°C and 15°C (Phase III). It confirmed that the alternation of high-low DO phases and aeration phase length control was selective enough to prevent the recovery of NOB, even at 15°C. It agrees with findings of Yang et al. (2007), who could maintain the nitrite pathway with temperatures as low as 12°C. In the subsequent period with uncontrolled high aeration, total nitrification recovered much faster at 15°C than at 20°C (Phase IV). An explanation for this phenomenon could be the higher nitrite accumulation at 15°C which provided more substrate for the growth of NOB. At 15°C, nitrate concentrations in the effluent reached a steady state after about 15 days. At 20°C, it gradually increased during the 33 days of uncontrolled full aeration without reaching a steady state (Figure 6-6B). Presumably some N was still removed over nitrite at 20°C at the end of Phase IV.

With intermittent aeration and aeration phase length control (Phase V), the nitrite pathway was rapidly recovered at 20°C. The clear decrease of the *Nitrospira* population and the stable N-removal of 95% indicated that after 20 days N was mainly removed over nitrite, even if some residual nitrate was still observed in the effluent. Two reasons may explain the rapid recovery of the nitrite pathway. First, intermittent aeration was supposed to act similarly on NOB growth rate as alternate high-low DO, but more efficient. After each pulse, some nitrite would remain and be denitrified in the subsequent unaerated phase instead of being oxidized by NOB. Hence, the impact on the growth rates should be multiplied by the number of pulses compared to a simple aeration phase length control. A crucial point was to ensure that nitrite was completely removed before the new aeration pulse started. For this reason, relatively long unaerated phases of 12.5 min between the aeration pulses were defined. Fux et al. (2006) implemented an automatic aeration control for intermittent aeration based on the oxidation–reduction potential (ORP) to make sure that all nitrite was

denitrified before restarting aeration. A second explanation for the rapid recovery of the nitrite pathway may be that the NOB population was still relatively low at 20°C at the end of Phase IV (Figure 6-6C). At 15°C, the *Nitrospira* population also decreased with intermittent aeration, but remained 3-4 times higher than at 20°C. N-removal reached a steady state at around 80%, with mainly nitrate in the effluent. This indicated that at 15°C intermittent aeration was not able to completely suppress nitrite oxidation. The most likely reason for this is the higher maximum growth rates of NOB compared to AOB at this temperature.

Over the whole study, only little nitrite accumulated with N-removal over nitrite. The highest concentration measured in the fully aerated reactor during the SBR cycle at day 99 was 0.13 mmol L^{-1} (Figure 6-4). During the full aeration period (Phase III), the effluent NO_x^- concentration was at both temperatures about 50% lower with N-removal over nitrite than with N-removal over nitrate (Figure 6-6B and Figure 6-7B). With intermittent aeration, no nitrite was detected during the SBR cycle (Figure SI 6-2A and B), despite of evidence for N-removal over nitrite, at least at 20°C. The low nitrite concentrations proofed that simultaneous nitrification and denitrification was more efficient by the nitrite pathway than the nitrate pathway. In a previous study, 70 – 100% higher denitrification rates have been observed for nitrite compared to nitrate (Chapter 5). Hence, this result was expected. However, a recent study by Bassin et al. (2012) with aerobic granular sludge and similar operating conditions reported significantly higher denitrification rates with nitrate than with nitrite. Hence, the observation of enhanced SND over nitrite should probably be considered as sludge specific. The accumulation of nitrite is known to have a negative impact on the phosphate uptake (Saito et al. 2004). Since nitrite did not accumulate in this study, it was always possible to maintain high P-removal performances in all different phases of the reactor operation.

N_2O emissions were under all four tested conditions higher with N-removal over nitrite than over nitrate. This agrees with two other recent studies on N_2O emissions (Ahn et al. 2011; Rodriguez-Caballero et al. 2013). Moreover, N_2O emissions were lower with intermittent aeration than with a DO of 30%. Low DO concentrations enhance the production of N_2O (Kampschreur et al. 2009; Wunderlin et al. 2012). With intermittent aeration the DO was either high or oxygen was completely absent. The reason for the N_2O peak right after aeration stop (Figure 6-8B) was most likely due the remaining oxygen during the first 1-2 minutes after aeration stop. Simultaneous nitrification and denitrification could still occur, but the N_2O produced by these processes was not stripped anymore by the aeration. This N_2O was only removed from the reactor at the beginning of the subsequent aeration pulse, explaining the emission peak right after the start of aeration. With N-removal over nitrate, a N_2O peak was observed during the first minutes of the feeding phase. This peak was most

likely due to heterotrophic denitrification, and the emission peak due to the stripping of this N_2O. With N-removal over nitrite, almost no nitrite remained at the end of the SBR cycle and therefore no heterotrophic denitrification took place during the subsequent feeding phase.

