Partial denitrification as nitrite provider for mainstream anammox

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Water and Environmental Engineering
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Picture on front page: K5 biofilm carriers. Photo by Felix W. Holmin.

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Populärvetenskaplig sammanfattning

Anammox-processen erbjuder en hållbar och energieffektiv metod för biologisk kväveavskiljning inom avloppsrening. Men bakterierna som utför arbetet kväver en källa till nitrit, och en möjlig lösning på detta är att kombinera metoden med partiell denitrifikation. Kan ett samarbete mellan dessa olika grupper av mikroorganismer upprättas, och vilken påverkan har typen av kolkälla som används i processen?

Biologisk kväveavskiljning är en viktig del av avloppsrening, då utsläpp av höga halter ammonium i miljön kan leda till både försurning och övergödning av mark och vatten. Metoden som ofta används idag för att behandla större delen av det inkommande avloppsvattnet, så kallad nitrifikation och denitrifikation, kräver dock mycket energi i form av luftning, och eventuella tillsatser av organiskt kol. Detta gör det till en kostsam process, som även bidrar till klimatförändring genom utsläpp av växthusgasen lustgas. I metoden omvandlas det inkommande kvävet, i form av ammonium, först till nitrat (nitrifikation) och sedan till kvävgas (denitrifikation) som kan släppas ut i atmosfären utan negativa konsekvenser.

Partiell denitrifikation och anammox (förkortat PDA) är en alternativ metod för biologisk kväveavskiljning som har potentialen att minska energikonsumtionen för avloppsreningsverk. Idén bygger på att endast hälften av det inkommande ammoniumet omvandlas till nitrat i nitrifikationssteget. I en PDA reaktor omvandlas sedan detta nitrat till nitrit (genom partiell denitrifikation), som sedan direkt används av anammoxbakterier för att bilda kvävgas av det återstående ammoniumet och nitriten. Svårigheterna i processen ligger främst i att ”stoppa” denitrifikation efter att nitriten bildats, och i att upprätthålla en samlevnad av rätt typer av mikroorganismer i reaktorn.

I detta examensarbete utforskades möjligheten att kombinera anammox med partiell denitrifikation för gemensam avskiljning av ammonium och nitrat i avloppsvatten. Efter tio veckors drift indikerade resultaten att de två grupperna av mikroorganismer som är nödvändiga kunde samexistera på bärarmaterialet i reaktorn, och därigenom arbeta tillsammans för att omvandla nitrat och ammonium till kvävgas. Avskiljningshasigheten visade även en generell trend av ökning mot slutet av experimentet, vilket tyder på att processen behöver en längre tid av drift för att uppnå maximal kapacitet. Utöver detta visade sig acetat vara en mer lämplig kolkälla för bakterierna jämfört med propionat, eftersom det resulterade både i högre avskiljningshastigheter och ett mer lämpligt mikrobiellt samhälle.

Genom att applicera PDA i behandling av huvudströms avloppsvatten kan behovet av luftning potentiellt minskas med 50 %, och behovet av organiskt kol med 80 %, i biologisk kväveavskiljning. På grund av detta undersöker R&D (research and development) företaget Sweden Water Research möjligheterna för framtida tillämpningar av PDA i nästa generationens avloppsreningsverk, där detta examensarbete utgör början. Till följd av den påvisade potentialen av processen i detta arbete kan de två använda labbskalereaktorerna fortsättas hållas i drift under en längre tidsperiod, för att ytterligare utforska processens prestation när systemet nått en högre nivå av stabilitet

Preface

This master's thesis has been performed on the request of Sweden Water Research, in collaboration with the Department for Chemical Engineering at Lund University. I am very grateful for being given the opportunity to study this topic, and it has certainly been both fun and educational. Hopefully, the product of this work may be of use for future endeavours regarding the PDA process, just as the experiences gained will undoubtedly aid me.

I would like to thank everyone at Water and Environmental Engineering at Lund University for the help provided during the experimental parts of the project (amongst others Stina Karlsson and Gertrud Persson), Sofia Lindström for reviewing my report, and AnoxKaldnes for providing the K5 carriers used in the experiments. Additionally, I would like to give special thanks to my supervisors, David Gustavsson and Per Falås, for all the aid and guidance provided throughout the project.

Finally, I want to thank my friends and family, both in Lund and Stockholm, for supporting me during my studies and making my time in Lund the amazing experience it has been.

Felix W. Holmin

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Summary

In traditional wastewater treatment, nitrogen is removed through the biological process combination of nitrification and denitrification. Although it is a well-established method, the need for aeration and possible addition of an external carbon source results in it being an energy-negative and costly process, thus making alternative methods for nitrogen removal compelling to examine. Anaerobic ammonium oxidation (anammox) has been presented as such an alternative, providing a "short-cut" through the nitrogen cycle by anaerobically oxidizing ammonium (NH_4^+) to nitrogen gas (N_2) using nitrite (NO_2^-) as elector acceptor. By coupling the anammox reaction to partial denitrification (PDA), where nitrate (NO_3^-) is reduced to nitrite, simultaneous nitrogen removal of ammonium and nitrate is possible under anoxic conditions and low concentrations of organic carbon.

To examine the start-up and operation of a PDA process treating synthetic mainstream wastewater, two continuous 2 L moving bed biofilm reactors (MBBRs) were operated in parallel for ten weeks. After being inoculated with K5 biofilm carriers (previously used for partial nitrification/anammox) provided by AnoxKaldnes, the reactors were fed with low concentration synthetic wastewater, with either acetate or propionate as carbon source at a chemical oxygen demand (COD) to NO_3^--N ratio of 2. Analysis of the influents and effluents were performed three times a week to determine the removal rates of the reactors. Furthermore, four ex-situ activity batch tests were performed during the ten weeks to determine the change of activity in the microbial populations on the biofilm carriers.

During the start-up period several modifications were made to the experimental setup, such as separating the carbon source influents, in order to achieve stable conditions and influent concentrations. Subsequently, the reactors displayed a general increase of PDA capabilities during the final five weeks, with total nitrogen removal rates increasing from 0.29 ± 0.01 to 0.47 ± 0.03 g N/(m^2*d) in the acetate fed reactor and from 0.17 ± 0.03 to 0.23 ± 0.03 g N/(m^2*d) in the propionate fed reactor. Additionally, the anammox contribution to nitrogen gas formation was calculated to approximately 91 % in the acetate fed reactor and 64 % in the propionate fed reactor in the final week of operation. The improved performance of the acetate fed reactor was mainly attributed to a higher denitrification rate achieved by less complex utilization of the carbon source, as well as a larger abundance of partial denitrifiers compared to complete denitrifiers on the biofilm carriers. Furthermore, the nitrate reducing activities, and thus the nitrogen removal rates, appeared to still be increasing during the final weeks, suggesting that the microorganisms had not yet reached a stable co-community of denitrifiers and anammox bacteria. Consequently, further operation is required to establish the full capacity of the process.

Sammanfattning

I traditionell avloppsrening avskiljs kväve via den biologiska processkombinationen av nitrifikation och denitrifikation. Denna väletablerade metod kräver dock mycket luftning samt eventuell tillsättning av extern kolkälla vilket resulterar i en energinegativ och dyr process, och det är därför av intresse att undersöka alternativa metoder för kväveavskiljning. Anaerob ammoniumodixation (anammox) är en sådan metod, där ammonium (NH_4^+) oxideras till kvävgas (N_2) med nitrit (NO_2^-) som elektronacceptor via en ”genväg” i kvävecykeln. Genom att kombinera anammox med partiell denitrifikation (PDA), där nitrat (NO_3^-) reduceras till nitrit, är kombinerad kväveavskiljning av nitrat och ammonium möjlig under anoxiska förhållanden och låga koncentrationer av organiskt kol.

För att undersöka uppstarten och potentialen av en PDA process startades två parallella 2 L MBBR-reaktorer (Moving Bed Biofilm Reactor) och hölls i drift under tio veckors tid. Efter inokulering med K5-biofilmsbärare (tidigare använda för partiell nitrifikation/anammox), tillhandahållna av AnoxKaldnes, tillfördes syntetiskt avloppsvatten med låga komponentkoncentrationer till de två reaktorerna, med antingen acetat eller propionat som organisk kolkälla i ett COD (chemical oxygen demand) till NO_3^--N förhållande av 2. Analyser av reaktorernas in- och utflöden utfördes tre gånger i veckan, och fyra aktivitetstest utfördes under de tio veckorna för att utvärdera förändringen av biofilmens denitrifikations- och anammoxaktivitet.

Under uppstartsperioden gjordes flera modifieringar av försöksuppställningen, såsom att tillföra kolkällan separat, för att uppnå stabila reaktorförhållanden och koncentrationer i inflödet. Därefter observerades en generell ökning av PDA-förmåga i båda reaktorer under de sista fem veckorna av experimentet, med en ökning av den totala kväveavskiljningshastigheten från 0.29 ± 0.01 till 0.47 ± 0.03 g N/(m^2*d) i den acetatmatade reaktorn och från 0.17 ± 0.03 till 0.23 ± 0.03 g N/(m^2*d) i den propionatmatade. Dessutom var det beräknade anammoxbidraget till kvävgasbildningen 91 % i den acetatmatade reaktorn och 64 % i den propionatmatade under den tionde veckan. Den högre prestationsförmågan av den acetatmatade reaktorn tillskrevs i första hand den högre denitrifikationshastigheten, möjliggjord av den mer lättnedbrytbara kolkällan, samt en högre partiell denitrifikationshastighet i förhållande till komplett denitrifikationshastighet, och därav en större tillgänglighet av nitrit för anammoxbakterierna. Utöver detta ökade också den nitratreducerande aktiviteten, och därigenom den totala kväveavskiljningshastigheten, fortfarande för biobärarna i båda reaktorerna under de sista veckorna, vilket indikerade att det mikrobiella samhället ännu inte nått stabilitet. Således krävs ytterligare drift för att fastställa processens fulla kapacitet.

List of Abbreviations

BNR – Biological nitrogen removal

COD – Chemical oxygen demand

DO – Dissolved oxygen

MBBR – Moving bed biofilm reactor

NLR – Total nitrogen loading rate

NRR – Total nitrogen removal rate

PD – Partial denitrification

PDA – Partial denitrification coupled to anammox

PNA – Partial nitrification coupled to anammox

VFA – Volatile fatty acid

VSS – Volatile suspended solids

WWTP – Wastewater treatment plant

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1 Introduction

In traditional wastewater treatment, the most common method of biological nitrogen removal (BNR) is the nitrification-denitrification process. This technique involves oxidising the nitrogen (present as ammonium, NH_4^+) to nitrate (NO_3^-) under oxic conditions (i.e. nitrification), followed by a reduction of nitrate to nitrogen gas (N_2) under anoxic conditions and with an organic carbon source available (i.e. denitrification) (Ferguson et al., 2007). When applying nitrification in wastewater treatment plants (WWTPs), the availability of oxygen results in oxidation of the organic carbon to CO_2. Consequently, it may be required to add additional organic carbon in order to achieve effective denitrification. This can, however, be avoided by first performing denitrification, followed by nitrification, and recirculating the wastewater. Although this is a well-established method of BNR, high aeration costs, sludge formation and possible nitrous oxide (N_2O) emissions make development of an alternative method appealing (Zhang et al., 2019; Chai et al., 2019).

In recent years, anaerobic ammonium oxidation (anammox) process has been investigated as a possible alternative to conventional BNR. It offers a method of BNR that would reduce the amount of organic carbon and aeration rate required (Kartal et al., 2010), which represents a large part of the cost for maintaining wastewater treatment plants (WWTP) today. The anammox reaction requires ammonium (NH_4^+) and nitrite (NO_2^-), as well as carbon dioxide (CO_2), to form biomass and nitrogen gas (N_2), which can then be released into the atmosphere. Since most of the nitrogen in wastewater is present in the form of ammonium, a source of nitrite is required to achieve BNR through anammox. Partial nitrification (PN), where ammonium is aerobically oxidised to nitrite, has been utilized in combination with anammox to provide the system with a source of nitrite, however this typically requires high temperature and concentration of ammonium in the wastewater to reach satisfactory performance (Cao et al., 2017). Thus, the combination of PN and anammox (i.e. PNA) may prove challenging when applied in mainstream wastewater treatment, which is characterized by low temperatures and concentrations.

Studies investigating denitrification, where nitrate (NO_3^-) is reduced to nitrogen gas (N_2), has recorded an accumulation of nitrite under certain conditions; a behaviour which appears to be impacted by the type of carbon source present (Cao et al., 2013; Le et al., 2019). Thus, partial denitrification (PD) may potentially be utilized as nitrite provider for the anammox reaction when treating mainstream wastewater. Combined with an initial pre-treatment step of nitrification, where half of the incoming ammonium is oxidised to nitrate, this process may reduce the need for aeration by 50 % and organic carbon by 80 % when compared to conventional BNR (Le et al., 2019b). Although the PNA process potentially reduces aeration and organic carbon needs for BNR by 60 % and 100 % respectively (Ma et al., 2020), mass balances of a full-scale WWTP has revealed that implementation of partial denitrification coupled to anammox (PDA) in mainstream wastewater treatment would require less oxygen compared to PNA, due to a portion of the organic carbon being consumed anaerobically in PDA (Cao et al., 2019a). The potential advantages of PDA make it an appealing process to study, and this Master's thesis will explore the possibility of running PDA continuously in a lab-scale moving bed biofilm reactor (MBBR) for nitrogen removal in synthetic wastewater, using carbon sources previously found to induce nitrite accumulation in denitrification.

1.1 Aim

The aim of this Master's thesis project was to examine the BNR capabilities of a PDA system, inoculated with biofilm carriers previously performing PNA. The effect on the PDA process when using either acetate or propionate as carbon source for the denitrification was investigated by operating two continuous lab-scale reactors in parallel. The stability of the systems was determined by operating the reactors for ten weeks including start-up. Furthermore, ex-situ activity batch tests were performed regularly to evaluate the PDA capabilities of the microbial populations on the carriers.

2 Theory

2.1 Biological nitrogen removal methods

2.1.1 Nitrification & Denitrification

During nitrification, autotrophic nitrifying microorganisms oxidise ammonium (NH_4^+) to nitrate (NO_3^-) under oxic conditions via the intermediate nitrite (NO_2^-) (Fig. 2.1). Nitrifiers are divided in two groups, based on the reactions they catalyse; ammonium oxidizing bacteria (AOB) catalyses the oxidation of ammonium (NH_4^+) to hydroxylamine (NH_2OH) and further to nitrite (NO_2^-), and nitrite oxidizing bacteria (NOB) catalyses the oxidation of nitrite to nitrate (NO_3^-) (Ferguson et al., 2007). Although a dissolved oxygen concentration below 1.0 mg/L has been shown to decrease both AOB and NOB activity, the decrease is larger for the nitrite oxidation rate (Garrido et al., 1997). Nitrification is usually followed by denitrification, where nitrate is reduced to nitrogen gas through the intermediates nitrite, nitric oxide (NO) and nitrous oxide (N_2O) (van Spanning et al., 2007). These reactions are catalysed by heterotrophic microorganisms referred to as denitrifiers and require anoxic conditions and an available source of organic carbon.

