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Microbial resource management for mainstream partial nitritation/anammox: Strategies to enhance the nitrogen conversion efficiency

Thesis submitted for the degree of doctor in bioscience engineering at the University of Antwerp to be defended by

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List of abbreviation

AerAOB	aerobic ammonium-oxidizing bacteria
Amo	ammonia monooxygenase
Anammox	anaerobic ammonium oxidation
AnAOB	anaerobic ammonium-oxidizing bacteria
AOA	ammonium-oxidizing archaea
ASO	anoxic sulfide oxidation
ASV	amplicon sequence variants
BABE	bioaugmented batch enhancement reactor
bCOD	biodegradable chemical oxygen demand
CAPEX	capital expenditures
COD	chemical oxygen demand
Comammox	complete ammonium oxidation
C-stage	carbon (redirection) stage in the STP
DNA	deoxyribonucleic acid
DO	dissolved oxygen
EC	electrical conductivity
EPS	extracellular polymeric substances
FA	free ammonia
FNA	free nitrous acid
Hao	hydroxylamine oxidoreductase
HBx	heterotrophic bacteria; x = preferential functionality
Hdh	hydrazine dehydrogenase
HRAS	high-rate activated sludge
HRT	hydraulic retention time
Hzs	hydrazine synthase
IFAS	integrated fixed film activated sludge
MSV	minimum settling velocity of sludge
Nap	periplasmic nitrate reductase
Nar	nitrate reductase
N/DN	nitrification/denitrification

Nir	nitrite reductase
Nit/DNit	nitritation/denitritation
NOB	nitrite-oxidizing bacteria
Nor	nitric oxide reductase
Nos	nitrous oxide reductase
N-stage	nitrogen (removal) stage in the STP
Nxr	nitrite oxidoreductase
OPEX	operational expenditures
OUT	operational taxonomic unit
PN/A	partial nitritation/anammox
PN	partial nitritation
PCR	polymerase chain reaction
RBC	rotating biological contactor
RNA	ribonucleic acid
SBR	sequencing batch reactor
SRT	sludge retention time
STP	sewage treatment plant
SVI	sludge volume index
TIN	total inorganic nitrogen
TNLR	total nitrogen loading rate
TSS	total suspended solids
UN	United Nations
VSS	volatile suspended solids
ΔT	temperature difference

Contents

Abstract

This thesis provides three potential ways to enhance the nitrogen removal efficiency of mainstream partial nitritation/anammox (PN/A), a key technology to enable energy-positive sewage treatment. In Chapter 1, the typical technologies to promote nitrogen removal efficiency are summarized.

In Chapters 2 and 3, the concept 'winter bioaugmentation with stored summer surplus sludge' is proposed. Applying that, a cost-effective sludge preservation strategy is required. The results demonstrated that nitrogen additives conditions (in the presence of nitrite or nitrate) resulted in a higher anammox bacteria (AnAOB) activity decay than no nitrogen additives conditions (without nitrite and nitrate). Preserving PN/A biomass without cooling and redox adjustment proved to be the cost-effective strategy. The reactivation of these stored sludges was also tested in low-temperature systems (15 and 10℃). Respectively 56% and 41% of granules activity compared to pre-storage activity (after Arrhenius-based temperature correction) could be recovered within a month (41% and 32% for flocs activity). Additionally, 85 – 87% of granules and 50 – 53% of flocs were retained in the systems. In the end, the stored AnAOB bioaugmentation was successfully validated in the lab (20℃). Therefore, this concept could compensate for the bacterial activity loss that occurs with ordinary sludge (mainstream system).

In Chapter 4, a return-sludge nursery concept, applying the sidestream nitritation and blending the resulting effluent with mainstream effluent to achieve an intermediate temperature and nitrogen concentrations, is proposed. That led to a 33 – 36% increase in nitrogen removal efficiency. Arrhenius' expectations (10 ℃ higher temperature, $\theta = 1.09$) could only explain 49 – 51% of the activity increase in the nursery reactor, pointing to the role of other factors, e.g., the ~400% elevated electrical conductivity (15 – 16%), the 56-335% higher effluent nitrogen concentrations (12 – 14%), and the synergy and unknown factors (20 – 23%). A relatively stable AnAOB community composition was detected during the nursery treatment. Thus, the return-sludge biostimulation approach could also enhance nitrogen efficiency in the mainstream.

In Chapter 5, the N_2O emissions, linked to three typical nitrite-oxidizing bacteria (NOB) suppression strategies (low dissolved oxygen (DO) level, free ammonia (FA), and free nitrous acids (FNA) treatments) were tested in a biofilm system. A low emerged DO level (~0.60 mg O_2 L^{-1}) was effective to suppress NOB activity and decrease N_2O emissions, but NOB adaptation

gradually appeared after 200 days of operation. Further NOB inhibition was successfully achieved by periodical (3 hours per week) FA (~30 mg NH_3-N L^{-1}) or FNA (~3 mg HNO_2-N L^{-1}) treatments. The FA treatment promoted N_2O production, while the FNA treatment had no effect. Thus, PN/A systems should be operated at relatively low DO levels with periodical FNA treatment.

In Chapter 6, the major findings proposed and the main conclusions drawn in this thesis are outlined. Beyond that, the possible design of a mainstream PN/A configuration that combined all described three technologies is demonstrated. Overall, the novel insights from this thesis potential to improving nitrogen removal efficiency in the mainstream.

Samenvatting

In dit proefschrift worden drie mogelijke manieren beschreven om het stikstofverwijderingsrendement van partiële nitritatie/anammox (PN/A) in de waterlijn te verbeteren, als kerntechnologie om energiepositieve rioolwaterzuivering mogelijk te maken. In hoofdstuk 1 wordt een overzicht gegeven van de typische technologieën om dit rendement te bevorderen.

In de hoofdstukken 2 en 3 wordt het concept "Inoculatie in de winter met opgeslagen overtollig zomerslib" voorgesteld. Essentieel hiervoor is een kosteneffectieve slibbewaringsstrategie. De resultaten toonden aan dat anoxische opslagomstandigheden (in de aanwezigheid van nitriet of nitraat en de afwezigheid van zuurstof) resulteerden in een hoger verlies aan activiteit van anammoxbacteriën (AnAOB) dan anaerobe omstandigheden (zonder zuurstof, nitriet en nitraat). Het bewaren van PN/A-biomassa zonder koeling en redoxaanpassing bleek de meest kosteneffectieve strategie te zijn. De reactivatie van deze opgeslagen slibsoorten werd ook getest in lage-temperatuursystemen (15 en 10℃). Respectievelijk 56% en 41% van de originele activiteit (voor opslag) van de korrels (na temperatuurcorrectie op basis van Arrhenius) kon binnen een maand worden teruggewonnen (41% en 32% voor de activiteit van de vlokken). Bovendien werd 85-87% van de korrels en 50-53% van de vlokken in deze systemen vastgehouden. Uiteindelijk werd de opgeslagen AnAOB-bioaugmentatie met succes gevalideerd in het lab (20℃). Dit concept zou dus het verlies aan bacteriële activiteit tijdens de winter, dat optreedt bij actief slib (hoofdstroomsysteem), kunnen compenseren.

In hoofdstuk 4 wordt een concept voor een retourslibkwekerij voorgesteld, waarbij nitritatie in de zijstroom wordt toegepast en het resulterende effluent wordt gemengd met hoofdstroom effluent om een intermediaire temperatuur en stikstofconcentraties te bereiken. Dit leidde tot een verhoging van het stikstofverwijderingsrendement met 33-36%. Het temperatuurseffect (~10℃ hoger, θ=1,09) kon slechts 49-51% van de activiteitstoename in de kweekreactor verklaren, wat wijst op de rol van andere factoren, bv. de ~400% verhoogde elektrische geleidbaarheid (15-16%), de 56-335% hogere effluentstikstofconcentraties (12-14%), en de synergie en onbekende factoren (20 - 23%). Er werd een relatief stabiele samenstelling van de AnAOB-gemeenschap geconstateerd tijdens de behandeling van de

kwekerij. De biostimulatie van het retourslib zou dus ook de stikstofefficiëntie in de hoofdstroom kunnen verbeteren.

In hoofdstuk 5 werden de N_2O-emissies gekoppeld aan drie typische onderdrukkingsstrategieën voor nitrietoxiderende bacteriën (NOB) (een laag opgelost zuurstofgehalte (DO), vrije ammoniak (FA) en vrij salpeterigzuur (FNA) behandelingen) getest in een biofilmsysteem. Een laag DO-niveau (~0,60 mg O_2 L^{-1}) was doeltreffend om de NOB-activiteit te onderdrukken en de N_2O-emissies te verlagen, maar na 200 dagen werking trad geleidelijk NOB-adaptatie op. Verdere NOB-remming werd met succes bereikt door periodieke (3 uur per week) behandelingen met FA (~30 mg NH_3-N L^{-1}) of FNA (~3 mg HNO_2-N L^{-1}). De FA-behandeling bevorderde de N_2O-productie terwijl de FNA-behandeling geen effect had. PN/A-systemen worden dus best geëxploiteerd bij relatief lage DO-niveaus met periodieke FNA-behandeling.

In hoofdstuk 6 worden de belangrijkste bevindingen en de belangrijkste conclusies van dit proefschrift uiteengezet. Daarnaast wordt het mogelijke ontwerp van een hoofdstroom PN/A-configuratie gedemonstreerd waarin alle drie beschreven technologieën worden gecombineerd. De nieuwe inzichten uit dit proefschrift leverde toonden potentieel om stikstofverwijderingsefficiëntie in de hoofdstroom te verbeteren.

Chapter 1

General introduction

1.1 Introduction

1.1.1 Ever increasing wastewater production

In recent years, the world population has exploded. According to the report of the United Nations in 2017, the current population counted 7.5 billion people (United Nations, 2017). As expected, the population will continue to grow this century and peak around 2100 at about 11.2 billion (United Nations, 2017). At the same time, more and more people are moving to urban areas. In 2009, for the first time in human history, there were more people living in urban areas than in rural areas (UN-Habitat, 2012). According to the United Nations' projections in 2018, the share of the urban population will continue to increase and account for 68% of the world population by 2050 (United Nations, 2018). As the world population continues to grow, the scale of cities will also expand year by year. Wastewater is generated in all aspects of life, such as bathing, washing clothes, cooking, etc. The large-scale volume of wastewater being discharged centrally provides a unique challenge for wastewater treatment. Reactive nitrogen is, among all, the main pollutant, mainly presents in the form of ammonium, with some amounts of nitrite and nitrate.

1.1.2 Toxic impact of nitrogen in wastewater

There are three main consequences of the discharge of nitrogen into the environment.

First, it will lead to eutrophication (Conley et al., 2009). Eutrophication refers to the excessive growth of phytoplankton and algae after nitrogen rich wastewater is discharged into the aquatic environment. Although they can produce oxygen (O_2) during the day, O_2 is consumed in large quantities at night, which leads to a decrease in the fish population and biodiversity loss (Camargo and Alonso, 2006).

Further, free ammonia, and to a lesser extent nitrite and nitrate are toxic to aquatic life and the human body (Randall and Tsui, 2002). Ammonia is toxic to all vertebrates causing convulsions, coma and death (Randall et al., 2002). Excessive intake of nitrate or nitrite in the human body can cause methemoglobin (i.e., a type of hemoglobin that cannot be combined with O_2) to accumulate in the blood, leading to human diseases, such as methemoglobinemia (Knobeloch et al., 2000). As this is more prevalent in babies, it is also called 'blue baby syndrome' (Ward et al., 2005). To protect the most sensitive aquatic life, Camargo and Alonso (2006)

reported that the concentration of ammonia, nitrite, and nitrate should stay below the range of 0.01 – 0.02, 0.08 – 0.35, and 2.9 – 3.6 mg N L^{-1}, respectively, in a long-term exposure.

Third, it will exacerbate climate change. In water bodies, the transformation of nitrogen is usually accompanied by the emission of nitrous oxide (N_2O) as the by-product (Beaulieu et al., 2011; Cooper et al., 2017; Strauss et al., 2004). N_2O is a potent greenhouse gas. It is the third most critical well-mixed greenhouse gas behind carbon dioxide (CO_2) and methane (CH_4), accounting for 6% of the total anthropogenic radiative forcing (0.17 W m^{-2}) (Cooper et al., 2017). On a 100-year time scale, its global warming potential is 265 times that of CO_2 (IPCC, 2013). In addition, N_2O is also the dominant stratospheric ozone (O_3) depleting substance emitted in the 21st century, since the products of photocatalytic N_2O cleavage to NO and NO_2 could reduce O_3 to O_2 (IPCC, 2013; Ravishankara et al., 2009). As N_2O emissions have increased in recent years, it has also raised more and more concerns.

Nitrogenous wastewater can be extremely harmful to the human environment, wildlife habitat, and animal and human health, indicating that proper treatment is essential. Wastewater treatment is a process of purifying wastewater into clean water for harmless discharge or even reuse. Although countries are aware of the importance of wastewater treatment, there is a wide gap in the percentage (the proportion of treated wastewater to total produced wastewater) of wastewater treatment in each country. Overall, the majority of wastewater can be treated in high-income countries (~70%), whereas only a small amount of wastewater receives any type of treatment in middle-income countries (28 – 38%) and low-income countries (~8%) (WWAP, 2017). Fortunately, more and more developing countries have implemented strict nitrogen discharge regulations. For example, China has applied strict nitrogen discharge standards (< 5 mg N L^{-1} of ammonium and 15 mg N L^{-1} of total nitrogen) (GB 18918-2002). Therefore, it is becoming increasingly more important to improve the efficiency of nitrogen removal in the sewage treatment plants (STP).

1.2 Typical nitrogen removal process

As shown in Figure – 1.1, nitrogen removal is the process converting reactive nitrogen (or called biologically available nitrogen, e.g., ammonium, nitrite, and nitrate) to nitrogen gas (N_2). Most of the nitrogenous compounds in wastewater are present in the reduced form of ammonium or as components of larger organic molecules. Therefore, most nitrogen removal in wastewater starts with ammonium (nitrogenous compounds released by the hydrolysis of larger organic

molecules are also ammonia nitrogen). There are three typical nitrogen removal processes, i.e., nitrification/denitrification (the conventional process), nitritation/denitritation (a shortcut from the conventional process), and nitritation/anammox (a new process showing great promise).

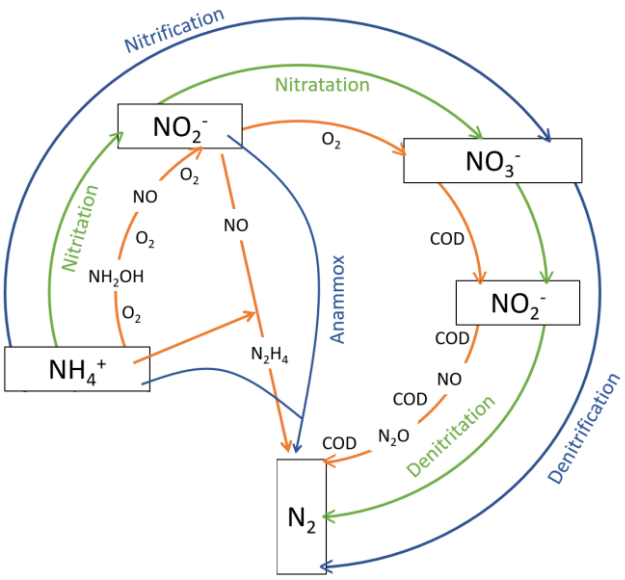


Figure – 1.1 The nitrogen removal processes from biological wastewater treatment.

1.2.1 Nitrification: nitritation/nitratation

Nitritation: The ammonium is converted to nitrite with O_2 by aerobic ammonia-oxidizing microorganisms, e.g., aerobic ammonia-oxidizing bacteria (AerAOB) (Koops and Pommerening-Röser, 2001), ammonia-oxidizing archaea (AOA) (Limpiyakorn et al., 2013), and potential complete ammonia-oxidizing bacteria (comammox bacteria) (Van Kessel et al., 2015). That is the first step of the nitrification, and the stoichiometric reaction is shown in Eq – 1.1 (Vlaeminck, 2009). During the nitritation process, there are three separate steps: i) $NH_4^+ + O_2 \rightarrow NH_2OH$ by the membrane-bound ammonia monooxygenase (*amo*) enzyme, ii) $NH_2OH + O_2 \rightarrow NO$ by the hydroxylamine oxidoreductase (*hao*) enzyme, and iii) $NO + O2 \rightarrow NO_2^-$ with no enzyme (Caranto and Lancester, 2017; Seuntjens, 2018a). These processes are usually accompanied by the emissions of N_2O (Figure – 1.2). There are two main routes for AerAOB to produce N_2O, i.e., the nitrifier denitrification route ($NO_2^- \rightarrow NO \rightarrow N_2O$) (Kampschreur et al., 2008) and the NH_2OH oxidation route (a byproduct of the oxidation process: $NH_2OH \rightarrow N_2O$) (Peng et al., 2014).

$$NH_4^+ + 1.382O_2 + 0.091HCO_3^- \rightarrow 0.982NO_2^- + 1.891H^+ + 1.036H_2O + 0.091CH_{1.4}O_{0.4}N_{0.2} \quad (Eq - 1.1)$$

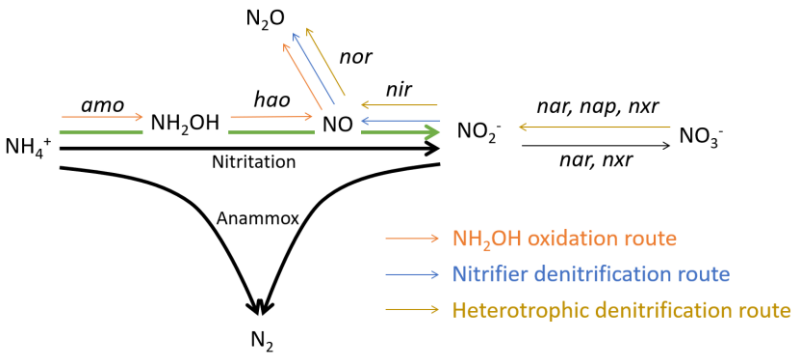


Figure – 1.2 The three N_2O formation pathways in the PN/A system. Enzymes involved: *amo*, ammonia monooxygenase; *hao*, hydroxylamine dehydrogenase; *nir*, nitrite reductase; nor, nitric oxide reductase; *nar*, nitrate reductase; *nxr*, nitrite oxidoreductase; *nap*, periplasmic nitrate reductase.

Nitratation: The produced nitrite (through the nitritation process) is oxidized to nitrate by nitrite-oxidizing microorganisms, which are mainly nitrite-oxidizing bacteria (NOB) (Speth et al., 2016). This process is executed in one step: NO_2^- + O_2 → NO_3^- by the nitrite oxidoreductase (*nxr*) enzyme. That is the second step of the nitrification, and the stoichiometric reaction is shown in Eq – 1.2 (Vlaeminck, 2009). According to previous studies (Freitag et al., 1987; Inamori et al., 1997), N_2O can also be released via NOB but this contribution is negligible compared to the emission rate of AerAOB (Inamori et al., 1997).

$$NO_2^- + 0.488O_2 + 0.003NH_4^+ + 0.010H^+ + 0.013HCO_3^- \rightarrow NO_3^- + 0.008H_2O + 0.013CH_{1.4}O_{0.4}N_{0.2} \quad (Eq - 1.2)$$

Nitrification (nitritation & nitratation): Nitrification consists of the combination of nitritation and nitratation. Among all the involved microorganisms, AerAOB (mainly genera *Nitrosomonas* and *Nitrosospira*), AOA, and NOB (mainly genera *Nitrospira*, Candidatus *Nitrotoga*, and *Nitrobacter*) recurrently play roles in nitrification. The existence of the comammox bacteria was proved in STPs (Daims et al., 2015; Gonzalez-Martinez et al., 2016; Sakoula et al., 2021; Van

Kessel et al., 2015). However, the importance of their role in wastewater treatment is still unclear.

1.2.2 Heterotrophic denitrification

Heterotrophic denitrification is a process to reduce nitrate to N_2 by heterotrophic bacteria ($HBNO_{x-}$). The stochiometric reaction is shown in Eq – 1.3 and this process includes two main steps (Speth et al., 2016): i) NO_3^- + COD → NO_2^- by the nitrate reductase (*nar*) enzyme, ii) NO_2^- → NO → N_2O → N_2 by the nitrite reductase (*nir*), nitric oxide reductase (*nor*), and nitrous oxide reductase (*nos*) enzymes, respectively (all processes rely on COD as an electron donor (Zumft, 1997)). Conditions with low COD/N ratio, high nitrite concentrations, high H_2S concentrations, or micro-aerobic conditions will result in incomplete denitrification, which in turn can lead to the emissions of N_2O (Bartacek et al., 2010; Seuntjens, 2018a). Heterotrophic denitrification is therefore another main N_2O production route (anaerobic reduction: NO_2^- or NO_3^- → N_2O) (Ma et al., 2017a). The $HBNO_{x-}$ are widespread and diverse in all the STPs (Agrawal et al., 2017; Thakur and Medhi, 2019; Zumft, 1997).

$$NO_3^- + 1.080CH_3OH \rightarrow 0.467N_2 + OH^- + 1.440H_2O + 0.760CO_2 + 0.325CH_{1.4}O_{0.4}N_{0.2} \quad (Eq - 1.3)$$

1.2.3 Nitrification/denitrification (N/DN)

The nitrification or denitrification process alone cannot complete the ammonium removal, so they are usually used together in wastewater treatment, i.e., the Nitrification/Denitrification technology (N/DN). The complete stoichiometric reaction of this technology is shown in Eq – 1.4 (Vlaeminck, 2009). In the application, the reactor configuration is mainly an anoxic zone and an aerobic zone alternately. The alternation between aerobic and anoxic zones (without oxygen, but with nitrite/nitrate as an electron acceptor), combined with sufficiently long sludge retention times (SRT), gave space for nitrifying bacteria to oxidize ammonium to nitrate, and denitrifying bacteria to finally reduce the produced nitrate to N_2 (the generated nitrate internally circulated to the first anoxic zone, which makes the best anoxic utilization of the incoming biodegradable chemical oxygen demand (bCOD)) (Figure – 1.3).

$$NH_4^+ + 1.856O_2 + 1.055CH_3OH + 0.104HCO_3^- \rightarrow 0.457N_2 + 0.918H^+ + 2.448H_2O + 0.743CO_2 + 0.421CH_{1.4}O_{0.4}N_{0.2} \quad (Eq - 1.4)$$

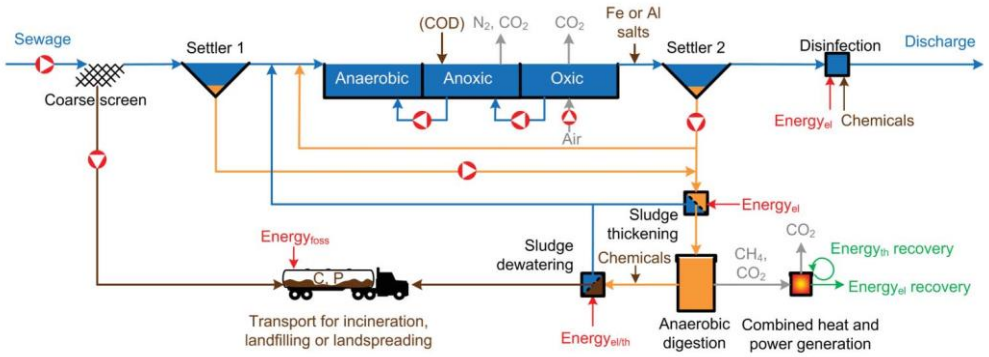


Figure – 1.3 Major pathways for water, energy, and materials in an advanced CAS sewage treatment plant with biological nutrient removal, sludge digestion, and disinfection. Subscripts 'el', 'th', and 'foss' refer to electrical, thermal, and fossil energy, respectively (Verstraete and Vlaeminck, 2011).

Even though this technology is used in most STPs (Bertanza, 1997; Tallec et al., 2006), it still has many drawbacks. First, a large amount of O_2 is required for the nitrification process. Second, an excess of organic carbon is needed in the reaction process to achieve sufficient denitrification. Third, the sludge production is also high since the heterotrophic biomass yield (Y_{max} = ± 0.5 g VSS g N^{-1}) is higher than for autotrophs (Y_{max} = ± 0.04 – 0.15 g VSS g N^{-1}) (Seuntjens, 2018a; Wiesmann, 1994). All the mentioned points make the N/DN technology one of the most expensive processes for nitrogen removal.

1.2.4 Nitritation/denitritation (Nit/DNit)

Since both nitrification and denitrification have the same intermediate product, i.e., nitrite, the shortcut Nitritation/Denitritation (Nit/DNit) is also applied in wastewater treatment (Corsino et al. 2016; Kulikowska and Bernat, 2013; Regmi et al., 2014). The complete stoichiometric reaction of this technology is shown in Eq – 1.5 (Vlaeminck, 2009). Compared with N/DN, less O_2 and COD are required since the ammonium is only converted to nitrite resulting in less biomass production. In addition, due to its higher reaction rates, a reduction in size of the

bioreactor can be realized (Fux and Siegrist, 2004). The reactor configuration will be the same as N/DN, e.g., alternating anoxic and aerobic zones. As ammonium is only needed to be oxidized to nitrite, the nitratation must be avoided. Thus, one of the critical operations is to inhibit the activity of NOB, thereby stopping the oxidation of ammonium at nitrite (Hellinga et al., 1998; Kornaros et al., 2010).

$$\mathrm{NH_4^+} + 1.382\mathrm{O_2} + 0.66\mathrm{CH_3OH} + 0.091\mathrm{H_2CO_3} \rightarrow 0.48\mathrm{N_2} + 0.982\mathrm{H^+} + 2.63\mathrm{H_2O} + 0.51\mathrm{CO_2} + 0.24\mathrm{CH_{1.4}O_{0.4}N_{0.2}} \quad (\mathrm{Eq}-1.5)$$

1.2.5 Anaerobic ammonium oxidation (anammox)

Anammox is a process that anoxically converts ammonium with nitrite to N_2 by the anaerobic ammonium-oxidizing bacteria (AnAOB). The complete stoichiometric reaction is shown in Eq – 1.6, which is literately taken from Strous et al. (1998), and it needs to point out that there is a small misbalance in the charge of H^+ (1.32 is adjusted to 0.116 in the present research). The reaction was updated by Lotti et al. (2014c) in 2014 by a high purity anammox culture, and it is shown in Eq – 1.7. There are three main steps in the process: i) NO_2^- → NO by *nir* enzyme, ii) NH_4^+ + NO → N_2H_4 by the hydrazine synthase (*hzs*) enzyme, and iii) N_2H_4 → N_2 by the hydrazine dehydrogenase (*hdh*) enzyme (Dietl, 2015; Maalcke et al., 2016; Speth et al., 2016). As shown in the stoichiometric equations, a small amount of nitrogenous byproduct (i.e., nitrate) is also formed during the anammox process (by the *nxr* enzyme) (Kartal et al., 2012).

$$\mathrm{NH_4^+} + 1.32\mathrm{NO_2^-} + 0.066\mathrm{HCO_3^-} + 0.116\mathrm{H^+} \rightarrow 1.025\mathrm{N_2} + 0.26\mathrm{NO_3^-} + 2.025\mathrm{H_2O} + 0.066\mathrm{CH_2O_{0.5}N_{0.15}} \quad (Eq-1.6)$$

$$\mathrm{NH_4^+} + 1.146\mathrm{NO_2^-} + 0.071\mathrm{HCO_3^-} + 0.057\mathrm{H^+} \rightarrow 0.986\mathrm{N_2} + 0.16\mathrm{NO_3^-} + 2.00\mathrm{H_2O} + 0.071\mathrm{CH_{1.74}O_{0.31}N_{0.20}} \quad (\mathrm{Eq}-1.7)$$

Till now, there are five candidate anammox genera, i.e., Candidatus *Brocadia*, Candidatus *Kuenenia*, Candidatus *Jettenia*, *Scalindua*, and *Anammoxoglobus* (Kartal et al., 2012; Kuenen, 2008). Except for *Scalindua*, which was only detected in marine or halophilic environments, the other four genera were encountered in STPs (Kartal et al., 2012; van de Vossenberg et al., 2013).

1.2.6 Partial nitritation/anammox (PN/A)

The nitrogen removal via anammox requires the presence of nitrite, produced through nitritation, so the nitrogen removal process combining anammox and nitritation, namely partial nitritation and anammox (PN/A) or deammonification, came into existence. The complete stoichiometric reaction is shown in Eq – 1.8. It is the combination of nitritation and anammox processes, which consists of aerobic ammonium-oxidizing bacteria (AerAOB), that oxidize roughly half of the ammonium to nitrite ('partial nitritation'), and AnAOB, that oxidize the produced nitrite and residual ammonium to N_2 ('anammox') (Agrawal et al., 2018). During this process, N_2O is also emitted (Peng et al., 2014) and since both functional bacteria (i.e., AerAOB and AnAOB) are autotrophic, the PN/A process requires only inorganic carbon as the carbon source and no external organic carbon is required (Van Hulle et al., 2010). Therefore, to limit the competition with heterotrophs (denitrifying bacteria), wastewater with a low bCOD/N (< 2) ratio is advised (Lackner et al., 2008). In addition, to avoid nitrite competition between AnAOB and NOB and O_2 competition between AerAOB and NOB, the activity of NOB should be inhibited.

$$\begin{aligned} &NH_4^+ + 0.793O_2 + 0.080HCO_3^- \\ &\quad \rightarrow 0.438N_2 + 0.111NO_3^- + 1.035H^+ + 1.458H_2O + 0.052CH_{1.4}O_{0.4}N_{0.2} \\ &\quad + 0.028CH_2O_{0.5}N_{0.15} \end{aligned} \qquad (Eq - 1.8)$$

1.2.7 Comparison of PN/A with N/DN and Nit/DNit

Table – 1.1 shows the comparison between PN/A, N/DN, and Nit/DNit in terms of bCOD/N ratio, aeration, and biomass sludge requirements. Considering the COD requirement, N/DN is suitable for treating carbon-rich wastewater, which requires a bCOD/N = 4.8. For the middle bCOD/N wastewater, the shortcut Nit/DNit (requires the bCOD/N = 2.9) is the available candidate. For the low bCOD/N wastewater, the PN/A technology is the best choice. In the aspect of aeration, the PN/A process only needs 0.9 kWh kg N^{-1} compared to 2.3 and 1.7 kWh kg N^{-1} in N/DN and Nit/DNit process, respectively. That is attributed to the characteristic of anammox which is an anaerobic process and therefore does not consume O_2 consumption. As far as biomass production is concerned, only 0.16 kg DW kg N^{-1} biomass is produced in the PN/A process, while in the N/DN and Nit/DNit processes, as much as 1.83 and 1.28 kg DW kg N^{-1} are produced, respectively. That is also important for the application since the management of

waste activated sludge is an energy-intensive and expensive task that can account for up to 50% of the total operating costs of STPs (Baek et al., 2016).

Therefore, compared with the N/DN process, the PN/A process can save 100% of the organic carbon requirement, 60% of the aeration demand, and 90% of the biomass production, which all lead to a decrease in operational costs. For example, the energy consumption had about a 45% decrease (i.e., from 2.66 to 1.50 kWh kg^{-1} N removed) in the Strass STPs (Strass, Austria), where the PN/A technology was used to treat the reject water (Schaubroeck et al., 2015). Even compared with the shortcut Nit/DNit technology, the PN/A process can still save 100% of the organic carbon requirement, 45% of the aeration demand, and 85% of the biomass production. That makes PN/A technology an economically and environmentally friendly process (Agrawal et al., 2018; Ali et al., 2016).

Table – 1.1 Comparison of PN/A, N/DN, and Nit/DNit in bCOD/N requirement, aeration demand, and biomass sludge production (Desloover, 2013; Seuntjens, 2018a; Vlaeminck, 2009). bCOD/N requirements include biomass growth and 20% aerobic COD loss (Matějů et al., 1992).

Technology	bCOD/N requirement (-)	Energy required for aeration (kWh kg N^{-1})	Biomass production (kg DW kg N^{-1})
N/DN	4.8	2.3	1.83
Nit/DNit	2.9	1.7	1.28
PN/A	0	0.9	0.16

1.3 Challenges of nitrogen removal in the mainstream PN/A process

PN/A systems have been successfully implemented worldwide in the sidestream (sludge line) (Lackner et al., 2014). Compared to the sidestream, which has a higher temperature (15 – 20℃ higher than the mainstream) and nitrogen concentration (more than 20 times higher than the mainstream), achieving efficient nitrogen removal in the mainstream is much more challenging. Even though promising, the PN/A technology still needs to overcome some challenges before application in the mainstream. There are many challenges of the implementation of PN/A technology in the mainstream, e.g., the COD/N ratio (Zhu et al., 2017c), H_2S concentration (Kouba et al., 2017), etc. Among them, the main challenges are the decrease in conversion rates due to low temperatures, and the control of competition between AerAOB, AnAOB, and NOB (Cao et al., 2017; Fernández et al., 2016; Lotti et al., 2014a).

1.3.1 Low temperature

The low temperature in the mainstream will lead to the decrease of functional bacterial activity, especially AnAOB. The optimum temperature for AnAOB is between 35 – 40 ℃ (Ma et al., 2019; Zhu et al., 2017b). However, the mainstream temperature fluctuates between 5 – 20 ℃ in moderate climate regions (Hendrickx et al., 2014). Specifically, in winter, the mainstream temperature could drop to about 10 – 12℃ in western Europe (20 – 22℃ in summer) according to the data in Nieuwveer STP (Breda, the Netherlands), which affects the AnAOB activity. According to the previous study, the effect of temperature on bacterial activity (and metabolic rate) can be easily demonstrated by a simplified Arrhenius equation (Eq – 1.9) (Gilbert et al., 2015). The typical θ values for anammox sludge ranges from 1.07 to 1.10 depending on the different systems (Gilbert et al., 2015; Liu et al., 2020; Sobotka et al., 2016; Vandekerckhove et al., 2020).

$$\mu_{T1} = \mu_{T2} \times \theta_{AnAOB}^{(T1-T2)} \qquad (Eq - 1.9)$$

In addition, the low growth rates of AnAOB also limit the application of the PN/A system in the mainstream. In the sidestream conditions, the AnAOB growth rate is about 0.07 d^{-1} at 32℃ (Strous et al., 1998). However, it decreases to 0.02 d^{-1} at 20℃ and only 0.005 d^{-1} at 10℃ (Lotti et al., 2014c) which will lead to effluent quality deterioration in the mainstream. Thus, the low temperature remains one of the main challenges for STPs. Especially in winter, high AnAOB abundance and SRT are required due to the reduced growth and removal rate of AnAOB. According to previous research, the anammox SRT should be as long as 70 days at 15℃ and it must increase to more than 100 days when temperature dropped to 10℃ (Pérez et al., 2014; Yang et al., 2017).

1.3.2 NOB competition

Different from the sidestream conditions in which the NOB can be well suppressed (Duan et al., 2019b), the suppression of NOB is more difficult in the mainstream. NOB are undesired in the mainstream PN/A system, as it causes competition in terms of i) O_2 availability through competition with AerAOB, ii) nitrite due to competition with AnAOB and iii) space, as both competition with AnAOB and AerAOB occur. When NOB oxidizes nitrite to nitrate under aerobic conditions, a higher total O_2 load is required to provide AnAOB with enough nitrite, and

at the same time, more nitrate is produced, which is considered to be a by-product of the PN/A process (Blum, 2018a).

Five typical NOB genera (i.e., *Nitrobacter*, *Nitrococcus*, *Nitrospira*, *Nitrispina*, and *Nitrotoga*) are known (Daims et al., 2001). According to Hao et al. (2002), NOB is more difficult to outcompete at lower ammonium loading rates or ammonium limiting conditions (due to the absence of a strong stratification of microbial populations in such systems) (Hoekstra, 2017). Furthermore, *Nitrospira* has not only a higher affinity for nitrite and O_2 but also a higher energy transfer efficiency than other genera that make it a competitive advantage under mainstream conditions (low temperature and ammonium concentrations) (Park et al., 2017). In other words, this genus has a competitive advantage under mainstream conditions, which increases the difficulty of NOB inhibition. Effective NOB suppression strategies are still one of the most critical challenges in the mainstream PN/A.

1.4 Enhancing anammox efficiency in the mainstream PN/A process

1.4.1 Bioaugmentation

Bioaugmentation, i.e., the addition of selected sludge or biomass to the receptor reactor to enhance the decomposition of specific pollutants (e.g., nitrogen), is a promising process that can improve the pollutant removal efficiency (Herrero and Stuckey, 2015). Based on the Arrhenius equation (Eq – 1.9), a ~2.5 times higher biomass load is needed due to the temperature decrease in the winter to achieve a similar nitrogen removal rate in the mainstream PN/A system (assuming that the temperature difference between summer and winter is 10℃ and θ is 1.10). Since merely extending the SRT will not be sufficient, bioaugmentation by the external AnAOB sludge is a suitable option.

AnAOB bioaugmentation was already explored as a remedy to recover system performance from inhibition or shocks, such as long-term oxytetracycline exposure (Jin et al., 2014) and high COD concentration (800 mg COD/L) inhibition (Tang et al., 2014). These conditions were tested on a lab scale, where the AnAOB source was not limited. Whereas, when the AnAOB bioaugmentation is applied in the full-scale STPs, as the slow growing bacterium, the source of them was limited. Cultivating the biomass in a separate enrichment reactor was another option (Herrero and Stuckey, 2015), but that was not cost-effective and wasted space in STP.

Mainstream biomass biostimulation with sidestream conditions (EssDe®) or seeding concepts from sidestream to mainstream (ANITA™Mox) were a good source for bioaugmentation in STPs (Christensson et al., 2013; González-Martínez et al., 2021; Szatkowska and Paulsrud, 2014). Given that there are too many niche differences between the two systems (e.g., temperature, predation, and nitrogen concentration), it is less likely to yield the appropriate AnAOB biomass (Head and Oleszkiewicz, 2004; Mannucci et al., 2015). Additionally, the significant temperature difference would affect the performance and bacterial activity (Head and Oleszkiewicz, 2004). In addition, even though the sidestream reactor would be a potential biomass source, long-term bioaugmentation with this sludge will be harmful to the sidestream system since it contributed to about 15 – 25% of the total ammonium removal in STPs.

Limitations on bioaugmentation will occur when the sludge to be seeded is grown in conditions differing from the seeding condition. Only if the appropriate biomass (i.e., less niche difference to the receptor biomass) is available, the AnAOB bioaugmentation can be applied. Storing excess AnAOB sludge, mainly harvested over summer (higher biomass growth rate due to the relatively high temperatures) and re-inoculate this in winter would be a promising alternative.

1.4.2 Biomass storage

A lot of different strategies were studied on long term storage of AnAOB. They can mainly be divided into four categories: i) the cryopreservation; ii) substrates or redox spikes; iii) the above-zero low temperatures (e.g., 4℃); vi) other methods.

Cryopreservation (-20 ℃, -80 ℃, and -200 ℃) with different cryoprotective agents (e.g., dimethyl sulfoxide) that prevent activity loss during sludge or culture collections storage has been extensively described (Rothrock et al., 2011; Viancelli et al., 2017). Viancelli et al. (2017) indicated that the activity of the AnAOB, which was stored for 120 days with glycerol or skimmed cow milk at -80℃ could be recovered. Rothrock et al. (2011) successfully preserved the AnAOB activity for 120 days after freezing in liquid nitrogen (-200℃) followed by lyophilization in skim milk media without glycerol. However, due to the cost of the cooling process and cryoprotective agents, this method is not often applied in full-scale STP.

In contrast, substrates or redox spikes are expected applicable for the bioaugmentation concept since they can well retain anammox activity and low operation cost. Redox buffer

(nitrite or nitrate) adjustment during sludge preservation was studied since it can effectively prevent sulfate reduction, avoiding H_2S production which is harmful to biomass (Vlaeminck et al., 2007). Wang et al. (2016c) demonstrated that about 30% of AnAOB activity was maintained after 180 days of preservation at 35℃ with weekly spikes of NH_4^+-N and NO_2^--N (50 mg N L^{-1} respectively). Vlaeminck et al. (2007) reported that high AnAOB activity was maintained after storage for five months at 20°C, when nitrate was periodically added. In addition, the above-zero low temperature has also proved beneficial for AnAOB preservation. Xing et al. (2016) revealed that anammox granules preserved at 4 ℃ without substrate addition had a lower decay rate and higher nitrogen removal capacity after storage comparing to granules stored at 20°C.

There were also some special approaches to preserve the AnAOB, e.g., consortia transformation strategy (Shi et al., 2020), immobilizing technique (Ali et al., 2014), and protective agents' addition (e.g., hydrazine, glycerol, skim milk) (Ganesan and Vadivelu, 2020; Rothrock et al. 2011). Shi et al. (2020) proposed a special AnAOB preservation strategy, in which the anammox consortia were preserved in the form of anoxic sulfide oxidation (ASO) microorganisms (an intermediate), and the microbial revised back to anammox consortia after storage. Ali et al. (2014) was successfully achieved reactivation of stored AnAOB sludge, which was immobilized in polyvinyl alcohol (6%, w/v) and sodium alginate (2%, w/v) gel. But these technologies are also not feasible for full-scale application because of the operational complexity and high cost.

1.4.3 Biostimulation

Biostimulation, the addition of nutrients or electron acceptors to activate the indigenous microbiota, can also be used to enhance the microbial contaminants degradation rates (Herrero and Stuckey, 2015). Many strategies had been used to biostimulate the AnAOB activity, which was suppressed by specific inhibitors. Zhang et al. (2015) successfully biostimulated the copper (II) inhibited AnAOB by low-intensity ultrasound. Li et al. (2020) also achieved biostimulation of a marine anammox bacteria-dominated bioprocess through the external addition of copper (II). However, these approaches were only executed in the lab-scale reactors. When taking cost, ease of operation, and environmental hazards into consideration, it is still debatable whether they can be applied to large-scale sewage treatment plants.

In the full-scale biological pollutant removal systems, biostimulation is usually used combined with bioaugmentation. Salem et al. (2003) created a bioaugmented batch enhancement reactor (BABE), a nitrification reactor located in the sludge return line, to enhance endogenous nitrifying bacteria that reached the mainstream from the sidestream system. In the BABE process, part of the return sludge was introduced into the BABE reactor to enrich nitrifying bacteria. The growth of nitrifying bacteria in the original return sludge ensured the consistency of the enriched nitrifying bacteria in the BABE reactor with the dominant nitrifying bacteria in the mainstream system. In addition, the short sludge retention time and low returned sludge ratio ensured the reactor operated at higher temperatures and high nitrogen levels, which stimulated the endogenous microorganisms and enhanced the nitrification efficiency (Berends et al., 2005; Salem et al. 2004).

However, the partial nitritation/anammox (PN/A) process has not been studied since AnAOB are more complicated than nitrification bacteria (e.g., low growth rate and sensitivity to external conditions change (Jetten et al., 2001; Lotti et al., 2014c)). A return-sludge nursery concept that blends sidestream (partial) nitritation effluent with mainstream effluent to achieve an intermediate temperature and nitrogen level (favorable conditions for anammox) to stimulate AnAOB sludge (retained from the mainstream waste sludge) deserves to be studied.

1.5 Suppressing NOB activity and N_2O emissions in the mainstream PN/A process

In mainstream PN/A systems, NOB suppression and N_2O mitigation are two important operational objectives. Achieving a balance between these two will be key in achieving a long-term stable and sustainable mainstream PN/A.

1.5.1 NOB suppression

Achieving a long-term stable PN/A process in the mainstream is challenging, which is mainly attributed to the low temperature (10 – 15℃ versus > 30℃) and low nitrogen concentration (< 70 mg N L^{-1} versus > 1000 mg N L^{-1}), which makes NOB suppression difficult (Laureni et al., 2016; Peng et al., 2020). There are two typical NOB suppression strategies: i) "ON/OFF" control, promoting the growth and activity of AerAOB, AnAOB, and tolerating the activity of nitrite- and nitrate-reducing heterotrophs while inhibiting the activity of NOB; ii) 'IN/OUT' control, which

leads to washout of unwanted microbes (e.g., NOB and heterotrophs) from the reactors while selectively retaining (and seeding) AerAOB and AnAOB (e.g., granules (Lotti et al., 2014a)) (Agrawal et al., 2018). To be more specific, the observed NOB growth rates (dX_{NOB}/dt), shown in Eq – 1.10 (Laureni et al., 2019; Wang et al., 2021), NOB suppression can be obtained using three main strategies, namely reduction of maximum bacterial growth rate ($\mu_{NOB,max}$), promotion of biomass decay rate (b_{NOB}), and washout of NOB microorganisms (through decrease of SRT).

$$\frac{dX_{NOB}}{X_{NOB} \cdot dt} = \left(\mu_{NOB,max} - b_{NOB} - \frac{1}{SRT}\right) \qquad (Eq - 1.10)$$

For the reduction of the bacteria growth rate ($\mu_{NOB,max}$), low DO concentrations (Joss et al., 2011; Ma et al., 2011; Ma et al., 2009; Wang et al., 2021) or intermittent aeration (Blackburne et al., 2008), as well as maintaining a residual ammonium concentration (Poot et al., 2016) were used. Continuous low DO levels (e.g., < 0.2 mg O_2 L^{-1}) have been successfully applied to improve the competitive advantage of AnAOB over NOB for nitrite and minimize the O_2 inhibition of AnAOB in the PN/A systems (Laureni et al., 2016; Morales et al., 2016). Intermittent aeration uses the character of the nitratational lag (minimum 15–30 min anoxic) (Gilbert et al., 2014; Kornaros et al., 2010) to completely consume the nitrite produced in the aerobic phase during the anoxic phase. The existence of an anoxic phase can also reduce the inhibition of AnAOB by O_2 as much as possible (Seuntjens et al., 2018b). Under normal circumstances, setting higher DO-setpoints (e.g., > 1.5 mg O_2 L^{-1}) can maximize the activity of AerAOB over NOB (Han et al., 2016; Regmi et al., 2014).

Regarding strategies to promote the decay rate of NOB, free ammonia (FA) (Wang et al., 2017) and free nitrite (FNA) (Peng et al., 2020) treatments have been successfully applied. Wang et al. (2017) reported that 210 mg of NH_3-N L^{-1} has a much lower biotoxicity to AerAOB than to NOB, which makes it possible to inhibit the activity of NOB by FA treatment. Peng et al. (2020) demonstrated that weekly FNA treatment (e.g., 2.0 mg of HNO_2-N L^{-1}, 4 hours) could favor AerAOB over NOB, while the influence of this treatment on the activity of AnAOB was negligible.

To decrease the SRT of NOB, the 'IN/OUT' control was applied (Agrawal et al., 2018) to reduce the SRT of NOB (e.g., flocs) while maintaining a higher SRT of AnAOB (e.g., biofilm, due to its low growth rate (Lotti et al., 2014c)). Meanwhile, flocculated SRT was short enough to

selectively flush NOB and retain AerAOB (Regmi et al., 2014). That can be easily implemented in so-called hybrid reactors, i.e., the integrated fixed film activated sludge (IFAS) reactor configuration, which is based on the coexistence of suspended (i.e., NOB) and attached growth (i.e., AnAOB and AerAOB) (Laureni et al., 2019; Peng et al., 2020). In addition, the two-stage PN//A system, which separates the partial nitritation and anammox process into two reactors, is another option. This strategy is necessary for the mainstream since the low temperature reduces the growth rates and activities of the functional microorganisms (Jin et al., 2019; Valenzuela-Heredia et al., 2021).

The strategies described above are usually accompanied by dynamic changes in DO levels, ammonium (potential during FA treatment), or nitrite concentrations (potential during FNA treatment), and these can be potentially powerful emitters of N_2O (Peng et al., 2014; Wunderlin et al., 2013). That led to the variation of N_2O emission (0.8 – 6.4% of the influent nitrogen loading) from the PN/A system (Ali et al., 2016; Domingo-Félez et al., 2014; Rathnayake et al., 2013).

1.5.2 N_2O emissions

As a strong greenhouse gas and an ozone-destroying gas, the emission of N_2O has attracted more and more attention. In the PN/A system, around 97.5% of the N_2O was emitted from the partial nitritation process (Okabe et al., 2011), thus the parameters which could affect the performance of partial nitritration or AerAOB activity would have effects on N_2O emissions.

For the effect of different DO levels on N_2O emissions, Liu et al. (2021) revealed that a short-term low DO levels increased the N_2O emission, whereas it would decrease in the long-term low DO operation ($P < 0.01$). In addition, the maximum N_2O emission was reported at pH 7.5, although the nitritation rate was constant between pH 6.5 and 8.5 (Rathnayake et al., 2015). The previous research also reported that the N_2O emissions were positively correlated with the ammonium (Jin et al., 2016) and nitrite concentrations (Peng et al., 2020). In addition, the effluent ammonium concentration control could decrease the N_2O emissions while achieving a high nitrogen removal rate (Wan et al., 2021).

The balance between high NOB suppression efficiency and low N_2O emissions rate should be made during the operation of mainstream PN/A. Only by studying the effects of different NOB suppression strategies on N_2O emissions can we choose an optimal operation strategy in the

mainstream PN/A system. Till now, that knowledge was still lacking, which limited the strategy choice in the PN/A system.