The DO signal appeared to be a valid and reliable criterion for the aeration phase length control. It agrees with results of a study by Yang et al. (2007), where the aeration phase length was also controlled based on the slope of DO. In other studies the real-time control of aeration and/or feeding was done based on the pH (Lemaire et al. 2008) or the oxidation–reduction potential (Fux et al. 2006). In the present study, the main reason for choosing a DO based aeration phase length control rather than a pH based control, was that the pH was regulated during the aerated phases. The drawback of the DO based control was, that the length of the non-aerated phases in intermittent aeration could not be real-time controlled, since DO gives no indication on denitrification. The pH signal neither was usable to figure out the moment when all nitrite was denitrified. A pH drop could be observed during the non-aerated phases. However, the signal was not reliable enough, probably because the bulk liquid was unmixed during the non-aerated phases.

6.5 Conclusion

This study showed that aeration phase length control combined with intermittent aeration or alternating high-low DO, is an efficient way to achieve N-removal over nitrite which is especially interesting for COD-limited systems. N-removal efficiencies of up to 95% were achieved with this way of reactor operation. At 20°C, N-removal over nitrite was achieved within 20 – 60 days and it was possible to switch from N-removal over nitrite to N-removal over nitrate and back again. At 15°C, the NOB population could be reduced, but nitrite oxidation could not be completely repressed. However, the combination of aeration phase length control and high-low DO was successful to maintain the nitrite pathway at 15°C where the maximum growth rate of NOB is clearly higher than the one of AOB.

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Supplementary information

Table SI 6-1: *Nitrospira* 16S rRNA gene concentrations measured with qPCR under different aeration conditions during the study period of 261 days.

Reactor	Aeration strategy	Temperature	Day	*Nitrospira* cn ng^{-1} of DNA
Parent reactor	alternating high-low DO	20°C	0	1779
	alternating high-low DO	20°C	42	4583
	alternating high-low DO	20°C	62	781
	alternating high-low DO	20°C	75	919
	alternating high-low DO	20°C	83	458
	alternating high-low DO	20°C	92	76
	alternating high-low DO	20°C	125	26
	alternating high-low DO	20°C	140	95
	alternating high-low DO	20°C	152	107
Reactor 1	alternating high-low DO	15°C	170	78
	alternating high-low DO	15°C	182	45
	constant high DO	15°C	191	40
	constant high DO	15°C	201	545
	constant high DO	15°C	212	586
	constant high DO	15°C	217	1936
	intermittent aeration	15°C	232	810
	intermittent aeration	15°C	251	303
	intermittent aeration	15°C	254	230
	intermittent aeration	15°C	258	337
Reactor 2	alternating high-low DO	20°C	170	75
	alternating high-low DO	20°C	182	91
	constant high DO	20°C	191	163
	constant high DO	20°C	201	203
	constant high DO	20°C	212	207
	constant high DO	20°C	217	473
	intermittent aeration	20°C	232	168
	intermittent aeration	20°C	251	99
	intermittent aeration	20°C	254	87
	intermittent aeration	20°C	258	86

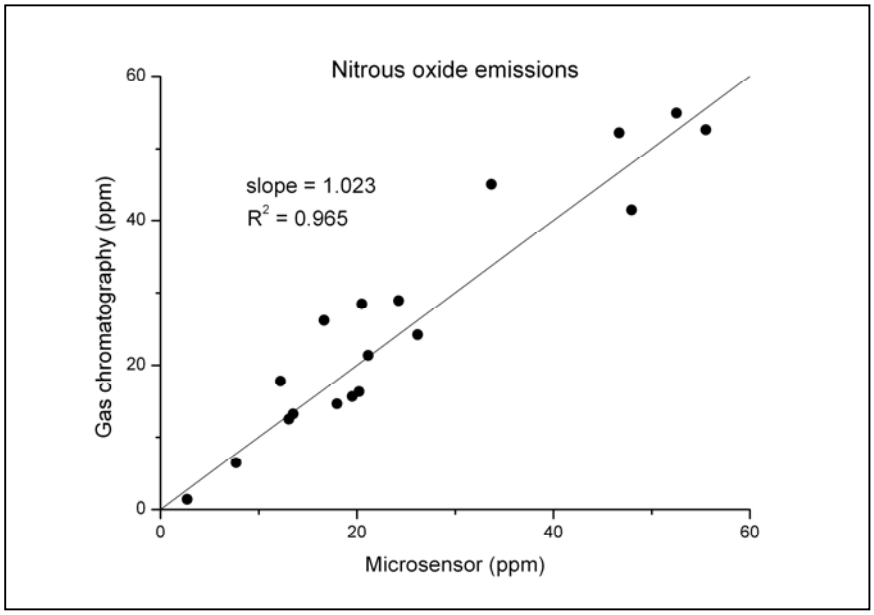

Figure SI 6-1: Comparison between gas chromatography and microsensor measurements for nitrous oxide in the gas phase