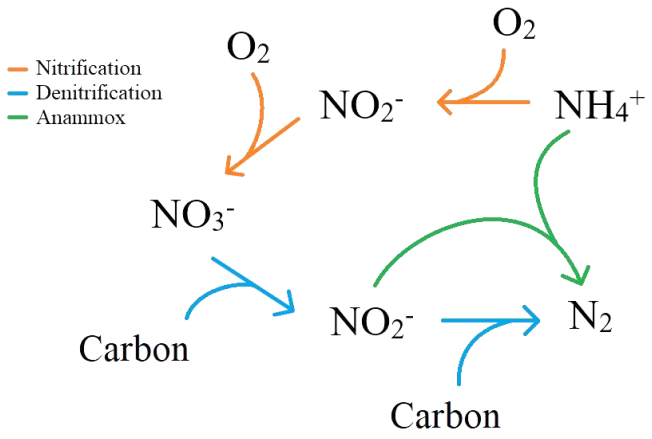

Figure 2.1. Simplified scheme of biological nitrogen removal in wastewater treatment, showing nitrification in orange, denitrification in blue and anammox in green.

2.1.2 Anammox

In the **an**aerobic **amm**onium **ox**idation reaction (anammox) ammonium is oxidised using nitrite as an electron acceptor (Eq. 2.1), resulting in the formation of nitrogen gas (Strous et al., 1998):

$$NH_4^+ + 1.32\,NO_2^- + 0.066\,HCO_3^- + 0.13\,H^+ \rightarrow 1.02\,N_2 + 0.26\,NO_3^- + 0.066\,CH_2O_{0.5}N_{0.15} + 2.03\,H_2O \quad (2.1)$$

Anammox was first theorized in 1977, and the first successful attempt at cultivating the bacteria catalysing the reaction was performed in a fluidized bed reactor during the 90s (Strous et al., 1998; Mulder et al., 1995). The reaction is catalysed by autotrophic bacteria and is achieved in anoxic conditions. This method of BNR presents an alternative approach for nitrogen removal in wastewater treatment and could potentially circumvent the need for both high aeration rates and the potential addition of an external carbon source required in conventional BNR, thus

changing the process from energy-negative to an energy-positive sewage treatment. (Kartal et al., 2010)

Anammox bacteria are characterised by low growth rates, a complicated cell plan (typically containing a large vacuolar cell organelle; the anammoxosome) and high substrate affinity (i.e. the ability for growth at low substrate concentrations), thus displaying the properties of a K-strategy microorganism (Kartal et al., 2012; Kartal & Keltjens 2016). Doubling times for the bacteria are usually at approximately 11 days at 30 – 33ºC, although more rapid growth (1.8 days at 37ºC) have been recorded (Op den Camp et al., 2007; Strous et al., 1998; Isaka et al., 2006). Their optimal temperature for growth is approximately 40ºC, although they may be adapted to lower temperatures, and they are active at a pH of approximately 6.7 – 8.3 (Op den Camp et al., 2007; Cho et al., 2020). Some anammox bacteria have been found to have a versatile metabolic capacity, with the number of anammox catabolism and respiration genes exceeding 200 (Strous et al., 2006). In addition, anammox bacteria are capable of staying dormant under unfavourable conditions and re-activate under favourable conditions (Cho et al., 2020). As part of the *Planctomycetes* phylum, six *Candidatus* genera have been found to include anammox active bacteria: *Brocadia*, *Kuenenia*, *Scalindua*, *Anammoxoglobus*, *Anammoximicrobium* and *Jettenia* (Kumwimba et al., 2020; Op den Camp et al., 2007).

Certain anammox bacteria have been found to possess organotrophic capabilities, thus being able to obtain electrons from organic compounds (Kartal et al., 2007a). Through a reaction called dissimilatory nitrate reduction to ammonium (DNRA), species such as *Kuenenia stuttgartiensis*, *Ananimoxoglobus propionicus* and *Brocadia fulgida* have been reported to reduce nitrate to ammonium via an intermediate step of nitrite, using volatile fatty acids (VFA) (including acetate, propionate and formate) as electron donors (Fig. 2.2) (Kartal et al., 2007b). The reduction of nitrite to ammonium is the rate limiting step in DNRA, consequently providing the microorganisms with both ammonium and nitrite, which may in turn be utilized for the ammonium oxidation reaction (Eq. 2.1) that they typically are known for. Although the reaction rate for DNRA may be 10 % of the ammonium oxidation reaction rate (Kartal et al., 2007a), and denitrification is favoured over DNRA in environments with a C/NO_3^- ratio below 12 (Friedl et al., 2018), it efficiently utilizes the redox potential of the organic carbon (by oxidising it completely to CO_2 and not producing biomass) and could possibly contribute to the nitrate-to-nitrite conversion in the PDA process (Shu et al., 2015).

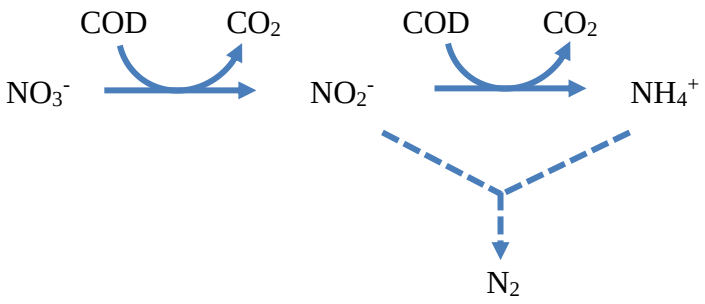

Figure 2.2. Simplified scheme of dissimilatory nitrate reduction to ammonium (DNRA) (solid lines) and anaerobic ammonium oxidation (dashed lines) performed by anammox bacteria.

2.1.3 Partial denitrification coupled to anammox

To implement anammox for wastewater treatment process, a source of nitrite (i.e. the electron acceptor in the reaction) is required. In the nitrification process, ammonium is initially oxidised to nitrite before being oxidised further to nitrate. Hence, partial nitrification (i.e. accumulation of the nitrite intermediate during nitrification; often referred to as partial nitrification-anammox) has been utilized as a source of nitrite for the anammox reaction when treating high concentration (500 – 1500 mg NH_4^+-N/L) and high temperature (~30°C) wastewater side streams (Lackner et al., 2014). The growth of NOB may be suppressed by for example a low dissolved oxygen (DO) concentration, as the AOB has a higher oxygen affinity, thus resulting in an incomplete nitrification with nitrite accumulation which may be exploited by anammox bacteria (Hao et al., 2002). However, the application of PNA to mainstream wastewater treatment, characterized by low temperatures (10 – 20°C) and low concentrations (30-100 mg NH_4^+-N), has proven to be challenging.

Insufficient suppression of NOB during PNA may result in increased residual concentrations of nitrate in the effluent, in addition to the nitrate formed as a by-product of the anammox reaction (Du et al., 2017b). Free ammonia and free nitrous acid acts as inhibitors on NOB growth, thus benefitting the process, however the low concentration of ammonium in the influent results in lower concentrations of these inhibitors (Cao et al., 2017). Furthermore, to achieve sufficient NOB suppression and effluent ammonium concentration at reduced temperatures, a low DO concentration is typically required in the reactor, resulting in a need for operating at low nitrogen loading rates (Pérez et al., 2014).

In denitrification, a nitrate reduction rate (NO_3^- → NO_2^-) which surpasses the nitrite reduction rate (NO_2^- → NO → N_2O → N_2) results in an accumulation of nitrite, which may be utilized as a reactant by anammox bacteria. A combination of partial denitrification and anammox (PDA) can therefore reduce the amount of carbon required for BNR in comparison with conventional nitrification-denitrification. Specific conditions are required in order to limit the reduction of nitrite to nitric oxide and achieve satisfactory rates of nitrite formation, and nitrate-to-nitrite conversion yields of 80 % has been reached in PD (Du et al., 2017a; Ma et al., 2017; Zhang et al., 2019). The following reactions describe partial denitrification (Eq. 2.2) and partial denitrification coupled to anammox (Eq. 2.3), with acetate as organic carbon source (Du et al., 2017a):

$$1.32\ NO_3^- + 0.55\ CH_3COO^- + 0.088\ NH_4^+ \rightarrow 1.32\ NO_2^- + 0.088\ C_5H_7NO_2 + 0.66\ HCO_3^- + 0.198\ H^+ + 0.264\ H_2O \quad (2.2)$$

$$NO_3^- + 0.519\ CH_3COO^- + 1.026\ NH_4^+ \rightarrow 0.926\ N_2 + 0.56\ HCO_3^- + 0.064\ H^+ + 0.083\ C_5H_7NO_{2(Denitrifying)} + 0.062\ CH_2O_{0.5}N_{0.15(Anammox)} + 2.16\ H_2O \quad (2.3)$$

Thus, the PDA process requires a microbial population of two groups of microorganisms: denitrifiers and anammox bacteria. The denitrifiers are a group of heterotrophic bacteria composed dominantly of bacteria of the *Proteobacteria* phylum, from genera such as *Pseudomonas*, *Ralstonia*, *Alcaligenes*, *Paracoccus*, *Rhodobacter*, *Rubrivivax*, *Thauera*, *Burkholderia*, *Bacullus*, and *Streptomyces* (Du et al., 2017a; Ginige et al., 2005; van Spanning et al., 2007). Although several of the bacterial species in the denitrifying community are capable of catalysing all denitrification reactions, certain species have been shown to favour specific reactions (Cao et al., 2013). Typically, the bacteria are capable of either a full reduction of nitrate (NO_3^-

→ N_2) with nitrite accumulation (type A), full reduction of nitrate without nitrite accumulation (type B) or only reduction of nitrate to nitrite (NO_3^- → NO_2^-) (type C).

The genes involved in denitrification are *narG* (encoding nitrate reductase), *nirS* and *nirK* (encoding independent nitrite reducing enzymes), *norB* (encoding the catalytic subunit of nitric oxide reductase) and *nosZ* (encoding nitrous oxide reductase) (Fig. 2.3) (Luvizotto et al., 2019). Denitrifying bacteria of the genus *Thauera* has been identified as dominant in the PDA system when acetate is used as substrate, and some species suppresses their *nirS* gene (coding for nitrite reductase) when nitrate is available, resulting in an increased accumulation of nitrite (Du et al., 2017b; Liu et al., 2013).

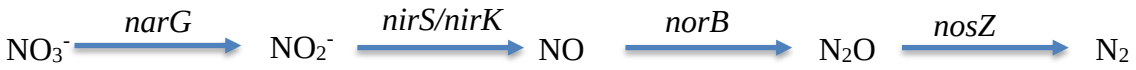

Figure 2.3. Intermediate steps of denitrification, as well as genes coding for the corresponding reductases of each reaction.

2.2 PDA process parameters

2.2.1 Carbon source

There are several factors that should be taken into consideration when designing a PDA process. The carbon source has a large effect on the denitrification, and various compounds has been investigated for their capability of increasing the nitrate reduction (NO_3^- → NO_2^-) in the reactor while decreasing nitrite reduction (NO_2^- → N_2), as well as their ability to increase the selective pressure for growth of bacteria favouring nitrate reduction over nitrite reduction. These two qualities results in an efficient supply of nitrite for the anammox bacteria, as well as providing a long-term stability to the system. (Le et al., 2019a)

Among the carbon sources examined are acetate, methanol, ethanol, glycerol, formic acid, propionic acid, n-butyric acid, isobutyric acid and valeric acid (Le et al., 2019a; Li et al., 2015; Du et al., 2017a). Acetic acid has been showed to result in the highest nitrate consumption rate in PD (6.32 mg NO_3^--N/(g MLSS*h)) followed by propionic acid. Since acetate is an end fermentation product it can be consumed directly by a wide range of bacteria, and thus promotes growth of a wide range of denitrifying bacteria. In contrast, when propionic acid is used as carbon source it must first be degraded into smaller VFAs before it can be utilized as a secondary carbon source for denitrification, meaning a longer period of acclimatization of both denitrifiers and propionic acid degrading microorganisms is required (Li et al., 2015).

The type of carbon source may also have an effect on the nitrate-to-nitrite transformation ratio (NTR) as a result of how the compound is processed within the bacterial cells. The enzymes involved in denitrification (nitrate reductase (NR) and nitrite reductase (NIR)) accepts electrons at different regions of the electron transport chain, and therefore a carbon source that donates its electrons in the upstream region, where NR accepts electrons, may increase the electron availability for NR in comparison with NIR. One such carbon source is acetate, which may help explain its advantage. (van Rijn et al., 1996; Le et al., 2019a)

2.2.2 Nitrate and COD concentration

In order to achieve a successful PDA process, adequate growth of both denitrifiers and anammox bacteria is required. Since denitrifiers generally have higher growth rates in comparison

with anammox active bacteria, limiting the carbon availability may reduce the growth of the heterotrophic denitrifiers and thus result in a stable population of both types of bacteria (Du et al., 2017a). Additionally, the COD (chemical oxygen demand) concentration will also affect the denitrifying bacterial community. A deficiency of COD, i.e. the electron donor, favours the reduction of nitrate to nitrite over the reduction of nitrite to nitrogen gas since it is a more energy efficient reaction, requiring two and three mole electrons per mole respectively (Li et al., 2016). Although a low COD/NO_3^--N ratio has shown to result in efficient nitrite accumulation and a selective pressure for partial denitrifiers, it may not be a crucial factor for partial denitrification once a suitable denitrifying community is acquired (Le et al., 2019a; Le et al., 2019b; Li et al., 2016). Instead, nitrate concentration has been identified as a key parameter for maintaining an efficient NTR, where a residual concentration above 2 mg N/L has been shown to facilitate partial denitrification and a concentration below results in nitrite reduction by denitrifiers (Le et al., 2019b). Thus, the COD/NO_3^--N ratio may instead be used as a method of controlling the nitrate reduction rate, and consequently the residual nitrate concentration and nitrite availability for anammox bacteria (Le et al., 2019a).

Previous studies have observed nitrite accumulation in denitrification with COD/NO_3^--N ratios < 8 (Cao et al., 2013; Le et al., 2019a). However, to decrease the rate of full denitrification, the PDA process requires that the nitrate reduction rate, which may be controlled by the COD/NO_3^--N ratio, is in balance with the anammox activity in the system. Consequently, ratios ranging between approximately 2 – 3 has frequently been selected when conducting PDA experiments (Xu et al., 2020; Du et al., 2017b; Cao et al., 2016; Ma et al., 2017). Furthermore, a COD/NH_4^+-N ratio (which is often equal to the COD/NO_3^--N ratio in PDA) above 5 has been found to reduce anammox activity (Zhu et al., 2017). Based on nitrate-to-nitrite reduction reaction stoichiometry (see Eq. 3.10 & 3.18 in section 3.4.5), the theoretical COD/NO_3^--N ratio required for PD is 1.7 and 1.5 when utilizing acetate or propionate, respectively, as carbon source.