1.6 Thesis outline and objectives

Nowadays, the anammox-related system has been wildly installed in the STPs to remove nitrogen all over the world (Lackner et al., 2014). However, most of them are applied in the sidestream, which has relatively high temperatures and ammonium concentrations. Limitations on enhancing the nitrogen removal efficiency under the relatively low temperature restrict the application of the PN/A system in the mainstream. That is why the PN/A process has still not replaced nitrification-denitrification technology. Thus, there is still research needed before the PN/A process can be fully applied to treat mainstream wastewater. In other words, converting as much ammonium into N_2 as possible, instead of byproducts (e.g., nitrate or N_2O) or residual in the system (i.e., ammonium), is the key for the success of mainstream PN/A.

This thesis aims to search for practical approaches to enhance the anammox efficiency under low temperatures. To be specific, the goals are i) winter bioaugmentation with the stored summer excess sludge, ii) anammox nursery reactor to biostimulate the anammox, and iii) the characteristics of the N_2O emissions during the typical NOB suppression strategies. The overview of the thesis chapters and their relationship is shown in Figure – 1.4.

In **Chapter 2**, different biomass preservation strategies based on different temperatures and redox adjustment are evaluated. To fit the practical application as closely as possible, granular and floccular sludge harvested from two full-scale PN/A installations were preserved. After the preservation, the activity after reactivation was also tested in the consecutive batch tests. In the end, the cost assessment was carried out to propose the most cost-effective sludge preservation strategy.

In **Chapter 3**, a novel concept of bioaugmentation with stored summer sludge for winter anammox assistance was proposed. The cost-effective biomass storage strategy (proposed in Chapter 2) provided adequate sludge for this concept application. Through the bioaugmentation, the biomass concentration in the mainstream is increased which lead to the improvement of the nitrogen removal efficiency. A framework of mainstream partial nitritation/anammox in four seasons was fully described. This research presented a new strategy to facilitate the implementation of high-performance nitrogen removal throughout winter.

As with the bioaugmentation strategy, another commonly used strategy for pollutant removal efficiency improvement, i.e., biostimulation, is updated in this thesis. In **Chapter 4**, a return-sludge nursery concept was proposed to biostimulate AnAOB with a mainstream niche. In this system, not only biostimulation, but also bioaugmentation are achieved. During this study, the potential mechanism that contribute to the enhancing of anammox efficiency was revealed. The results provided another new idea on improving the nitrogen removal capacity of STPs under low-temperature conditions.

In **Chapter 5**, the characteristics of N_2O emissions linked to typical NOB suppression strategies were studied in a biofilm reactor. Three different NOB suppression approaches (i.e., low DO levels, FA and FNA treatment) have already been successfully applied, whereas characteristics of the N_2O emissions linked to these approaches were seldomly validated. The balance between NOB suppression and the related N_2O emissions will determine the strategy choice in the application.

In the end, a general discussion and outlook for further research is presented in **Chapter 6** based on the schematic overview of the thesis chapters. The potential application of the two newly proposed concepts (i.e., winter bioaugmentation with stored summer sludge and return-sludge nursery reactor biostimulation) were validated. At the same time, the fitness to combine NOB suppression strategized with these two concepts are also demonstrated.

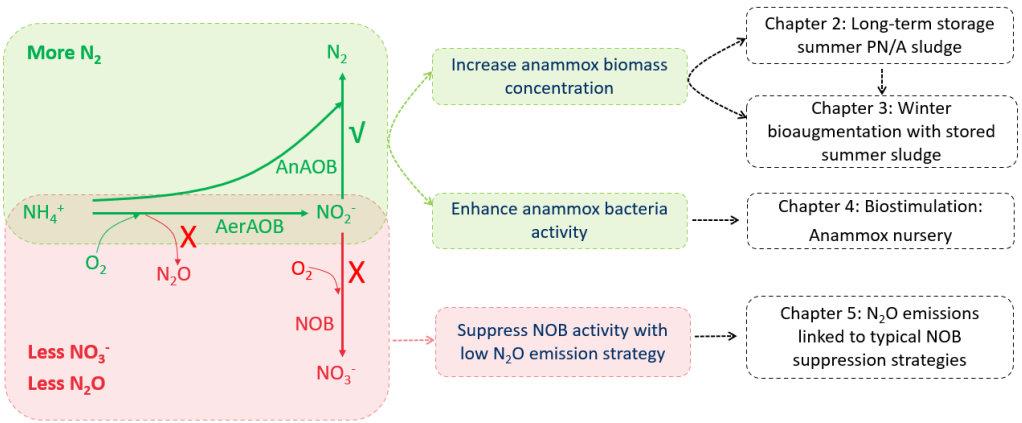


Figure – 1.4 Schematic overview of the thesis chapters. Processes that are desired to be enhanced are displayed in green and the processes that are desired to be suppressed in red.

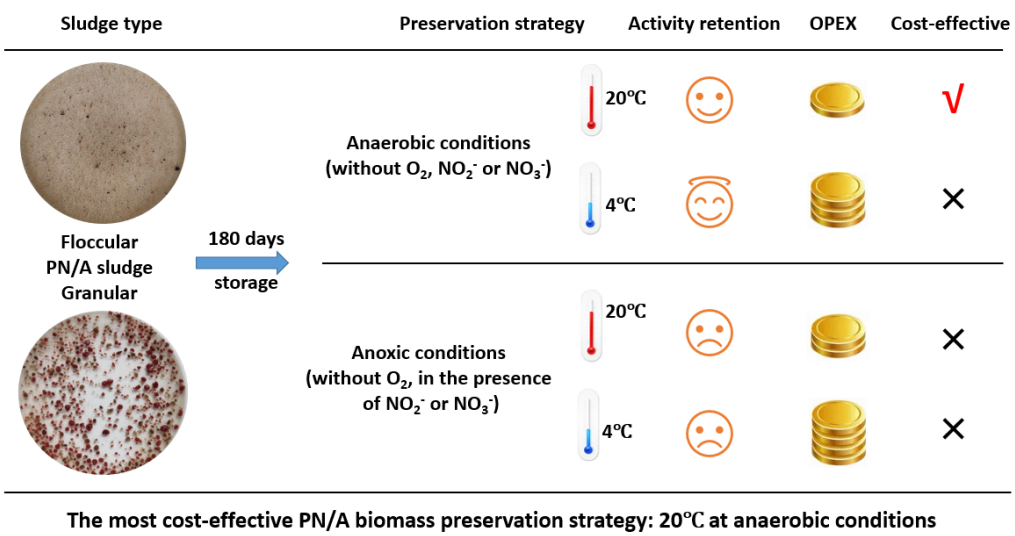
Chapter 2

Storage without nitrite or nitrate enables the long-term preservation of full-scale partial nitritation/anammox sludge

Redrafted from Zhu, W., Van Tendeloo, M., Xie, Y., Timmer, M.J., Peng, L., Vlaeminck, S.E, 2022. Storage without nitrite or nitrate enables the long-term preservation of full-scale partial nitritation/anammox sludge. *Science of the Total Environment*. 806, 151330.

Abstract

Bioaugmentation with summer harvested sludge during winter could compensate for bacterial activity loss but requires that sludge activity can be restored after storage. This study assesses the effect of temperature and redox adjustment during the storage over 180 days of partial nitritation/anammox (PN/A) granular resp. floccular sludge from potato processing resp. sludge reject water treatment. With nitrogen additives conditions (in the presence of nitrite or nitrate) resulted in a loss of 80–100% of the anammox bacteria (AnAOB) activity capacity at 20 °C and 4 °C, while no nitrogen additives conditions (without nitrite and nitrate) lost only 45–63%. Storage at 20 °C was more cost-effective compared to 4 °C, and this was confirmed in the sludge reactivation experiment (20 °C). Furthermore, AnAOB activity correlated negatively with the electrical conductivity level ($R2 > 0.85$, $p < 0.05$), so strong salinity increases should be avoided. No significant differences were found in the activity capacity of aerobic ammonia-oxidizing bacteria (AerAOB) under different storage conditions ($p > 0.1$). The relative abundance of dominant AnAOB (Candidatus *Brocadia*) and AerAOB genera (*Nitrosomonas*) remained constant in both sludges. In conclusion, preserving PN/A biomass without cooling and nitrite or nitrate addition proved to be a cost-effective strategy.



2.1 Introduction

Around 25 years after conceptually proposing the importance of anaerobic ammonia oxidation (anammox) bacteria (AnAOB) in energypositive sewage treatment (Mulder et al., 1995), a current hot topic in wastewater treatment is the implementation of so-called mainstream partial nitritation/anammox (PN/A) or deammonification. Due to the lower energy and carbon demand as well as the lower N_2O emission compared to conventional nitrification/denitrification, it is an economical and environmental-friendly process (Agrawal et al., 2018; Ali et al., 2016) which consists of aerobic ammonium-oxidizing bacteria (AerAOB), that oxidize roughly half of the NH_4^+ to NO_2^- ('partial nitritation') and AnAOB, that oxidize the produced NO_2^- and residual NH_4^+ to N_2 ('anammox') (Agrawal et al., 2018).

PN/A systems have been successfully implemented in the reject water line (sidestream) all over the world (Lackner et al., 2014). Compared to the sidestream, which has a higher temperature (15–20 °C higher) and nitrogen concentration (more than 20 times), achieving efficient nitrogen removal in the mainstream is much more challenging. Especially during the winter period, as the mainstream temperature could drop to about 10–12 °C in western Europe (20–22 °C in summer) according to the data in Nieuwveer sewage treatment plant (STP) (Breda, the Netherlands). This temperature decrease significantly reduces the activity of functional bacteria, especially for the AnAOB. Lotti et al. (2014) reported a growth rate of 0.02 d^{-1} at 20 °C and only 0.005 d^{-1} at 10 °C. As an extended sludge retention time in winter will not be sufficient because of the low growth rates, this problem may be tackled by storing excess PN/A sludge, mainly harvested over summer, and reinoculated this in winter.

Long-term preservation to maintain enough and activated sludge for at least 180 days is essential for the successful application of this novel concept. Different commonly used storage methods were deemed unfeasible for this bioaugmentation concept. Cryopreservation (−20 °C, −80 °C and −200 °C) with various cryoprotective agents (e.g., dimethyl sulfoxide) that prevent activity loss during sludge or culture collections storage has been extensively described (Rothrock et al., 2011; Viancelli et al., 2017), the cooling process and cryoprotective agents are expensive which limit its application at full-scale. Likewise, immobilizing technique (Ali et al., 2014), consortia transformation strategy (Shi et al., 2020), and protective agents addition (e.g., hydrazine, glycerol, skim milk) (Ganesan and Vadivelu, 2020; Rothrock et al., 2011; Vlaeminck et al., 2007) is also not feasible for full-scale application because of the operational complexity and high cost, even though many of them have proved useful.

In contrast, substrates or redox spikes are expected to be applicable for the bio-augmentation concept since they could well retain anammox activity and low operation cost. The addition of redox (nitrite or nitrate) during sludge preservation was investigated, as it may be effective in preventing sulfate reduction, which produces H_2S, harmful to biomass (Vlaeminck et al., 2007). Wang et al. (2016c) demonstrated that about 30% of AnAOB activity was maintained after 180 days of preservation at 35 °C with weekly supplying NH_4^+-N and NO_2^--N (50 mg N L^{-1} respectively). Ali et al. (2014) stored anammox sludge with 3 mM molybdate (inhibitor of sulfate reduction) and a regular supply of NH_4^+ and NO_2^- at room temperature and maintained 65% of the activity after five months of storage. Considering that both NH_4^+ and NO_2^- are substrates for AnAOB, periodical adding them could well avoid starvation.

Besides redox stabilization, the low temperature has also been proved beneficial for anammox sludge preservation. Vlaeminck et al. (2007) reported that 4 °C without nitrate storage addition was a recommended strategy for PN/A biofilm storage over five months (maintained 55% AnAOB activity). Xing et al. (2016) also revealed that anammox granules preserved at 4 °C without substrate addition had a lower decay rate and higher nitrogen removal capacity after storage compared to granules stored at 20 °C.

To evaluate the feasibility of the novel winter bioaugmentation concept, essential information about PN/A sludge storage and reactivation is currently still lacking. Firstly, various preservation methods have been applied in several studies, but there is no study comparing all these different preservation methods, which is essential for a fair comparison, as results can be influenced by the microbial communitycomposition and sludge types. Secondly, most studies only assess the preservation of anammox or nitrifying sludge, not PN/A sludge. Thirdly, room or lower temperature reactivation is important since it has more implementation potential for its lower required amount of energy (heat), whereas sludge reactivation has only been widely studied at the optimum temperature. The preserved sludge in previous studies could be reactivated at the optimum temperature (30–40 °C) from days to weeks (Ali et al., 2014; Viancelli et al., 2017).

The overall objective of this study is to find a cost-effective and simple operation strategy to store PN/A sludge on a large scale. Therefore, three parts were examined: i) the effect of temperature and redox stabilization over 180 days' storage of PN/A granules and flocs derived from full-scale STPs, ii) the potential of the stored biomass reactivation after 180 days, and iii) the OPEX (operating expenses or expenditure) and cost-effective analysis of different

preservation strategies. During the biomass storage, physicochemical characteristics and community composition are closely monitored. The findings will have good guiding significance for the storage of PN/A sludge on a large scale and provide the cost-effective biomass source for the concept of winter bioaugmentation with the stored summer sludge.

2.2 Materials and methods

2.2.1 PN/A sludge source and characterization

To verify if the proposed strategy in this study would be feasible and stable for different sludge types, floccular sludge (with a biomass concentration of 9.0 ± 0.2 g VSS L^{-1}) and granular sludge (13.6 ± 0.9 g VSS L^{-1}) were collected from full-scale sludge reject water (990 m^3, Breda, The Netherlands) and potato-processing wastewater (600 m^3, Olburgen, The Netherlands) PN/A installations, respectively. The initial average temperatures of the seed systems were about 32℃. The characteristics of both sludges are shown in Table – 2.1.

Table – 2.1 The initial characteristic of floccular and granular sludge

Sludge	Activity (mg NH_4^+-N g^{-1} VSS d^{-1}) (NOB: mg NO_3^--N g^{-1} VSS d^{-1})			Heme c (AU·L g^{-1} VSS)	Size – d (0.5) (μm) [a]	Color
	AnAOB	AerAOB	NOB			
Floccular	119.28	98.25	9.86	0.0575	57.60	Gray
Granular	78.83	18.80	12.24	0.3030	1237	Red

a, The particle size distribution of floccular and granular sludge was measured using a Mastersizer 2000 (Malvern Instruments, UK) and the software Image J (National Institutes of Health, USA).

2.2.2 Sludge storage procedure and strategies

The sludge was stored under different storage conditions for 180 days in 5 L plastic containers (0.4 L of headspace, with a small opening to avoid pressure build-up by gas (e.g., N_2 and H_2S) production). Four preservation strategies were tested (Table – 2.2): 1) no N spike, i.e., sludge storage under no nitrogen additives conditions (without redox buffer), 2) NO_2^- + NH_4^+ spikes (substrates for AnAOB), 3) NO_3^- + NH_4^+ spikes (The decay of biomass was accompanied by ammonium production. However, the produced ammonium could be consumed in the

presence of nitrate according to our pre-test. Thus, ammonium was added to ensure adequate supply in this strategy.), and 4) NO_3^- spikes (redox buffer to alleviate sulfate reduction). Two preservation strategies (i.e., 'no N spike' and 'NO_2^- + NH_4^+) were also additionally tested at 4 °C for floccular sludge. Only floccular sludge was chosen to test the strategy of 4 °C because the activity maintenance had already been proved in the previous study (Vlaeminck et al., 2007), and the high cooling cost was also not expected to be applied in the biomass storage on the large scale. The concentration of ammonium, nitrite, and nitrate was maintained between 30 and 180 mg N L^{-1} by regular addition of NH_4Cl, $NaNO_2$, and $NaNO_3$, respectively. The determination of NO_2^--N concentration was based on previous research (Talan et al., 2021). For NH_4^+-N and NO_3^--N, their concentrations were consistent with NO_2^--N since no inhibition occurred at the concentration lower than 200 mg N L^{-1} (Strous et al., 1999a; Zhu et al., 2017a). All storage experiments were performed in duplicate, and the mean values are reported.

During storage, the pH was controlled manually within 7.2–8.0 by 1 M HCl and NaOH addition. The dissolved oxygen (DO) concentration was lower than 0.01 mg O_2 L^{-1} in all the storage containers. The storage vessels were mixed five times per week.

2.2.3 Single and consecutive batch activity tests

2.2.3.1 Single batch tests: maximum activities

Activity batch tests were performed to determine the maximum activity of AnAOB every 30 days. After mixing, about 20 mL of sludge was harvested from each container and stored at 20 ± 1 °C for one day to make sure that all the batch tests were carried out at the same temperature. The sludge was washed four times and diluted to a biomass concentration of 1.0 g VSS L^{-1} with a buffer solution containing only $NaHCO_3$ (0.4 g L^{-1}) and trace elements (1 mL L^{-1}) (Van de Graaf et al., 1995). Ammonium and nitrite (50 mg N L^{-1}) were spiked in all Erlenmeyer flasks only at the beginning of the batch tests. The AerAOB and NOB activity was determined in flasks exposed to the air (by air stone), whereas the flasks to determine AnAOB activity were sealed with rubber stops after 15 min flushing with N_2 (to provide anoxic conditions). The flasks were incubated on a shaker (200 rpm) at 20 ± 1 °C, and pH was adapted to 7.5 (with HCl) at the start of the tests. The biomass concentration was only measured at the start of each test. DO and pH levels were also followed during the whole test. The DO concentrations for aerobic tests were higher than 7.0 mg O_2 L^{-1} and pH values were around 7.5. All the batch tests were

performed in duplicate, and the average values were reported. To monitor the nitrogen conversion, samples were taken periodically (every hour) and stored at 4 °C until analysis. The maximum specific anammox activity was determined from the maximum slope of the curve indicated by the decrease of NH_4^+-N over time, divided by the biomass concentration in the flask.

2.2.3.2 Consecutive batch tests: bacterial activity reactivation

After 180 days, consecutive batch tests (i.e., 20 spikes over 7 days) were performed to assess the activities' reactivation at 20℃. Sludge was pretreated and spiked every 12 h (after washing) to avoid substrate limitation or accumulation. The substrate conversion rate was quantified every other cycle (7 in total) by measuring the NH_4^+-N, NO_2^--N, and NO_3^--N concentration. The biomass concentration was only determined at the start of each test. The activity recovery percentage was defined as the maximum activity obtained in these seven days divided by the initial activities (before storage).

2.2.4 Analytical procedures

Liquid and microbial samples were taken periodically from the storage containers (mixed before sampling). In addition, during the batch tests, liquid samples were taken from the Erlenmeyer flasks regularly. After filtering by 0.2 μm syringe filter (CHROMAFIL Xtra PVDF) and storing at 4 °C, NH_4^+-N, NO_2^--N, and NO_3^--N were measured with a San^{++} Automated Wet Chemistry Analyzer (SKALAR, the Netherlands). H_2S in the liquid was measured using Spectroquant test kits (Merck, Germany). The biomass concentration was followed over time using volatile suspended solids (VSS) measurements (APHA, 2005). Handheld meters were used to monitor pH, electrical conductivity (EC), and DO concentration (Hach HQ30d, USA). The detailed information about extracellular polymeric substances (EPS) extraction and heme c measurement is shown in S – 2.1 (Supporting Information). The calculation of free nitrous acid (FNA) and free ammonia (FA) are shown in S – 2.2 (Supporting information). The V4 region of the 16S rRNA gene was sequenced for the following-up analysis by Novogene Europe (United Kingdom) after the genomic DNA was extracted using a Powerfecal kit (Qiagen, Germany) in the lab. The detailed method is presented in S – 2.3 of the Supporting Information.

Table – 2.2 Overview of the PN/A sludge storage strategies.

Sludge	Strategy	NO_3^--N	NO_2^--N (mg N L^{-1}) [a]	NH_4^+-N	Temperature (°C)	Sludge concentration (g VSS L^{-1})
Floccular Sludge	1) No N spike	-	-	-	20	8.98 ± 0.05
	1) No N spike	-	-	-	4	8.95 ± 0.21
	2) NO_2^-+NH_4^+	-	30-180	30-180	20	9.06 ± 0.13
	2) NO_2^-+NH_4^+	-	30-180	30-180	4	9.38 ± 0.02
	3) NO_3^-+NH_4^+	30-180	-	30-180	20	8.59 ± 0.29
	4) NO_3^-	30-180	-	-	20	8.64 ± 0.29
Granular sludge	1) No N spike	-	-	-	20	14.08 ± 0.21
	2) NO_2^-+NH_4^+	-	30-180	30-180	20	12.24 ± 0.23
	3) NO_3^-+NH_4^+	30-180	-	30-180	20	13.25 ± 0.07
	4) NO_3^-	30-180	-	-	20	13.86 ± 0.62

Note: a. The range was controlled during the storage, i.e., when the nitrogen concentration was decreased to 30 mg N L^{-1}, nitrogen will be added into the tank to achieve a concentration of 180 mg N L^{-1}.

2.2.5 Bacterial activity capacity calculation

During the sludge preservation process, AnAOB activity and biomass concentration will decrease. Since only specific AnAOB activities were measured in batch tests (monthly), volumetric removal rates of nitrogen were not available. Thus, an accurate parameter, the bacterial activity capacity (R_c) that combines these two factors (i.e., activity decrease and biomass decay), is proposed.

$$R_c = r_b * C_x * V_t \qquad (Eq - 2.1)$$

R_c is the bacterial activity capacity [mg NH_4^+-N d^{-1}] for AerAOB/AnAOB and [mg NO_3^--N d^{-1}] for NOB; r_b is the maximum bacterial (AnAOB, AerAOB, and NOB) activity [mg NH_4^+-N g^{-1} VSS d^{-1}] for AerAOB/AnAOB and [mg NO_3^--N g^{-1} VSS d^{-1}] for NOB; C_x is the biomass concentration [g VSS L^{-1}]; and V_t is the sludge volume [L].

The decay rate (d^{-1}) of bacterial activity capacity depends on activity decay (decrease in sludge-specific activity) and cell death (decrease in biomass concentration), and is calculated according to Hao et al. (2009). The bacterial decay rates are calculated through the exponential plots. The exponent of the fitted curve (four significant digits) is considered as the corresponding decay rate.

2.2.6 Cost assessment

As far as the authors know, this is the first research to assess the cost-effectiveness during the PN/A sludge preservation process. Room temperature is assumed to be constant at 20 °C, thus only the 4 °C conditions need to consume electricity to cool down. During the whole biomass preservation period, the main OPEX would be nitrogen compounds addition (NH_4Cl, $NaNO_2$, and $NaNO_3$), pH control (HCl and NaOH), and potential cooling (20 °C → 4 °C). Mixing cost is negligible compared to the other operational factors (< 0.5% of the total cost).

OPEX per specific AnAOB activity (€ (kg NH_4^+-N $d^{-1})^{-1}$) is put forward to analyze the cost-effectiveness of different biomass preservation strategies. Its calculation process is the whole OPEX (over 180 days, € ton VSS^{-1}) divided by the specific AnAOB activity at Day 180 (kg NH_4^+- N ton VSS^{-1} d^{-1}). The strategy which has the lowest value is regarded as the most cost-effective preservation method.

2.3 Results and discussion

2.3.1 No nitrogen additives conditions maintained higher bacterial activity capacity than nitrogen additives conditions

2.3.1.1 AnAOB activity capacity

Over time, the AnAOB activity capacity decreased for both sludge types and for all storage conditions due to a strong decrease in AnAOB activity and a mild decrease in biomass concentration (Figure – 2.1).

Considering only the temperature variable, sludge stored at 4 °C outperformed sludge stored at 20 °C in terms of AnAOB activity capacity retention. The AnAOB activity capacity of the 'no N spike' at 20 °C and 4 °C was respectively 1.95 and 2.98 kg NH_4^+-N d^{-1} on Day 180 for the floccular sludge. For the 'NO_2^- + NH_4^+' storage method, this difference was even more pronounced: 0.05 and 1.15 kg NH_4^+-N d^{-1} at 20 °C and 4 °C, respectively. According to Adav et al. (2007) and Gao et al. (2012), the endogenous respiration and cell lysis process that microorganisms use to sustain activity during storage decelerates at low storage temperatures, which could explain the lower drop in biomass concentration. Taking the 'no N spike' as an example, only 7.5% of biomass concentration loss was observed at 4 °C compared to 30.7% loss at 20 °C in floccular sludge. Although there was little variation between activity preservation (59.47% at 4 °C versus 52.36% at 20 °C), 'no N spike (4 °C)' still maintained much higher activity capacity than that at 20 °C.

For the storage strategies at 20 °C, sludge stored without any nitrogen addition ('no N spike (20 °C)') retained a higher AnAOB activity capacity (37–49%) compared to sludge with redox (nitrite or nitrate) adjustment (0–20%) in both floccular and granular sludges. This is contrary to many previous studies, stating that, at 20 °C, NO_3^--N addition is the best storage strategy (Engelbrecht et al., 2016; Vlaeminck et al., 2007). Firstly, pH control under no nitrogen additives conditions in the present research might benefit the AnAOB activity capacity maintenance. The sludge consumed alkalinity during biomass decay and starvation, which led to a pH decrease (Anjali and Sabumon, 2014). The low pH values could increase FNA concentration (Figure – S2.1) that probably inhibited AnAOB activity (Strous et al., 1999b). Secondly, the glycogen (one of the intracellular polymers that microbial store in cells when there was an adequate supply of substrates) might serve only as a maintenance energy source for microorganisms (Ma and

Wang, 2018). However, it possibly also could be used by the dissimilatory nitrate/nitrite reduction to ammonium process (as electron donor) to generate energy under nitrogen additives condition stress, which increased the decay rate of biomass. That could promote it to have much better resistance, tolerance, and self-adaptation to starvation.

Specific for 'NO_2^- + NH_4^+ (20 °C), the addition of substrates during storage resulted in the loss of almost all AnAOB activity over time, even though AnAOB preferentially oxidizes both ammonium and nitrite into N_2 if present (Ganesan and Vadivelu, 2020). On one hand, only nitrogen compounds were provided during the preservation process while their metabolic activity requires many other elements (e.g., inorganic carbon, trace elements, etc.) (Ma et al., 2015; Van de Graaf et al., 1995). With nitrogen as the only substrate, the starvation of microbes possibly is more serious than that without nitrogen addition (Lu et al., 2018). On the other hand, the EC levels increased rapidly (e.g., 17.53 mS cm^{-1} in 'NO_2^- + NH_4^+ (20 °C)' versus 5.09 mS cm^{-1} in 'no N spike (20 °C)' on Day 180 for floccular sludge), which was also harmful to AnAOB (Section 3.4). Additionally, the higher nitrite concentration range (30–180 mg N L^{-1}) compared to previous studies (e.g., 50–70 mg N L^{-1} (Ali et al., 2014; Wang et al., 2016c)) was probably another reason since the free nitrous acid (FNA) concentration (0-0.18 mg FNA-N L^{-1}) was higher than the reported IC_{50} (50% activity inhibition concentration, 11 µg HNO_2^--N L^{-1}) (Fernández et al., 2012). However, 'NO_2^- + NH_4^+' was still unlikely suitable for biomass preservation even at the concentration of 50 mg N L^{-1} as studied by Ganesan and Vadivelu (2020).

In the present research, NO_3^--N was supplied as a redox buffer to prevent sulfate reduction. During the whole preservation period, no NO_2^--N accumulation was observed. Low temperature (4 °C) and NO_2^--N could also play the same role to suppress sulfate reduction (Vlaeminck et al., 2007). A higher H_2S concentration after 180 days of preservation was detected in the 'no N spike (20 °C)' compared to the other conditions (i.e., 'no N spike (4 °C)' and nitrogen additives conditions) for both floccular (0.23 versus < 0.19 mg S L^{-1}) and granular (0.08 versus < 0.06 mg S L^{-1}) sludge (Figure – 2.2). This confirmed the inhibition of sulfate reduction under low temperature conditions or in the presence of NO_3^--N and NO_2^--N. However, this H_2S concentration probably did not affect the AnAOB activity according to previous research (Jin et al., 2013b).

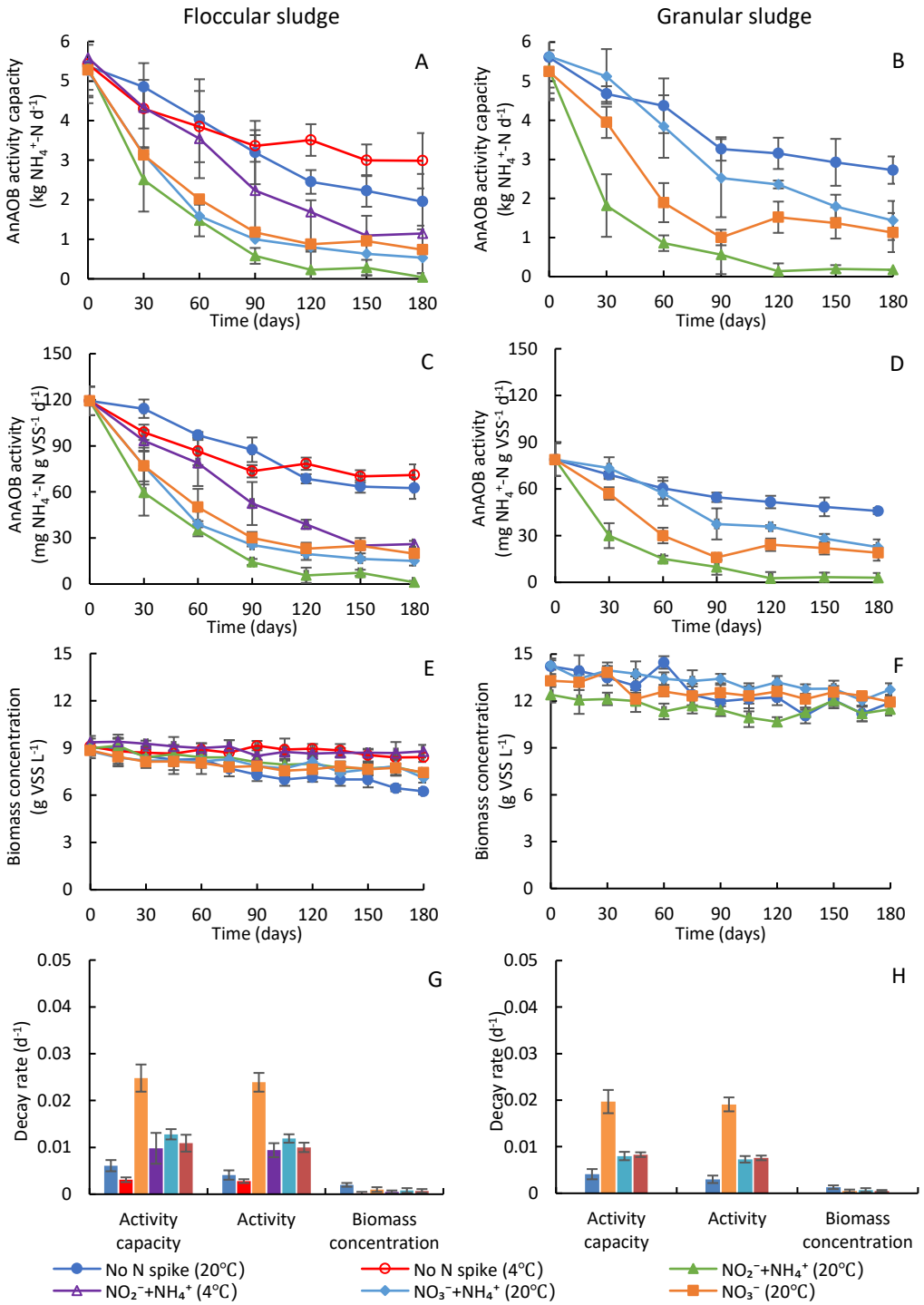


Figure – 2.1 Change of AnAOB activity capacity (A, B), AnAOB activity (C, D), biomass concentration (E, F), and the relevant decay rates (G, H) over 180 days. A, C, E, G: floccular sludge; B, D, F, H: granular sludge.

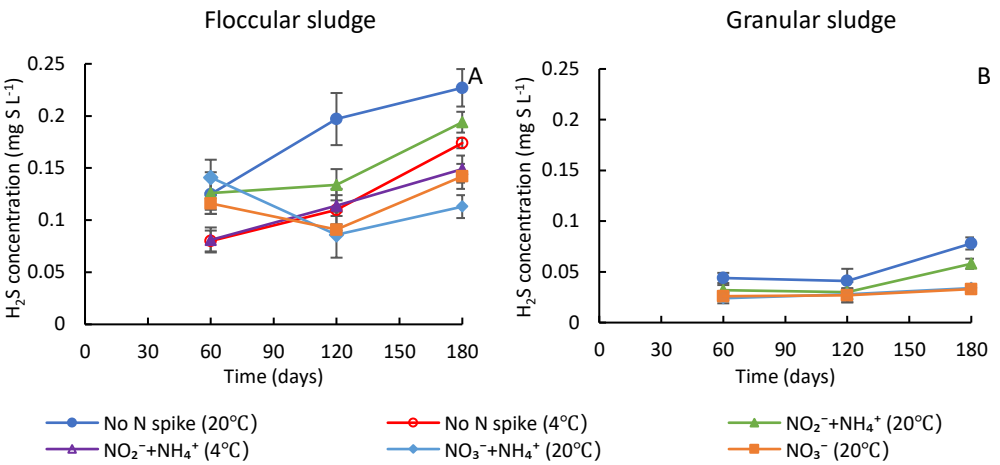


Figure – 2.2 Change of H_2S concentration (in water phase) over time. A: Floccular sludge; B: Granular sludge

In short, for both sludge types, the AnAOB activity decay rates under no nitrogen additives conditions (i.e., 'no N spike') (0.0028–0.0041 d^{-1}) were more than twice as low compared to under nitrogen additives conditions (i.e., 'NO_2^- + NH_4^+', 'NO_3^- + NH_4^+', and 'NO_3^-') (0.0094–0.0239 d^{-1}). The results were in line with Ma and Wang (2018), who got an AnAOB activity decay rate of 0.0049 d^{-1} in no nitrogen additives conditions and 0.0129 d^{-1} in nitrogen additives conditions after 60 days of storage. The biomass decay was much less critical than activity decay during the sludge storage under different storage strategies (3–20 times lower, Figure – 2.1G/H).

2.3.1.2 AerAOB and NOB activity capacity

The floccular sludge stored at 4 °C ('no N spike (4 °C)' and 'NO_2^- + NH_4^+ (4 °C)') maintained the highest AerAOB activity capacity (75% and 66% of the initial value, respectively) after 180 days of storage (Figure – 2.3). Floccular sludge stored at 20 °C without N spike ('No N spike (20 °C)') or with nitrate (and ammonium) spikes ('NO_3^- + NH_4^+ (20 °C)' and 'NO_3^- (20 °C)') also retained more than 50% of the AerAOB activity capacity. Sludge spiked with nitrite and ammonium ('NO_2^- + NH_4^+ (20 °C)'), in contrast, lost more than 70% of its activity capacity. Similarly, in the granular sludge, about ~65% of the AerAOB activity capacity could be retained in all conditions, except for 'NO_2^- + NH_4^+ (20 °C)' (28%).

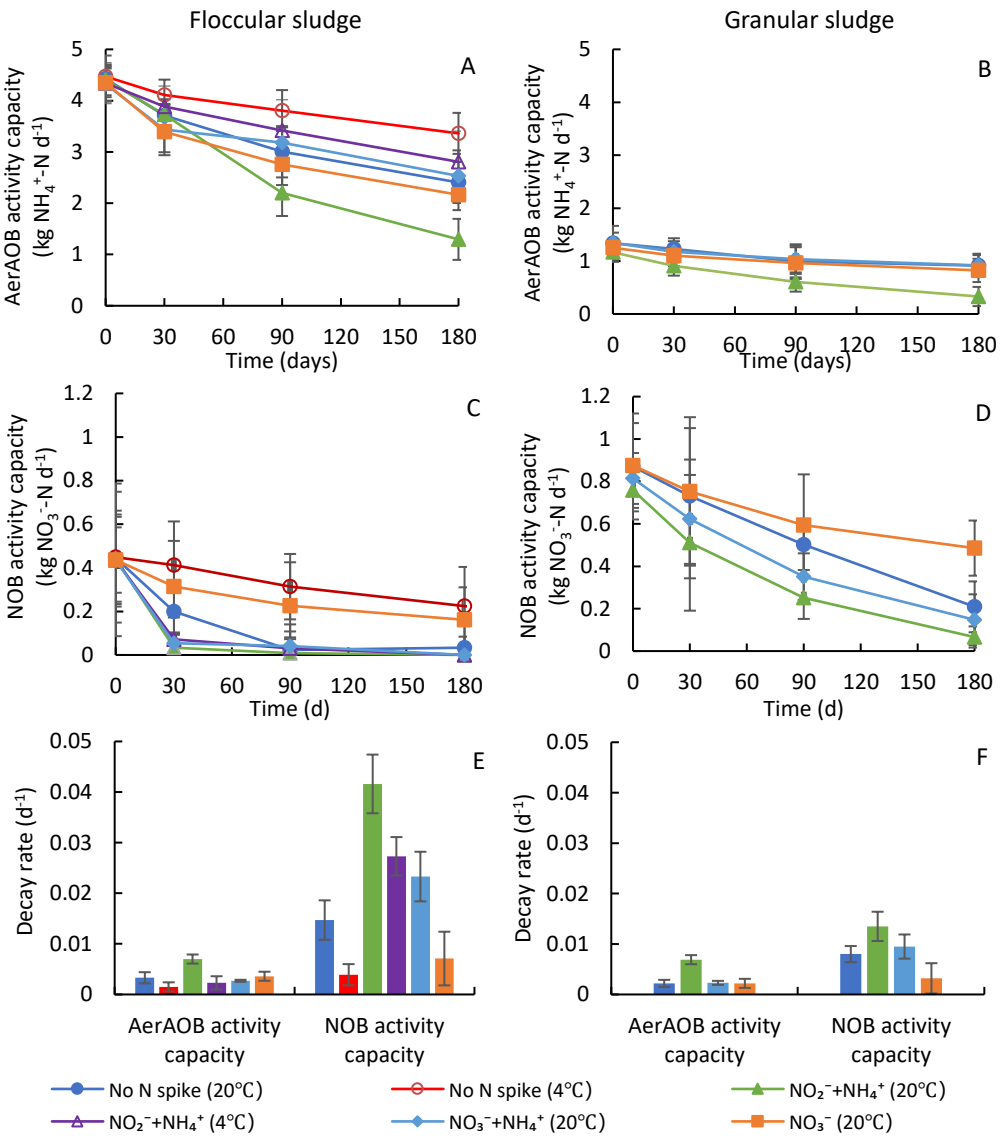


Figure – 2.3 Change of AerAOB activity capacity (A, B), NOB activity capacity (C, D), and their decay rates (E, F) under different storage conditions. A, C, E: floccular sludge; B, D, F: granular sludge.

The difference in AerAOB activity capacity between the different storage strategies was similar to that on AnAOB activity capacity retention, which might attribute to the change of EC levels, FNA and/or FA concentrations, and nitrite concentration range mentioned above (Section 2.3.1.1). Specific for low temperature, a high AerAOB activity capacity could be retained at 4 °C (3.36 and 2.81 kg NH_4^+-N d^{-1} for 'no N spike (4 °C)' and 'NO_2^- + NH_4^+ (4 °C)'

compared to that 2.40 and 1.30 kg NH_4^+-N d^{-1} at 20 °C), which was confirmed by Gao et al. (2012) who reported that 4 °C was significantly better to store aerobic granules compared to room temperature.

NOB was more sensitive to starvation compared to AerAOB. A very low NOB activity capacity was obtained after 180 days of storage except for the 'no N spike (4 °C)' (floccular sludge) and 'NO_3^- (20 °C)' (floccular and granular sludge) treatments. 'NO_2^- + NH_4^+ (20 °C)' had the lowest NOB activity capacity, which was followed by 'NO_2^- + NH_4^+ (4 °C)' and 'NO_3^- + NH_4^+ (20 °C)' for both floccular and granular sludge. The high FA concentration (0–4 mg L^{-1}, Figure – S2.1) in the storage vessels might lead to the low NOB activity capacity maintenance. According to Vadivelu et al. (2007), a concentration of 1–6 mg FA-N L^{-1} eliminated the NOB in PN/A sludge during reactor treatment. That was also proven by the higher NOB decay rate compared to AerAOB at identical preservation conditions (e.g., 0.003 d^{-1} versus 0.015 d^{-1} in 'no N spike (20 °C)' of floccular sludge). NOB suppression during storage will be beneficial for PN/A application.

The AerAOB had the lowest decay rates compared to AnAOB and NOB. That was likely cause AerAOB promoted cellular adaptation to starvation by stabilizing intracellular macromolecular levels (Ma et al., 2017b). Apart from that, three physiological advantages might also have attributed to this better survival (Geets et al., 2006): i) AerAOB has stable catabolic cellular components (e.g., energy-generating enzymes), which corresponded to their fast reactivation (Section 2.3.2) ii) AerAOB has a lower maintenance-energy demand than other bacteria, and iii) signaling pathways involved in starvation survival would be initiated in AerAOB during storage.

2.3.2 Both AnAOB and AerAOB of no nitrogen additives stored sludge showed best reactivation

2.3.2.1 AnAOB activity reactivation

The AnAOB activity increased during the first 3–4 days of reactivation but decreased afterward (Figure – 2.4). This decline can be attributed to the biomass loss during the reactivation in the flasks (the medium was replaced every day).

The preserved sludge under no nitrogen additives conditions (i.e., 'no N spike') could recover faster (activity doubled after 2–3 days) compared to the sludge stored under nitrogen additives conditions and showed a higher recovery percentage (87–114% versus 6–50%) at 20 °C (Figure – 2.4E/F). Without substrate being supplied during preservation (i.e., 'no N spike'), AnAOB

might still maintain activity through the internal endogenous metabolism and intercellular substrates from biomass decay by inducing starvation proteins (especially enzymes related to the PN/A process) (Ma and Wang, 2018). In addition, the nitrogen additives storage conditions changed rapidly (pH adjustment and nitrate or nitrite addition periodically, EC increased gradually) could probably harm AnAOB, which consequently affected its reactivation. Ma et al. (2017b) demonstrated that AnAOB sludge starved under no nitrogen additives conditions (for 40 h), almost completely recovered within 6 h whereas sludge stored in nitrogen additives conditions could only be reactivated for 34%.

The recovery percentage of 'NO_3^- + NH_4^+ (20 °C)' was higher than 'NO_2^- + NH_4^+ (20 °C)', which might also attribute to the toxicity of high EC levels. In addition, even the 'NO_2^- + NH_4^+ (20 °C)' supplied substrates for AnAOB, the relatively higher nitrite concentration possibly toxic to AnAOB (Carvajal-Arroyo et al., 2014). Whereas the partial denitrification probably occurred in 'NO_3^- + NH_4^+ (20 °C)' based on the COD released by biomass decay. That process could produce a low concentration of nitrite for AnAOB.

Even though the preserved biomass at both 4 °C and 20 °C could be reactivated, 4 °C conditions had a higher recovery percentage than 20 °C (114% versus 99% in 'no N spike' and 17% versus 6% in 'NO_2^- + NH_4^+' for floccular sludge). That was in line with the performance during the biomass storage for which 4 °C could maintain higher AnAOB activity after 180 days of preservation.

Sludge reactivation is essential for the full-scale application of stored sludge in the concept of 'winter bioaugmentation with stored summer sludge'. Mainstream inoculation and high sludge retention time (SRT) control may be sufficient to achieve AnAOB reactivation, which is in order of days (i.e., 3–4 days), while the SRT is in order of weeks (i.e., ~4 weeks, based on the original reactors of both sludges). So, there is a good chance that a dedicated reactivation tank would not be needed in practice. Even if a reactivation reactor would be needed, its volume would be limited as several batches of sludge can be reactivated sequentially, which can save space. Additionally, compared to the present research, the reactivation temperature in the full-scale application was closer to the cultivation temperature (30℃ versus 20℃), which also might benefit the sludge activity recovery (less temperature difference between two systems) (Head and Oleszkiewicz, 2004).

Thirdly, room or lower temperature reactivation is important since it has more implementation potential for its lower required amount of energy (heat), whereas sludge

reactivation has only been widely studied at the optimum temperature. The preserved sludge in previous studies could be reactivated at the optimum temperature (30–40 °C) from days to weeks (Ali et al., 2014; Viancelli et al., 2017).

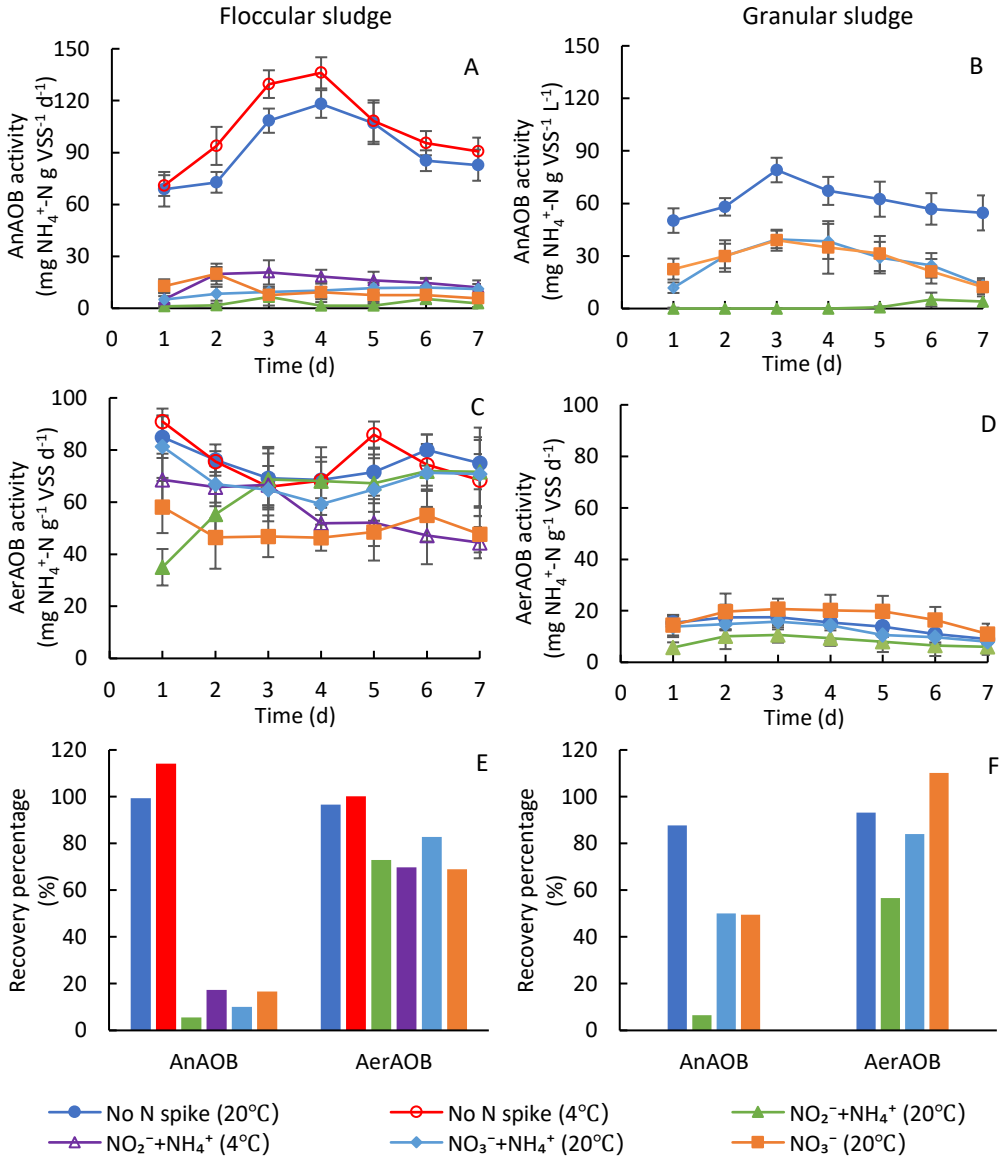


Figure – 2.4 AnAOB activity (A, B) and AerAOB activity (C, D) reactivation after 180 days of storage in consecutive batch activity tests, and the recovery percentage (E, F). A, C, E: floccular sludge; B, D, F: granular sludge. Column represents the recovery percentage, and the color corresponds to the one in the legend.

2.3.2.2 AerAOB activity reactivation

Even though sufficient AerAOB activity in principle can be obtained through inoculation of activated sludge from other conventional sewage treatment plants, the AerAOB preservation and reactivation together with AnAOB could still have some benefits. However, this is causing the activated sludge inoculation would introduce high levels of potential NOB activity, potentially connecting the NOB to AerAOB rather than the AerAOB to AnAOB, adding extra competition for nitrite. NOB suppression remains the main challenge for mainstream PN/A application (Peng et al., 2020), and the yearly introduction of new NOB could hamper the overall performance by disturbing the microbial balance.

AerAOB had an overall higher activity recovery percentage than AnAOB at 20 °C (56–110% vs. 5–114%) (Figure – 2.4E/F). Except for the sludge stored in 'no N spike' which recovered 93–100% of activity, more than 57% of activity was reactivated for the other sludges. For the recovery of AerAOB activity, that sensitivity difference compared to AnAOB could be explained by the 10-times higher growth rate of AerAOB (0.04 h^{-1} for AerAOB and 0.003 h^{-1} for AnAOB at 32–33 °C (Jetten et al., 2001)). Similar to the present research, Gao et al. (2012) reported that AerAOB activity could be fully restored within ten days after eight months of storage from almost 0 for all different storage conditions. The present research results indicated that even when AerAOB was stored together with AnAOB (PN/A sludge), they were still easy to be reactivated.

