Development of a kinetically engineered microbial community for Nitrite Shunt as a B-stage process using different Aeration Strategies

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A DISSERTATION SUBMITTED TO THE FACULTY OF GRADUATE STUDIES IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

GRADUATE PROGRAM IN CIVIL ENGINEERING
YORK UNIVERSITY
TORONTO, ONTARIO

December 2022

ABSTRACT

Nowadays, depleted energy resources, increasing worldwide energy demand and global climate change has been witnessed. In accordance, wastewater treatment plants (WWTPs) have prioritized minimizing its energy use, maximizing resources recovery, while efficiently treating the received wastewater. Shortcut BNR (SBNR) has been proposed as an energy-efficient nutrients removal process towards lowering the energy use of the current WWTPs.

Nonetheless, full-scale implementation of SBNR in mainstream conditions has been hindered by the major challenge of nitrite oxidizing bacteria (NOB out-selection. To address such a key bottleneck, this dissertation proposes, for the first time, a novel kinetic-adaptation based strategy to engineer the microbial community to maintain NOB out-selection at mainstream conditions. The successful implementation of such a strategy and its underlying mechanisms was demonstrated and investigated for more than 400 days. In result, an ammonia removal efficiency of 99.4±0.4% and nitrite accumulation rate of 87.4±0.6% under low DO levels of 0.1–0.2 mg/L was reached.

Afterwards, the potential to employ the developed strategy to perform mainstream nitrite shunt was investigated considering the limited carbon availability in the A-stage effluent, its fractionation, and the applied aeration strategy. At carbon to nitrogen (C:N) ratio as low as 6.0, ammonia, COD and total inorganic nitrogen (TIN) removal efficiencies of 99.2±0.7, 94.0±0.1 and 93.2±1.6% were successfully achieved under continuous low DO aeration strategy. Investigations revealed that maintaining NOB suppression played a key role in achieving high TIN without the need for external carbon addition. Two more aeration strategies were investigated, low DO intermittent aeration and high DO intermittent aeration. At C:N ratio as low as 6, higher TIN removal of 95.8±0.9% was achieved at low DO compared to high DO which achieved a TIN removal of 73.8±1.7%. Therefore, it was concluded that the developed kinetic-adaptation strategy can be utilized along with different aeration strategies with slight advantage to low DO intermittent aeration for its higher TIN removal with limited carbon.

The findings of this dissertation present a novel strategy that blaze a trail to overcome the major bottleneck of NOB out-selection to implement nitrite shunt at mainstream as energy and resources efficient B-stage process.

DEDICATION

"To my wife Rana, daughter Sophia and my unborn son. Thank you for being in my life. Thank you for being the main source of my inspiration. Hope one day, I'll make you proud as much as you always make me."

ACKNOWLEDGEMENT

Alhamdulillah, praise to ALLAH the Almighty, the Most Gracious and the Most Merciful who empowered me with strength and knowledge to accomplish this work.

Dr. Ahmed ElDyasti: I would like to express my sincere gratitude for your support academically and even more outside of professional life. We’ve come a long way since the day you welcomed me to Canada. I am hopeful that our journey together will continue beyond this milestone with a future filled with blessings and success. I will always be thankful for the opportunity you have given me and the support you have provided.

My dearest wife Rana: You always stick by my side, no matter how hard it is or how long it takes. You lifted me up when I was down, you encouraged me when I was disappointed, you always saw the light at the end of tunnel even when I couldn’t see it. You’ve always put me first in bad times and good times. I couldn’t have done it without you. Thank you for being you, thank you for being my best friend, my backbone and lifetime partner.

My beautiful daughter Sophia (Sophsoph): Thank you for bringing joy to my heart, beauty to my world, love to my life. No matter how hard my day was, a smile from you was always able to turn my whole day upside down. Coming back home every day to play with you was the best reward that always kept me motivated. No matter how much you mature, you’ll always be my little girl and I’m forever thankful for that.

My family: I am really thankful for your continuous support and unbounded love. A thousand miles might separate us physically, but I never felt that you are not with me. I am grateful for **My Dad** for being a role model. I know that you have always put our needs before yours and I’ll be forever thankful for that. **My Mum** for her endless prayers, I was never worried before any big milestone because I knew that your prayers would guide me. I'd like to thank **Moustafa, Marwan, Suzie, Rowan, Meezo** and **Kinda** for being in my life. As well, I'd like to extend my gratitude to my second family, **Dodo Khaled, Nana Manal, Riham, Raghda, Rim, Omar,** and **Hany**. I love and miss each one of you.

Ahmed Alsayed: My PhD journey companion. You've always supported me in bad times before good times. Your impact on me was beyond your significant contribution to this dissertation. Our long discussions made me a better person academically and personally. Thank you for keeping with my nonsense and never getting tired of our long conversations. I'll always be grateful for your valuable advice. I am thankful that I've gained a lifetime friend along with my PhD degree.

Moustafa Aboutabikh, Hadil and Feesho: Thank you for making Canada feel like home. Thank you for making us feel that we live with our family.

My friends: Thank you for all the fun we have had, the late night coffees, the laughter during the bad times and the valuable advice you provided. Thank you **Fergala, Zaki, Nader, Abdelahmeed, Waleed, Khaled, Hassan, Kholy.**

My friends in Egypt: I am thankful for your virtual presence in my life. Despite the long distance you were always here for me. Thank you **Abdelmeguid (Gydo)** for your continuous support, you have always been a brother to me. Also, thanks to **Rani, Magdi, Sheikh, Shawki, , Moneim, Aly, Mohamed Amir (Buffalo), Saad (Bolbol), Nader, Omar, Tarawy, Amin**, and **Hamami**.

iWATER Team: Thanks for the fruitful discussion and for your continuous support. Special thanks to **Parin** and **Parnian** for always being good friends.

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ACRONYMS

A/B	Adsorption/Bio-oxidation
A2O	Anaerobic/Anoxic/Aerobic
AAA	Alternating Activated Adsorption
ABAC	Ammonia Based Aeration Control
AD	Anaerobic Digestor
AFBR	Anaerobic Fixed Bed Reactor
Anammox	ANaerobic AMMonia OXidizing bacteria
AO	Anaerobic/Aerobic
AOAO	Anaerobic/Aerobic/Anoxic/Aerobic
AOB	Ammonia Oxidizing Bacteria
ARE	Ammonia Removal Efficiency
AT	Anaerobic Treatment
AvN	Ammonia vs NOx control
bCOD	Biodegradable COD
BES	BioElectrochemical System
BNR	Biological Nitrogen Removal
C:N	Carbon to Nitrogen
CANON	Completely Autotrophic Nitrogen removal Over Nitrite process
CAS	Conventional Activated Sludge
CEPT	Chemically Enhanced Primary Treatment
CHP	Combined Heat and Power
COD	Chemical Oxygen Demand

CSTR	Continuously Stirred Tank Reactor
DEMON	Deammonification process
DGAO	Denitrifying Glycogen Accumulating Organisms
DO	Dissolved Oxygen
DPAO	Denitrifying Phosphorus Accumulating Organisms
EBPR	Enhanced Biological Phosphorus Removal
EIA	Energy Information Administration
FA	Free Ammonia
FNA	Free Nitrous Acid
HiCS	High-Rate Contact Stabilization
HRAS	High-Rate Activated Sludge
HRT	Hydraulic Retention Time
$\mathbf{K_o}$	Oxygen half saturation coefficient
$\mathbf{K_{o,AOB}}$	AOB Oxygen half saturation coefficient
$\mathbf{K_{o,NOB}}$	NOB Oxygen half saturation coefficient
MBR	Membrane BioReactor
MFC	Mass Flowrate Controller
MLE	Modified Ludzack– Ettinger
NAR	Nitrite Accumulation Rate
nbCOD	Non-Biodegradable COD
nbpCOD	Non-Biodegradable Particulate COD
nbsCOD	Non-Biodegradable Soluble COD
$\mathbf{NH_4}$	Ammonia

NLR	Nitrogen Loading Rate
NO_2	Nitrite
NO_3	Nitrate
NOB	Nitrite Oxidizing Bacteria
OLAND	Oxygen Limited Autotrophic Nitrification Denitrification process
PAO	Phosphorus Accumulating Organisms
pCOD	Particulate COD
PdNA	Partial DeNitrification-Anammox
PID	Proportion-Integral-Derivative
PLC	Programmable Logic Controller
R.A	Relative Abundance
RAS	Return Activated Sludge
rbCOD	Readily Biodegradable COD
sbCOD	Slowly Biodegradable COD
SBNR	Shortcut Biological Nitrogen Removal
SBR	Sequential Batch Reactor
sCOD	Soluble COD
SHARON	Single reactor for High activity Ammonia Removal Over Nitrite process
SNAP	Single stage Nitrogen removal using Anammox and Partial nitrification process
SND	Simultaneous Nitrification/Denitrification
SNR	Specific Nitrification Rate
SRT	Solids Retention Time
SS	Suspended Solids

SVI	Sludge Volume Index
TCF	Trillion Cubic Feet
TIN	Total Inorganic Nitrogen
TSS	Total Suspended Solids
UN-FAO	Food and Agriculture Organization of the United Nations
VER	Volume Exchange Ratio
VSS	Volatile Suspended Solids
WAS	Wasted Activated Sludge
WRF	Water Reclamation Facility
WRRFs	Water Resources Recovery Facilities
WW	WasteWater
WWTPs	WasteWater Treatment Plants

Chapter 1: Introduction and Dissertation Motivation

1.1. Introduction

Recently, environmental engineering has gained numerous modern advances due to depleted energy resources, increasing worldwide energy demand and global climate change, mainly caused by utilization of fossil fuels which can be detrimental to economic development, particularly in areas of high-energy consumption such as North America. According to the U.S. energy Information Administration (EIA), the global consumption of dry natural gas has increased from 50 trillion cubic feet (TCF) to 122 TCF between 1980 to 2013 with North America being the highest contributor with 33 TCF (more than 25% of the global consumption). Furthermore, wastewater treatment plants (WWTPs) in North America consume 4% of these 33 TCF to meet its energy requirement. Thus, reducing this energy use has been a topic of great interest lately which led to a paradigm shift towards the vision of WWTPs from energy-intensive facilities with a sole objective of removing pollutants to facilities which encompass an immense latent energy and resources that are able to turn them into self-sufficient or even net positive facilities. Therewith, the total dependence on conventional activated sludge (CAS) as the main process for wastewater treatment has been reassessed resulting in the emergence of the water resources recover facilities (WRRFs) concept. The WRRF concept relies upon maximizing the energy recovery/minimizing the energy use while efficiently treating the received streams and meeting the required effluent standards.

One of the widely adopted schemes for WRRF is the Adsorption/ Bio-oxidation (A/B) scheme where carbon, typically expressed as chemical oxygen demand (COD), and nitrogen removal are separated unlike in CAS. In such a scheme, the A-stage is designed solely for improving organics capture and directing it to the solids stream before its biological oxidation, whereas the B-stage is dedicated for nutrients treatment. The objective of A-stage is to achieve higher energy recovery through maximizing COD capture, while the B-stage's goal is to develop novel nitrogen removal technologies that are able to reduce the energy use of conventional biological nitrogen removal (BNR) to avoid nullifying the energy gain achieved in the A-stage. Thus, shortcut BNR (SBNR) has been proposed as a suitable B-stage system to lower the aeration requirements and by consequence reduce the energy use while requiring less carbon demand which enables maximizing COD redirection to energy/resource recovery processes. This dissertation develops, for the first time, a strategy for the successful implementation of a robust short-cut BNR process (i.e., nitrite shunt) and demonstrates that, with this strategy, SBNR can be effectively implemented in the B-stage. This strategy relies on kinetically engineering the microbial community in B-stage process.

1.2. Technical Background

Wastewater treatment is a complex process comprising several physical/chemical/biological processes with an objective of removing different pollutants before employing the treated wastewater for other purposes such as replenishing surface or groundwater, irrigation and in some cases of advanced treatment for drinking water. Those pollutants can be categorized into solids, carbonaceous matter (COD), nitrogenous and phosphorus compounds (nutrients). Typically, raw wastewater from collection systems is preliminary treated using physical processes, i.e., screens and girt removal chambers to remove coarse and heavy solids material to protect downstream processes. Afterwards, wastewater is passed to the primary clarifier to remove settleable solids by sedimentation and floatable materials by skimming. In primary treatment, around 50 to 70% suspended solids (SS) and 25 to 40% of COD are removed, while inorganic nutrients remain unremoved.

1.2.1. Conventional activated sludge (CAS) as the core biological process in energy intensive WWTPs

Conventionally, the effluent of the primary treatment is passed to an activated sludge (i.e., CAS) process comprising an aeration tank and a secondary clarifier whereas the collected primary sludge is directed to the solids stream (side stream) for further sludge handling. In the aeration tank of the CAS, aerobic heterotrophic bacteria consume the remaining COD using oxygen as the terminal electron acceptor, afterwards in the secondary clarifier the sludge is given enough time to settle. Part of the settled sludge is returned back to the aeration tank to maintain a high biomass concentration, whereas the excess sludge is directed to the solids stream. The performance of the CAS process relies remarkably on the solids retention time (SRT) employed in the aeration tank. It has been reported that a minimum SRT of 2-5 days is required to allow for COD hydrolysis and utilization, however, typically the actual SRT applied in CAS process is longer than the minimum required SRT and can reach more than 10 days (Metcalf & Eddy, 2013). The prolonged SRT is to allow nitrification to occur in the aeration tank in order to increase the system capacity in removing nitrogenous compounds as will be discussed in the latter sections. CAS is an efficient process in terms of COD removal as an effluent COD of less than 4% of the influent COD can be achieved at appropriate SRTs (Batstone et al., 2015).

Nonetheless, CAS is an energy, and cost intensive process as it is estimated that the treatment of 1 m^3 of wastewater (WW) consumes around 1080 – 2160 kJ (McCarty et al., 2011a). As such, according to the food and agriculture organization of the United Nations (UN-FAO), in 2009, 26.61 billion of m^3 WW was treated and if an average of 0.45 kWh/m^3 WW treated is considered, that means that around 12 x 10^9 kWh of electricity is consumed per year for wastewater treatment (Liu et al., 2018). Interestingly, it was reported that aeration by itself contributes to around 50%

of the total energy consumption which triggered extensive research on how to improve the aeration efficiency and exploring new technologies that require less aeration demand in order to decrease the aeration-associated energy consumption (Henderson, 2002). Herewith it is worth mentioning that integrating nitrification within the CAS process results in an increase in the energy consumption by around 30% as it was observed that the energy consumption required for aeration rose from 0.305 to 0.405 kWh/m^3 WW treated after implementing nitrification in the aeration tank of the CAS process (Monteith et al., 2007). Moreover, the second challenge associated with CAS process is its relatively high sludge production as it is estimated that each Kg of COD removed produces around 0.3-0.5 Kg of dry biomass in the form of wasted activated sludge (WAS). In 2010, it was reported that the European Union produced 19 million tons of dry WAS against 8 million tons in the U.S (Liu et al., 2018). In addition, it was reported that sludge production increased drastically from 40 million tons in 2016 to 60-90 million tons in 2020. These high volumes of produced sludge represent a major barrier towards the efforts of reducing the energy footprint of WWTPs since it is estimated that sludge post-treatment contributes to around 30% of the total energy consumption of WWTPs besides the fact that these high volumes increase the capital and operational cost associated with the WWTPs (Henderson, 2002).

Even though CAS based WWTPs are energy intensive, wastewater encompasses an enormous latent source of energy in the form of COD which can be converted to biogas through the anaerobic digestion of biosolids. In particular, it is estimated that the theoretical potential recoverable energy from 1 g COD is around 14.7 – 17.8 KJ (Heidrich et al., 2011). As stated previously, the average energy consumption of the CAS is 0.45 kWh/m^3 WW treated (1620 KJ/ m^3 WW treated) and if the average COD content in municipal WW of 500 mg COD/L is considered, that means that the average energy consumption of CAS is 3.24 KJ/g COD. Hence, the potential energy in WW is 4.5-

5.5 times higher than the energy consumed during its treatment. However, the current configuration of WWTPs relying on the CAS process is incapable of recovering a big portion of the latent energy in WW. In CAS, energy is recovered through anaerobic digestion in the solid streams where collected primary and secondary sludge are digested and converted into biogas. Thus, the amount of energy recovered depends on the amount of the COD captured in the mainstream line and directed to the anaerobic digester (AD). Unfortunately, COD in the aeration tank of the CAS process is mainly removed through mineralization where the COD is oxidized to carbon dioxide which has no combustion value for energy recovery and only small portion is captured and directed to the AD. The percentage of captured COD varies in the range of 18 – 26% of the influent COD according to the applied SRT in the aeration tank with higher COD captured at lower SRT (Alloul et al., 2018; Sancho et al., 2019). On the other hand, in the primary clarifier all the COD removed is captured and directed to the AD which accounts for around 40% of the influent COD. Hence, it can be assumed that the total COD captured and directed to the AD from both the primary and secondary clarifiers is in the range of 58 – 66% of the total influent COD. However, only about 20 – 65 % of the COD captured can be destructed in the AD and ends up into biogas depending on the sludge characteristics. In particular, it has been reported that the primary sludge conversion into methane efficiency is around 40 – 65% against only 20 – 35% for the secondary sludge (Alloul et al., 2018; Sancho et al., 2019; Wan et al., 2016). Therefore, if the maximum conversion efficiencies are assumed, then the CAS process has the ability to convert 35% of the total influent COD into biogas. Theoretically, about 13.9 KJ of chemical energy can be obtained from one gram of biogas, thus the total chemical energy that can be recovered in CAS process is 4.87 KJ per gram of influent COD (Heidrich et al., 2011). Consequently, the produced biogas can be converted into electricity using the combined heat and power (CHP) technologies in which around 35% of the chemical energy is converted to electrical energy. Thus, it can be concluded that in CAS process a maximum of 1.7 KJ is recovered per gram of COD which is around 50% of the total energy consumption (3.24 KJ/g COD), however in practice the actual percentage of recovered energy is much lower in most of the cases. Collectively, the low energy recovery capacity of CAS process can be referred to the high loss of COD through mineralization in the aeration tank and consequently the low COD capture efficiency.

1.2.2. The A/B process to consolidate the shift towards Water Resources Recovery Facilities (WRRFs)

All the aforementioned challenges and shortcomings of CAS has driven research towards the implementation of new technologies that can overcame these challenges which have blazed a trail for the emergence of the new concept of Water Resources Recovery Facilities (WRRFs). The WRRF concept embraces the following objectives in order to reduce the energy use of WWTPs towards achieving a net-zero consumption or even net-positive production WWTPs: (i) lowering the aeration energy use and consequently reducing the energy consumption, (ii) increasing the COD capture by reducing mineralization and subsequently increasing energy recovery, and (iii) minimizing the sludge production and consequently reducing the cost of the sludge treatment. In pursuit of these objectives, several configurations have been proposed, among which the A/B process is the most prominent since it is compatible with the existing infrastructure and has already been applied at full scale in many countries (Meerburg, 2016). As discussed previously, the long SRT applied in CAS process in order to allow more time for biological nitrogen removal results in high COD mineralization and consequently less COD capture. Moreover, it results in high aeration associated energy consumption which increases the energy and cost of WW treatment and lowers its potential energy recovery. Thus, in A/B process, COD and nitrogen removal are separated with the A-stage designed solely for maximizing COD capture and directing it to the solids stream before its biological oxidation whereas B-stage is dedicated for nutrients treatment. In the previous proposal, the A-stage aims to achieve higher energy recovery through higher COD capture and B-stage aims to develop novel nitrogen removal technologies that are able to reduce the energy use of conventional biological nitrogen removal (BNR) to avoid nullifying the energy gain achieved in the A stage. In summary, the WRRFs objective relies upon maximizing the energy recovery of the carbon stream while minimizing the energy consumption of the nitrogen removal systems creating a condition where the energy gain in the carbon stream can offset the energy consumption of the following nitrogen stream **(Figure 1.1**).

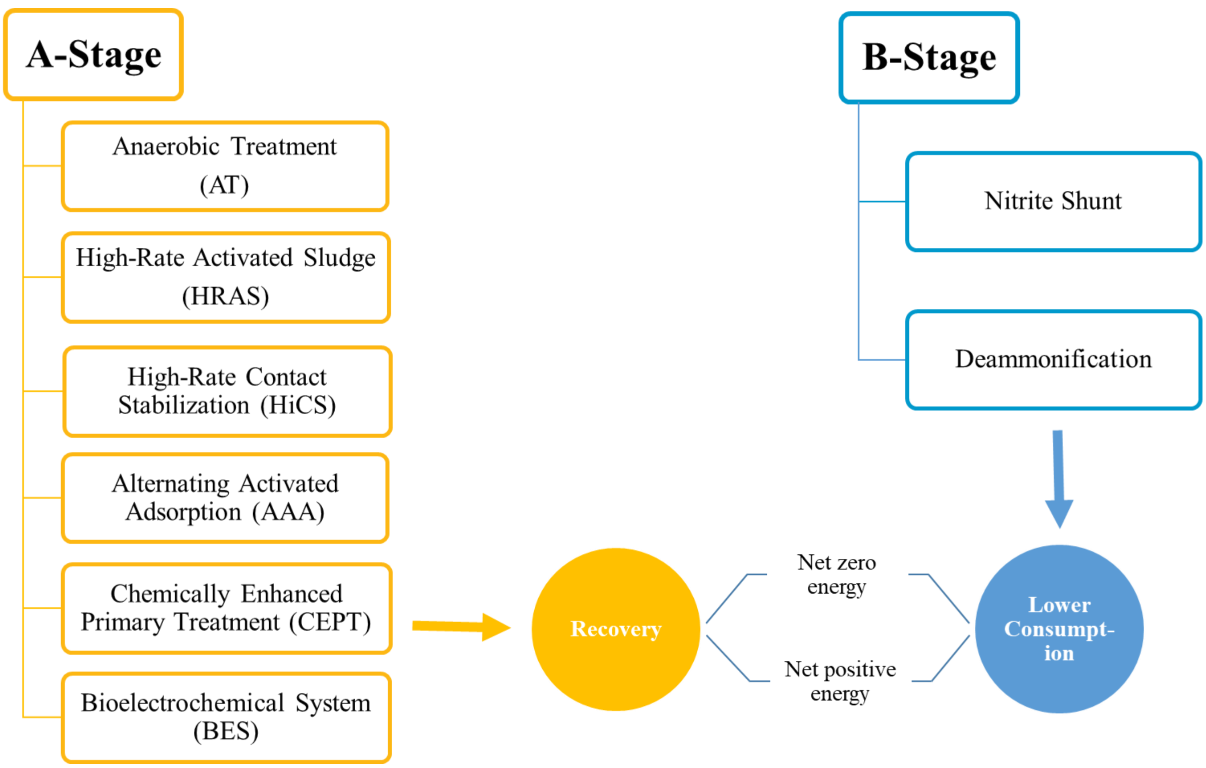

Figure 1.1: A-B scheme in Water resources recovery facilities (WRRFs)

Several - stage technologies have been proposed in the literature with various potentials including: (i) High Rate Activated Sludge (HRAS) (Ge et al., 2017; Jimenez et al., 2015; Kinyua et al., 2017;

Meerburg, 2016), (ii) High Rate Contact/Stabilization (HRCS) (Dai et al., 2018; Meerburg et al., 2016; Rahman et al., 2017), (iii) Alternating Activated Adsorption (AAA) (AlSayed et al., 2022; Wett et al., 2020), (iv) Anaerobic Treatment (AT) (Delgado Vela et al., 2015; McCarty et al., 2011b; Smith et al., 2012; Wan et al., 2016) , (v) Enhanced Biological Phosphorus Removal (EBPR) (Chan et al., 2017; Ma et al., 2011; Yang et al., 2017), (vi) Chemically Enhanced Primary Treatment (CEPT) (Aiyuk et al., 2004; Parker et al., 2001; Shewa et al., 2020), and (vii) bio-electrochemical systems (BES) (Santoro et al., 2017; Schievano et al., 2018; Zou and He, 2018).