2.3 MBBR

In a moving bed biofilm reactor (MBBR), a suspended plastic media known as carriers are used to increase the amount of biomass contained in the reactor. The microorganisms form biofilms on the carriers, which are moving freely, and the solution is mixed to ensure homogeneity. Aeration or mechanical mixing is used to accomplish agitation, and the MBBR is used frequently in both industrial and municipal wastewater treatment (Ødegaard & Jahren, 2000). Due to the bacterial growth on the carriers, an increased amount of biomass can be retained in the reactor, leading to higher load capacities and lower reactor volumes.

When employing MBBR for PDA processes, the anoxic-carrier biofilm has been found to have a larger potential for promoting nitrate-to-nitrite conversion compared to flocculent sludge, as the *narG* gene (encoding nitrate reductase) can be enriched in the microbial community (Li et al., 2019). In turn, this increased conversion may enhance the growth of anammox bacteria by increasing nitrite availability, as well as assist in managing the nitrate formed in the anammox reaction, thus creating a more efficient process (Li et al., 2019).

2.4 Nitrous oxide by-product of PDA

As nitrite is further reduced as part of denitrification, it takes an intermediate form of nitrous oxide (N_2O). This compound is a well-known greenhouse gas, and substantial amounts are emitted from wastewater treatment plants (Chai et al., 2019). Elevated concentrations of NO_2^- has been found to increase the accumulation of NO and N_2O, due to inhibitory effects on

enzymes involved in denitrification (Schulthess et al., 1995). The increased nitrite accumulation in partial denitrification could thus be a cause for concern regarding N_2O emissions, however in a well-functioning one-stage PDA process the nitrite produced by the denitrifiers is promptly consumed by the anammox bacteria. Consequently, by maintaining a low concentration of nitrite in the reactor, as well as limiting the extent of full denitrification taking place, the formation and emission of N_2O may be minimized.

The COD/NO_3^--N ratio is an important parameter for reducing the accumulation of nitrous oxide. In a study by Du *et al.* (2016), significant formation of the gas has been observed when performing PD with a COD/NO_3^--N ratio above 4. In addition, the accumulation of the gas increased rapidly as the nitrate was depleted and the nitrite reduction increased in the batch reactor (Du et al., 2016). Thus, a continuously run PD may be able to avoid significant nitrous oxide formation, due to the constant addition of nitrate.

2.5 One-stage and two-stage PDA

PDA may be operated either as a one-stage or two-stage process. During two-stage PDA, the biological processes of partial denitrification and anammox are performed in two separate reactors, thus avoiding the competition for space between the two groups of microorganisms (You et al., 2020). In a study by Cao *et al*. (2019), > 95 % nitrogen removal was achieved in a two-stage PDA process operated at temperatures ranging from 18.7 – 27.8^oC and influent NO_3^- concentrations of 20 – 40 mg N/L, with an anammox contribution towards nitrogen removal of 78.2 % (Cao et al., 2019b). Additionally, relatively low N_2O formation was observed during the experiment, despite the elevated concentrations of nitrite in the effluent of the PD reactor.

In contrast, a one-stage PDA process utilizes one reactor for both nitrate-to-nitrite conversion and nitrogen removal by anammox, consequently reducing the infrastructure cost and simplifying operations (Du et al., 2019). Furthermore, the nitrate formed in the anammox reaction may be directly utilized by the denitrifiers in the reactor, without the need for recirculating the effluent. Nitrogen removal efficiencies of 97 % has been achieved in one-stage PDA at temperatures of 22 – 28^oC, with anammox contributions towards nitrogen removal of 90 % (Cao et al., 2016). With a stable co-community of denitrifiers and anammox bacteria in a one-stage PDA process, efficient nitrogen removal is thus possible while simultaneously avoiding the need for accumulating higher nitrite concentrations, which (as stated in section 2.4) may contribute to both denitrification inhibition and N_2O formation.

3 Method

This section is comprised of four parts, one for each of the three types of experiments performed (i.e. continuous PDA experiment, ex-situ batch activity tests and COD determination of biomass), and one describing the calculations and mass balances used to process the results obtained in the experiments.

3.1 Continuous PDA experiment

3.1.1 Experimental setup

In the continuous experiment, two 2 L reactors were operated in a water bath at 17.1 ± 0.4°C (Fig. 3.1). As light has been found to have a negative effect on anammox activity (van de Graaf et al., 1996), the reactors were covered in aluminium foil. Overhead paddle stirrers (operating at 45 rpm) were mounted above the reactors and nitrogen gas was used to purge the reactors of oxygen, ensuring a homogenous anoxic environment. In addition, plastic wrapping was used to isolate the reactors, limiting the backflow of air into the overhead volumes. The reactors were inoculated with 225 AnoxKaldnes K5 biofilm carriers in each reactor, which had previously been used for PNA in sludge liquor treatment at Sjölunda WWTP in Malmö, Sweden (Christensson et al., 2013). Each reactor was fed with synthetic wastewater from 25 L tanks during weekdays and 50 L tanks during weekends using a peristaltic pump, and the effluents were collected in a 200 L tank. When refilling and emptying the tanks, they were rinsed with hot water to minimize biofilm formation.

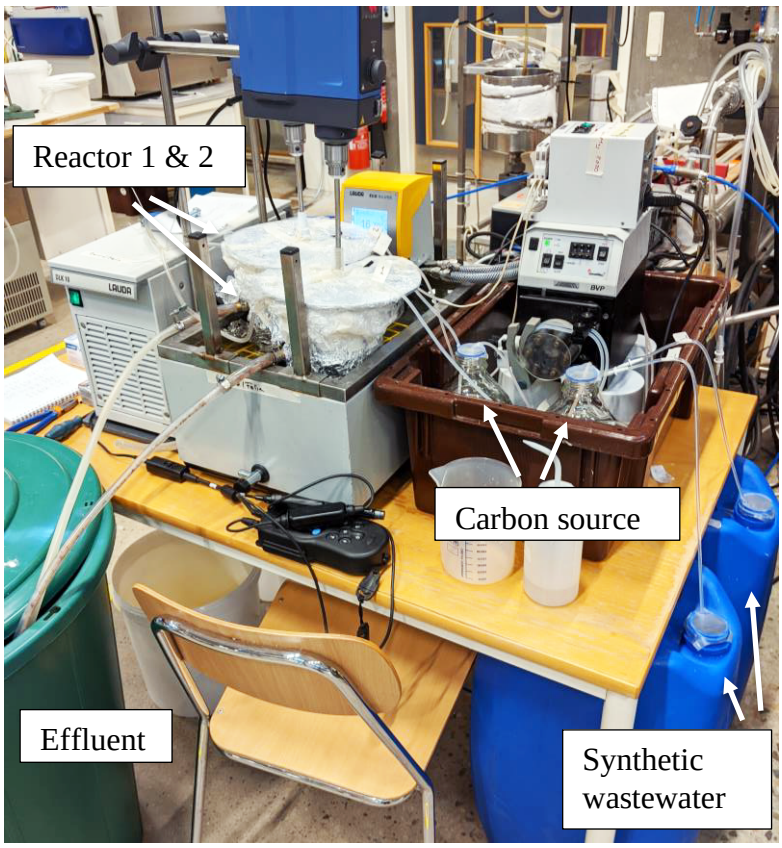

Figure 3.1. Picture of the experimental setup of the continuous PDA experiment, with separate feeds for synthetic wastewater and carbon source.

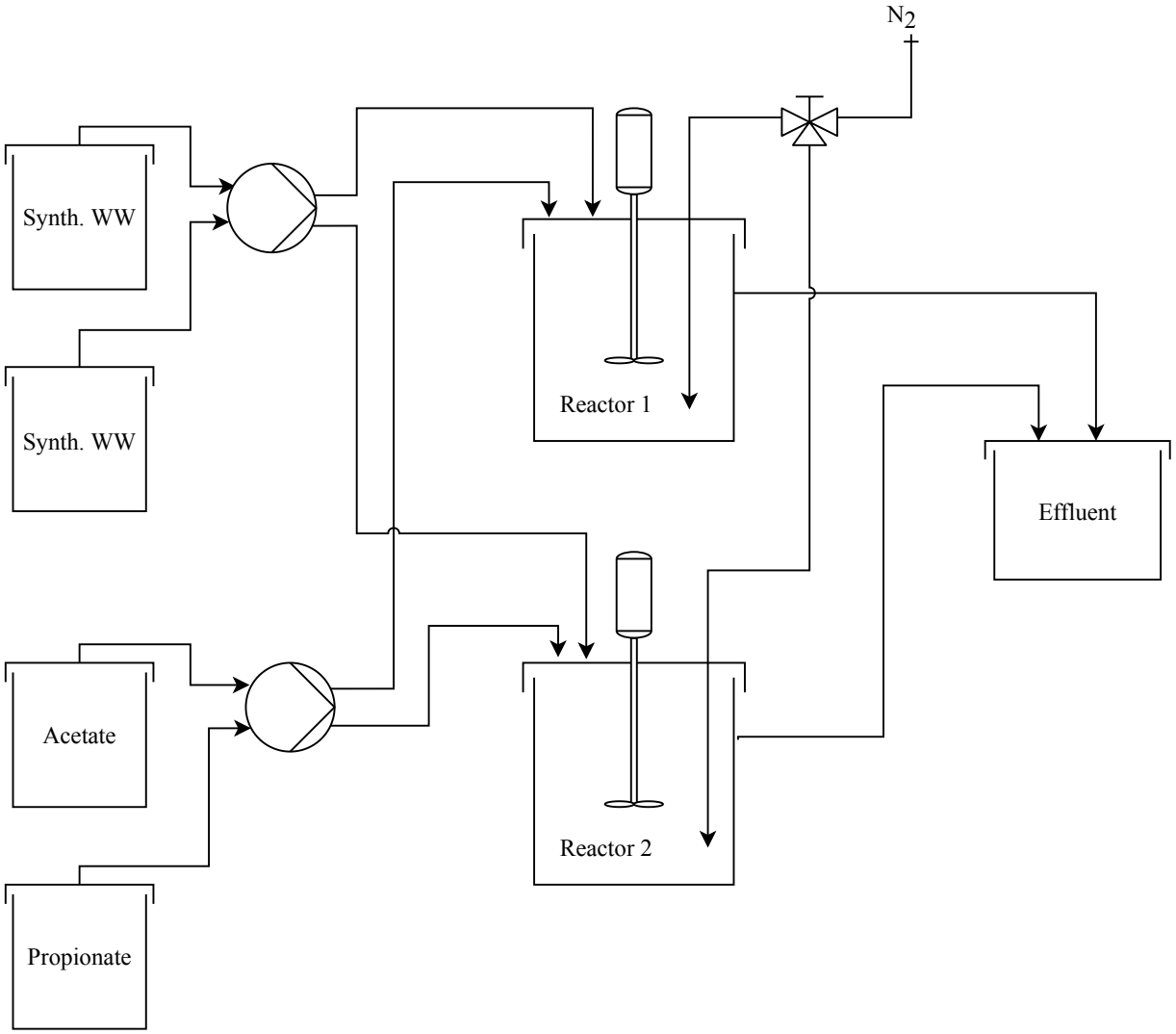

Figure 3.2. Process flow diagram of the experimental setup of the continuous PDA experiment, with separate feeds for synthetic wastewater and carbon source.

During the initial five weeks (Period I), the synthetic wastewater fed to reactor 1 (referred to as R1) and reactor 2 (referred to as R2) contained acetate and propionic acid respectively, and during weeks 6 – 10 (Period II) the carbon sources were added to the reactors from separate bottles, using a peristaltic pump (Fig. 3.1 & 3.2). At the start of the experiment, an initial total nitrogen loading rate (NLR) of 0.2 g N/(m^2*day) and a COD/NO_3-N ratio of 1 was used as the microorganisms acclimated. The NLR was increased by 0.2 g N/(m^2*day) steps, by increasing the flow rate, until it reached approximately 1.0 g N/(m^2*day) on day 5, where it was maintained for the remainder of the experiment. Additionally, on day 5 the COD/NO_3-N ratio was increased to 2. The influent conditions during Period II is presented in Table 3.1.

Table 3.1. Average influent conditions for reactors 1 and 2 during Period II of the continuous PDA experiment.

Influent conditions, Period II [grand mean ± standard deviation]				
	NH_4^+ (mg N/L)	**NO_3^- (mg N/L)**	**COD/NO_3^--N**	**NLR (g N/(m^2*d))**
Reactor 1	18.8 ± 0.7	18.9 ± 0.5	2.07 ± 0.18	0.88 ± 0.07
Reactor 2	18.8 ± 0.8	18.9 ± 0.5	1.87 ± 0.13	0.89 ± 0.07

3.1.2 Sampling and analysis

Throughout the experiment, DO concentration, pH and temperature were measured in the reactors during weekdays, using a HACH digital multimeter (HQ40D) with a HACH LDO101 sensor and a WTW pH320 pH meter with a WTW SenTix 41 pH electrode. Furthermore, influent flow rates were measured daily by collecting influent in a graduated cylinder for a measured amount of time, and the pump speed was adjusted when required. Samples for COD and nitrogen species concentration measurements were taken three times per week from the influent tanks and reactors. COD was measured in unfiltered influent and reactor samples, and dissolved COD (DCOD) was measured in filtered reactor samples (0.45 μm filter), using HACH cuvettes (LCK314 [15 – 150 mg COD/L] & LCK1414 [5 – 60 mg COD/L]). In addition, ion chromatography (Metrohm Eco IC) was used to determine NH_4^+, NO_2^- and NO_3^- concentrations of filtered influent and reactor samples.

3.1.3 Synthetic wastewater

The synthetic wastewater was prepared with NO_3^--N and NH_4^+-N concentrations of 20 mg N/L and 20 mg N/L respectively, to achieve a total nitrogen concentration of 40 mg/L. Acetate or propionic acid was used as carbon source in reactor 1 and 2 respectively, with a COD/NO_3-N ratio of 1 during week 1, and 2 during week 2 – 10. The carbon sources were added to the synthetic wastewater during the first 5 weeks and prepared in separate solutions of 750 mg COD/L during the subsequent weeks.