2.3.3 Dominant genera retained their relative abundance over the storage period

Since ammonium was always present due to biomass decay, the sludge stored with only nitrate spikes (i.e., 'NO_3^- (20 °C)') resembled the conditions in 'NO_3^- + NH_4^+ (20 °C)', which resulted in a similar bacterial activity capacity. Therefore, only the samples from the 'NO_3^- + NH_4^+ (20 °C)' strategy were analyzed.

The Shannon index decreased over time in the floccular sludge (e.g., from 7.125 on day 0 to 5.498 on day 180 in 'no N spike (20 °C)'), meaning that the diversity of the communities decreased during storage (Table – S2.1). The DO levels during the whole experiments were lower than 0.01 mg O_2 L^{-1}, causing some species related to aerobic processes to be eliminated as they might not have adapted to the new conditions. In addition, a salinity condition was created due to a gradual increase of the EC value during preservation (Section 2.3.4), which

possibly also affected the diversity according to the finding of He et al. (2019). That was also the possible explanation for the higher diversity at 4 °C (lower EC levels) than 20 °C. The index exhibited opposite results in granular sludge (e.g., from 6.096 on day 0 to 6.777 on day 180) (Table – S2.1). In both sludges, 'NO_2^- + NH_4^+ (20 °C) groups had the lowest diversity, which probably indicated that the substrates addition strategy was not suitable for biomass preservation at the community level.

The microbial community composition at phylum level is shown in Figure – S2.2A/B. There were twenty main microbial phyla (relative abundance ≥ 0.1% in at least one sample) detected in all samples, Planctomycetes, Proteobacteria, Bacteroidetes, and Chloroflexi among them accounted for more than 87% and 83% in floccular and granular sludge, respectively. For floccular sludge, the relative abundance of Planctomycetes (the phylum to which AnAOB belong) (14.7–38.2%), decreased under all different strategies except for the 4 °C groups and 'NO_3^- + NH_4^+ (20 °C)', suggesting that low temperature or nitrate addition could help AnAOB to tolerate starvation to some extent. Proteobacteria (27.1–40.8%), containing nitrite reductase genes (*nir*) and possibly almost all AerAOB (Wu et al., 2020), showed a trend of decrease under all the conditions. Contrary to that, Chloroflexi (2.2–7.0%) which are facultative anaerobes that widely existing in autotrophic systems (Chen et al., 2016), increased in relative abundance with increasing preservation time. Different from floccular sludge, granular sludge had a rather constant relative abundance of Planctomycetes (~30%) and Proteobacteria (~22%). According to Wang et al. (2018a), this might be due to the greater resistance of the microbial community of the granules to external change compared to the flocs. In addition, the heterotrophic bacteria, Firmicutes (2.4–5.1%) their relative abundance remained rather unchanged for the different groups and sludge types.

The taxonomic results of the dominant nitrogen removal-related bacteria on genus level are shown in Figure – S2.2C/D. The common AnAOB genera, Candidatus *Brocadia*, Candidatus *Kuenenia*, and Candidatus *Jettenia*, were identified in both floccular and granular sludge. Representative microbial genera were also found for AerAOB (*Nitrosomonas*) and NOB (*Nitrospira* and Candidatus *Nitrotoga*). Candidatus *Brocadia* (belonging to the Planctomycetes) dominated the microbial community in both floccular and granular sludge over the 180 days preservation with a relative abundance of 15% and 32%, respectively. For the floccular sludge, the relative abundance of Candidatus *Brocadia* increased (up to 20–30%), whereas it was stable for granular sludge (25–31%). The characteristics of floccular and granular (different particle

size distribution) probably caused this difference. Ma and Wang (2018) also reported a stable relative abundance of AnAOB in granular sludge (dominated by Candidatus *Kuenenia*). The relative abundance of *Nitrosomonas*, the only identified AerAOB, was stable around 2–6% and 1–2% in the floccular and granular sludge, respectively. The lower relative abundance in the granular sludge corresponded with the lower AerAOB activity capacity (Section 2.3.1.2). A low relative abundance of representative NOB genera (*Nitrospira* < 0.6% and Candidatus *Nitrotoga* < 0.1%) was found in both sludge types. *Denitratisoma*, another nitrogen removal related genus following the denitrifying route, was abundant in all different storage conditions (7–14% and 3–6% in floccular and granular sludge, respectively), which could convert NO_2^--N or NO_3^--N (redox in the present study) to N_2 (Vlaeminck et al., 2007). It was likely one of the reasons for the reduction of redox. Moreover, according to Kartal et al. (2007), AnAOB could reduce NO_3^--N or NO_2^--N to NH_4^+-N through dissimilatory reduction. That is probably another possible route for redox removal, but that was not proved in this study.

In the present research, the granular sludge had higher AnAOB abundance, but their specific activity was lower. That is probably attributed to the kinetic limitation and morphology, since the diffusion efficiency of the substrates may limit the actual nitrogen removal rate. If the middle of the granular has anammox cell, the availability of substrates to them is lower.

2.3.4 Properties of sludges during preservation

2.3.4.1 EC level increased and negatively correlated with AnAOB activity

The EC value (Figure – S2.3) increased over time for all storage conditions due to biomass decay (releasing ions like PO_4^{3-}, NH_4^+, etc.), pH control (i.e., HCl or NaOH addition), and addition of chemicals (i.e., NH_4Cl, $NaNO_2$, or $NaNO_3$ addition). Under no nitrogen additives storage conditions, it only slightly increased over 180 days as no chemicals were added. In contrast, the EC value of the sludge stored in nitrogen additives conditions rapidly increased, especially for 'NO_2^- + NH_4^+ (20 °C)' probably due to the redox buffer and acid addition. There are two possible reasons to redox was consumed: i) the existence of denitrifiers (Section 2.3.3) and ii) the occurrence of dissimilatory reduction (Kartal et al., 2007). That could also explain the lower EC levels of the sludge stored at 4 °C compared to 20 °C. The levels in nitrogen additives conditions were close to or even higher than the maximum inhibition value (IC_{50}, 14.6 mS cm^{-1} (Lin et al., 2020)) after 180 days of storage. Besides, salinity suppression might be more serious on starved

biomass compared to fresh biomass. A negative correlation between EC values and AnAOB activity (R^2 = 0.8513, $p < 0.05$ for floccular sludge and R^2 = 0.8863, $p < 0.05$ for granular sludge) was found (Figure – 5A/B).

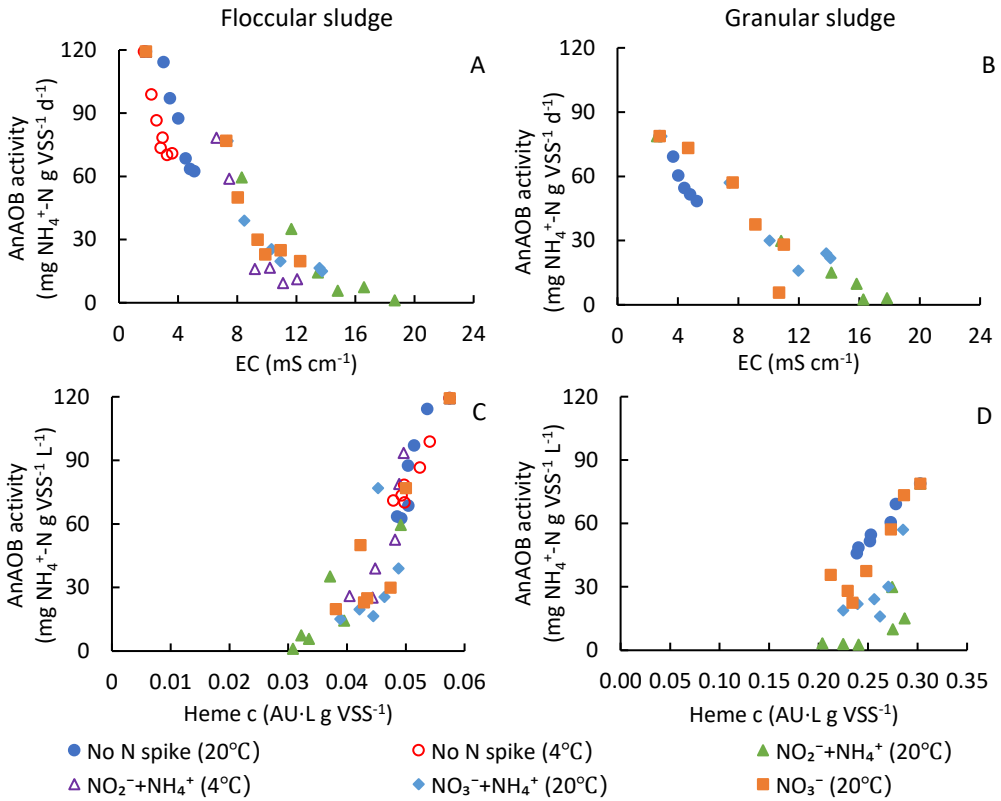


Figure – 2.5 The correlation between EC values/heme c and the AnAOB activity over the 180 days biomass preservation. A, C: floccular sludge; B, D: granular sludge.

2.3.4.2 Heme c decreased

Proteins containing heme c are commonly referred to as cytochrome c. Unlike other heme derivatives, heme c is covalently bound to the protein backbone and plays an important role in electron transfer and electron storage (Kleingardner and Bren, 2015). Most cytochromes act as electron carriers and cycle between the reduced (ferrous) and oxidized (ferric) oxidation states to perform single electron transfer (Bowman and Bren, 2008). Normally, heme c was regarded as a good indicator of anammox performance evaluation (Kartal and Keltjens, 2016; Ma et al., 2019). Heme c concentration gradually decreased during the sludge preservation (Figure – S2.4). Even there was a correlation between AnAOB activity and the heme c for both floccular (R^2 =

0.8286) and granular (R^2 = 0.4703) sludge, they were not significant (p > 0.1, analyzed by one-way ANOVA). Although a significant positive correlation has been proven during reactor operation (Ma et al., 2019), this rule was likely not applicable in the period of biomass preservation. Ma and Wang (2018) reported that heme c remained stable during the anammox starvation period, although AnAOB activity gradually decreased. That difference might attribute to the variance in sludge type (anammox versus PN/A sludge) and dominant genera (Candidatus *Kuenenia* versus Candidatus *Brocadia*).

2.3.4.3 EPS decreased

Regardless of the storage strategy, the EPS content in floccular and granular sludge gradually decreased (Figure – S2.5). That was in line with Zhang and Bishop (2003) who demonstrated that EPS could be secreted from cells to serve as the energy source for the functional bacteria when suffering from starvation. Gao et al. (2012) also found a significant reduction of EPS in aerobic granular after storage. According to Figure – S2.5, the low temperature probably reduced the EPS hydrolysis rate, whereas the redox adjustment did not affect that.

2.3.4.4 Morphology

The preserved sludge surface was gradually covered by a black layer in 'no N Spike (20℃)', indicating sulfate reduction to H_2S, which was also indicated by the smell of rotten eggs. The black color disappeared and turned reddish within 2 - 3 days during the reactivation process which coincided with the increased AnAOB activity. Sludge stored at 4℃ or with redox stabilization had a similar color change that lighter than 'no N spike (20℃)'. It is still unclear where the methane formed.

2.3.5 No nitrogen additives and without cooling conditions is a cost-effective sludge storage strategy

An OPEX analysis (Figure – 2.6) demonstrated that the sludge stored under 'no N spike (20 °C)' has the lowest operational expense. Only 104 € ton VSS^{-1} and 22 € ton VSS^{-1} are needed when storing floccular and granular sludge over 180 days, respectively, which is considerably lower than the storage costs in the continuous presence of redox buffers (at least around 340 € ton VSS^{-1}). For 'no N spike (20 °C)', pH control (base addition) is the main expense. Next to this,

there are no additional costs (e.g., reagents and cooling, etc.). The storage condition which could maintain the highest anammox activity, 'no N spike (4 °C)', has the lowest cost in pH control, whereas the cooling price is extremely high (752 € ton VSS^{-1}). This leads to the total cost of 'no N spike (4 °C)' being more than 20 times higher than 'no N spike (20 °C)'. Considering the higher residual anammox activity after storage, 'no N spike (4 °C)' is suitable for storing a small amount of sludge in the lab.

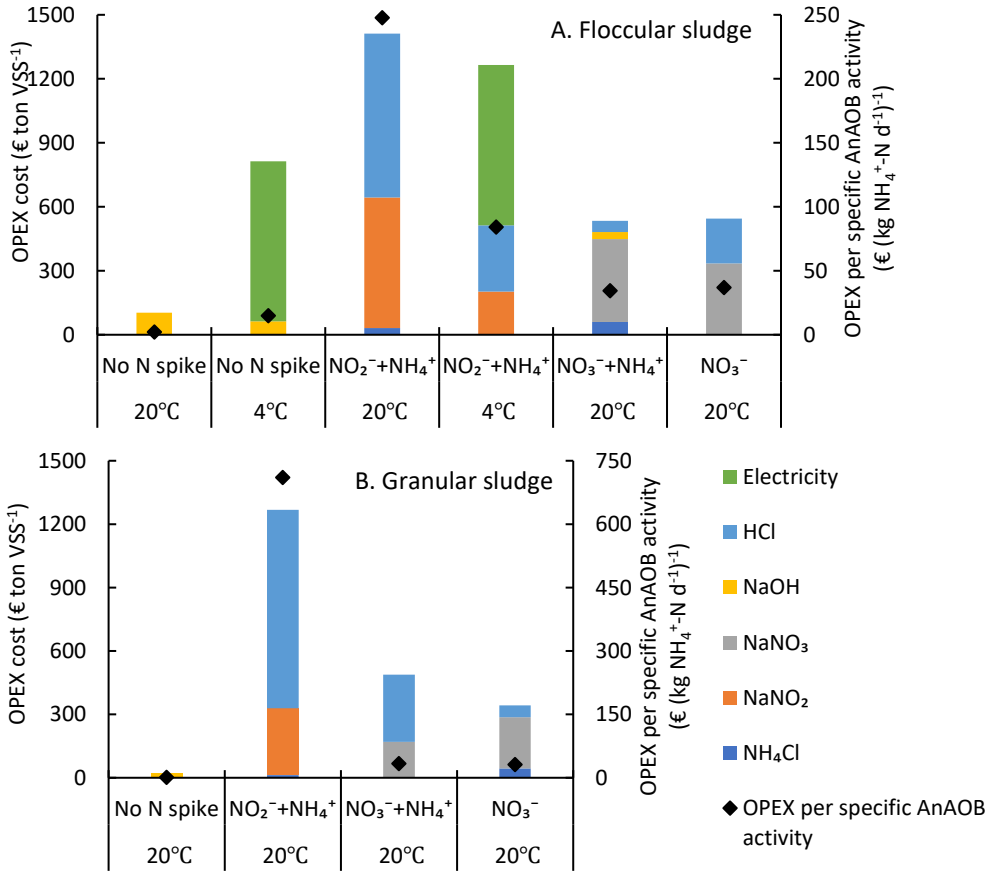


Figure – 2.6 OPEX and cost-effectiveness analysis for 180 days of storage. Note: (1) Electricity price was according to the price level of Antwerp in 2021, i.e., 0.1 € kW^{-1} h^{-1}; (2) The price of the chemicals was based on Alibaba.com (2020-01-30); (3) Electricity consumption for mixing, pH dosage, etc. was not included since they were negligible (i.e., < 0.5%).

The cost of pH control (i.e., acid addition) during the sludge storage is higher when applying nitrogen additives conditions compared to no nitrogen additives conditions. This may be due to the occurrence of denitrification or denitritation, gradually consuming H^+ and increasing pH. In addition, the redox adjustment is another major expense. Even though 'NO_3^- + NH_4^+ (20 °C)'

costs less for pH control than 'NO_3^- (20 °C)', it could consume more redox buffer due to the possible occurrence of both anammox and denitrification. Both strategies have a much lower cost than 'NO_2^- + NH_4^+ (20 °C)', but are still at least 5 times higher than that of 'no N spike (20 °C)'.

The results of the OPEX per specific AnAOB activity (cost-effective analysis) reveal that only 2.3 and 0.6 (€ (kg NH_4^+-N $d^{-1})^{-1}$) are needed in the 'no N spike (20 °C)' storage strategy for the floccular and granular sludge. That is more than 15 times lower than the nitrogen additives storage strategies. 'no N spike (4 °C)' is the second best option even though it needs extremely high cooling costs. That proves the no nitrogen additives condition is more cost-effective for PN/A sludge storage than nitrogen additives ones, especially for the large scale.

2.3.6 Research application and prospect

The findings on the anammox-related parameters (e.g., AnAOB activity) are generic and can be extrapolated to any other sludge. While biomass decay is specific to the sludge tested (e.g., composition and proportion of functional microbes) and should not be extrapolated directly, the dominant genus in this study is Candidatus *Brocadia*, a typical anammox genus that is also present in many other studied sludges (Ali et al., 2014; Oshiki et al., 2011). For another common AnAOB genus, Candidatus *Kuenenia*, similar findings to the present research were also found by other researchers (Ma and Wang, 2018; Ma et al., 2017b). Thus, the conclusions proposed in the present research are most likely also applicable for other PN/A sludges with different dominant genera. Additionally, for the effect of proportions of functional microbes on the biomass decay, convergence is also expected between different PN/A sludge since most of the microbes in PN/A sludge are heterotrophs (85–90% in the mainstream versus 67–84% in this study) (Henze et al., 2000; Lotti et al., 2015c; Strous et al., 1998).

According to the results of this study, the 4 °C without N spike ('no N spike (4 °C)') storage strategy was the most effective in maintaining the highest biomass activity capacity after long-term storage, which was in line with the finding of Xing et al. (2016). This strategy is recommended for preserving small amounts of sludge in the laboratory, but it is too costly for large-scale applications. For the winter bioaugmentation concept in the STPs, the 20 °C without N spike ('no N spike (20 °C)') strategy deserves to be chosen, since it is the most cost-effective storage strategy.

Before the full-scale application, there are still several aspects that need to be studied. Firstly, whether the activity of stored sludge can be reactivated quickly at low temperature (<15°C) as it is essential for the winter bioaugmentation application (stored sludge directly bioaugmented into the mainstream reactor). Secondly, the effect of EC value on the AnAOB activity during the preservation should be tested separately (e.g., testing the osmotic effect with NaCl). The addition of Na^+ (or Cl^-) probably also affects the AnAOB sludge during storage. The extra control following the same EC profile should be set, with a gradual dose of NaCl over time to avoid the salt shock. Thirdly, whether the 'NO_2^- + NH_4^+' is suitable for biomass preservation in the presence of other elements that the metabolic activity requires. Finally, a pilot-scale system should also be established to verify the stability of the concept. Using the preservation strategy in large-scale sludge remains a challenge.

2.4 Conclusions

No nitrogen additives storage conditions resulted in a higher preservation of AnAOB activity capacity (37–55%) than nitrogen additives conditions during storage for 180 days. Despite the higher activity capacity retention at 4 °C, 'no N spike (20 °C)' is recommended to preserve sludge on a large scale, since it has the lowest value in OPEX per specific AnAOB activity (i.e., OPEX/anammox activity on Day 180, 2.3 and 0.6 (€ (kg NH_4^+-N $d^{-1})^{-1}$) for floccular and granular sludge, respectively) making it the most cost-effective alternative. Successful biomass reactivation (20 °C) and maintaining the predominant AnAOB genus (Candidatus *Brocadia*) furthermore strengthen the potential of this approach. An exponential negative correlation between the electrical conductivity and the AnAOB activity was also found, so strong salinity increases should be avoided.

Acknowledgements

Dr. Jolien De Paepe, Dr. Wannes Van Beeck and Dr. Jef Wens from the University of Antwerp, are appreciated for their assistance on manuscript revision and numerous discussions. Dr. Willie Driessen from Paques Technology B.V. (Balk, the Netherlands) and Harry Vrins from STP Nieuwveer (Breda, the Netherlands) are acknowledged for granular and floccular sludge harvesting.

Supporting Information

S – 2.1 Extraction of extracellular polymeric substances (EPS) and measurement procedure of heme c

Extraction of extracellular polymeric substances (EPS): EPS fractions were extracted using a heat extraction method modified after Van Winckel et al. (2019a) and Li and Yang (2007). The pellet was resuspended using 25% ringer solution (pH = 7.0) which was pre-heated at 60℃ after the sludge was centrifuged for 5 min at 4000 g, and the supernatant recovered. After vortexing for 1 min at 60 ℃ and removing the supernatant, the left pellet was resuspended one more time using 25% ringer solution (pH = 7.0) which was pre-heated at 60℃ after the sludge was centrifuged for 10 min at 4000 g. After vortexing for 1 min at 60 ℃, the pellet was subsequently used to extract the EPS fraction with a 30 min incubation at 60 ℃ and centrifugation for 15 min at 4000 g. The extraction was standardized on 25 mg TSS (calculated according to the biomass concentration in liquid samples). EPS fractions were filtered through a 0.2 µm syringe filter (CHROMAFIL Xtra PVDF) and stored at -20℃. The EPS fractions were analyzed for chemical oxygen demand (COD). Ringer solution (g L^{-1}): NaCl, 9; KCl, 0.42; $CaCl_2$, 0.48; $NaHCO_3$, 0.20.

Measurement procedure of heme c: Heme c, regarded as a proxy for AnAOB activity (Kartal and Keltjens 2016), is determined according to Berry and Trumpower (1987) and Sinclair et al. (1999). Centrifuge 10 mL of a representative sample of mixed liquor at 10000 r/min for 5 min. Make sure the biomass concentration is around 2.5 g VSS/L. Besides, add a blank at this period. Then discard the supernatant and fill it up with NaOH (1 mol/L) back to 10 mL. Add dry sodium dithionite to give a concentration of 20 g/L, then mix or shake to resuspend the pellet and dissolve the dithionite. Using a water bath to heat these samples to 70 ℃ and keep the temperature for 10 min. Mix occasionally (3 – 4 times) during this period. After that, cool down the samples at room temperature for 10 min. Mix occasionally (3 – 4 times) during this period, too. In the end, samples were measured extinctions in clear solutions at 535, 550, and 570 nm using the 3-wavelength method of the photometer after centrifuging (1000 r/min for 5 min). The ratio of absorbance to biomass concentration is regarded as heme c level in this work. The unit is (AU·L)/g VSS.

S – 2.2 Calculation of free nitrous acid (FNA, Eq-S2.1) and free ammonia (FA, Eq-S2.2) (Anthonisen et al. 1976).

$$\mathrm{FNA} = \frac{17}{14} \times \frac{\mathrm{C}_{\mathrm{NH}_4^+ - \mathrm{N}} \times 10^{\mathrm{pH}}}{\left[\exp\left(\frac{6334}{273 + \mathrm{T}}\right) + 10^{\mathrm{pH}}\right]} \qquad (\mathrm{Eq} - \mathrm{S2.1})$$

$$\mathrm{FA} = \frac{47}{14} \times \frac{\mathrm{C}_{\mathrm{NO}_2^- - \mathrm{N}}}{\left[\exp\left(\frac{-2300}{273 + \mathrm{T}}\right) \times 10^{\mathrm{pH}}\right] + 1} \qquad (\mathrm{Eq} - \mathrm{S2.2})$$

where $C_{NH_4^+ - N}$ is the concentration of total ammonium [mg NH_4^+-N L^{-1}]; $C_{NO_2^- - N}$ is the concentration of total nitrite [mg NO_2^--N L^{-1}]; T is the temperature of the liquid sample [°C].

S – 2.3 Microbiome analysis

To test the microbial community shift during the sludge storage period, the V4 region of the 16S rRNA gene was used after the genomic DNA was extracted using Powerfecal kit (Qiagen, Germany) following the manufacturer's instructions. The DNA concentrations were measured by Qbit3 fluorimeter (Thermofisher Scientific, United States). The extracted DNA samples were stored at -20℃ until shipped to Novogene Europe (United Kingdom) for amplicon sequencing. After amplifying by polymerase chain reaction (PCR) using the set of forwarding V4 forward 515f (GTGCCAGCMGCCGCGGTAA) and reverse 806r (GGACTACHVGGGTWTCTAAT) primers. Their libraries were pooled and sequenced paired end in a MiSeqbenchtop sequencer (Illumina). The sequencing reads were clustered contigs into operational taxonomic units (OTUs) at 97% sequences similarity. The follow-up analysis using Qiime software (V1.7.0) was performed by Novogene Europe (United Kingdom). The detailed method was based on Alloul et al. (2021).

Alpha-diversity indices were also calculated by Qiime software (V1.7.0) based on the OTUs results to determine the Shannon index, and a higher value represents a richer microbial community diversity.

Figure – S2.1 FNA and FA concentration during storage (the highest FA or FNA concentration in the strategies which are not shown here were close to 0). A, C: Floccular sludge; B, D: Granular sludge.

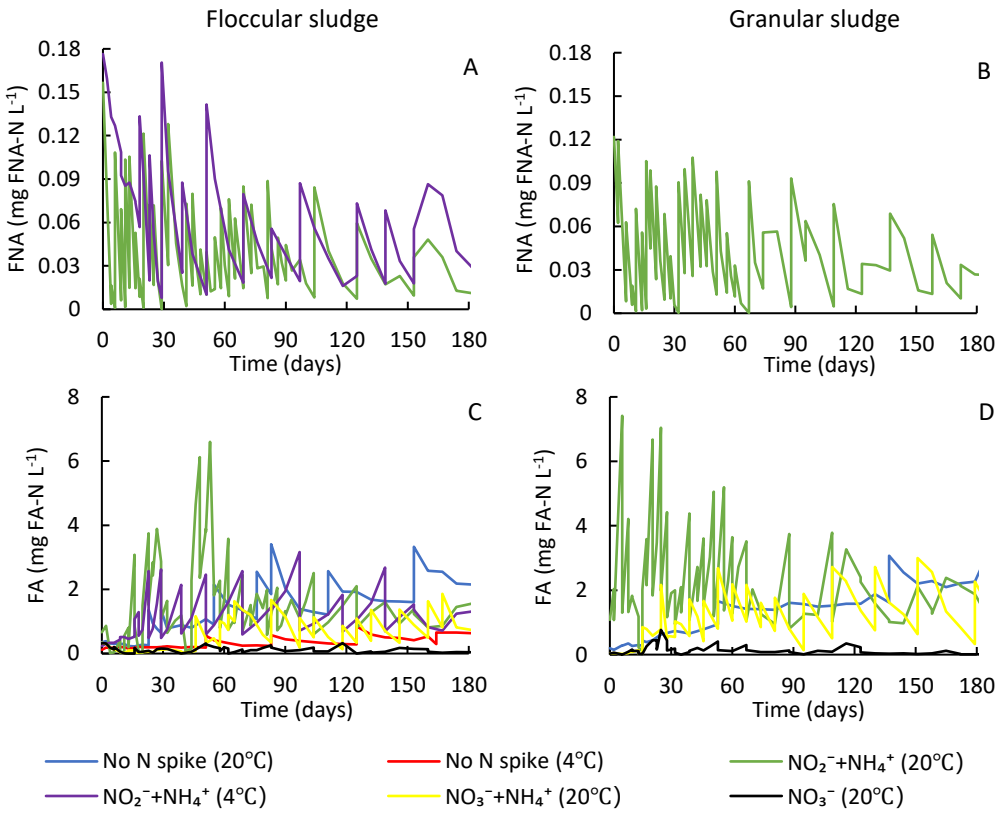


Figure – S2.2 The microbial community during long-term PN/A sludge storage at phylum (A, B) and genus (C, D) levels, expressed relatively over the total community. A, C: Floccular sludge; B, D: Granular sludge. At the genus level, only the dominant nitrogen removal related bacteria (AnAOB (orange), AerAOB (green), NOB (blue), and denitrifying bacteria (purple)) were shown.

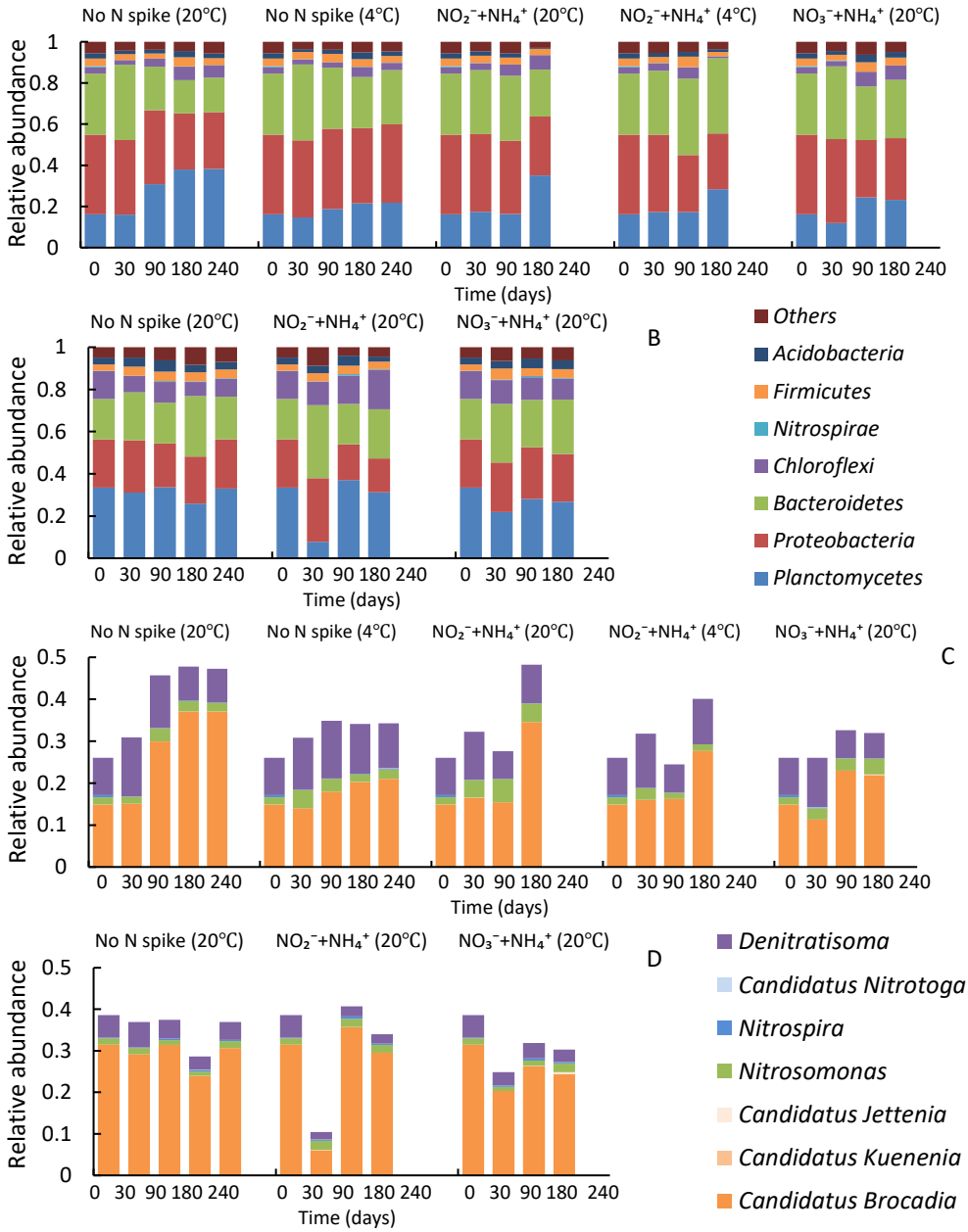


Table – S2.1 Shannon index over time under different storage conditions.

Sludge type	Strategy	Shannon index					Trend
		Day 0	Day 30	Day 90	Day 180	Day 240	
Floccular sludge	No N spike (20℃)	7.125	5.984	5.873	5.852	5.498	
	No N spike (4℃)	7.125	6.462	5.959	6.586	5.959	
	NO_2^-+NH_4^+ (20℃)	7.125	6.645	6.138	4.392	-	
	NO_2^-+NH_4^+ (4℃)	7.125	6.341	6.071	4.681	-	
	NO_3^-+NH_4^+ (20℃)	7.125	6.345	6.445	6.365	-	
Granular sludge	No N spike (20℃)	6.096	6.281	6.269	6.854	6.777	
	NO_2^-+NH_4^+ (20℃)	6.096	7.189	5.659	5.653	-	
	NO_3^-+NH_4^+ (20℃)	6.096	6.733	6.695	6.706	-	

Figure – S2.3 Change of EC value level over 180 days' preservation. A, Floccular sludge; B, Granular sludge.

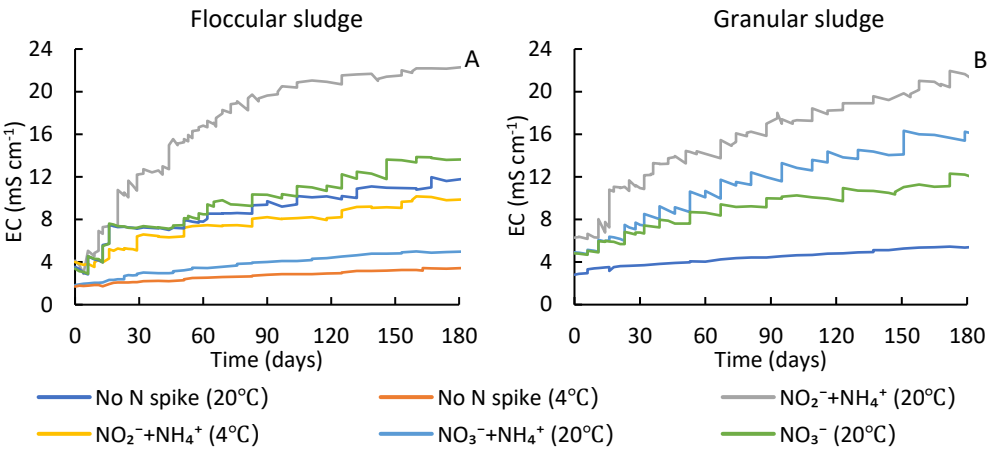


Figure – S2.4 Change of heme c level over 180 days' preservation. A, Floccular sludge; B, D: Granular sludge.

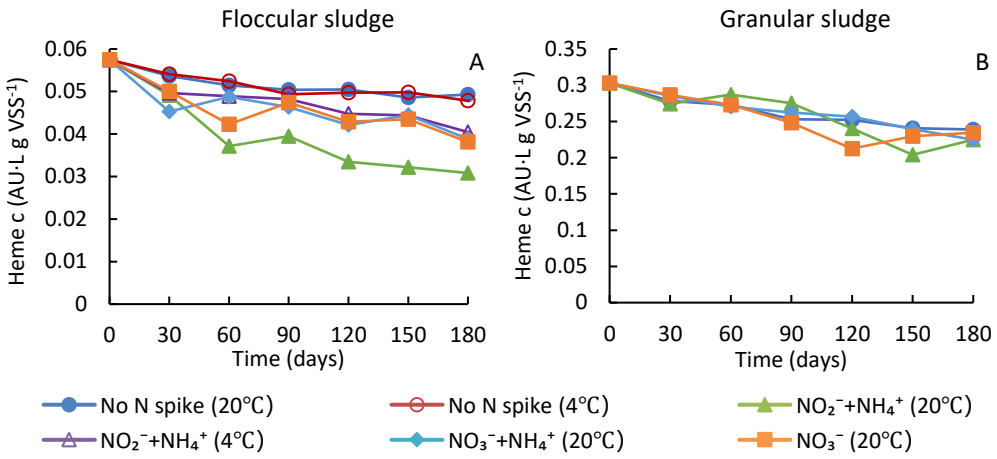
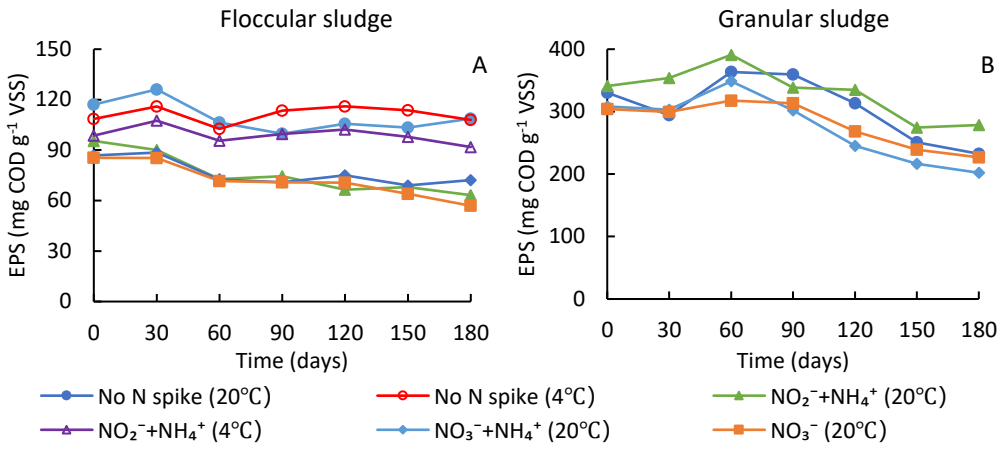


Figure – S2.5 The variation of EPS content over time. A: Floccular sludge; B: Granular sludge



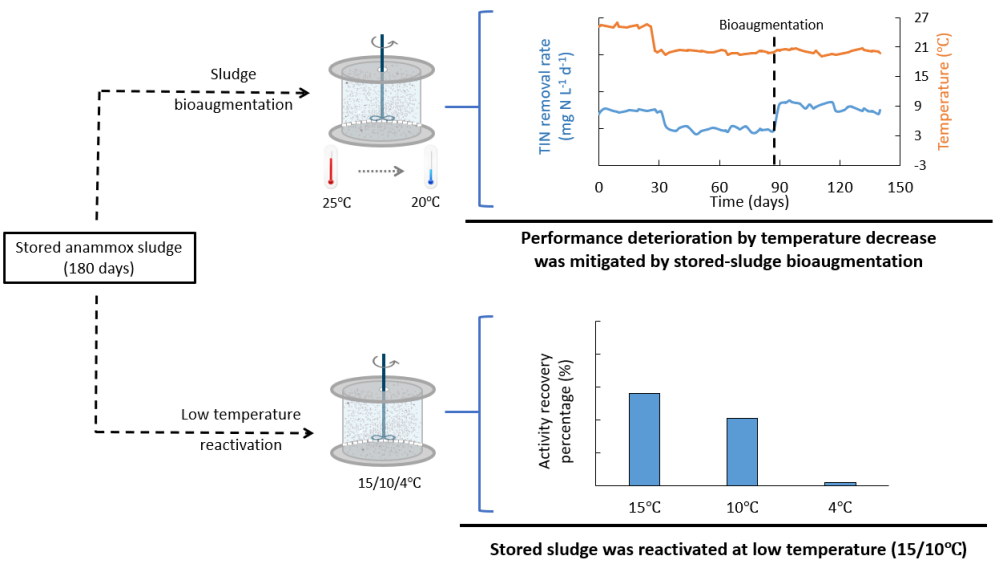
Chapter 3

Towards mainstream partial nitritation/anammox in four seasons: Feasibility of bioaugmentation with stored summer sludge for winter anammox assistance

Redrafted from Zhu, W., Van Tendeloo, M., Alloul, A., Vlaeminck, S.E. Towards mainstream partial nitritation/anammox in four seasons: Feasibility of bioaugmentation with stored summer sludge for winter anammox assistance. *Bioresource Technology*. Revised.

Abstract

The strong effect of low temperatures on anammox bacteria challenges its application over the winter in temperate climates. Winter bioaugmentation with stored summer surplus sludge is a potential solution to guarantee sufficient nitrogen removal in winter. Firstly, the systems for which nitrogen removal deteriorated by the temperature decrease (25℃→20℃) could be fully restored with 118–220% stored sludges (6-month) bioaugmentation. Secondly, the reactivation of these stored sludges was also tested in a lower temperature system (15/10°C). Compared to the activity before storage, between 56% - 41% of the activity of granular sludge was restored within one month, and 41% - 32% for floccular sludge. Additionally, 85–87% of granules and 50–53% of flocs were retained in the systems. After reactivation (15/10°C), a more specialized community was formed (diversity decreased) with Candidatus *Brocadia* still dominant in terms of relative abundance. Capital and operating expenditures (CAPEX, OPEX) were neglectable compared to sewage treatment costs (0.19-0.36%).



3.1 Introduction

Partial nitritation/anammox (PN/A) is a commonly applied wastewater treatment technology for ammonium removal in the sidestream (i.e., sludge line) (Lackner et al., 2014). It is based on the conversion of ammonium to nitrogen gas through the activity of aerobic and anoxic ammonium-oxidizing bacteria (AerAOB and AnAOB). For mainstream (i.e., water line) applications, PN/A can reduce the COD-supplementation requirements, oxygen demand, and excess sludge production by respectively 100%, 60%, and 80% compared to traditional nitrification/denitrification (Cao et al., 2017).

In temperate climate regions, the temperature of the mainstream fluctuates between 5 – 20 ℃ (Hendrickx et al., 2014). Specifically, in winter, temperatures can be below 10℃, which affects the microbial activity. The effluent quality of the PN/A reactor is sensitive to low temperatures due to the low growth rates of AnAOB (the maximal specific growth rates are 0.02 d^{-1} at 20℃ and only 0.005 d^{-1} at 10℃ (Lotti et al., 2014c)), leading to effluent quality deterioration in winter. Low temperatures, therefore, remain a major challenge for applying PN/A in sewage treatment plants (STP).

Low temperatures in winter reduce the activity of AnAOB. Current STP uses several operational strategies to mitigate the reduced activity such as sludge retention times (SRT) and hydraulic retention times (HRT) adjustment (Guo et al., 2013; Ha et al., 2010). Prolonging the HRT is an effective strategy yet requires larger reactor volumes. Merely extending the sludge retention time (SRT) in winter will not be sufficient as it requires a roughly 2.5 times increase in SRT (low AnAOB growth rate). The problem might be solved by bioaugmentation. That strategy has been applied in the nitrification/denitrification process to mitigate the suppression of low temperature (Head and Oleszkiewicz, 2004; Figdore et al., 2018). For AnAOB, bioaugmentation was already explored as a remedy to recover system performance from inhibition or shock (e.g., oxytetracycline and high COD concentration) (Jin et al., 2014; Tang et al., 2014). To overcome low-temperature effects on the AnAOB system by bioaugmentation, only the sidestream sludge was used. However, the temperature differences (> 30℃ in the sidestream versus < 15℃ in the mainstream) between the two systems would strongly affect the activity of the bioaugmented biomass (Head and Oleszkiewicz, 2004). Biomass loss from the sidestream reactor would also be harmful to the STP as it contributes to 15-25% of the total ammonium removal. Podmirseg et al. (2010), for example, showed that bioaugmentation influenced the microbial community composition of both systems (sidestream and

mainstream). Bioaugmentation would promote the relative abundance of the expected genera (e.g., Candidatus *Brocadia*) (Chen et al., 2015; Figdore et al., 2018). Although the reactor performance improves after bioaugmentation (Chen et al., 2015), the positive effects are only maintained for a short period (Patureau et al. 2001). This might be attributed to competition, inhibition, predation, or the presence of bacteriophages caused by the addition of exogenous microorganisms (Herrero and Stuckey, 2015; van Veen et al., 1997).

Stored anammox biomass was applied to start up the bioreactor or to restore the biological process after a disturbance or inhibition (Wenjie et al., 2014). The 'anammox bioaugmentation of stored AnAOB biomass to relieve the effect of low temperature' concept, to the authors' knowledge, has not been explored thus far. The reactivation of stored sludge at low temperatures is critical as the stored sludge is directly exposed to low temperatures during winter bioaugmentation. To verify that, the long-term reactivation of stored biomass in bioreactors at low temperatures, which has also never been tested before, was carried out in the present research.

The overall objective of this research was to provide evidence for AnAOB bioaugmentation as a remedy for temperature drops on winter days. To achieve this, three parts were examined: i) validation of the stored biomass bioaugmentation mitigation concept at a moderate temperature difference (25 to 20°C), ii) the effects of different reactor temperatures (15, 10, and 4°C) on the stored summer sludge's activity recovery, biomass retention, and community shift, iii) concept evaluation through a cost estimation (capital (CAPEX) and operating (OPEX) expenditures) based on the data from Nieuwveer STP (Breda, the Netherlands) and compared to normal operation. The findings present a new strategy to facilitate the implementation of high-performance nitrogen removal through winter.

3.2 Materials and methods

3.2.1 Characteristics of the preserved AnAOB sludge

Sidestream sludge was used as inoculum for the experiments because it is a good proxy for mainstream sludge due to species (e.g., the AnAOB, AerAOB, and NOB which were dominated by Candidatus *Brocadia*, *Nitrosomonas*, and *Nitrospira,* respectively) (Laureni et al., 2016) and composition similarities (e.g., heterotrophs dominated 67-84% of the total community in the present research and 80-90% in mainstream studies) (Lotti et al., 2015b; Yang et al., 2018). Both

floccular (harvested from the 990 m^3 sidestream PN/A installation in Breda (the Netherlands) with the particle size 0 – 0.45 mm) and granular (harvested from the 600 m^3 potato-processing wastewater line in Olburgen (the Netherlands) with the particle size 0.2 – 3.0 mm) are exploited. AnAOB sludges were dominated by Candidatus *Brocadia*, and their initial AnAOB activity was 119.28 ± 6.11 and 78.83 ± 5.79 mg NH_4^+-N g^{-1} VSS d^{-1} at 20 ℃, respectively for floccular and granular sludges. The initial average temperatures of the seed systems were about 32℃. The sludges were stored for 6-month at cost-effective conditions: without cooling and no nitrogen additives conditions (without nitrate and nitrite)) (Zhu et al., 2022).

Before bioaugmentation, the sludge was washed four times with a buffer solution which consisted of tap water spiked with $NaHCO_3$ (0.4 g L^{-1}) and trace elements solution A/B (1 ml L^{-1}) to remove the COD, phosphorus, and ammonium produced by biomass decay during sludge preservation. The composition of trace element solution A/B is based on Van de Graaf et al. (1995).

3.2.2 Setups for validation of the stored biomass bioaugmentation mitigation concept

The stored biomass bioaugmentation mitigation concept is the use of sludge stored from the reactor that is bioaugmented when the reactor (the same reactor as the sludge harvested) temperature is decreased, thus restoring the nitrogen removal performance to the state before the temperature drops.

Two cylindrical sequencing batch reactors (SBR), inoculated with either floccular (R1) or granular (R2) sludge, were operated for 140 days. The working volume of the reactors was 2.25 L (total volume of 2.5 L). The targeted nitrogen loading rate and volume exchange ratio were 320 mg N L^{-1} d^{-1} and 33%, respectively, leading to a cycle time of 2 h and an HRT of 6 h. Each cycle included 80 minutes of continuous feeding and reaction, 30 minutes of settling, 6 minutes of decantation, and 4 minutes of idle time. The sequencing batch mode was achieved through timers (EverFlourish EMT757-F, Germany) which controlled influent/effluent pumps (SEKO R1/R7, United States) and overhead stirrers (200 rpm) (Velp Scientific ES, Italy). There was no sensor control during the experiments.

The overall experiment is divided into three stages. During stage – I (Days 1 – 27), the reactors were operated at 25.2 ± 0.3℃. During stage – II (Days 28 – 62 for R1; Days 28 – 86 for R2), the temperature was decreased to 20.1 ± 0.4℃ to get a steady state. During stage – III (Days 63 – 140 for R1; Days 87 – 140 for R2), bioaugmentation with stored summer AnAOB biomass (two

times for R1 and one time for R2), aiming to offset the performance deterioration caused by temperature decrease in stage – II.

The initial biomass concentrations were 0.98 ± 0.07 and 1.28 ± 0.10 g VSS L^{-1} for R1 (floccular sludge) and R2 (granular sludge), respectively. After each bioaugmentation, the biomass concentration in the reactors increased by a percentage of about 100%. Steady state was achieved before each new bioaugmentation. The SRT was not controlled but monitored by measuring volatile suspended solid (VSS) concentration in the effluent during the whole test.

3.2.3 Stored-sludge low-temperature reactivation

Stored-sludge reactivation contains two main parts: AnAOB activity recovery and the bioaugmented biomass retention in the system. Multiple cylindrical SBRs, with a working volume of 1.2 L (volume exchange ratio is 33%), were operated for about 35 days to reactivate the stored sludge under different temperatures (No biological replicates were included, yet variation was accounted for by sampling at different time points 30-35 days in a row). The cycle composition, reactor operation, and targeted nitrogen loading rate were the same as R1 and R2. After preserving for six months, the stored floccular and granular sludge (same to Section – 2.2) were bioaugmented into SBRs which operated at 15.3 ± 0.4 °C, 10.4 ± 0.4 ℃, and 3.9 ± 0.15 ℃, respectively. Their initial biomass concentrations were 2.3 ± 0.3, 4.0 ± 0.6, and 6.4 ± 0.2 g VSS L^{-1}, respectively. The higher biomass concentration was chosen at a lower temperature since that could improve the tolerance and resilience of biomass to some content (Jin et al., 2013a).