Since the focus of this thesis proposal is on nitrogen removal within WRRFs, B-stage will be thoroughly discussed in the next section taking in consideration all the aspects that affects its performance, whereas A-stage will only be considered briefly within its effect on the subsequent B-stage system.

1.2.3. B-stage systems within WRRFs

Conventionally, BNR via nitrification and denitrification has been employed in WWTPs to reduce the nitrogenous content of the effluent. Nitrification is a two-step process where ammonia is first oxidized to nitrite carried out by ammonia oxidizing bacteria (AOB) followed by the further oxidation of nitrite to nitrate performed by nitrite oxidizing bacteria (NOB). According to the nitrification stoichiometry, as shown in **Figure 1.2**, about 4.7 g O_2 are consumed per gram of ammonia oxidized, 3.42 g O_2 of which are consumed to support the ammonia oxidation while the remaining 1.15 g O_2 are consumed in the later nitrite oxidation (Soliman and Eldyasti, 2016a). Similarly, denitrification is a two -step process which involves the reduction of the produced nitrate to nitrogen gas with nitrite as an intermediate compound as well carried out heterotrophic denitrifying bacteria. Theoretically, around 2.86 g COD are required to completely reduce 1 gm of nitrate where 1.71 g COD are consumed to denitrify 1 gm of nitrite. However, in practice it was estimated that COD:N ratio in the range of 6-10 is required to achieve complete denitrification (Jimenez et al., 2014a). Commonly in CAS-WWTPs, the COD supply for denitrification is provided either through the sCOD in the influent in the pre-anoxic systems or externally in the form of methanol in the post anoxic systems. As such, conventional BNR requires high oxygen and carbon requirements which makes it an energy and cost intensive process contradicting with the central objective of the WRRF concept of achieving self-sufficient or net positive operation of WWTPs. Hence to reduce the aeration and carbon requirements of nitrogen removal, two main processes have been developed, i.e., nitrite shunt and deammonification.

Nitrite shunt also called nitritation/denitritation or partial nitrification/denitrification relies on halting the ammonia oxidation at the nitrite stage through a process called partial nitrification followed by direct reduction of nitrite to nitrogen gas through conventional denitritation. Compared to conventional BNR, nitrite shunt implies 25% savings in term of oxygen due to skipping nitrite oxidation to nitrate, consequently reducing the total energy required by 60% as well it implies 40% reduction in COD requirements due to skipping nitrate reduction to nitrite, as shown in **Figure 1.2**. Additionally, it results in a significant decrease of the sludge production in nitrification and denitrification processes by 35% and 55%, respectively (Ge et al., 2014).

On the other hand, in the deammonification process -also called nitritation/ anammox- only half of the ammonia is oxidized to nitrite through partial nitrification then the remaining half of the ammonia is oxidized anaerobically to nitrogen gas using the produced nitrite as an electron acceptor carried out by Anaerobic ammonium oxidizing (Anammox) bacteria. In term of savings compared to conventional BNR, deammonification has the advantage of saving 62.5% of the oxygen demand due to oxidizing only half of the ammonia to nitrite and skipping its further oxidation to nitrate, it as well does not require any carbon addition as the process is completely autotrophic resulting in 100% savings in COD demand **(Figure 1.2).**

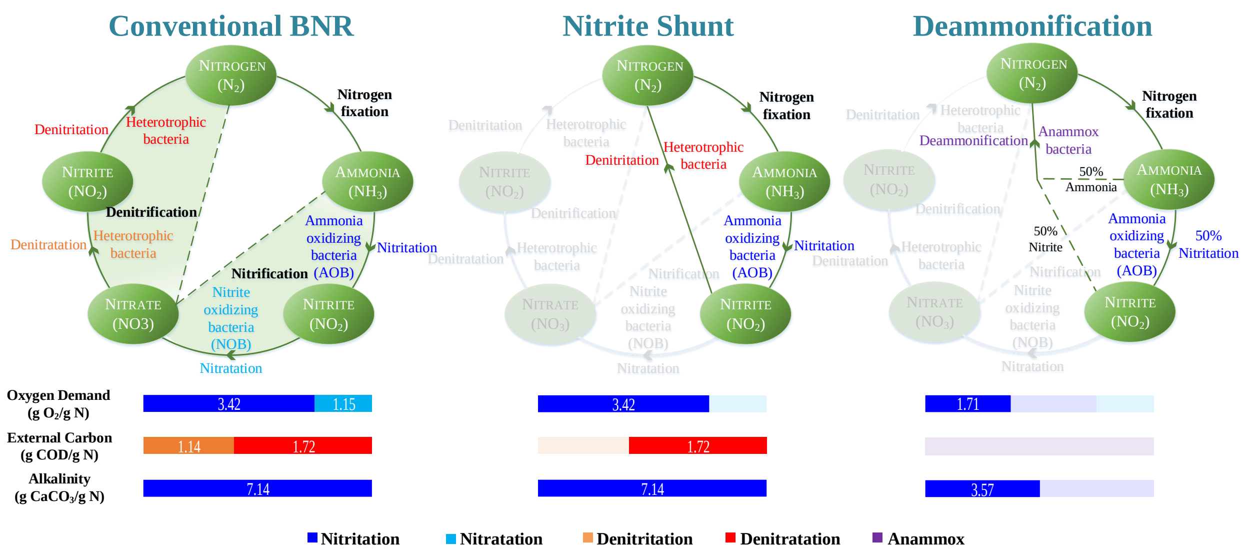

Figure 1.2: Comparison between conventional and shortcut nitrogen removal over the nitrogen cycle and the corresponding stoichiometric savings calculations

However, here it is worth mentioning that these theoretical savings calculations are based on the perfect implementation of the subject process and overlook the impact of the implementation challenges. For instance, in nitrite shunt, it assumes that carbon is completely used for denitrification and no COD oxidation will take place in the aerobic zone while for deammonification its assumes that complete carbon removal will take place prior to the nitrogen removal step which is impossible from a practical standpoint. Otherwise, carbon oxidation in the aerobic zones will utilize more aeration energy, produce more biomass, increase COD requirements which will alter the savings calculation. Moreover, it neglects the effect of the challenge of suppressing NOB at mainstream conditions hindering the implementation of both nitrite shunt and deammonification (Kirim et al., 2022). Furthermore, it disregards the effect of the slow growth of the Anammox bacteria at mainstream conditions which requires the addition of an anammox retention mechanism such as hydrocyclones to allow for adequate SRT and consequently increase the process cost. Thus, basing the comparison between the different nitrogen removal processes only on the theoretical saving calculations would lead to erroneous conclusions. However, the evaluation of each process on the basis of the mechanisms of overcoming the challenges facing its implementation and the actual resulting savings of its application in full-scale would be a better route. Thus, in the following section, the challenges impeding the application of the nitrite shunt process as a B-stage in mainstream will be discussed.

1.3. Research Gaps

As discussed in the previous section, the shift to more energy efficient WWTPs relies on achieving resource and energy savings in the nitrogen removal step. Nitrite shunt is proposed as a suitable B-stage process in the A/B scheme since it implies savings in the aeration (energy) and COD requirements (resources) compared to the conventional BNR processes. As described previously, nitrite shunt and deammonification share the same first step of partial nitrification (halting the ammonia oxidation at the nitrite stage). However, one of the advantages of the nitrite shunt process over deammonification is that the second step is carried out by ordinary heterotrophic bacteria unlike in deammonification where the slow growing Anammox bacteria is responsible for the second step which might require additional operational costs to retain its activity. Nonetheless, heterotrophic bacteria require organic carbon that can be utilized as electron donor in the denitrification step as opposed to deammonification which is a completely autotrophic process. Such organic carbon requirement if not available in the influent and must be externally supplied would result in lower resource savings compared to deammonification. Encouragingly, the different A-stage technologies in WRRFs are characterized by their lower COD removal compared to CAS process. Such lower removal implies that higher COD concentrations in the effluent of the

A-stage are directed to B-stage to support the second step of nitrite shunt. Thus, the employment of nitrite shunt as a B-stage might represent a key solution to benefit from the A-stage effluent COD to further decrease its COD requirement. However, several challenges can impede the use of this available COD for denitrification such as COD oxidation in the aerobic zone, effect of influent COD fractionation on denitrification and others as will be described in **section 1.3.2**.

Moreover, a critical factor contributing to the energy and resources savings of nitrite shunt is the assumption that nitrogen removal will be performed through nitrite pathway. Such an assumption requires the suppression of NOB activity to avoid nitrite oxidation to nitrate which would require additional aeration and COD for its subsequent denitrification. Nonetheless, despite the considerable efforts devoted and the significant progress achieved, NOB out-selection in the mainstream of WWTPs remains in the cradle as will be discussed in **section 1.3.1.**

1.3.1. NOB out-selection in mainstream conditions challenge

As stated previously, the first step of nitrite shunt is partial nitrification. which depends upon stimulating AOBs' growth while inhibiting or out-selecting NOBs. Several strategies have been employed for NOB out-selection including: (i) maintaining low dissolved oxygen (DO) concentrations, (ii) maintaining high free ammonia (FA) and free nitrous acid (FNA) concentrations in the liquid (iii) controlling the reaction at high temperature levels, (iv) maintaining high pH, (v) applying short SRTs. The first strategy of relying on low DO concentration for NOB inhibition is based on the differences between the Monod saturation constant of oxygen for AOB (0.3 mg/L) and NOB (1.1 mg/L) indicating the higher affinity of oxygen for AOB over NOB, thus AOBs are more prone to maintain their activity at oxygen limitation conditions (Wiesmann, 1994). The second strategy of controlling free ammonia (FA) and free nitrous acid (FNA) concentrations is an effective strategy for NOB out-selection as high

FA and FNA have an inhibitory effect on both AOB and NOB but at different concentrations. The inhibition limit of FA for NOB is 0.1-1.0 mg N/L, whereas AOB can maintain its activity at these levels and no inhibition is observed until concentrations of 10-150 mg N/L of FA are reached (Anthonisen et al., 1976). Similarly, NOB is more sensitive to free nitrous acid compared to AOB since it was reported that low concentrations of FNA in the range 0.01-0.2 mg-N/L started to inhibit NOB while FNA concentration as high as 0.4-1.7 mg-N/L resulted in only a 50% reduction in AOB activity (Zhou et al., 2011). Thus, controlling FA and FNA concentrations in the reactor at the levels of NOB inhibition and lower than those of AOBs inhibition should result in NOB suppression. The third strategy of controlling the reaction at high temperature is based on the fact that AOB has higher maximum specific growth rate (μ_{max}) than that of NOBs when the temperature is increased over 24 °C, while low temperatures lead to NOB domination over AOB, hence partial nitrification reactors are commonly operated at a temperature of range between 30-35 °C (Rodriguez-Sanchez et al., 2014). Moreover, maintaining a high pH in the reactor is a strategy used to suppress NOB since it has been reported that the optimal pH for *Nitrosomonas sp.* (the reported dominant AOB species in partial nitrification reactors) ranges between 7.9 and 8.2, while for *Nitrobacter sp.* (the reported dominant NOB species in partial nitrification systems) it ranges between 7.2 and 7.6 (Alleman, 1985). Here it is worth mentioning that high pH and temperature increase the concentrations of FA and FNA which result in more inhibition to NOB activity allowing AOBs to outcompete NOBs in partial nitrification systems. Lastly, shortening SRT as a strategy to wash out relies on the fact AOB has a 50% less doubling time (7-8 h) compared to NOB (10-13h) (Soliman and Eldyasti, 2018). Hence, controlling SRT in a range shorter than NOB retention time but longer than AOB retention time would result in NOB being continuously washed out from the system.

These strategies have been studied extensively and some of them have already been implemented at full scale facilities. In fact up till now, more than 110 full scale shortcut BNR systems have been in operation worldwide treating wastewater with high nitrogen content such as reject water, landfill leachate and industrial wastewater using different configurations including Completely Autotrophic Nitrogen removal Over Nitrite (CANON) process, Single reactor for High activity Ammonia Removal Over Nitrite (SHARON) process, Deammonification (DEMON) process, Oxygen Limited Autotrophic Nitrification Denitrification (OLAND) process, Single stage Nitrogen removal using Anammox and Partial nitrification (SNAP) process, and others (Soliman and Eldyasti, 2018).

Although all the previous strategies have been proven to be successful in achieving partial nitrification, their application is still limited to solids stream line and their implementation in mainstream line is still hindered by several challenges. The main difference between mainstream and solids stream is that mainstream has higher flowrates, low nitrogen content and low temperature which challenge the application of NOB washout strategies. For instance, relying on FA and FNA inhibition for suppressing NOB is no longer a viable strategy since FA and FNA concentrations are a factor of the ammonia concentration, temperature and pH as shown in the following two equations:

$$\boldsymbol{FA\ \left(\frac{mg}{L}\right) = \frac{17}{14}\ X\ \frac{NH_3-N\ X\ 10^{pH}}{10^{pH}+\exp\left(\frac{6344}{273+T}\right)}} \quad \textbf{(1.1)}$$

$$\boldsymbol{FNA\ \left(\frac{mg}{L}\right) = \frac{46}{14}\ X\ \frac{NO_2-N}{10^{pH}\ X\ \exp\left(-\frac{2300}{273+T}\right)}} \quad \textbf{(1.2)}$$

As shown in the equations, high FA and FNA concentrations requires high ammonia concentrations, which are not available in mainstream wastewater, in fact ammonium concentrations in mainstream are up to 50 times lower than those of anaerobic digester effluent. Moreover, another two strategies which are ruled out due to mainstream conditions are high temperature and high pH since it is considered unpractical from a cost perspective to heat the liquid or to add chemicals to elevate the pH due to the high mainstream flowrates. Therefore, the only remaining strategies which can be employed in mainstream lines are short SRTs and low DO concentrations.

However, in recent studies NOB out selection by low DO has been challenged by the fact that oxygen affinity (K_o) differs according to several factors. For instance, it has been reported that K_o may be affected by sludge form and floc size, advection, diffusion, different species of AOB and NOB as well as operational conditions such as pH, temperature, nitrogen loading rate (NLR), HRT, and SRT (Arnaldos et al., 2015; Ni et al., 2009; Puyol et al., 2013; Regmi et al., 2022a; Tomaszewski et al., 2017; Yao et al., 2015). Moreover, it was reported that in mainstream conditions *Nitrospira sp.* which has higher oxygen affinity compared to *Nitrobacter sp.* might be the abundant species of NOBs unlike in side stream conditions where *Nitrobacter sp.* is the dominant species (Al-Omari et al., 2015). The previous factors resulted in discrepancies in the reported K_o values for AOB and NOB with some studies reporting $K_{o,AOB}$ higher than $K_{o,NOB}$ while other studies reporting the reverse (Blackburne et al., 2008; Cui et al., 2020; Guisasola et al., 2005; Regmi et al., 2014; L. Wang et al., 2021; Z. Wang et al., 2021; Yu et al., 2020). Such discrepancies can explain the challenge of inhibiting NOB growth at low DO concentrations. Several strategies have been proposed to overcome such a challenge including maintaining an effluent ammonia residual, intermittent aeration, short aerobic SRT, nitrifiers adaptation, bioaugmentation and others (Al-Omari et al., 2015; Gilbert et al., 2014; Keene et al., 2017; Regmi et al., 2022b, 2014; Wett et al., 2015) . However, despite these many strategies, stable NOB out-selection in partial nitrification at mainstream condition is yet to be achieved (Kirim et al., 2022). In fact, due to the challenges facing NOB out-selection, recent studies have abandoned efforts to suppress NOB and shifted towards systems that does not require inhibition such as partial denitrification-anammox (PdNA) (Fofana et al., 2022; Ladipo-Obasa et al., 2022; Mccullough et al., 2022). However, such systems would result in losing some of the process savings achieved in partial nitrification systems. Hence, extensive research should be directed towards developing a feasible strategy to achieve stable mainstream NOB out-selection.

1.3.2. COD requirements in nitrite shunt process

Along with the challenge of its first step, nitrite shunt encounters an additional challenge in its subsequent step. Although nitrite shunt's second step is denitritation which is a conventional step, it requires a theoretical 1.71 g of COD per g of nitrite produced which would increase the process costs if this required amount of COD is not readily available in the influent and have to be externally provided. Moreover, it has been found that in practice the required COD for denitritation is higher than the theoretical value and a carbon to nitrogen (C:N) ratio in the range of 2.5-6 is required for complete denitritation (Mccullough et al., 2022). Encouragingly, one of the key challenges facing the A-stage process in WRRFs is its lower COD removal compared to CAS which means that higher COD concentrations in the effluent of the A-stage are directed to B-stage which might be sufficient for nitrite denitritation. In fact, the reported C: N ratio in the effluent of CEPT, HRAS, and HRCS are 3-6, 0.67-8, and 2.7-7.1, respectively (Liu et al., 2018; Rahman et al., 2019; Xu et al., 2015). Thus, employing nitrite shunt following an A-stage system might represent a key solution for the low COD removal efficiency of A-stage systems and the lack of substrate for denitritation in nitrite shunt. However, here it is worth pointing out that only a fraction of the total COD in the influent can be used by heterotrophic bacteria as a substrate for nitrite denitrification. In fact, TCOD can be classified in two main categories, (i) biodegradable COD (bCOD) which can be divided to readily biodegradable COD (rbCOD) and slowly biodegradable COD (sbCOD), and (ii) non-biodegradable COD (nbCOD) which also can be divided to non-biodegradable soluble COD (nbsCOD) and non-biodegradable particulate COD (nbpCOD). It has been reported that heterotrophic bacteria can use only rbCOD as a substrate for denitrification (Ni et al., 2017). Whereas sbCOD requires prior hydrolysis for the heterotrophic bacteria to be able to utilize it. Thus, nitrite shunt is highly dependent on the COD composition and particularly the rbCOD fraction in the influent wastewater.

Furthermore, the challenge is aggravated by the fact that the first step of nitrite shunt requires the oxidation of ammonia to nitrite in aerobic conditions which would result in the loss of influent COD required for denitrification through oxidation in the case of post-denitrification. To overcome this challenge, several configurations have been suggested to perform denitrification prior to COD oxidation in the subsequent step i.e., pre-denitrification such as the modified Ludzack– Ettinger (MLE) process. In such a process, nitrite/nitrate produced in the aerobic zones by nitrification/partial nitrification is recycled back to the pre-denitrification compartment for denitrification/denitritation. This process would enable the utilization of the COD that is available in the influent for denitrification before its oxidation in the subsequent aerobic zone. However, such processes require a continuous internal mixing regime which increases the process cost and complicates the process operation. Other alternative strategies have been suggested for the efficient utilization of the influent COD without the need of internal mixing through simultaneous nitrification/denitrification (SND) in continuous aeration process or through using intermittent aeration (anoxic/oxic cycling).

The first strategy relies on the reports about the possibility of simultaneous nitrification/denitrification (SND) at low DO concentrations (Jimenez et al., 2011). Thus, this strategy adopts operating the system through continuous aeration at low DO levels. Low DO would enable heterotrophic denitrifies to utilize the oxidized nitrite produced by AOBs as an electron acceptor and influent COD as electron donor simultaneously. Therefore, on the one hand the system benefits from the easier and more practical method of aeration compared to other aeration methods while efficiently using influent COD for denitrification. On the other hand, the combination of low DO and the pressure imposed on NOB by the constant utilization of the nitrite produced (NOB substrate) by heterotrophs would help reduce NOB growth and might result in its activity suppression. However, the exact mechanisms of SND and to what extent it can occur in a nitrite shunt system are not well reported. For SND to take place at low DO, there must be an available electron donor for denitrification in the aerobic zone. It was suggested that this electron donor source can be derived from: (i) the rbCOD available in the A-stage effluent, (ii) the rbCOD resulting from the hydrolysis of the influent pCOD in the aerobic zones, (iii) the rbCOD from endogenous decay, and (iv) internally stored carbon in a pre-anaerobic zone which can be performed by denitrifying phosphorus accumulating organisms (DPAO), denitrifying glycogen accumulating organisms (DGAO), or other heterotrophic bacteria (Giraldo et al., 2012; Klaus and Bott, 2020; Mino et al., n.d.; Rubio-Rincón et al., 2017; Tsuneda et al., 2006; van Loosdrecht et al., 1997; Van Loosdrecht and Henze, 1999). Thus, further examination should be directed towards determining the amount of nitrogen removal that can be achieved through SND in nitrite shunt process using continuous aeration.

The second strategy for efficient COD utilization without the need of internal mixing is operating the system using intermittent aeration. Intermittent aeration is performed by cycling anoxic/oxic conditions either in time (turning the aeration off and on in intervals) or in space (providing dedicated anoxic and oxic compartments). Despite of being more complex, intermittent aeration provide the advantage of having dedicated anoxic zones/phase to perform the denitrification step in nitrite shunt and does not rely solely on the occurrence of SND in the aerobic zone which can help increase COD utilization efficiency. Moreover, it has been reported that NOB exhibits a period of reduced growth rate (lag phase) following anoxic periods, this phenomena is called transient anoxia (Gilbert et al., 2014; Kornaros et al., 2010). Consequently, adopting an oxygen feeding strategy based on cyclic oxic/anoxic conditions (intermittent aeration) would impose additional pressure on NOB growth. Nonetheless, there have been contradictory approaches in the literature towards the DO concentration levels in the oxic zones of the intermittent aeration. Some studies adopt controlling the DO at moderate levels (1.0-1.5 mg/L) while others suggest operating at lower DO concentrations (0.2-0.8 mg/L). The rationale behind the first approach is that *Nitrospira sp.* which has higher oxygen affinity compared to *Nitrobacter sp*. might be the abundant species of NOBs in mainstream conditions and by consequence no advantage will be provided to AOBs at low DO. In contrast, the limited DO concentration would affect the AOB nitrification activity resulting in lower nitrogen removal rates. Thus, operating at moderate DO would ensure maintaining high AOB rates and consequently high nitrogen removal rate. As for NOB out-selection, this approach relies on transient anoxia to reduce NOB growth and short aerobic SRT to increase their washout. Contrarily, the second approach adopts running the aerobic zones of the intermittent aeration at low DO concentrations in order to minimize the aeration-associated energy consumption and consequently maximize the energy gain from the carbon stream. This approach is motivated by the reports that AOB adaptation to low DO would result in achieving higher ammonia oxidation. Hence, it can be concluded that the effect of DO in the aerobic zones of intermittent aeration on AOB activity in mainstream conditions has not been fully understood yet. Hence, further studies that examine nitrification/denitrification rates at different DO concentrations are still needed.

All in all, it can be concluded from this section that adequate B-stage design is vital to optimize the use of the available influent COD before its oxidation. Therefore, further investigation needs to be carried out to target the achievement of complete nitrogen removal through nitrite shunt relying on the available COD in the A-stage effluent (at low C:N ratios).

1.4. Dissertation Motivation and Objectives

Although, the implementation of the nitrite shunt as a B-stage in the A/B scheme would potentially help transforming the current WWTPs from energy-consumers to energy-exporters, two main issues are to be addressed to enable its practical application. The first issue is the inability to suppress NOB activity in mainstream conditions which results in higher energy and resources consumption as explained in section **1.3.1.** While the second challenge is the efficient utilization of influent COD for denitritation which impedes the achievement of nitrite shunt at low C:N ratio without the need of external carbon addition as explained in **section 1.3.2.** Thus, this dissertation aims at developing a novel strategy to implement a robust and resource-efficient nitrite shunt process. This process can effectively utilize the COD available in A-stage effluent to perform complete nitrogen removal towards its implementation in the A/B scheme in the next generation wastewater treatment plants.