The full composition of the wastewater was as follows (per litre): 0.0764 g NH_4Cl, 0.144 g KNO_3, either 0.0586 g CH_3COONa or 0.0265 g $C_3H_6O_2$ (week 2 – 5), 0.68 g KH_2PO_4, 0.014 g $CaCl_2$*$2H_2O$, 0.09 g $MgSO_4$*$7H_2O$, 1.25 mL Trace element solution A and 1.25 mL Trace element solution B (Xu et al., 2020; Ma et al., 2017). The pH was adjusted to 7.0 ± 0.1 by adding approximately 40-50 mL 1 M NaOH solution. Trace solution A and B were prepared according to van Loosdrecht et al. (2016) (based on van der Graaf et al., 1996) with the following trace elements per litre trace solution: Trace solution A: 5 g EDTA, 9.14 g $FeSO_4$*$7H_2O$; Trace solution B: 15 g EDTA, 0.43 g $ZnSO_4$*$7H_2O$, 0.24 g $CoCl_2$*$6H_2O$, 0.99 g $MnCl_2$*$4H_2O$, 0.25 g $CuSO_4$*$5H_2O$, 0.22 g $NaMoO_4$*$2H_2O$, 0.19 g $NiCl_2$*$6H_2O$, 0.21 g $NaSeO_4$*$10H_2O$, 0.014 g H_3BO_4 (van Loosdrecht et al., 2016; van de Graaf et al., 1996).

3.2 Ex-situ activity batch tests

Six conical flasks (300 mL) were placed in a water bath at 20°C, and 240 mL of varying substrate solutions were added to each flask. Dispersion tubes connected to a nitrogen gas valve were used to continuously purge the flasks, achieving anoxic conditions, and reactor temperatures were maintained at approximately 20°C by addition/removal of water to the water bath.

Thirty biofilm carriers (ten carriers in activity test 1) were added to each flask, and the timer started. The carriers used were taken from reactors 1 and 2 of the continuous experiment (section 3.1) and returned to the corresponding reactor after finishing the test.

An initial concentration of 75 mg N/L for the respective nitrogen species in all reactors was chosen based on Stefansdottir (2014), to ensure an independency of initial nitrite concentration in the anammox activity tests. The reactors used for nitrate reduction (NA_A and NA_P) and nitrite reduction (NI_A and NI_P) contained a carbon source at a COD/NO_x^--N ratio of 4 (Table 3.2). The two reactors used for anammox activity (AMX_A and AMX_P) contained carbonate at a CO_3^{2+}/NH_4^+ molar ratio of 0.7 (approximately 10 times larger than the stoichiometric requirement for the anammox reaction) as suggested by van Loosdrecht et al. (2016). In addition, all reactors contained 22 mM KH_2PO_4 buffer (as suggested by Stefansdottir (2014)) and the following nutrients: 0.014 g $CaCl_2*2H_2O$, 0.09 g $MgSO_4*7H_2O$, 1.25 mL Trace element solution A and 1.25 mL Trace element solution B.

Table 3.2. Targeted concentrations of various substrates in each batch reactor during the activity tests.

Carrier origin and substrates in activity test reactors

	Reactor					
	NA_A	**NA_P**	**NI_A**	**NI_P**	**AMX_A**	**AMX_P**
NO_3^- (mg N/L)	75	75				
NO_2^- (mg N/L)			75	75	75	75
NH_4^+ (mg N/L)					75	75
Acetate (mg COD/L)	300		300			
Propionate (mg COD/L)		300		300		
$NaHCO_3$ (mg/L)					315	315
Carriers taken from:	R1	R2	R1	R2	R1	R2

Samples of 10 mL were taken from each flask at minutes 1, 60, 120, 180 and 240, and immediately filtered using 0.45 µm filters. Carriers were removed from each flask when necessary (one after 60 minutes, two after 120 minutes and one after 180 minutes), in order to maintain the carrier-to-volume ratio.

The samples from minute 1 and 240 from NA_A, NA_P, NI_A and NI_P were analysed for COD content using Hach-Lange cuvettes, and all samples were analysed for NH_4^+, NO_2^- and NO_3^- concentrations using ion chromatography. Additionally, temperatures and DO were measured during the experiments, and the initial and final pH was measured.

After the experiments, the measured concentrations of nitrate (reactors NA_x), nitrite (reactors NI_x) and nitrite and ammonium (reactors AMX_x) were plotted against time in order to determine the reduction rates of the various compounds. Subsequently, they were used to calculate the specific removal rates, which were plotted against the days of the experiment, thus displaying how the activities of the biofilm carriers changed over the course of the continuous PDA experiment.

3.3 Chemical oxygen demand of biomass

The COD of the biomass was approximated measuring both COD and volatile suspended solids (VSS) on a solution containing biomass used for inoculation and finding a ratio. The biofilm on one of the carriers obtained from Sjölunda WWTP (previously stored in a refrigerator) was scraped off and suspended in 40 mL water. Three filter papers (pore size 1.6 µm) were labelled and their weight recorded. Triplicate samples (10 mL each) of the biomass solution was filtered using the labelled filters, which were then dried in an oven at 105°C for one hour. After cooling down in a desiccator for 30 minutes, the weight of the filters was recorded. Subsequently, the filters were placed in an oven at 550°C for one hour to remove the volatile fraction of the samples, and once again placed in a desiccator for 30 minutes before their weight was recorded. The VSS (in g/L) could be determined by the following expression,

$$VSS = \frac{m_{105\ C^o} - m_{550\ C^0}}{0.01\ L} \qquad [g\ VSS \cdot L^{-1}] \tag{3.1}$$

After measuring the COD concentration (in mg/L) in the remaining 10 mL biomass solution, the following conversion constant, COD_{VSS}, could be determined by dividing the measured concentrations,

$$COD_{VSS} = \frac{{}^{COD}/_{1000\ {}^{mg}/_{g}}}{VSS_{mean}} \qquad [g\ COD \cdot g\ VSS^{-1}] \tag{3.2}$$

This conversion constant was utilized to convert the suspended COD measured in the continuous experiment from g COD to g VSS.

3.4 Calculations

3.4.1 Carrier surface area

Values provided by AnoxKaldnes was utilized to determine the surface area of each K5 biofilm carrier, according to the following equation,

$$A = \frac{800\ {}^{m^2}/_{m^3}}{331\ 000\ {}^{carriers}/_{m^3}} = 2.417 \cdot 10^{-3}\ {}^{m^2}/_{carrier} \tag{3.3}$$

where 800 m^2/m^3 is the protected surface area of the K5 carriers and 331 000 is the number of carriers in one cubic metre.

3.4.2 Loading and removal rates

In order to calculate the total nitrogen loading rate (NLR) the following expression was used,

$$NLR = \frac{c_N \cdot Q \cdot 24\ {}^{h}/_{d}}{A \cdot 225\ carriers} \qquad [g \cdot (m^2 \cdot d)^{-1}] \tag{3.4}$$

where c_N is the total nitrogen concentration of the influent (g/L), Q the total volumetric flow rate (L/h), 24 the number of hours per day (h/d), A the surface area per carrier (m^2/carrier), and 225 the number of carriers in each reactor (carriers).

To calculate the total nitrogen removal rate, NH_4^+ removal rate, NO_3^- removal rate, NO_2^- accumulation rate and effluent COD accumulation rate, the following expression was used,

$$Removal/accumulation\,rate = \frac{\Delta c_x \cdot Q \cdot 24\ {}^{h}/_{d}}{A \cdot 225\ carriers} \qquad [g \cdot (m^2 \cdot d)^{-1}] \tag{3.5}$$

where Δc_x is the change in the concentration of interest (g/L), Q the total volumetric flow rate (L/h), 24 the number of hours per day (h/d), A the surface area per carrier (m^2/carrier), and 225 the number of carriers in each reactor (carriers).

During the initial phase of the PDA experiment, the total volumetric flow rate, Q, was equivalent to the volumetric flow rate of the synthetic wastewater, Q_N. However, after adding a separate inflow for the carbon source, Q_C, the total volumetric flow rate used was the sum of Q_N and Q_C.

3.4.3 COD/NO_3^--N ratio

The influent COD/NO_3^--N ratio was initially calculated by dividing the COD concentration in the influent with the nitrate concentration of the influent. When the COD was fed separately, however, the following expression was used to compensate for the differing volumetric flow rates of the influents,

$$\frac{COD}{NO_3^- - N} = \frac{Q_C \cdot c_c}{Q_N \cdot c_{NO3}} \tag{3.6}$$

where Q_c is the volumetric flow rate of carbon source solution (L/h), Q_N the volumetric flow rate of the synthetic wastewater (L/h), c_C the COD concentration in the carbon source solution (mg/L), and c_{NO3} the nitrate concentration in the synthetic wastewater (mg/L).

3.4.4 Ex-situ activity batch tests

After plotting the concentrations of the species in the batch reactors against time, the slopes of the linear trendlines were used to calculate the activities of the biofilm carriers, r_x, according to the following equation,

$$r_x = -\frac{slope_x}{A} \cdot \frac{0.23\ L}{30\ carriers} \cdot \frac{1440\ {}^{min}/_{d}}{1000\ {}^{mg}/_{g}} \qquad [g \cdot (m^2 \cdot d)^{-1}] \tag{3.7}$$

where $slope_x$ is the slope of the trendline of the corresponding concentration plot (mg N/(L*min)), A the surface area per carrier (m^2/carrier), 0.23 L the batch reactor volume (initially 240 mL with 10 mL removed at time zero), 30 the number of carriers in each reactor (carrier) (note that 10 carriers were used in the first activity test), 1 440 the number of minutes per day (min/d), and 1000 the number of milligrams per gram (mg/g). Note that when samples were

taken from the reactor, thus reducing the volume, carriers were removed to maintain the ratio of volume/carriers throughout the experiment.

3.4.5 Mass balances

Acetate carbon source

When balancing the stoichiometry of the heterotrophic denitrification, the redox reaction can initially be divided in reduction and oxidation half reactions before being combined into the balanced reaction. Assuming a biomass yield in the heterotrophic NO_3^- reduction ($NO_3^- \rightarrow NO_2^-$) of 0.25 g biomass/g acetate (0.13 mol/mol), a biomass composition of $C_5H_7NO_2$, and that ammonium is used as nitrogen source for biomass formation, the redox reaction can be balanced as follows (Strohm et al., 2007):

Reduction half reaction:

$$NO_3^- + 2H^+ + 2e^- \rightarrow NO_2^- + H_2O \quad (3.8)$$

Oxidation half reaction:

$$CH_3COO^- + 0.13NH_4^+ + 0.96H_2O \rightarrow 0.13C_5H_7NO_2 + 1.35CO_2 + 4.53H^+ + 5.4e^- \quad (3.9)$$

Balanced NO_3^- reduction [(Eq. 3.8) + (Eq. 3.9)]:

$$NO_3^- + 0.37CH_3COO^- + 0.048NH_4^+ + 0.32H^+ \rightarrow NO_2^-0.048C_5H_7NO_2 + 0.5CO_2 + 0.64H_2O \quad (3.10)$$

In the same manner, the heterotrophic NO_2^- reduction ($NO_2^- \rightarrow N_2$) can be balanced as follows, assuming the same biomass composition, a biomass yield of 0.36 g biomass/g acetate (0.19 mol/mol), no accumulation of intermediates (NO or N_2O), and that ammonium is used as nitrogen source for biomass formation (Strohm et al., 2007):

Reduction half reaction:

$$NO_2^- + 4H^+ + 3e^- \rightarrow 0.5N_2 + 2H_2O \quad (3.11)$$

Oxidation half reaction:

$$CH_3COO^- + 0.19NH_4^+ + 0.48H_2O \rightarrow 0.19C_5H_7NO_2 + 1.05CO_2 + 3.39H^+ + 4.2e^- \quad (3.12)$$

Balanced NO_2^- reduction [(Eq. 3.11) + (Eq. 3.12)]:

$$NO_2^- + 0.71CH_3COO^- + 0.14NH_4^+ + 1.58H^+ \rightarrow 0.5N_2 + 0.14C_5H_7NO_2 + 0.75CO_2 + 1.66H_2O \quad (3.13)$$

The anammox reaction oxidises ammonium with nitrite as electron acceptor while using carbon dioxide as carbon source for biomass formation. Assuming a biomass composition of $CH_2O_{0.5}N_{0.15}$, it can be described by the following reaction:

$$NH_4^+ + 1.32\ NO_2^- + 0.066\ HCO_3^- + 0.13\ H^+ \rightarrow 1.02\ N_2 + 0.26\ NO_3^- + 0.066\ CH_2O_{0.5}N_{0.15} + 2.03\ H_2O \quad (3.14)$$

Using reactions (Eq. 3.10), (Eq. 3.13) and (Eq. 3.14), the nitrate removal rate (NO_3^--RR), ammonium removal rate (NH_4^+-RR) and nitrite accumulation rate (NO_2^--AR) (in units of mg N/(m^2*d)) can be described by the following system of equations when using acetate as carbon source:

$$NO_3^- - RR = R_1 - 0.26R_3 \quad (3.15)$$

$$NH_4^+ - RR = 0.048R_1 + 0.14R_2 + R_3 \quad (3.16)$$

$$NO_2^- - AR = R_1 - R_2 - 1.32R_3 \quad (3.17)$$

Where R_1 is the reaction rate of the first part of denitrification, R_2 is the reaction rate of the second half of denitrification and R_3 is the anammox reaction rate.

Propionate carbon source

Assuming the same biomass yield for propionate as for acetate (in g biomass/g carbon source) in the first and second half of the denitrification reactions, it corresponds to approximately 0.16 mol biomass/mol propionate and 0.23 mol biomass/mol propionate in the heterotrophic NO_3^- and NO_2^- reduction reactions respectively. Note that these yields most likely are not the same for the two carbon sources. However, slight variations do not affect the overall nitrogen flow greatly since only a small part of the nitrogen is used for assimilation (e.g. 0.03 mol NH_4^+ compared to 1 mol NO_3^- in Eq. 3.18). With similar assumptions as above, the reactions can be described as follows:

$$NO_3^- + 0.19C_2H_5COO^- + 0.030NH_4^+ + 0.16H^+ \rightarrow NO_2^-0.030C_5H_7NO_2 + 0.41CO_2 + 0.50H_2O \quad (3.18)$$

$$NO_2^- + 0.32C_2H_5COO^- + 0.073NH_4^+ + 1.25H^+ \rightarrow 0.5N_2 + 0.073C_5H_7NO_2 + 0.59CO_2 + 1.31H_2O \quad (3.19)$$

Using these reactions, as well as the anammox reaction (Eq. 3.14), the following system of equations can be used to describe the NO_3^--RR, NH_4^+-RR and NO_2^--AR (in units of mg N/(m^2*d)) when propionate is utilized as carbon source:

$$NO_3^- - RR = R_1 - 0.26R_3 \quad (3.20)$$

$$NH_4^+ - RR = 0.030R_1 + 0.073R_2 + R_3 \quad (3.21)$$

$$NO_2^- - AR = R_1 - R_2 - 1.32R_3 \quad (3.22)$$

Where R_1 is the reaction rate of the first part of denitrification, R_2 is the reaction rate of the second half of denitrification and R_3 is the anammox reaction rate.