3.2.4 Reactors operation and synthetic wastewater composition

Except the NH_4^+-N and NO_2^--N, synthetic feed consisted of tap water spiked with KH_2PO_4-P (3.2 mg L^{-1}), $NaHCO_3$ (350 mg L^{-1}), $MgSO_4 \cdot 7H_2O$-Mg (2.2 mg L^{-1}), $CaCl_2 \cdot 2H_2O$ (2 mg L^{-1}), and trace element solution A/B (0.5 ml L^{-1}). The influent was deoxygenated by N_2 purging, followed by manually pH adjusting to 6.9 – 7.0 with the addition of 3 M HCl. The pH of the reactors was not controlled, yet the influent low pH lowered the pH of the reactors to 7.2-7.5 (pH rises due to anammox process). An N_2 gas balloon was installed on the influent vessel to balance the pressure inside since it was always air-tight.

3.2.5 Analytical procedures

Liquid (6 ml) and microbial samples (6 ml) were taken periodically from the influent, effluent, and reactors. After filtering by 0.2 µm syringe filters (CHROMAFIL Xtra PVDF, Germany) and storing at 4°C, NH_4^+-N, NO_2^--N, and NO_3^--N were measured with a San^{++} Automated Wet Chemistry Analyzer (SKALAR, the Netherlands). The biomass concentration was followed over time using volatile suspended solids (VSS) measurements (APHA, 1998). A handheld meter was used to monitor pH (Hach HQ30d, USA).

3.2.6 Microbiome analysis

To test the microbial community shift during the sludge reactivation period at different temperatures, the V4 region of the 16S rRNA gene was used after the genomic DNA was extracted using Powerfecal kit (Qiagen, Germany) following the manufacturer's instructions. The extracted DNA samples were stored at -20℃ until shipped to a commercial company (Novogene Europe, United Kingdom) for amplicon sequencing analysis. The set of forwarding 515f (GTGCCAGCMGCCGCGGTAA) and reverse 806r (GGACTACHVGGGTWTCTAAT) primers were used to amplify the V4 hypervariable region of the 16S rRNA gene by polymerase chain reaction (PCR) (Kozich et al., 2013). The amplicon sequencing libraries were pooled and sequenced in an Illumina. paired-end platform. After sequencing, the raw reads were quality filtered, chimeric sequences were removed and ASVs were generated. Subsequently, microbial community analysis was performed by Novogene using Qiime software (V1.7.0). For phylogenetical determination, the SSURef database from SILVA (http://www.arb-silva.de/) was used. Relative abundances of ASVs were reported as % total sequencing reads count. The detail method was based on Alloul et al. (2021). To compare the community diversity, Shannon, Chao1, and Coverage indices were calculated. The data have been deposited with links to BioProject accession number PRJNA778735 in the NCBI BioProject database.

3.2.7 Calculations

3.2.7.1 Specific anammox activity, nitrogen conversion rate, and sludge retention time

The nitrogen removal rate (NRR, mg N L^{-1} d^{-1}), specific AnAOB activity (SAA_T, mg NH_4^+-N g^{-1} VSS d^{-1}), and sludge retention time (SRT, d) were calculated according to the following equations.

$$\mathrm{NRR} = \frac{\mathrm{Q_{in}}}{\mathrm{V}} \times (\mathrm{N_{in}} - \mathrm{N_{out}}) \qquad (\mathrm{Eq} - 3.1)$$

$$\mathrm{SAA_T} = \frac{\mathrm{ARR}}{\mathrm{VSS_{reactor}}} \qquad (\mathrm{Eq} - 3.2)$$

$$\mathrm{SRT} = \frac{\mathrm{VSS_{reactor}} \times \mathrm{V}}{\mathrm{VSS_{effluent}} \times \mathrm{Q_{out}}} \qquad (\mathrm{Eq} - 3.3)$$

Q_{in} and Q_{out} are the flow rate of influent and effluent, respectively [L d^{-1}]; V is the reactor volume [L]; N_{in} and N_{out} are the nitrogen concentration of influent and effluent, respectively [mg N L^{-1}]; ARR is the ammonium removal rate [mg N L^{-1} d^{-1}]; $VSS_{reactor}$ and $VSS_{effluent}$ are the biomass concentration in reactor and effluent, respectively [g VSS L^{-1}].

3.2.7.2 Arrhenius equation

The microbial temperature effect is normally described by a simplified Arrhenius equation (Eq – 3.4) (Gilbert et al., 2015).

$$\mathrm{SAA_T} = \mathrm{SAA_{20℃}} \times \theta_{\mathrm{AnAOB}}^{(\mathrm{T}-20℃)} \qquad (\mathrm{Eq} - 3.4)$$

SAA_T and $SAA_{20℃}$ are the measured specific AnAOB activity at T and 20℃, respectively [mg NH_4^+-N g^{-1} VSS d^{-1}]; θ is the temperature coefficient [unitless]; T is the respective temperature of the measured system [℃]. A θ value of 1.07 to 1.10 was chosen based on previous research (Gilbert et al., 2015; Liu et al., 2020; Sobotka et al., 2016; Vandekerckhove et al., 2020).

3.2.7.3 The percentage of activity recovery and biomass retention in reactors

The activity recovery percentage (p) is defined as the measured specific AnAOB activity (SAA_T) divided by the expected specific AnAOB activity ($SAA_{T,excepted}$, normalized to T℃ based on Arrhenius) (Eq – 3.5 and Eq – 3.6). The biomass retention percentage is the ratio of biomass concentration in the end (average the last three samples) divided by the initial value (Day 0).

$$\mathrm{p} = \frac{\mathrm{SAA_T}}{\mathrm{SAA_{T,excepted}}} \qquad (\mathrm{Eq} - 3.5)$$

$$\mathrm{SAA_{T,excepted}} = \mathrm{SAA_{20,initial}} \times \theta_{\mathrm{AnAOB}}^{(\mathrm{T}-20℃)} \qquad (\mathrm{Eq} - 3.6)$$

$SAA_{20,initial}$ the initial specific AnAOB activity at 20℃ before the sludge storage [mg NH_4^+-N g^{-1} VSS d^{-1}]. The values were respectively 119.28 ± 9.38 and 78.83 ± 10.54 mg NH_4^+-N g^{-1} VSS L^{-1} for floccular and granular sludge.

3.3 Results and discussion

3.3.1 Validation of stored AnAOB bioaugmentation at moderate temperature differences

The objective of this section is to validate the bioaugmentation mitigation concept at a moderate temperature difference (25 to 20°C). After 6-month preservation, the AnAOB activity in stored flocs and granules decreased to 62.45 ± 3.89 and 45.79 ± 4.21 mg NH_4^+-N g^{-1} VSS d^{-1}, respectively (Zhu et al., 2022). R1 (flocs bioaugmentation) and R2 (granules bioaugmentation) were run for 140 days to verify the feasibility of stored summer sludge bioaugmentation to restore the nitrogen removal performance of the reactor after temperature reduction. The nitrogen removal performance and sludge retention properties were compared throughout the experiment (Figure – 3.1).

3.3.1.1 220% flocs bioaugmentation mitigated the effect of 5.1℃ decrease

Before reducing the temperature from 25.2 ± 0.3℃ to 20.1 ± 0.4℃, the effluent quality of NH_4^+-N, NO_2^--N, and NO_3^--N remained stable (4.4 ± 1.3 mg L^{-1}, 5.3 ± 1.2 mg L^{-1}, and 9.0 ± 1.2 mg L^{-1}) with removal (NO_3^--N production) rates of respectively 122 ± 3.8, 160 ± 4.2, and 32 ± 1.8 mg N L^{-1} d^{-1}. The temperature change on Day 28 resulted in a sudden drop of the total inorganic nitrogen (TIN) removal rate from 250 ± 7.8 to 173 ± 24.0 mg N L^{-1} d^{-1} (Figure – 3.1C). The SRT was increased from 9.8 ± 0.6 to 16.0 ± 0.4 d. To restore the effluent quality to the level prior to the temperature decrease, 1.20 g VSS L^{-1} stored flocs were bioaugmented into R1 (106% of the initial biomass concentration, leading to the increase from 1.13 to 2.33 g VSS L^{-1}) (Figure – 3.1E). The TIN removal rate improved significantly, peaking at 259 mg N L^{-1} d^{-1} on Day 73. In the following days, the TIN removal rate gradually decreased due to biomass washout (2.33 → 1.63 g VSS L^{-1}). To further mitigate the influence of temperature drop, a second bioaugmentation (114% of the initial biomass concentration, leading to the increase from 1.63 to 2.93 g VSS L^{-1}) was conducted on Day 89. Biomass washout still occurred, yet adaptation to the receptor system appeared after 30 days (TIN removal rate stable at 246 ± 8.2 mg N L^{-1} d^{-1}). After bioaugmentation, the rapid SRT decrease was followed by a gradual increase (e.g., SRT from

9.3 to 13.6 d after the first bioaugmentation). The impacts caused by temperature decrease were offset by a two times stored flocs bioaugmentation.

3.3.1.2 118% granules bioaugmentation mitigated the effect of 5.1℃ decrease

Acclimatization has been regarded as a promising solution to mitigate the inhibition of low temperatures (De Cocker et al., 2018). The reactor was, therefore, operated for a longer period at 20.1 ± 0.4℃ (60 days in R2 versus 36 days in R1) to study the potential of adaptation to low temperatures before bioaugmentation. After approximately 60 days of operating at the relatively low temperature (20℃ versus 25℃), no obvious increase of the TIN removal rate was found (stabilized at 147 ± 9.4 (222 ± 5.8 mg N L^{-1} d^{-1} at 25℃). The SRT increased to 22.0 ± 0.4 from 12.8 ± 0.3 d. On Day 87, 1.57 g VSS L^{-1} stored granules (118% of the initial biomass concentration, leading to the increase from 1.33 to 2.90 g VSS L^{-1}) were supplemented into R2 (Figure – 3.1F). The TIN removal rate immediately increased to 236 ± 15.0 mg N L^{-1} d^{-1}. As opposed to the R1 (flocs bioaugmentation), the bioaugmented granules could be successfully retained in the reactor with a low biomass washout within 40 days, resulting in a stable TIN removal rate. The SRT change after the stored granules bioaugmentation was the same as flocs (increased immediately followed by the gradual decrease).

3.3.1.3 Stored sludge bioaugmentation restored the performance deterioration

A 5.1℃ temperature drop resulted in a sudden TIN removal rate reduction for the R1 (flocs bioaugmentation) and R2 (granules bioaugmentation) by respectively 31% and 34%. That was consistent with previous research (Lotti et al., 2014c; Lotti et al., 2015c). It is might a consequence of the higher energy barrier for enzymatic reactions (Tian et al., 2019). Because of the temperature drop, the nitrogen removal rate decreased rapidly, thereby, exceeding the nitrogen discharge limits. As shown above, exogenous bioaugmentation with stored biomass is a promising solution to mitigate the impacts of temperature drop. The biomass adaptation to the low temperature can be excluded since more than two months of steady state was observed before bioaugmentation (R2, granules bioaugmentation). Furthermore, more than one month of stable performance after the inoculation suggesting the enhancement could last a certain period.

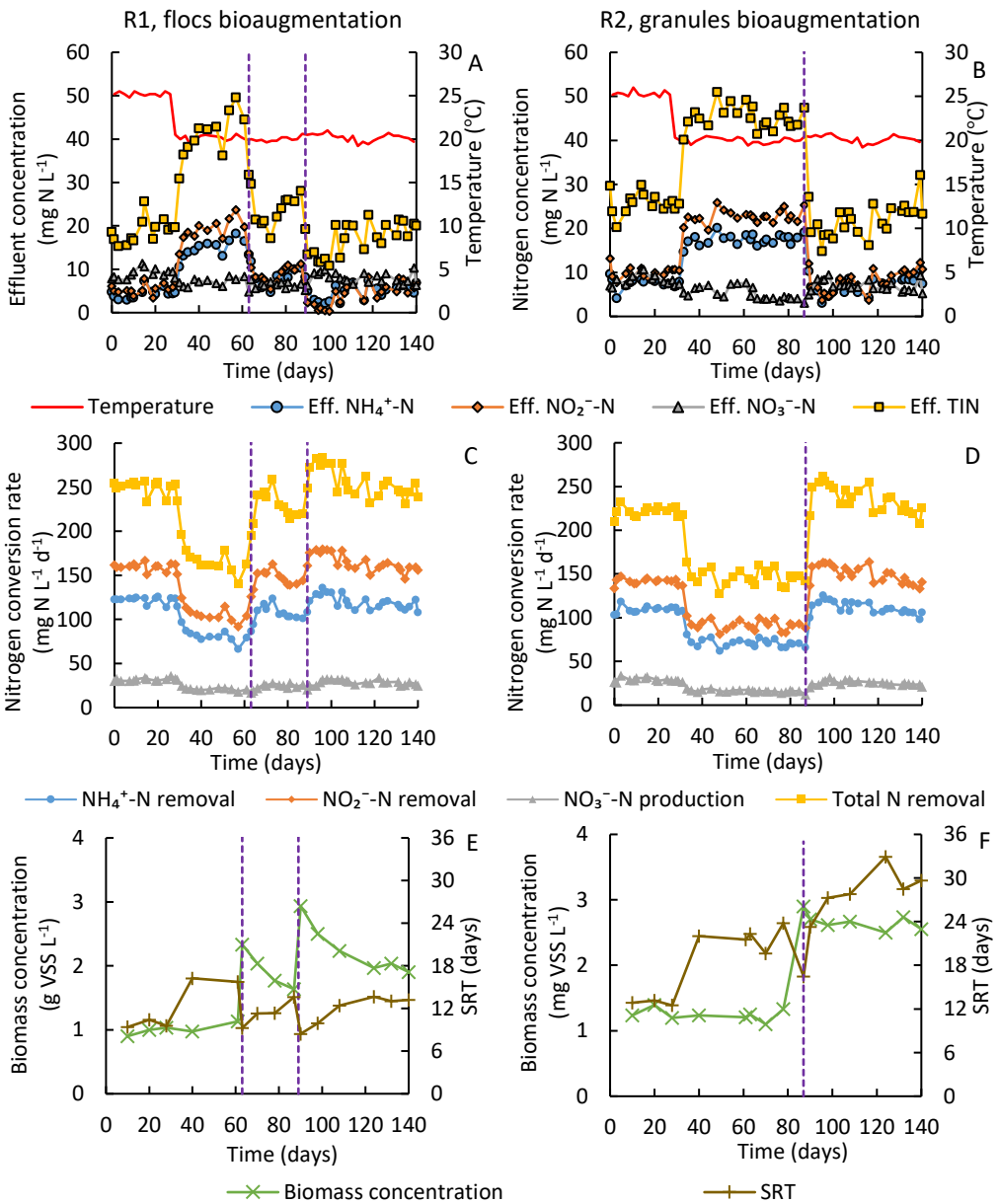


Figure – 3.1 Reactor performance in flocs system (A, C, E) and granules system (B, D, F). A, B: Effluent nitrogen concentrations and temperature; C, D: Nitrogen conversion rate; E, F: Biomass concentration and SRT.

A high sludge retention time was essential to maintain AnAOB in the reactor (low growth rate and biomass yield (Jetten et al., 2001; Lotti et al., 2014c)). Compared to the R2, almost twice the number of stored flocs (220% of the initial flocs was bioaugmented in R1 vs. 118% of

the initial granules was bioaugmented in R2) were required to mitigate the effect of the 5.1℃ temperature drop. That was mainly due to higher floc washout in the effluent relative to the R2 (13.3 ± 0.3 d of SRT in R1 vs. 30.3 ± 2.3 d of SRT in R2). Two possible reasons explained that: i) Granules have a better sedimentation performance (higher biomass density) and settling properties (Abma et al., 2007) than flocs, especially after storage (Xu et al., 2020), and ii) flocs were more susceptible to low temperature (more activity lost) since the higher activity was recovered in granules (Fig.3A). That can probably be attributed to the existence of the polymeric matrix that has a protective role in granular sludge to improve the bacterial cold tolerance (Jin et al., 2013a). There was no effort to retain the sludge in systems which was the worst case. In practice, it was easier to maintain AnAOB since the cyclone or screen was used (Van Winckel et al., 2019b). Additionally, recycling can be used to avoid so much stored sludge washing out.

After bioaugmentation, the SRT dropped at first, followed by an increase and a final stabilization. Pei et al. (2015) also reported that a bioaugmentation could reduce the SRT of nitrifiers. A solids balance analysis demonstrated that the SRT values of R1 and R2 were stabilized at 13 ± 0.3 and 30 ± 2.3 days, respectively. Thus, the granules (with a longer SRT than flocs) achieved a higher mass of sludge in the reactor. The SRT in R2 after bioaugmentation was higher than before, which was essential for the newly bioaugmented biomass to ensure long-term retention in the received system. The results were in line with the finding of R1, indicating a certain percentage of the bioaugmented biomass could eventually remain and grow in the receptor reactors.

3.3.2 Stored-sludge could reactivate (activity recovery and biomass retention) at low-temperature systems

Whether the inoculants can show activity and be retained directly in the low-temperature conditions after the storage is another challenge to the success of the novel stored summer biomass bioaugmentation concept. This section had the objective to assess the reactivation performance of the stored biomass (flocs and granules) at three different temperatures (15℃/10℃/4℃) reactors (Figure – 3.2).

3.3.2.1 AnAOB activity recovered in 15℃/10℃ flocs-/granules- based reactors

The stored flocs and granules were reactivated rapidly (five days) for the reactor run at 15.3 ± 0.4°C. After five days, the specific AnAOB activity was already stable, showing a value of 28.3 ± 2.5 mg NH_4^+-N g^{-1} VSS d^{-1} for the granules. For the flocs, on the other hand, the AnAOB activity decreased from 39.9 mg NH_4^+-N g^{-1} VSS d^{-1} on Day 7 to 27.9 mg NH_4^+-N g^{-1} VSS d^{-1} on Day 33. The reactor operated at 10.4 ± 0.4℃ required a longer reactivation time (defined as the time from Day 0 to the day when the maximum specific AnAOB is reached) for both sludge types (seven days at 10℃ versus five days at 15°C). For the floc-based reactor, the specific AnAOB activity followed the same trend as the 15°C reactors, which a gradual decrease of activity after seven days. For the granules, the specific AnAOB activity decreased after 20 days of reactivation. The AnAOB activity was neglectable in the initial ten days in reactors at 3.9 ± 0.15℃. In the following 20 days, nearly no specific AnAOB activity was detected.

Rapid recovery of the AnAOB activity is one of the essential factors for successful bioaugmentation. With decreasing temperature, the recovery percentage (i.e., the measured AnAOB activity divided by the expected activity (after Arrhenius-based temperature correction, θ = 1.10)) decreased. For the flocs, 41 ± 5.7% of AnAOB activity could be recovered at 15℃ and 32 ± 6.7% at 10℃ within a month (Figure – 3.3). The granules showed a similar trend with a recovery percentage of 56 ± 4.9% to 41 ± 3.0% at respectively 15℃ and 10℃.

As opposed to the research reactivating the stored anammox sludge at high temperature (33 – 37℃) where the specific AnAOB activity could be completely recovered within a month (Ali et al., 2014; Magrí et al., 2012), only a certain percentage of AnAOB activity was restored at a lower temperature in the current study. Several reasons attributed to that. Firstly, the key enzyme activities in AnAOB (e.g., nitrite reductase (*nir*)) were probably suppressed at low temperature which might contribute to the inhibition of the anammoxosome and the cell nucleus, which in turn limit the specific AnAOB activity (Zhang et al. 2019). Secondly, the AnAOB growth rate at 10 – 15℃ had a ± 8-time decrease compared to that at 33 – 37℃ (after Arrhenius-based temperature correction with the temperature difference of 22°C). There was a lack of sufficient growth at the reduced temperatures. Thirdly, due to the decay during the sludge storage (biomass and AnAOB activity decay was 0.0041 and 0.002 d^{-1}, respectively for floccular sludge, and the values were 0.003 and 0.0013 d^{-1} for granular sludge (Zhu et al., 2022)), the longer biomass preservation time of the present research compared with other studies (180 days vs. 30 – 150 days in Ali et al. (2014) and Magrí et al. (2012)) might result in the lower

recovery percentage. Moreover, the Arrhenius coefficient might not be constant at low temperature (< 15℃) possibly due to two rate-determining enzymes with different temperature optima (Isaka et al., 2008; Lotti et al., 2015c), which could also affect the expected AnAOB activity calculated in the present research.

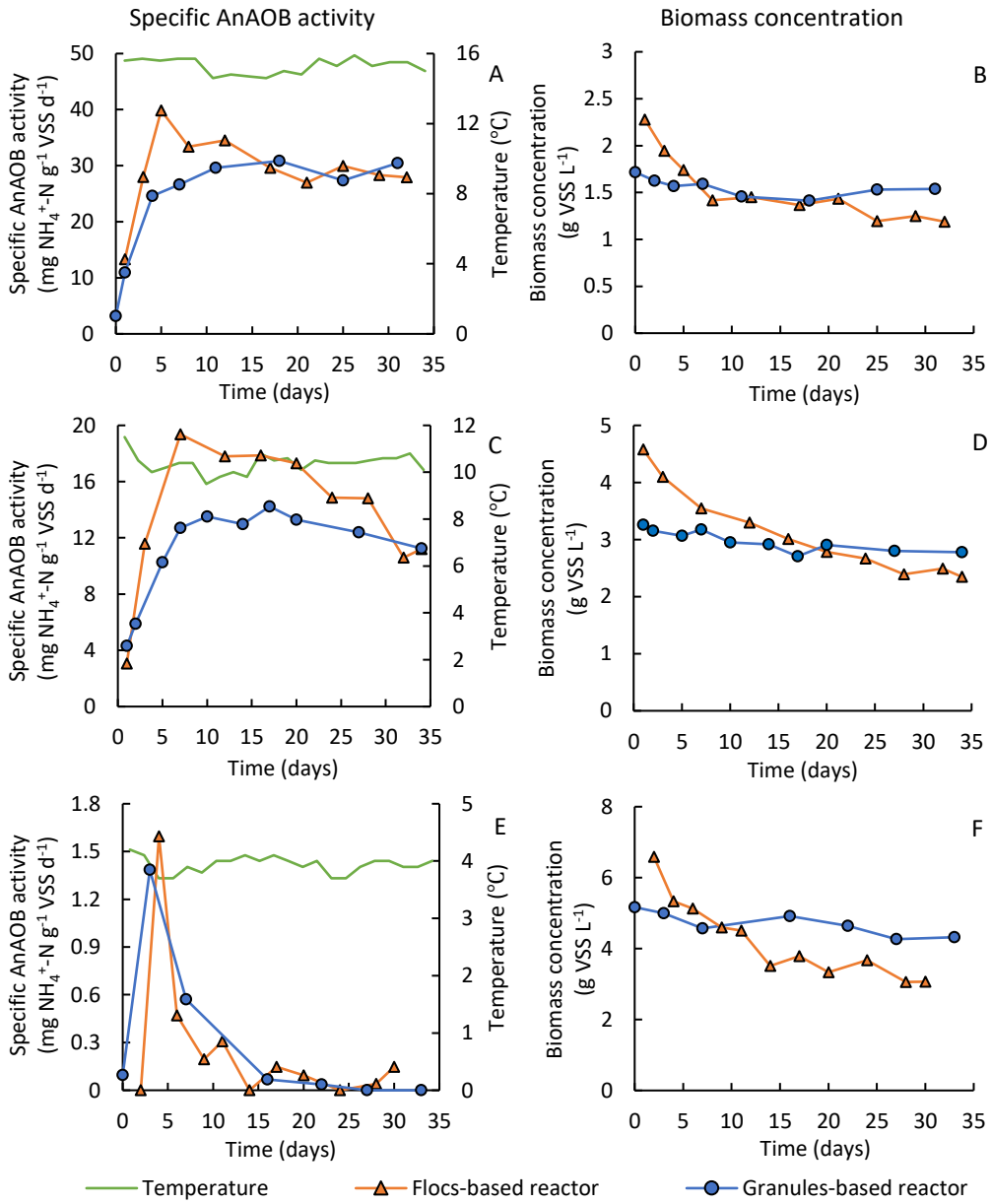


Figure – 3.2 Reactivation of stored sludge (six months) under different temperatures. A, B: 15 ℃; C, D: 10 ℃; E, F: 4 ℃.

3.3.2.2 Higher biomass retention was achieved in granules-based reactors

As shown in Figure – 3.2B, at 15℃, the biomass concentration in the flocs-based reactor dropped from 2.28 to 1.19 g VSS L^{-1} over 30 days compared to 1.72 to 1.54 g VSS L^{-1} for the granular-based reactor. At 10℃, ± 49% of the bioaugmented (on Day 0) flocs were washed out within a month. But for the granules, the biomass concentration remained stable (3.0 ± 0.2 g VSS L^{-1}). Reactivation at 4℃ caused more sludge washout from the reactors compared to 15℃ and 10℃. That was consistent with Guo et al. (2010), who found the cold temperature could impact the settling characteristics of biomass during the research at varying temperatures (5-30℃). Regarding the biomass retention percentage (Figure – 3.3B), for granules, 84.9 ± 3.4% to 86.6 ± 4.1% of sludge could be retained, whereas the values were only 49.7±5.4% to 53.1±1.5% for flocs. The stable biomass concentration and AnAOB activity (Section – 3.2.1) after 30 days reactivation suggesting partial inoculated bacteria acclimated and proliferation in the receptor system successfully.

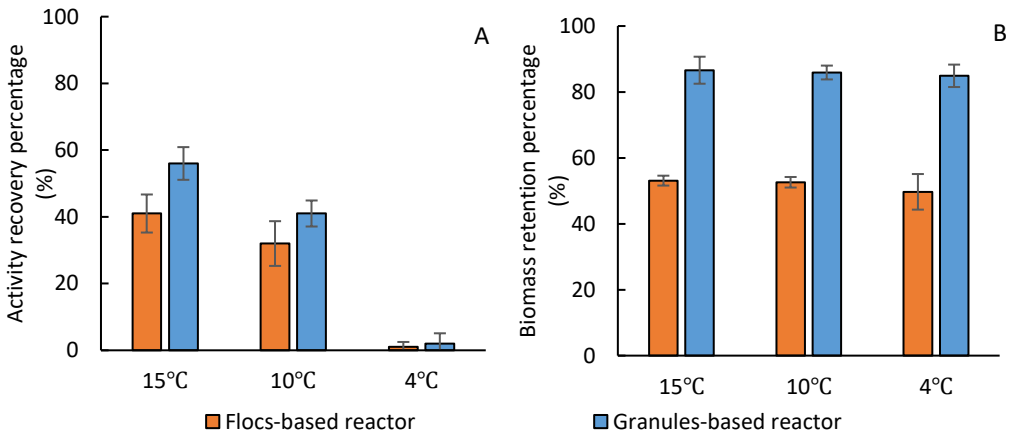


Figure – 3.3 The percentage of AnAOB activity recovery (A) and biomass retention (B) under different temperatures.

Compared with the pre-adapted strain or consortia used in other bioaugmentation studies (El Fantroussi and Agathos, 2005), the stored AnAOB sludge (both flocs and granules were suitable even though about two times flocs were needed) is preferably bioaugmented directly into the receptor system without any pretreatment steps, which is then easy-operation and economic-effective. Negligible AnAOB activity detected at 4℃ would hinder this concept using at lower temperatures (e.g., lower than 5℃) since the AnAOB activity could not be recovered

even though enough biomass was retained in the system (49.7% and 84.9% respectively for flocs and granules).

3.3.3 The abundance of AnAOB genera can be promoted at 15℃ and 10℃

This section had the objective to assess the microbial community shifts as the relative AnAOB abundance is essential for biomass reactivation under low temperatures. Approximately 95% of the metabolically active community abundance was classified into 8 phyla (Fig. 4A/B), of which Planctomycetota, Proteobacteria, Bacteroidetes, and Chloroflexi were the most abundant phyla in both floccular and granular sludges. For both stored sludges, the relative abundance of Planctomycetota in the reactors increased at higher reactivation temperature (e.g., 49%, 40%, and 38% for floccular sludge at 15, 10, and 4℃, respectively). Hence, too low temperatures (e.g., 4℃) could not achieve reactivation of the stored anammox sludge. The relationship between the relative abundance of Planctomycetota and reactor temperature was in line with Akaboci et al. (2018).

Three representative AnAOB genera (Candidatus *Brocadia*, Candidatus *Kuenenia*, and Candidatus *Jettenia*) were detected in the stored sludge. Candidatus *Brocadia* dominated the community in floccular and granular sludge, with a relative abundance (expressed relatively over the total community) of 37% and 24%, respectively, representing approximately 99% of Planctomycetota (Fig. 4C/D). After 30 days' reactivation, Candidatus *Brocadia* was still dominant at 15℃ with a relative abundance of 48% and 54% for flocs and granules, respectively (versus 38% and 42% at 10℃, respectively). The relative abundance of the other AnAOB genera also increased in all systems but still with a low level (e.g., Candidatus *Kuenenia* increased to 0.7% from almost 0 in 15℃ flocs-based reactor). For the 4°C reactors, the relative abundance of three AnAOB genera was similar to the values before reactivation for both sludges. This contrasted with Reeve et al. (2016), who found no clear community shifts during the reactivation period. The difference might attribute to the recovery temperature (28℃ vs. 4 – 15°C in the present research) and the biomass source (pilot-scale system with relatively simple influent vs. full-scale system with complex influent composition). The relative abundance of the denitrifier-related genus, *Denitratisoma*, was present in reactors. This is likely due to the occurrence of endogenous respiration, releasing COD for the heterotrophic bacteria (Contreras et al., 2011).

Even though the reactivation temperature (15 and 10℃) was much lower than their optimum (35°C) (Hu et al., 2013), AnAOB could still be enriched in the system compared to the other bacteria presented in sludge. That was possibly due to the low DO and COD concentration conditions (compared to the influent of their parent reactors) which decreased the growth rates of certain bacteria (e.g., heterotrophic bacteria). The higher AnAOB recovery percentage at 15℃ compared to 10℃ (Section – 3.2) was in line with the higher relative abundance of the dominant genus. Even after 30 days' reactivation, the community in all four reactors was still dominated by Candidatus *Brocadia*. This competitive advantage of Candidatus *Brocadia* over other AnAOB at low temperatures was also reported by Hendrickx et al. (2014). At 4℃, the community composition was relatively stable in both floccular- and granular- based reactivation reactor. All the bacteria present in the sludge might have a lower metabolic activity at that temperature, which led to the slow changes in the most abundant genera in the short term.

The Shannon, Chao1, and coverage indices were used to reflect microbial diversity. The goods coverage of all samples was above 0.995, indicating that the sequences in the samples were detected with high probability and the results of diversity analysis had high reliability and authenticity. In addition, Shannon and Chao1 indices were generally showed the same trend of decreasing as dropping off the reactivation temperatures, which demonstrated the higher microbial diversity. Under relatively high temperature conditions, there is more opportunity to reconstitute bacteria that favor the settlement of AnAOB. This was in line with Wang et al. (2022), who reported that the community could resist the effect of low temperature by increasing the diversity of microbes. But the dominant species were weakened. Compared to the stored sludge, microbial diversity decreased after reactivation in the 10°C and 15°C reactors. Certain species were probably removed from the system by selection (e.g., washout due to lower SRT and worse settling properties) and competition caused by temperature (metabolism and growth rate), thereby, triggering changes in diversity. Apart from that, DO levels (< 0.05 mg O_2 L^{-1}) in the reactors were lower than that in STP, some species related to aerobic processes could not adapt to the conditions were eliminated. Moreover, compared to the complex influent in a full-scale STP system, the simple influent composition might eliminate certain bacteria (e.g., heterotrophic bacteria, due to the absence of COD). That possibly explained the decrease of the diversity in the process of anammox sludge reactivation. Therefore, the enhanced AnAOB activity due to activity reactivation was accompanied by a more specialized

(less diversity) and dominant community for anammox, which promoted a more efficient anammox process.

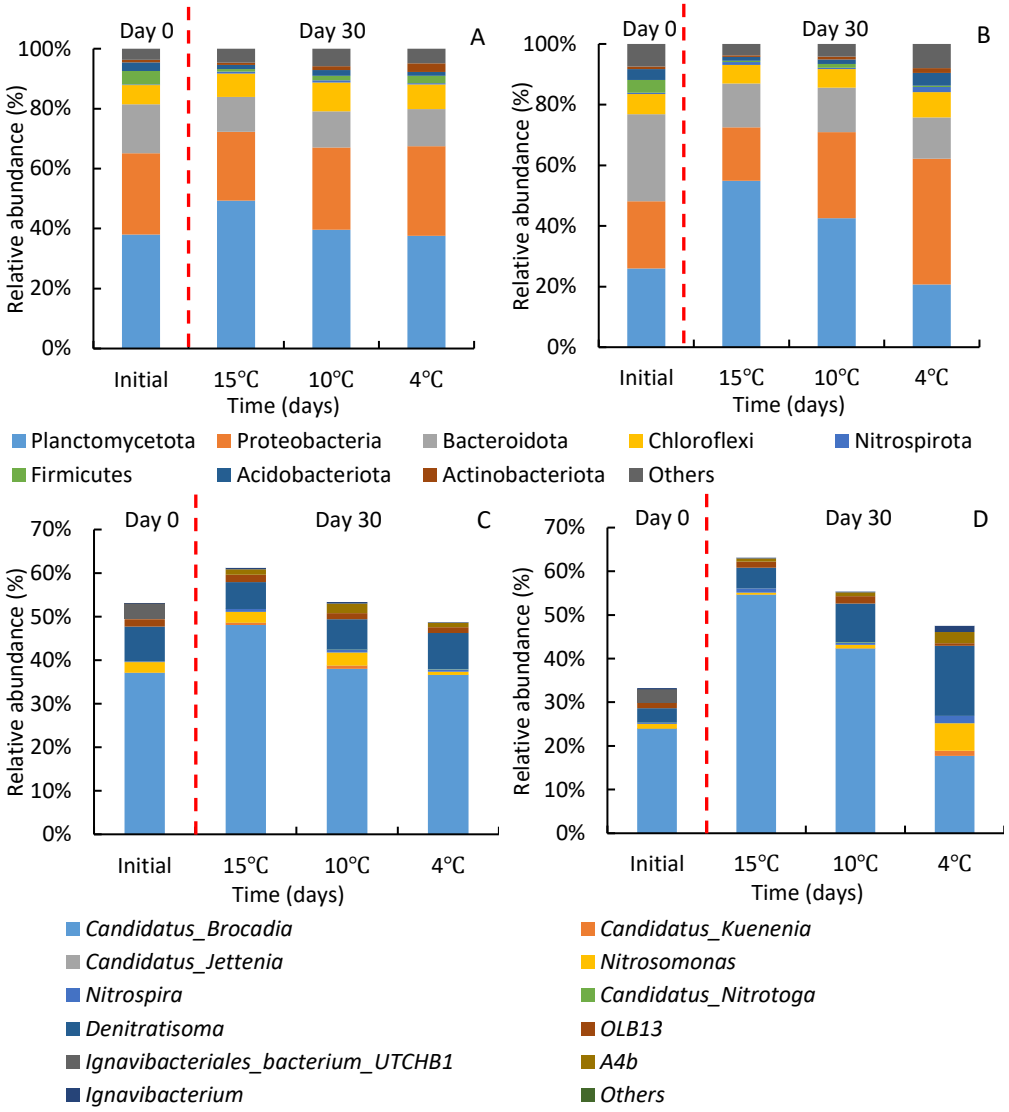


Figure – 3.4 The microbial community during the stored sludge reactivation at phyla (A, B) and genus levels (C, D), expressed relatively over the total community (A, C: Flocs-based reactor; B, D: Granules-based reactor). At the genus level, only the dominant nitrogen removal related bacteria (AnAOB (orange), AerAOB (green), NOB (blue), and denitrifying bacteria (purple)) were shown.

3.3.4 The stored summer sludge bioaugmentation concept is economic-effective

The proposed 'bioaugmentation with stored summer sludge for winter anammox assistance' concept was shown in Fig.5A.

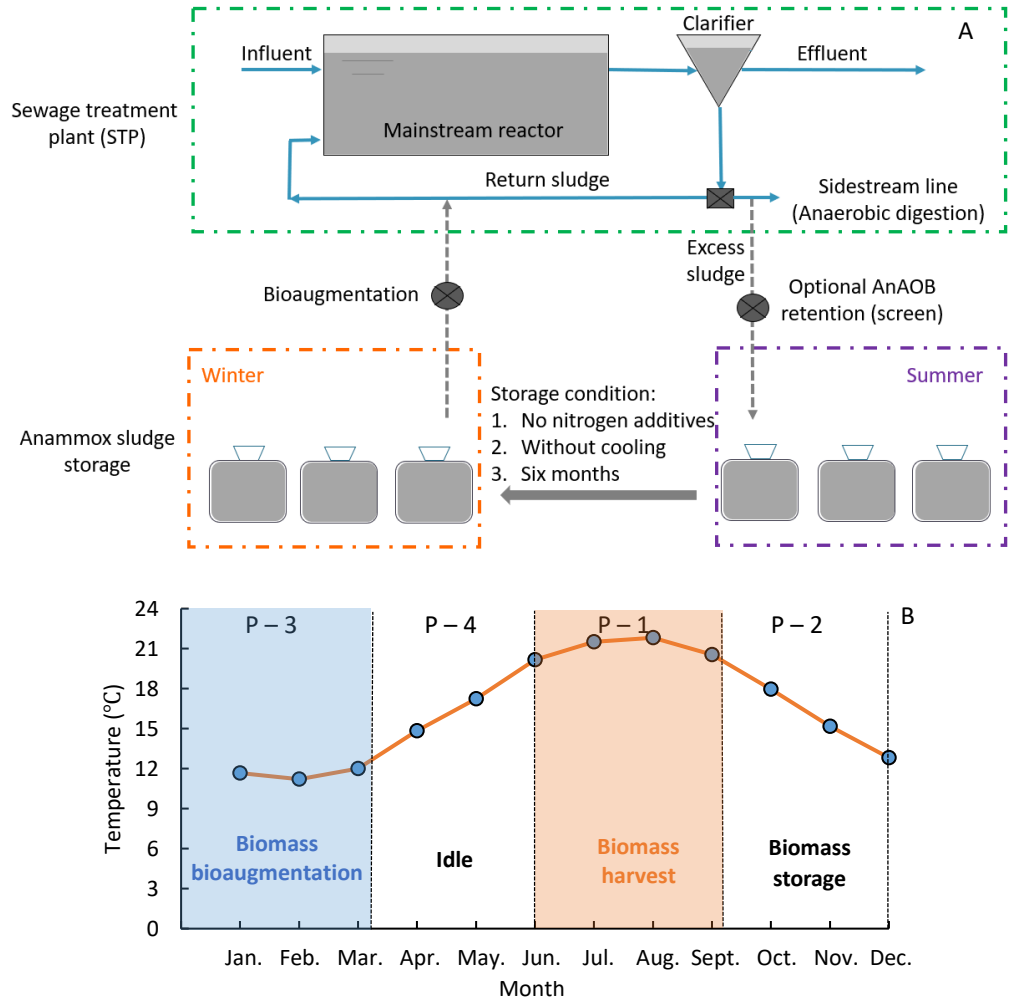


Figure – 3.5 Schematic of the winter bioaugmentation concept (A) and the potential harvest/preservation/bioaugmentation period (B). P – 1, sludge harvest; P – 2, sludge preservation; P – 3, sludge bioaugmentation; P – 4, idle. Temperature data was based on the average value within 2016/01 – 2021/06 from Nieuwveer STP (Breda, the Netherlands).

The average temperature of the mainstream wastewater changes between 11.2℃ and 21.8℃ during the whole year (Nieuwveer STP). We assume to harvest sludge when temperatures are higher than 20℃ and bioaugment sludge when temperatures are below 13℃. A year, thus,

consists of four periods: (P – 1) sludge harvest (July, August, and September), (P – 2) sludge storage (October, November, and December), (P – 3) sludge bioaugmentation (January, February, and March), and (P – 4) idle (April, May, and June) (Figure – 3.5B). Thus, the average biomass preservation period is assumed to 6-month on average.

The biomass concentration in the mainstream system is assumed to be the same as that in summer (2.3 g TSS L^{-1}), due to the low growth rate of AnAOB sludge in the winter period (0.02 d^{-1} at 20℃ and only 0.005 d^{-1} at 10℃ (Lotti et al., 2014c)). The increase in biomass concentration in winter could only be attributed to bioaugmentation. In winter, it is assumed that there is no biomass growth and loss in the mainstream. The cost assessment of this concept is shown in Table – 1. The estimated OPEX is 0.031-0.040 € IE^{-1} $year^{-1}$. This is mainly attributed to the base consumption during storage. According to our previous research, the pH during biomass storage should be maintained within 7.2-8.0 to avoid enhanced decay rates due to low pH (Sun et al., 2020).

Table – 3.1 The cost assessment of the concept 'bioaugmentation with stored summer sludge for winter anammox assistance'.

	Parameters	Value	Unit
Stored sludge	Required sludge	239-306	ton TSS
	Available sludge in summer (90 days) [a]	436	Ton TSS
	Storage volume	3,677-10,200	m^3
Cost	OPEX (NaOH)	0.031-0.040	€ IE^{-1} $year^{-1}$ [b]
	CAPEX (Cement tank)	0.025-0.069	€ IE^{-1} $year^{-1}$
	Total cost	0.056-0.109	€ IE^{-1} $year^{-1}$
	Percentage of concept cost to the total cost of STP [c]	0.19-0.36	%

Note: a, AnAOB yield is 0.122 g VSS g-1 NH_4^+-N (Lotti et al., 2015a)

b, IE represents 'inhabitant equivalents'.

c, The sewage treatment cost in STP is ~30 € IE^{-1} $year^{-1}$ in high-income countries.

The CAPEX, which is the cost of building the storage tank (cement tank (Meerburg et al., 2016)), is 0.025-0.069 € IE^{-1} $year^{-1}$. Thus, the total cost of this concept was 0.056-0.109 € IE^{-1} $year^{-1}$. That was neglectable (0.19-0.36%) compared to sewage treatment cost (30 € IE^{-1} $year^{-1}$ in high-income EU countries) (Zessner et al., 2010). Due to the generic unit '€ IE^{-1} $year^{-1}$' being

proposed in the present research, the cost assessment results could be extrapolated to other STPs located in the high-income EU countries in temperate regions (e.g., the Netherlands). Additionally, to extrapolate the concept to other temperate regions (avoid the influences of socioeconomic status), the percentage of concept cost to the total cost of STP is also put forward in this study, which shows the cost-effectiveness of the concept more visually.

In full-scale applications, COD is present in the wastewater, which leads to certain nitrogen being removed by the denitrifier, resulting in a reduction in the production of AnAOB biomass. However, the present research assumptions are based on a system where mainstream PN/A is already implemented full-scale. The incoming bCOD/N ratio is expected lower than 2, which is in line with the expected ratio of pre-treated sewage (e.g., high-rate activated sludge) (Laureni et al., 2019; Malovanyy et al., 2015). That means maximally 50% of the total nitrogen could be removed by denitrification, and the residual nitrogen removal (to meet the discharge standards) might be attributed to anammox. Lower bCOD/N ratios mean even more total nitrogen removal via PN/A. The produced sludge in summer (436 ton TSS) is enough for the 'winter bioaugmentation concept' (require 239-306 ton TSS) (Table – 1). Based on that, 3677-10200 m^3 of excess sludge needs to be stored (this range comes from the different biomass concentrations during storage: 30 g TSS L^{-1} (the tested value in pre-test) versus 65 g TSS L^{-1} (the feasible B-sludge thickening level in Nieuwveer STP)).

3.3.5 Application and outlook

Maintaining sufficient AnAOB at low temperatures is still one of the main challenges (Kumwimba et al., 2020; Liu et al., 2020). Even though some studies achieved high nitrogen removal efficiency at relatively low temperature, e.g., Ma et al. (2013) (e.g., 2.3 kg N m^3 d^{-1} under 16℃ in up-flow anaerobic sludge blanket reactor), there was still a drop in temperature during winter which would result in the decrease of AnAOB activity. So, more biomass is needed in winter (e.g., 77% more sludge is required at 10 ℃ versus 16℃ based on Arrhenius with θ = 1.10) to treat the incoming loading rate. Due to the low growth rate of AnAOB (Strous et al., 1998), the 'winter bioaugmentation concept' proposed in the present research should be a feasible strategy to solve that. Except for the low temperature, achieving stable partial nitritation (provide stable nitrite for anammox) is another challenge for mainstream anammox application, which also needs to be tested next. In addition, rainy seasons are also challenging for STP due to the low nitrogen concentration, high flow rate, and low HRT. In this case, the

sewage treatment efficiency is reduced and there is a risk of biomass washout. This might also be mitigated with sludge supply to the mainstream with stock biomass.

According to the authors' knowledge, this was the first time that this novel concept was studied. Overall, the present study provided a new insight to alleviate the lower activity and slower metabolism in winter. Nonetheless, further additional studies on substantial areas are required before it is globally used in the application. First, different bioaugmentation dosage percentages (amount of the biomass in the receptor system) and inoculation frequencies are also interesting to explore. Repeating bioaugmentation (gradually increasing the sludge concentration in the reactor) on a weekly or monthly basis could be an alternative strategy to guarantee the 'critical biomass concentration' in the reactor since the temperature decreased gradually during autumn and winter. Second, avoiding the supplemented sludge loss from the system was another challenge (e.g., immobilization, flocculant). According to the present research, granular sludge was a good option, but it is not always available in the application. The use of a screen might be the option to retain granules in the mainstream. Third, after this first proof of concept at 20°C, it is recommended to test this at 15°C or lower temperature in future experiments. Finally, the shift of the community composition in the receptor system during the bioaugmentation process also needs to be tested in the future.

3.4 Conclusions

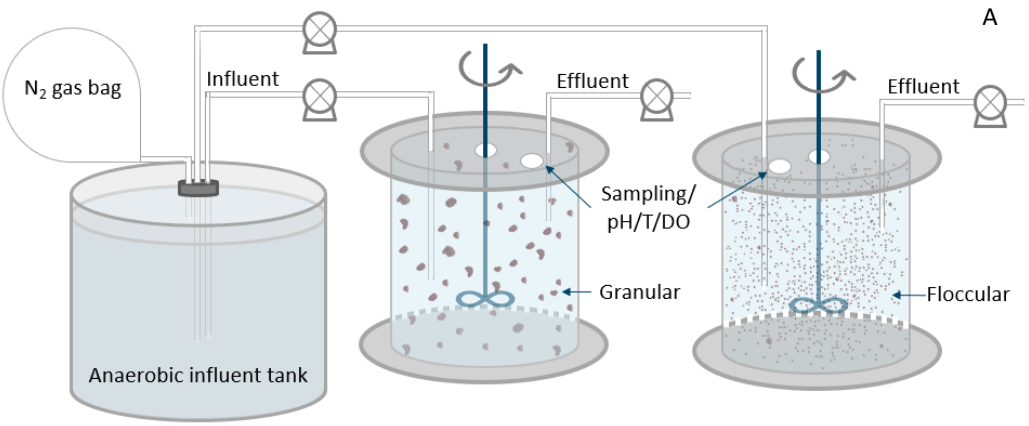
The potential for winter bioaugmentation with the stored summer sludge is demonstrated for the first time. The effect of a 5.1℃ temperature decreasing could be alleviated effectively by the stored-sludge bioaugmentation (118 – 220% of initial biomass). Moreover, the stored-sludge could be reactivated efficiently (AnAOB activity recovery and biomass retention) after bioaugmented into low-temperature reactors. Additionally, a specialized community was formed in the system (less diversity with a higher relative abundance of dominant genera (Candidatus *Brocadia*)). In the end, this concept revealed the economic feasibility of the application. It presents a new countermeasure to enhance the nitrogen-removal performance on winter days.

Acknowledgements

Dr. Bart Joosse, from Waterschap Brabantse Delta (the Netherlands), was kindly acknowledged for his helping in collecting the operation data in Nieuwveer STP (Breda, the Netherlands).

Supporting Information

Figure – S3.1 A, schematic diagram of the reactivation/bioaugmentation SBRs; B, the sequencing batch mode and DO/pH change (the steady-state of R2 (granules bioaugmentation) at 25℃ as an example here) during one complete cycle.