In the view of the above-mentioned technical challenges facing the development of the nitrite shunt process, the specific objectives for this dissertation are as following:

<u>Objective 1:</u> Developing a novel strategy to suppress NOB activity at mainstream conditions:

It has been more than 20 years since shortcut BNR was proposed as a revolutionary process towards achieving net zero or net positive wastewater treatment. SBNR has been implemented successfully in the solids line of more than 110 full-scale WWTPs around the world. However, side stream represents only less than 30% of the total ammonia in a WWTP, and SBNR implementation in mainstream lines is still hindered by the major bottleneck of NOB out-selection at mainstream conditions.

Thus, the first objective of this dissertation is to develop a novel strategy to suppress NOB activity at mainstream conditions benefiting from the reports of AOB adaptation to low DO concentrations. Moreover, in order to develop a fundamental understanding about NOB suppression mechanism, the effect of the adaptation strategy on AOB and NOB nitrification rates as well as AOB and NOB oxygen half saturation coefficients will be studied. This step is necessary to move forward towards the implementation of the SBNR process in full-scale plants.

<u>Objective 2:</u> Evaluating nitrite shunt process as a B-stage in the A/B stage scheme for low carbon to nitrogen (C:N) wastewater using continuous aeration at low DO concentrations

The most valuable resource in wastewater, other than the water, is the chemical energy embedded in the influent carbon. Thus, the objective of the nitrite shunt process as a B-stage is to minimize its organic carbon utilization, so more carbon can be captured and diverted in the A-stage. However, the nitrite shunt carbon requirement is highly dependent on its adequate design in order to ensure high carbon utilization efficiency. As discussed in **section 1.3.2**, several C:N requirements have been reported for nitrite shunt in the literature with values reaching up to 14. However, the C:N ratio in the reported A-stage effluent is less than the previous ratios and ranges between 2-7.

Thus, the second objective of this dissertation is to develop a nitrite shunt process that can achieve high nitrogen removal rates (total nitrogen effluent below the discharge limit) at low C:N ratios. In the pursuit of this objective, the developed nitrite shunt system will benefit from the adaptation strategy developed in objective 1 to suppress NOB activity which would decrease the COD requirements. Moreover, the developed system will be operated using continuous aeration at low DO concentrations to eliminate the use of internal mixing regime as discussed in **section 1.3.2.** Moreover, the effect of continuous aeration at low DO concentrations on total inorganic nitrogen (TIN) removal, COD removal, nitrite accumulation rates (NAR), and AOB and NOB activities will be studied.

<u>Objective 3</u>: Studying the effect of C:N ratio and COD fractionation on the nitrite shunt process

In the A/B scheme, carbon and nitrogen removal is decoupled. The objective of the high-rate A-stage is to remove carbon either through capture or redirection. Moreover, an additional goal for A-stage is to provide a controlled carbon effluent for B-stage which would enable the removal of the influent nitrogen. Here it is worth noting that this controlled carbon effluent is not only in terms of the total COD load but also the COD fractionation (ratio between sCOD and pCOD).

Thus, the third objective of this dissertation is to study the effect of different C:N ratios and COD fractionation on the performance of nitrite shunt process. Based on the results of this step, A-stage can be controlled to achieve a desired C:N ratio with a desired COD fractionation. This step would help balancing the carbon that that is being captured in A-stage with the remaining carbon that is being fed to B-stage.

<u>Objective 4:</u> Evaluating nitrite shunt process as a B-stage in the A/B stage scheme for low carbon to nitrogen (C:N) wastewater using intermittent aeration at different DO concentrations

Intermittent aeration has been used widely as an aeration control strategy for mainstream SBNR processes. Compared to continuous aeration, it offers the advantage of dedicated anoxic zones for the denitritation step which can help improve the influent COD utilization and lower the C:N requirements for complete nitrogen removal. Moreover, it can benefit from the reported lag phase of NOB following anoxic conditions which help add an additional pressure towards NOB out-selection. However, as described in **section 1.3.2**., there is no consensus on the DO level in the aerobic zones of intermittent aeration.

Thus, the fourth objective of this dissertation is to study the effect of different DO levels in the aerobic zones of intermittent aeration on nitrite shunt performance. Moreover, the effect of high and low DO levels on total inorganic nitrogen (TIN) removal, COD removal, nitrite accumulation rates (NAR), and AOB and NOB activities will be studied. Additionally, the effect of COD fractionation on the denitritation rates in the anaerobic zones of intermittent aeration will be tested.

1.5. Dissertation Layout

The overall objective of this dissertation is to develop a robust and efficient nitrite shunt process which can be implemented as a B-stage system in the A/B scheme in the next generation WRRFs.

To that end, this dissertation comprises five chapters. In **chapter 1**, an introduction is provided to discuss the current paradigm shift from energy intensive to more sustainable and efficient facilities. The literature research gaps are identified, and the dissertation rationale and objectives are discussed.

Chapter 2 works on addressing **Objective 1** towards achieving a stable NOB out-selection at mainstream conditions which is a significant barrier to the implementation of the SBNR technologies. It details the development of a NOB out-selection kinetic-adaptation strategy in an SBR at low DO concentrations. It was hypothesized that lowering the DO concentration in a stepwise fashion and transitioning gradually from side stream to mainstream conditions allowing the biomass sufficient acclimation time would result in NOB suppression at mainstream conditions. Over 410 days of operation, ammonia removal efficiency (ARE), nitrite accumulation rate (NAR), AOB and NOB maximum activity, oxygen half saturation coefficients, and relative abundance were monitored as measure of the success of the kinetic adaptation strategy. The objective of this chapter is to overcome the mainstream NOB out-selection bottleneck towards the implementation of the SBNR technologies in full-scale plants.

Afterwards, in **Chapter 3**, the resulting biomass from the previous chapter system was used to seed a B-sage nitrite shunt system operated using continuous aeration at low DO concentrations. It details the operation of the system targeting nitrogen removal at low C:N ratios to address **Objective 2**. Over 140 days of operation, total inorganic nitrogen (TIN), COD removal, ammonia removal efficiency (ARE), nitrite accumulation rate (NAR), AOB and NOB maximum activity, denitrification rates were used as a measure of the success of the developed system. Moreover, to address **Objective 3**, the system was operated at different C:N ratio and different COD fractionation. The effect of these conditions on TIN removal and NAR was studied as a measure of their effect on nitrite shunt performance. The results of this chapter would help facilitate the scale-up of nitrite shunt process as a B-stage in the A/B scheme. Moreover, it would help control the A-stage effluent to achieve the desired B-stage influent characteristics.

In **Chapter 4,** a different aeration pattern for nitrite shunt was tested i.e., intermittent aeration. The system was operated at 2 different DO levels in the aerobic phase of intermittent aeration, (i) moderate DO concentrations (1.5 mg/L) and (ii) low DO concentrations (0.2 mg/L). Over 140 days of operation, the effect of the different DO concentrations on nitrite shunt performance was monitored to address **Objective 4**. Total inorganic nitrogen (TIN), COD removal, ammonia removal efficiency (ARE), nitrite accumulation rate (NAR), AOB and NOB maximum activity, denitrification rates were used as indications for nitrite shunt performance. The objective of this chapter is to explore an alternative aeration pattern for the implementation of nitrite shunt as a B-stage system. Moreover, it aims to assess the performance of the system at the different DO levels.

Finally, the last chapter of this dissertation (**Chapter 5**) provides the overall conclusions and key findings obtained in this dissertation and discusses the recommendations for future work direction.

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Chapter 2: Implementation of a Kinetic-Based Adaptation Strategy for NOB-out-selection under Low DO Concentrations at Mainstream conditions

ABSTRACT

Although shortcut biological nitrogen removal has been implemented at full scale facilities in the solids stream, its implementation in mainstream line is still hindered by the challenges facing NOB inhibition in mainstreams conditions. To this end, this study proposes a kinetic adaptation strategy to achieve complete mainstream partial nitrification as a first step of nitrite shunt process. This novel adaptation strategy relies on lowering the DO concentration in a stepwise fashion and transitioning gradually from side stream to mainstream conditions allowing the biomass sufficient acclimation time.

The experiment was performed in an SBR operated at 8 phases with different DO and nitrogen loading rates combination to assess the ability of maintaining NOB out-selection in mainstream conditions through the adaptation strategy. In result, at mainstream conditions, the SBR was able to maintain an ammonia removal efficiency of 99.4 ± 0.4% and nitrite accumulation rate of 87.4 ± 0.6% under low DO levels (0.1–0.2 mg/L). The adaptation strategy resulted in NOB activity suppression and AOB maximum activity was 5 times higher than that of NOB. Moreover, amplicon sequencing revealed that the relative abundance of *Nitrosomonas* (the dominant AOB species) was 40% vs 3.7% for *Nitrospira* (the dominant NOB species) compared to 1.3 and 1.8% in the seeding sludge proving the success of the adaptation strategy in NOB out-selection. Lastly, the adaptation strategy allowed the bioreactor to be operated at a DO level higher than $K_{o,AOB}$ but lower than $K_{o,NOB}$ which played a pivotal role in NOB inhibition. These results blaze a trail for the implementation of shortcut biological nitrogen removal systems in full-scale plants by overcoming the NOB out-selection bottleneck.

2.1. Introduction

Due to depleted energy resources, increasing worldwide energy demand and global climate change, there has been a wide interest in developing novel wastewater treatment technologies able to meet the required effluent standards with minimum energy usage. In fact, it is estimated that the treatment of 1 m^3 of wastewater using conventional processes consumes around 0.3-0.6 kWh (McCarty et al., 2011a). As such, according to the UN-FAO, in 2009, 26.61 billion of m^3 wastewater (WW) were treated which means that around $12x10^9$ kWh of electricity are consumed per year for wastewater treatment (Liu et al., 2018). Interestingly, it was reported that aeration by itself contributes to around 50% of this total energy use (Henderson, 2002). Moreover, integrating nitrification within the activated sludge process results in an increase in the energy consumption by around 30% (Monteith et al., 2007). Thus, extensive research should be directed towards nitrogen removal technologies that require less aeration demand.

To this end, partial nitrification has been suggested for biological nitrogen removal as it implies 25% oxygen savings and consequently results in 60% reduction in the total energy required (Soliman and Eldyasti, 2018). Partial nitrification relies on halting ammonia oxidation at the nitrite step through inhibiting or washing out nitrite oxidizing bacteria (NOBs). Several strategies have been employed for NOB out-selection including: (i) low DO concentrations, (ii) high free ammonia (FA) and free nitrous acid (FNA) (iii) high temperature, (iv) high pH, and (v) short SRTs (Soliman and Eldyasti, 2018). These strategies have been studied extensively and some of them have already been implemented at full scale facilities. In fact, up till now, more than 110 full scale shortcut BNR systems have been in operation worldwide treating wastewater with high nitrogen content such as reject water, landfill leachate and industrial wastewater using different configurations including Completely Autotrophic Nitrogen removal Over Nitrite (CANON) process, Single reactor for High activity Ammonia Removal Over Nitrite (SHARON) process, Deammonification (DEMON) process, Oxygen Limited Autotrophic Nitrification Denitrification (OLAND) process, Single stage Nitrogen removal using Anammox and Partial nitrification (SNAP) process, and others (Mao et al., 2017).

Although all the previous strategies have been proven successful to achieve partial nitrification, their application is still limited to the side stream. Meanwhile, their implementation in mainstream is still hindered by several challenges (Klaus et al., 2020a). These challenges stem from the fact that the mainstream has higher flowrates, low nitrogen content and low temperature compared with the side stream. This is the main difference between mainstream and solids stream which challenge the application of most of the NOB washout strategies including high FA and FNA, high temperature and high pH. Thus, it is justified to conclude that short SRTs and low DO concentrations are the only possible strategies to achieve mainstream partial nitrification.

NOB out selection by low DO is reliant on the widely reported observation that that the oxygen half-saturation coefficient for AOB ($K_{o,AOB}$) is lower than that of NOB ($K_{o,NOB}$). This indicates AOBs higher affinity for oxygen compared to NOBs. Thus, it can be implied that AOBs are more prone to maintain their activity at oxygen limitation conditions (Wiesmann, 1994). Therefore, low DO partial nitrification can be achieved by enriching a nitrifying community in which $K_{o,AOB}$ is lower than $K_{o,NOB}$ by exposing it to DO concentrations higher than $K_{o,AOB}$ but lower than $K_{o,NOB}$. Notwithstanding, there have been conflicting reports about the values of $K_{o,AOB}$ and $K_{o,NOB}$ as shown in **Table 2.1**. Some studies have reported a K_o for AOB lower than that of NOB (Blackburne et al., 2008; Guisasola et al., 2005; Hunik, 1993; Nowak et al., 1995; Z. Wang et al., 2021; Wiesmann, 1994; Yu et al., 2020), while in other studies, the opposite has been reported (Cui et al., 2020; Daebel et al., 2007; Manser et al., 2005; Regmi et al., 2014; L. Wang et al., 2021).

Table 2.1: A survey on oxygen half saturation coefficient for AOB ($K_{o,AOB}$) and NOB ($K_{o,NOB}$) at different operational conditions in the literature

Reference	$K_{o,AOB}$ (mg O_2/L)	$K_{o,NOB}$ (mg O_2/L)	DO (mg/L)	Reactor config.	Temp. (ºC)	NLR (kg/m^3/d)	NH_4 conc. (mg N/L)	HRT (d)	SRT (d)
(Hunik, 1993)	**0.16**	**1.1**	-	-	-	-	-	-	-
(Wiesmann, 1994)	**0.3**	**1.1**	-	-	-	-	-	-	-
(Nowak et al., 1995)	**0.3**	**0.6**	3.0	-	28	-	360	-	25
(Manser et al., 2005)	**0.79**	**0.47**	2.5-3.0	CSTR	Ambient	-	-	-	20
	0.18	**0.13**	2.5-3.0	MBR	Ambient				20
(Guisasola et al., 2005)	**0.74**	**1.75**	3.0	CSTR	25	0.475	1900	3-5	25
(Daebel et al., 2007)	**0.51-0.77**	**0.19-0.29**	2.5-3.0	CSTR	Ambient	-	-	-	20
	0.31-0.57	**0.14-0.51**	2.5-3.0	MBR	Ambient	-	-	-	20
(Blackburne et al., 2008)	**0.033**	**0.43**	0.4	CSTR	19-23	0.42	1000	2.38	-
(Regmi et al., 2014)	**1.16**	**0.16**	>1.5	CSTR	25	0.24	29.7	0.125	4-8
(Cui et al., 2020)	**0.31**	**0.24**	2.5	SBR	18-29		55.5		40
(Yu et al., 2020)	**0.28**	**0.39**	4.0	SBR	17-22	0.6	300	0.5	20
(L. Wang et al., 2021)	**0.39**	**0.29**	2.0	CSTR	-	0.04-0.06	25-37	0.58	15
	0.29	**0.09**	0.5	CSTR	-	0.04-0.06	25-37	0.58	15
(Z. Wang et al., 2021)	0.24	0.38	0.2-0.4	SBR	22	1.25	1250	1	15

CSTR: Continuously stirred tank reactor. MBR: Membrane bioreactor. SBR: Sequential batch reactor

These discrepancies in the reported K_o values explain the reported challenge of the out-selection of NOBs at mainstream lines using low DO concentrations. However, it is worth noting that stoichiometric coefficients are not fixed constants, and their values could be affected by different factors. For instance, it has been reported that K_o may be affected by sludge form and floc size, advection, diffusion, different species of AOB and NOB as well as operational conditions such as pH, temperature, nitrogen loading rate (NLR), HRT, and SRT (Arnaldos et al., 2015; Ni et al., 2009; Puyol et al., 2013; Regmi et al., 2022a; Tomaszewski et al., 2017; Yao et al., 2015). Thus, it is imperative to understand the reason behind these discrepancies, since enriching a nitrifying community, in which $K_{o,AOB}$ is lower than $K_{o,NOB}$, is the key to achieve mainstream partial nitrification.

Interestingly, it has been reported that the adaptation of some nitrifiers to new conditions such as low DO might result in lower K_o values (Keene et al., 2017; Regmi et al., 2022a; Yu et al., 2020). In fact, several studies in the literature have suggested that achieving high ammonia removal at low DO requires AOB adaptation to the limited DO conditions (Fitzgerald et al., 2015; Kirim et al., 2022; Liu and Wang, 2013; Regmi et al., 2022a). In agreement, the majority of the studies which reported a $K_{o,AOB}$ lower than $K_{o,NOB}$ at low DO comprised a gradual decrease from high to low DO concentrations over a long time. This brings an adaptation period to enhance the bacterial tolerance in acclimating to the induced stressful changes ((Blackburne et al., 2008; Keene et al., 2017; Z. Wang et al., 2021). For instance, it was reported that, in a pilot scale fed with primary effluent and operated for a period of 16 months, a stepwise decrease in DO concentration from 0.78 to 0.33 mg O_2/L while allowing at least 3 weeks for community adaptation between each DO reduction, resulted in a decrease in the $K_{o,AOB}$ from 1.38 to 0.3 mg O_2/L (Keene et al., 2017). Similar pattern was observed in another study performed in an SBR where $K_{o,AOB}$ dropped from 0.63 to 0.24 mg O_2/L following a decrease in DO concentrations from 1.5-2.0 to 0.2-0.4 mg O_2/L after more than 100 days of operation at the low DO (Z. Wang et al., 2021). Furthermore, Blackburne et al. 2008 reported the lowest $K_{o,AOB}$ in the literature of 0.033 mg O_2/L. This study was performed in a CSTR and operated in 11 phases with different DO concentrations and NLRs (Blackburne et al., 2008). The different phases comprised a stepwise decrease in DO from 4 mg O_2/L on the first phase to 0.4 mg O_2/L in the last phase which resulted in a $K_{o,AOB}$ much lower than $K_{o,NOB}$ of 0.033 and 0.43 mg O_2/L, respectively. Here, it is worthmentionning that the former two studies were conducted with high ammonia concentration in the influent which is not present in mainstream conditions. However, AOB adaptability to low DO after a period of operation was also reported in other mainstream studies (Fitzgerald et al., 2015; Kirim et al., 2022; Liu and Wang, 2013; Regmi et al., 2022).

Moreover, it was suggested in the literature that achieving stable NOB washout does not only rely on operating at low DO concentrations but also a factor of the combination between NLR and DO concentrations (Blackburne et al., 2008). It was demonstrated that operating the reactor at a DO and dilution rate combination above the NOB growth rate would result in NOB washout and consequently nitrite accumulation (Blackburne et al., 2008). Whereas a combination of DO and dilution rate below NOB growth rate should result in nitrate dominance in the effluent. Furthermore, in a study operated at a SBR at low DO concentrations, it was reported that each NLR change was accompanied by a drop in ammonia removal efficiency. However, after a period of operation at the same NLR, AOB was able to adapt and recover its activity and high ammonia removal was restored (Soliman and Eldyasti, 2016a). The previous results suggest that AOB might be sensitive as well to sudden changes in NLR distinctly at low DO concentrations. Thus, it can be hypothesized that inducing changes in DO and NLRs following a stepwise fashion to allow AOB enough time adapt should provide it a kinetic advantage to maintain its activity and allow it to outcompete NOB at low DO concentrations.

Therefore, in this study, a novel adaptation strategy is introduced in an SBR targeting the achievement of complete partial nitrification as a first step of nitrite shunt process in mainstream lines. The strategy adopts lowering the DO concentrations in a stepwise fashion while transitioning gradually from side stream to mainstream NLRs allowing AOB enough time to adapt to each new condition before moving to the next. The SBR was operated at 8 phases with different DO and NLRs combination to assess the ability of maintaining NOB out-selection in mainstream conditions through the adaptation strategy. In order to evaluate the adaptation of the microbial community to low DO and NLR, batch tests were conducted periodically to monitor AOB and NOB maximum activity. Moreover, batch kinetic experiments were performed at the end of each phase at different DO concentrations to determine the oxygen half saturation coefficients of both AOBs and NOBs and evaluate the success of the adaptation strategy in enriching a nitrifying community in which $K_{o,AOB}$ is lower than $K_{o,NOB}$. Lastly, the succession in nitrifying organisms as a result of the adaptation strategy was assessed using 16S rRNA gene amplicon sequencing.

2.2. Materials and Methods

2.2.1. Reactor Setup

The experiment was performed in an SBR, depcited in **Figure 2.1a**, with a working volume of 5 L. The reactor was fed from the feeding tank using a peristaltic pump (Masterflex L/S Digital Pump System with Easy-Load II Pump Head, Germany). The effluent was discharged from the middle of the tank during the decant phase and collected into a discharge tank to monitor sludge washout and determine the solid retention time (SRT). The experiment was operated and controlled using a control device (Biostat® A Benchtop Fermenter & Bioreactor, Goettingen, Germany). The SBR was equipped with a variable speed mixer and sensors to monitor DO, pH, and temperature which can be controlled using the control device. The sensors were connected to a programmable logic controller (PLC) and then used for proportion-integral-derivative (PID) or PI control.

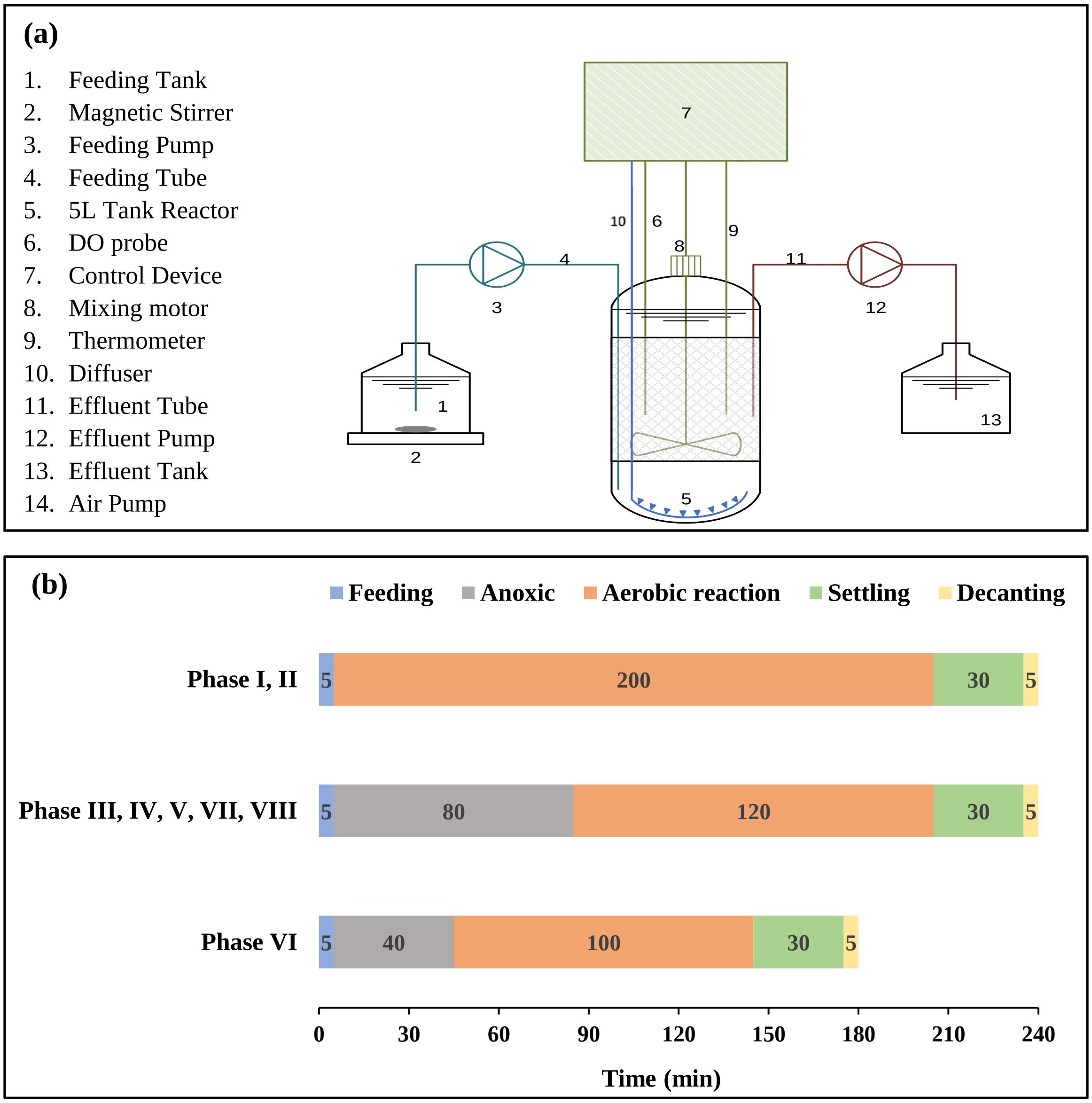

Figure 2.1: (a) Schematic diagram of the SBR configuration and (b) the SBR's cycle duration during different phases of operation

2.2.2. Operating Conditions

The SBR was inoculated with return activated sludge (RAS) from the Humber Municipal Wastewater Treatment Plant, Toronto, Canada. The reactor was fed with synthetic solution devoid of any organic carbon source and operated in eight phases with different operational conditions as shown in **Table 2.2**. The first two phases (Startup period) aimed at achieving complete nitrification to enrich the nitrifying bacteria and washout heterotrophic bacteria under carbon starvation conditions, thus the reactor was operated at high DO levels (2.5-4.0 mg/L). The SBR had a total cycle duration of 4 h consisting of 5 mins of feeding, 200 aerobic reaction phase, 30 mins of settling and 5 mins of decanting as shown in **Figure 2.1b**. Starting from phase III, DO limitations conditions were introduced in the system to suppress NOB activity and start nitrite accumulation.