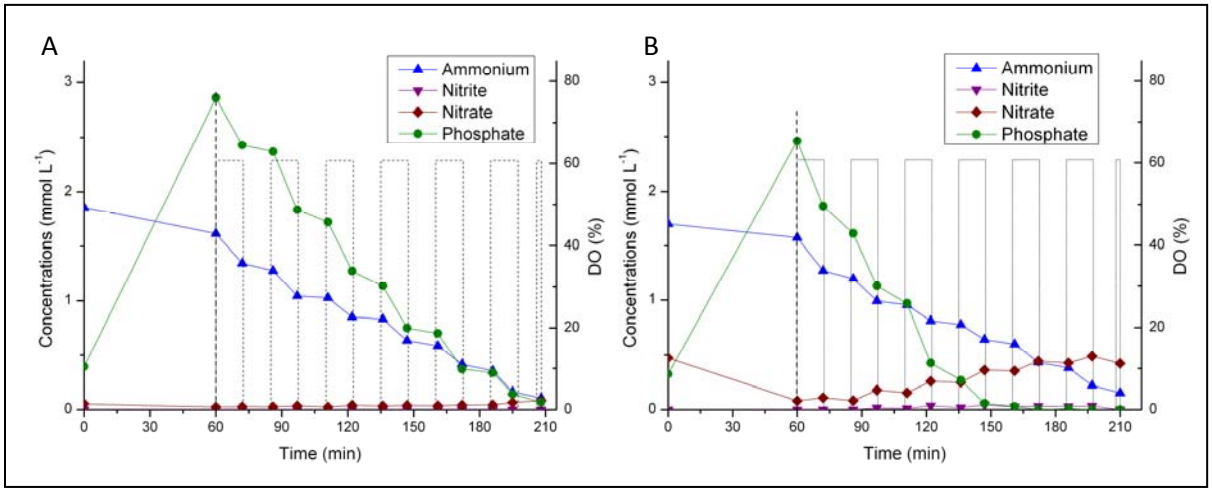

Figure SI 6-2: SBR cycles at day 258 at **(A)** 20°C and **(B)** 15°C with intermittent aeration. Concentrations at time 0 were calculated based on the effluent concentrations of the previous cycle and the influent concentrations. Aeration started after 60 min of plug-flow feeding. The dashed lines show schematically the aeration strategy.

Concluding remarks and Outlook

7

7. Concluding remarks and outlook

7.1 Startup of AGS-SBR

This thesis showed that under optimal conditions the startup of aerobic granular sludge (AGS) in sequencing batch reactors (SBR) could be achieved in less than 30 days. The comparison of seven parameters in parallel using a Plackett-Burman experimental design showed that the following tested conditions are optimal for a rapid startup of AGS-SBRs with good biological nutrient removal (BNR): (i) the alternation of high and low dissolved oxygen (DO) phases during aeration, (ii) a settling strategy avoiding too high biomass washout, (iii) the adaptation of the pollution load in the early stage of the startup in order to ensure that all soluble COD was consumed before the aeration phase, (iv) higher temperature (20°C), and (v) a neutral pH. The strategy was reproducible and the granular sludge as well as the BNR performances were stable. Compared to startup durations reported in literature of 60 - 150 days (de Kreuk et al. 2005a; Xavier et al. 2007; Gonzalez-Gil and Holliger 2011), the suggested startup strategy is a considerable improvement.

A particularly sensitive parameter appeared to be the settling time. In some experimental runs a massive wash-out of biomass was observed during the periods in which the settling time was decreased. Mainly with settling times <10min wash-out increased, resulting usually in a decrease of BNR. Hence, some experimental runs showed higher removal performances in the middle of the 45-days test phase than at the end. On the other hand, it has been observed that the sludge volume index (SVI) improved rapidly with all configurations; sometimes even before the sludge became completely granular. It can be concluded, that a too rapid decrease of the settling time is not necessary and should even be avoided. Presumably a better solution would be to couple the settling time to the sludge wash-out and to discharge every day a constant amount of sludge as for example applied by Sheng et al. (2010).