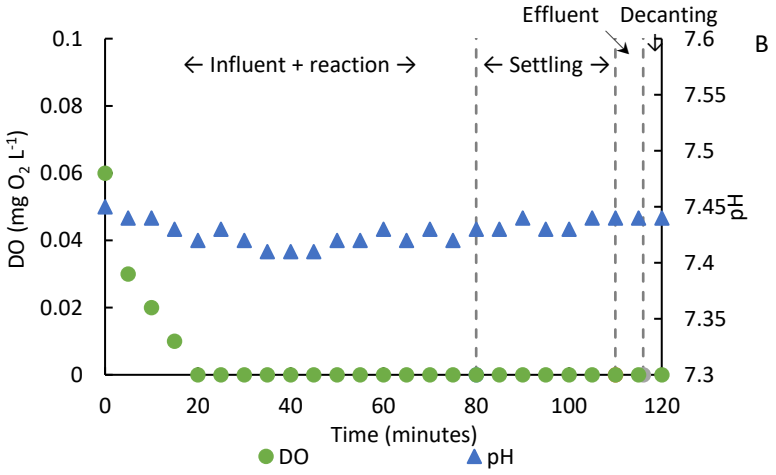


Table – S3.1 The effect of reactivation temperature on the activity recovery of stored AnAOB sludge

Sludge	Temperature	Reactivation time [a]	Specific AnAOB activity		Recovery percentage [d]
			Expected for 100% recovery [b]	Measured [c]	
	℃	d	mg NH_4^+-N g^{-1} VSS d^{-1}		%
Flocs	15.3 ± 0.4	5	76.2 ± 5.6	31.3 ± 4.3	41.1 ± 5.7
	10.4 ± 0.4	7	47.8 ± 3.8	15.5 ± 3.2	32.4 ± 6.7
	3.9 ± 0.15	-[d]	25.7 ± 2.0	0.3 ± 0.5	1.2 ± 1.5
Granules	15.3 ± 0.4	4	50.4 ± 6.7	28.3 ± 2.5	56.2 ± 4.9
	10.4 ± 0.4	7	31.6 ± 4.2	12.9 ± 0.9	40.8 ± 3.0
	3.9 ± 0.15	-[d]	17.0 ± 2.3	0.3 ± 0.5	1.8 ± 3.1

Note: a. Reactivation time is defined as the time from Day 0 to the day when the maximum specific AnAOB is reached.

b. The expected AnAOB activity was calculated according to the Arrhenius equation (θ = 1.10). The initial AnAOB activity at 20℃ was 119.28 ± 9.38 and 78.83 ± 10.54 mg NH_4^+-N g^{-1} VSS L^{-1}, respectively. And they were the potential maximum AnAOB activities that were measured in batch tests. The activities were normalized to the same temperature as the measured value.

c. The average values after the recovery period were reported.

d. Not applicable.

Table – S3.2 The biomass retention performance during the sludge reactivation under different temperatures

Sludge	Temperature	MSV [a]	SVI_{30} [b]	Biomass concentration		Biomass retention percentage [d]
				Initial	End [c]	
	℃	mm min^{-1}	mL g^{-1} VSS	g VSS L^{-1}		%
Flocs	15.3 ± 0.4	2.2	86.4	2.28 ± 0.12	1.21 ± 0.03	53.1 ± 1.5
	10.4 ± 0.4	2.2	89.6	4.58 ± 0.21	2.41 ± 0.07	52.6 ± 1.6
	3.9 ± 0.15	2.2	102.4	6.58 ± 0.22	3.27 ± 0.35	49.7 ± 5.4
Granules	15.3 ± 0.4	2.2	35.3	1.72 ± 0.09	1.49 ± 0.07	86.6 ± 4.1
	10.4 ± 0.4	2.2	39.7	3.26 ± 0.17	2.80 ± 0.07	85.9 ± 2.1
	3.9 ± 0.15	2.2	45.1	5.17 ± 0.23	4.39 ± 0.20	84.9 ± 3.4

Note: a. MSV: Minimum settling velocity of sludge (mm min^{-1}).

b. The SVI_{30} was measured at the beginning of the reactivation under different temperatures (SVI_{30} refers to the sludge volume index at 30 min).

c. The values were averaged for the last three biomass concentration measurements.

d. It was calculated as follows: The biomass concentration in the end (average of the last three samples) divided by the initial value

Table – S3.3 The change of alpha diversity indices during the stored reactivation under different temperatures.

Sludge	Condition	Shannon	Chao1	Goods_coverage
Floccular sludge	Initial [a]	5.873	2108.171	0.995
	15℃	5.716	1169.828	0.998
	10℃	6.413	1213.477	0.996
	4℃	6.342	1484.662	0.998
Granular sludge	Initial [a]	6.854	2492.272	0.995
	15℃	4.964	730.235	0.999
	10℃	5.702	1077.796	0.997
	4℃	6.782	921.536	0.999

Note: a, The sludge was preserved at no nitrogen additives and without cooling conditions for 180 days.

Table – S3.4 Wastewater properties and operational parameters from Nieuwveer STP (Breda, the Netherlands) and the cost assessment of the bioaugmentation concept. Assumption: i) no biomass growth and loss in the mainstream in winter, ii) all the biomass concentration increases in winter results from bioaugmentation, and iii) the nitrogen loading and effluent nitrogen concentration are the same over the whole year.

	Parameters	Value	Unit	Source or deduction
Facts and assumptions (mainstream)	Population equivalents	~485,000	Inhabitant	Nieuwveer STP
	Reactor volume	28,000	m^3	Nieuwveer STP
	Wastewater flowrate	148,500	$m^3\ d^{-1}$	Nieuwveer STP
	Influent nitrogen concentration	0.022	kg N m^{-3}	Nieuwveer STP
	Sewage treatment cost in STP	30 [a]	€ IE^{-1} $year^{-1}$ [b]	Zessner et al. (2010)
	Summer temperature	21.3 ± 0.7	℃	Nieuwveer STP
	Winter temperature	11.6 ± 0.4	℃	Nieuwveer STP
	Summer biomass concentration	2.3 ± 0.3	kg TSS m^{-3}	Nieuwveer STP
	Required winter biomass concentration	~5.8	kg TSS m^{-3}	$5.8 = (1.1^{(21.3-11.6)}) \times 2.3$
	Arrhenius temperature coefficient	1.1	unitless	Vandekerckhove et al. (2020)
	AnAOB activity recovery	32-41	%	Range of AnAOB activity recovery percentage of stored sludge at 10℃ (Section – 3.2 of the present research).
Sludge storage tank	Stored sludge biomass concentration	0.030-0.065	ton TSS m^{-3}	* 0.030 ton TSS m^{-3}: Sludge stored at this level had a similar performance as 0.01 ton VSS m^{-3} (Zhu et al., 2022) * 0.065 ton TSS m^{-3}: Feasible B-sludge thickening level[c]

	Required sludge [d]	239-306	ton TSS	239 = ((5.8 - 2.3) x 28000) ÷ 32% 306 = ((5.8 - 2.3) x 28000) ÷ 41%
	Available sludge in summer (90 days)	436	ton TSS	* AnAOB: 148500 m^3 d^{-1} x (0.022 kg TN m^{-3} x (1 ÷ (1 + 1.32-0.26)) x (0.122 kg AnAOB-VSS kg^{-1} NH_4^+-N ÷ 0.8) x 90 d = 21.8 ton AnAOB-TSS * Total biomass: 21.8 AnAOB-TSS ÷ 5% = 436 ton TSS
	Storage volume	3,677-10,200	m^3	3677 = (239 ÷ 0.065) 10200 = (306 ÷ 0.03)
	Opex NaOH for storage over 180 days (without cooling or N additives)	63.5	€ ton^{-1} TSS	22-105 € ton^{-1} TSS over 180 days [e]. The average value was assumed.
	Capex sludge holding tank	35	€ m^{-3}	31-39 € m^{-3} based on Meerburg et al. (2016), and the average value was assumed.
	Depreciation time	15	Years	(Meerburg et al., 2016)
	Belgian Bank Lending Rate	4.5	%	4-5% was expected by personal communication from ING bank (https://www.ing.be/en/business). The average value was assumed.
Total cost	Opex (NaOH)	0.031-0.040	€ IE^{-1} $year^{-1}$	0.031 = 239 x 63.5 ÷ 485000 0.040 = 306 x 63.5 ÷ 485000
	Capex (Cement tank) [f]	0.025-0.069	€ IE^{-1} $year^{-1}$	0.025 = (35x3677x0.045) ÷ (1-(1+0.045)$^{-15}$)÷485000 0.069 = (35x10200x0.045)÷(1-(1+0.045)$^{-15}$)÷485000
	Total cost	0.056-0.109	€ IE^{-1} $year^{-1}$	0.056 = 0.031 + 0.025 0.109 = 0.069 + 0.040
	Percentage of sewage treatment cost in STP	0.19-0.36	%	0.19 = 0.056 ÷ 30 0.36 = 0.109 ÷ 30

Note: a, The highest value was taken and assumed costs in Austria approximate to those in the Netherlands. In low-income countries, e.g., the Danube and Black Sea catchment areas, there was at a maximum 30% lower than in Austria.

b, IE means 'inhabitant equivalents'.

c, Personal communication from Bart Joosse (Waterschap Brabantse Delta, the Netherlands)

d, Assumption and facts: i) VSS/TSS ratio is 0.8, ii) all the nitrogen is removed by anammox in mainstream PN/A system, iii) AnAOB yield is 0.122 g VSS g-1 NH_4^+-N (Lotti et al., 2015a), and iv) AnAOB species account for ~5% of the mainstream sludge (Lotti et al., 2015; Yang et al., 2018) (That was also proved by the floccular sludge used in the present study (more similar to mainstream sludge). AnAOB activity = 0.119 kg NH_4^+-N kg^{-1} VSS d^{-1} = 0.119 x (1+1.32-0.26) x 0.8 kg TN g^{-1} TSS d^{-1} = 0.196 kg TN kg^{-1} TSS d^{-1} (relative abundance of AnAOB genera is 16%) = 1.225 kg TN kg^{-1} AnAOB-TSS d^{-1}; mainstream AnAOB activity in summer = (148500 m^3 d^{-1} x 0.022 kg TN m^{-3}) ÷ (2.3 kg TSS m^{-3} x 28000 m^3) = 0.051 kg TN kg^{-1} TSS d^{-1}; the relative abundance of the AnAOB in mainstream = (0.051 kg TN kg^{-1} TSS d^{-1} ÷ 1.225 kg TN kg^{-1} AnAOB-TSS d^{-1} = 4.2%).

e, NaOH was used to control the pH, and a 50% NaOH solution was used (€ 200 ton^{-1} 50% NaOH solution (Vandekerckhove et al., 2018)). The range was attributed to the different biomass decay rates of floccular and granular sludge in our previous research (Zhu et al., 2022).

f, The cost of the mixer (Meerburg et al., 2016) compared to the cost of the storage tank only accounts for 10%, so it was not taken up in this first cost-assessment.

Chapter 4

Feasibility of a return-sludge nursery reactor to biostimulate mainstream anammox bacteria

Publication of a redrafted version of this chapter is intended: Zhu, W., Van Tendeloo, M., Alloul, A., Vlaeminck, S.E. Feasibility of a return-sludge nursery reactor to biostimulate mainstream anammox bacteria. Manuscript in preparation.

Abstract

Low temperatures challenge the application potential of mainstream anammox. To overcome that, a return-sludge nursery concept, bridging conditions between sidestream and mainstream for anammox activity boosting, is proposed to biostimulate anammox bacteria with a mainstream niche. The idea is to apply sidestream nitritation, and blend the resulting effluent with mainstream effluent to achieve an intermediate temperature (25°C) and intermediate nitrogen concentrations. Different nursery frequencies (versus the total duration of the nursery mode) were tested (0.5 – 2 days nursery per 3.5 – 14 days). Results showed that the total nitrogen removal rates at mainstream increased with 33 to **–** 38% after nursery treatment. There were no considerable effects of the nursery frequency on efficiency enhancement. Arrhenius' expectations (high-temperature enhancement) could only explain 49 – 51% of the activity increase in the nursery reactor ($\theta = 1.09$), pointing at the role of other factors, e.g., the ~400% elevated electrical conductivity (15 – 16%), the 56-335% higher effluent nitrogen concentrations (12 – 14%), and the synergy and unknown factors (20 – 23%). A relatively stable anammox community composition, dominated by Candidatus *Brocadia*, was detected during the nursery treatment. This mainstream return-sludge biostimulation approach boosted anammox activity and sludge growth, which is a promising alternative for sidestream-to-mainstream bioaugmentation.

4.1 Introduction

After almost 25 years, the anaerobic ammonium oxidation (anammox) related process (e.g., partial nitritation/anammox; PN/A) is still key in scientific research on nitrogen removal due to its cost-effectiveness and energy-efficiency compared to conventional processes (e.g., nitrification/denitrification or nitritation/denitritation) (Seuntjens et al., 2018c; Strous et al., 1998).

As for other biological processes, AnAOB is extremely sensitive to temperature changes (Lotti et al., 2015c). Especially in winter, the nitrogen removal efficiency in the mainstream (i.e., water treatment line) decreases due to the drop in temperature. Several strategies have been proposed to improve the activity of AnAOB and the efficiency of nitrogen removal in winter. Chemical additions (e.g., glycine betaine), for example, have been successfully used to restore the activity of AnAOB at low temperatures (Jin et al., 2013a; Zhu et al., 2017b). The associated operational costs and environmental risks, however, prevent full-scale implementation. Recently, Kouba et al. (2021) reported that a cold-shock (35℃ → 5℃ (8h) → 15℃) could achieve 136% higher AnAOB activity than the control (35℃ → 15℃). Rapidly cooling large amounts of sludge to 5°C is, however, not cost-effective even in winter. Periodic temperature shocks (about two weeks at 30℃ per month) has also been shown to enhance the total nitrogen removal by 19.1% in a 20℃ reactor (Ma et al., 2020). It is a promising strategy, yet a long-term heat shock is unlikely to be achieved in winter.

Biostimulation, the addition of nutrients, energy sources or electron acceptors to enhance the activity of microbes, is a potential way that can be used in the sewage treatment plants (STP) (El Fantroussi and Agathos, 2005). The reject water, which is typically at higher temperatures (15 – 20℃ higher) and nitrogen concentrations (> 20 times) compared to the mainstream, could be a reasonable source to biostimulate the microbes activity in the mainstream.

Mainstream biomass biostimulation with sidestream conditions (EssDe®) or seeding concepts from sidestream to mainstream (ANITA™Mox) are good options to operate in STP (Christensson et al., 2013; González-Martínez et al., 2021; Szatkowska and Paulsrud, 2014). Limitations (e.g., biomass washout, low bacterial activity, etc.), however, occur when the seeded biomass was cultured in one condition and then bioaugmented into another (Head and Oleszkiewicz, 2004). The microorganisms cultured at high temperature and nitrogen

concentrations hardly showed activity after bioaugmenting into low temperature (temperature difference > 10°C) and nitrogen concentrations conditions (Abeysinghe et al., 2002). This could have been attributed to the diversity in niches between the two systems (e.g., temperature, predation, and nitrogen concentration). Yielding the 'right' AnAOB community is, therefore, less likely (Mannucci et al., 2015). Intermediate temperatures and nitrogen concentrations conditions, not that different from the mainstream (e.g., Bioaugmentation batch enhanced reactor (BABE)), have shown to be effective to improve the nitrification activity (Salem et al., 2003).

For partial nitritation/anammox (PN/A), no similar research exists whatsoever. In this research, we propose a reactor to blend sidestream (partial) nitritation effluent with mainstream effluent. This would enable to achieve intermediate temperatures and nitrogen concentrations, conditions more favorable for anammox. According to the authors' knowledge, this study is the first research about the AnAOB biostimulation using the anammox nursery reactor.

The proposed implementation of the anammox nursery concept in STP is shown in Figure – 4.1. Three lines flow into the nursery reactor: AnAOB granules (retained from the waste sludge by screen, e.g., 250 µm size (Van Winckel et al., 2019b)), reject water (high temperature with a high concentration of nitrite and optionally ammonium), and B-stage effluent (used for temperature control in nursery reactor). In this way, an environment is created that is as similar as possible to the mainstream conditions (less temperature difference) while ensuring that anammox activity can be biostimulated (relatively higher temperature and substrates concentration).

Thus, the overall objective of this research is to systematically test the effectiveness and sustainability of the nursery reactor concept. Three parts were assessed: i) the performance of the nursery reactor for enhancing the mainstream anammox efficiency under four time ratios of nursery versus mainstream exposure (i.e., nursery frequency = nursery time/(nursery + mainstream)), ii) the change of biomass growth rate and the microbial community caused by the biostimulation in nursery conditions, iii) the analysis of the mechanism that caused the performance enhancement behind the biostimulation (the potential enhancement factors). The results of the present study provide a new idea on how to improve the nitrogen removal capacity of STPs under low-temperature conditions.

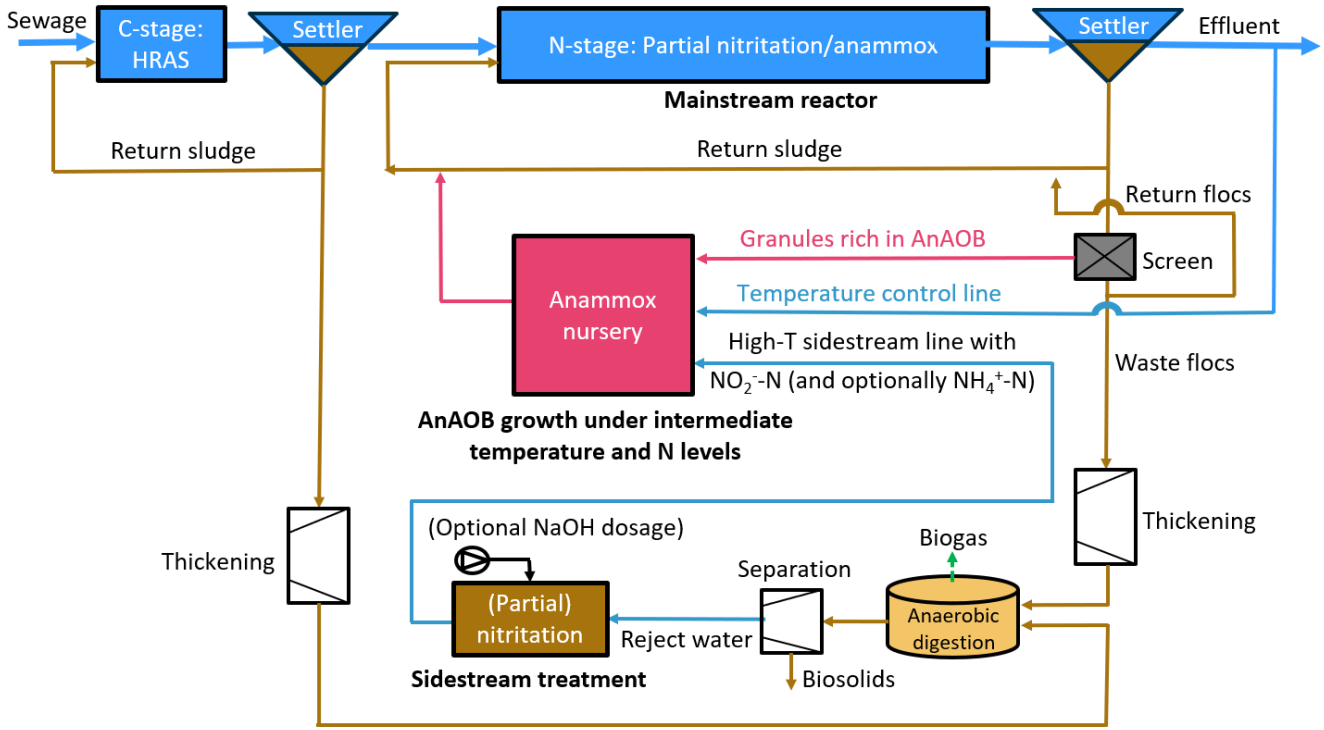


Figure – 4.1 Proposed implementation of the anammox nursery concept in an energy-positive sewage treatment plant. HRAS refers to high rate activated sludge. The concept was based on Nieuwveer STP which consisted of C-stage (carbon and phosphorus removal) and N-stage (nitrogen removal). 30% of granules and 72% of AnAOB activity were assumed to calculate the flow rate retained by the screen (250 µm) (Van Winckel et al. 2019b).

4.2 Materials and methods

4.2.1 Anammox nursery reactor setup

To simulate the anammox nursery reactor in the lab, two parallel anoxic sequencing batch reactors (SBR) (i.e., R1 and R2, Figure – S4.1) were inoculated with anammox sludge (detailed information was shown in Section – 2.2) and operated mainly at mainstream conditions (15℃) on synthetic autotrophic wastewater (NO_2^- and NH_4^+ = 38 resp. 29 mg N L^{-1}). In a recurrent manner, nursery treatments were imposed to both reactors, at 25℃ and increased influent concentrations (NO_2^- and NH_4^+ = 320 resp. 240 mg N L^{-1}). The heating and cooling process commonly lasted ~3.3 and ~3.7 hours, respectively (Figure – S4.2).

Two SBRs were operated for 500 days. The effective volume of SBRs is 2.25 L (total volume of 2.5 L) with a volume exchange ratio of 33%. Each mainstream cycle lasted 2 hours, including 80 minutes of continuous feeding (and reaction), 30 minutes of settling, 6 minutes of decantation, and 4 minutes of idle time. That led to the targeted nitrogen loading rate of 248 mg N L^{-1} d^{-1} and resulted in a hydraulic retention time (HRT) of 6 h. During nursery treatment, the cycle time of 6 and 4 hours (320 and 200 minutes of continuous feeding and reaction, respectively) were chosen in R1 and R2, respectively, which was based on their AnAOB activity (to avoid both starvation and nitrite suppression). The sequencing batch mode was achieved through timers (EverFlourish EMT757-F, Germany) which was controlled by influent/effluent pumps (SEKO R1/R7, United States) and overhead stirrers (200 rpm) (Velp Scientific ES, Italy). The pH value (Hach HQ30d, United States) in the reactors was controlled in 7.3 – 7.5 (Figure – S4.3) by the influent pH control and reactors HCl dosing (0.1 mM) (Thermofisher Scientific FH10, United States).

4.2.2 Biomass source

The biomass inoculated into the two SBRs was the mixture of floccular (~50%) and granular sludges (~50%). The flocs were harvested from a full-scale DEMON® configuration (990 m^3, Breda, The Netherlands) with the particles size range within 0 – 1 mm (57.6 µm on average). The granular sludge was collected from a potato-processing wastewater treatment PN/A system (600 m^3, Olburgen, The Netherlands) with the particles size range within 0.45 – 2 mm (1237 µm on average). The initial average temperatures of the seed systems were about 32℃. After AnAOB enrichment at 20℃, the reactors decreased to 15℃ via two different pathways

(R1: 20℃ → 5℃ cold-shock for 6h → 15℃; R2: 20℃ → 15℃). The operation in R1 tested the effectiveness of the cold shock on anammox activity (Figure – S4.7 and S – 4.2 of the supporting information). The low temperatures were achieved by recirculating-chiller (Thermo-fisher Scientific thermoChill II-a, United States).

4.2.3 Experimental operation

Feasible nursery exposure ratio (the ratio of nursery sludge retention time to the overall time (nursery retention time plus the mainstream retention time) of 1/7 were estimated based on data from Nieuwveer STP (Breda, the Netherlands) and Strass STP (Strass, Austria) (Figure – S4.4 and S – 4.1 of the Supporting Information). The overall experiment was divided into three stages. In Stage-I (day 0 – 95), high nursery exposure ratio (2/7) was tested in both reactors to verify this novel concept. In Stage-II (day 96 – 275), the exposure ratio decreased to 1/7. Different nursery frequencies were tested at this ratio, including 2/14 (R1 and R2), 1/7 (R1) and 0.5/3.5 (R2). In Stage-III (day 276 – 470), the potential enhancement factors (i.e., high temperature, high effluent nitrogen concentrations, high EC value) under the nursery conditions were tested (treatment frequencies were 1/7 and 0.5/3.5 respectively in R1 and R2). The detailed information is shown in Table – 4.1 and 4.2.

For the enhancement caused by the temperature increase in the nursery reactor, it was assumed to follow the Arrhenius equation (Section – 2.7). During the test to determine the effect of effluent nitrogen concentration (the nitrogen concentration to which the bacteria are exposed in the continuous feed reactor), the effluent nitrogen concentration during nursery was decreased to that of the mainstream conditions (from ~24 to ~18 mg NH_4^+-N L^{-1} in R1 and from ~47 to ~13 mg NH_4^+-N L^{-1} in R2). To determine the effect of higher EC levels under nursery conditions (~4.4 mS cm^{-1} versus 1.1 mS cm^{-1}), the reactor was operated under mainstream conditions with high EC values, which was the same as nursery conditions (The concentration of ions other than NH_4^+ and NO_2^- was increased to the level of the nursery conditions, and the EC value gap between the obtained and the nursery conditions was supplemented with NaCl (because the NH_4^+ and NO_2^- was dosed in the form of NH_4Cl and $NaNO_2$).

Table – 4.1 Overview of all the operations in R1 and R2 during the 500 days. 25% of the total nitrogen load was assumed in sidestream (Figdore et al., 2018) which led to the nursery frequency = 1/7 (nursery/ (nursery + mainstream) = 1/7)

R1	Description	Day[1]	Temperature	Frequency	N load	N load ratio[2]	Effluent NH_4^+-N	EC
			°C		g N/L/d	Nursery/total	mg N L^{-1}	mS cm^{-1}
/	Baseline	1 – 56	15	/	0.27	/	18 ± 1	1.2
1 – a	High exposure ratio	57 – 105	25	2/7	0.75	53%	30 ± 19	4.4
/	Baseline	106 – 138	15	/	0.27	/	17 ± 2	1.1
1 – b	Low frequency	139 – 190	25	2/14	0.75	32%	21 ± 9	4.3
/	Baseline	191 – 233	15	/	0.27	/	16 ± 1	1.1
1 – c	High frequency	234 – 276	25	1/7	0.75	32%	27 ± 6	4.5
/	Baseline	277 – 317	15	/	0.27	/	17 ± 1	1.2
2	Low nitrogen loading	318 – 353	25	1/7	0.375	19%	4 ± 2	2.6
/	Baseline	354 – 372	15	/	0.27	/	16 ± 1	1.1
3	Low effluent nitrogen	373 – 401	25	1/7	0.62	28%	15 ± 4	4.4
/	Baseline	402 – 415	15	/	0.27	/	17 ±1	1.2
4	High EC level	416 – 444	15	1/7	0.27	/	14 ± 1	4.6
/	Baseline	445 – 500	15	/	0.27	/	17 ± 1	1.2

Note: 1. The period included the time of the lasting enhancement after bioaugmentation (3 – 14 days which depended on the nursery length).

2. The ratio meant the proportion of the N loading of the sidestream in the total N loading of the STP.

Table – 4.2 Overview of all the operations in R1 and R2 during the 500 days. 25% of the total nitrogen load was assumed in sidestream (Figdore et al., 2018) which led to the nursery frequency = 1/7 (nursery/ (nursery + mainstream) = 1/7)

R2	Description	Day[1]	Temperature	Frequency	N load	N load ratio[2]	Effluent NH_4^+-N	EC
			°C		g N/L/d	Nursery/total	mg N L^{-1}	mS cm^{-1}
/	Baseline	1 – 30	15	/	0.27	/	13 ± 1	1.1
1 – a	High exposure ratio	31 – 68	25	2/7	1.12	62%	30 ± 26	4.3
/	Baseline	69 – 97	15	/	0.27	/	12 ± 2	1.0
1 – b	Low frequency	98 – 172	25	2/14	1.12	41%	47 ± 10	4.3
/	Baseline	173 – 232	15	/	0.27	/	14 ± 1	1.0
1 – c	High frequency	233 – 276	25	0.5/3.5	1.12	41%	57 ± 4	4.4
/	Baseline	277 – 316	15	/	0.27	/	14 ± 1	1.0
2	Low Nitrogen loading	317 – 353	25	0.5/3.5	0.56	25%	3 ± 2	2.5
/	Baseline	354 – 372	15	/	0.27	/	13 ± 1	1.1
3	Low effluent nitrogen	373 – 400	25	0.5/3.5	0.79	31%	13 ± 4	4.3
/	Baseline	401 – 414	15	/	0.27	/	14 ± 1	1.0
4	High EC level	415 – 444	15	0.5/3.5	0.27	/	11 ± 1	4.5
/	Baseline	445 – 500	15	/	0.27	/	14 ± 1	1.1

Note: 1. The period included the time of the lasting enhancement after bioaugmentation (3 – 14 days which depended on the nursery length).

2. The ratio meant the proportion of the N loading of the sidestream in the total N loading of the STP.

4.2.4 Synthetic autotrophic wastewater

The composition and characteristics of the synthetic wastewater at mainstream and nursery conditions are shown in Table – 4.3 (the data of mainstream and sidestream in STP is shown in Table – S4.1). Trace element solution A/B was prepared according to Van de Graaf et al. (1995). The synthetic wastewater was deoxygenated by N_2 purging for 25 minutes, followed by pH adjustment of 6.9 – 7.0. That led to the DO in reactors being lower than 0.05 mg O_2 L^{-1}.

Table – 4.3 Composition of the synthetic wastewater at mainstream and nursery conditions. The calculation was based on the data from Nieuwveer STP 2016-2021 (Breda, the Netherlands) and assumed 57% of NH_4^+-N in sidestream was oxidized into NO_2^--N by partial nitritation (PN).

Chemical	Unit	Influent	
		Mainstream conditions	Nursery conditions
K_2HPO_4	mg P/L	3.2	50
$NaHCO_3$	mg HCO_3/L	350	1500
$MgSO_4 \cdot 7H_2O$	mg Mg/L	2.2	20
$CaCl_2 \cdot 2H_2O$	mg Ca/L	2	19.60
NH_4Cl-N	mg N/L	28	240
$NaNO_2$-N	mg N/L	36	320
TIN	mg N/L	62	560
Trace-A	ml/L	0.5	1.5
Trace-B	ml/L	0.5	1.5
EC	mS/cm	1.4	4.5
pH	-	7.0	7.0
Temperature	℃	15	25

4.2.5 Analytical procedures

Liquid samples were collected daily from the influent and effluent, and were stored at 4°C after filtering by 0.2 µm syringe filters (CHROMAFIL Xtra PVDF, Germany). Ammonium, nitrite, and nitrate were determined with a San^{++} Automated Wet Chemistry Analyzer (SKALAR, the Netherlands) and Ion Chromatography (Column: Metrosep A Supp 5- 150/4.0) (Metrohm – Eco IC, Switzerland). The microbial samples were collected weekly from reactors to measure the biomass concentration by volatile suspended solids (VSS) measurements (APHA, 1998). Electrical conductivity (EC) was measured by a handheld meter (Hach HQ30d, United States). The microbiome analysis was following the procedure described in Alloul et al. (2021) that a V4

region based on the 16S rRNA gene amplicon sequence variants (ASVs) were used (detail information was shown in S – 4.2 of the Supporting Information).

4.2.6 Calculations

4.2.6.1 Arrhenius equation

The effect of temperature on bacterial activity or metabolic rate is demonstrated by a simplified Arrhenius equation (Eq – 4.1) (Gilbert et al., 2015).

$$SAA_T = SAA_{max,15℃} \times \theta_{AnAOB}^{(T-15℃)} \quad (Eq-4.1)$$

SAA_T is the reference r_{max} at T°C [mg NH_4^+-N g^{-1} VSS d^{-1}]; $r_{max,\ 15℃}$ is the specific anammox activity at 15℃ [mg NH_4^+-N g^{-1} VSS d^{-1}]; θ is the temperature coefficient [unitless]; T is the respective temperature of the nursery reactor [℃]. The typical θ for anammox sludge range from 1.07 – 1.10 (Gilbert et al., 2015; Liu et al., 2020; Sobotka et al., 2016; Vandekerckhove et al., 2020).

4.2.6.2 SRT, growth rate, and SAA calculation

The calculation of sludge retention time (SRT), AnAOB growth rate (μ), and specific anammox activity (SAA) is shown from Eq – 4.2 to Eq – 4.4.

$$SRT = \frac{VSS_{reactor} \times V}{VSS_{effluent} \times Q_{effluent}} \quad (Eq-4.2)$$

$$\mu = \frac{1}{SRT} \quad (Eq-4.3)$$

$$SAA = \frac{ARR}{VSS_{reactor}} \quad (Eq-4.4)$$

$VSS_{reactor}$ and $VSS_{effluent}$ are the biomass concentration in reactor and effluent, respectively [g VSS L^{-1}]; V is the reactor volume [L]; $Q_{effluent}$ is the effluent flowrate [L d^{-1}]; ARR is the ammonium removal rate in the reactor [mg NH_4^+-N L^{-1} d^{-1}].

4.2.6.3 The temperature correction factor

The temperature correction factor (K) that corrected the biomass growth rate to mainstream (15℃) is shown in Eq – 4.5.

$$K = \frac{\left(t_{nursery} \times \theta^{\Delta T}\right) + (t_{mainstream} \times 1)}{(t_{nursery} + t_{mainstream})} \quad (Eq - 4.5)$$

$t_{nursery}$ and $t_{mainstream}$ is the length of nursery and mainstream [d]; ΔT is the temperature difference between two conditions [℃].

4.2.6.4 The enhancement determination

For the extra enhancement determination (E) caused by the potential factors (i.e., effluent nitrogen concentration, EC value), specific anammox activity of nursery reactor is divided by the normalized mainstream activity (normalized to 25°C by Arrhenius equation) (Eq – 4.6). The effect enhancement caused by temperature increase was based on Arrhenius.

$$E = \frac{SAA_{nursery}}{(SAA_{mainstream} \times \theta^{\Delta T})} \quad (Eq - 4.6)$$

$SAA_{nursery}$ and $SAA_{mainstream}$ are the specific anammox activity under nursery conditions and mainstream conditions, respectively [mg NH_4^+-N g^{-1} VSS d^{-1}]; ΔT is the temperature difference between mainstream and nursery conditions [℃].

4.3 Results and discussion

4.3.1 Anammox nursery reactor boosts AnAOB

4.3.1.1 The nitrogen removal rate was improved

This experiment has the objective to test the effect of nursery frequency (i.e., 1/7, 2/14, and 0.5/3.5) on day 0 to 275. The nitrogen conversion rates at mainstream conditions are presented in Figure – 4.2 (detailed information on reactor performance is shown in Figure – S4.5). During the nursery exposure ratio of 2/7 (i.e., nursery length/(the total duration of the nursery mode) = 2/7), the total nitrogen (TN) removal rates at mainstream was 56 – 68% higher

than the baseline (Figure – 4.2). This improvement lasted for 14 days (the number of days from the last nursery treatment to the TN removal rates back to baseline) after stopping the nursery treatment. Then, the nursery exposure ratio was decreased to 1/7. Different nursery frequencies were tested for this ratio, including 2/14 (R1 and R2), 1/7 (R1), and 0.5/3.5 (R2), which all promoted the mainstream TN removal rates with 33 – 36%, 38%, and 36%, respectively (Table – 4.3). But the enhancement gradually disappeared after 10 days, 6 days, and 3 days after the treatments, respectively.

The results confirmed that nursery treatment considerably boosted the mainstream nitrogen removal rate (33 – 38%). No influence of nursery frequency on the nitrogen removal rate enhancement was observed. For full-scale STP, high frequency is, therefore recommended because a lower frequency (e.g., 2/14) requires a larger reactor size (the biomass stay in the reactor for a longer time).

The lasting nitrogen removal improvement faded away after 3-14 days, and the performance of anammox returned to its original state (baseline). We could, hence, conclude that the nursery treatment was the sole cause of the improvement in nitrogen removal. In full-scale STP, the retained AnAOB sludge would flow in/out the nursery reactor continuously, yielding probably a longer-lasting yet lower performance-enhancing effect. Tan et al. (2016) revealed that the sidestream bioaugmentation has the risk to disrupt native microbes and break down the system. As a return-sludge biostimulation system, no external microorganisms were bioaugmented directly into the mainstream and no microorganisms were harvested from sidestream which can avoid the disturbances to the system. Zhang et al. (2018) reported that the bioaugmentation-exchange mode (the same amount of reactor biomass was discharged after bioaugmenting a certain amount of highly activated sludge), a similar process as nursery reactor, was superior to biomass bioaugmentation to alleviate oxytetracycline suppression.

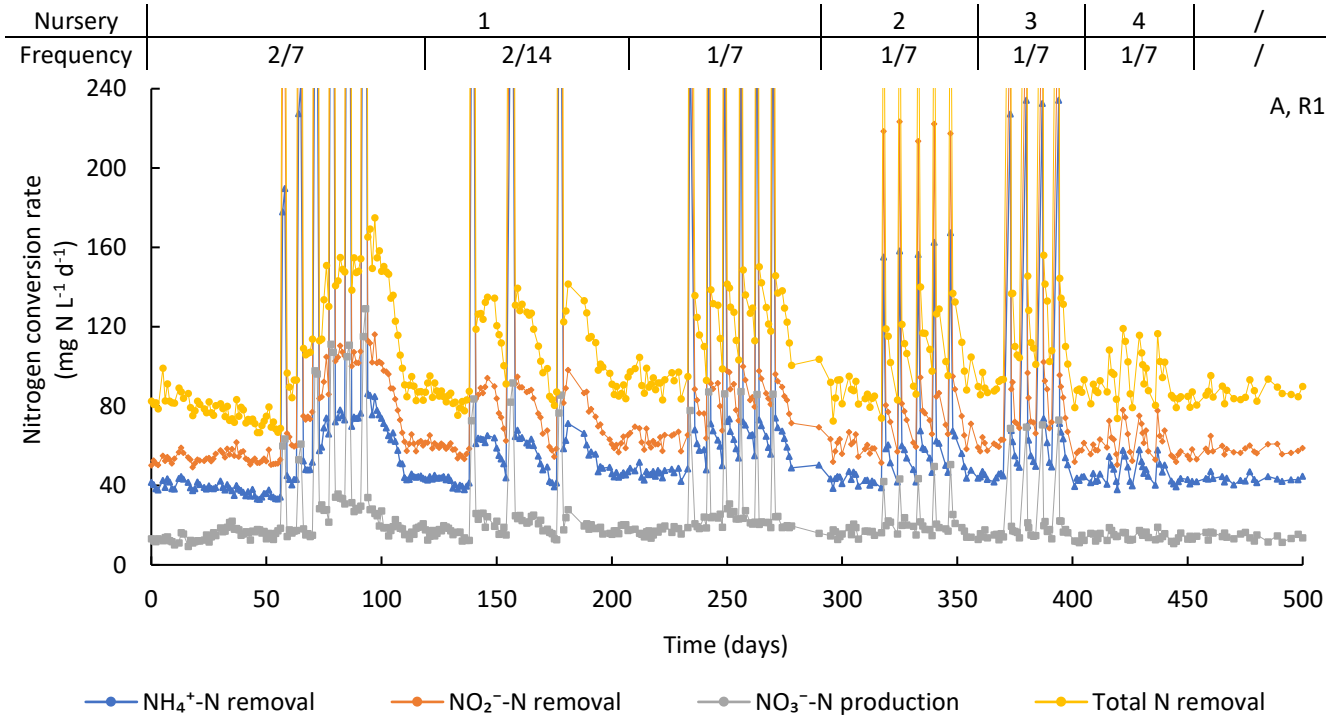


Figure – 4.2 (A) The nitrogen removal rates change in R1 over 500 days. The peaks in removal rates correspond to the nursery treatment.

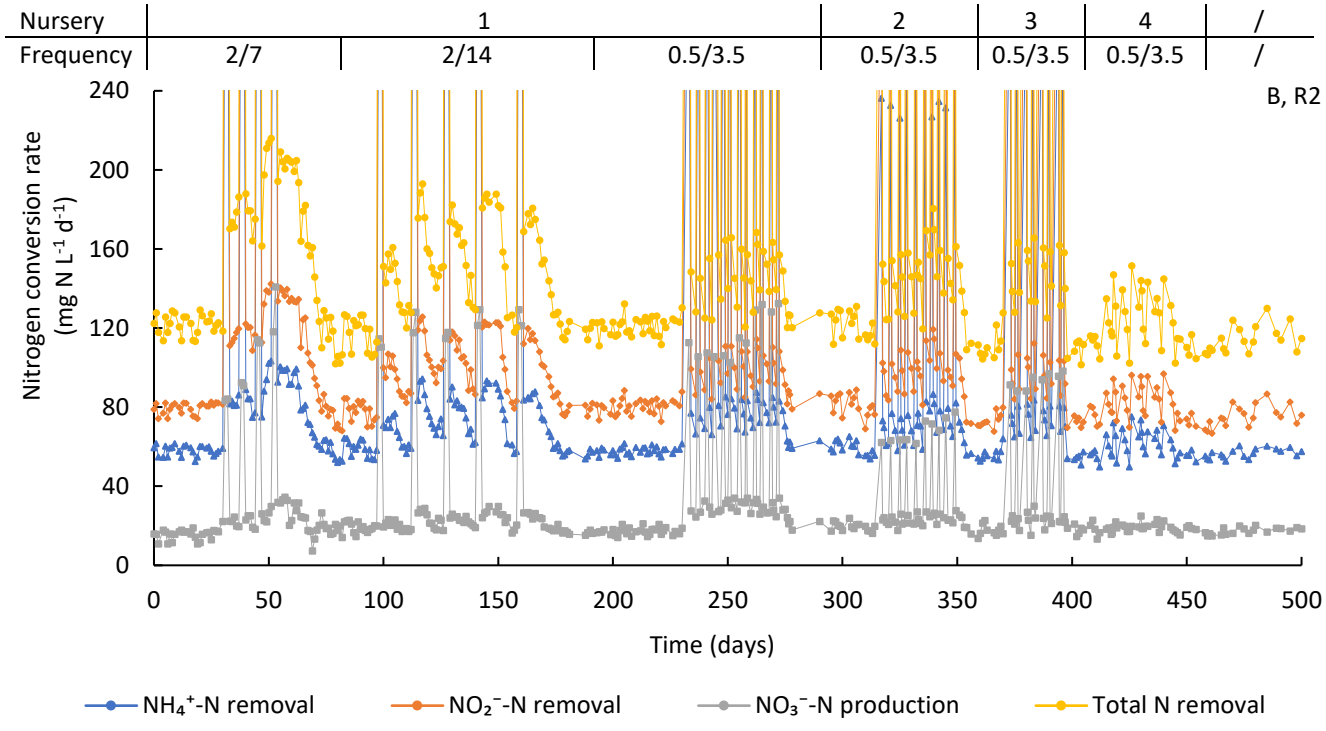


Figure – 4.2 (B) The nitrogen removal rates change in R2 over 500 days. The peaks in removal rates correspond to the nursery treatment.

4.3.1.2 The microbial growth rate was promoted

During the nursery treatment, the biomass concentration in the reactor increased even though more sludge was washed out with the effluent (Figure – 3A/B). Thus, more biomass was produced in the reactor. For the nursery exposure ratio of 2/7, the biomass concentration increased with an increment of ~4.2% (R1) and ~2.6% (R2) per nursery treatment, respectively. Even though the nursery exposure ratio decreased to 1/7 in the following tests, an increment of 2.8% and 1.8% per nursery treatment was still detected in R1 and R2, respectively, which indicated that the nursery condition could promote biomass growth.

Table – 4.3 The enhancement of TN removal rate and microbial growth rate (after temperature normalization) facilitated by the nursery.

Nursery	TN removal rate (mg N L^{-1} d^{-1})			Growth rate at 15℃ (d^{-1})		
	Before[1]	After[1]	Enhancement[2]	Before[1]	Nursery[3]	Enhancement
R1						
1 – a	78±7	131±26	68%	0.043±0.004	0.046-0.052	7.0-20.9%
1 – b	86±5	117±17	36%	0.043±0.008	0.045-0.048	4.7-11.6%
1 – c	92±6	127±14	38%	0.042±0.005	0.044-0.047	4.8-11.9%
2	86±7	111±16	29%	0.043±0.003	0.045-0.048	4.7-11.6%
3	90±4	121±20	34%	0.044±0.002	0.046-0.049	4.5-11.4%
4	88±4	94±11	7%	0.044±0.001	0.044-0.046	0-4.5%
R2						
1 – a	122±5	190±17	56%	0.041±0.003	0.043-0.049	4.9-19.5%
1 – b	116±9	154±18	33%	0.042±0.004	0.043-0.046	2.4-9.5%
1 – c	121±5	164±14	36%	0.041±0.003	0.043-0.046	4.9-12.2%
2	122±7	147±15	21%	0.042±0.005	0.044-0.047	4.8-11.9%
3	112±7	145±16	30%	0.041±0.003	0.042-0.045	2.4-9.8%
4	111±5	122±10	10%	0.038±0.001	0.038-0.040	0-5.4%

Note: 1. 'Before' and 'After' meant the values before and after the nursery, respectively. They are the average value of all the samples during the nursery treatment.

2. Enhancement was based on the TN removal rate or growth rate (after temperature correction) after nursery versus before (refer to mainstream).

3. The growth rate was temperature corrected according to Arrhenius (θ = 1.07-1.10). But this was not applicable for TN removal rate.

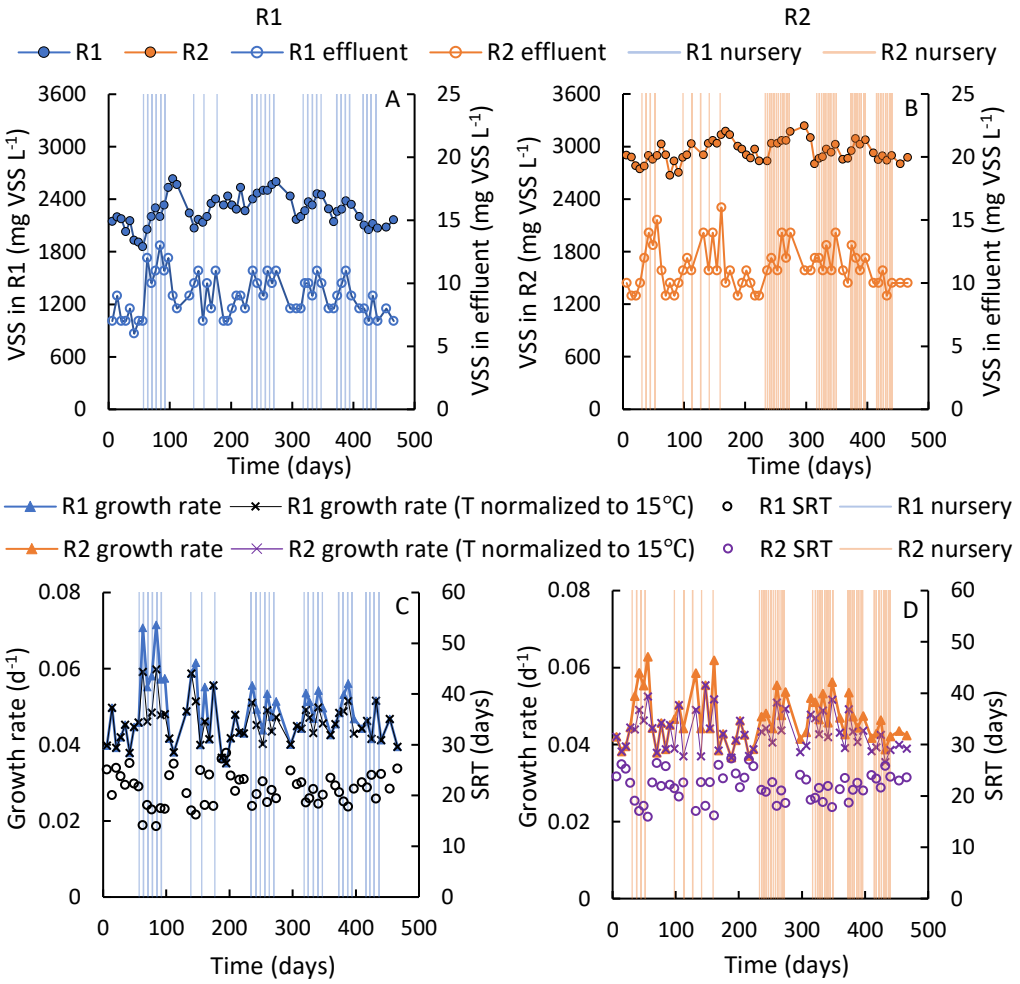


Figure – 4.3 Change of the biomass concentration and the growth rate over time. A, C: R1; B, D: R2. The temperature correction factor (K) in C and D was calculated based on the θ value of 1.085 (average of the range 1.07-1.10).

To verify the main reason for the growth rate promotion, the temperature-normalized biomass growth rate (Eq – 4.5) is shown in Figure – 4.3 and Table – 4.3. The relatively stable values (less than 12% fluctuation at the nursery exposure ratio of 1/7) demonstrated that the increase in biomass concentration was mainly due to the temperature increase during the nursery. Stopping the nursery treatment resulted in a decrease in the biomass concentration in both reactors. The temperature decrease did not only reduce the growth rate of the microorganisms but also affected the sedimentation capacity of the sludge (Cui et al., 2014; Lotti et al., 2015c), which caused more biomass washout after nursery treatment. In addition,

the biomass growth rate and AnAOB specific activity showed a correlation. However, the growth rate improvement did not completely follow the activity enhancement, which meant anammox activity biostimulation was not only enhanced by the higher temperature, but also by other underlying factors (Section – 3.2).

Except biostimulation (adding nutrients/substrates to stimulate bacteria activity), bioaugmentation (adding microorganisms to improve pollutants removal efficiency) (Liu et al., 2017) seemed also to play a role in the enhancement of mainstream nitrogen removal in the nursery concept. Different from the sidestream sludge bioaugmentation process (Figdore et al., 2018; Mannucci et al., 2015) that the mainstream performance improvement mainly attributed to the increase of biomass concentration (bioaugmentation), the mainstream efficiency enhancement caused by nursery reactor was mainly attributed to the improvement in AnAOB activity.

4.3.1.3 Anammox community kept stable

Three AnAOB genera Candidatus *Brocadia*, Candidatus *Kuenenia*, and Candidatus *Jettenia* were detected in both reactors, with Candidatus *Brocadia* being the most dominant (~0.3 in R1 and ~0.4 in R2) (Figure – S4.7), a relative abundance less than 0.01 was detected for the other two genera. At the nursery exposure ratio of 2/7, the relative abundance of the dominant genus Candidatus *Brocadia* increased to 0.41 and 0.51 in R1 and R2 compared to baseline (0.29 in R1 and 0.39 in R2). Then, during the whole experiment at the nursery exposure ratio of 1/7, the anammox community was relatively stable (less than 10% fluctuation). Candidatus *Brocadia* was again dominant (e.g., 0.35 versus 0.33 at the nursery frequency 1/7 (R1) and 0.50 versus 0.46 at the nursery frequency 0.5/3.5 (R2)). This observation, therefore, confirms that the nursery concept does not lead to a community shift (e.g., versus EssDe®).