DO was decreased to 0.3-0.4 mg/L, ammonia conc. and HRT were not changed to ensure NOB inhibition under side stream conditions. Moreover, starting from phase III, an anoxic phase was introduced to the SBR cycle before the aerobic reaction phase. Hence, starting at phase III and onwards, the SBR cycle consisted of 5 mins of feeding, 80 mins anoxic period, 120 aerobic reaction phase, 30 mins of settling and 5 mins of decanting. Afterwards, in each phase, ammonia and DO concentrations and HRT were decreased gradually to transition towards mainstream conditions as shown in **Table 2.2**. During all phases of operation, the SBR was operated at ambient temperature 20 ± 2 ºC and a pH of 7.2-7.6.

Table 2.2: Detailed operational conditions during the different phases of operation

Phase	Time (Days)	Ammonia conc. (mg N/L)	DO (mg/L)	HRT (h)	Nitrogen Loading Rate NLR (kg/m^3/d)	Volume Exchange Ratio (%)
I	1-45	**145.1±12.2**	**2.5-4.0**	16	0.225	25
II	46-75	**250.4±5.4**	**2.5-4.0**	16	0.375	25
III	76-131	**251.1±4.3**	**0.3-0.4**	16	0.375	25
IV	132-187	**199.5±2.9**	**0.2-0.3**	16	0.3	25
V	188-243	**150.6±2.1**	**0.1-0.2**	16	0.225	25
VI	244-271	**100.3±2.1**	**0.1-0.2**	12	0.2	25
VII	272-327	**100.1±0.9**	**0.1-0.2**	12	0.2	33
VIII	328-411	**49.3±2.1**	**0.1-0.2**	6	0.2	67

2.2.3. AOB and NOB activity tests

Every 2 weeks, a microbial activity batch test was conducted to monitor AOB and NOB maximum activity. A 2 L sample was collected from the SBR at the end of the aerobic reaction phase, centrifuged and washed with a nutrient solution. The washing was repeated twice to ensure that residual ammonia, nitrite, or nitrate are washed out before starting the test. Afterwards, the sample was transferred to a 2 L reactor and spiked with NH_4Cl and $NaNO_2$ to achieve an initial concentration of 30-40 mg NH_4-N/L and 15-20 mg NO_2-N/L. The reactor was operated at a DO > 4.0 mg/L using an air pump and a diffuser. DO concentrations were monitored using a DO probe (Hach LDO, CO). The pH was controlled to be in the same range as the parent reactor. Samples were collected every 10 mins and analyzed for ammonia, nitrate, nitrite, VSS and TSS. The test was conducted until all the ammonia in the reactor has been depleted. The AOB maximum activity rate (mg N/mg VSS/d) was calculated as the slope of the NOx-N production normalized over average VSS. NOB maximum activity rate (mg N/mg VSS/d) was calculated as the slope of the NO_3-N production normalized over average VSS, as described in (Klaus et al., 2020a), as shown in Eq. (2.1) and Eq. (2.2), respectively.

$$\boldsymbol{r_{AOB}\ (mg\ N/mg\ VSS/d) = \frac{r_{NO_2} + r_{NO_3}}{VSS_{avg.}}} \tag{2.1}$$

$$\boldsymbol{r_{NOB}\ (mg\ N/mg\ VSS/d) = \frac{r_{NO_3}}{VSS_{avg.}}} \tag{2.2}$$

Where $\boldsymbol{r_{NO_2}}$ is the nitrite rate measured in the batch test and calculated as the change in NO_2 over time, $\boldsymbol{r_{NO_3}}$ is the nitrate rate measured in the batch test and calculated as the change in NO_3 over time, and $\boldsymbol{VSS_{avg.}}$ is the average VSS in the batch reactor during the test period.

2.2.4. AOB and NOB Oxygen half saturation coefficients measurements

At the end of each phase, 6 batch tests were conducted at different DO concentrations of 0.1, 0.3, 0.6, 1.2, 2 and 2.8 mg/L to determine AOB and NOB oxygen half saturation coefficients ($K_{o,AOB}$ and $K_{o,NOB}$). At the beginning of each test, pre-calculated amounts of NH_4Cl and $NaNO_2$ stock solutions were added, to achieve an initial ammonium and nitrite concentrations of 30-40 mg NH_4-N/L and 15-20 mg NO_2-N/L, respectively. Afterwards, the DO setpoint was adjusted to the targeted value. Samples were collected at 10 min intervals and analyzed for ammonia, nitrate, nitrite, VSS and TSS until all the ammonia has been consumed. AOB and NOB rates were calculated as described in the previous section for each test and then plotted to a Monod Curve to estimate $K_{o,AOB}$ and $K_{o,NOB}$ using the values recorded for the six different DO concentrations.

2.2.5. Analytical methods

Influent and effluent were collected 3 days a week, filtered through 0.45 μm syringe filter and analyzed for ammonia, nitrate, and nitrite using HACH TNTplus kits and a HACH DR3900 spectrophotometer (HACH Loveland, CO). Samples from inside the reactor and effluent were collected twice a week and analyzed for total and volatile suspended solids (TSS and VSS) using standard methods 2540D and 2540E respectively (APHA, 2012). Temperature, pH, and DO were monitored using online sensors equipped in the SBR.

The ammonia removal efficiency (ARE) and nitrite accumulation rate (NAR) were calculated according to Eq. (2.3) and Eq. (2.4) as follows:

$$\boldsymbol{ARE}\ (\%) = \frac{(NH_3-N)_{inf} - (NH_3-N)_{eff}}{(NH_3-N)_{inf}}\ \boldsymbol{X\ 100} \qquad \textbf{(2.3)}$$

$$\boldsymbol{NAR}\ (\%) = \frac{(NO_2-N)_{eff}}{(NO_2-N)_{eff} + (NO_3-N)_{eff}}\ \boldsymbol{X\ 100} \qquad \textbf{(2.4)}$$

2.2.6. DNA extraction and amplicon sequencing

Total genomic DNA was extracted using the DNeasy PowerSoil Pro Kit (Qiagen) according to the manufacturer´s protocol. The final DNA concentrations were measured using a Nanodrop ND-1000 UV spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA).

Afterwards, purified DNA was used to amplify the V3-V4 region of the 16S rRNA gene by PCR. 50 ng of DNA was used as template with 1U of Taq, 1x buffer, 1.5 mM $MgCl_2$, 0.4 mg/mL BSA, 0.2 mM dNTPs, and 5 pmoles each of 341F (CCTACGGGNGGCWGCAG) and 806R (GGACTACNVGGGTWTCTAAT) Illumina adapted primers, as described in Bartram *et al.* (doi: 10.1128/AEM.02772-10) The reaction was carried out at 94 ºC for 5 minutes, 5 cycles of 94 ºC for 30 seconds, 47 ºC for 30 seconds and 72 ºC for 40 seconds, followed by 25 cycles of 94 ºC for 30 seconds, 50 ºC for 30 seconds and 72 ºC for 40 seconds, with a final extension of 72 ºC for 10 minutes.

The resulting PCR products were visualized on a 1.5% agarose gel. Positive amplicons were normalized by eye, pooled, and sequenced on the Illumina MiSeq sequencer (Illumina Incorporated, San Diego CA). Reads were processed using DADA2 (Callahan et al., 2016). First, Cutadapt was used to filter and trim adapter sequences and PCR primers from the raw reads with a minimum quality score of 30 and a minimum read length of 100bp (Martin, 2011). Sequence variants were then resolved from the trimmed raw reads using DADA2, an accurate sample inference pipeline from 16S amplicon data. The DNA sequence reads were filtered and trimmed based on the quality of the reads, error rates were learned, and sequence variants were determined by DADA2. Bimeras were removed and taxonomy was assigned using the RDP classifier against the SILVA database version 1.3.8.

2.3. Results

2.3.1. SBR performance

In Phase I and II, the SBR was operated at high DO concentrations of 2.5-4.0 mg/L. The ammonia concentrations were 150 and 250 mg NH_4-N/L which corresponded to a NLR of 0.225 and 0.375 kg/m^3/d, in Phase I and II, respectively. During both phases, as illustrated in **Figure 2.2**, all the ammonia was oxidized to nitrate with no nitrite accumulation indicating the occurrence of complete nitrification which is referred to the absence of any NOB inhibition conditions.

In Phase III, DO limitations conditions (0.3-0.4 mg/L) were introduced to the reactor to suppress NOB activity and halt the ammonia oxidation at the nitrite phase. Moreover, starting from this phase, an anoxic period was introduced to the SBR cycle before the aerobic reaction period to benefit from the reported reduced growth rate (lag phase) of NOB following anoxic periods, i.e., transient anoxia (Kirim et al., 2022). During the first couple of days of operation, a 23% decrease in ARE was observed which can be referred to the sudden drop in DO concentrations from 2.5 to 0.4 mg/L. However, after less than 2 weeks of operation, AOB was able to adapt to the new DO condition and restore its activity and ARE improved to reach back 99.9% with effluent ammonia as low as 0.3 mg NH_4-N/L. In the meantime, a gradual increase in nitrite concentrations was observed until a nitrite accumulation rate (NAR) of 80% was reached by the end of this phase implying successful NOB inhibition. This inhibition can be attributed to the low DO conc. as well as the high ammonia concentration and nitrogen loading rate (NLR) which were equal 250.4 ± 4.2 mg NH_4-N/L and 0.375 kg/m^3/d, respectively.

The previous conditions are more suitable for side stream, thus in order to transition gradually to mainstream conditions, ammonia concentrations were decreased to 199.5 ± 2.9 mg NH_4-N/L in the following phase (Phase IV) which corresponds to an NLR of 0.3 ($kg/m^3/d$). In addition, DO concentrations were slightly decreased to 0.2-0.3 mg/L to monitor AOB and NOB activity at limited DO conditions. The previous changes in the operating conditions were accompanied by a 7% decrease in the nitrite accumulation rate which was equal to 74% after 5 days of operation. However, after 2 weeks of operation, NAR reached back to 80%. By the end of Phase IV and after 8 weeks of operation under the new conditions, NAR was further improved and reached 87%. Interestingly, ammonia removal was not affected by the new operational conditions and ammonia removal efficiency (ARE) average at 99.7 ± 0.1%. The same pattern was observed in Phase V when ammonia and DO concentrations were further decreased to 150.6 ± 2.1 mg NH_4-N/L and 0.1-0.2 mg/L, respectively. A 10% decrease in the NAR to 79% followed by a recovery in the next couple of weeks reaching 94.5% by the end of the phase after 8 weeks of operation. Similarly, ARE was not affected and the SBR was able to remove almost all ammonia during the whole period of operation.

In Phase VI, ammonia concentration was reduced to 100.3 ± 2.1 mg NH_4-N/L and HRT was also decreased from 16 to 12 h to shift more towards mainstream condition as well as to maintain a NLR close to that of the previous phase in order to prevent any shock effect on the biomass. Although ammonia concentration was dropped by a 33%, decreasing the HRT resulted in a NLR decrease from 0.225 to 0.2 $kg/m^3/d$ only (~ 12% decrease). The HRT was decreased by shortening the cycle duration from 4 to 3 h while keeping the volume exchange ratio (VER) constant at 25%. To accommodate for the previous change, the anoxic phase was shortened to 40 mins instead of 80 mins and the aerobic reaction phase was also reduced to 100 mins. Thus, the new SBR cycle duration consisted of 5 mins of feeding, 40 mins anoxic period, 100 aerobic reaction phase, 30 mins of settling and 5 mins of decanting, as shown in **Figure 1b**. These changes resulted in a drastic decline in NAR from 94.5 to 47.6% after 4 weeks of operation. Since the volume exchange ratio was kept constant at 25%, the initial ammonia concentration inside the reactor at the beginning of the cycle in Phase VI was around 25 mg NH_4-N/L compared to 37.5 mg NH_4-N/L in Phase V. Consequently, it might have been kinetically disadvantageous to AOB ability to outcompete NOB at such low ammonia concentrations.

Therefore, in the next phase (Phase VII), the total cycle duration was increased back to 4 h while the volume exchange ratio was increased to 33% to maintain the same HRT of 12 h. Moreover, feed ammonia concentration, NLR, and DO concentration were all kept constant. By increasing the VER to 33.3% and maintaining the feed ammonia concentration at 100 mg NH_4-N/L, the initial ammonia concentration was equal to 33.3 mg NH_4-N/L compared to 25 mg NH_4-N/L in Phase VI. Interestingly, after only 5 days of operation, NAR increased by 15% compared to that achieved at the end of Phase VI. Moreover, operating the SBR with the new VER, NAR kept increasing until it reached 89% after 8 weeks of operation with nitrite concentration of up to 84.9 mg NO_2-N/L compared to 45.4 mg NO_2-N/L at the end of the previous phase. Lastly, in Phase VIII, ammonia concentration was decreased to 49.3 ± 2.1 mg NH_4-N/L and HRT to 6 h to mimic mainstream conditions which resulted in an NLR of 0.2 kg/m^3/d which was equal to that of the previous phase. In this phase, the HRT was decreased by increasing the VER to 66.67% which corresponded to an initial ammonia concentration inside the reactor of 33.3 mg NH_4-N/L which is equal to that of Phase VII. Despite the decrease in the feed ammonia concentration from 100.1 ± 0.9 to 49.3 ± 2.1 mg NH_4-N/L, no drop in NAR was observed when transitioning to this phase. During the 84 days of operation in Phase VIII, NAR averaged at 87.4 ± 0.6% with nitrite concentrations up to 44.8 mg NO_2-N/L and nitrate concentrations as low as 5.2 mg NO_3-N/L. Moreover, ammonia removal efficiency (ARE) averaged at 99.4 ± 0.4% with negligible concentrations of ammonia in the effluent.

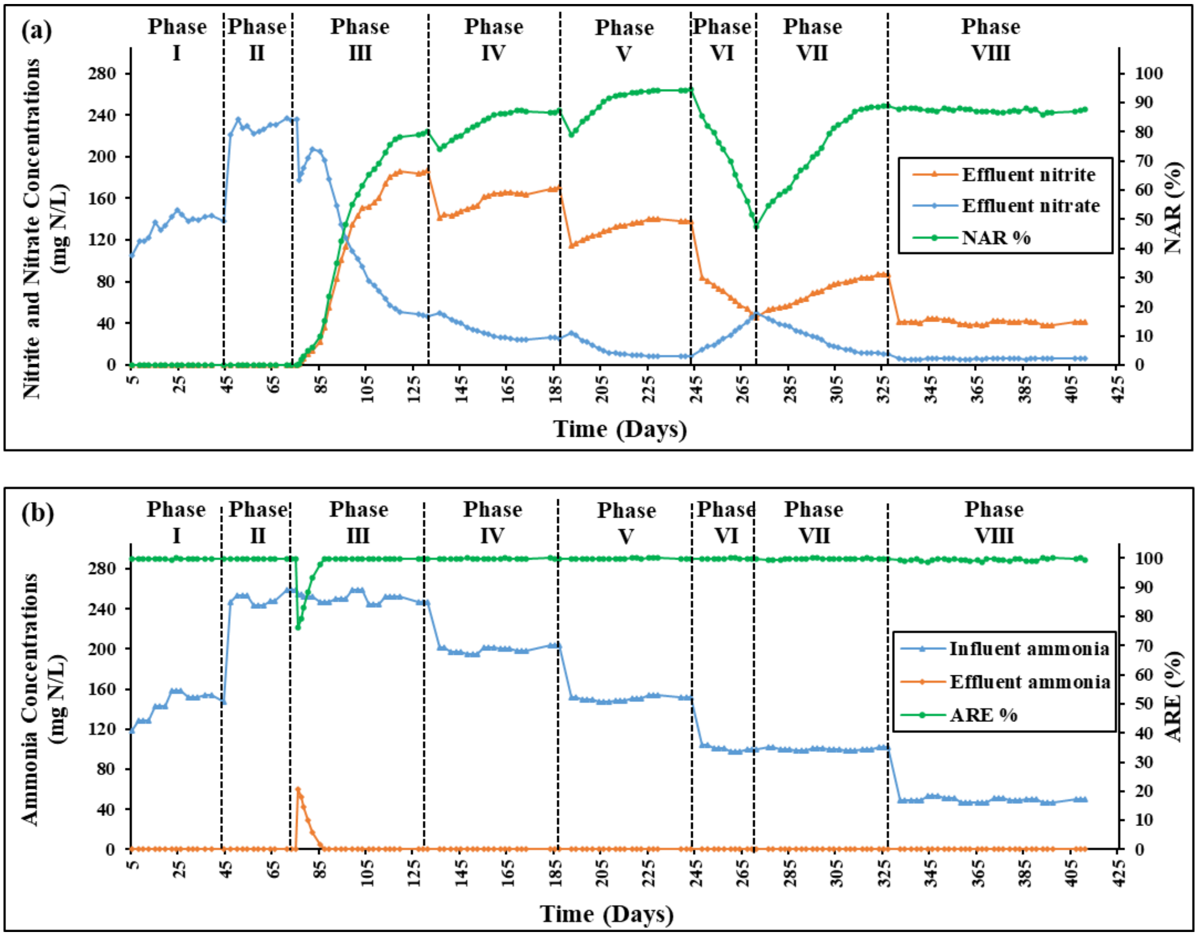

Figure 2.2: SBR performance during the different phases of operation: (a) effluent nitrite and nitrate concentrations and nitrite accumulation rates (NAR), and (b) Influent and effluent ammonia concentrations and ammonia removal efficiencies

2.3.2. AOB and NOB activity

As described in section 2.3., a batch test was conducted every 2 weeks to monitor AOB and NOB maximum activity (r_{AOB} and r_{NOB}). After 8 days of operation, NOB activity was 1.6 times higher than that of AOB with a r_{NOB} of 0.197 mg N/mg VSS/d compared to a r_{AOB} of 0.120 mg N/mg VSS/d as shown in **Figure 2.3a**. In the next few weeks, NOB and AOB activity continued to increase gradually reaching a maximum activity of 1.039 and 0.614 mg N/mg VSS/d, respectively by the end of Phase II.

In Phase III, a sudden decrease in both AOB and NOB activity was observed of 30 and 40%, respectively after 8 days of operation. The previous drop in activity is attributed to the sudden decrease in DO concentrations from 2.5-4.0 to 0.3-0.4 mg/L. However, after 2 weeks of operation, AOB was able to adapt to the new DO conditions and its activity increased from 0.421 to 1.146 mg N/mg VSS/d. On the other hand, NOB activity continued to decrease reaching a maximum activity of 0.538 mg N/mg VSS/d by the end of Phase III implying its inability of NOB to adapt to the low DO. Whereas AOB activity kept increasing and reached a maximum activity of 1.890 mg N/mg VSS/d by the end of this phase which explains the high NAR achieved.

A similar pattern was observed in Phases IV and V where AOB activity dropped for the first two weeks of operation following DO decrease by 18 and 8%, respectively. The previous decrease in activity can explain the observed drop in NAR in the beginning of each of the two phases. Nonetheless, AOB was able to adapt to the new conditions and recover its activity and a 28 and 22% increase in r_{AOB} were observed in the following 2 weeks for Phase IV and Phase V, respectively. Afterwards, r_{AOB} continued increasing in the following four weeks but at a lower rate until it reached 2.316 and 3.008 mg N/mg VSS/d by the end of Phase IV and Phase V, respectively. On the other hand, NOB was not able to adapt to the new conditions, and its activity kept decreasing gradually during the whole period of operation and r_{NOB} was equal to 0.388 mg N/mg VSS/d by the end of Phase V. As such, r_{AOB} was 8 times higher than r_{NOB} by the end of Phase V which explains the high NAR of 94.5% reached at the end of Phase V.

In Phase VI, as explained in the previous section, the drop in the initial ammonia concentration inside the SBR at the beginning of the cycle resulted in NAR deterioration 94.5 to 47.6%. This decline in NAR was accompanied by a severe decline in AOB activity and r_{AOB} dropped by more than 50% from 3.008 to 1.489 mg N/mg VSS/d after 4 weeks of operation. On the other hand, NOB was able to recover a part of its activity and r_{NOB} climbed from 0.388 to 0.783 mg N/mg VSS/d by the end of this phase. Nevertheless, despite NOB partial activity recovery, AOB maximum activity was still 1.9 times higher than that of NOB which explains the 47.6% NAR in the effluent.

Following an increase in the initial ammonia concentration in Phase VII, an 8% increase in r_{AOB} and a 9% decrease in r_{NOB} was observed after the first 2 weeks of operation which was accompanied by an increase in NAR to 60%. Moreover, during the following 6 weeks AOB activity kept increasing whereas a gradual decrease in NOB activity was observed. By the end of Phase VII, r_{AOB} rose back to 2.127 mg N/mg VSS/d which was 5.3 times higher than r_{NOB} which dropped to 0.405 mg N/mg VSS/d. In Phase VIII, when the initial ammonia concentration was kept constant, no substantial change was observed for both AOB and NOB activity despite the decrease in the feed ammonia concentration.

After the startup period and during 336 days of operation, NOB activity was successfully suppressed except for a short period of time in Phase VI when the initial ammonia concentration was decreased. It was noteworthy that NOB was not able to adapt, and its activity kept decreasing gradually in each phase, while AOB was able to adapt to each new operating conditions after a short period of decrease in activity and its activity kept rosing until the end of each phase.