Anammox contribution

After using experimentally determined values for NO_3^--RR, NH_4^+-RR and NO_2^--AR and solving the equation systems (Eq. 3.15 – 3.17) and (Eq. 3.20 – 3.22) for R_1, R_2 and R_3 when using

the two carbon sources, the following expression can be used to determine the percental contribution of the anammox reaction to nitrogen gas formation in the two reactors:

$$Anammox\ contribution = \frac{1.02R_3}{1.02R_3 + 0.5R_2} \cdot 100 \qquad [\%] \tag{3.23}$$

Theoretical biomass formation

Using the obtained reaction rates R_1, R_2 and R_3, the biomass (i.e. VSS) formation, μ_x, (in g VSS/(m^2*d)) can be calculated by using the following expressions when acetate and propionate is used as carbon source:

$$\mu_{ace} = 0.048R_1 \cdot \frac{113g \cdot mol^{-1}}{14\ g \cdot mol^{-1}} + 0.14R_2 \cdot \frac{113g \cdot mol^{-1}}{14\ g \cdot mol^{-1}} + 0.066R_3 \cdot \frac{24.1g \cdot mol^{-1}}{14\ g \cdot mol^{-1}} \tag{3.24}$$

$$\mu_{pro} = 0.030R_1 \cdot \frac{113g \cdot mol^{-1}}{14\ g \cdot mol^{-1}} + 0.073R_2 \cdot \frac{113g \cdot mol^{-1}}{14\ g \cdot mol^{-1}} + 0.066R_3 \cdot \frac{24.1g \cdot mol^{-1}}{14\ g \cdot mol^{-1}} \tag{3.25}$$

Where 113 g/mol is the molar mass of the denitrifiers, 24.1 g/mol is the molar mass of the anammox bacteria and 14 g/mol is the molar mass of nitrogen.

4 Results and discussion

4.1 Ex-situ activity batch tests

A general overview of the results from the activity tests are presented in Table 4.1. As the slopes of the NO_x^- concentrations (i.e. the removal rates) in the activity test of reactors NA_x during week 1 had low R^2 values, the number of carriers in each reactor was increased from 10 to 30 in the following activity tests.

Nitrate and nitrite reduction

As seen in Figure 4.1, the denitrification activity of the biofilm carriers increased throughout the continuous experiment. This indicates that the microbial communities adapted to introduction of COD and nitrate, increasing the amount of denitrifiers in the biofilm. In the initial activity tests measuring nitrate reduction, there were no nitrite accumulation when using either carbon source. However, after six weeks nitrite started to accumulate in the NA_A reactor (Fig. 4.2). The linear increase in nitrite indicates that the growing denitrifying population on the carriers were either only of type A denitrifiers (capable of nitrate reduction with nitrite accumulation), or a combination of type A and type C denitrifiers (capable of only reducing nitrate to nitrite). Thus, a selective pressure for the desired types of denitrifiers had been established in the continuous experiment, caused by the low COD/NO_3^--N ratio, which is crucial for the PDA process.

The nitrite reduction rate generally decreased over the ten weeks (Fig. 4.1). Although there was no observed nitrite accumulation in NA_P, this indicates that the growing denitrifying population on both the R1 and R2 carriers favoured reducing nitrate over nitrite, thus increasing the PDA capacity of the carriers. However, the difference in nitrate reducing activity and nitrite reducing activity (i.e. nitrite accumulation potential) was greater for the carriers utilizing acetate as carbon source compared to propionate, consequently making them more suited for PDA.

It is important to note that during the activity test of week 1, there was residual ammonium (from the reject water where the carriers were stored) present in the NI_A and NI_P reactors. This almost certainly resulted in a higher nitrite reducing activity, due to anammox activity in the reactors. Nevertheless, the carriers from both R1 and R2 did not display a significant increase in nitrite reducing activity as the denitrifying population increased throughout the continuous experiment.

Table 4.1. Activities of each reactor in the four ex-situ activity batch tests, including R^2 value of the trendline used to calculate the activity, and ratio of consumed COD/consumed NO_x^- or consumed NO_2^-/consumed NH_4^+. (1)Theoretical ratio from reaction stoichiometry is 1.32.

Ex-situ activity batch tests results

NA_A: Nitrate reduction / acetate carriers
NA_P: Nitrate reduction / propionate carriers
NI_A: Nitrite reduction / acetate carriers
NI_P: Nitrite reduction / propionate carriers
AMX_A: Anammox / acetate carriers
AMX_P: Anammox / propionate carriers

		Week 1	Week 4	Week 6	Week 10
NA_A	NO_3^- Removal (g N/(m^2*d))	0.410	0.418	0.592	1.036
	R^2	0.215	0.944	0.966	0.991
	$COD_{consumed}/NO_3^-{}_{consumed}$	3.03	5.22	4.72	2.46
NA_P	NO_3^- Removal (g N/(m^2*d))	0.211	0.276	0.394	0.769
	R^2	0.465	0.859	0.948	0.990
	$COD_{consumed}/NO_3^-{}_{consumed}$	8.28	5.68	4.39	4.90
NI_A	NO_2^- Removal (g N/(m^2*d))	0.713	0.253	0.302	0.181
	R^2	0.937	0.775	0.823	0.926
	$COD_{consumed}/NO_2^-{}_{consumed}$	5.26	4.10	5.03	3.50
NI_P	NO_2^- Removal (g N/(m^2*d))	0.298	0.165	0.205	0.074
	R^2	0.925	0.579	0.672	0.794
	$COD_{consumed}/NO_2^-{}_{consumed}$	5.84	5.52	4.21	2.37
AMX_A	NH_4^+ Removal (g N/(m^2*d))	1.097	0.793	0.765	0.270
	R^2	0.997	0.969	0.938	0.978
	NO_2^- Removal (g N/(m^2*d))	1.673	1.077	1.059	0.334
	R^2	0.999	0.993	0.970	0.956
	TN consumption (g N/(m^2*d))	2.770	1.870	1.824	0.604
	$NO_2^-{}_{consumption}/NH_4^+{}_{consumption}$ (1)	1.53	1.36	1.38	1.24
AMX_P	NH_4^+ Removal (g N/(m^2*d))	1.097	0.816	0.576	0.086
	R^2	0.997	0.957	0.967	0.762
	NO_2^- Removal (g N/(m^2*d))	1.673	1.117	0.785	0.135
	R^2	0.999	0.990	0.992	0.968
	TN consumption (g N/(m^2*d))	2.770	1.933	1.360	0.221
	$NO_2^-{}_{consumption}/NH_4^+{}_{consumption}$ (1)	1.53	1.37	1.36	1.56

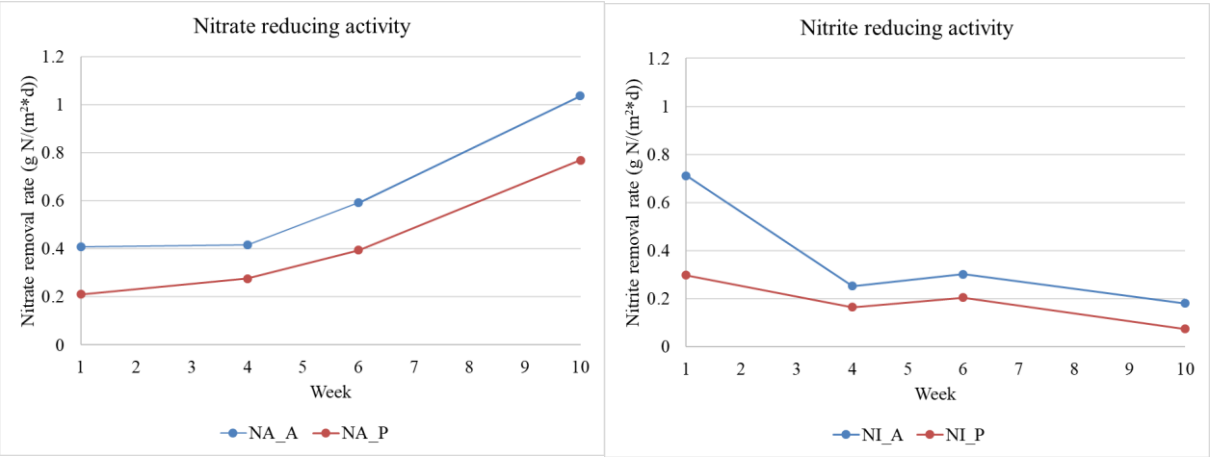

Figure 4.1. Nitrate reducing activity of the biofilm carriers in reactors NA_A and NA_P (left), and nitrite reducing activity of the biofilm carriers in reactors NI_A and NI_P (right), during the four ex-situ activity batch tests plotted against the time of the continuous experiment.

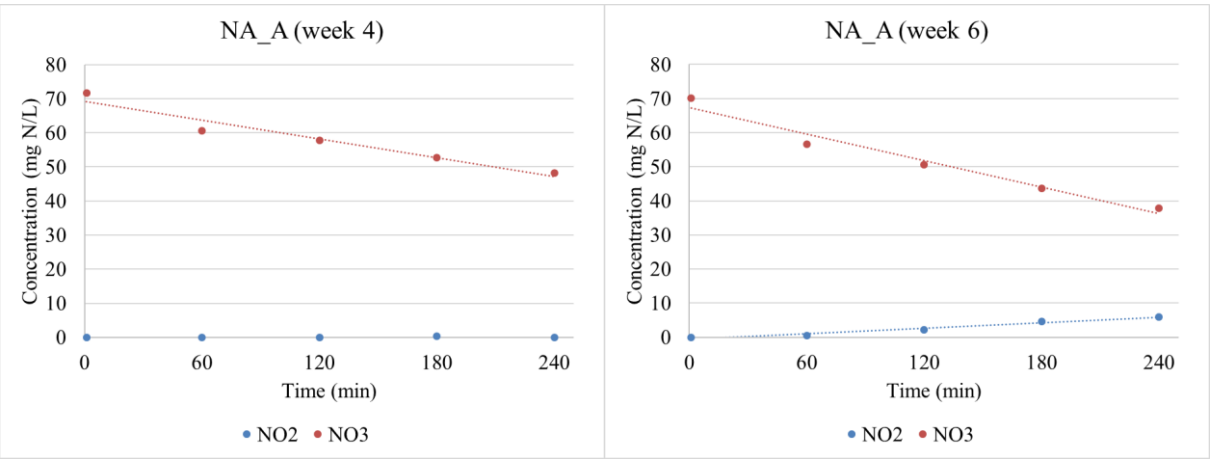

Figure 4.2. Nitrate (NO3) and nitrite (NO2) concentration plots from reactor NA_A (i.e. nitrate reduction with acetate as carbon source) in the ex-situ activity batch tests of weeks 4 & 6.

Anammox activity

During the ten weeks of the continuous experiment, the anammox activity of the biofilm carrier decreased (Fig. 4.3). This indicates that the growing denitrifying population out-competed the over abundant anammox bacteria already present on the carriers, resulting in a more balanced community of the two groups of bacteria. At week 10, the anammox activity of the biofilm carriers was found to be 0.60 g N/(m^2*d) and 0.22 g N/(m^2*d) in AMX_A and AMX_P respectively, where 0.27 g N/(m^2*d) and 0.086 g N/(m^2*d) of these rates were due to ammonium removal (Table 4.1). Comparing these values to the ammonium removal rates of the continuous experiment at week 10 (0.22 g N/(m^2*d) and 0.078 g N/(m^2*d) for R1 and R2 respectively (Table 4.2)), the anammox bacteria appears to be working at close to full capacity in the continuous experiment at this time. Thus, should the anammox activity decrease further, there would be a reduction in the performance of the PDA reactors.

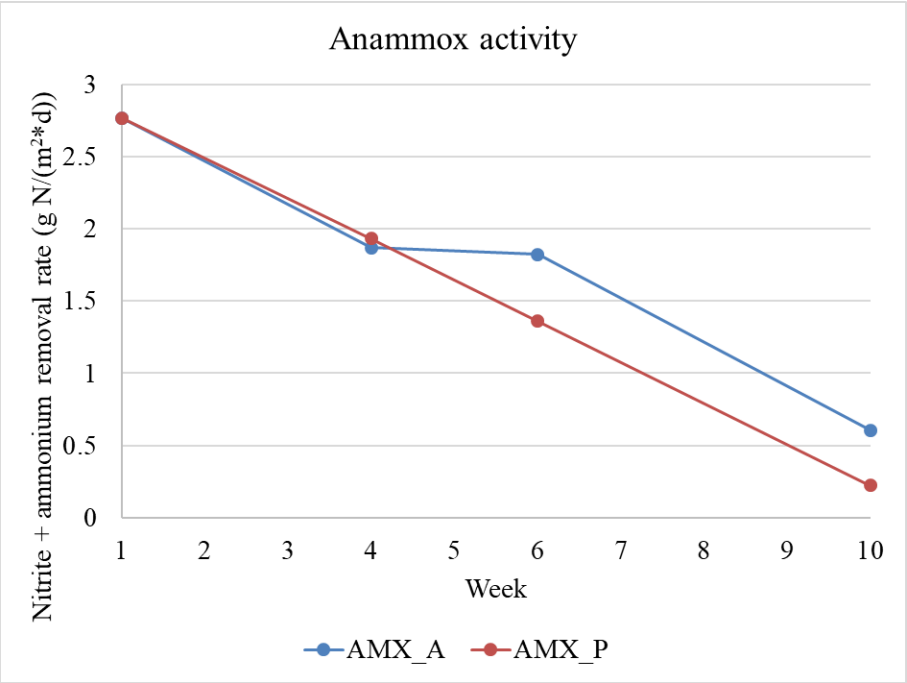

Figure 4.3. Anammox activity of the biofilm carriers in reactors AMX_A and AMX_P during the four ex-situ activity batch tests, plotted against the time of the continuous experiment.

4.2 PDA experiment

4.2.1 Period I

During the initial five weeks of the PDA experiment there were quite large variations in the performance of the two reactors, which was partly caused by uneven conditions in the reactors. The main two disturbances were high oxygen concentration in the reactors (Fig. 4.4) and fluctuating influent concentrations (Fig. 4.5).

Since the biofilm carriers have previously been used for PNA, the initial microbial community is partly composed of nitrifiers (catalysing aerobic oxidation of ammonium). Consequently, the inadequate/uneven nitrogen purging (due to clogging of the gas dispersion tubes) during the first four weeks may have resulted in these microbes being partly responsible for reduction in ammonium, as opposed to the anammox bacteria. In addition, the presence of oxygen in the reactors may have resulted in the carbon source being oxidised by bacteria using oxygen as the electron acceptor, instead of nitrate, thus limiting the carbon available for denitrification.