A ~14% higher relative abundance of Candidatus *Brocadia* was observed in R2 compared to R1 (0.42 ± 0.05 versus 0.28 ± 0.04). This was also reflected in the higher AnAOB activity (± 30%). The difference in community composition of R1 and the R2 baseline might be attributed to the cold-shock treatment of R1 (S – 4.2 of the Supporting Information and Figure – S4.6), which decreased the relative abundance of the predominant genus and AnAOB activity (Figure – S4.7).

The microbial diversity (refer to Shannon index) was also relatively stable, even though a small decrease appeared after nursery biostimulation (6.37 ± 0.28 versus 6.73 ± 0.32 in R1 and 5.47 ± 0.22 versus 5.97 ± 0.19) at a frequency of 1/7. The decrease in diversity was in line with

Okonkwo et al. (2020), who reported that even a short-term temperature differences (ΔT = 20℃, 48h) lead to a decrease in microbial diversity. The nursery condition likely selected the species by sludge washout (effluent) and competition (temperature-based metabolism). In the nursery concept, only 1/7 of the time was run at a high temperature, which limited the shift of the community. A more specialized community might be formed if the longer nursery exposure ratio was applied (e.g., nursery exposure ratio = 2/7, the Shannon index was only 5.94 (R1) and 4.97 (R2)).

According to Zhang et al. (2018), the abundance of functional microorganisms with niche differentiation would affect the survival of the inoculated microbial (e.g., less sludge adaptation and more biomass washout (Abeysinghe et al., 2002)). That was probably ascribed to the interactions between indigenous and inoculated microorganisms (e.g., competition, maladjustment, and microbial ecosystem imbalance), which commonly would lead to the failure of bioaugmentation (Liu et al., 2017). This problem could be tackled by the nursery concept proposed in the present research since a relatively stable community was maintained, which is the advantage of the nursery reactor over other external biomass bioaugmentation technologies. Bioaugmentation with sludge (less niche difference to mainstream) ensured the consistency of the biostimulated AnAOB in the nursery reactor, with the dominant AnAOB in the mainstream system. For the mainstream bioaugmentation with the sidestream biomass, an obvious increase of the relative abundance of nitrifiers and community diversity was found (Stenström and la Cour Jansen, 2017), which led to a more niche difference between the two reactors. Podmirseg et al. (2010) revealed that not only the mainstream but also the sidestream community underwent a considerable shift after bioaugmentation.

Even the BABE process, a concept similar to the nitrification nursery system, reported a rise in microbial diversity (Gatti et al., 2015). Unlike the present study, part of the return sludge flowed directly into the sidestream reactor to mix the microorganisms living in the separation system in the BABE process. But for the nursery concept, a system that was independent of the sidestream and mainstream was created.

4.3.2 Operating factors contribute to the biostimulation

4.3.2.1 Temperature

Compared to the mainstream conditions, the specific anammox activity under nursery conditions was about 271% and 261% higher in R1 (nursery frequency of 1/7) and R2 (nursery frequency of 0.5/3.5), respectively (Figure – 4.4). The effect of temperature on AnAOB activity has been broadly studied in the past years (Gilbert et al., 2015; Lotti et al., 2015c; Zhu et al., 2017b). In general, the relationship of the specific anammox activity under different temperatures follows the Arrhenius equation (Gilbert et al., 2015; Seuntjens et al., 2018d). It enables to estimate the activity enhancing from the mere temperature increase. Based on a typical temperature correction value (θ = 1.07-1.10, $\Delta T \approx 10$℃), a specific anammox activity was expected to be 97 to 159% higher under nursery conditions.

The low temperature could severely affect the anammox process as it might inhibit the growth rate, microbial activity, substrate utilization, and even the settling capacity of AnAOB (Cui et al., 2014). In the ScanDeNi process (Rosén and Huijbregsen, 2003), all of the return sludge flowed into the sidestream reactor, which resulted in a significant drop in temperature and substrate concentration. Compared with ScanDeNi, the BABE process was used by many STP because moderate temperature and substrates conditions for nitrifiers were maintained (Salem et al., 2003). Compared to nitrifiers, AnAOB were even more sensitive to temperature change (θ was 1.07 – 1.10 for AnAOB versus 1.02 – 1.10 for nitrifiers) (Gilbert et al., 2015). A temperature increase would, therefore, benefit AnAOB more than AerAOB due that with low growth rate (0.02 d^{-1} at 20℃ and only 0.005 d^{-1} at 10℃) (Lotti et al., 2014c, 2015)) in the nursery concept.

A smaller temperature difference (ΔT) from the mainstream would limit the temperature shock to microorganisms (Figdore et al., 2018; Wett et al., 2011). When the ΔT was higher than 10℃, cold-shock occurred for bacteria. At these conditions, a lag phase appeared, bacterial growth rate was suppressed, and specific proteins (cold-shock proteins) might be produced (Mannucci et al., 2015) which caused a reduction of bacterial activity. This was in line with the finding of Head and Oleszkiewicz (2004), who reported that nitrifiers cultured at elevated temperatures (i.e., 20, 25, and 30°C) suffered a considerable decrease in nitrification performance after inoculating into the 10℃ conditions. Additionally, Plaza et al. (2001) indicated a greater ΔT affected the bioaugmentation efficiency in a pilot-scale activated sludge

system. Compared to the sidestream reactor, nursery conditions have an intermediate temperature difference (> 15°C versus ~10°C) which played an important role in bacteria biostimulation.

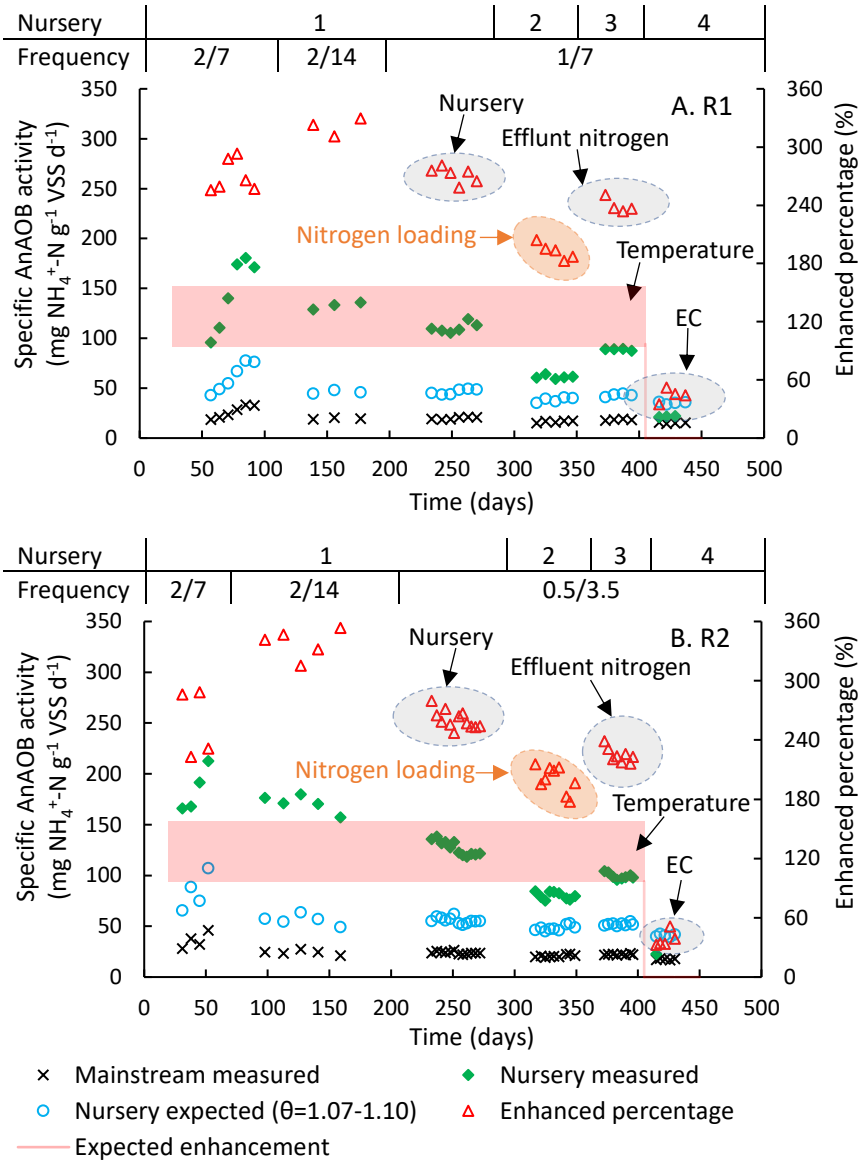


Figure – 4.4 Measured and expected specific AnAOB activity under nursery conditions. For every nursery bio-stimulation, the mainstream activity of one day before the treatment was given, and the expected activity was obtained by applying an Arrhenius equation (θ = 1.07-1.10). Expected enhancement meant the assumption that only the elevated temperature played a role in anammox reactivation. A, R1; B, R2.

After temperature correction, there was a 112 to 174% (R1) and 87 to 164% (R2) surplus enhancement. That meant not only the higher temperature (~35 to 61% of the total enhancement, Figure – S4.8) but also other factors were playing as well (~39 to 65%), such as the higher effluent nitrogen concentrations and EC levels (Table – 4.1).

4.3.2.2 Effluent nitrogen concentration

Next to the temperature difference, the effluent nitrogen concentration under nursery and mainstream conditions was also different (24 ± 8 versus 18 ± 1 mg NH_4^+-N L^{-1} in R1 and 47 ± 13 versus 13 ± 1 mg NH_4^+-N L^{-1} in R2) (Table – 4.1). The effect of effluent nitrogen concentration on nursery reactor was, therefore, tested during nursery conditions. After decreasing the effluent nitrogen concentration to that of the mainstream values, there was a 239% (R1) and 225% (R2) activity enhancement (Figure – 4.4). Compared to the activity enhanced under nursery conditions (Nursery – 3), the effluent nitrogen concentration contributed to a 32% (R1) and 36% (R2) activity increase. That contributed to 12% (R1) and 14% (R2) of the total enhancement in the nursery conditions (Figure – S4.8). In addition, 4.7-11.6% and 4.8-11.9% bacterial growth rate (normalized by Arrhenius equation) increase were found in R1 and R2 during these tests (Table – 4.3).

The effluent nitrogen concentration played an important role in the nitrogen removal efficiency. According to Wang et al. (2019), the nitrogen removal efficiency decreased after reducing the residual effluent nitrogen concentration. That was probably because the substrates limitation conditions could reduce the microbial growth rate/activity, and/or lead to the deterioration of granules (Hu et al., 2013; Laureni et al., 2015). That was proved by Zięba and Janiak (2017), who found a nitrogen-rich condition was an important parameter to maximize the bacterial yield. The higher target compound (NH_4^+-N and NO_2^--N) concentrations in the nursery reactor provided the unlimited substrates for AnAOB, which was beneficial for the AnAOB activity (34% versus 38% in R1 and 30% versus 36% in R2) and microbial growth rate (9.5% versus 4.7-11.6% in R1 and 9.8% versus 4.8-11.9% in R2) recovery compared to the mainstream conditions (Table – 4.3).

Excluding the promotive effects of temperature (97-159%) (Section – 3.1) and effluent nitrogen concentration (32% (R1) and 36% (R2)), a further 80-142% (R1) and 66-128% (R2) of the increase in activity was unexplained. The higher EC values (referred to the concentration of the nutrients) were expected to contribute to that.

4.3.2.3 The EC level

Next to the difference in temperature and effluent nitrogen concentration, the EC levels (referred to chemicals concentration, e.g., HCO_3^-, PO_4^{3-}, Mg^{2+}, etc.) were also different between nursery and mainstream conditions (Table – 4.1). The roles of high EC levels under nursery conditions were therefore determined. As shown in Figure – 4.4, after increasing the EC to the level of nursery conditions, an average of 44% (R1) and 38% (R2) activity increase was found.

The EC levels is an essential parameter in wastewater treatment. During the previous bioaugmentation research, low levels commonly would lead to failure that was ascribed to the severe microbial competition (Martín-Hernández et al., 2012). High EC supplementation improved the performance of the bacteria, which might attribute to the increased growth rate and resistance to external conditions (Ramadan et al., 1990). Even though a relatively stable bacterial growth rate was demonstrated under high EC levels in the present research (0-4.5% increase in R1 and 0-5.4% increase in R2, Table – 4.3), the obvious increase in the nitrogen removal rate suggested the benefits of EC levels in nursery reactors.

Excluding the contribution of high temperature, effluent nitrogen concentration, and EC levels, there was still 36-98% (R1) and 28-90% (R2) surplus AnAOB activity increase. That surplus effect was possibly attributed to the synergy of these three factors and/or some unknown factors. Wang et al. (2018b) found that the activity of anammox was affected by the joint roles of temperature and nitrogen concentration, which demonstrates the existence of synergistic effects. The unknown factors that were crucial for the AnAOB growth were also found during the anammox bioaugmentation by Tang et al. (2011).

Thus, four factors contributed to the performance enhancement caused by nursery conditions. High temperature (25 versus 15℃), high effluent nitrogen concentration (21 – 57 versus 12 – 18 mg NH_4^+-N L^{-1}), high EC levels (4.3 – 4.6 versus 1.0 – 1.2 mS cm^{-1}), and the synergy and unknown factors, which contributed to 35 – 60%, 12 – 14%, 15 – 16%, and 10 – 36%, respectively (Figure – S4.8).

4.3.3 Implication and outlook

4.3.3.1 Potential application advantages

The nursery reactor provides an environment similar to mainstream conditions (fewer adaptation problems (Parker and Wanner, 2007)) while ensuring that anammox activity is

boosted (higher temperature, nitrogen, and chemicals concentration, etc.). In the nursery reactor, not only the AnAOB activity is enhanced (biostimulation), but also the anammox biomass is enriched (bioaugmentation). Moreover, the relatively stable community (less competition and predation) may make this technique a better choice than the sidestream sludge bioaugmentation approach. Since the retained sludge flows into the nursery reactor continuously in the full-scale STP, the nitrogen removal rate can be enhanced stably instead of fluctuant as simulated in the present research.

After a long-term operation in the mainstream, the morphological changes of biomass were not obvious. Although the average particle size decreased, other properties (e.g., color, settling ability, etc.) remained stable. This indicates, to some extent, the stability of the system.

4.3.3.2 Outlook

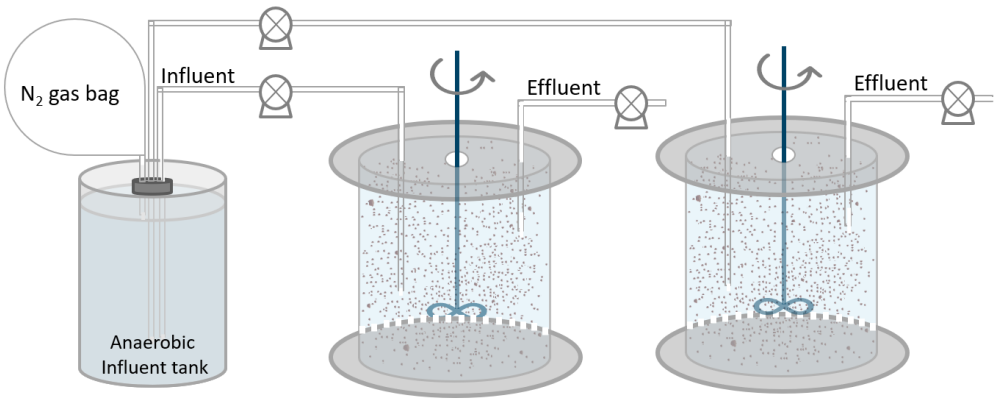
Although promising, there are still some research gaps for the anammox nursery concept. First, the effect of lower mainstream temperature (e.g., < 10℃) on the enhancement by nursery reactor should be tested. According to Salem et al. (2003) and Stenström and la Cour Jansen (2016), the greater nitrification enhancement appeared under the lower mainstream temperature when operating the BABE system. However, the consistency of the situation in the anammox nursery reactor still needs to be investigated. Second, the effect of the temperature difference (ΔT) between the mainstream and the nursery reactor on the enhancement of nitrogen removal is also worth investigating. The variation in ΔT corresponds to different nursery exposure ratios (e.g., 1/7 in this study), which also leads to variations in costs (capital and operating expenses, i.e., CAPEX and OPEX). Third, the performance of anammox nursery to treat the real wastewater instead of the synthetic wastewater. The complex influent composition and the fluctuation of the temperature may influence the enhancement performance. Fourth, whether the anammox nursery works at the STPs with low sidestream loading rate (e.g., 10%). Even though a low value (19%) within the typical range of STPs (15 – 25%) (Tan and Shuai, 2015) presented the promising results in the present research (S – 4.3 of Supporting Information), the operation at a very low nitrogen loading in the sidestream is still needs further research. In the end, the cost assessment is still missing here due to the limited data about the full-scale mainstream PN/A system.

4.4 Conclusions

This study showed that the novel nursery reactor concept has the potential to enhance mainstream anammox performance and that the nursery frequency showed no influence on the performance enhancement. The temperature increases of about 10°C were the main contributor (~50%) to the nitrogen removal efficiency enhancement and it also caused more biomass production in nursery conditions. The remaining ~50% was due to the higher effluent nitrogen concentrations, EC levels, and their synergy or some unknown factors. The mainstream return-sludge nursery approach, the combination of biostimulation and bioaugmentation, seemed promising, as the anammox community remained relatively stable (dominated by *Candidatus* Brocadia), and maybe a good alternative compared to sidestream to mainstream bioaugmentation approaches.

Supporting Information

Figure – S4.1 Scheme of the reactor setup.



- ✓ Reactor: 2 SBR reactors (2.25L with volume exchange ratio of 33%)
- ✓ Biomass concentration: ± 3 g VSS/L
- ✓ DO: < 0.05 $mgO_2\,L^{-1}$
- ✓ Temperature: 15℃ (mainstream); 25℃ (nursery)
- ✓ Nursery exposure ratio: 1/7

Figure – S4.2 The heating and cooling process during the transformation between nursery and mainstream conditions. Take the temperature change of R1 from day – 241 to day – 243 as an example.

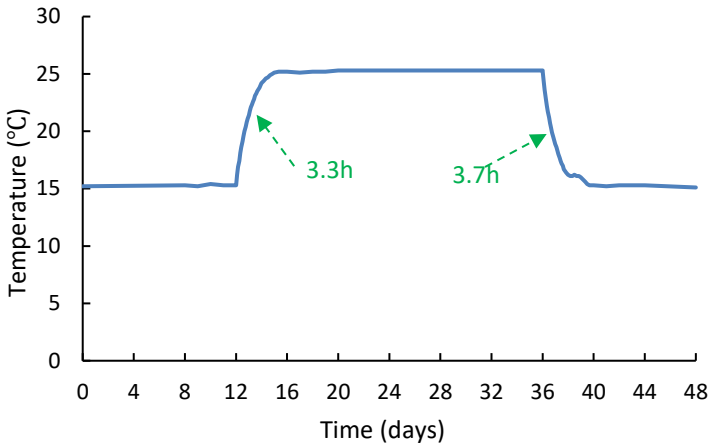


Figure – S4.3 The change of reactor temperature (A + B) and pH (C + D) over the 500 days experiment. A, C: R1; B, D: R2.

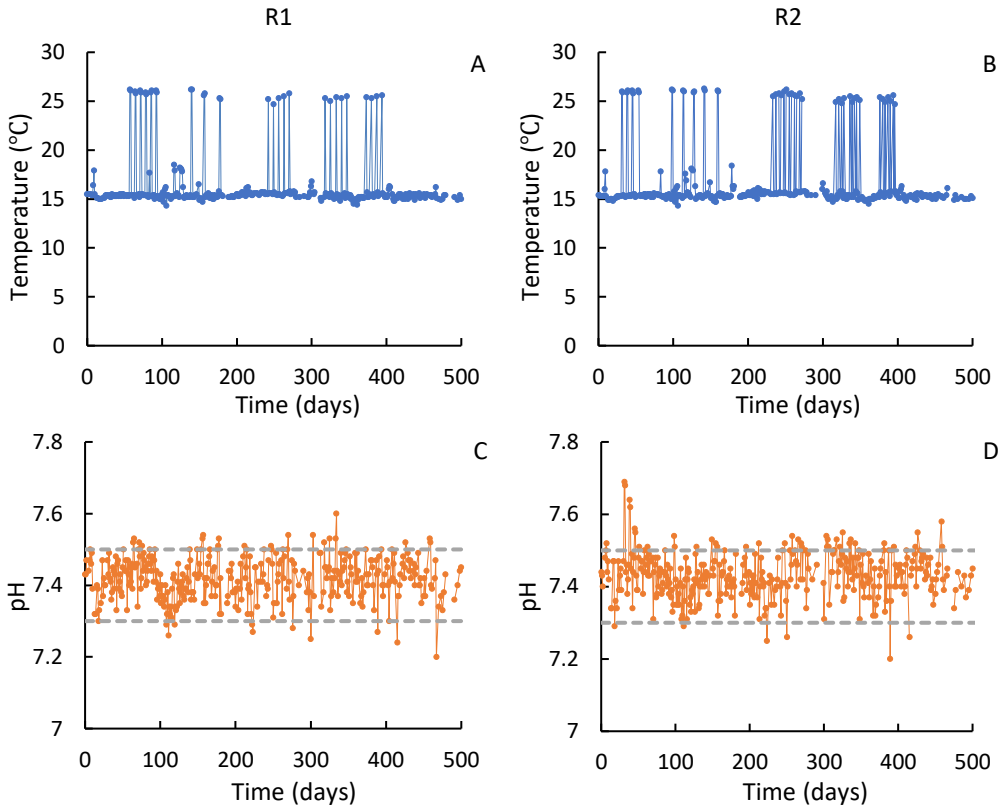
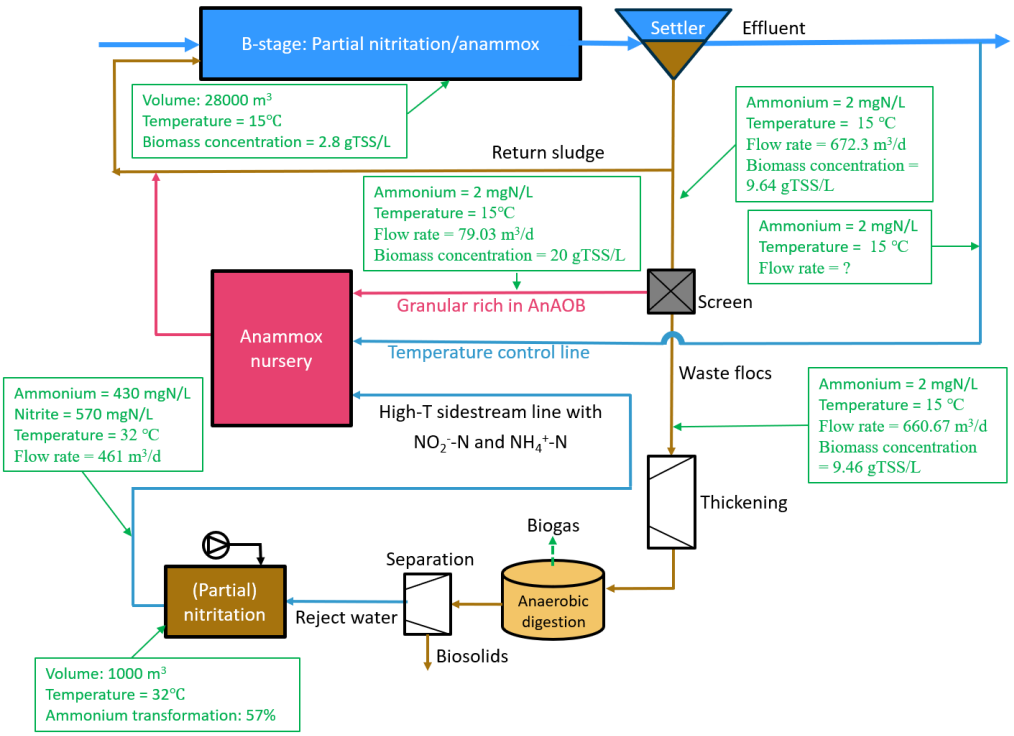


Figure – S4.4 The operational data (green) during the calculation of nursery frequency. The values were based on Strass WWTP (Austria), Nieuwveer STP (the Netherlands), and Van Winckel et al. (2019b).



S – 4.1 The calculation of the nursery exposure ratio (1/7)

Assumptions:

i) all the rejected water flows into the nursery reactor, ii) sidestream PN reactor will transform 57% of ammonium to nitrite, iii) the temperatures of mainstream, nursery reactor, and sidestream are 15 ℃, 25 ℃, and 32℃, iv) VSS/TSS = 0.9, v) the AnAOB activity at 15℃ was 25 mg NH_4^+-N/g VSS/d (19 – 31 mg NH_4^+-N/g VSS/d from R1 and R2 of the present research, and the average was reported), and vi) Arrhenius temperature constant (θ) was 1.07 – 1.10 (Gilbert et al., 2015; Liu et al., 2020; Sobotka et al., 2016; Vandekerckhove et al., 2020).

Calculation process:

Step 1: The flow rate of the temperature control line (Q):

(79.03 m^3/d + Q) × (25℃-15℃) = 461 m^3/d × (32℃-25℃), Q = 243.67 m^3/d

Step 2: NH_4^+-N concentration (X) and NO_2^--N concentration (Y) in nursery reactor:

79 m^3/d × 2 mg N/L + 244 m^3/d × 2 mg N/L + 461 m^3/d × 430 mg N/L = (79 + 244 + 461) m^3/d × X, X = 254 mg NH_4^+-N/L

461 m^3/d × 570 mg N/L = (79 m^3/d + 244 m^3/d + 461 m^3/d) × Y, Y = 335 mg NO_2^--N/L

Step 3: The total ammonium loading of the nursery reactor (L):

L = 254 mg NH_4^+-N/L × (79 + 244 + 461) m^3/d = 199 kg NH_4^+-N/d

Step 4: The biomass concentration (C_x) in nursery reactor:

C_x = [20 g TSS/L × 79 m^3/d ÷ (79 + 461 + 244) m^3/d] = 2.02 g TSS/L ≈ 1.82 g VSS/L

Step 5: The excepted AnAOB activity at nursery condition ($SAA_{25℃}$):

$SAA_{25℃}$ = 25 mg NH_4^+-N/g VSS/d × 1.07 (or 1.10)$^{(25℃-15℃)}$ = 0.049 to 0.065 g NH_4^+-N/g VSS/d

Step 6: The volume of nursery reactor (V):

V = 199 kg NH_4^+-N/d ÷ (0.049 g NH_4^+-N/g VSS/d (or 0.065) × 1.82 gVSS/L) = 1682 to 2231 m^3

Step 7: The biomass retention time in the nursing reactor (SRT-1):

SRT-1 = 1682 m^3 (or 2231 m^3) ÷ (79 m^3/d + 244 m^3/d + 461 m^3/d) = 2.1 to 2.8 d

Step 8: The biomass retention time in the mainstream (SRT-2):

SRT-2 = 28200 m^3 × 2.8 g TSS/L ÷ (672.3 m^3/d × 9.64 g TSS/L) = 12.2 d

Step 9: The nursery exposure ratio (R):

R = 2.1 d (or 2.8 d) ÷ (12.2 d + 2.8 d) = 1/6.8 to 1/5.4

The residence time of AnAOB in the nursery reactor was not fixed (it can be shorted by increasing the flow rate of the waste sludge (return certain flocs from waste sludge)). But the

nursery exposure ratio (residence time in nursery reactor divide by the overall time) was always constant. Thus, the nursery exposure ratio was 1/6.8 (~1/7), the worst case was considered in the present research.

Table – S4.1 Composition of the wastewater at mainstream and nursery conditions. The data was estimated based on data from both Nieuwveer STP (Breda, the Netherlands) and Strass STP (Strass, Austria).

Chemical	Unit	In practice (STP)	
		Reject water	N-Stage effluent[a]
K_2HPO_4	mg P/L	104	1.7
$NaHCO_3$	mg HCO_3/L	3000	39
$MgSO_4 \cdot 7H_2O$	mg Mg/L	16.10	0
$CaCl_2 \cdot 2H_2O$	mg Ca/L	39.18	0
NH_4Cl-N	mg N/L	1000	1.6
$NaNO_2$-N	mg N/L	0	0.54
TIN	mg N/L	1000	11
Trace-A	ml/L	2	0
Trace-B	ml/L	2	0
EC	mS/cm	6.0	1.5 - 2.0
pH	-	6.5 - 7.0	7.0
Temperature	℃	33	15

Note: a, Nieuwveer is a two-stage STP receiving municipal sewage with a flow rate of about 75,000 m^3 d^{-1} (~485,000 population equivalents). The C-stage is the high-rate activated sludge (HRAS) treatment that redirects a fraction of the incoming COD to the anaerobic digester. The N-stage treats the effluent of the C-stage to remove the nitrogen via nitrification/denitrification (N/DN).

Figure – S4.5 The influent and effluent nitrogen concentration, and the conversion ratios in R1 and R2. A, C, E: R1; B, D, F: R2. The referred theoretical conversion ratios were based on Strous et al. (1998) and Lotti et al. (2014b).

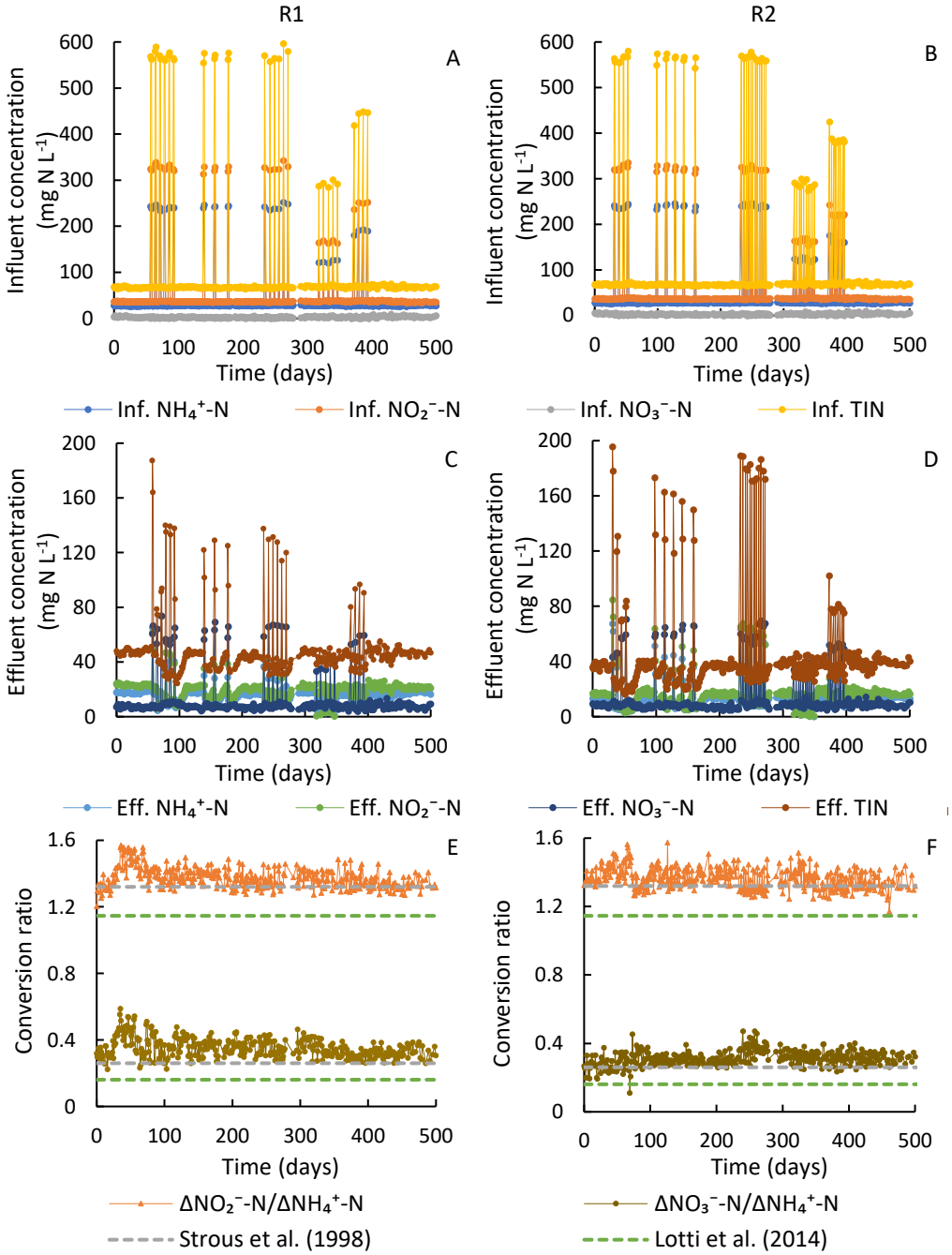
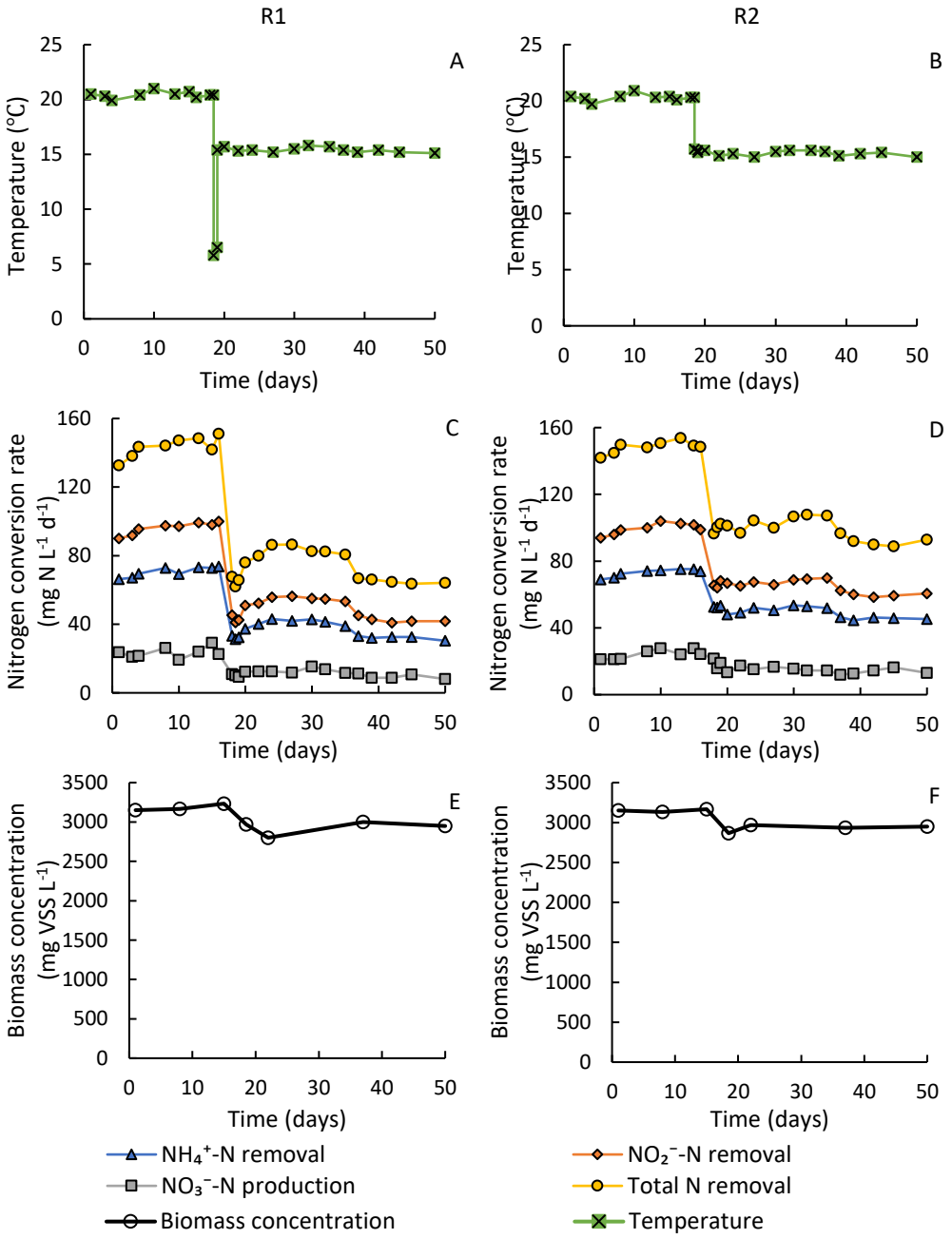


Figure – S4.6 The cold-shock (5℃, 6h) pre-experiment for anammox sludge. A, C, E: R1; B, D, F: R2.



S – 4.2 Biomass cold-shock in R1

For the effectiveness of the bacteria cold-shock (R1: 20℃ → 5℃ (6h) → 15℃), instead of promoting, it inhibited the activity to some extent (Figure – S4.5). This result contradicted the finding of Kouba et al. (2021), who found the cold shock (35℃ → 5℃ (8h) → 15℃) could significantly promote the nitrogen removal performance (136 ± 101% enhancement). Different temperature differences during the cold-shock (Δ30℃ versus Δ15℃) and the different temperatures of the original reactors (35℃ versus 20℃) might explain that. In addition, the different biomass types (PN/A sludge versus anammox) could also be a potential reason. More research is still needed to verify that.

According to the cold-shock test in the present research (Figure – S4.5), the microorganisms' activity would be inhibited (± 25%) when the temperature difference (ΔT) between two reactors was 15℃ (73.04 ± 9.16 versus 98.96 ± 6.22 mg N L^{-1} d^{-1} in TN removal rate).

Figure – S4.7 The community diversity changes and the relative abundance of the dominant nitrogen removal-related genera (AnAOB (orange), AerAOB (green), NOB (blue), and denitrifier (purple)), expressed relatively over the total community. A, R1; B, R2. '/' meant the baseline.

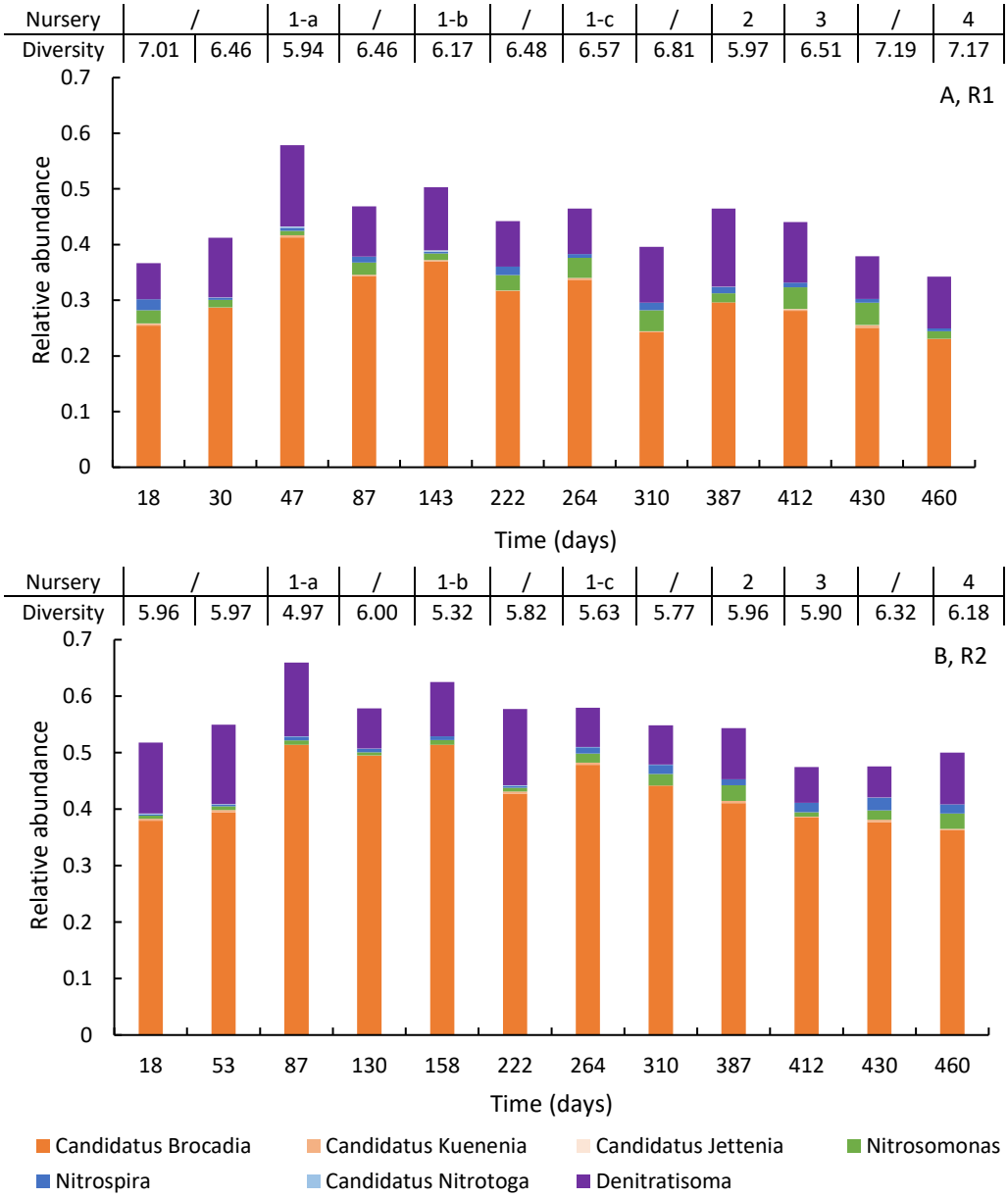
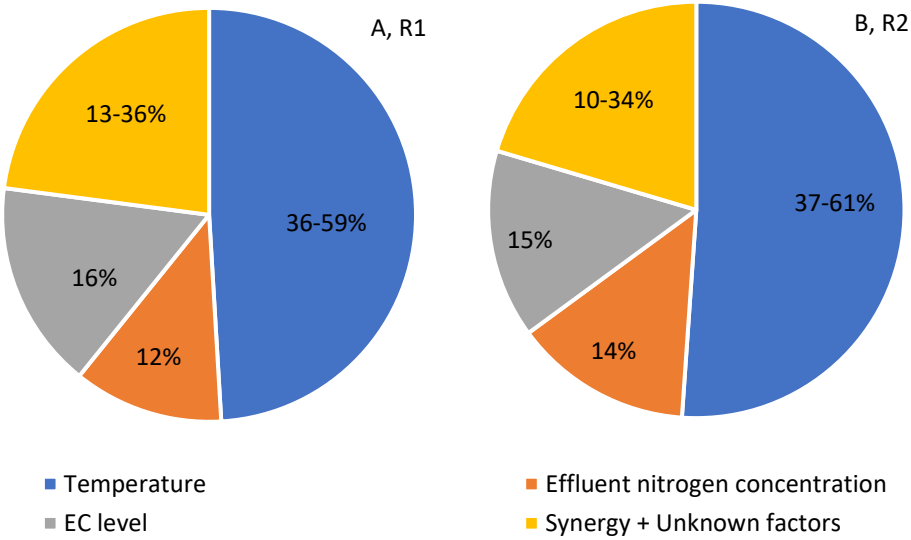


Figure – S4.8 The contribution percentage (%) of four factors to the AnAOB activity enhancement at nursery conditions. A, R1; B, R2.



S – 4.3 The nursery performance at the low loading rate

The nitrogen load used during the nursery biostimulation was 7 – 16% higher than those, which would be employed in the application (15 – 25% of the total nitrogen load in the sidestream (Tan and Shuai 2015)). That was due to the AnAOB activity in lab-scale was higher than that in the pilot- or full-scale application (caused by fluctuation of temperature and complex sewage composition) (Seuntjens et al. 2016). To avoid starvation in the present research, the nitrogen loading rate was 7 – 15% higher. During the nursery treatment – 2, half of the nitrogen loading rate was applied.

The results demonstrated that, the anammox performance could also be considerably promoted (± 200% enhancement) in the nursery reactor even though the nitrogen load of the sidestream (versus the total nitrogen load of STP) decreased to 19% (Figure – 4.4A). The growth rate was also enhanced with a percentage of 4.7-11.6% (Table – 4.3). That was expected since the effluent nitrogen concentration only contributed 12 – 14% to the performance enhancement.

Chapter 5

Characterization of N_2O emissions linked to nitrite-oxidizing bacteria suppression strategies in a biofilm reactor

Publication of a redrafted version of this chapter is intended: Zhu, W., Van Tendeloo, M., De Paepe, J., Vlaeminck, S.E. Characterization of N_2O emissions linked to nitrite-oxidizing bacteria suppression strategies in a biofilm reactor. Manuscript in preparation.

Abstract

In mainstream partial nitritation/anammox (PN/A), inhibition of nitrite-oxidizing bacteria (NOB) and mitigation of nitrous oxide (N_2O) emissions are two important operational goals. In this study, the N_2O emissions, linked to three typical NOB suppression strategies (low dissolved oxygen (DO) concentration, free ammonia (FA), and free nitrous acids (FNA) treatments) were tested in a covered rotating biological contactor (RBC) system. A low emerged DO level (~0.60 mg O_2 L^{-1}) was effective to suppress NOB activity and decrease N_2O emissions, but NOB adaptation gradually appeared after 200 days of operation. Further NOB suppression was successfully achieved by periodical (3 hours per week) FA (29.3 ± 2.6 mg NH_3-N L^{-1}) or FNA (3.1 ± 0.3 mg HNO_2-N L^{-1}) treatments. However, FA treatment led to the promotion of N_2O production, whereas FNA treatments did not have an effect. Thus, PN/A systems should be operated at relatively low DO levels with periodical FNA treatment, which could also avoid high N_2O emissions caused by applying NOB suppression strategies.

5.1 Introduction

Nitrogen removal is the key to alleviating eutrophication in wastewater treatment. The process of Partial nitritation/anammox (PN/A) has been widely implemented in the sidestream (i.e., sludge line) of sewage treatment plants (STP) due to its advantages over the traditional nitrogen removal technologies (e.g., nitrification/denitrification), such as the lower energy and carbon demand as well as the reduced sludge production (Agrawal et al., 2018; Ali et al., 2016; Lackner et al., 2014). The saved organic carbon (not needed by functional bacteria in PN/A) can be instead valorized in anaerobic digestion yielding (bio)energy (Jimenez et al., 2015). The PN/A process is executed by two functional bacteria groups, aerobic ammonium-oxidizing bacteria (AerAOB), oxidizing roughly half of the NH_4^+ to NO_2^- ('partial nitritation') and anaerobic ammonium-oxidizing bacteria (AnAOB), oxidizing the produced NO_2^- and residual NH_4^+ to N_2 ('anammox') (Agrawal et al., 2018). During the PN/A process, there is a group of unwanted microorganisms, nitrite-oxidizing bacteria (NOB), which can oxidize the produced nitrite to nitrate (competing for nitrite with AnAOB and for O_2 with AerAOB).

Even though PN/A holds the potential for considerable energy savings in STPs, the nitrous oxide (N_2O) emission from the process gains increased attention (Blum et al., 2018b; Ma et al., 2017a). N_2O is a potent greenhouse gas (~265-fold stronger in global warming potential than carbon dioxide (CO_2)) and a strong ozone-depleting substance (IPCC, 2013; Ravishankara et al., 2009). N_2O emitted by STPs may account for ~26% of the greenhouse gas footprint of the entire water chain and accounts for ~3% of the total estimated anthropogenic N_2O emissions (Kampschreur et al., 2009). Although the N_2O produced by AnAOB has been found negligible (~0.20% (Lotti et al., 2014b)), AerAOB are an important contributor of N_2O (Ma et al., 2017), thus potentially challenging the sustainability of nitrogen removal by PN/A. There are two main routes for AerAOB to produce N_2O, i.e., the nitrifier denitrification route ($NO_2^- \rightarrow NO \rightarrow N_2O$) (Kampschreur et al., 2008) and the NH_2OH oxidation route (a side product of the incomplete oxidation process: $NH_4^+ \rightarrow NH_2OH \rightarrow NO_2^-$) (Peng et al., 2014). Thus, the NO_2^--N and DO levels are the most critical factors affecting N_2O production. In addition, there is a third N_2O production route, i.e., heterotrophic denitrification (anaerobic reduction: NO_2^- or $NO_3^- \rightarrow N_2O$), during the nitrogen removal process in wastewater when certain organic carbon is present (Ma et al., 2017a).