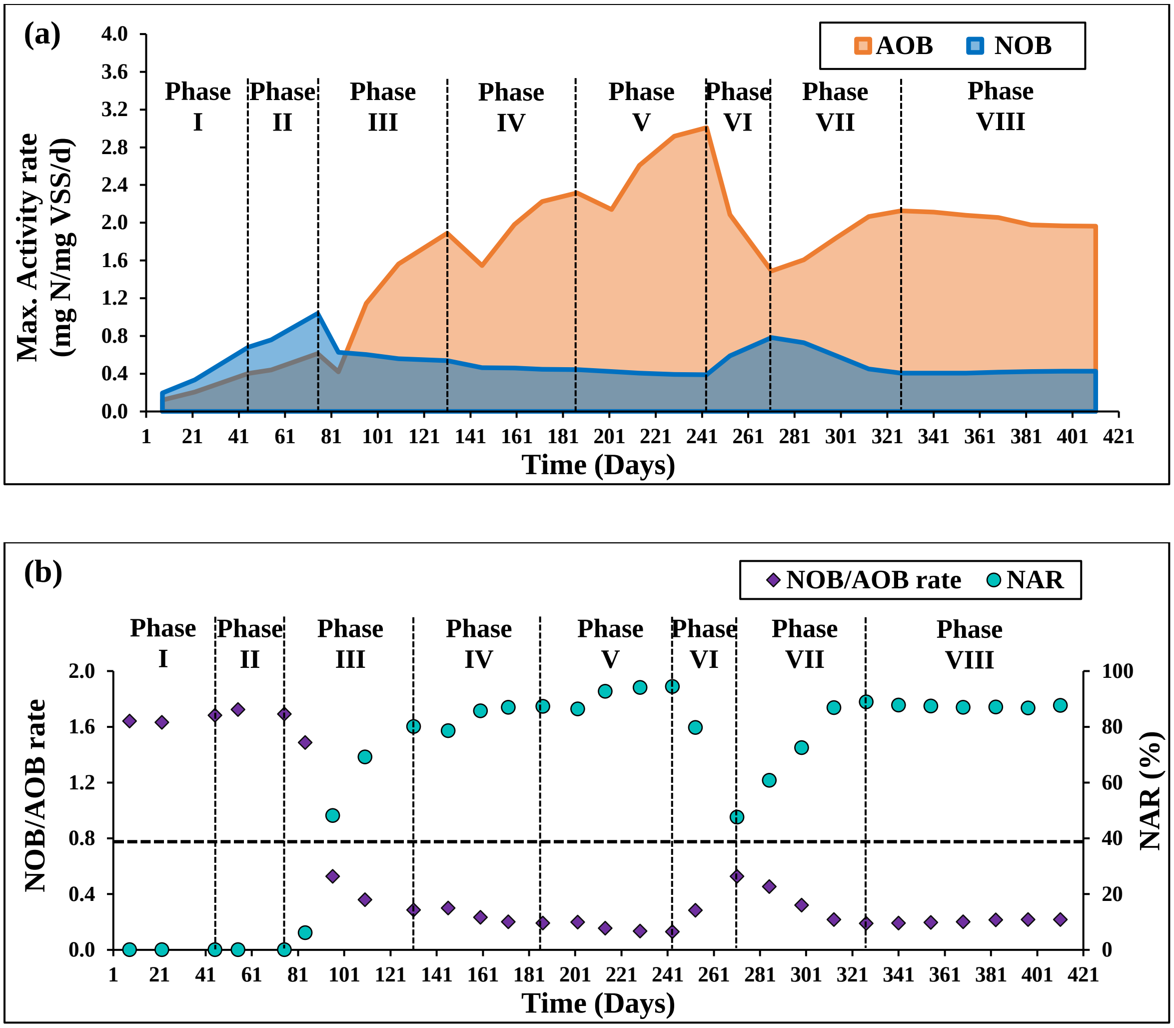

Figure 2.3: (a) AOB and NOB maximum activities during the different phases of operation, and (b) Comparison between NOB divided by AOB maximum activity rate and nitrite accumulation rate (NAR) where the horizontal dotted line is the theoretical NOB/AOB rate for full nitrification

2.3.3. AOB and NOB Oxygen half saturation coefficients

In order to develop a complete understanding of AOB and NOB behavior at low DO, 6 batch tests were conducted at different DO concentrations at the end of each phase to determine AOB and NOB oxygen half saturation coefficients ($K_{o,AOB}$ and $K_{o,NOB.}$). As illustrated in **Figure 2.4,** during the complete nitrification period (Phase I and II), $K_{o,NOB}$ was slightly higher than $K_{o,AOB}$, however it did not affect NOB activity since the SBR was operated at much higher DO concentrations in these two phases. In fact, $K_{o,NOB}$ and $K_{o,AOB}$ were equal to 0.55 and 0.44 mg O_2/L, respectively, whereas DO concentration in the SBR was in the range of 2.5-4.0 mg/L which explains the absence of any nitrite build-up in the effluent since no inhibitory was applied to NOBs.

In Phase III, with the introduction of lower DO concentration (0.3-0.4 mg/L), a drop in both $K_{o,AOB}$ and $K_{o,NOB}$ was observed and were equal to 0.29 and 0.38 mg O_2/L, respectively. Moreover, with further decrease in DO concentration in the following phases, AOB was able to adapt, and its oxygen half saturation coefficient kept decreasing at each phase reaching a K_o of 0.13 mg O_2/L at Phase V when the SBR was operated at DO levels of 0.1-0.2 mg/L. Contrarily, NOB had a K_o of 0.31 mg O_2/L at the end of Phase V which was higher than the operating DO in the SBR which explains the NOB inability to recover its activity resulting in a high nitrite accumulation during the different phases.

Following the partial nitrification disturbance that occurred in Phase VI and resulted in a deterioration in the NAR, AOB was able to adapt and restore its activity at low DO concentration in the last two phases and its K_o was equal to 0.16 mg O_2/L by the end of Phase VIII. On the other hand, after increasing the initial ammonia conc., $K_{o,NOB}$ was equal to 0.3 mg O_2/L which was almost similar to its value in Phase V which explains the NAR recovery after its deterioration in Phase VI.

All in all, it was noteworthy that during all partial nitrification phases, $K_{o,AOB}$ was always lower than the operated DO inside the reactor in contrast to $K_{o,NOB}$ which demonstrates AOB ability to adapt to low DO conditions and explains NOB activity suppression.

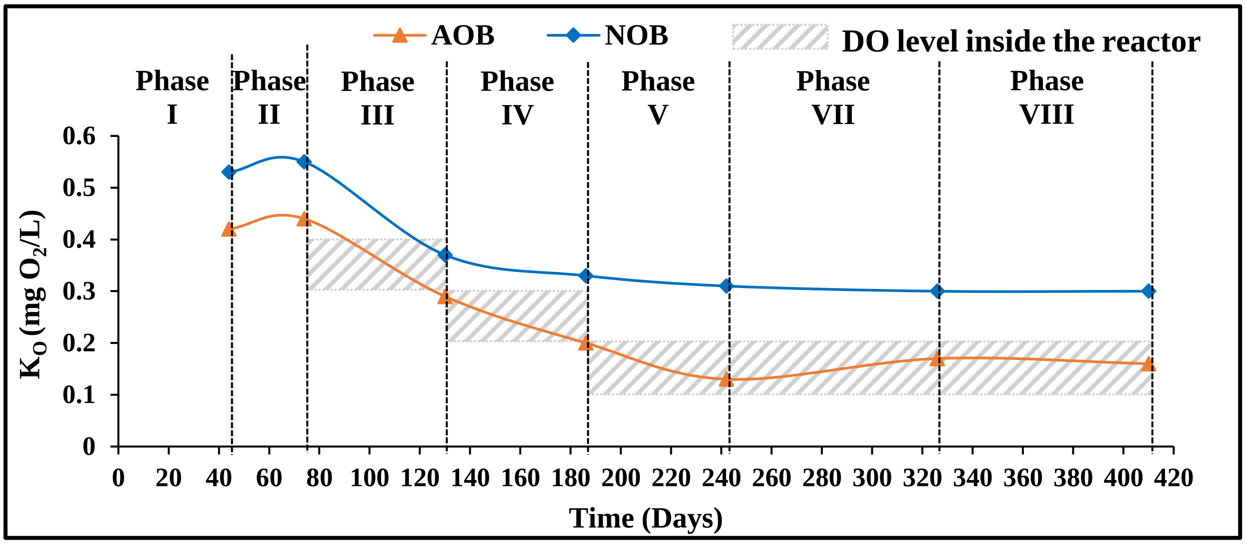

Figure 2.4: Plot between different oxygen half-saturation coefficients of AOB and NOB ($K_{O,AOB}$ and $K_{O,NOB}$) obtained at each phase
*DO levels in Phase I and II was 2.5-4.0 mg/L

2.3.4. Solids Measurements and SRT

During all different phases of operation, a sample was taken from the effluent of the 6th cycle, 2 times a week and analyzed for TSS and VSS. SRT was not controlled but was calculated for each phase. As shown in **Figure 2.5a**, in Phase I, the biomass inventory experienced a constant drop until the end of the phase which is attributed to the washout of the heterotrophic bacteria under carbon starvation conditions. In phase II, increasing the NLR resulted in a slight increase in the VSS and TSS which averaged at 1988 ± 184 mg VSS/L and 3689 ± 184 mg TSS/L, respectively. In Phase I and II, SRT was 29.9 ± 0.8 and 20.3 ± 1.4 days, respectively as illustrated in **Figure 2.5b**, which is suitable for side stream conditions. In the following phase, the decrease in DO concentrations from 2.5 to 0.4 mg O_2/L resulted in a decrease in both TSS and VSS concentrations and at the end of Phase III, they averaged at 1692 ± 137 mg VSS/L and 2870 ± 304 mg TSS/L, respectively. A similar pattern has been observed in the next two phases where the decrease in the DO concentrations was accompanied by a decrease in the biomass concentrations. This drop in biomass concentrations might be attributed to inability of some strains to adapt to the harsh conditions induced inside the reactor which resulted in their washout which can be observed in the elevated effluent TSS and VSS. The SRT in Phase III, IV, and V averaged at 16.5 ± 1.9, 12.8 ± 1.4, and 9.1 ± 0.6 days, respectively. In the last three phases, no notable change in the biomass concentration was observed which can be explained by the maintenance of a constant DO of 0.1-0.2 mg/L. During these three phases, VSS and TSS average at 460 ± 15 mg VSS/L and 539 ± 16 mg VSS/L. In Phase VI, VII, and VIII, SRT averaged at 8.4 ± 0.5, 5.0 ± 0.3, and 4.9 ± 0.4 days, respectively.

Notably, the ratio between VSS/TSS in the startup phases averaged at 0.55 compared to 0.75 in the seeding sludge which indicates the increase in inactive biomass inside the reactor. The drop in VSS/TSS ratio can be explained by the absence of organic carbon source for heterotrophic bacteria in the feed which is combined with the high SRT resulted in an increase in the portion of inactive bacteria. However, with the decrease in SRT and biomass concentrations, the ratio of VSS/TSS kept increasing and averaged at 0.85 at the last phase.

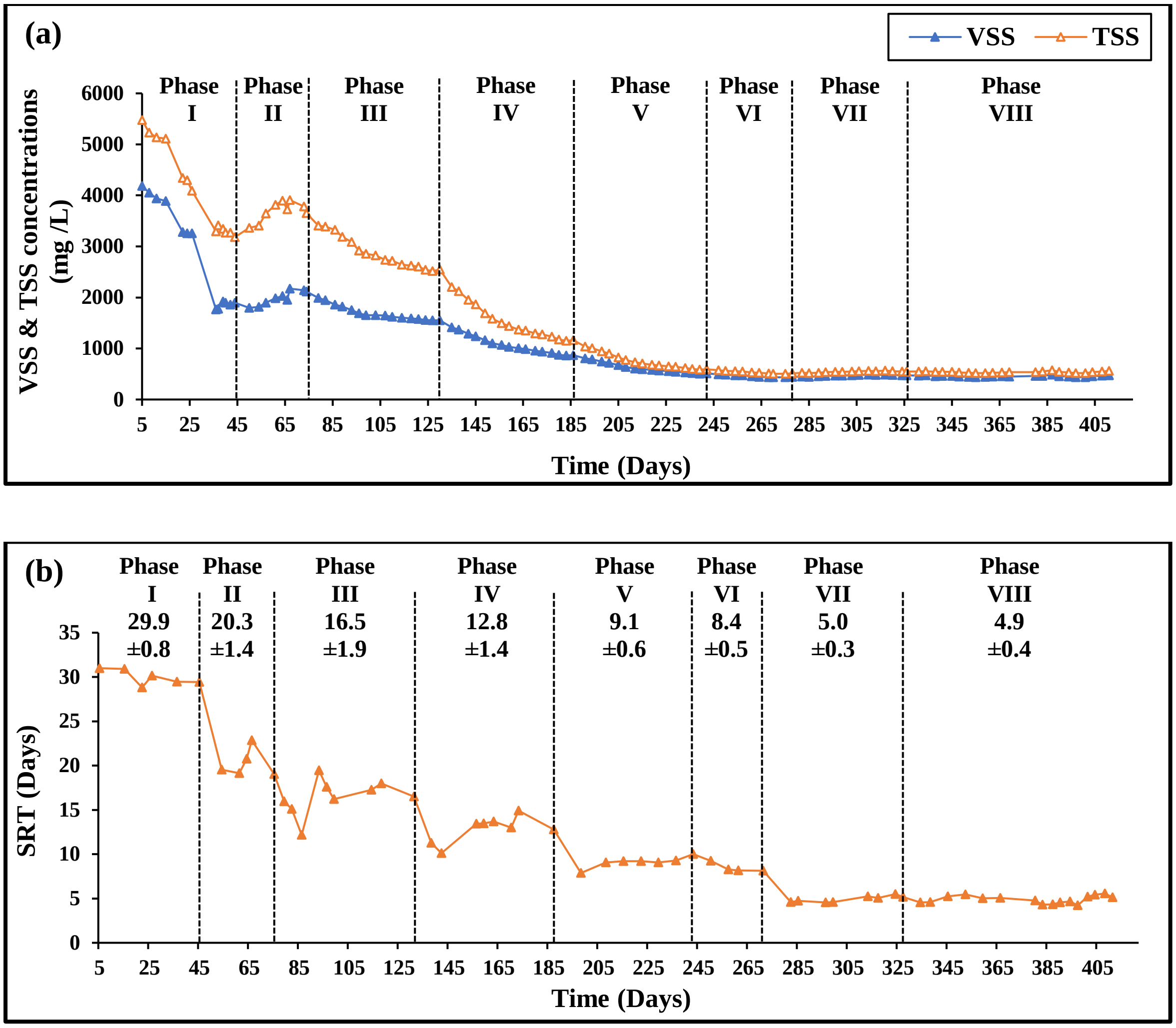

Figure 2.5: Solids measurements during the different phases of operation: (a) Total suspended solids (TSS) and Volatile suspended solids (VSS), (b) SRT

2.3.5. Microbial community dynamics

In order to monitor the evolution of the nitrifying community, a sample was taken at the end of each phase and analyzed using amplicon sequencing. As illustrated in **Figure 2.6**, *Nitrosomonas* and *Nitrospira* were the dominant species of AOB and NOB, respectively, during all phases of operation. In Phase I and II which were operated at high DO concentrations, a steady increase in the relative abundance (R.A.) of both *Nitrosomonas* and *Nitrospira* was observed. By the end of Phase II, *Nitrosomonas* and *Nitrospira* R.A. were 5.2 and 5.5 times higher than their R.A. in the seeding sludge and had a value of 6.8 and 9.9%, respectively. In phase III, following the transition to low DO, a severe drop in *Nitrospira* R.A. to 1.9% was observed. On the other hand, *Nitrosomonas'* abundance did not exhibit any decline, contrarily, its R.A. slightly increased to 7.2%. A similar pattern was observed in the following two phases where *Nitrospira* R.A. kept decreasing till it reached 1.5% while *Nitrosomonas* R.A. kept climbing until it reached 15.8% by the end of phase V. Nevertheless, in Phase VI, a reversal in *Nitrospira* abundance was observed and its RA climbed to 13.7% which explains the drop in NAR and nitrate build-up in the effluent observed in this phase. Whereas *Nitrosomonas* R.A. kept increasing and reached 21.7% by the end of this phase despite the observed drop in NAR. In the following two phases, the operational conditions change resulted in a successful NOB out-selection which was reflected in the decrease in *Nitrospira* R.A. and increase in that of *Nitrosomonas*. At mainstream conditions, *Nitrosomonas* (AOB dominant species) R.A. was almost 11 times higher than that of *Nitropsira* (NOB dominant species) and had values of 40.1 and 3.7%, respectively which demonstrates the success of the adaptation strategy in NOB out-selection.

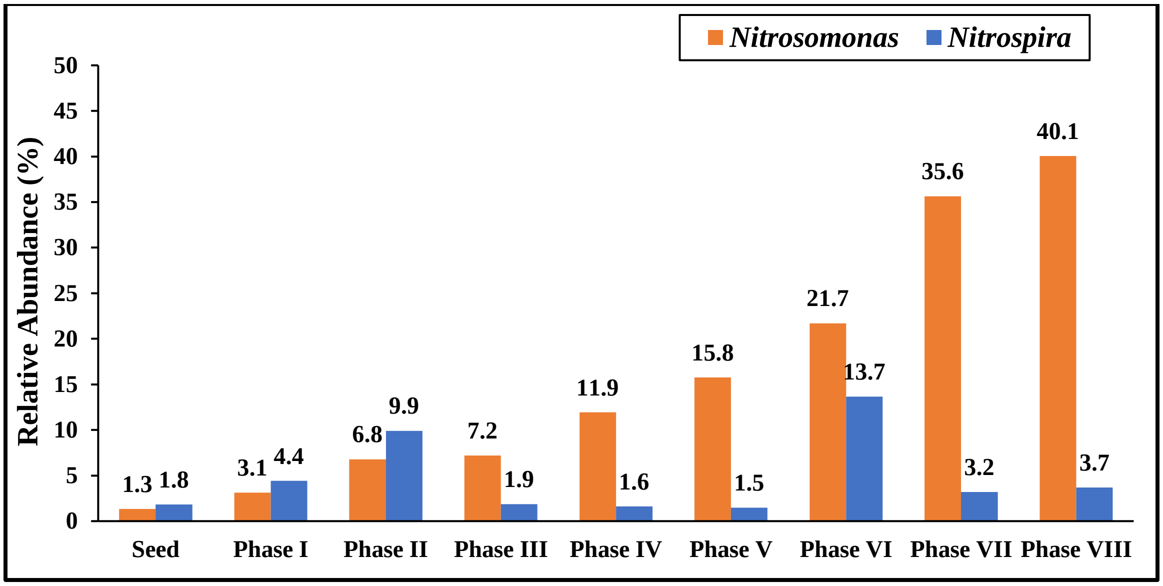

Figure 2.6: Change in relative abundances of the dominant AOB and NOB species during the different phases of operation

2.4. Discussion

Phase I and Phase II (start-up period) aimed at enriching nitrifying bacteria (AOB and NOB) and washout heterotrophic bacteria under carbon starvation phase, thus no DO limitation conditions were applied in the reactor. As a result, in the startup phases, no nitrite build up was observed inside the SBR with all the ammonia being oxidized to nitrate. In agreement, microbial activity tests revealed that both AOB and NOB kept increasing gradually during the first two phases and by the end of Phase II, r_{NOB} and r_{AOB} were 1.039 and 0.614 mg N/mg VSS/d, respectively, compared to 0.197 and 0.120 mg N/mg VSS/d at the beginning of the experiment implying the success of enriching nitrifiers. The theoretical NOB/AOB maximum activity ratio value to achieve complete nitrification is 0.78 (Dold et al., 2015). Thus, for partial nitrification NOB/AOB ratio should be expected to be lower than the theoretical value and any values above 0.78 indicates that no NOB suppression is occurring. In agreement, as illustrated in **Figure 3b,** the NOB/AOB ratio was above the theoretical line in Phase I and II during all days of operation indicating the occurrence of complete nitrification. Moreover, during Phase I, a loss in VSS and TSS was observed during the first few weeks of operation which can be attributed to the washout of heterotrophic bacteria due to the absence of any organic carbon source in the feed. However, in Phase II, a slight increase back in the VSS and TSS was observed which is attributed to the increase in the ammonia concentration (nitrifiers' substrate) and the reduction in biomass washout indicating the success of washing out heterotrophic bacteria. The previous observation is supported by amplicon sequencing which revealed that the relative abundance of *Nitrospira* and *Nitrosomonas* increased to 9.9 and 6.8% by the end of Phase II compared to 1.8 and 1.3% in the seeding sludge, respectively, implying the success of enriching nitrifiers. During the startup period, $K_{o,NOB}$ was slightly higher than $K_{o,AOB}$, however no NOB activity suppression was taking place since the DO concentrations inside the reactor were much higher than K_o for both NOB and AOB.

The objective of Phase III was to observe the effect of low DO on AOB and NOB. Thus, all the operational conditions were kept constant when transiting from Phase II to III except for the DO concentrations which were lowered from 2.5 to 0.3-0.4 mg/L. Moreover, it was anticipated that sides stream conditions, i.e., high NH_4-N concentrations and NLR, and long HRT would provide AOB kinetic advantage in outcompeting NOB for oxygen. The transition from high to low DO resulted in a decrease in ARE during the first few days of operation. Microbial activity tests showed a decrease in AOB maximum activity following the DO decrease which explains the drop in ARE. However, after a period of operation, r_{AOB} started increasing back and kept increasing till the end of the phase which resulted in the restoration of high ARE. A similar pattern was reported in other studies in the literature following a drop in DO concentrations which was attributed to AOB adaptation. For instance, in an SBR, it was reported that the drop in DO concentrations from 1.5-2.0 to 0.2-0.4 mg/L was accompanied with a temporary decrease in AOB activity which led to elevated ammonia concentrations in the effluent (Z. Wang et al., 2021). However, after a period of operation, AOB was able to restore its activity and improved ammonia oxidation was observed due to the adaptation of AOB to the low DO. A similar behavior was reported in another study performed in a CSTR after a sudden decrease in DO concentrations from 4 to 0.25 mg/L (Blackburne et al., 2008). On the other hand, a similar drop in NOB maximum activity was observed following the decrease in DO concentrations. However, NOB was not able to recover its activity which resulted in the nitrite build-up in the effluent. As a result of AOB adaptation and NOB inability to recover its activity, NOB/AOB ratio kept decreasing and by the end of the phase, it was equal to 0.28. The previous value is much lower than the theoretical value for complete nitrification which confirms the occurrence of partial nitrification and explains the high NAR achieved. The increase in NAR was further demonstrated by the sequencing data which showed that *Nitrospira* relative abundance by the end this phase decreased to 1.9% compared to 9.9% in the previous phase. while *Nitrosomonas* relative abundance increased to 7.2%. Moreover, $K_{o,NOB}$ by the end of this phase was 0.38 mg O_2/L which was close to the operating DO of 0.4 mg/L whereas $K_{o,AOB}$ was lower and had a value of 0.29 mg O_2/L. Thus, it can be concluded that a combination of low DO concentrations and high ammonia concentrations and NLR was proved successful in providing AOB a kinetic advantage over NOB resulting in successful NOB suppression.

In the following two phases (Phase IV and V), the DO and ammonia concentrations were further decreased in a stepwise fashion to initiate the gradual transition from side stream to mainstream conditions while allowing AOB enough time to adapt to each new combination of DO and NLR. A similar pattern was observed in both phases of a slight drop in AOB maximum activity in the first few days of operation resulting a slight drop in NAR which is referred to AOB sensitivity to new DO and NLR conditions. Followed by an increase in AOB activity in the next two weeks of operation and a further increase in the following four weeks but at lower rates resulting in a gradual increase in NAR which is attributed to AOB adaptation to the new conditions. Contrarily, NOB activity kept decreasing during the whole period of operation of Phase IV and V indicating the success of maintaining NOB inhibition at lower NLRs. The NOB/AOB ratio kept decreasing in the two phases except for a short period at the beginning of each phase where AOB was experiencing a short period of reduced activity. By the end of Phase V, the NOB/AOB ratio was 0.13 which corresponded to a NAR of 94.5%. During the two phases, AOB adaptation resulted in a steady decrease in its K_o value and was lower than the SBR operating DO in both phases. On the other hand, NOB inability to adapt to the new NLR and DO combination was reflected in its K_o value being higher than the DO inside the SBR in both phases resulting in lower nitrate accumulation in the effluent. In agreement, a steady increase in *Nitrosomonas* relative abundance was observed in Phase IV and V while *Nitrospira* relative abundance was further decreased.