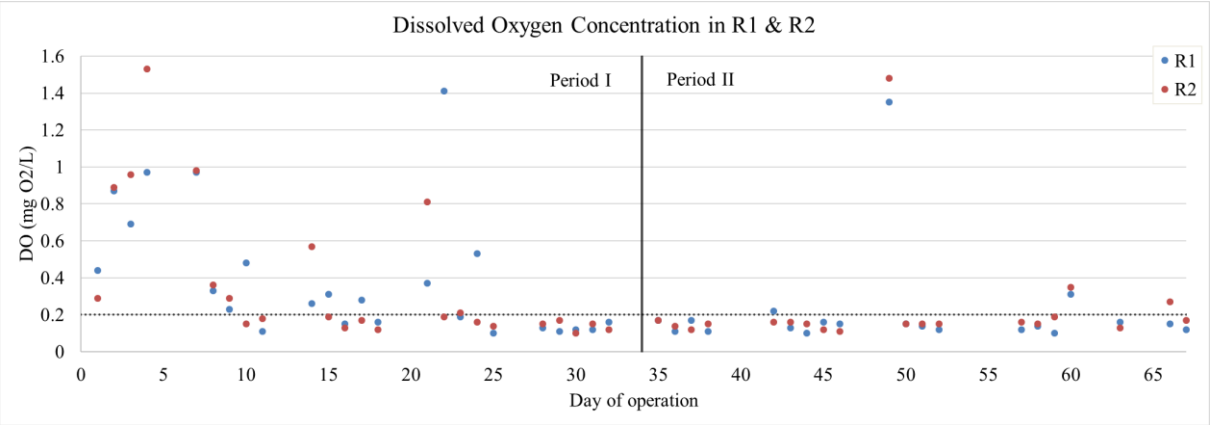

Figure 4.4. Dissolved oxygen (DO) concentration in R1 and R2 during the continuous PDA experiment, with the targeted maximum DO concentration (0.2 mg/L) displayed as a dotted black line.

The uneven influent concentrations during the initial five weeks were caused by bacterial growth in the influent tanks. Since the 25 L (weekdays) and 50 L (weekends) influent tanks were kept at room temperature, the microbial contamination in the tanks proved to be significant and could not be avoided by washing the tanks between use. Consequently, the already low concentration of COD was reduced each day after preparing the synthetic wastewater, leading to substantial differences in the COD/NO_3^--N ratio of the influent. In addition, after incorporating 30 minutes of nitrogen purging to the wastewater preparation to reduce the dissolved oxygen in the influent, apparent growth of denitrifiers in the influent tanks resulted in a relatively high concentration of nitrite in the influent. For example, during day 30 (week 5) the nitrite concentration in the influent was 11.2 mg N/L and 8.7 mg N/L in R1 and R2 respectively, leading to a high total nitrogen removal rate in the reactors due to anammox activity without the need for partial denitrification.

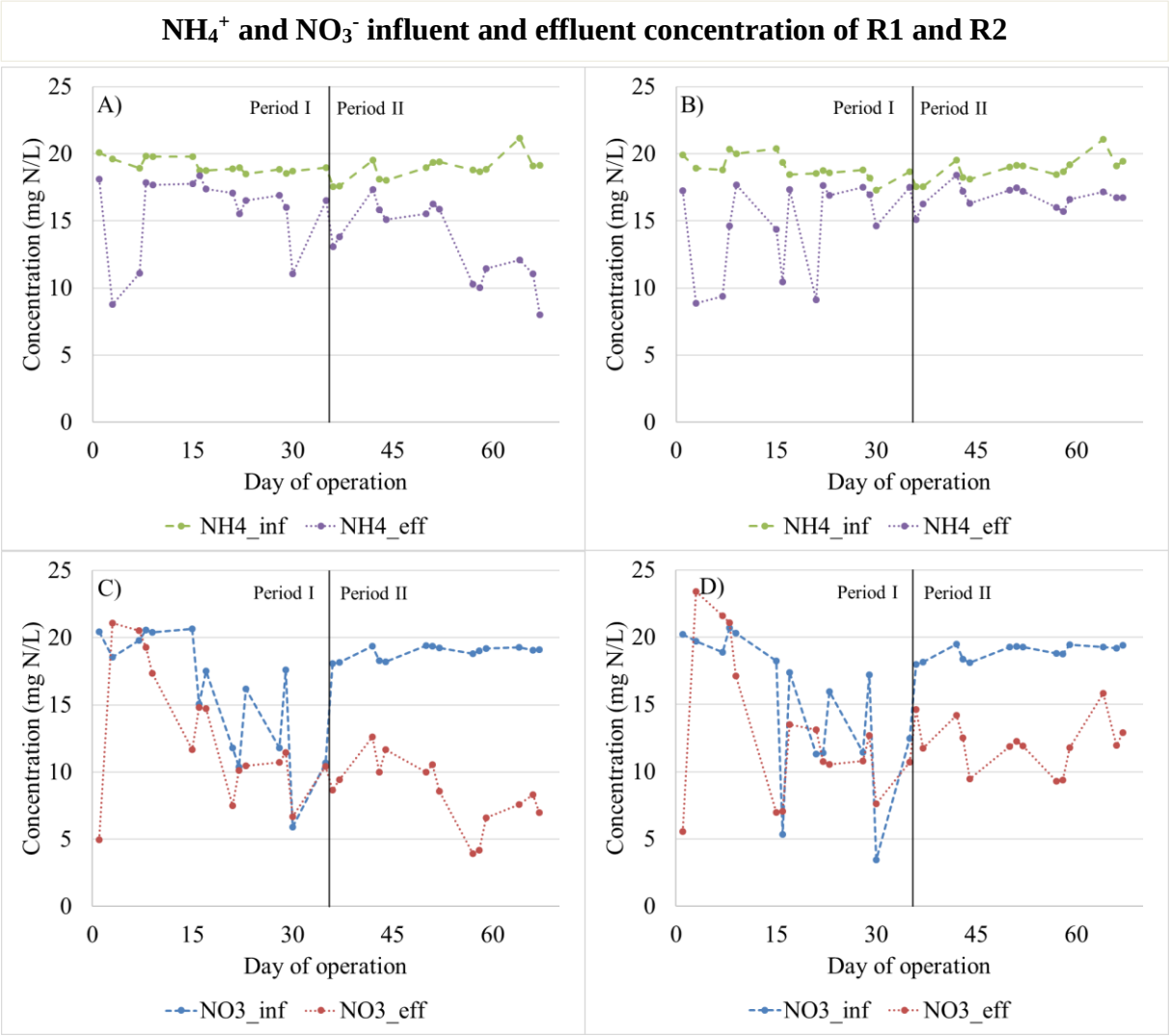

Figure 4.5. Influent and effluent concentrations during the continuous PDA experiment, showing A) ammonium in R1, B) ammonium in R2, C) nitrate in R1, and D) nitrate in R2.

Due to the initial difficulties of the PDA experiment, the focus during Period I was mainly to establish a functioning experimental setup and methodology for operation, to ensure a steady process with stable influent and reactor conditions, in order to achieve a period where PDA could be examined. The initial elevated concentration of dissolved oxygen was overcome by adding more plastic wrapping to the reactors, finding a suitable gas flow for purging the reactors, and identifying appropriate sparging stones. In addition, the synthetic wastewater was purged with nitrogen gas to reduce oxygen entering the system through the substrate and to reduce microbial activity in the influent tanks. The disturbance of fluctuations in the influent concentrations was largely eliminated by separating the carbon source from the synthetic wastewater and feeding it to the reactors separately. Furthermore, procedures for minimizing the impact and frequency of for example leaking tubes, uneven gas distribution and tube blockage were established.

Period II

Biomass

The COD of the biomass was found to be approximately 1.5 g COD/g VSS. When applying this conversion constant to the suspended COD of the effluents of R1 (acetate) and R2 (propionate), the biomass content of the effluents, and consequently the biomass yield (biomass per loaded carbon) could be determined and compared to the theoretical growth yield during Period II (calculated from the mass balances of Section 3.4.5). As seen in Figure 4.6, the experimentally determined biomass yield was elevated during week 6 for both R1 and R2, followed by relatively stable yields of approximately 0.23 ± 0.19 g VSS/g COD_{Load} in R1 and 0.23 ± 0.09 g VSS/g COD_{Load} in R2 during weeks 7 – 10.

Due to elevated experimental biomass yields (i.e. high effluent concentrations of biomass at low carbon loading) on individual days during Period I (as well as during week 6), it can be reasoned that a loss of biomass had occurred prior to Period II. In contrast, during week 9 the theoretical growth yield was approximately 4.4 times larger than the experimental yield in R1 and 1.7 times larger in R2 (Fig.4.7), indicating that a portion of the biomass formed was retained in the biofilms of the reactors.

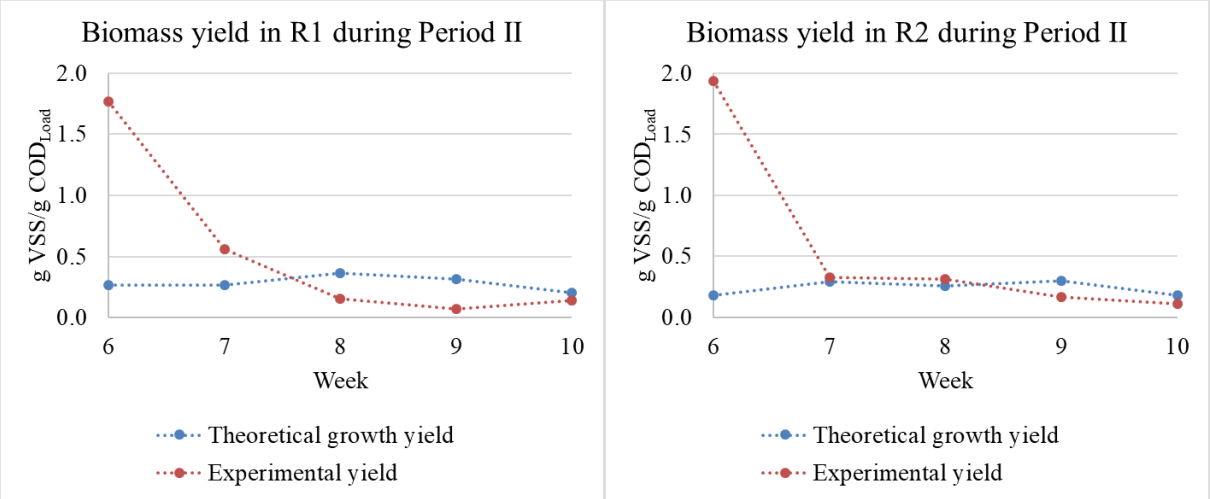

Figure 4.6. Average experimentally determined biomass yield and theoretically calculated biomass yield of the two reactors (R1: acetate, R2: propionate) during the continuous PDA experiment.

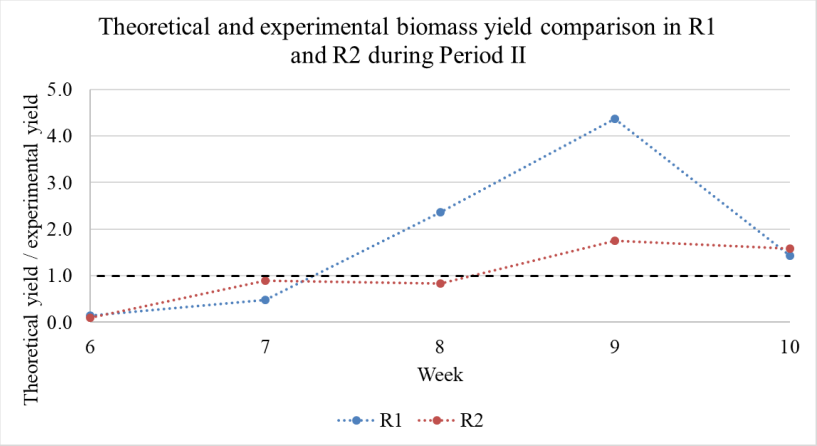

Figure 4.7. Theoretical growth yield of biomass divided by averaged experimentally determined biomass yield of R1 and R2 during Period II of the continuous PDA experiment, with a dashed black line to indicate where the yields are equal.

Interaction between denitrifiers and anammox bacteria

As seen in Figures 4.8 & 4.9, the average nitrate removal rates generally improved during Period II, with a final rate of 0.27 ± 0.02 g N/(m^2*d) and 0.15 ± 0.04 g N/(m^2*d) in R1 and R2 respectively. In addition, the average ammonium removal rates in both reactors increased during the period, reaching 0.22 ± 0.01 g N/(m^2*d) and 0.08 ± 0.02 g N/(m^2*d) for R1 and R2 respectively in week 10. The increased denitrification therefore resulted in higher rates of nitrite formation in both reactors, indicating growth of the desired types of denitrifiers.

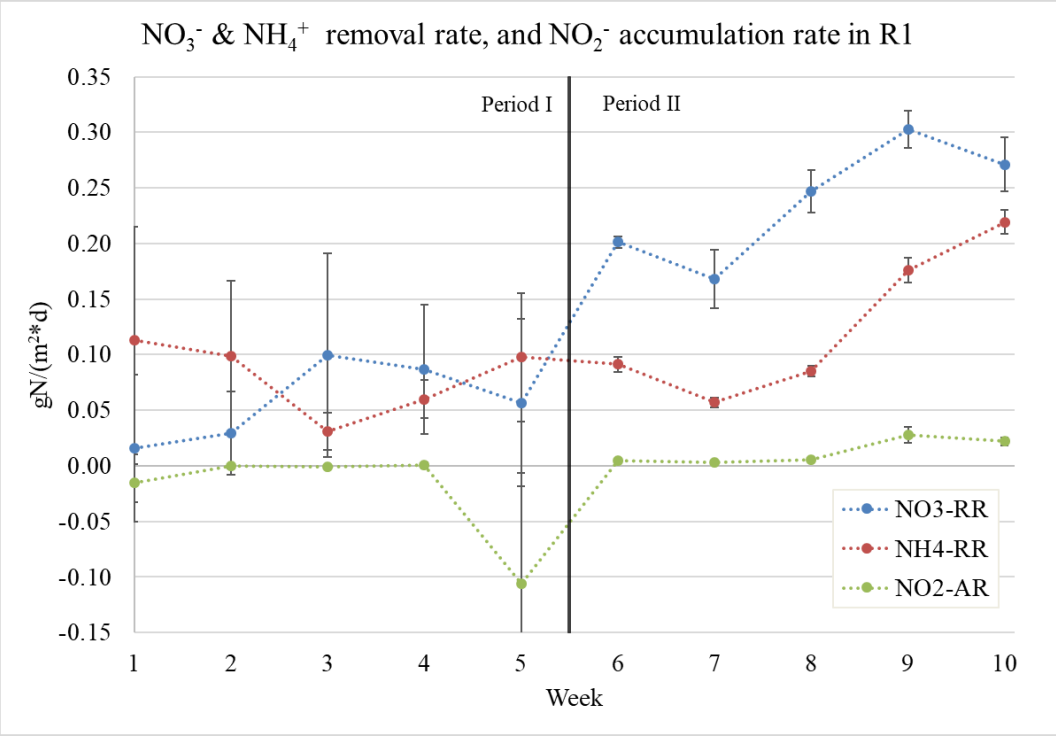

Figure 4.8. Average nitrate removal rate (NO3-RR), ammonium removal rate (NH4-RR) and nitrite accumulation rate (NO2-AR) in reactor 1 (acetate as carbon source) during the continuous PDA experiment.