Achieving a long-term stable PN/A process in the mainstream (i.e., waterline) is challenging, which is mainly attributed to the low temperature (10 – 15°C versus > 30°C) and low incoming nitrogen concentration (< 70 mg N L^{-1} versus > 1000 mg N L^{-1}) making NOB suppression difficult (Laureni et al., 2016; Peng et al., 2020). The key goal is that the activities of AnAOB and AerAOB can be maintained while NOB activity is effectively inhibited. To achieve that, cooperation between AnAOB, AerAOB, and the inhibition of NOB is required. Till now, some strategies that relied on the physiological characteristics of these three bacteria were tried. To be more specific, the observed NOB growth rates (dX_{NOB}/dt), shown in Eq – 5.1 (Laureni et al., 2019; Wang et al., 2021), NOB suppression can be obtained using three main strategies, e.g., reducing bacterial growth rate ($\mu_{NOB,max}$), promoting biomass decay rate (b_{NOB}), and increasing NOB's washout (sludge retention time (SRT) decrease).

$$\frac{dX_{NOB}}{X_{NOB} \cdot dt} = \left(\mu_{NOB,max} - b_{NOB} - \frac{1}{SRT}\right) \qquad (Eq - 5.1)$$

To reduce $\mu_{NOB,max}$, scientists commonly use low dissolved oxygen (DO) concentration (Ma et al., 2011; Ma et al., 2009) or intermittent aeration (Blackburne et al., 2008). A lower DO can suppress NOB because the half-saturation constants for O_2 of NOB are higher compared to AerAOB, which means that NOB lose more activity as DO decreases (Blackburne et al., 2008; Picioreanu et al., 1997). To promote the b_{NOB}, free ammonia (FA) (Wang et al., 2017), free nitrous acid (FNA) (Peng et al., 2020), or alternating FA/FNA shock strategies (Duan et al., 2019a) were successfully applied. The low FA and FNA level (due to low nitrogen concentration and temperature) in the mainstream limits the application of NOB suppression, but this could be tackled by return-sludge treatment (high FA and FNA conditions could be achieved in sidestream) (Peng et al., 2020). To decrease the SRT of NOB, the 'SRT control' was applied to remove NOB (e.g., flocs) while maintaining AnAOB (e.g., biofilm) (Agrawal et al., 2018). Thus, the integrated fixed-film activated sludge reactor (IFAS), where AnAOB grown on carries (long-biofilm) and NOB commonly on flocs (short-floc), or the two-stage PN/A process, has been used (Peng et al., 2020; Seuntjens et al., 2018d).

The strategies described above are usually accompanied by high NH_4^+-N (potentially during FA treatment) and high NO_2^--N concentrations (potentially during FNA treatment) change, and these can be potentially powerful emitters of N_2O (Peng et al., 2014; Wunderlin et al., 2013). That led to the variation of N_2O emission (0.8 – 6.4% of the influent nitrogen loading) from the

PN/A system (Ali et al., 2016; Domingo-Félez et al., 2014; Rathnayake et al., 2013). Only by studying the influences of different NOB suppression strategies on N_2O emissions can we choose an optimal strategy that can effectively suppress NOB while limit N_2O emissions. Till now, that knowledge was still lacking.

The overall objective of the present research is to test the effectiveness of typical NOB suppression strategies and the accompanying N_2O emissions. To achieve that, the N_2O emissions linking to three common NOB strategies, i.e., low DO levels, FA treatment, and FNA treatment, are characterized.

5.2 Materials and methods

5.2.1 Rotating biological contactor (RBC)

A rotating biological contactor (RBC) with mature PN/A biofilm was operated at 21 ± 0.6°C for 550 days. The detailed information of this reactor was shown in Van Tendeloo et al. (2021) (Figure – 5.1). The discs' submersion level was fixed at 50%, which corresponded to an effective volume of 51.1 L. With a disc rotation speed of 1.8 rpm, a consecutive exposure of 17 s emerged and submerged condition was achieved. The RBC was covered by an airtight overhead cover, creating a controlled headspace. The O_2 concentration in this headspace (i.e., the emerged DO level) was controlled by the influent flow rates of N_2 (~312 L h^{-1}) and compressed air (~18 L h^{-1}) to the headspace except for day 401 to 450 when the effect of different emerged DO levels was assessed. The emerged DO was fixed at ~0.60 mg O_2 L^{-1} (except for day 401 to 450 which ranged within 0.19 – 1.84 mg O_2 L^{-1}) to test the effect of the emerged DO levels. The emerged DO levels meant the O2 concentration in the liquid film around the biofilm, which was approximately derived from the gas phase measurement in the present research (the corresponding O2 level, which could be dissolved in the liquid film, linked to O2 in the gas phase). The microorganisms always take up the O2 from the liquid film. According to our previous research, 85–89% of the O2 input was directly absorbed during the air exposure of the discs (air phase) (Courtens et al., 2014). The off-gas was actively pumped out at 330 L h^{-1} by a gas analyzer (Emerson Rosemount CT5800 Continuous Gas Analyzer, United States) after being dehumidified by a gas cooler (Bühler Technologies, Germany).

The synthetic mainstream wastewater was made from tap water supplemented with $(NH_4)_2SO_4$ (47.6 ± 2.3 mg N L^{-1}), $NaHCO_3$ (5 mg HCO_3^- mg^{-1} N), KH_2PO_4 (7.5 mg P L^{-1}), and trace

elements solutions A/B (0.01 ml L^{-1}) (Van de Graaf et al. 1995). The influent flow rate (Watson-Marlow 323, United Kingdom) was kept at ~120 L d^{-1} in a continuous mode, resulting in a nitrogen loading rate of 105.2 ± 5.2 mg N L^{-1} d^{-1}.

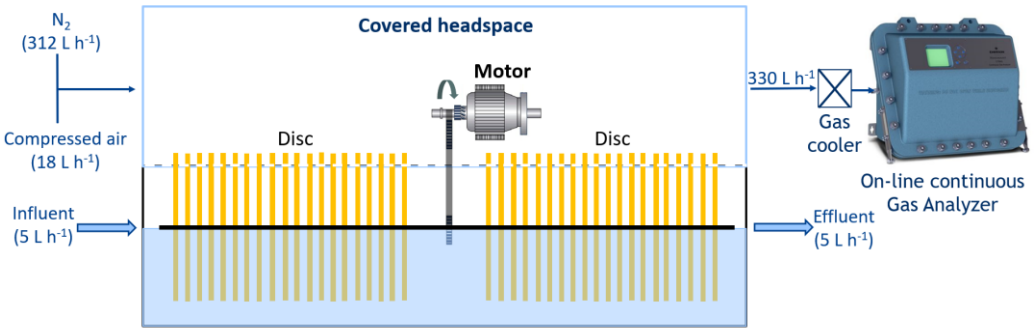


Figure – 5.1 Schematic of the RBC configuration (with overhead cover).

5.2.2 Overall Experimental Plan

The experiment was divided into four phases.

5.2.2.1 Phase – I: Long-term operation at low emerged DO level

In Phase – I (from day 0 to 400), the long-term stability of mainstream PN/A under low emerged DO level was studied. Because of the high initial NOB activity, the emerged DO level was reduced from ~0.80 O_2 L^{-1} to ~0.60 mg O_2 L^{-1} on Day 26 to suppress NOB activity. Afterwards, a period of up to 374 days was used to verify the stability of NOB inhibition by a low emerged DO level.

5.2.2.2 Phase – II: Effect of different emerged DO levels on the N_2O emission

In Phase – II (from day 401 to 450), the effect of five different emerged DO levels (0.19, 0.60, 0.91, 1.43, and 1.84 mg O_2 L^{-1}) on the PN/A performance and N_2O emission was investigated by adapting the influent flow rates of N_2 (from 8.8 L h^{-1} to 11.4L h^{-1}) and compressed air (from 0 L h^{-1} to 2.8 L h^{-1}) to the headspace. Each DO level was tested for only 2 days to avoid a strong (irreversible) increase in NOB activity at higher emerged DO conditions. Subsequently, the emerged DO level was returned to ~ 0.60 mg O_2 L^{-1}. When the NH_4^+-N and NO_3^--N conversion rates were recovered to the benchmark, the next emerged DO level was tested.

5.2.2.3 Phase – III: Effect of FA treatment on the N_2O emission

In Phase – III (from day 451 to 497), FA was used as a stressor for biofilm treatments. In total, three FA treatments (3 hours per treatment) were performed with a treatment interval of 7 days (day 453, 460, and 467). The FA treatment condition (29.3 ± 2.6 mg NH_3-N L^{-1} achieved at a pH of ~8.0, the temperature of ~21℃, and NH_4^+–N concentration of ~735 mg N L^{-1}) was chosen based on Van Tendeloo et al. (2021). After the RBC recovered from the FA treatment, the isolated effect of high pH (8.0, 3h) and NH_4^+–N concentration (735 mg N L^{-1}, 3h) shock were tested on day 488 – 490 and day 480 – 484 respectively to verify the effect of these two factors on the N_2O emissions. The emerged DO level was stable at ~0.60 mg O_2 L^{-1} throughout the phase.

5.2.2.4 Phase – IV: Effect of FNA treatment on the N_2O emission

In Phase – IV (from day 498 to 550), three FNA biofilm treatments were applied. The FNA treatments lasted for 3 hours and were performed with a treatment interval of 7 days (day 498, 505, and 512). The FNA treatment condition (3.1 ± 0.3 mg HNO_2-N L^{-1} achieved at a pH of ~6.0, the temperature of ~21℃, and NO_2^-–N concentration of ~1205 mg N L^{-1}) was chosen according to Peng et al. (2020). After the RBC recovered from the FNA treatment, the isolated effect of low pH (6.0, 3h) and high NO_2^-–N concentration (1205 mg N L^{-1}, 3h) shock were tested on day 531 – 536 and 540 – 547, respectively, to verify the effect of these two factors on the N_2O emission. During the whole period, the emerged DO was also stable at ~0.60 mg O_2 L^{-1}.

5.2.3 Online N_2O emission monitoring

The off-gas was analysed with an online N_2O gas analyzer (Emerson Rosemount CT5800 Continuous Gas Analyzer, United States) with a range of 0 – 500 ppm (the lowest detection (LOD) was 2 ppm). The data was logged every 30 s. Zero and span calibration were accomplished by calibrating the N_2O analyzer measurements against N_2 of instrument gas purity and N_2O reference gas. The sampling flow rate was set at 330 L h^{-1} by a vacuum pump (KNF Laboport, the Netherlands) which equalled the inlet flow rate (N_2 and compressed air) to maintain the gas balance.

The N_2O emission factor (%) was calculated according to the following equation (Eq – 5.2 to 5.4):

$$\mathrm{N_2O\ emission\ factor} = \mathrm{N_2O - N\ emitted} \div \mathrm{NH_4^+ - N\ converted} \quad (\mathrm{Eq} - 5.2)$$

$$\mathrm{N_2O - N\ emitted} = \sum\left(\mathrm{C_{N_2O\ gas}} \times \mathrm{Q_{sampling\ pump}} \times \Delta\mathrm{t}\right) \quad (\mathrm{Eq} - 5.3)$$

$$\mathrm{NH_4^+ - N\ converted} = \mathrm{NH_4^+ - N\ conversion\ rate} \times \mathrm{Q_{influent}} \times \Delta\mathrm{t} \quad (\mathrm{Eq} - 5.4)$$

where N_2O-N emitted represents the released N_2O over a certain amount of time [mg N_2O-N]; NH_4^+-N converted represents the converted NH_4^+-N over a certain amount of time [mg NH_4^+-N]; C_{N2Ogas} represents the point N_2O concentration in the off-gas [mg N_2O-N L^{-1}]; $Q_{\text{sampling pump}}$ represents the flow rate of the gas sampling pump [330 L h^{-1}]; Δt represents the time interval by which the off-gas N_2O concentration was recorded [h]; NH_4^+-N conversion rate is measured by daily sampling [mg N L^{-1} d^{-1}]; $Q_{influent}$ represents the flow rate of the influent (L h^{-1}). The N_2O concentration in the off-gas in ppmv was converted to mg N_2O-N L^{-1} based on the volume occupied by 1 mole of an ideal gas at standard temperature and pressure (0°C and 101.3 kPa), which is 22.4 L and corrected for temperature of the gas sample (24°C).

The N_2O emission factor is calculated based on the average N_2O emitted within one day (continuous N_2O measurement (per 30 seconds) and daily water samples (for NH_4^+-N converted measurement)). During the tests of the DO influences, the data was averaged over the whole period (from the moment that the DO value changed until the moment that it shifted back to 0.6 mg O_2 L^{-1}). The background values for the indoor N_2O were also measured, which was almost negligible.

5.2.4 Analytical procedures

Liquid samples were taken periodically from the influent and effluent to determine the PN/A performance. After filtering through a 0.2 µm syringe filter (CHROMAFIL Xtra PVDF, Germany) and storing at 4°C, NH_4^+-N, NO_2^--N, and NO_3^--N concentrations were measured with a San^{++} Automated Wet Chemistry Analyzer (SKALAR, the Netherlands) and Ion Chromatography with column of Metrosep A Supp 5- 150/4.0 (Metrohm – Eco IC, Switzerland). The pH and DO values were measured using handheld meters (Hach HQ30d, United States).

The p-value obtained from the analysis of variance (ANOVA) was compared with a significance level of 0.05 to assess the level of variation. A p-value below 0.05 was considered acceptable evidence of a significant difference. The FA and FNA concentrations were calculated

according to the equations in S – 5.1 (Supporting information). The relative nitrite consumption by AnAOB and NOB was determined using a mass balance applying that in literature described stoichiometric values for AerAOB, NOB (Barnes and Bliss, 1983) and AnAOB (Lotti et al., 2014b; Strous et al., 1998).

5.2.5 Calculations: The relative NO_2^- consumption by AnAOB and NOB

In the PN/A system, assuming the decreased total nitrogen is converted to N_2 by the anammox process (the feed did not contain COD in the present study). During the anammox process, the stoichiometric ratio of removed NO_2^--N/NH_4^+-N is 1.23 (the average of the value reported by Strous et al. (1998) (1.32) and Lotti et al. (2014b) (1.146)) and the stoichiometric ratio of produced NO_3^--N/NH_4^+-N is 0.21 (the average of the value reported by Strous et al. (1998) (0.26) and Lotti et al. (2014b) (0.161)). The percentage of NO_2^- consumed by AnAOB (P(AnAOB)), NOB (P(NOB)), and excess (P(excess)) are shown in Eq-5.5 to Eq-5.10 (TN presents the total nitrogen).

$$\mathrm{P\ (A_nAOB)} = \frac{\mathrm{NO_2^-\ consumed\ by\ A_nAOB}}{\mathrm{Influent\ NO_2^- + NO_2^-\ produced\ by\ A_{er}AOB}} \quad (\mathrm{Eq-5.5})$$

$$\mathrm{P(excess)} = \frac{\mathrm{Remained\ NO_2^-}}{\mathrm{Influent\ NO_2^- + NO_2^-\ produced\ by\ A_{er}AOB}} \quad (\mathrm{Eq-5.6})$$

$$\mathrm{P(NOB)} = 1 - \mathrm{P(A_nAOB)} - \mathrm{P(excess)} \quad (\mathrm{Eq-5.7})$$

$$\mathrm{NO_2^-\ produced\ by\ A_{er}AOB} = \mathrm{NH_4^+\ removed - NH_4^+\ consumed\ by\ A_NAOB} \quad (\mathrm{Eq-5.8})$$

$$\mathrm{NO_2^-\ consumed\ by\ A_nAOB} = \frac{1.23}{1 + 1.23 - 0.21} \times \mathrm{TN\ removed} \quad (\mathrm{Eq-5.9})$$

$$\mathrm{NH_4^+\ consumed\ by\ A_{\mathit{n}}AOB} = \frac{1}{1 + 1.23 - 0.21} \times \mathrm{TN\ removed} \quad (\mathrm{Eq-5.10})$$

5.3 Results and discussion

5.3.1 Long-term operation at low emerged DO levels

5.3.1.1 Low emerged DO level effectively suppressed NOB

Long-term stability is essential for the successful implementation of PN/A in full-scale installations. To assess the long-term stability, the performance of the RBC was evaluated for 400 days at a low emerged DO level. Initially, the emerged DO level was set at ~0.80 mg O_2 L^{-1}. This, however, boosted NOB activity resulting in a gradual increase in produced NO_3^--N and a nitrite consumption by NOB of more than 50% (Figure – 5.2 and Figure – S5.1). To suppress NOB, the emerged DO level was decreased to 0.60 ± 0.02 mg O_2 L^{-1} by changing the ratio between the influent flow rates of N_2 (from 8.8 L h^{-1} to 11.4L h^{-1}) and compressed air (from 0.2 L h^{-1} to 2.8 L h^{-1}) on day 26. Between days 26 and 200, the NO_3^--N production rate (from 33.8 to 5.8 mg N L^{-1} d^{-1}) and the percentage of NO_2^--N consumed by NOB (from 52.1% to ~0%) gradually decreased (Figure – 5.2), indicating that NOB activity was effectively reduced by the lower emerged DO. That is in line with our previous research (Van Tendeloo et al., 2021) that strict O_2 control could achieve complete NOB suppression.

The NH_4^+-N removal rate remained stable (71.7 ± 5.7 mg NH_4^+-N L^{-1} d^{-1}) between day 26 and 200 (Figure – 5.2A), which demonstrates that an emerged DO level of ~0.60 mg O_2 L^{-1} not only effectively suppressed NOB activity but also maintained AerAOB and AnAOB activity. In previous studies, DO levels in a biofilm PN/A system were controlled below 0.20 mg O_2 L^{-1} (to maintain enough AnAOB and) to suppress NOB, but this strategy jeopardized AerAOB activity, thereby limiting the overall nitrogen removal rate (Joss et al., 2011; Wang et al. 2021). The biofilm in RBC faced an intermittent aeration mode, switching between anoxic (i.e., DO of 0 mg O_2 L^{-1}) and low DO (i.e., DO of 0.60 mg O_2 L^{-1}) every 17 seconds (i.e., transient anoxia). This might balance the regular supply of O_2 for AerAOB to produce NO_2^--N, followed by the removal of the produced NO_2^--N during the anoxic period by AnAOB while suppressing NOB activity. The relatively high DO level (~0.60 versus ≤ 0.20 mg O_2 L^{-1}) in the present research could not only increase the activity of AerAOB but also make the operation simple and reduce cost (by obviating O_2 removal).

Phase	Phase – I	Phase – II
Emerged DO level	~ 0.60 mg O_2 L^{-1}	0.15 – 1.84
Eff. NH_4^+-N	13.9 ± 3.5 mg N L^{-1}	12.9 ± 5.3

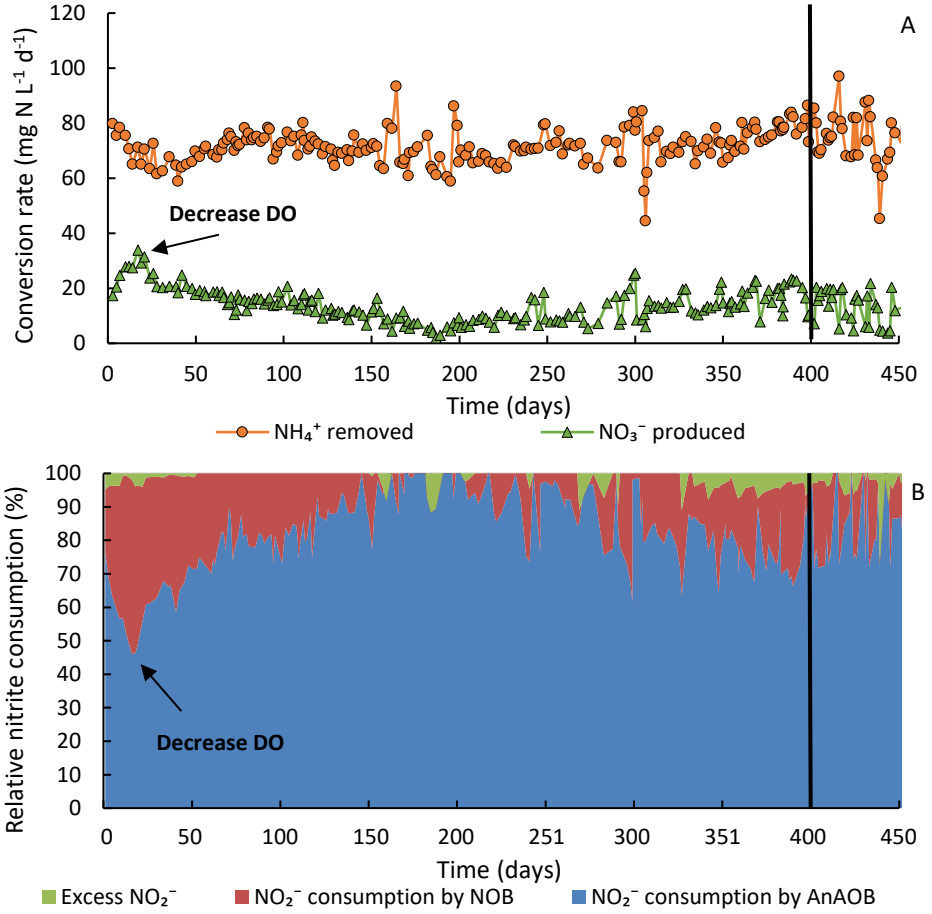


Figure – 5.2 Reactor performance in Phase – I (day 0 to 400) and Phase – II (day 401 to 450). A, volumetric NH_4^+-N removal and NO_3^--N production rate; B, relative NO_2^--N consumption by AnAOB and NOB, and residual NO_2^--N.

After 200 days of operation at an emerged DO level of ~0.60 mg O_2 L^{-1}, the nitrite consumption by NOB started to increase (e.g., 0% at day 204 versus 30% at day 392) (Figure – 5.2). This might be due to the adaptation of the suppressed NOB to the low DO conditions (i.e., NOB became more efficient in competition with AerAOB for O_2 and NO_2^--N). Microbial community analysis (S – 5.2 of the Supporting Information) revealed enrichment in *Nitrospira* (from 0.8% before the DO change to 4.7% on day 402, Figure – S5.2), a NOB genus with a higher affinity to O_2 than others (e.g., *Nitrobacter* and Candidatus *Nitrotoga*) (Wang et al., 2021), in the PN/A biofilm. At the same time, NOB are also likely to maintain structural and genetic

integrity by repairing and minimizing damage to the cellular infrastructure at low DO levels (Duan et al., 2019a). The adaptation of NOB to low DO levels after the long-term operation (> 200 days) has also been reported by Cao et al. (2018) and Liu and Wang (2013).

5.3.1.2 Lower emerged DO levels reduced N_2O emission

To investigate the effect of the emerged DO level on the N_2O emission of the RBC, the emerged DO level was varied from 0.19 up to 1.84 mg O_2 L^{-1} between day 401 and day 450. At an emerged DO level of ~0.60 mg O_2 L^{-1}, the average N_2O emission factor was 1.92 ± 0.47% which is within the typical range for a PN/A system (0.1 – 2.4% reviewed by Ali et al. (2016)). As shown in Figure – 5.3, the N_2O emission factor increased with increasing emerged DO level. At a DO of 0.19 mg O_2 L^{-1}, the emission factor was 1.59 ± 0.05%, while at a DO of 1.84 mg O_2 L^{-1}, it was 2.48 ± 0.20%. One-way ANOVA analysis showed that there was a significant positive correlation (R^2 = 0.9994, $p < 0.05$) between the emerged DO level and N_2O emission factor at DO < 0.91 mg O_2 L^{-1}. At higher emerged DO levels, the correlation was no longer significant (R^2 = 0.9224, $p > 0.05$).

Since the nitrifier denitrification N_2O production route is linked to the NO_2^--N concentration (Ma et al., 2017a; Okabe et al., 2011), it possibly had only limited contribution to the N_2O emission in our study because of the low NO_2^--N concentration (0.6 ± 0.8 mg N L^{-1}). Furthermore, heterotrophic denitrification was likely negligible since it could only dominate when heterotrophy was stimulated in the presence of COD (the feed did not contain COD in the present study) (Ma et al., 2017a). Therefore, N_2O emission might be mainly attributed to the NH_2OH oxidation pathway. The positive correlation between the DO level and the N_2O emission via the NH_2OH oxidation route was also demonstrated in previous research (Ni et al., 2014; Wunderlin et al., 2013). Hence, a low DO level is recommended to decrease N_2O emissions from PN/A systems.

The emission of N_2O was dynamic (the average values were used to calculate the N_2O emission factor) and was, amongst others, influenced by changes in operating conditions in the reactor. As shown in Figure – S5.3, a shift in emerged DO level caused an immediate change in the emitted N_2O rate. Even when the DO level was not changed, the N_2O concentration in the headspace varied. This might be caused by NO_2^--N accumulation, light, varying temperature (± 1℃) and microbial respiration, varying gas pressure, or carbon fixation rate (Lotti et al., 2014a). Qiu et al. (2021) demonstrated that even at a constant nitritation activity, the observed N_2O production rate was dynamic.

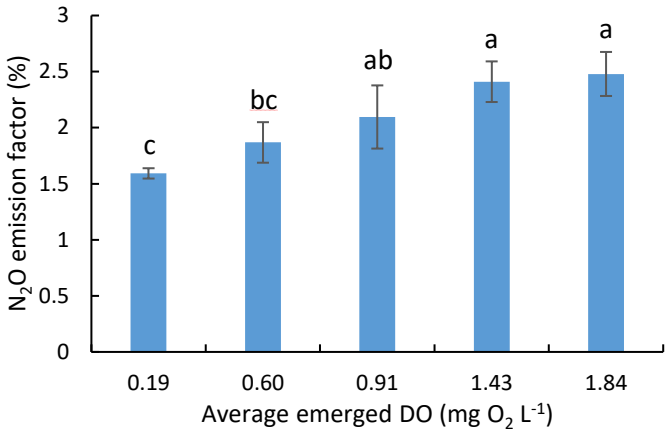


Figure – 5.3 The average N_2O emission factor under different emerged DO levels. The erwror bars depict the standard deviations (n = 2). Significant differences are marked with a letter.

5.3.2 FA treatment on PN/A biofilm

5.3.2.1 NOB suppression was further improved by FA treatment

The long-term operation can trigger adaptation of NOB at low emerged DO conditions (Section 3.1). In Phase – III (from day 451 to day 497), FA treatment (29.3 ± 2.6 mg NH_3-N L^{-1}) was therefore implemented based on a low emerged DO level (~0.60 mg O_2 L^{-1}) to suppress NOB activity (Figure – 5.4). Before the first FA treatment, an average of 17.5 ± 9.3% of the NO_2^--N was consumed by NOB. This value decreased to 0% on day 455 (two days after the first FA treatment), showing that NOB activity was suppressed by FA treatment. Two following FA treatments kept the NOB activity low between day 453 and day 473 (e.g., 1.9% on day 470). The effectiveness of FA treatment is consistent with our previous paper (Van Tendeloo et al., 2021). After the last FA treatment, the inhibited NOB activity gradually restored (e.g., 22.7% of the NO_2^--N was consumed by the NOB on day 480, 12 days after the third FA treatment), which is also in line with Duan et al. (2019a) who found that stopping FA treatment disrupted the NO_2^--N shunt that was established. To conclude, low-DO adapted NOB was successfully inhibited by FA treatments, but repetitive periodical treatment (3 hours per week) is required to sustain the suppression.

Phase	Phase – III	Phase – IV
DO level	~ 0.60 mg O_2 L^{-1}	
Eff. NH_4^+-N	13.3 ± 3.8 mg N L^{-1}	
FA	30 mg NH_3-N L^{-1}	/
FNA	/	3.0 mg HNO_2-N L^{-1}

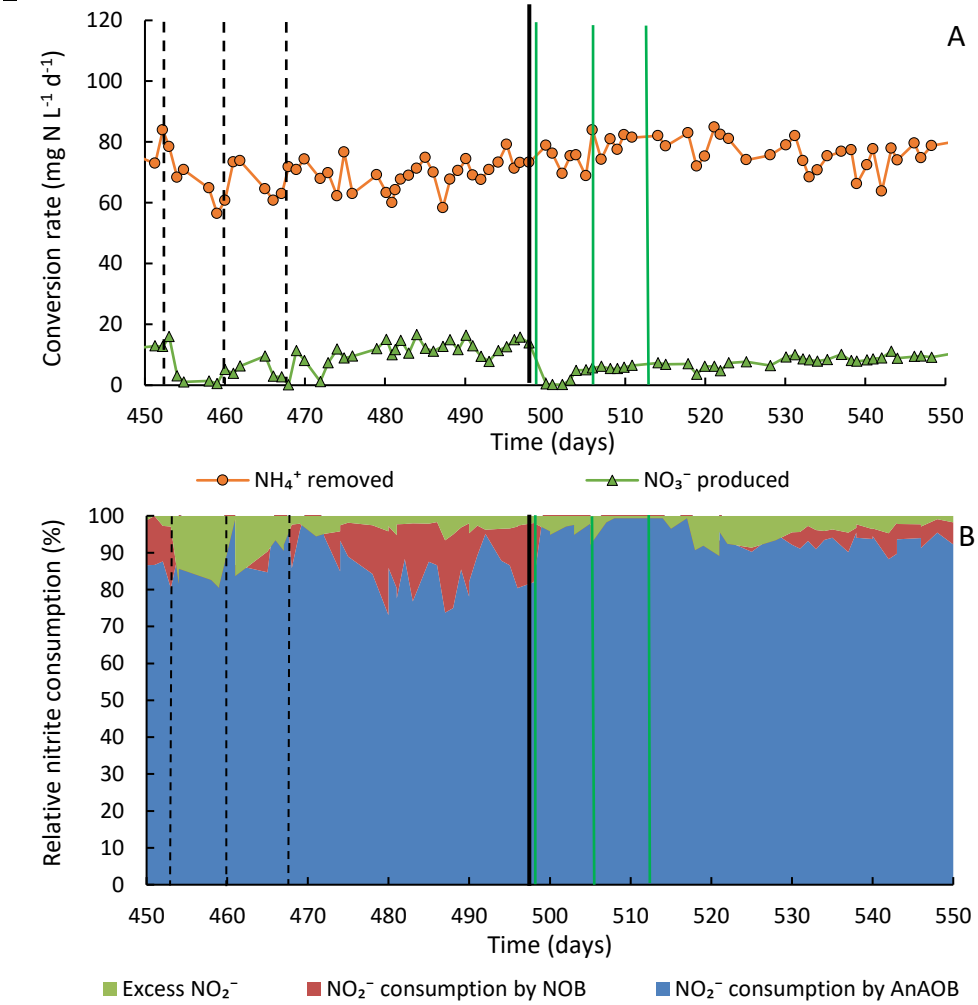


Figure – 5.4 Reactor performance in Phase – III (day 451 to 497) and Phase – IV (day 498 to 551). A, volumetric NH_4^+-N removal and NO_3^--N production rate; B, relative NO_2^--N consumption by AnAOB and NOB, and residual NO_2^--N (in the effluent). The black dashed lines indicate FA treatment, whereas FNA treatments are indicated with a green solid line.

During FA treatment, the NH_4^+-N removal rate decreased (68.2 ± 6.1 versus 76.9 ± 4.6 mg N L^{-1} d^{-1}), indicating that also AerAOB activity was influenced by the FA treatment (30 mg NH_3-N L^{-1}). The residual NO_2^--N concentration increased (9.1 ± 6.7% versus < 5% before FA treatment) which indicates that FA probably had a stronger suppression on NOB than on AerAOB. A more

selective inhibition on NOB guaranteed a stable operation of the PN/A system. Seuntjens et al. (2018d) also used a similar FA level (30 mg NH_3-N L^{-1}, 3 hours) to suppress NOB and reported that FA treatment as the sole strategy could not inhibit NOB (increase in relative activity ratio between AerAOB and NOB). That suggests the combination of FA treatment with a low DO level is critical to obtain NOB suppression.

5.3.2.2 FA treatment increased the N_2O emission

N_2O concentrations in the air-phase of the RBC system were measured to assess the effect of FA treatment in low-DO conditions on the N_2O emission. Before the FA treatment (benchmark, day 350 – 400), the N_2O emission factor was 1.65 ± 0.10%. It rapidly increased to 2.33 ± 0.36%, 2.40 ± 0.45%, and 2.15 ± 0.30% (they stayed at that level until the next treatment) after each FA treatment (day 453, 460, and 467), respectively (Figure – 5.5), revealing that FA treatment promoted N_2O emission from the RBC system. After the final FA treatment, the N_2O emission factor gradually decreased back to the benchmark (which was reached within a week). To distinguish the effect of FA treatment from the effect of a high NH_4^+-N concentration and high pH, a high pH (~8.0, 3h) and NH_4^+–N concentration (~735 mg N L^{-1}, 3h) shock were tested on day 488 – 490 and day 480 – 484. pH did not affect the N_2O emission factor (1.68 ± 0.08%), whereas the NH_4^+-N concentration shock promoted the emission of N_2O (2.27 ± 0.13% versus 1.65 ± 0.10% from the benchmark), suggesting that the high NH_4^+-N concentration might be the major contributor to the high N_2O emission of FA treatment. Kampschreur et al. (2009) reported that N_2O emission increased with increasing NH_4^+-N concentration in full-scale STP.

DO and NO_2^--N levels are two important factors affecting N_2O production by AerAOB (Peng et al. 2014, Wunderlin et al. 2013). The DO levels were always constant during the tests thus the residual NO_2^--N levels were likely the main influencing factor since it was positively correlated with the NO_2^--N levels (Harris et al., 2015; Peng et al., 2015). After the FA treatment, due to the residual NO_2^--N increase (10.7 ± 6.7% versus 4.2 ± 1.7%), the nitrifier denitrification N_2O production pathway might also play a role. The nitrifier denitrification route was nitrite-sensitive because the expression of copper-containing NO_2^- reductase (NirK) is regulated by a nitrite-sensitive transcription repressor protein (Beaumont et al., 2004). The lower residual NO_2^--N after the third FA treatment corresponded to the lower N_2O emission factor, which suggested the role of residual NO_2^--N. Due to the operation being kept constant after the FA treatment (e.g., influent NH_4^+-N and the emerged DO level), the N_2O produced through the

NH_2OH oxidation route was likely also the same as before. Thus, the N_2O emission increased after the FA treatment might be caused by an increase nitrifier denitrification in the present research.

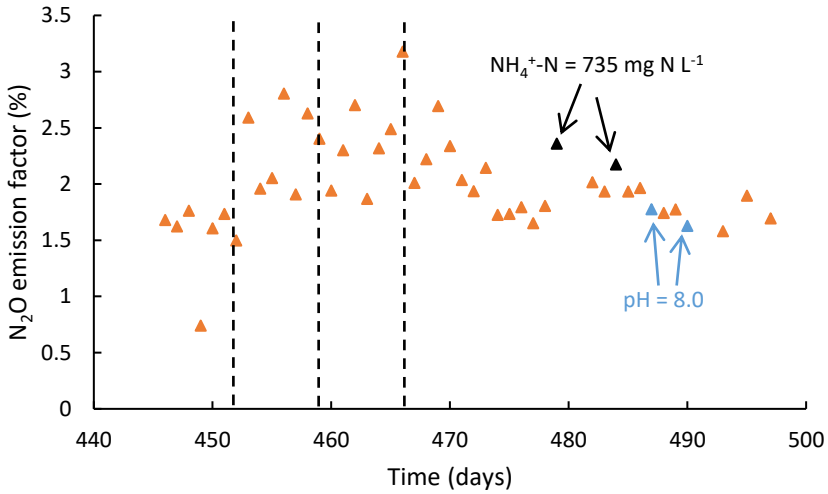


Figure – 5.5 Changes in N_2O emission factors under FA treatment and associated NH_4^+-N (735 mg N L^{-1}) or pH shock (8.0). The black dashed lines represent FA treatments while black and blue triangles indicated the NH_4^+-N and pH shock respectively.

The N_2O emission was also dynamic during the FA treatments (Figure – S5.4). At the beginning of the FA treatment, the N_2O concentration in the air phase rapidly increased (e.g., from 1.03 to 4.23 ppmv within 30 minutes during the first FA treatment). Subsequently, the N_2O production gradually decreased (e.g., from 4.23 to 2.31 ppmv). After 3 hours of FA treatment, a peak appeared which was followed by a decrease in N_2O production. Even after 8 hours, the N_2O emission was still higher than that before the FA treatment. The presence of high residual NH_4^+-N concentration might increase the NH_4^+-N oxidation rate, which probably yielded more intermediates (e.g., HNO, a byproduct during the oxidation process: $NH_2OH \rightarrow N_2O$) (Colliver and Stephenson, 2000; Law et al., 2012). The NH_4^+-N shock caused a rapid increase in N_2O concentration (e.g., from 4.02 to 24.06 ppmv in the first shock, Figure – S5.5). Even though the value gradually decreased to 16.15 ppmv after 3 hours, which might be explained by the decrease in NH_4^+-N concentration (Table – S5.1), that was still more than 10 times higher than the benchmark. At high NH_4^+-N levels, not only the NH_2OH oxidation route in AerAOB is promoted, but also N_2O emissions from AnAOB were reported to be enhanced (Jin et al., 2016).

The high pH (8.0) shock had a limited influence on N_2O emission (the drop during the shock can be attributed to the cessation of influent). The influence of pH on N_2O emissions was likely directly related to the effect on the bacterial enzymes and nitrogen compounds rather than leading to a shift in bacterial communities (not long enough for a change). A pH range of 6 – 8 did not expect to affect the ammonia oxidation rate, which was expected not to affect N_2O emissions. It was agreed by Ribera-Guardia and Pijuan (2017) that N_2O emission was not influenced by gradually decreasing the pH (8.0 → 6.5).

5.3.3 FNA treatment on PN/A biofilm

5.3.3.1 NOB suppression was further improved by FNA treatment

After stopping the FA treatment, the percentage of NO_2^--N consumed by NOB increased to 13.5 ± 5.6 %, showing the recovery of NOB activity. In Phase – IV (from day 498 – 550), FNA treatment (3.1 ± 0.3 mg HNO_2-N L^{-1}) was applied in combination with low emerged DO levels (~0.60 mg O_2 L^{-1}) (Figure – 5.4) to suppress NOB activity. The FNA treatment rapidly inhibited the NOB activity, as evidenced by a decrease in the percentage of nitrite consumed by NOB (0%). That was agreed by Wang et al. (2014), who revealed that FNA treatment (0.24 − 1.35 mg of HNO_2^--N L^{-1}) was significantly more biocidal to NOB than AerAOB. After the last FNA treatment (day 512), the inhibited NOB activity was suppressed for the following 15 days (e.g., only 4.1% of NO_2^- was consumed by the NOB on day 527). That was different from FA treatment that led to the rapid recovery of NOB activity after treatment (~6 versus ~15 days). To conclude, low-DO adapted NOB were successfully suppressed by FNA treatment, but repetitive periodical treatment is also required to sustain the suppression. Wang et al. (2016a) also reported that low DO conditions (0.30 − 0.80 mg O_2 L^{-1}) combined with the FNA treatment successfully suppressed NOB in the mainstream PN/A system. This finding was contradicting our previous results (Van Tendeloo et al., 2021) in which the FNA treatments failed to completely suppress NOB activity. That might be attributed to the difference of the reactor setup (without overhead cover), operating conditions (higher emerged (~4.5 mg O_2 L^{-1}) and submerged (~1.0 mg O_2 L^{-1}) DO levels), and the community composition (e.g., dominant NOB genus was *Nitrotoga* versus *Nitrospira* in the present research).

After the FNA treatment, the NH_4^+-N removal rate did not differ from the value before treatment (77.9 ± 5.5 versus 76.9 ± 4.6 mg N L^{-1} d^{-1}). In addition, almost no residual NO_2^--N (0.8

± 0.6 mg N L^{-1}) was presented in the RBC system. Both results indicated that AnAOB and AerAOB activities were not affected by FNA treatment. Peng et al. (2020) reported on the other hand that FNA treatment exerted an inhibitory impact on both AerAOB and NOB, while the inhibition on NOB was stronger. This difference might be attributed to the higher stability of the biofilm structure in the RBC system.

5.3.3.2 FNA treatment did not affect N_2O emission

The N_2O emission factor after FNA treatment (based on the low-DO conditions) was also tested. Its value was 1.73 ± 0.27%, 1.64 ± 0.24%, and 1.64 ± 0.34% after each FNA treatment (the average value until the next treatment), respectively (Figure – 5.6). There was no difference comparing to the benchmark (1.72 ± 0.16%) before the first FNA treatment. That differed from the FA treatment (promoted N_2O emissions). To distinguish the effect of FNA treatment on N_2O emission from the effect of a high NO_2^--N concentration and low pH, a low pH (~6.0, 3h) and high NO_2^-–N concentration (~1205 mg N L^{-1}, 3h) shock were tested on day 531 – 536 and day 540 – 547. Both the pH (1.68 ± 0.23%) and NO_2^--N concentration (1.70 ± 0.16%) did not affect the N_2O emission factor (1.65 ± 0.10% in the benchmark), corresponding to the fact that FNA treatment does not affect N_2O emissions.

Even if the high NO_2^--N during the FNA treatment (3h) promoted the N_2O emission (Figure – S5.10), the NO_2^--N concentration was diluted (to < 1.0 mg N L^{-1}) within two hours. After the FNA treatment, there is no change in the residual NO_2^--N (Figure – 6.6, 2.6 ± 2.2% versus 4.2 ± 1.7%) which demonstrated the nitrifier denitrification N_2O production pathway likely still did not play a role. That was different from the results after the FA treatment (increased residual NO_2^--N concentration promoted N_2O emission). In addition, the operation was also constant to that before the treatment indicating similar N_2O emissions from the PN/A system.

As mentioned above, the N_2O emissions were always dynamic, including during the FNA treatment process (Figure – S5.6). During the FNA treatment, the N_2O production gradually decreased (e.g., from 4.15 to 3.27 ppmv within 3 hours in the first FNA treatment). During the FNA treatment, due to the presence of high NO_2^--N, the nitrifier denitrification was likely the primary N_2O production pathway (Harris et al., 2015; Peng et al., 2015). According to the previous research, the nitrifier denitrification process was stimulated at moderate NO_2^--N range (0 − 50 mg of N L^{-1}) (Peng et al., 2015), whereas the inhibition appeared at high concentrations (500 − 1000 mg of N L^{-1}) (Law et al., 2013). The NO_2^--N level used in the present research (1205

mg of N L^{-1}) is already beyond the reported suppression range, which might change the detoxification mechanism of NirK, altering the stimulation threshold of NO_2^--N to N_2O emissions (Wang et al., 2016b). That could explain the limited influences of FNA treatment on N_2O emissions. After 3 hours of FNA treatment, the production gradually returned to the benchmark. Due to the sensitivity of N_2O emission to high NO_2^--N concentration being relatively large (Tallec et al., 2006), the N_2O production rapidly increased at the beginning of the NO_2^- shock (e.g., from 3.91 to 6.33 ppmv within 10 minutes in the first NO_2^- shock, Figure – S5.7). Subsequently, the N_2O production gradually increased (e.g., from 6.33 to 10.91 ppmv). That might be explained by the previous findings (Colliver and Stephenson, 2000; Ma et al., 2017a) that the NO_2^--N concentration increase could promote the AerAOB denitrification rate (effectively reducing NO_2^--N to N_2O). Regarding the low pH (6.0) shock, its impact on N_2O emissions was also limited, which was like the high pH (8.0) shock.

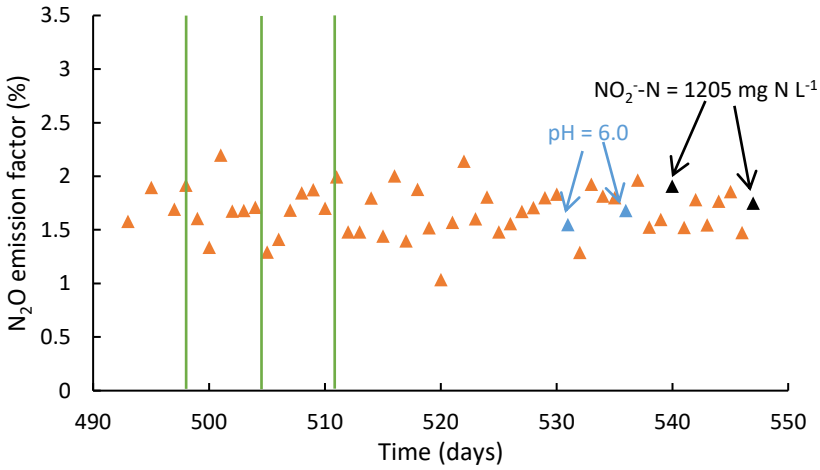


Figure – 5.6 Changes in N_2O emission factors under FNA treatment and associated NO_2^--N (~1205 mg N L^{-1}) or pH shock (~6.0). The green solid lines represent FNA treatments.

5.3.4 Implication and outlook

Regarding the suppression of NOB activity in mainstream PN/A systems, engineers have conducted a lot of research, but studies of N_2O emissions from this process are still lacking. Strategies to suppress NOB are always accompanied by changes in operating parameters (e.g., pH, ammonium or nitrite concentration, temperature, etc.), which can lead to dynamic conditions that probably promote N_2O emissions (Kampschreus et al., 2009). Low DO control is a feasible strategy to suppress NOB activity while mitigating N_2O emissions. However, NOB

adaptation occurred at low dissolved oxygen levels in long-term operation (> 200 days). Therefore, in addition to controlling low DO level, FNA treatment should be performed periodically because it stabilizes N_2O emissions while suppressing NOB activity (although FA treatment can also effectively inhibit the activity of NOB, it will promote the emission of N_2O). That is similar to Peng et al. (2020), who noted that FNA treatment produced less N_2O compared to FA treatment in an integrated fixed membrane activated sludge (IFAS) PN/A system. Therefore, the findings in the present study are a beneficial supplement to the application of PN/A in the mainstream. However, a part of the N_2O emitted by the system was probably in the effluent. It was not considered in the present research, and the follow-up study should include it to make the full carbon footprint.

RBC is a type of biofilm-only system, which has higher stability than the other reactors. RBC is probably not a preferred system if it applies in mainstream anammox. But if we would use them, several units that are coupled in series or parallel can be applied to treat the large flow wastewater. It would be at least technologically feasible or durable to cover them, mainly to lower the O_2 levels in the headspace (even the gas control is economically not feasible). Even though the biomass growth configuration was different from the floccular and granular system (attached growth versus suspended growth), the findings of the present research are generic and can be extrapolated to several types of biofilm-based systems (e.g., integrated fixed-film activated sludge (IFAS), moving bed biofilm reactor (MBBR), etc.) (Van Tendeloo et al., 2021). At the level of N_2O emissions, the type of system or the type of growth main not matter too much since it is mainly determined by the nitrite accumulation, O_2 level, and operational parameters.

5.4 Conclusion

In mainstream PN/A systems, NOB suppression and N_2O mitigation are two important operational objectives. Achieving a balance between these two will be key in achieving the long-term stable mainstream PN/A. This research aimed to characterize the N_2O emissions behind the effective NOB suppression strategies in the long term. Low emerged DO conditions effectively suppressed NOB activity as well mitigated the N_2O emission (there was a significant positive correlation ($R^2 = 0.9994$, $p < 0.05$) between the emerged DO levels and N_2O emission factor), whereas the adaptation of NOB occurred in the long-term operation (> 200 days). The low DO-adapted NOB activity could be further inhibited by repetitive periodical FA or FNA

treatment. When characterizing the N_2O emissions linked to them, FNA treatment did not affect N_2O emissions (~1.67% versus 1.72% of removed NH_4^+-N), and was, hence, more advantageous than FA treatment (promoted the N_2O emissions, ~2.29% versus ~1.65% of removed NH_4^+-N)). Thus, to effectively inhibit NOB while controlling N_2O emissions, the PN/A system should be applied at relatively low DO conditions together with repetitive periodical FNA treatment.