After reaching a DO level of 0.1-0.2 in Phase V which is the target DO level, it was kept constant for the remainder of the experiment. On the other hand, ammonia concentrations were still higher than the anticipated mainstream concentrations, thus a further decrease in ammonia concentrations was introduced in Phase VI. Moreover, HRT was simultaneously decreased to avoid a significant drop in NLR which might have a negative affect AOB activity. Nonetheless, these changes resulted in a substantial drop in AOB activity and a steady increase in NOB activity which was accompanied by a severe decrease in NAR. Accordingly, the NOB/AOB ratio experienced a steady increase and reached 0.53 by the end of this phase which was close to the theoretical threshold for complete nitrification of 0.78 resulting in a NAR of 47.5%. Accordingly, *Nitrospira* exhibited an increase in its R.A. in this phase from 1.5 to 13.7% which explains NOB restoration of its activity and the observed decrease in NAR. Interestingly, *Nitrosomonas* R.A. kept increasing in this phase, which implies that the observed drop was not due to AOB washout but rather due to inability to outcompete NOB. Here, it is worth noting that the DO concentrations were kept constant in this phase and NLR was only decreased from 0.225 to 0.2 kg/m^3/d, thus AOB sensitivity to the change in DO and NLR cannot explain the lower NAR. The first possible explanation for such a drop might be the decrease in the anoxic phase from 80 to 40 mins which might have not been enough to introduce a lag phase for NOB activity or the shorter anoxic phase might have allowed NOB to recover its activity faster and oxidize some of the accumulated nitrite before the end of the reaction time. However, here it is worthmentionning that some studies in the literature reported NOB inhibition following an anoxic phase as short as less than 10 mins (Klaus et al., 2020a; Regmi et al., 2014, 2015). Nonetheless, in these studies the total cycle time (anoxic + aerobic duration) was between 12-16 mins before another anoxic phase is introduced compared to 100 mins in this study which allows NOB more time to recover its activity. The second explanation for the previous drop might be the low initial ammonia concentration inside the SBR at the beginning of the cycle. In fact, in Phase VI, the decrease in feed ammonia concentrations resulted in a sudden decrease in the initial ammonia concentrations inside the reactor from 37.5 to 25 NH_4-N/L. Consequently, this low initial ammonia concentration might have posed a challenge on AOB ability to outcompete NOB. Overall, it can be concluded that the combination of low initial ammonia concentrations and shorter anoxic period was not enough to completely suppress NOB activity.

Therefore, in the next phase (Phase VII), all the operational conditions were kept constant except for the VER which was increased from 25 to 33% to ensure whether the drop in the NAR was due to the decrease in the initial ammonia conc. inside the reactor at the beginning of the cycle or not. Interestingly, the increase in initial ammonia concentrations resulted in a gradual increase in AOB maximum activity and a decrease in that of NOB. By the end of Phase VII, the NOB/AOB ratio was 0.19 compared to 0.53 in the last phase resulting in an increase in NAR from 47.5 to 89%. The SBR ability to restore its high NAR was also driven by the increase in *Nitrosomonas* relative abundance and decrease in that of *Nitrospira* to 35.6 and 3.2%, respectively. The previous results indicate that initial ammonia concentration inside the SBR at the beginning of the cycle might have played an important role in suppressing NOB activity and achieving a high NAR.

In order to further validate the previous hypothesis, in Phase VIII, while decreasing the ammonia concentrations to transition to mainstream concentrations, HRT was decreased and VER was increased to maintain a similar NLR and initial ammonia concentration inside the SBR as in the previous phases. Interestingly, no drop in AOB maximum activity or NAR in the beginning of the phase was observed unlike in the previous phases which reinforces the previous hypothesis that the initial ammonia concentration inside the reactor might be the controlling parameter rather than the feed ammonia concentration.

At mainstream conditions, the SBR was able to maintain a 99.4 ± 0.4% ARE and an 87.4 ± 0.6% NAR using the kinetic adaptation strategy. NOB activity was successfully suppressed during the whole period of operation and AOB maximum activity was 5 times higher than that of NOB. The NOB/AOB ratio in this phase averaged at 0.21 which is much lower than the theoretical threshold for full nitrification confirming the occurrence of partial nitrification. In a step-fed SBR treating the effluent of an A-stage MBBR, it was reported that a NOB/AOB ratio of 0.28 resulted in a NAR of 62-70% (Xu et al., 2017). In the previous study, the SBR was operated using intermittent aeration with DO concentrations in the aerobic phases of 1.4-1.6 mg/L and at a temperature of 30 ± 1 ºC. Despite the high temperature and DO concentrations, AOB and NOB maximum activity were 0.63 and 0.18 mg N/mg VSS/d which were much less than those obtained in the current study of 1.97 and 0.42 mg N/mg VSS/d. However, it is worth noting that in the current study the SBR was fed with a solution devoid of any organic carbon substrate which resulted in higher dominance of nitrifiers in the biomass. Similarly, it was reported in a CSTR operated at low DO (0.1-0.4 mg/L) that a NOB/AOB of 0.28 resulted in a NAR of 64% (Jimenez et al., 2014b). However, in the previous study, AOB and NOB maximum activity were as low as 0.023 and 0.06 mg N/mg VSS/d despite that the average temperature was 29.2 ºC.

The adaptation strategy was proven successful in enriching a nitrifying community in which $K_{o,AOB}$ is lower than $K_{o,NOB}$. Thus, AOB was provided a kinetic advantage over NOB through operating the SBR at a DO level higher than its K_o but lower than that of NOB which explains the high NAR achieved in this experiment. In agreement, it was reported in a study performed in an SBR, that at high influent ammonia concentrations, a NAR of 80% was achieved. However, when the SBR was shifted to low ammonia concentrations, the NAR was reduced to zero despite that $K_{o,AOB}$ was 1.35 mg/L which was lower than $K_{o,NOB}$ of 1.95 mg/L. In this experiment, the DO concentrations inside the SBR was maintained at a level higher than both $K_{o,AOB}$ and $K_{o,NOB}$ (above 4 mg/L) which eliminated the kinetic advantage of AOB over NOB (Yu et al., 2020). Similar pattern was observed in another study performed in an SBR fed with the effluent of a pilot scale HRAS plant (Keene et al., 2017). Although $K_{o,AOB}$ (0.63 mg/L) was lower than $K_{o,NOB}$ (1.01), a NAR of only 15.3% was achieved which was due to operating the SBR at DO levels higher than both AOB and NOB K_o of 1.5-2.0 mg/L. Thus, it can be concluded that maintaining stable partial nitrification does not only rely on $K_{o,AOB}$ being lower than $K_{o,NOB}$ but also on operating at DO levels higher than $K_{o,AOB}$ but lower than that of NOB.

Furthermore, the amplicon sequencing demonstrated the success of out-selecting NOB since the relative abundance of the dominant AOB species was almost 11 times higher than that of the dominant NOB species. During all phases of operation, *Nitrosomonas* and *Nitrospira* were the dominant species of AOB and NOB, respectively, while *Nitrobacter* species of NOB was not detected. Similar finding was reported in other mainstream studies (Keene et al., 2017; Wang et al., 2019; Xu et al., 2017; Yu et al., 2020). It was suggested that *Nitrospira* are mostly present at low nitrite and DO concentrations unlike *Nitrobacter* which commonly prefers high concentrations of oxygen and nitrite (Huang et al., 2010; Nowka et al., 2015). Moreover, *Nitrobacter* was not detected in the seeding sludge used to inoculate the SBR unlike *Nitrospira* which might have as well contributed to its presence during the different phases of operation.

Overall, the results obtained in this study provide evidence that NOB out-selection can be achieved at low DO concentration through the adaptation strategy. These findings pave the way towards scaling-up of mainstream shortcut biological nitrogen removal processes by overcoming a major bottleneck (i.e., NOB out-selection in mainstream).

2.5. Conclusion

Despite the reported challenges of NOB suppression at mainstream conditions, this study was able to achieve successful complete partial nitrification under low DO levels (0.1–0.2 mg/L). The SBR was able to maintain an ammonia removal efficiency (ARE) of 99.4 ± 0.4% and nitrite accumulation rate (NAR) of 87.4 ± 0.6% at mainstream conditions using a kinetic adaptation strategy. The adaptation strategy relied on lowering the DO concentration in a stepwise fashion and transitioning gradually from side stream to mainstream conditions allowing the biomass enough time to adapt to each new condition before moving to the next. Microbial activity tests revealed that at each DO level decrease, after a short period of activity decline, AOB was able to adapt and restore its high activity. Contrarily, NOB demonstrated an inability to recover, and its activity kept declining during all phases of operation. In the last phase, which was operated at mainstream conditions, AOB maximum activity was 5 times higher than that of NOB. Moreover, it was demonstrated that the initial ammonia concentration inside the reactor at the beginning of the SBR cycle might be the controlling parameter for NOB inhibition rather than the feed ammonia concentration.

Furthermore, AOB and NOB oxygen half saturation coefficients tests revealed that AOB was able to adapt to lower DO concentration and its oxygen half saturation coefficient kept decreasing at each phase reaching a K_o of 0.16 mg/L at mainstream conditions when the SBR was operated at DO levels of 0.1-0.2 mg/L. Contrarily, NOB had a K_o of 0.3 mg/L at the mainstream phase which was higher than the operating DO in the SBR which elucidates the NOB inability to recover its activity resulting in a high nitrite accumulation. Thus, it was concluded that controlling the DO inside the reactor at a level higher than $K_{o,AOB}$ but lower than $K_{o,NOB}$ played a pivotal role in NOB inhibition.

Lastly, the adaptation strategy resulted in an increase in *Nitrosomonas* relative abundance to 40% vs 3.7% for *Nitrospira* compared to 1.3 and 1.8% in the seeding sludge which demonstrates the success of NOB out-selection.

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Chapter 3: Evaluation of the nitrite shunt process as a B-stage in the A/B stage scheme for low carbon to nitrogen (C:N) wastewater using continuous aeration at low DO concentrations

ABSTRACT

Recently, the concept of water resources recovery facilities (WRRFs) has emerged to facilitate the shift from energy consuming wastewater treatment facilities to sustainable and energy efficient ones. The two-stage A/B process scheme is one of the the widely adopted schemes in WRRFs. In such a scheme, the A-stage's purpose is carbon removal and maximizing resources recovery, whereas the B-stage aims at nutrient removal using less energy and resources to avoid nullifying the gain achieved in the A-stage. For this purpose, the nitrite shunt process has been proposed as a B-stage alternative; however, its implementation has been hindered by the challenges facing NOB out-selection at mainstream conditions.

Thus, in this study, it is targeted to demonstrate the successful maintenance of a stable nitrite shunt at mainstream conditions. Owing to the pre-adaptation of the nitrifying sludge to low DO, the system was able to maintain an ammonia, COD, and total inorganic nitrogen (TIN) removal efficiencies of 99.2 ± 0.7, 94.0 ± 0.1 and 93.2 ± 1.6% at a C:N ratio as low as 6.0 ± 0.2. Amplicon sequencing revealed that NOB genus had a total relative abundance of 0.25-0.41% during different phases of operation compared to 2.1-2.5% for AOB which resulted in an average nitrite accumulation rate of 88.4 ± 3.4% during the entire study. It was demonstrated that the combination of low DO concentrations (0.2 mg/L), short aerobic SRT (3.7 ± 0.4 d), high heterotrophic denitrifiers activity rates were a key in achieving NOB suppression. Moreover, the effect of influent C:N ratio and COD fractionation on TIN removal and NOB out-selection was studied. , It was demonstrated that pCOD correlated positively with both TIN removal and NOB out-selection

3.1. Introduction

Recently, there has been a paradigm shift towards the vision of wastewater treatment plants (WWTPs) from being intensive energy consumer facilities with a sole objective of removing pollutants to the water resources recover facilities (WRRFs) concept. WRRFs are facilities which encompass an immense latent energy and resources that are able to turn them into self-sufficient or even net positive facilities. The WRRF concept relies upon maximizing the energy recovery/minimizing the energy use while efficiently treating the received streams and meeting the required effluent standards. Therewith, the total dependence on conventional activated sludge (CAS) process for wastewater treatment has been reassessed. Several configurations have been proposed for WRRF, among which the A/B process stands-out since it is compatible with the existing infrastructure and has already been applied at full scale in many countries (Meerburg, 2016). In such a scheme, the A-stage is designed for maximizing COD capture and redirecting it to the solids stream for recovery, whereas B-stage is dedicated for nutrients treatment. The objective of A-stage is to achieve higher energy recovery, while that of B-stage is to develop removal technologies that are able to reduce the energy use of conventional processes to avoid nullifying the energy gain achieved in the A-stage.

Several technologies have been proposed for the A-stage including high-rate activated sludge (HRAS), high-rate contact stabilization (HiCS), and the alternating activated adsorption (AAA) (Wett et al., 2020). In all of these technologies, high-rate conditions (low SRTs and low HRTs) are applied which minimize organic loss by oxidation and promotes COD capture by biosorption (Rahman et al., 2020). As a result, it was demonstrated that 2-4 times more solids production can be achieved through A-stage systems while organics oxidation can be reduced by up to 70%. This would yield 2.5-3.5 times higher biogas production compared to CAS (Meerburg et al., 2015).

On the other hand, shortcut biological nitrogen removal (SBNR) which comprises two main processes i.e., nitrite shunt (partial nitrification/denitritation) and deammonification (partial nitrification/anammox) has been proposed for B-stage (Roots et al., 2020a, 2020b). Compared to conventional BNR, SBNR implies 25 and 62.5% savings in term of oxygen and 40 and 100% savings in COD demand for nitrite shunt and deammonification, respectively (Soliman and Eldyasti, 2016b). Even though deammonification might appear as a the most suitable process as a B-stage, few studies argued the opposite based on other factors that might arise during implementation (Mccullough et al., 2022). To elaborate, for the nitrite shunt process, the 40% COD savings assume that all the COD have been used solely for denitritation and no COD oxidation has taken place during the process which is impractical given the low rates conditions applied in B-stage process. Similarly, for deammonification process, the COD savings calculation, in addition to the no COD oxidation during partial nitrification assumption, assume a partial nitrification effluent of exactly 1:1.32 ammonium: nitrite molar ratio with complete absence of nitrate concentrations which is required for subsequent anammox process. The previous assumption disregards the COD that would be required to reduce any additional oxidized nitrogen compounds as well as the COD required to denitrify the nitrate produced in the anammox process. Moreover, the oxygen savings calculations in both processes assume that the same oxygen concentrations are used in conventional and partial nitrification while in fact most of partial nitrification strategies adopt applying low DO concentrations which would completely alter the calculations and should result in more oxygen savings. Moreover, these calculations do not take into consideration the challenge facing the implementation of deammonification process. The implementation of the deammonification process is typically challenged by the slow growth of the anammox bacteria. This requires the use of an additional anammox retention mechanism such as hydrocyclones to allow for adequate solids retention time (SRT) (Kirim et al., 2022). Thus, it can be concluded that evaluating the nitrogen removal processes solely based on these theoretical calculations would not be conclusive.

In the A/B scheme, a crucial factor that affects the selection of the B-stage process in WWRF is the performance of the A-stage since the A-stage effluent is the B-stage influent. The A-stage performance determines the ratio of carbon to nitrogen (C:N) ratio of the B-stage influent which has been reported to have a significant effect on the performance on SBNR processes. In fact, high C:N ratio impedes the advantage of AOB and anammox bacteria over the heterotrophic bacteria which would result in lower nitrogen removal rates (Li et al., 2018). However, anammox might be more sensitive to high C:N ratios due to their slow growth which results in denitrifying bacteria outcompeting them for nitrite (Jenni et al., 2014). For instance, it was reported that anammox could not be sustained at a C:N ratio above 2 (Lackner et al., 2008). As well, it was suggested that the biodegradable COD to nitrogen ratio should be maintained at a level below 0.5 for mainstream anammox (Daigger, 2014). Nonetheless, the reported C:N ratio for A-stage systems in the literature are higher than the suggested C:N ratio for mainstream anammox due to the high-rate conditions applied in these systems which results in lower COD removal compared to conventional activated sludge. In fact, the reported C:N ratio in the effluent of A-stage processes ranged from 0.67 to 8 (Liu et al., 2018; Rahman et al., 2019; Xu et al., 2015). On the other hand, one of the disadvantages encountering nitrite shunt process is its requirement of organic carbon to reduce the nitrite produced during partial nitrification which would increase the process costs if the required amount of COD is not readily available in the influent and have to be externally provided. Encouragingly, the relatively higher COD directed to the B-stage from the A-stage systems can benefit the nitrite shunt process. In fact, employing nitrite shunt following an A-stage system might represent a key solution for the relatively low COD removal efficiency of A-stage systems and the lack of substrate for denitritation in nitrite shunt.

Nevertheless, the implementation of nitrite shunt as a B-stage system at full-scale is hindered by two main challenges. The first challenge is NOB-out selection in mainstream lines due to the lower ammonia concentrations and temperature compared to side stream (Gholami-Shiri et al., 2021). However, it was demonstrated in the previous chapter that using a kinetic-adaptation strategy can play a pivotal role in NOB out-selection in mainstream lines. Moreover, the presence of denitrifies in the nitrite shunt would add an additional pressure on NOB through their competition for nitrite (NOB substrate) which can potentially increase NOB out-selection (Klaus et al., 2019). The second challenge facing the implementation of nitrite shunt is the availability of readily biodegradable COD (rbCOD) for the denitritation step. Although the theoretical COD requirement for denitritation is 1.71 g COD/g NO_2, it has been reported that heterotrophic denitrifies can only use readily biodegradable COD as their substrate (Daigger, 2014). Thus, taking into consideration that the COD directed from the A-stage to the B-stage contains a non-biodegradable fraction, the actual C:N ratio required for denitritation would be higher than the theoretical value. In fact, it was reported in a study in the literature that the total COD to nitrogen ratio required for complete nitrogen removal is 5 and 3.25 for complete denitrification and denitritation, respectively (Mccullough et al., 2022). Hence, it can be concluded that not only the C:N ratio of the A-stage effluent affects the B-stage performance but also the fractionation of the effluent COD. Moreover, since the first step of nitrite shunt is an aerobic process (partial nitrification), a big portion (if not all) of the rbCOD would be oxidized by aerobic heterotrophic bacteria before it can be used be denitrifies in the second step. In fact, in the previous study, to calculate the C:N ratio required for nitrogen removal it was assumed that no COD oxidation is occurring. Hence, in practice, these values would be much higher and depend on the efficiency of the nitrogen removal process in minimizing COD utilization through oxidation.

A key solution to reduce COD oxidation and increase COD utilization for denitrification is simultaneous nitrification denitrification (SND). It has been reported that at low DO levels (0.2-0.7 mg/L), SND can take place in the aerobic phase (Jimenez et al., 2011). However, for SND to occur, an available substrate for denitrifies should be present (rbCOD) in the aerobic phase which can come from (i) rbCOD present in the A-stage effluent which has not been oxidized by aerobic heterotrophic bacteria, (ii) the hydrolysis of slowly biodegradable COD (sbCOD), (iii) endogenous decay products, and (iv) internal storage products (Bernat and Wojnowska-Baryła, 2007; Mino et al., n.d.; Tsuneda et al., 2006; van Loosdrecht et al., 1997; Van Loosdrecht and Henze, 1999; Zeng et al., 2003a). Thus, it has been hypothesized that sbCOD in the B-stage influent might be more beneficial for nitrite shunt process utilizing SND (Klaus et al., 2020). However, despite SND role in reducing COD utilization through oxidation, it was reported in a SBR study that SND was able to achieve a N removal of only 50% at a C:N of 6 and a C:N ratio of 12 was required to reach 80% removal. Here, it is worthmentionning that in this study conventional SND (via nitrate pathway) was taking place which explains the high C:N required. The previous results were in agreement with the reports suggesting a C:N ranging from 6-10 is required for complete denitrification in conventional SND. However, applying SND via nitrite pathway should result in higher nitrogen removal at lower C:N ratios.

Therefore, the objective of this study is to test the application of nitrite shunt in mainstream lines as a B-stage system in the A/B scheme using low C:N ratio. The experiment will be operated in a SBR using continuous aeration since it is commonly more practical for full-scale plants to implement compared to intermittent aeration. An anoxic zone will be introduced in the SBR cycle before the aerobic phase to provide denitrifying bacteria an advantage for utilizing any remaining rbCOD from the A-stage effluent as an electron donor to reduce any oxidized nitrogen compound before its oxidation by aerobic heterotrophs in the subsequent aerobic phase. The aerobic phase will be operated at low DO concentrations to allow SND to occur which would add an additional pressure on NOB through the consumption of nitrite by ordinary heterotrophs for denitritation. In order to achieve higher ammonia oxidation rates, the SBR will be seeded with a previously adapted to low DO nitrifying sludge in which $K_{o,AOB}$ was much lower than $K_{o,NOB}$. Moreover, the effect of influent C:N ratio and COD fractionation on total inorganic nitrogen (TIN) removal will be evaluated through operating the SBR at 3 different scenarios. Furthermore, the effect of low DO on AOB and NOB at the different C:N ratios and COD fractionation will be monitored using batch kinetic tests.

3.2. Materials and Methods

3.2.1. Experimental Setup and operation

The experiment was operated in an SBR with a working volume of 5L. The SBR had a total cycle duration of 4 h which was divided to 5 mins of feeding, 60 mins anoxic reaction, 140 mins aerobic reaction, 30 mins of settling, and 5 mins of wastage. The flow rates of feeding and decanting were controlled by peristaltic pumps. The experiment was controlled and monitored using a control device (Biostat® A Benchtop Fermenter & Bioreactor, Goettingen, Germany). The SBR was equipped with a variable speed mixer and sensors to monitor DO, pH, and temperature which can be controlled using the control device. The sensors were connected to a programmable logic controller (PLC) and then used for proportion-integral-derivative (PID) or PI control.

The SBR was operated for 140 days including three successive phases. The operational condition for each phase is shown in **Table 3.1**. The DO concentration in the aerobic phase was controlled at 0.2 mg/L through a PI controller in which DO controls the air flowrate via a mass flowrate controller (MFC). The hydraulic retention time (HRT) was controlled at 6 h. The total SRT was targeted at around 6 days and was controlled by discharging excess sludge from the bottom of the SBR during the settling time. During the entire study, the SBR was operated at ambient temperature 20 ± 2 ºC and a pH of 7.2-7.6. The experimental purpose of Phase I and Phase II was to investigate the effect of soluble COD (sCOD) on nitrite shunt at different C:N ratios. Whereas Phase III aimed at exploring the effect of particulate COD (pCOD) on nitrite shunt.