Hence, the startup strategy suggested in the present thesis could presumably be further improved with a better adapted settling time strategy. However, even with strategies focusing exclusively on the granulation without taking into account BNR, the formation of granular sludge took 2 - 3 weeks (de Kreuk et al. 2005b; Li and Li 2009; Ebrahimi et al. 2010). Therefore, a further considerable improvement of the startup seems unrealistic. With regard to a full-scale application, the adaptation of the pollution load might be the most complicated parameter to handle. Unlike in lab-scale experiments, the COD concentration in the real wastewater is not constant and a mixing phase with

N_2 gas unrealistic. Hence it could be relatively difficult to estimate how much wastewater to add per cycle and after how long the aeration should be started. If mechanical mixing is possible, *in-situ* measurement could solve the problem.

Concerning the bacterial community composition during the startup phase, a general shift of the predominant populations was observed. In the flocculent inoculum sludge taken from a continuous flow reactor (CFR), *Intrasporangiaceae* and *Sphingobacteriales* were predominant, whereas in the early-stage granular sludge, *Dechloromonas* and *Zoogloea* were the most abundant populations. This shift was observed in all runs and was related to the general change in conditions between full-scale CFR and controlled lab-scale AGS-SBR, rather than to the operation conditions in the different experimental runs. However, it has been observed that polyphosphate-accumulating organisms (PAO) and glycogen-accumulating organisms (GAO) related populations were favored by the adaptation of the pollutant load in the early stage of the startup in order to ensure that all soluble COD was consumed before the aeration phase. Besides the pollution load, temperature and pH had also a significant impact on the global bacterial community structure.

The question of the predominant PAO could not be completely elucidated. The well-known *Rhodocyclus*-affiliated *Candidatus* Accumulibacter phosphatis was neither in the inoculum sludge nor in most granular samples predominant. Nevertheless high P-removal performances were observed in several runs. This was rather surprising since in former AGS-SBR startups in our laboratory with the same inoculation sludge P-removal could directly by linked to the appearance of *Candidatus* Accumulibacter phosphatis (Ebrahimi et al. 2010; Gonzalez-Gil and Holliger 2011; Weissbrodt et al. 2013). Recently some other populations, among them *Intrasporangiaceae*- (Hanada et al. 2002; Kong et al. 2002) and *Dechloromonas*-affiliated (Kong et al. 2007) ones, have been hypothesized to act as PAO. Hence, it was presumed that *Intrasporangiaceae* were the predominant PAO in the inoculum sludge and *Dechloromonas* in the granular sludge. However, it cannot be excluded that others, not yet identified as PAO populations were responsible for P-removal. No pure cultures of PAO exist so far, explaining the lack of knowledge in this field. Most hypotheses on putative PAO are derived from observations with enriched cultures. The only metagenomic study so far is on *Candidatus* Accumulibacter phosphatis (Martín et al. 2006).

7.1.1 Remarks on methods used to investigate startup

Two methods with mathematical background have been used in the startup study: (i) Plackett-Burman experimental design and (ii) multivariate statistical analyses. Such tools based on statistics can be very helpful; however they have to be well understood for efficient use and correct interpretation.

7.1.1.1 Plackett-Burman experimental design

A Plackett-Burman design was used to test the impact of operation conditions on the startup. This experimental design allows investigating a maximum of parameters with a minimum of experimental runs. Therefore, this design is perfectly adapted for time-consuming bioreactor studies, where it is important to minimize the number of experimental runs. On the other hand, a minimum of experiments means also a minimum of information, no control runs and a high impact of every single result. It presupposes a careful planning of the study including a sensible choice of parameters and conditions, and the anticipation of possible problems. Interactions of parameters have to be excluded as much as possible, as well as test conditions leading to extreme results or even failure of an experiment. Hence, a conservative choice of conditions is required. On the other hand, the two conditions for each parameter should be different enough, to potentially have an impact on the result. In the startup study, except for the settling time, all conditions were well chosen. The decrease of the settling time was slightly too optimistic and resulted in higher wash-out than expected.

7.1.1.2 Multivariate statistical analysis

Multiple factor analysis (MFA) based on principal component analysis and the Spearman's rank coefficient was used to determine correlations between bacterial communities and environmental variables (operation conditions and performances). The advantage of multivariate analyses is that the whole dataset can be analyzed together. However, the limits of these methods, mainly concerning their interpretation, have to be known. The MFA is a projection of the different variables on a two dimensional graph. The two axes correspond to the principle components of the dataset, explaining a maximum of variance between data. In the case of the present thesis, about 30% of the total variance was explained by the MFA plot. The Spearman's rank correlation gives a numerical correlation between variables, but it is only a correlation based on rank coefficients. Hence, both results cannot be completely interpreted as numerical correlations between variables. Nevertheless, these methods are very helpful to classify the data in correlated groups or to find strongly related variables.