Supporting Information

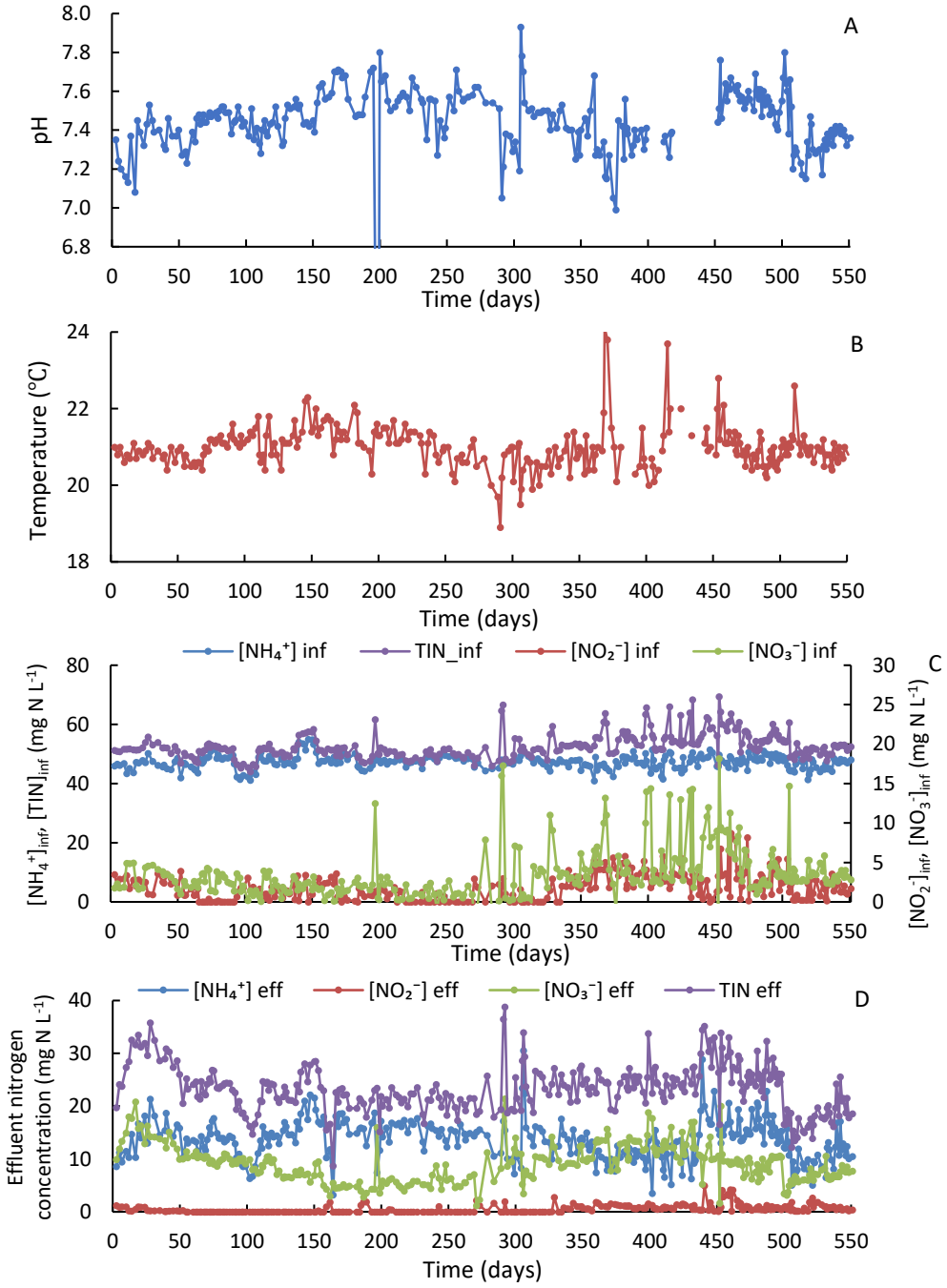
S – 5.1 Calculation of free ammonia (FA, Eq – S5.1) and free nitrous acid (FNA, Eq – S5.2) (Anthonisen et al. 1976).

$$\mathrm{FA\ [mg\ NH_3 - N\ L^{-1}]} = \frac{17}{14} \times \frac{\mathrm{C_{NH_4^+ - N}} \times 10^{\mathrm{pH}}}{\left[\exp\left(\frac{6334}{273 + \mathrm{T}}\right) + 10^{\mathrm{pH}}\right]} \qquad (\mathrm{Eq - S5.1})$$

$$\mathrm{FNA\ [mg\ HNO_2 - N\ L^{-1}]} = \frac{47}{14} \times \frac{\mathrm{C_{NO_2^- - N}}}{\left[\exp\left(\frac{-2300}{273 + \mathrm{T}}\right) \times 10^{\mathrm{pH}}\right] + 1} \qquad (\mathrm{Eq - S5.2})$$

where $C_{NH_4^+ - N}$ is the concentration of total ammonium [mg NH_4^+-N L^{-1}]; $C_{NO_2^- - N}$ is the concentration of total nitrite [mg NO_2^--N L^{-1}]; T is the temperature of the liquid sample [°C].

Figure – S5.1 The pH (A) and temperature (B) profile and influent (C) and effluent (D) nitrogen concentrations of the RBC system over 550 days. The nitrite and nitrate in the influent are not intended, the presence may be due to activity in the influent vessel and nitrate in the tap water.



S – 5.2 Microbial community analysis

The microbial community was analyzed using 16S rRNA gene amplicon sequencing, following the procedure described in Alloul et al. (2021). Briefly, biomass samples were taken from multiple disks and pooled it in one sample, centrifuged and the pellet was stored at -20°C prior to DNA extraction. DNA was extracted using the Powerfecal kit (Qiagen, Germany) following the manufacturer's instructions and the DNA concentration was measured using a Qbit3 fluorimeter (Thermofisher Scientific, United States).

DNA extracts were sent out to Novogene Europe (United Kingdom) for sequencing of the V4 region of the 16S rRNA gene on a MiSeq benchtop sequencer after DNA amplification by polymerase chain reaction (PCR) using the set of V4 forward 515f (GTGCCAGCMGCCGCGGTAA) and reverse 806r (GGACTACHVGGGTWTCTAAT) primers. The sequencing reads were clustered into operational taxonomic units (OTUs) at 97% sequence similarity. The data was processed by Novogene Europe with Qiime software (V1.7.0).

Figure – S5.2 The relative abundance of the key genera involved in nitrogen conversions. AnAOB (orange – yellow); AerAOB (gray); NOB (blue); Denitrifier (green). The day of -14 was the sample previous this study (operated at the DO level of 0.8 mg O_2 L^{-1}).

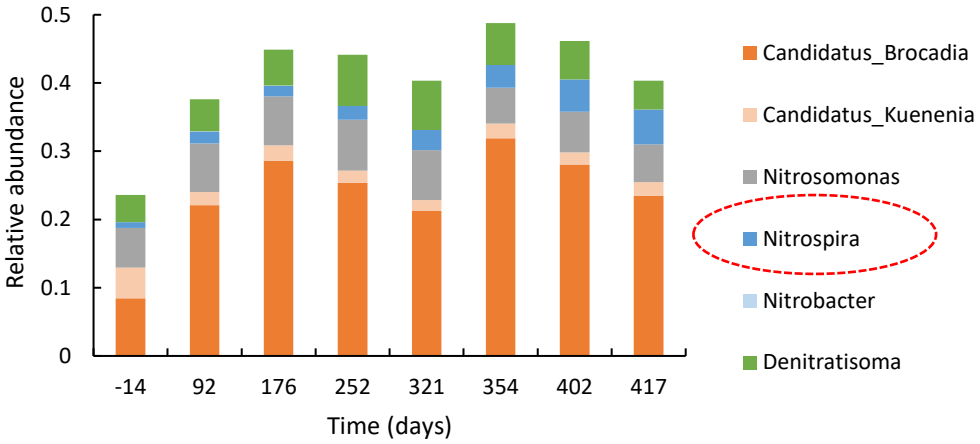


Figure – S5.3 N_2O emission dynamics under different emerged DO levels.

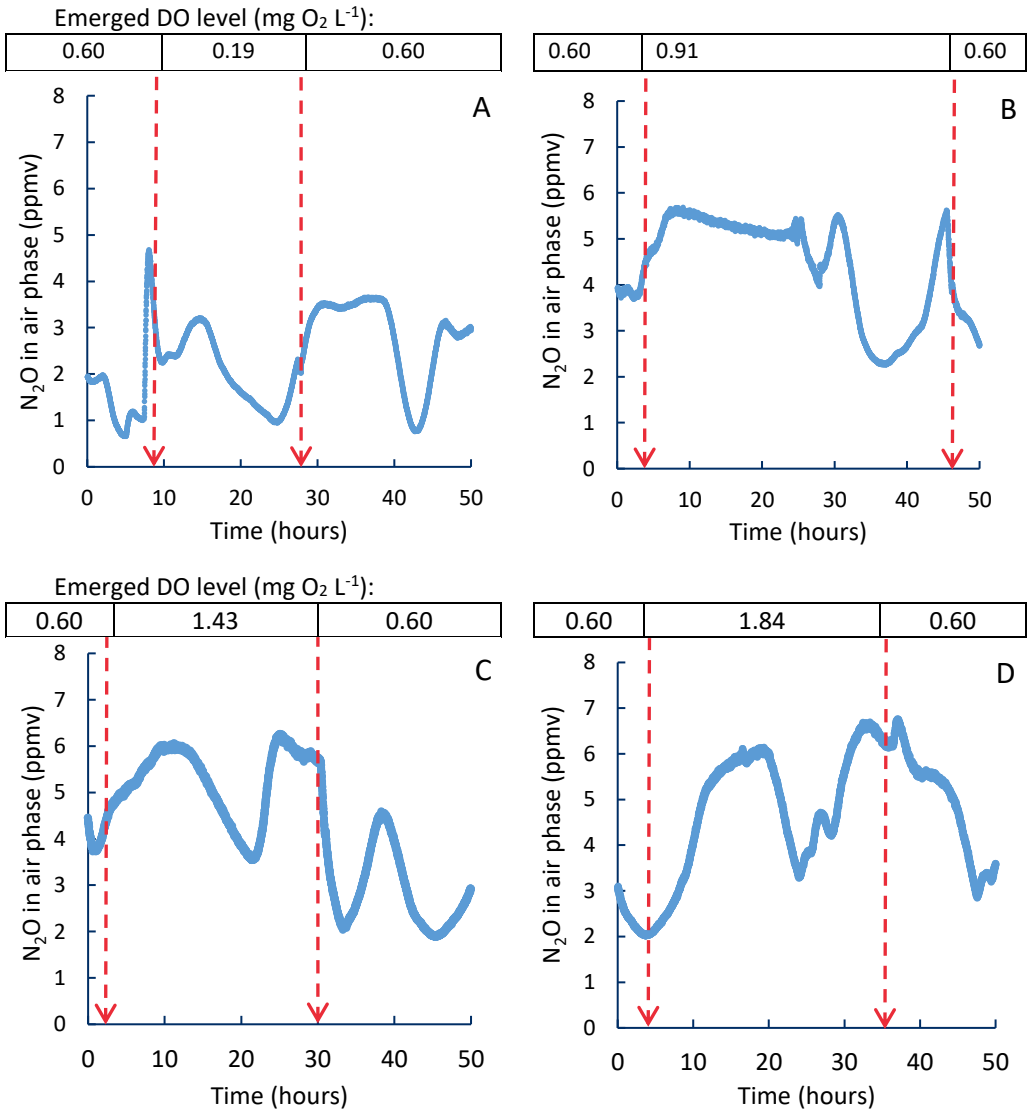


Table – S5.1 The nitrogen concentration comparison before and after FA/FNA treatment.

Treatment [a]	NH_4^+-N (mg N L^{-1})		NO_2^--N (mg N L^{-1})		NO_3^--N (mg N L^{-1})	
	Before	After	Before	After	Before	After
FA (30 mg FA-N L^{-1})	759.9 ± 28.6	747.4 ± 28.1	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
NH_4^+-N (735 mg N L^{-1})	768.2 ± 12.9	712.7 ± 3.3	1.5 ± 0.2	1.4 ± 0.1	7.6 ± 0.2	7.7 ± 0.4
pH (8.0)	16.6 ± 2.7	0.2 ± 0.3	0.8 ± 0.1	0.5 ± 0.1	5.2 ± 0.1	5.6 ± 0.1
FNA (3.0 mg FNA-N L^{-1})	23.8 ± 4.1	23.6 ± 2.6	1265.1 ± 28.4	1244.8 ± 33.2	10.8 ± 0.5	11.9 ± 0.4
NO_2^--N (1205 mg N L^{-1})	10.9 ± 1.8	10.6 ± 0.6	1225.1 ± 32.5	1152.7 ± 35.6	7.9 ± 0.2	7.2 ± 0.2
pH (6.0)	13.1 ± 0.1	9.4 ± 0.2	0.5 ± 0.1	1.2 ± 0.1	9.5 ± 0.3	9.6 ± 0.5

Note: a, All the treatment lasted 3 hours.

Figure – S5.4 N_2O emission dynamics during FA treatment (day 453, 460, and 467).

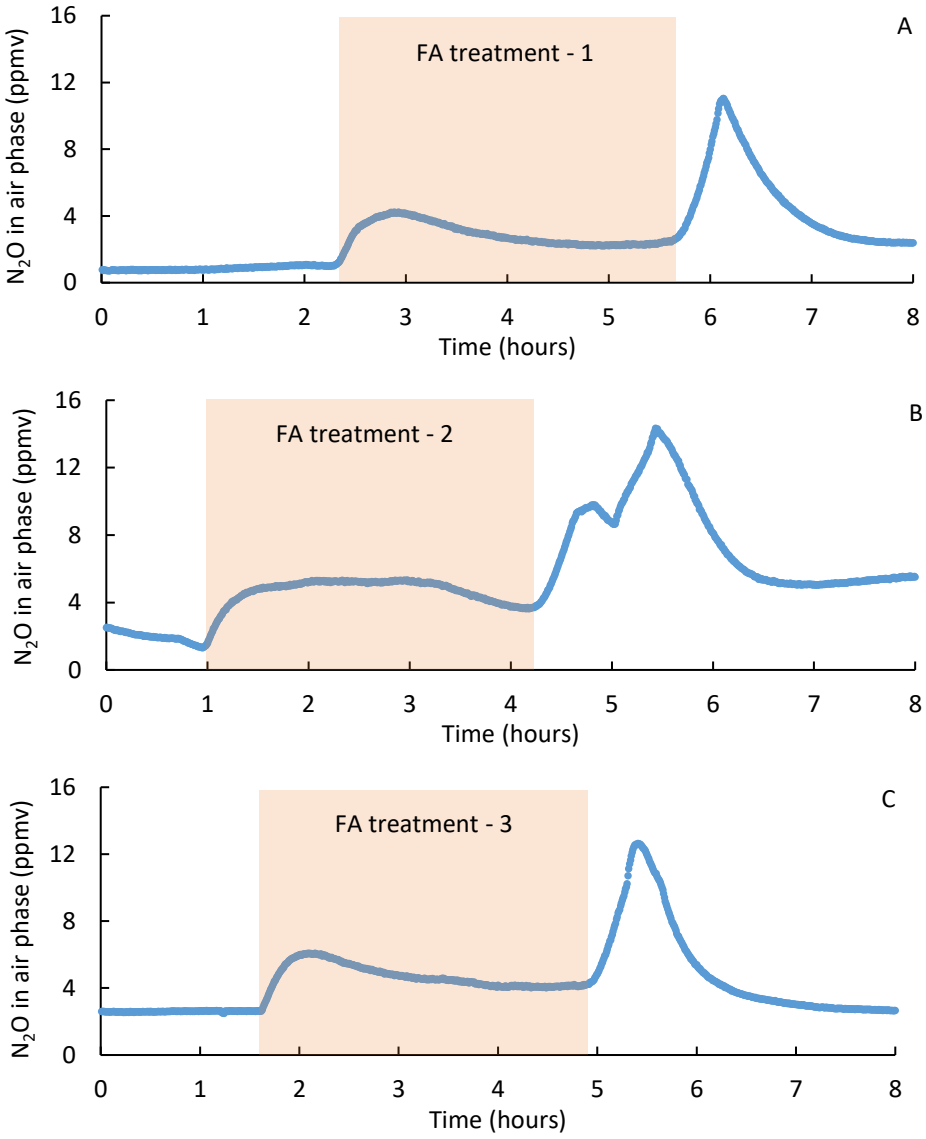


Figure – S5.5 N_2O emission dynamics during NH_4^+-N (735 mg N L^{-1}) (A+B) and high pH (8.0) (C+D) shock.

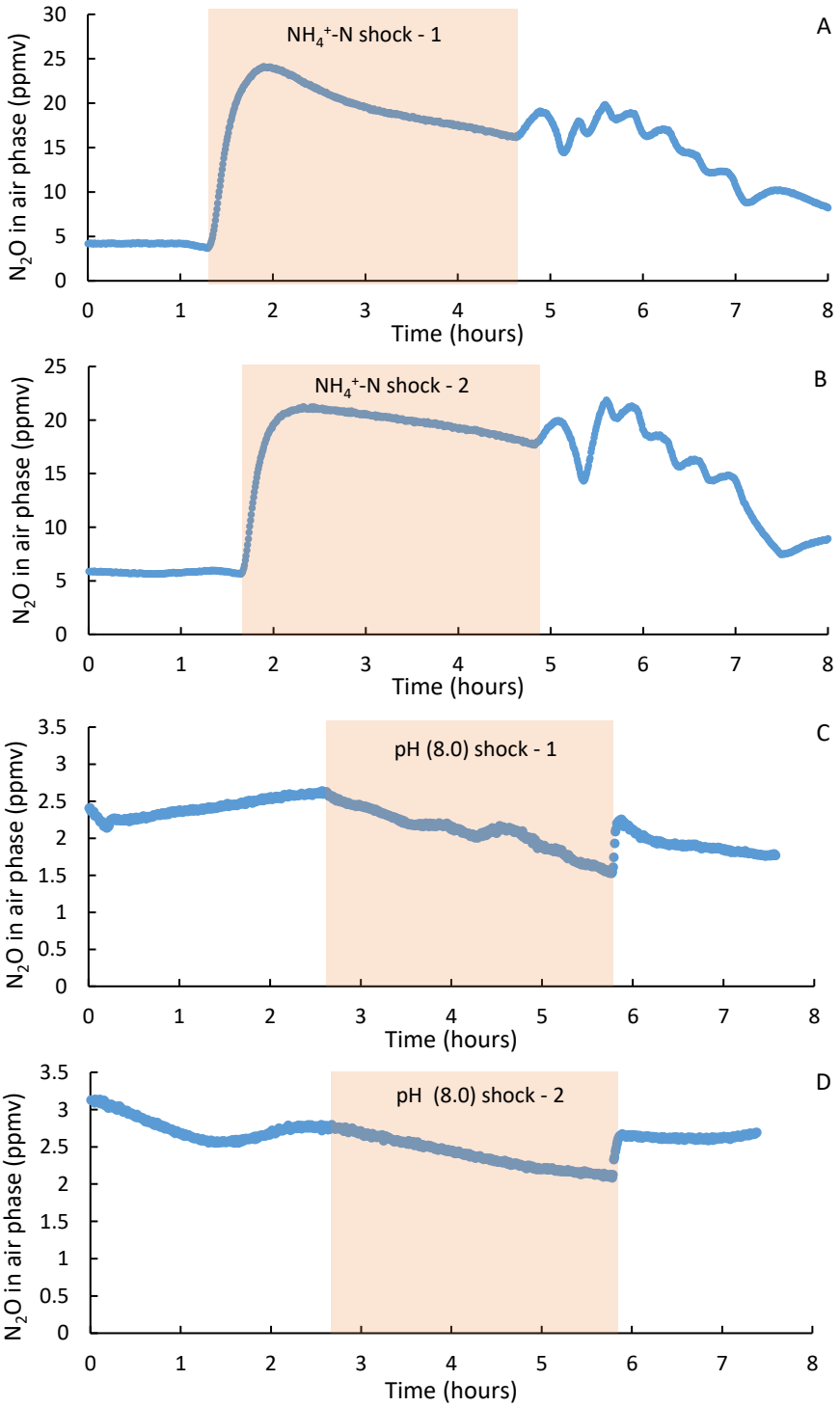


Figure – S5.6 N_2O emission dynamics during FNA treatment (day 498, 505, and 512).

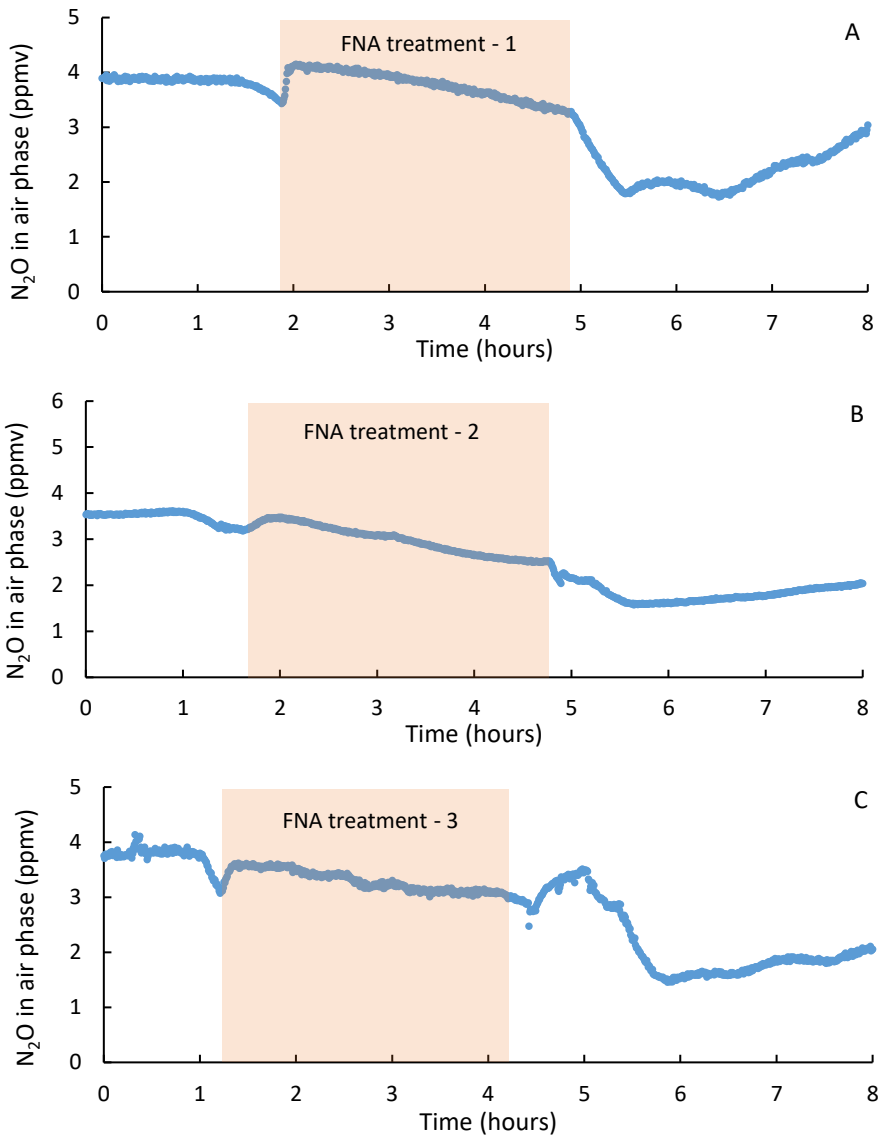
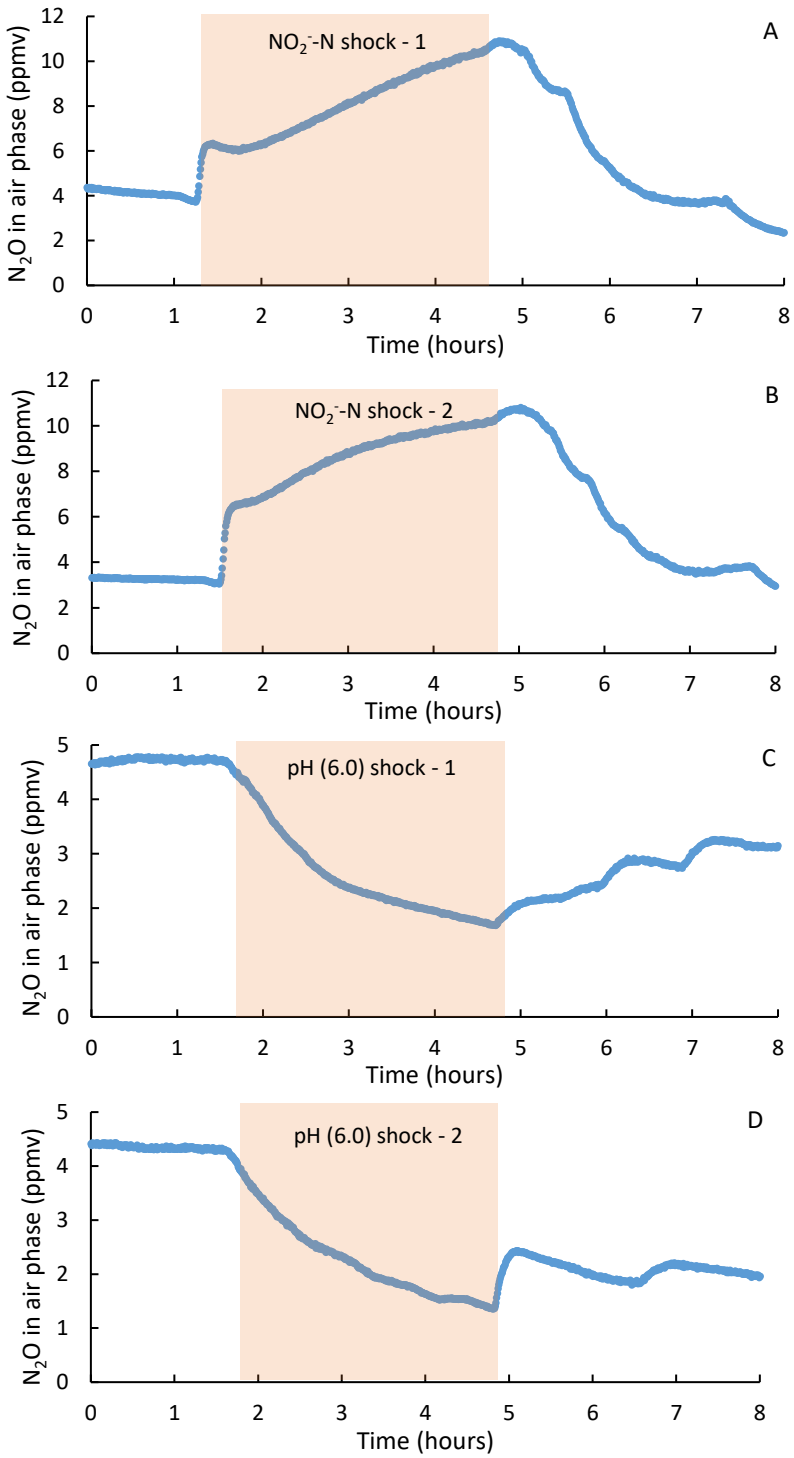


Figure – S5.7 N_2O emission dynamics during NH_4^+-N (~1205 mg N L^{-1}) (A+B) and low pH (~6.0) (C+D) shock.



Chapter 6

General discussion and outlook

6.1 Major findings

This thesis aims to develop the mainstream partial nitritation/anammox (PN/A) technology via proposing the enhancement approaches of nitrogen removal efficiency (i.e., increasing the AnAOB biomass concentration, stimulating the AnAOB activity, and suppressing nitrate and N_2O production). New strategies to facilitate the implementation of the mainstream PN/A are presented.

Chapter 2: Less is more: Substrate-free storage at room temperature enables the long-term preservation of full-scale partial nitritation/anammox sludge

Chapter 3: Towards mainstream partial nitritation/anammox in four seasons: Feasibility of bioaugmentation with stored summer sludge for winter anammox assistance

In these two chapters, the novel concept, i.e., the feasibility of bioaugmentation with stored summer sludge for winter anammox assistance, are validated (Figure – 6.1b). In Chapter 2, a cost-effective biomass preservation strategy was proposed. During this research, the effect of temperature and redox adjustment during the storage of PN/A sludge was assessed. The results showed that nitrogen additives storage conditions (in the presence of nitrite or nitrate) could maintain much higher AnAOB activity than no nitrogen additives conditions (without nitrite and nitrate). Preservation of PN/A biomass without cooling and redox adjustment proved to be a cost-effective strategy taking operating costs into account.

With this cost-effective biomass storage strategy, sufficient anammox sludge (stored summer excess sludge) can be bioaugmented during the winter. The AnAOB activity recovery and biomass retention performance at low-temperature systems were tested to mimic the process that the stored sludge is bioaugmented into the mainstream in winter. The results showed that not only the activity of AnAOB could be well activated at 15 and 10°C, but also that the biomass could be retained. Comparing different sludge types, granular sludge was more suitable for this concept, such sludge being able to reactivate 9 – 15% higher AnAOB activity and retain ~35% more biomass. In the end, this concept demonstrates the economic feasibility of the application. It presents a new countermeasure to enhance the nitrogen-removal performance on winter days.

Chapter 4: Feasibility of a return-sludge nursery reactor to biostimulate mainstream anammox bacteria

In Chapter 4, a return-sludge nursery concept was proposed to biostimulate anammox bacteria with a mainstream niche (Figure – 6.1c). In this new concept, the sidestream nitritation was applied and the resulting effluent is blended with mainstream effluent to achieve a condition that had intermediate temperature and nitrogen concentrations. Results revealed that the total nitrogen removal rates at mainstream increased considerably after nursery treatment. In addition to the relatively high temperature and nitrogen concentrations, the high electrical conductivity, and the potential synergy between these three factors and unknown factors could also contribute to the enhancement. In addition, the biomass growth rate in the nursery reactor was encouraged due to the increase in temperature. Therefore, this mainstream return-sludge biostimulation approach boosted anammox activity and sludge growth, which is a promising alternative to sidestream-to-mainstream bioaugmentation.

Chapter 5: Characterization of N_2O emissions linked to nitrite-oxidizing bacteria suppression strategies in a biofilm reactor

Except for the above-mentioned two direct efficiency-enhancing strategies, the characterization of the indirect methods (typically NOB suppression) is also assessed in Chapter 5 (Figure – 6.1d). In this chapter, three typical NOB suppression strategies (low DO levels, FA, and FNA treatments) and their related N_2O emissions were tested in a mainstream PN/A biofilm reactor. The results showed that long-term inhibition of NOB could not be achieved by low DO levels alone, due to the adaptive nature of NOB. Low DO levels should be used combined with the FA or FNA treatments. Taking the N_2O emissions into consideration, mainstream PN/A systems should be operated at relatively low DO conditions with periodic FNA treatment, which can achieve long-term NOB suppression while also limiting N_2O emissions.

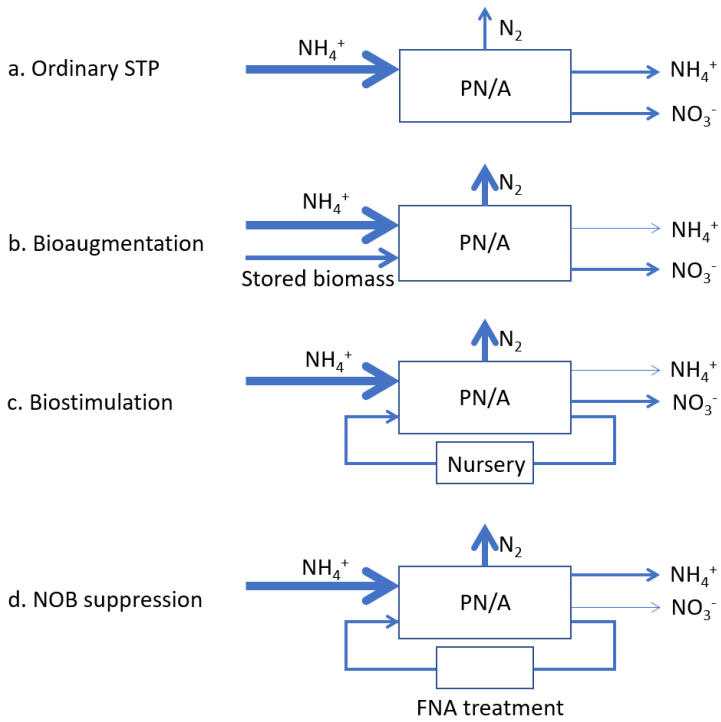


Figure – 6.1 Overview of three routes to enhance nitrogen removal efficiency

6.2 Practical considerations and potential bottlenecks

6.2.1 'Winter bioaugmentation' concept can only be suitable for temperate regions

For the concept of 'winter bioaugmentation with stored summer excess sludge' (Chapter 2 and 3), there needs to be a sufficient temperature difference between winter and summer. In this way, the AnAOB sludge has a higher growth rate in summer compared to winter (e.g., ~2.5 times higher if the temperature difference is 10℃). The sludge in summer is surplus, resulting in a large amount of excess sludge. That provides a source of AnAOB sludge that can be stored into the winter when it can be bioaugmented for mainstream PN/A systems.

For the STPs in the tropical regions, the nitrogen removal rate remains essentially constant due to the small temperature differences throughout the year. Even though the stable temperature in tropical areas limits the application of this concept, it is still a good practice for STPs to store certain amount of biomass to overcome the potential calamity or any issue occurred (e.g., re-startup the system, increase the biomass capacity, etc.). Since the temperature commonly higher than 20℃ (e.g., 25-30℃), the biomass decay and the loss of AnAOB activity during the

biomass storage need to be studied at higher temperature in the following up research. For the linked N_2O emissions, there should be no concern on it during the biomass storage period since no NO_2^- accumulation and operational parameters change (Zhu et al., 2021). About the effect of temperature on N_2O emissions, a higher N_2O production as the increase of temperature by Reino et al. (2017). But more research is needed to reveal the mechanisms. For the STPs in the frigid zone, even though there is enough temperature difference between winter and summer, the mainstream temperature in winter is lower than 5℃ also limits the application of this technology. This is because the study in Chapter 3 shows that when the stored sludge is bioaugmented into a reactor at 4°C, it can only show very little activity (1 – 2% of the initial AnAOB activity). Therefore, this process is only suitable for temperate regions.

6.2.2 The need for a sidestream nitritation unit

For the 'return-sludge anammox nursery' (Chapter 4) and 'return-sludge FNA treatment' technology (Chapter 5), a sidestream partial nitritation unit (PN) is required. That could incur extra operational costs due to the installation of the sludge treatment unit (only for STPs without sidestream) and chemical addition (acid addition to achieve the set FNA level). For STPs with sidestream PN/A system, PN should be used instead (to provide sufficient nitrite concentration for the return-sludge FNA treatment unit). Even taking these costs into consideration, the return sludge FNA treatment is probably still economically favorable due to the savings in methanol addition, aeration energy, and sludge handling (Duan et al., 2018; Wang et al., 2017).

6.2.3 The potential of high N_2O emissions

Due to the sidestream PN/A unit is replaced by the PN, a high nitrite concentration appears (e.g., > 650 mg N L^{-1} assuming 65% nitritation in PN). According to the previous research, the N_2O emissions are positively correlated with the nitrite concentration, which significantly promotes the N_2O emissions from the sidestream PN unit levels (Harris et al., 2015; Peng et al., 2015). Thus, the optimization of the operational conditions which can minimize the N_2O production from the sidestream PN unit still needs study.

6.2.4 Nitrogen removal versus nitrogen recovery

Nitrogen recovery is another option to purify wastewater. It is technically and economically easier and more feasible when the nitrogen concentration is higher (e.g., reject water). There are many different physical/chemical methods to recover nitrogen, e.g., air or steam stripping, struvite precipitation (Batstone et al., 2015; Lema and Martinez, 2017). For the wastewater with low nitrogen concentration (e.g., mainstream), nitrogen recovery becomes economically unfeasible. Thus, this wastewater should focus on nitrogen removal. In the present thesis, the rejected water was used by the return sludge FNA treatment or anammox biostimulation, which is another type of nitrogen recycling.

6.3 Proposed design of a mainstream PN/A configuration

Urban sewage has a high organic carbon to nitrogen ratio (COD/N), which might facilitate the development of heterotrophic bacteria (Hao et al., 2002; Ni et al., 2009; Strous et al. 1998). In the STPs, in order to improve the carbon and nitrogen removal efficiency and avoid the competition among microorganisms, the C/N-stage configuration will be used (Figure 6.3). In this configuration, the organic carbon removal process is separated from the nitrogen removal process.

6.3.1 C-stage: Carbon removal process

Since the PN/A process favors low COD/N conditions, organic carbon is removed in the primary C-stage before the nitrogenous wastewater flows into the PN/A reactor. The C-stage focuses on organic carbon removal and redirects a fraction of the incoming organic carbon to the anaerobic digester. Organic carbon can be removed by high-rate activated sludge (HRAS), which can be operated at low sludge retention time (SRT, < 2 days) and high carbon sludge loading rates (> 2 g bCOD g VSS^{-1} d^{-1}) to obtain young and easily digestible sludge (methane yield is 50 – 60%) (Meerburg, 2016; Seuntjens, 2018a). Therefore, the C-stage can maximize energy-positive wastewater treatment by removing the organic carbon from sewage along with methane production in the anaerobic digestion reactor. At the same time, the high HRAS growth rate provides adequate nitrogen loading in the sidestream, which can be used to stimulate AnAOB (nursery reactor biostimulation) and suppress NOB (return-sludge FNA treatment).

6.3.2 N-stage: Single-stage or two-stage partial nitritation/anammox?

The effluent from the C-stage will flow into the N-stage for nitrogen removal. There are two main processes in N-stage, partial nitritation (aerobic process) and anammox (anoxic process). Based on the previous literature, these two processes can be performed either in a single reactor or in two separated reactors.

6.3.2.1 A single-stage partial nitritation/anammox configuration

A potential efficiency enhancement configuration (with methods proposed in this thesis) based on the single-stage PN/A is shown in Figure – 6.2. Thus, a screen should be applied to separate the flocs and granules (Van Winckel et al., 2019b). Because the granules are rich in AnAOB, they flow into the anammox nursery reactor for biostimulation. Partial flocs (rich in AerAOB and NOB) are streamed into another reactor for NOB suppression by FNA treatment. In summer, the excess sludge can be preserved, waiting for the mainstream bioaugmentation in winter.

In a single-stage PN/A system, probably less N_2O is produced because of low nitrite accumulation (Wang et al., 2016b). In addition, the requirement for only one reactor results in lower capital expenditures (CAPEX) and a smaller footprint in STPs. These advantages contributed to the wide application of single-stage PN/A systems (Lackner et al., 2014; Sui et al., 2021). However, more frequent and intense competition between microorganisms (e.g., competition for nitrite between AnAOB and NOB) and more complicated operational functionalities (e.g., DO setpoints setting and the SRT separation) maybe occur in the single-stage configuration (Laureni et al., 2015; Regmi et al., 2014). Specific to the application of the technologies proposed in this thesis, the performance of AnAOB biostimulation and NOB inhibition will be limited by the efficiency of the screen. In addition, the excess sludge produced in summer might also rich in AerAOB and heterotrophic bacteria (a small amount of COD is present), unwanted in the concept ‘winter bioaugmentation’ since this merely increases the volume of stored sludge and the cost for sludge storage. Therefore, a two-stage PN/A configuration is recommended in this thesis.

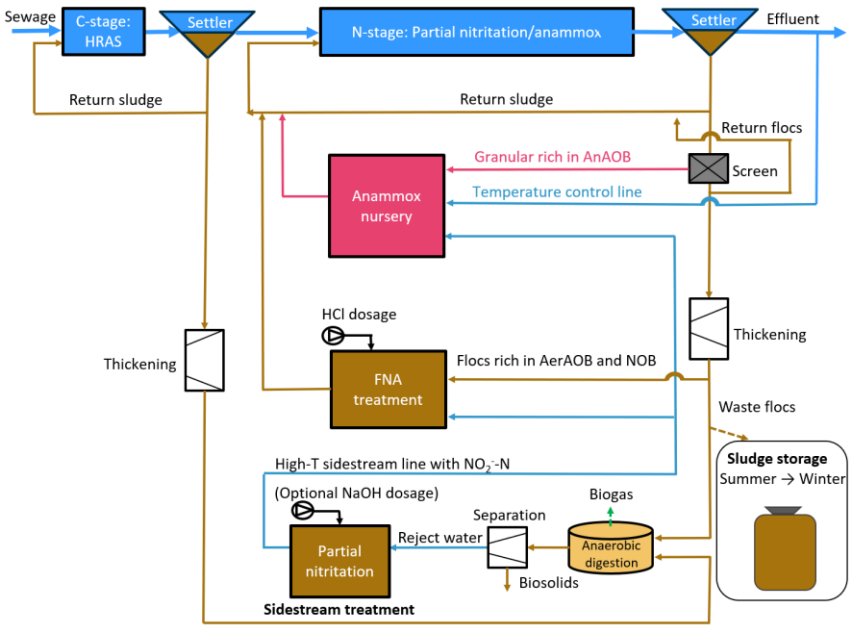


Figure – 6.2 The proposed design of a single-stage PN/A configuration for the future. (1). NOB suppression and AnAOB biostimulation are carried out by return-sludge FNA treatment and biostimulation, respectively. (2) Partial nitritation (with more than 65% nitritation) replaced the PN/A installation in the sidestream, and the NaOH addition was only needed in the 100% nitrite scenario. (3). HRAS refers to the high rate activated sludge.

6.3.2.2 A two-stage partial nitritation/anammox configuration

Separating the partial nitritation and anammox processes into two reactors minimizes competition for the AnAOB (anoxic conditions, no or low bCOD) and maximizes the efficiency of the anammox process. In the mainstream partial nitritation reactor, about half of the ammonium will be converted to nitrite. To inhibit its continued oxidation to nitrate, the activity of NOB should be suppressed. According to the research in **Chapter 5**, aeration control (low DO levels) can be used to suppress the NOB activity in a partial nitritation reactor. But the adaptation will occur in the long term. Therefore, FNA treatment is also required (research in **Chapter 5**)). Therefore, a return-sludge treatment with FNA, which is produced through a partial nitritation reactor of the sidestream, is applied.

After the mainstream partial nitritation process, the sewage that contains ammonium and nitrite is continually treated by the mainstream anammox process. The anoxic conditions of the reactor benefit the AnAOB. The low efficiency caused by the relatively low temperature in the

mainstream can be enhanced by the return-sludge nursery biostimulation (research in **Chapter 4**). In summer, excess anammox sludge will be produced due to the relatively high temperature and can be preserved with the cost-effective strategy (room temperature without cooling and substrates addition researched in **Chapter 2**). During the winter period, the stored sludge is bioaugmented into the mainstream anammox reactor in order to increase the biomass concentration (research in **Chapter 3**).

With this proposed configuration, the efficiency of anammox is improved, especially in winter, and can be further improved by bioaugmentation by the stored summer sludge. At the same time, the NOB activity is inhibited by a strategy (FNA treatment) with low N_2O emissions.

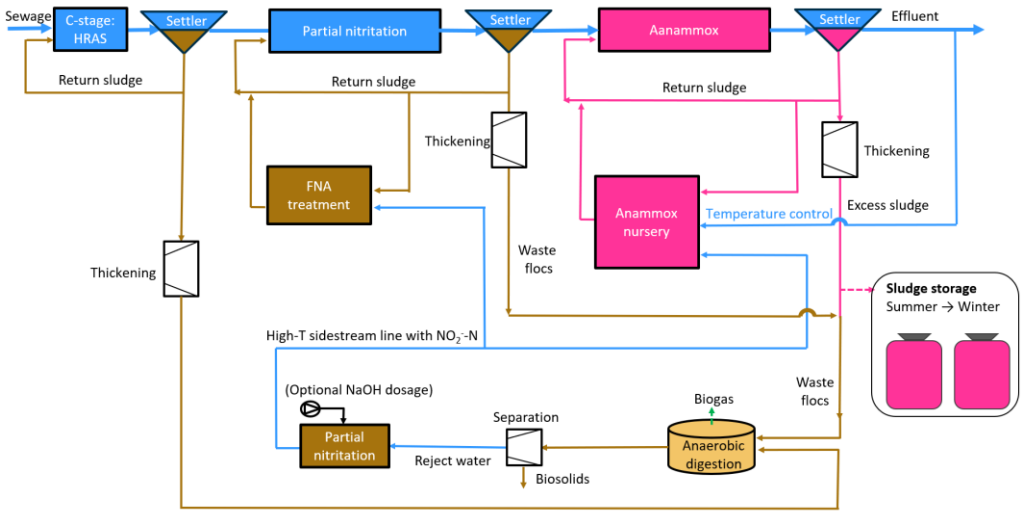


Figure – 6.3 The proposed design of a two-stage PN/A configuration for the future. (1). Partial return-sludge of the mainstream partial nitritation reactor is treated by FNA. (2). AnAOB activity in the mainstream reactor is biostimulated by a return-sludge nursery reactor. (3). Partial nitritation (with more than 65% nitritation) replace the PN/A installation in the sidestream, and the NaOH addition is only needed in the 100% nitrite scenario. (4). FNA treatment reactor refers to the contact tank is part of the return sludge line is mixed with the FNA-rich liquid from the partial nitritation unit. (5). HRAS refers to the high rate activated sludge.

6.3.3 Strengths and bottlenecks

Through the proposed configuration, the aerobic and anoxic processes are separated. It is easier to provide optimal operating conditions to satisfy both AerAOB and AnAOB, which is hard to achieve in the single-stage PN/A system. Firstly, the AnAOB can probably avoid the

inhibition caused by O_2 exposure. According to Seuntjens et al. (2018b), AnAOB showed complete inhibition until micro-aerobic conditions were reached again (< 0.02 mg O_2 L^{-1}). Higher DO setpoints (ON/OFF control) can be applied in the mainstream partial nitritation reactor to promote the activity of AerAOB over NOB (Regmi et al., 2014). Secondly, NOB suppression and AnAOB biostimulation can be achieved separately. Thus, there is no inhibition of FNA treatment on AnAOB (Peng et al., 2020). Finally, the novel 'winter bioaugmentation' concept might be applied in a more cost- and space-effective way (e.g., high purity AnAOB sludge from anoxic anammox reactor is harvested in summer). This can reduce the operating expenses and footprint for sludge preservation.

Even though promising, there are defects of this configuration. Firstly, due to the limitation data of the mainstream two-stage PN/A process, the technically possible analysis (heat and flow balance between FNA treatment line and anammox nursery line) was not carried out in the present research. On one hand, a larger footprint is needed when comparing with the conventional one-stage PN/A system (Seuntjens, 2018a). The newly added devices, namely the FNA treatment reactor and the anammox nursery reactor, take up some space in the STPs while improving the PN/A efficiency. On the other hand, more N_2O might produce in a partial nitritation reactor due to the higher nitrite concentration accumulation (Kampschreur et al., 2008; Lotti et al., 2014a). During the mainstream partial nitritation process, ~30 mg NO_2^--N L^{-1} of nitrite is produced, which leads to high N_2O emissions (Peng et al., 2015). Furthermore, available construction space is still a major limitation to the application of bioaugmentation (with stored summer excess sludge).

6.4 Outlook and perspectives: From challenging to promising

After more than 20 years' research, more and more challenges are being overcome in the mainstream PN/A system. On one hand, efficient and cost-effective NOB suppression strategies were proposed (Duan et al., 2019a; Ma et al., 2017c; Peng et al., 2020; Wang et al., 2014), such as the return-sludge FNA treatment applied in this thesis. This can effectively avoid the occurrence of side reactions, thus achieving long-term stable operation of PN/A systems. On the other hand, strategies to improve the efficiency of AnAOB have been extensively studied (Liu et al., 2014; Yu et al., 2013), such as the bioaugmentation and biostimulation concepts presented in this thesis. These can help to increase nitrogen removal efficiency. All these efforts have made the application of mainstream PN/A technology more and more promising. After

overcoming the newly discovered issues, the application of PN/A in the mainstream will be closer.

Firstly, the optimization of the operational conditions which can minimize the N_2O production still needs study. The separated partial nitritation reactor in the two-stage PN/A configuration appear to allow further development of aeration control strategies to reduce the associated N_2O emissions. Additionally, the mechanisms behind N_2O emissions also require more investigation.

Secondly, there is a small amount of nitrate production after the PN/A process. Due to the wastewater discharge standard limiting the effluent of total nitrogen, the appropriate strategies to remove the nitrate from the effluent also deserve more study.

Thirdly, how effective is the performance of the proposed configuration in the treatment of real sewage as opposed to synthetic wastewater is also need study. The complex influent composition and the fluctuation of the temperature may affect the performance of AnAOB enhancement and NOB suppression. Additionally, the detailed information about the heat and substances balances between sidestream, FNA treatment reactor, and anammox nursery system also need to be studied in the future.

6.5 Conclusions

Three ways to improve the efficiency of mainstream denitrification are demonstrated in this thesis. The winter bioaugmentation strategy promotes anammox efficiency by increasing the biomass concentration in winter (Chapter 3). Bioaugmentation of summer excess sludge stored using cost-effective sludge preservation methods (no substrate or redox addition at room temperature) can satisfy this (Chapter 2). The return-sludge biostimulation strategy enhances the AnAOB activity by an anammox nursery reactor, which applies sidestream nitritation, and blends the resulting effluent with mainstream effluent to achieve an intermediate temperature and nitrogen concentrations (Chapter 4). The third method is indirect, using the return-sludge FNA treatment (i.e., with less N_2O emissions) to inhibit NOB activity and reduce the conversion of ammonium to nitrate (Chapter 5). Through the proposed two-stage PN/A configuration (Figure 6.3), all these three technologies can be combined to enhance the efficiency of STPs and achieve the required effluent quality. Due to the high concentration of nitrite produce in a partial nitritation reactor, which may result in high N_2O emissions, more research on N_2O migration strategies is needed in the future.

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Curriculum Vitae

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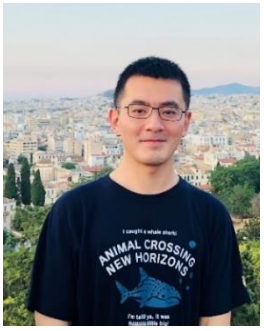

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Publications

1. Weiqiang Zhu, Michiel Van Tendeloo, Yankai Xie, Marijn Juliaan Timmer, Lai Peng, Siegfried E. Vlaeminck*, 2022. Storage without nitrite or nitrate enables the long-term preservation of full-scale partial nitritation/anammox sludge. *Science of the Total Environment,* 806, 151330.

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5. Lai Peng*, Yankai Xie, Wannes Van Beeck, **Weiqiang Zhu**, Michiel Van Tendeloo, Tom Tytgat, Sarah Lebeer, Siegfried E Vlaeminck*. Return sludge treatment with endogenous free nitrous acid limits nitrate production and N_2O emission for mainstream partial nitritation/anammox. *Environmental Science & Technology*, 2020, 54(9): 5822-5831.

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2. Van Tendeloo, M., Xie, Y., Van Beeck, **W., Zhu**, W., Lebeer, S. and Vlaeminck, S. E. Biofilm-based mainstream partial nitritation/anammox with complete suppression of nitrite oxidizing bacteria by continuously low oxygen levels. *Poster*, 2020-12 (07-10), IWA Biofilms 2020, University of Notre Dame, USA. (Fully virtual conference)

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