Table 3.1: Detailed operational conditions during the different phases of operation

Phase	Total SRT (Days)	Aerobic SRT (Days)	VSS (mg/L)	TSS (mg/L)	VSS/TSS (g/g)	Aerobic Phase DO (mg/L)
I	5.9 ± 0.5	3.5 ± 0.3	1294 ± 359	1415 ± 373	0.91 ± 0.03	0.2
II	6.2 ± 0.4	3.6 ± 0.2	1517 ± 336	1668 ± 348	0.91 ± 0.02	0.2
III	6.3 ± 0.7	3.7 ± 0.4	2048 ± 153	2228 ± 167	0.92 ± 0.01	0.2

3.2.2. Seed sludge and synthetic feed

The SBR was inoculated with a previously adapted nitrifying sludge to low DO. The seed sludge was enriched in an SBR fed with synthetic wastewater devoid of any carbon source through a kinetic-adaptation strategy which allowed for a high AOB dominance compared to NOB at mainstream conditions. The relative abundance of the dominant AOB species in the seed sludge was higher than that of NOB by 11 times. Moreover, the enriched sludge had a $K_{o,AOB}$ of 0.15 mg/L and $K_{o,NOB}$ of 0.3 mg/L.

The SBR was continuously fed with synthetic wastewater. The feeding solution was prepared in a daily basis using deionized water combined with concentrated stock solutions of ammonium chloride as the source of ammonia, sodium carbonate for alkalinity, sodium acetate as the source of sCOD, and Milk powder, starch, and yeast as the source of pCOD. The trace concentrated stock solutions (1mL/1000mL) contained 990 mg $MnCl_2 \cdot 4H_2O$/L, 500 mg $FeSo_4 \cdot 7H_2O$/L, 430 mg $ZnSo_4 \cdot 7H_2O$/L and the mineral salt stock solution contained 190 mg $NiCl_2 \cdot 6H_2O$/L, 220 mg $Na_2MoO_4 \cdot 2H_2O$/L, 250 mg $CuSo_4 \cdot 5H_2O$/L, 240 mg $CoCl_2 \cdot 6H_2O$/L, 210 mg $MnCl_2 \cdot 4H_2O$/L, 19 mg $H_3BO_4 \cdot 7H_2O$/L, and 5 g EDTA/L. The detailed description of the synthetic wastewater composition is listed in **Table 3.2**.

Table 3.2: The characteristics of the synthetic wastewater during the 3 phases of operation

Phase	**Time (Days)**	**Ammonia conc. (mg N/L)**	**sCOD (mg/L)**	**TCOD (mg/L)**	**C:N (-)**	**sCOD/TCOD (%)**
I	1-42	40.9 ± 1.5	126 ± 12	126 ± 12	3.1 ± 0.2	100
II	43-84	40.2 ± 0.9	242 ± 8	242 ± 8	6.0 ± 0.2	100
III	85-140	39.7 ± 0.8	118 ± 9	238 ± 11	6.1 ± 0.3	49 ± 2.8

3.2.3. AOB and NOB activity measurements

In order to monitor AOB and NOB maximum activities rate, a Specific nitrification rate (SNR) batch test was performed every 2 weeks. The test was conducted by collecting a 2 L sample from the SBR during the aeration phase. The sample was then washed two times with synthetic wastewater and centrifuged. The supernatant was discharged, and the settled sludge was transferred to a 2L batch reactor. The batch reactor was then aerated for 30 mins to ensure all excess COD (if available) has been oxidized. Afterwards, a synthetic stock solution was added to the reactor to reach an initial ammonia concentration of 30-40 mg NH_4-N/L and an initial nitrite concentration of 15-20 mg NO_2-N/L. DO concentrations were maintained at concentrations above 4 mg/L using an air pump and a diffuser to ensure that no oxygen limitation is inhibiting AOB and NOB activity. DO concentrations were monitored using a DO probe (Hach LDO, CO). The test was conducted until all the ammonia was oxidized and samples were taken every 15 mins and analyzed for ammonia, nitrite, nitrate, TSS, and VSS. The slopes of the linear responses of NO_x-N (NO_3-N + NO_2-N) production and of NO_3-N production were calculated. The AOB rates (mg N/g VSS/h) were then calculated by dividing the NO_x-N production slope by the batch test average VSS concentrations, and the NOB rates (mg N/g VSS/h) were calculated by dividing the NO_3-N production slope by the batch test average VSS concentrations.

3.2.4. Denitrification rates measurements

In order to monitor denitrification rates during the different phases of operation, a batch test was performed every 2 weeks. The test was conducted by collecting a 2 L sample from the SBR during the anoxic phase. The sample was then washed two times with synthetic wastewater and centrifuged. The supernatant was discharged, and the settled sludge was transferred to a 2L anoxic, sealed batch reactor. Prior to the start of the test, N_2 gas was purged in the batch reactor until the reactor became anoxic as measured by the DO probe. Afterwards, a synthetic stock solution was added to the reactor to reach an initial nitrate concentration of 20-30 mg NO_3-N/L and an initial nitrite concentration of 15-20 mg NO_2-N/L. Acetate was added in excess to the synthetic solution to ensure that no COD limitation is inhibiting denitrification. The test was conducted for 2 hours, and samples were taken every 15 mins and analyzed for sCOD, ammonia nitrite, nitrate, TSS, and VSS. The slopes of the linear responses of NO_x-N (NO_3-N + NO_2-N) consumption and of NO_3-N consumption were calculated. The NO_2-N denitrification rates which indicate the reduction rate from nitrite to nitrogen gas (mg N/g VSS/h) were then calculated by dividing the NO_x-N consumption slope by the batch test average VSS concentrations, as shown in Eq. (3.1). Whereas the NO_3-N denitrification rates which indicates the reduction rate from nitrate to nitrite (mg N/g

VSS/h) were calculated by dividing the NO_3-N consumption slope by the batch test average VSS as shown in Eq. (3.2)

$$\boldsymbol{NO_2 - N\ den.rate}\ \left(\frac{\boldsymbol{mg\ N}}{\boldsymbol{g\ VSS\ .\ d}}\right) = -\frac{\boldsymbol{r_{NO_2} + r_{NO_3}}}{\boldsymbol{VSS_{avg.}}} \qquad \textbf{(3.1)}$$

$$\boldsymbol{NO_3 - N\ den.rate}\ \left(\frac{\boldsymbol{mg\ N}}{\boldsymbol{g\ VSS\ .\ d}}\right) = -\frac{\boldsymbol{r_{NO_3}}}{\boldsymbol{VSS_{avg.}}} \qquad \textbf{(3.2)}$$

Where $\boldsymbol{r_{NO_2}}$ is the nitrite rate measured in the batch test and calculated as the change in NO_2 over time, $\boldsymbol{r_{NO_3}}$ is the nitrate rate measured in the batch test and calculated as the change in NO_3 over time, and $\boldsymbol{VSS_{avg.}}$ is the average VSS in the batch reactor during the test period.

3.2.5. DNA extraction and amplicon sequencing

Three samples were taken from the SBR at each phase of operation to monitor the change in the microbial community dynamics. The DNA was extracted from the biomass samples using the DNeasy PowerSoil Pro Kit (Qiagen) following the protocol provided by the manufacturer. The quality and quantity of the extracted DNA were checked by gel electrophoresis and spectrophotometer analysis (NanoDrop Technologies, Wilmington, DE, USA), respectively.

Amplicon sequencing of the extracted DNA was conducted using Illumina MiSeq sequencer (Illumina Incorporated, San Diego CA). Firstly, the 16S rRNA gene library was prepared following the workflow outlined by Illumina. The Illumina adapted primers 341F (5’-CCTACGGGNGGCWGCAG-3’) and 806R (5’-GGACTACNVGGGTWTCTAAT-3’) were used to amplify the V3-V4 regions of the 16S rRNA genes, through polymerase chain reaction (PCR). The obtained PCR products visualized on a 1.5% agarose gel. Afterwards, positive amplicons were normalized by eye, pooled together in equimolar concentrations, and sequenced on the Illumina

MiSeq sequencer (Illumina Incorporated, San Diego CA) in paired-end mode according to manufacturer's protocol.

Raw reads were processed using DADA2 (Callahan et al., 2016). First, Cutadapt was used to filter and trim adapter sequences and PCR primers from the raw reads with a minimum quality score of 30 and a minimum read length of 100bp (Martin, 2011). Sequence variants were then resolved from the trimmed raw reads using DADA2, an accurate sample inference pipeline from 16S amplicon data. The DNA sequence reads were filtered and trimmed based on the quality of the reads, error rates were learned, and sequence variants were determined by DADA2. Bimeras were removed and taxonomy was assigned using the RDP classifier against the SILVA database version 1.3.8.

3.2.6. Analytical methods

Influent and effluent samples were collected 3 days a week, and analyzed for ammonia, nitrate, and nitrite using HACH TNTplus kits and a HACH DR3900 spectrophotometer (HACH Loveland, CO). Total and soluble COD were measured using HACH testing kits, a HACH DRB200 digital digester, and a HACH DR3900 spectrophotometer (HACH, Loveland, CO, USA). Samples from inside the reactor and from the effluent were collected twice a week and analyzed for total and volatile suspended solids (TSS and VSS) using standard methods 2540D and 2540E, respectively (APHA, 2012). Nutrient and sCOD samples were filtered through 0.45 μm and 1.5 μm filters, respectively. Temperature, pH, and DO were monitored using online sensors equipped in the SBR.

3.2.7. Calculations

The ammonia removal efficiency (ARE) and nitrite accumulation rate (NAR) were calculated according to Eq. (3.3) and Eq. (3.4) as follows:

$$\boldsymbol{ARE}\ (\%) = \frac{(NH_3-N)_{inf} - (NH_3-N)_{eff}}{(NH_3-N)_{inf}}\ \boldsymbol{X\ 100} \tag{3.3}$$

$$\boldsymbol{NAR}\ (\%) = \frac{(NO_2-N)_{eff}}{(NO_2-N)_{eff} + (NO_3-N)_{eff}}\ \boldsymbol{X\ 100} \tag{3.4}$$

The TIN removal efficiency (%) and COD/TIN removal ratio (g/g) were calculated according to Eq. (3.5) and Eq. (3.6) as follows:

$$\boldsymbol{TIN\ removal\ efficiency}\ (\%) = \frac{(NH_4-N)_{inf} - [(NH_4-N)_{eff} + (NO_3-N)_{eff} + (NO_2-N)_{eff}]}{(NH_4-N)_{inf}}\ \boldsymbol{x\ 100} \tag{3.5}$$

$$\boldsymbol{COD/TIN\ removal\ ratio}\ \left(\frac{g}{g}\right) = \frac{(TCOD)_{inf} - (TCOD)_{eff}}{(NH_4-N)_{inf} - [(NH_4-N)_{eff} + (NO_3-N)_{eff} + (NO_2-N)_{eff}]} \tag{3.6}$$

The Simultaneous Nitrification Denitrification (SND) rate (mg N/L) was calculated as the total inorganic nitrogen (TIN) removed across the aerobic phase in the typical SBR cycle as shown in Eq. (3.7)

$$\boldsymbol{SND}\ \left(\frac{mg\ N}{L}\right) = \boldsymbol{TIN}_{end\ of\ anoxic\ phase} - \boldsymbol{TIN}_{end\ of\ aerbic\ phase} \tag{3.7}$$

3.3. Results and Discussion

The objective of Phase I and Phase II was to investigate the effect of sCOD at different C:N ratio on nitrite shunt. Thus, the SBR feed in those 2 phases contained only sCOD as the sole source of organic carbon. The sCOD concentrations in Phases I and II were 126 ± 12 and 242 ± 8 mg/L which corresponded to a C:N of 3.1 ± 0.2 and 6.0 ± 0.2, respectively. On the other hand, Phase III aimed at exploring the effect of pCOD. Hence, in this phase, pCOD in the form of milk powder, starch, and yeast was introduced in the feed. To build a valid comparison, total COD was kept at the range of 238 ± 11 to maintain a similar C:N ratio as that of Phase II. The sCOD in Phase III represented a 49 ± 2.8% of the TCOD which is close to the reported fractionation of A-stage effluent (Klaus and Bott, 2020; Rahman et al., 2019; Regmi et al., 2014).

3.3.1. Overall SBR performance

As described in the material and methods section, the SBR was inoculated with nitrifying sludge from an SBR which was operated for 410 days with synthetic wastewater devoid of any organic carbon sources which resulted in a poor COD and TIN removal in the first couple of days operation as shown in **Figure 3.1**. However, afterwards, a gradual increase in both COD and TIN removal was observed from 6.8% and 3.8% after the first day to 73.5% and 85.1% at the 10^{th} day, respectively. The previous increase in SBR performance was accompanied by an increase in the VSS and TSS from 472 and 563 mg/L to 1039 and 1156 mg/L, respectively after 10 days of operation, as illustrated in **Figure 3.2**. The improved COD and TIN removal and solids increase can be referred to the enrichment of heterotrophic bacteria due to the presence of organic carbon. On the other hand, a reverse trend was observed for ammonia removal efficiency (ARE) resulting in higher effluent ammonia concentrations which may be explained by the competition between the newly enriched heterotrophs and nitrifiers for oxygen. However, after 4 weeks of operation under the same conditions, high ARE were restored implying AOB adaptability. The adaptation of AOB at low DO and the increase in its activity at the same operational conditions have been previously reported in several studies in the literature (Blackburne et al., 2008; Keene et al., 2017). Overall, the SBR was able to achieve an average ARE, COD removal, and TIN removal of 96.1 ± 4.2, 92.2 ± 2.4, and 73.4 ± 2.7%, respectively at a C:N ratio of 3.1 ± 0.2.

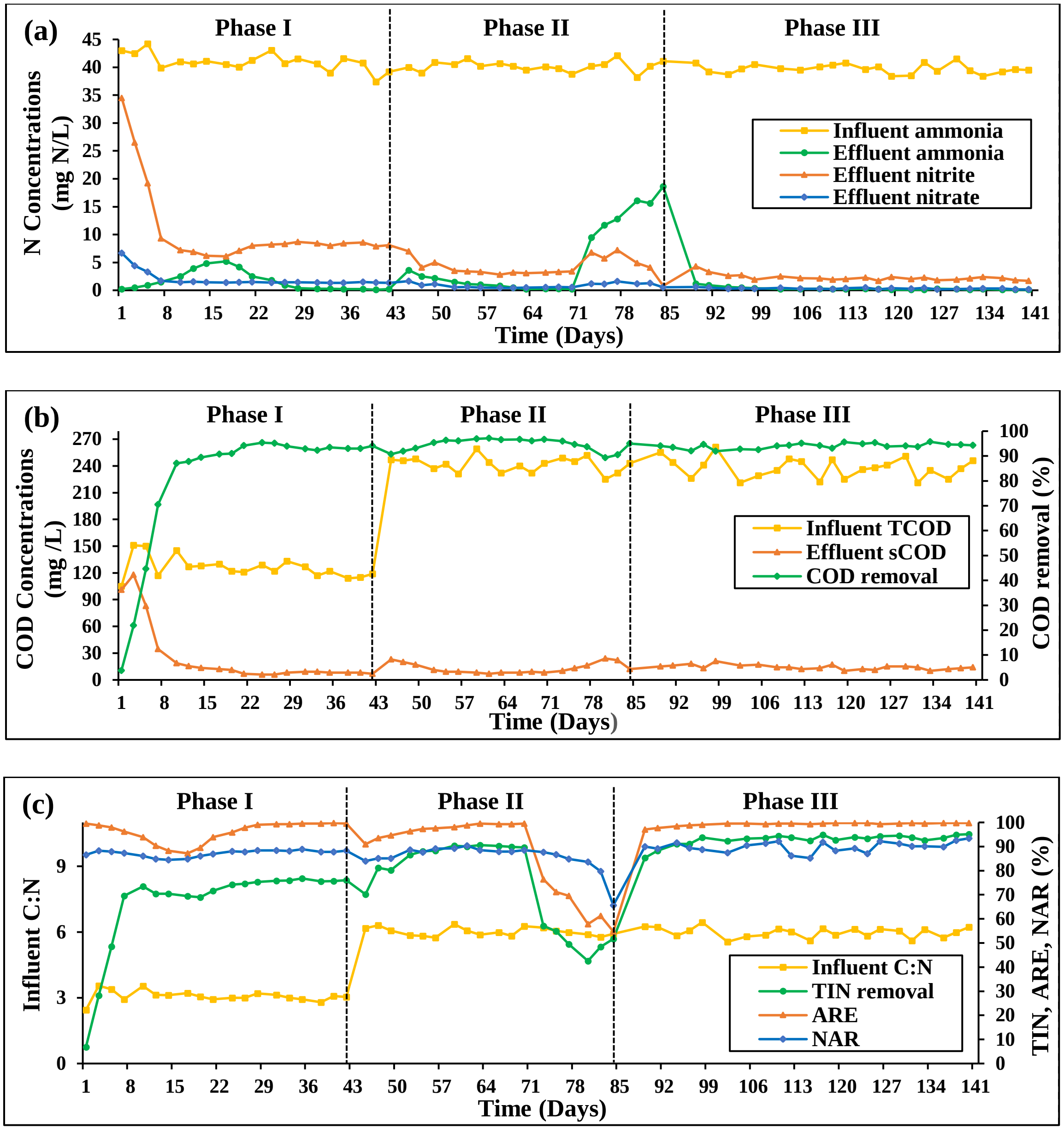

Figure 3.1: SBR performance during the different phases of operation: (a) Influent ammonia, effluent ammonia, effluent nitrite, and effluent nitrate concentrations, (b) Influent TCOD and effluent sCOD concentrations and COD removal efficiencies, and (c) Influent C:N, TIN removal efficiency, ammonia removal efficiency (ARE), and nitrite accumulation rate (NAR)

Although the theoretical COD requirements for denitritation and complete denitrification are 1.71 and 2.82 g COD/g N, respectively, the SBR was not able to completely denitrify all the oxidized nitrogen compound at a C:N of 3. However, as described previously, the theoretical values assume that no COD is being utilized by aerobic heterotrophs which is possible in anoxic/anaerobic conditions but impractical in aerobic zones. As shown in **Figure 3.3a**, in the anoxic phase, all the oxidized nitrogen from the previous cycle was completely denitrified by heterotrophic denitrifies using the influent sCOD as an electron donor. However, at the start of the aerobic phase, a big portion of the influent COD was oxidized by aerobic heterotrophs since they have a clear advantage for soluble substrate which was the sole source of carbon in this phase. By the end of this phase, ammonia, nitrite, nitrate concentrations in the effluent were 0.2, 8.2, 1.2 mg N/L, respectively, corresponding to a TIN of 8.6 mg N/L which is still considered high for stringent discharge limits.

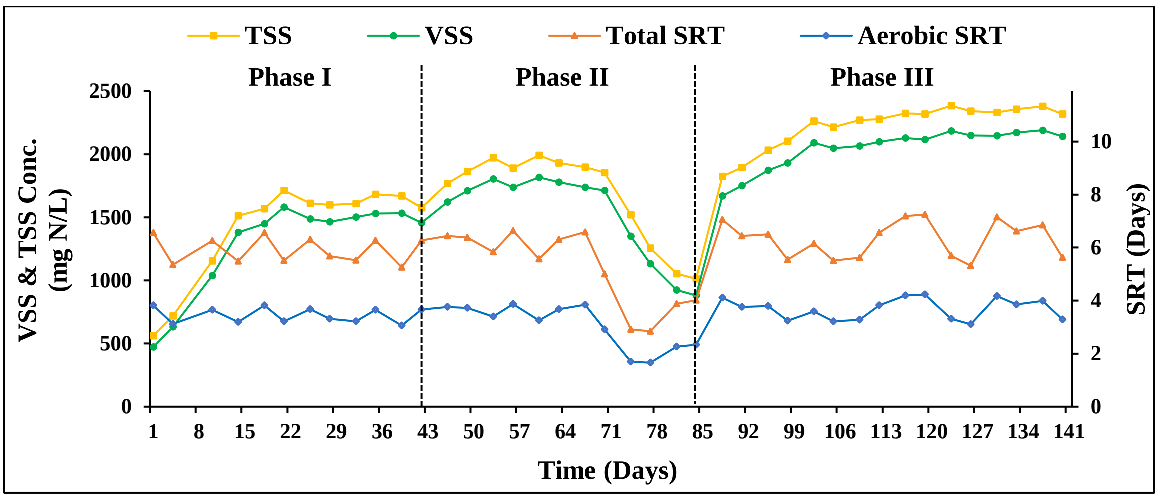

Figure 3.2: The SBR total suspended solids (TSS) concentrations, volatile suspended solids (VSS) concentrations, total SRT, and aerobic SRT variations during the different phases of operation

In the following phase, the influent COD was increased to reach a C:N of 6 but still only soluble COD was provided in the feed. Similar to the last phase, this increase in influent COD resulted in a slight increase in VSS and TSS concentrations which was accompanied by a drop in the ARE during the first couple days of operation. However, after a period of operation, AOB was able to restore its activity and improved ARE was observed until it reached back 97%. The increase in the feed COD resulted in an increased TIN removal of up to 90%. However, complete nitrogen removal was not attained during this phase despite the increase in C:N ratio which is attributed to COD oxidation during the aerobic phase. Approaching the end of the phase, a severe drop in VSS and TSS was observed which resulted in a drastic drop in ARE and TIN removal. The deterioration in performance can be referred to the poor settleability observed during the settling phase which was supported by the high SVI_{30} values (577 ± 21 mL/g) observed. Such a significantly high SVI may be attributed to the proliferation of filamentous bacteria (sludge bulking) which was reported to occur at SVI_{30} above 120 mL/g (Henze et al., 2008; Metcalf & Eddy, 2013). Interestingly, during this period of operation, an error in sludge wastage calculations resulted in lower SRT in the SBR of 3.41 ± 0.54 and 1.98 ± 0.54 for total and aerobic SRT, respectively. Thus, it can be hypothesized that the combination of low SRT and low DO might have resulted in the proliferation of filamentous bacteria which have been reported to occur in such conditions (Henze et al., 2008). However, further investigation should be performed to confirm whether this poor settleability was due to the drop in SRT or due to the presence of such high soluble COD concentrations. In phase II (excluding the period of data disruption by the poor settleability), an average ARE, COD removal, and TIN removal of 97.1 ± 2.6, 95.3 ± 2.1, and 86.3 ± 5.9. Hence, it can be concluded that doubling the influent C:N resulted in an increase of only 18% in TIN removal. Such a low increase percentage can be attributed to the competition between aerobic heterotrophs and denitrifies for COD in the aerobic phase. Moreover, since rbCOD (acetate) was the only source of COD in this phase, it might have provided aerobic heterotrophs an advantage to utilize it for oxidation. Interestingly, ARE was not affected by the increase in COD concentrations which implies that AOB was able to maintain its ability to compete with aerobic heterotrophs for oxygen despite the low DO concentrations. It can be hypothesized that the previous adaptation of AOB to low DO and its low K_o played an important role in its ability to maintain its high activity.

Additionally, a high COD removal was attained in this phase despite the increase in influent COD concentrations which is attributed to the presence of COD in its simplest form in the feed (rbCOD).

In Phase III, a C:N ratio of 6 was maintained, however pCOD was introduced in the feed to investigate its effect on the SBR performance. Thus, the sCOD/TCOD in this phase was 49.5 ± 2.8% compared to 100% in Phase II. The introduction of pCOD in the feed resulted in an 8% increase in TIN removal despite maintaining the same influent TCOD concentrations. It is hypothesized that pCOD is not readily available for aerobic heterotrophs consumption at the beginning of the aeration phase since prior hydrolysis is required. Thus, pCOD hydrolysis allows more time for ammonia conversion to oxidized nitrogen compounds (nitrate and nitrite) and consequently provides denitrifies an advantage for utilizing COD further downstream in the aeration phase. This observation was in accordance with the previously reported data in the literature. Klaus et al. 2020 suggested that pCOD can be more beneficial for denitrifies than rbCOD in B-stage and that it should lead to increased TIN removal (Klaus et al., 2020). Moreover, the introduction of pCOD resulted in a slight drop in COD removal, however still a high COD removal efficiency of 94 ± 1% was maintained during this phase. Such a drop can be explained by the added complexity in the form of COD source (pCOD) compared to the last phase where only acetate (rbCOD) was used as carbon source. Interestingly, no significant drop in ARE was observed in this phase unlike the last 2 phases which implies that AOB activity was not affected by the introduction of pCOD. Contrarily, ARE was improved during phase III and reached an average of 99.2 ± 0.7% compared to 97.1 ± 2.6% in Phase II.