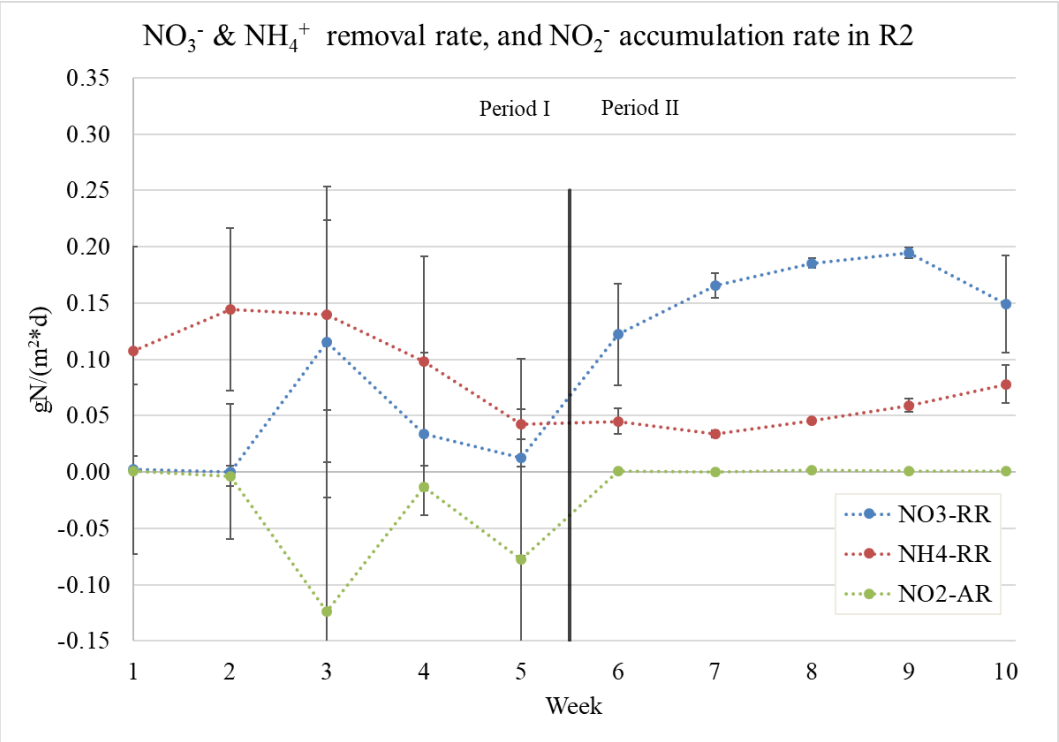

Figure 4.9. Average nitrate removal rate (NO3-RR), ammonium removal rate (NH4-RR) and nitrite accumulation rate (NO2-AR) in reactor 2 (propionate as carbon source) during the continuous PDA experiment.

As suggested by literature (Li et al., 2015), higher nitrate-to-nitrite conversion is observed with acetate as carbon source compared to propionate. The more rapid increase in ammonium removal rate in R1 suggests that is the case here as well, with nitrite accumulating in the effluent during the last two weeks. Since removal of ammonium is primarily performed by anammox bacteria (only a small part of ammonium is used for biomass assimilation, see Eq. 3.10, 3.13, 3.18 & 3.19) which requires nitrite from the denitrifiers, the increased ammonium removal rate suggests that the interaction between the partial denitrifiers and anammox bacteria was improving. The estimated contribution of anammox to the total amount of nitrogen gas formed (Fig. 4.10), calculated from the mass balances of the denitrification and anammox reactions (Section 3.4.5), reveals that the efficiency of the partial denitrification and anammox coupling was increasing from week 7, with a final contribution of 91 % and 64 % to the total amount of N_2 formed in R1 and R2 respectively. In recent studies, anammox contributions to N_2 formation in PDA of 70-94 % has been observed when utilizing acetate as carbon source, thus confirming the validity of these results (Xu et al., 2020; Ma et al., 2017; Du et al., 2017b).

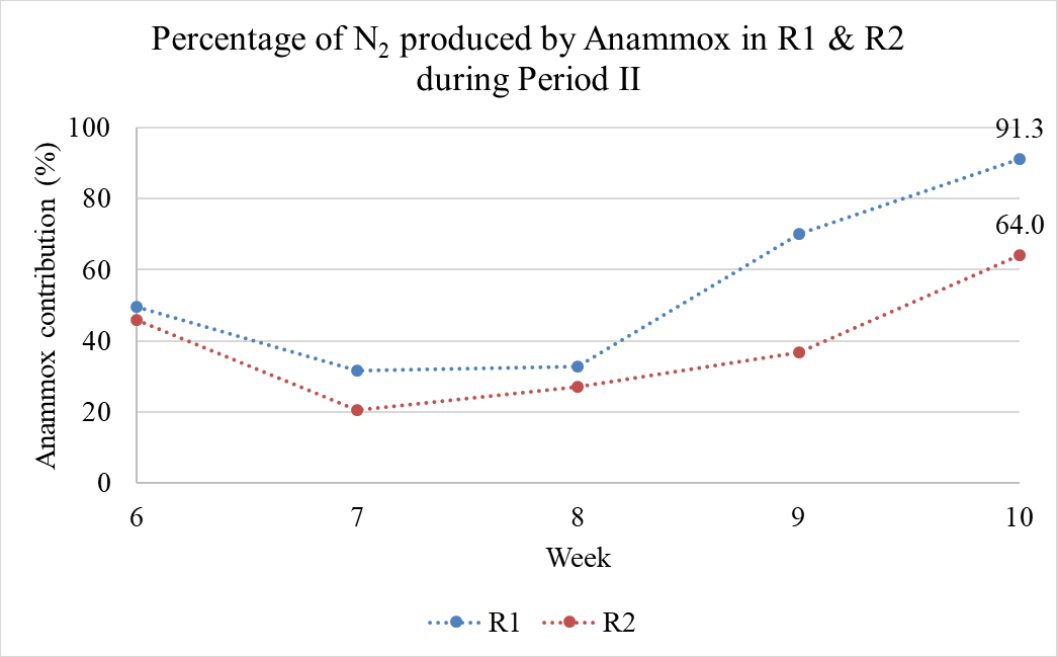

Figure 4.10. Anammox contribution to nitrogen gas formation during Period II of the continuous PDA experiment.

Total nitrogen removal

The addition of a second feed to the reactors, containing the carbon sources, resulted in more stable concentrations in the influent by limiting bacterial growth in the influent tanks. However, it also made the influent conditions somewhat susceptible to variations in the flow rates, which resulted in nitrogen loading rates ranging between approximately 0.80 – 1.0 g N/(m^2*d) (Fig. 4.11 & 4.12). Nevertheless, the average total nitrogen removal rate generally increased during this period for both reactors, indicating that the microbial communities on the carriers were acclimating to the substrates throughout the period.

In the acetate-fed reactor, there was an initial decrease in the average nitrogen removal rate from week six to seven in Period II. During week 6 there was also an elevated concentration of biomass in the effluent, indicating that there may have been a loss of biomass retained in the reactor, consequently decreasing the activity. However, after week 7 the average nitrogen removal rate increased throughout the remainder of the experiment, suggesting that there was a selective pressure favouring the growth of desired bacteria. At week 10 the average nitrogen removal rate had reached a value of 0.47 ± 0.03 g N/(m^2*d), thus increasing 63 % from week 6.

The propionate-fed reactor displayed an increase in the total nitrogen removal rate during Period II, with a slight decrease in week 10. As seen in Figure 4.9, this decrease was due to a lower denitrification rate, resulting in a final total nitrogen removal rate of 0.23 ± 0.03 g N/(m^2*d) (a 36 % increase from week 6). The slower nitrogen removal rate compared to R1 may be due to the fact that propionate (unlike acetate) is not an end-fermentation product, and thus more complex metabolic pathways are required when utilizing it as carbon source. While acetate may be directly converted to acetyl-CoA and enter the TCA cycle, propionate is converted to propionyl-CoA followed by succinyl-CoA by a series of reactions, before entering the TCA cycle, which results in a lower denitrification rate (Xu Y. , 1996). Since nitrogen removal

in the reactor is caused by either anammox, requiring nitrite from partial denitrification, or by full denitrification, a low denitrification rate directly acts as a bottleneck for the nitrogen removal rate.

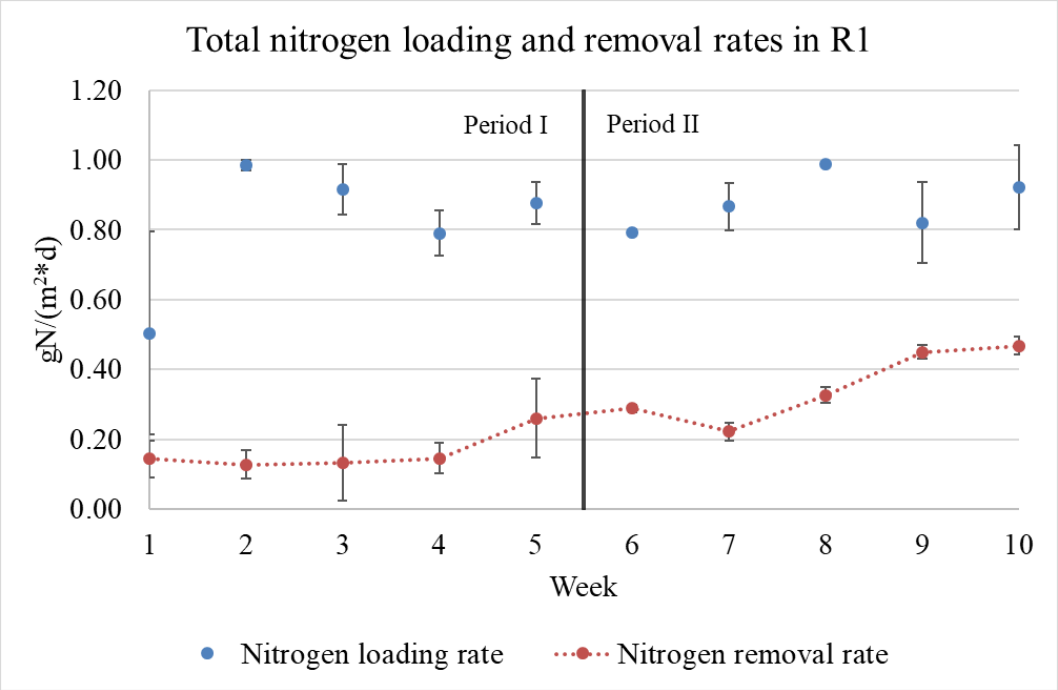

Figure 4.11. Average total nitrogen loading rate (NLR) and total nitrogen removal rate (NRR) in R1 (acetate as carbon source) during the continuous PDA experiment.

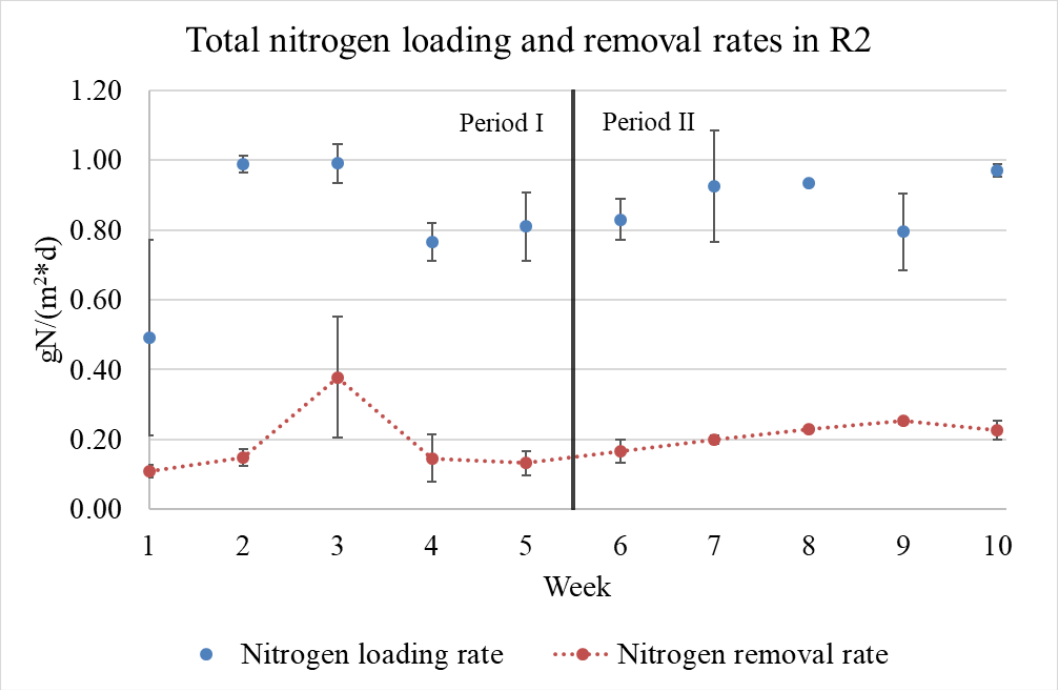

Figure 4.12. Average total nitrogen loading rate (NLR) and total nitrogen removal rate (NRR) in R2 (propionate as carbon source) during the continuous PDA experiment.

Overall performance of the two reactors

In the second period of the experiment, a general increase in the performance of both reactors was observed (Fig. 4.13). The nitrogen removal was approximately twice as high in the reactor fed with acetate compared to propionate in the final week (Table 4.2), a behaviour which was attributed to two reasons; the denitrification rate and the composition of the denitrifying population. The activity tests (where COD was more readily available) confirmed that the denitrification rate was lower for the biofilm carriers using propionate as carbon source, possibly due to it requiring more complex metabolism to be degraded as opposed to the easily consumed acetate. Additionally, the abundance of partial denitrifiers relative to full denitrifiers may have been greater for the acetate fed biofilm carriers, as seen by the anammox contribution to nitrogen removal. This would consequently provide more nitrite for the anammox reaction, which may explain the superior nitrogen removal rate. Furthermore, the denitrification rate in the propionate fed reactor appeared to be more negatively impacted by the low availability of COD. Due to the higher demand of organic carbon in the nitrite reduction part of denitrification, complete denitrification in the propionate fed reactor may have exhausted the available carbon intended for partial denitrification. The lower capability of nitrite accumulation for denitrifiers using propionate observed in this experiment corresponds with other studies, where long-term utilization of propionate has resulted in denitrifiers with a higher abundance of the *nirK* gene (encoding a nitrite reductase) compared to acetate (Li et al., 2015).