In the present thesis, correlations during the startup phase were studied, including samples from the inoculum sludge until day 45. Therefore, it is not further surprising that the shift from flocculent to granular sludge dominates the statistical analyses. An approach to eliminate this general shift would be to consider only samples with granular sludge. The focus would then be less on the startup, but more on the comparison between AGS-SBR operated under different conditions. Presumably, more correlations between single bacterial populations and environmental variables would be detected.

7.2 Biological nutrient removal

The present thesis showed the potential of aeration control for the optimization of BNR by AGS-SBR. In combined N- and enhanced biological P-removal (EBPR) systems, the COD concentration in the influent is often the limiting parameter (Kuba et al. 1996; Keller et al. 1997), resulting in limited denitrification. It could be proven here that aeration strategies promoting alternating nitrification and denitrification (AND) significantly improved N-removal compared to simultaneous nitrification and denitrification (SND) strategies. With SND glycogen-accumulating organisms (GAO) are presumed to be the main denitrifyiers. Polyphosphate-accumulating organisms (PAO) are more abundant in the outer layer of granules (Lemaire et al. 2008), which is aerated under SND conditions. The introduction of low DO phases or even anoxic phases in an early stage of the total aeration period when orthophosphate was still present probably enhanced denitrifying P-removal and led to COD savings. The highest N-removal efficiency has been achieved with intermittent aeration. The short mixing times with intermittent aeration of only about one third of the total cycle time were not problematic for the stability of the granules.

Aeration control has not only shown to be able to enhance denitrifying P-removal, but also to achieve N-removal over nitrite. With the nitrite pathway an even higher N-removal in COD-limited AGS-SBR was achieved than with enhanced denitrifying P-removal. Aeration phase length control combined with intermittent aeration or alternating high-low DO, was an efficient way to achieve N-removal over nitrite. Intermittent aeration or alternating high-low DO was presumed to enhance the effect of classical aeration phase length control, were aeration is stopped automatically when ammonium oxidation is upon completion. Every single aeration pulse should have a similar effect as aeration phase length control. With this way of reactor operation, N-removal efficiencies of up to 95% were achieved. At 20°C, N-removal over nitrite was achieved within 20 – 60 days and it was possible to switch from N-removal over nitrite to N-removal over nitrate and back again. At 15°C, the nitrite-oxidizing bacteria population could be reduced, but nitrite oxidation could not be completely repressed. However, the combination of aeration phase length control and high-low DO was

successful to maintain the nitrite pathway at 15°C where the maximum growth rate of nitrite-oxidizing bacteria is clearly higher than the one of ammonium-oxidizing bacteria.

Intermittent aeration is a realistic aeration strategy for full-scale application. The crucial point to achieve partial nitrification over time is the automated control of the length of the different phases. Ideally, the aerated and the anoxic phases should be both real-time controlled by *in-situ* measurements: the aerated phases to make sure to stop aeration when ammonium oxidation is upon completion and the unaerated phases to make sure to denitrify all NO_2^- before the next aeration pulse. Based on the results from this thesis, with such an aeration control it should be possible to achieve the N-removal over nitrite in summer with higher temperatures and to maintain it in winter.

A further step in the improvement of N-removal with AGS-SBR treating urban wastewater would be the integration of anammox in granular sludge as recently suggested by Winkler et al. (2012).

In the context of BNR the emission of nitrous oxide (N_2O) was also investigated. The emission measurements agreed with information available in literature (Kampschreur et al. 2009) on trigger factors for N_2O production: low DO, low COD and the presence of nitrite led to higher COD emissions. With intermittent aeration less N_2O emissions were observed compared to constant low DO. The emissions were highly variable according to the operation conditions. Between 0.7% and 13% of the influent N load was emitted as N_2O gas.

The problem of N_2O emissions from WWTP is a topic of growing interest. In the context of biofilm based wastewater treatment techniques it is particularly complex. It is known that N_2O production can take place during nitrification, denitrification and also by the reaction of nitrite with hydroxylamine. In AGS under typical SND conditions all three pathways are possible simultaneously. Hence, more research is needed in this field to elucidate the conditions to minimize N_2O emissions with AGS-SBR.

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