Table 4.2. Average effluent nitrate and ammonium concentrations, total nitrogen removal rate (NRR), ammonium removal rate (NH_4^+-RR), nitrate removal rate (NO_3^--RR), nitrite accumulation rate (NO_2^--AR), total nitrogen (TN) removal, anammox contribution to nitrogen gas formation and total percentage of influent nitrogen removed via anammox in reactors 1 (acetate) and 2 (propionate) during the final week of the continuous PDA experiment.

Reactor performance, week 10 [weekly mean ± standard deviation]		
	Reactor 1 (acetate)	**Reactor 2 (propionate)**
Effluent NH_4^+ (mg N/L)	10.4 ± 1.7	16.9 ± 0.2
Effluent NO_3^- (mg N/L)	7.6 ± 0.5	13.6 ± 1.6
NRR (g N/(m^2*d))	0.47 ± 0.03	0.23 ± 0.03
NH_4^+-RR (g N/(m^2*d))	0.22 ± 0.01	0.08 ± 0.02
NO_3^--RR (g N/(m^2*d))	0.27 ± 0.02	0.15 ± 0.04
NO_2^--AR (g N/(m^2*d))	0.024 ± 0.004	0
TN removal (%)	51.4 ± 5.0	23.4 ± 3.1
Anammox contribution to N_2 (%)	91.3	64.0
Nitrogen removed by anammox (%)	46.9	14.9

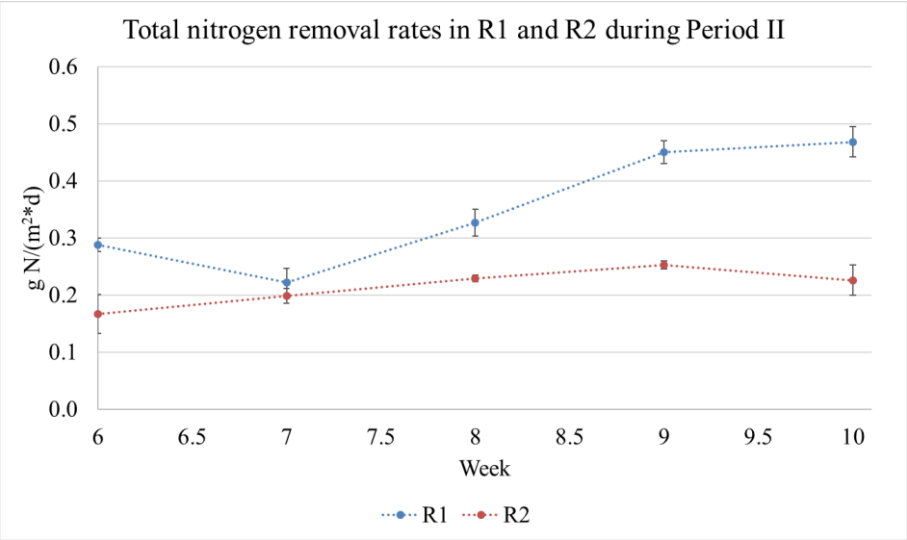

*Figure 4.13. Average total nitrogen removal rates in R1 (acetate) and R2 (propionate) during Period II of the continuous PDA experiment, utilizing total nitrogen loading rates varying between 0.8 – 1.0 g N/(m^2*d).*

Combining the percentage of total nitrogen removed with the anammox contribution to this expected nitrogen gas formation (Fig. 4.10), a final PDA efficiency (percentage of total nitrogen removed by PDA) of 47 % and 15 % can be determined for R1 and R2 respectively (Fig. 4.14). Although this is still relatively low efficiencies, they appear to still be increasing, indicating that the processes have not yet reached their steady states of operation.

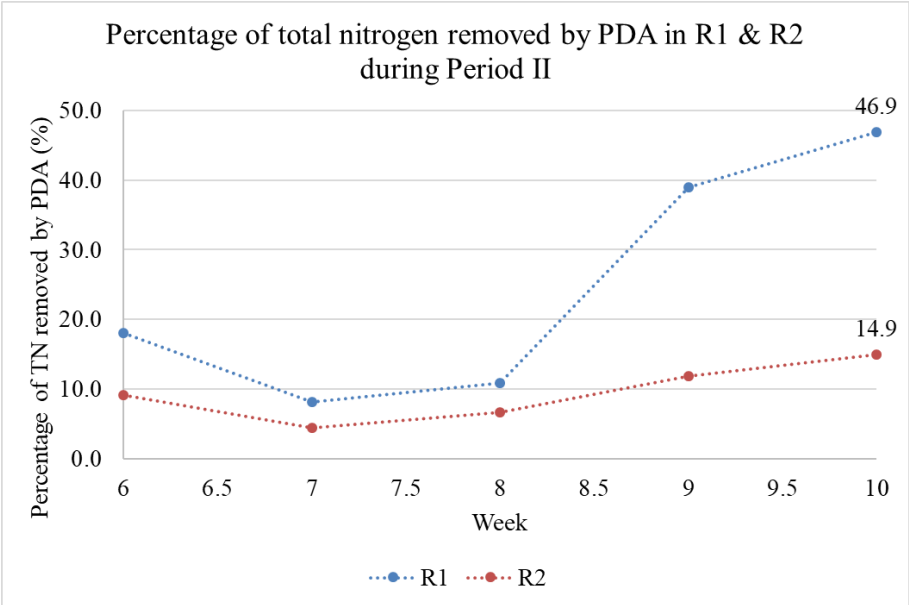

Figure 4.14. Percentage of total influent nitrogen removed via anammox (coupled to partial denitrification) in reactors 1 (acetate carbon source) and 2 (propionate carbon source) during Period II of the continuous PDA experiment.

The PDA process has previously been found to be capable of nitrogen removal efficiencies of up to 97 % when performed in a sequencing batch reactor and a granule-based upflow anaerobic sludge bed reactor, both using acetate as carbon source (Xu et al., 2020; Cao et al., 2016). An

important aspect in an MBBR process is the biofilm formation on the carrier media. Should the biofilm grow excessively it may drastically reduce the surface area of the carriers, as well as introduce diffusion limitations to the activity of the bacteria. The carriers used to inoculate the reactors in this experiment appeared to initially have a high abundance of biofilm, and toward the final weeks the individual carriers had developed considerable variations in biofilm quantity, thus explaining the fluctuating biomass concentration of the effluent. This most likely affected the performance of both reactors, resulting in the lower efficiencies compared to alternative PDA processes. However, this experiment was performed for 67 days, approximately half of which were under unstable conditions, while PDA processes have previously been found to require up to 130 days to reach stability (Ma et al., 2017). Both reactors in this experiment demonstrated a successful cooperation between partial denitrifiers and anammox bacteria, with anammox contributions to TN removal by 91 % and 64 % in R1 and R2 respectively. In addition, both the removal rates and the anammox contribution in the reactors displayed a trend of increasing, thus indicating that the processes had not yet reached their maximum performance at the final week of the experiment.

Similar removal rates to the ones observed during the final week of this experiment (0.47 g $N/(m^2*d)$ and 0.23 g $N/(m^2*d)$ for R1 and R2 respectively) have been achieved when utilizing PNA. Malovanyy et al. (2015) operated a deammonification MBBR at 20°C and achieved nitrogen removal rates of 0.04 – 0.13 g $N/(m^2*d)$, while Kowalski et al. (2019) reached values of 0.45 g $N/(m^2*d)$ at the same temperature. However, in both studies a lower nitrogen loading rate was used, resulting in a total nitrogen removal of 19 – 40 % and 77 % respectively. Furthermore, Gustavsson et al. (2020) maintained an NRR of approximately 0.45 g $N/(m^2*d)$, with a relative nitrate formation around 40 %, during a 33-month PNA pilot study for mainstream wastewater treatment at temperatures of 10 – 23°C (peak NRR of 0.66 g $N/(m^2*d)$). Comparing these values to the NRR measured at week 10, a PDA process utilizing acetate as carbon source appears to be comparative to PNA. While PDA requires a preceding step where half of the ammonium is oxidised to nitrate, a PNA process can be applied directly to ammonium-containing wastewater but may require a subsequent step of denitrification to account for residual nitrate in the effluent (Gustavsson et al., 2020). Additionally, the removal rates in this experiment do not seem to have reached their maximum capabilities, as they continuously increased during Period II. The last two weeks of operation of R1 indicated that residual nitrite in the effluent could be a by-product of a high rate of partial denitrification in PDA, thus investigating the introduction of a final anammox polishing step may be of interest to further limit effluent nitrogen. However, in general PNA faces difficulties with inevitable accumulation of residual NO_3^- in the effluent, which a well performing PDA process does not (Du et al., 2019).

When introducing a preceding nitrification step before the PDA reactor, to oxidise half of the incoming nitrogen (present as NH_4^+) to nitrate, the reactor volume is dependent on the loading rates used for the PDA reactor. Running the PDA reactor at an NLR of 0.5 g $N/(m^2*d)$ (i.e. approximately the removal rate of R1) and allowing half of the incoming wastewater to bypass the nitrification reactor, the two-step process may be operated as displayed in Figure 4.15. With a nitrification MBBR half the size of the PDA reactor, the ammonium and organic carbon loading rates in this reactor would be 0.5 g $N/(m^2*d)$ and 1.0 g $COD/(m^2*d)$. Thus, with a DO concentration > 3 mg O_2/L in the nitrification reactor, the ammonium removal would be sufficient for ~100 % conversion, consequently providing the PDA reactor with an influent composed of NO_3^- and NH_4^+ in a 1:1 ratio (Hem et al., 1994).

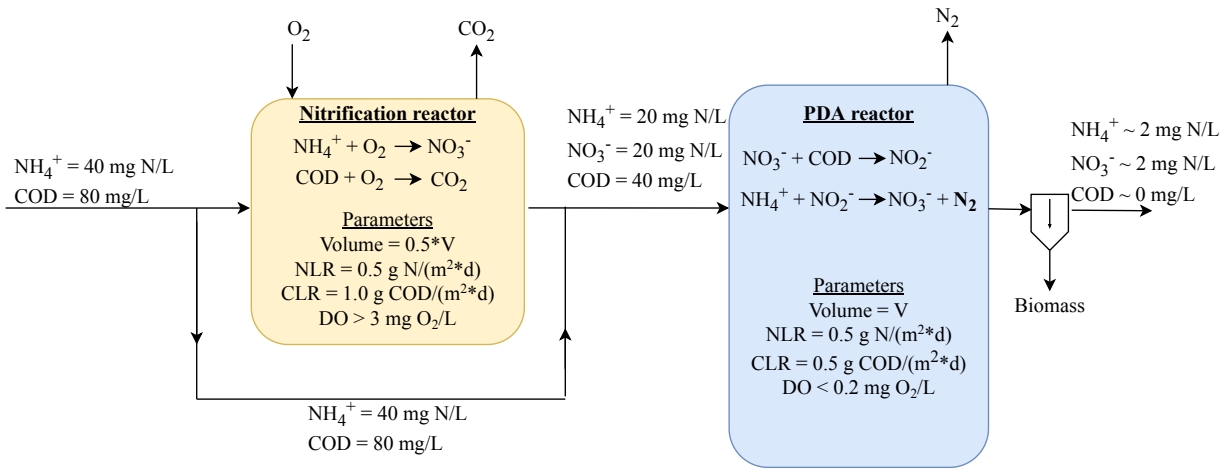

Figure 4.15. A proposed, simplified two-step process, using two MBBRs for BNR in low concentration and temperature wastewater. Half of the incoming wastewater enters an aerated nitrification reactor where NH_4^+ is oxidised to NO_3^-, while the other half bypasses the reactor. The effluent is then combined with the bypassing stream and fed to the PDA reactor for nitrogen removal. (CLR: organic carbon loading rate, NLR: total nitrogen loading rate, DO: dissolved oxygen)

5 Conclusions

The processes of partial denitrification and anammox have been coupled and operated continuously for ten weeks in two one-stage MBBRs, utilizing either acetate or propionate as carbon source for the heterotrophic denitrification.

- By maintaining anoxic conditions and providing nitrate and carbon, in the form of acetate or propionate, growth of heterotrophic denitrifiers was induced in the biofilm on the K5 carriers. At week 10, the nitrate reducing activities of the biofilm carriers reached 1.04 g N/(m^2*d) (acetate) and 0.77 g N/(m^2*d) (propionate) at 20°C, using a COD/NO_3^--ratio of approximately 4 in ex-situ batch activity tests.
- The nitrite reducing activities of the growing denitrifying populations decreased during the second period of the experiment. This indicates that a selective pressure for partial denitrifiers was successfully established in the two PDA reactors, by maintaining a COD/NO_3^--ratio of approximately 2.
- The impact of the selective pressure for partial denitrification was greater for the biofilm carriers with acetate as carbon source compared to propionate.
- The mean total nitrogen removal rate of the final week was 0.47 ± 0.03 g N/(m^2*d) in reactor 1 (acetate) and 0.23 ± 0.03 g N/(m^2*d) in reactor 2 (propionate). The lower removal rate in R2 could be attributed to two factors; a lower abundance of partial denitrifiers and a lower denitrification rate.
- The coupling of partial denitrifications and anammox was successfully established in reactor 1 (acetate) and semi-successfully in reactor 2 (propionate), with an anammox contribution to nitrogen gas formation of 91 % and 64 % respectively. In addition, the values displayed a trend of increasing during the final weeks of the experiment, thus indicating that the microbial communities had not yet reached their steady-state ratio of partial denitrifiers and anammox bacteria.
- Although a longer operational time is required to determine the feasibility of applying PDA for biological nitrogen removal in wastewater treatment, this experiment has demonstrated the possibility of coupling the activities of partial denitrifiers and anammox bacteria in a one-stage MBBR process for simultaneous nitrate and ammonium removal.

6 Future work

A longer period of operation is required to fully investigate the performance of the PDA process when stable removal rates have been achieved. Further operation of the reactors used in this experiment would probably also reveal the stability of the developed co-communities present at the final week of experiment. By examining the impulse or step responses to changes in reactor conditions, such as influent concentrations or temperature changes, the robustness of the process may be evaluated once a steady state of operation is attained. Additionally, it would be of interest to observe if higher efficiencies could be achieved by lowering the loading rates.

Further studies regarding partial denitrification utilizing propionate as carbon source could give insight as to how nitrite reduction might be avoided, and provide techniques for increasing the selective pressure for growth of the desired types of denitrifiers. Genetic analysis of the bacteria may provide information regarding the nitrate-to-nitrite conversion, by determining the abundance of *nirS* and *nirK* genes in the growing denitrifying population. It is also of interest to study how the process is affected by using a combination of VFAs as carbon source, which would likely be the case when implementing the process for wastewater treatment. In addition, computer modelling of the biofilm carriers could provide information regarding for example transport limitations of the biofilm.

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