Overall, the SBR in Phase III was able to maintain an average ARE, TIN removal and COD removal of 99.2 ± 0.7, 93.2 ± 1.6, and 94.0 ± 0.1%, respectively. The effluent ammonia, nitrite, and nitrate concentrations were as low as 0.3 ± 0.2, 2.3 ± 0.6, 0.3 ± 0.1 mg N/L which corresponds to a TIN concentrations of 2.8 ± 0.9 mg N/L which is an acceptable value according to the stringent discharge limits. The influent wastewater characteristics in this phase were 238 ± 11 mg/L, 118 ± 9 mg/L, and 40.2 ± 0.9 mg N/L for TCOD, sCOD, and NH_4-N corresponding to a C:N ratio of 6.1 ± 0.3 which is close to the reported effluent quality of A-stage systems (Liu et al., 2018; Rahman et al., 2019). As shown in **Table 3.3**, a similar C:N ratio was applied in the James R. Dilorio Water Reclamation Facility (JD WRF), a full-scale plant configured as a modified Johannesburg process (anaerobic/anoxic/aerobic) (Regmi et al., 2022a). The aerobic CSTRs were operated at low DO concentrations of 0.37 ± 0.27 mg/L using continuous aeration controlled by Ammonia vs. NO_x (AVN). The TIN removal achieved in JD WRF was 76% which is lower than the TIN achieved at this study of 93.2 ± 1.6% despite the presence of an internal mixed liquor recycle (IMLR) system and operating at the same C:N ratio. A possible explanation for the lower TIN removal is that it was reported that there was no NOB inhibition in the full-scale plant which indicates that nitrogen removal occurred through nitrate pathway unlike this study where a NAR of 89.9 ± 2.1% was achieved. Similarly, it was reported in another study performed at low DO concentrations (0.3 mg/L) that the absence of NOB out-selection resulted in a TIN removal of 86 ± 2.9% at a C:N ratio as high as 12.6 (Klaus and Bott, 2020). Thus, it can be concluded that NOB out-selection is crucial to achieve high TIN removal at low C:N ratio since less COD is required for nitrite denitrification compared to nitrate. In agreement, it was reported in a plug flow reactor configured as anaerobic/aerobic/anoxic/aerobic (AOAO) that an increase in NAR from 4.0 ± 1.5% to 89.3 ± 3.3% resulted in an increase in TIN removal from 73.9 ± 4.1% to 93.7 ± 2.2% despite a slight decrease in C:N ratio (Feng et al., 2021). On the other hand, at the Southwest Water Reclamation Facility (WRF), a NAR of 64% resulted in a TIN removal of 91.3% at a C:N ratio as low as 7 (Jimenez et al., 2020). In this full-scale plant, nutrient-rich side stream generated from dewatering anaerobically digested waste activated sludge was returned to the mainstream which might have resulted in the high NAR observed combined with the relatively high temperature (26.5 ºC) and low aerobic SRT (3.7 ± 0.4 days). Furthermore, in a CSTR intermittent aeration study, a C:N of 12.3 ± 0.9 was required to achieve a TIN removal of 89 ± 11% despite achieving a high NAR of 60 ± 22. However, it is worth noting that in the previous study, DO at the aerobic phase was controlled at high levels of 1.6 mg/L which might have resulted in higher COD loss through oxidation since SND is not expected at this value.

In addition, in the present study, despite the low DO concentrations, the SBR was able to maintain a high ARE of 99.2 ± 0.7% at a short total and aerobic SRT of 6.3 ± 0.7 and 3.7 ± 0.4 days, respectively which was lower than those of the other literature studies as shown in Table 3. A factor that might have contributed to such a high ARE at short SRT is the previous adaptation of the nitrifying sludge to the low DO. In agreement, it was suggested in the literature that to achieve higher ammonia removal rates at lower DO setpoints, either the SRT needs to be increased and/or the nitrifying community to be adapted to low DO concentrations (Giraldo et al., 2012; Keene et al., 2017; Klaus and Bott, 2020; H. D. Park et al., 2002).

Table 3. 3: Comparison of operational conditions, influent characteristics, and performance between mainstream nitrite shunt studies

Reference	(Regmi et al., 2022)	(Klaus and Bott, 2020)	(Klaus and Bott, 2020	(Jimenez et al., 2020)	(Regmi et al., 2014)	This Study
Operational Parameters						
Reactor configuration	CSTR A2O	CSTR MLE	CSTR A2O	CSTR A/O	CSTR	SBR A/O
Aeration pattern	Continuous AvN	Intermittent AvN	Continuous DO control	Continuous DO control	Intermittent AvN	Continuous DO control
Temperature (ºC)	-	-	-	25 ± 2.5	25	20 ± 2
DO (mg/L)	0.37 ± 0.27	1.5	0.3	0.1-0.4	1.6	0.2
TSS (mg/L)	2.2 ± 0.4	3.1 ± 0.3	4.4 ± 0.2	3.3	3.9 ± 0.4	2.2 ± 0.2
Total SRT (Days)	10.1 ± 1.6	18.2 ± 1.6	22.1 ± 2.6	5.2 ± 1.5	4.8 ± 1.4	6.3 ± 0.7
Aerobic SRT (Days)	7.7 ± 1.4	-	-	3.9 ±1.1	3.2	3.7 ± 0.4
HRT (h)	-	11	11	6	3	6
Influent characteristics						
TCOD (mg/L)	292.5	478 ± 34	452 ± 83	400 ± 65	306 ± 87	238 ± 11
sCOD (mg/L)	-	236 ± 52	221 ± 50	120 ± 20	-	118 ± 9
sCOD/TCOD (%)	-	49.4	48.9	30	-	49 ± 2.8
TKN (mg/L)	-	40.8 ± 2.9	40.8 ± 2.9	30 ± 5.6	-	40.2 ± 0.9
NH_4-N (mg/L)	48.8	35.0 ± 1.2	35.8 ± 2.5	23 ± 4.5	29.7 ± 3.9	40.2 ± 0.9
COD/NH_4-N (-)	6.0 ± 1.6	13.6 ± 0.9	12.6	7	12.3 ± 0.9	6.1 ± 0.3
Performance						
TIN (%)	76	89.4 ± 2.1	86.0 ± 2.9	91.3	89 ± 11	93.2 ± 1.6
ARE (%)	95	> 97	> 97	92.2		99.2 ± 0.7
NAR (%)	-	< 5	< 5	64	60 ± 22	89.9 ± 2.1
Effluent characteristics						
NH_4-N (mg/L)	2.6	0.37 ± 0.49	0.37 ± 0.49	1.8	-	0.3 ± 0.2
NO_3-N (mg/L)				0.41	-	0.3 ± 0.1
NO_2-N (mg/L)		0.07 ± 0.07	0.07 ± 0.07	0.23	-	2.3 ± 0.6
TIN (mg/L)	11.7	3.7	5	2.44	3.3	2.8 ± 0.9

CSTR: Continuously stirred tank reactor. SBR: Sequential batch reactor. A2O: Anaerobic/anoxic/aerobic. MLE: Modified Ludzack Ettinger. A/O: Anaerobic/Aerobic. AvN: Ammonia vs. NO_x control.

3.3.2. NOB out-selection in the SBR

In this study, nitrite accumulation rate in the effluent (NAR) and ex-situ AOB and NOB maximum activity tests were used as indicators for NOB out-selection. As explained in the materials and methods, every 2 weeks a batch test was performed to monitor AOB and NOB maximum activity (**Figure 3.3a**). Dold et al. 2015 demonstrated that in a full nitrification system where all the ammonia is oxidized to nitrate (no NOB inhibition), the ratio between NOB maximum activity to AOB maximum activity (NOB/AOB rate) should be around 0.78 (Dold et al., 2015). Moreover, it was reported that lower NOB/AOB ratio (0.2-0.3) should indicate that NOB activity is suppressed and nitrite shunt is taking place in the system (Jimenez et al., 2020). Thus, the average NOB/AOB rate was calculated for each phase, as illustrated in **Figure 3.3b**.

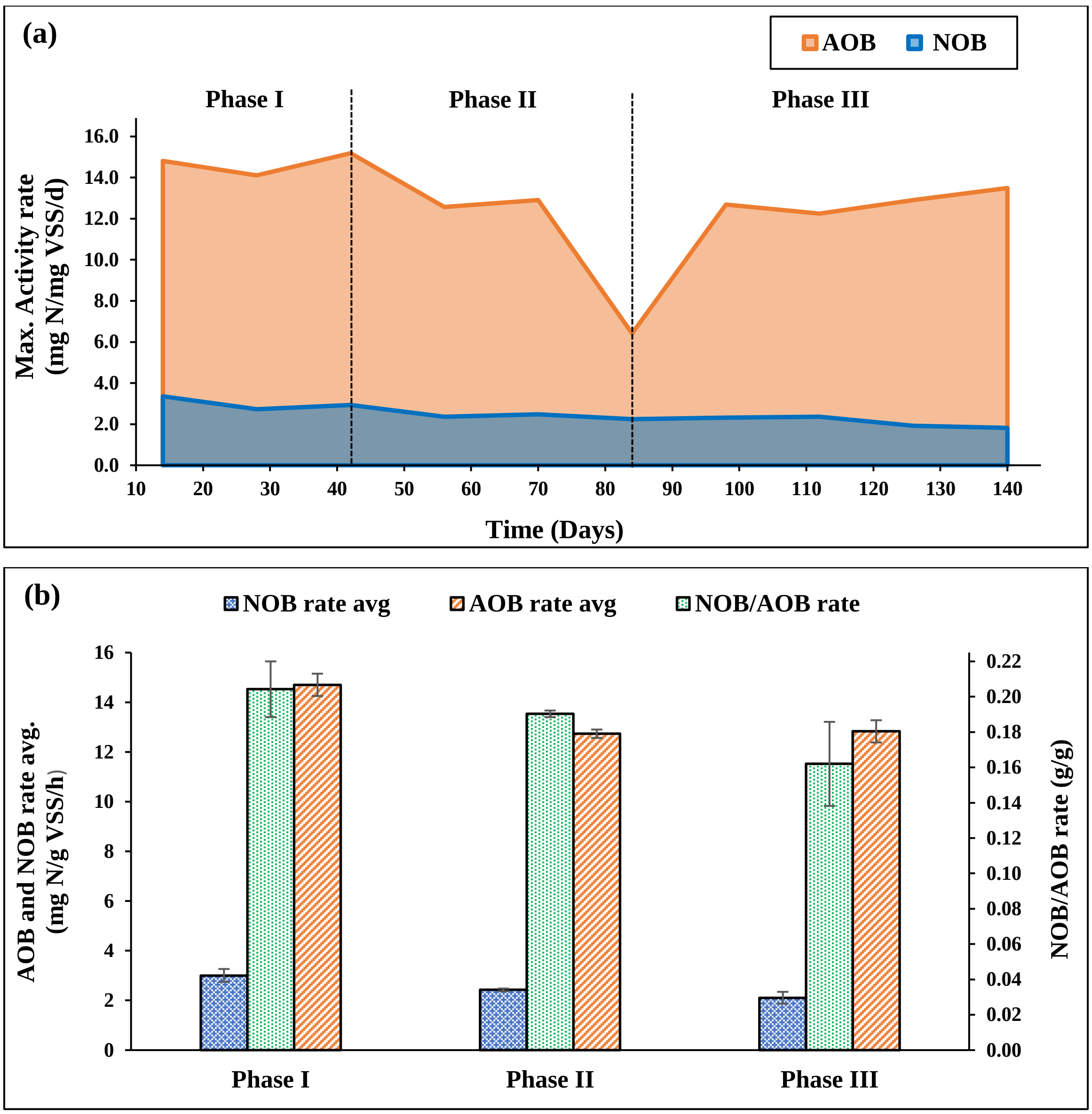

Figure 3.3: (a) variations in AOB and NOB maximum activities, and (b) Comparison between the average NOB maximum activity rate, the average AOB maximum activity rate, and the average ratio between NOB/AOB during the different phases of operation rates (Error bars represent standard deviation)

Overall, a high AOB activity was maintained during all 3 phases compared to NOB activity which resulted in average NAR of 88.4 ± 3.4% during the whole period of operation (excluding the period of filamentous bacteria proliferation). The average NOB/AOB ratio over all phases was 0.18 ± 0.03 which is within the range suggested for nitrite shunt occurrence. NOB suppression in this study can be referred to the previous adaptation of the seed sludge, the low DO, and the short SRT. One of the main challenges of operating the system at low DO is maintaining a high AOB activity to achieve complete ammonia oxidation especially when the system is running at low SRT. Therefore, in this study, the system was seeded by a nitrifying sludge previously adapted to low DO in an SBR operated for 410 days with no organic carbon source. AOB maximum activity in the seed sludge was as high as 80 mg N/g VSS/h and its K_o was as low as 0.15 mg/L which indicates its high ability in oxidizing ammonia at low DO concentrations. Moreover, the seed sludge was enriched through a kinetic adaptation strategy which resulted in high dominance of *Nitrosomonas* (AOB dominant species) compared to *Nitrospira* (NOB dominant species). Thus, to maintain such dominance, the SBR was operated at low DO (0.1-0.2 mg/L) and short aerobic SRT of 3.7 ± 0.4 days which is within the reported range of NOB out-selection at 20ºC (Hellinga et al., 1998). It is hypothesized that low DO in the aerobic phase would allow denitrifying heterotrophs to use the nitrite produced by AOB (NOB substrate) as an electron acceptor for denitrification through SND. Consequently, the competition between denitrifying heterotrophs and NOB for nitrite would result in reduced NOB growth which combined with the short SRT could result in NOB washout (Cao et al., 2017; Jimenez et al., 2020). Moreover, a pre-anoxic phase was included in the SBR cycle to benefit from the reported lag in NOB activity following anoxic conditions (transient anoxia) (Gilbert et al., 2014; Malovanyy et al., 2015).

The success of the previous strategy was demonstrated by the ex-situ activity tests which showed that NOB activity was much lower than that of AOB during the different phases of operation. Moreover, the average AOB maximum activity was 13.4 ± 1.0 mg N/g VSS/h during the entire study which is higher than the reported values in the literature. For instance, it was reported that the average AOB maximum activity in a pilot scale operated at low DO concentrations (0.2-0.3 mg/L) was 3.5 ± 0.25 mg N/g VSS/h (Klaus and Bott, 2020). Hence, in the previous study to achieve complete ammonia removal, the system was operated at a SRT as high as 20 days and by consequence no NOB out-selection was observed. The average NOB maximum activity in the pilot scale was 2.85 ± 0.35 mg N/g VSS/h which was slightly higher than NOB maximum activity in this study of 2.5 ± 0.5 mg N/g VSS/h. However, the average NOB/AOB rate in the pilot scale was 0.81 ± 0.05 (close to the reported value of full nitrification system) which was much higher than that achieved in the present study of 0.18 ± 0.03 explaining the difference in the reported NAR between the two studies. A slightly higher AOB maximum activity of 4.1 mg N/g VSS/h was reported in a full-scale study operated at a DO of 0.37 ± 0.27 mg/L (Regmi et al., 2022a). Thus, an ARE of 95% was achieved at a lower SRT of 10.1 ± 1.6 days. However, NOB maximum activity was as high as 4.16 mg N/g VSS/h which implies that no NOB inhibition was taking place leading to nitrite accumulation. In another full-scale study operated at DO concentrations of 0.1-0.4 mg/L, the AOB maximum activity was as low as 0.94 mg N/g VSS/h (Jimenez et al., 2020). In the previous study, the SRT was controlled at 5.2 ± 1.5 days which resulted in effluent ammonia concentrations of 1.8 mg N/L despite the low influent ammonia concentrations of 23 mg N/L and the high solids concentrations in the system. However, the combination of short SRT, low DO and moderately high temperature led to NOB activity suppression. It was reported that NOB/AOB rate was 0.27 (within the reported NOB suppression range) which resulted in NAR of 64% in the effluent. Therefore, it can be concluded that the high AOB maximum activity achieved in this study allowed the system to be operated at low DO levels and short aerobic SRT which led to NOB inhibition and resulted in high NAR.

3.3.3. Effect of C:N ratio and COD fractionation on TIN removal

In an optimal nitrite sunt process, TIN removal occurs through the oxidation of ammonia to nitrite carried out by AOB in aerobic conditions (partial nitrification) followed by the reduction of the produced nitrite to nitrogen gas using COD as an electron donor in anoxic conditions carried out by heterotrophic bacteria (denitritation). However, in practice, the presence of aerobic heterotrophs results in influent COD oxidation challenging the execution of the second denitritation step due to the absence of electron donor if the anoxic phase were to follow the aerobic phases. Thus, in this study, the SBR was operated using a pre-anoxic phase followed by an aerobic reaction phase. While it is expected that influent oxidation takes place in the aerobic phase, there are two possible routes for the denitrification/denitritation of the oxidized nitrogen compounds. The first route is through the denitrification of the remaining nitrate and nitrite from the previous cycle during the anoxic phase using the influent rbCOD. Whereas the second route is the denitrification of the newly oxidized nitrogen compounds during the aerobic phase using any remaining influent rbCOD or the hydrolyzed pCOD through SND. Since the SBR was operated at low DO concentrations of 0.2 mg/L (within the reported DO range for SND), a combination of both routes is expected during all 3 phases. Hence, to develop an understanding about the mechanisms of TIN removal and investigate the effect of C:N ratio and COD fractionation, samples were taken every 20 mins from the SBR during a typical cycle for all 3 phases. The samples were analyzed for ammonia, nitrite, nitrate, sCOD, TSS and VSS as shown in **Figure 3.4**. Moreover, the ratio of COD/TIN removal was computed during the different phases of operation to monitor the efficiency of the influent

COD utilization for TIN removal as illustrated in **Figure 3.5**. Lower COD/TIN removal ratio indicates that a bigger portion of the influent COD was used for denitrification/denitritation, and a lower portion was used through COD oxidation which should result in higher TIN removal.

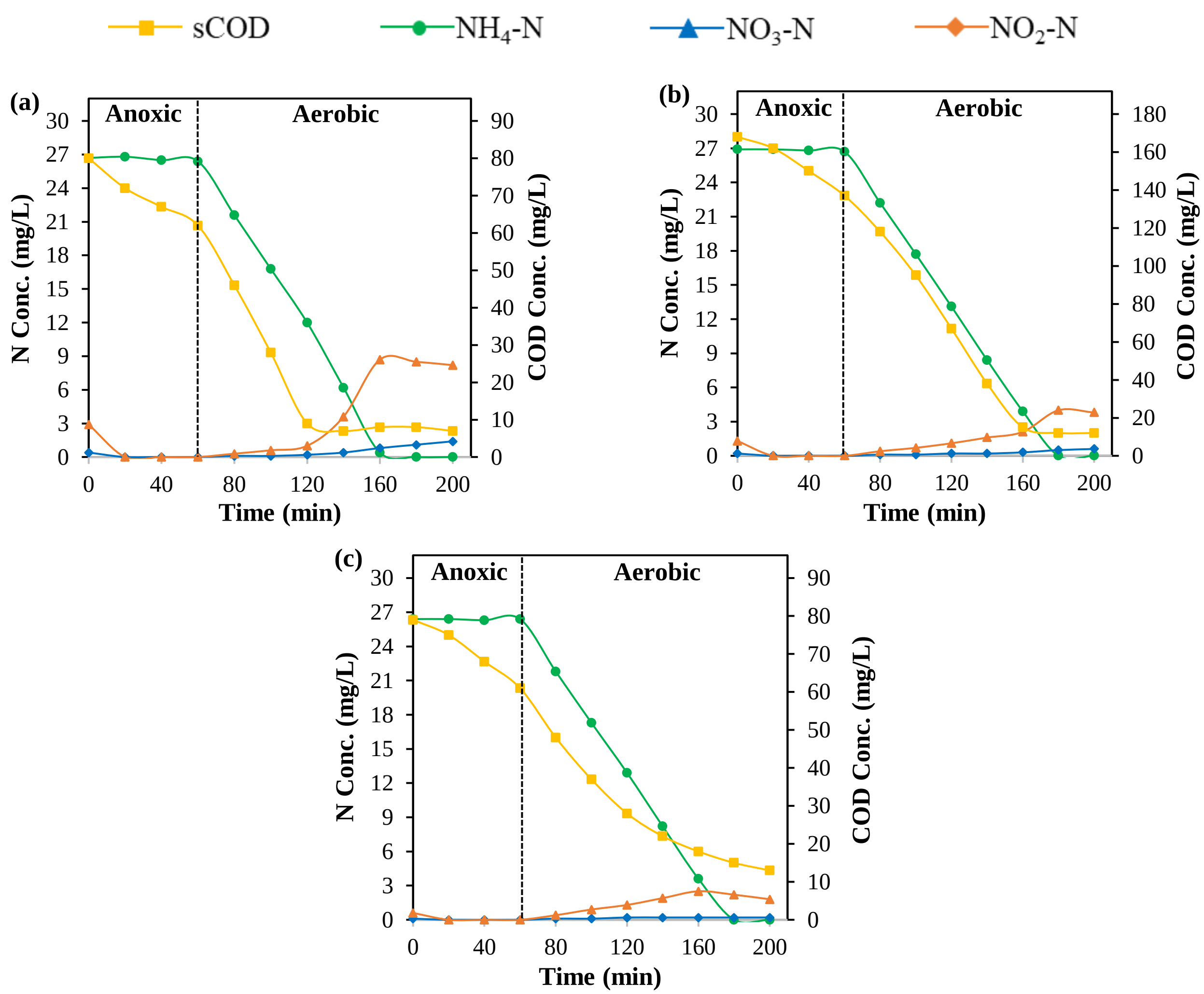

Figure 3.4: Variations of sCOD, ammonia, nitrate, and nitrite concentrations in a typical SBR cycle, (a) Phase I, (b) Phase II, and (c) Phase III

During Phase I, TIN removal was the lowest of an average of 73.4 ± 2.7%. As shown in **Figure 3.4a**, all the remaining nitrite and nitrate concentrations from the previous cycle were denitrified using influent sCOD in the anoxic zone. The previous observation highlights the importance of the pre-anoxic zone in providing the denitrifying biomass with an advantage of utilizing the rbCOD before its oxidation in the following aerobic zone. The samples taken over a typical SBR cycle showed that the pre-anoxic phase was not limited by influent COD and only around 22% of the influent COD was consumed in this phase. Such results suggest that SND was the main route for TIN removal which is expected since the reactor was run in an SBR mode with no internal recirculation, consequently the nitrite and nitrate concentrations in the pre-anoxic phase was limited to those which remains after the wasting phase. Moreover, it was noteworthy that all the COD was utilized before complete ammonia removal which implies that the addition of a post-anoxic zone would not result in an increase in TIN removal. However, here it is worthmentionning that it has been recently suggested in the literature that some heterotrophs might have the ability to internally store carbon during the aerobic zones operated at low DO concentrations and subsequently use it for post anoxic zone for denitrification (Feng et al., 2021; Regmi, 2022). Thus, further investigation should be conducted to test if the addition of a post anoxic phase would result in an improved TIN removal efficiency.

Furthermore, in Phase I, the average COD/TIN removal was 3.6 ± 0.6 as shown in **Figure 3.5**. Compared to the literature the ratio of COD/TIN removal achieved in this phase is significantly low which indicates that COD was used efficiently for denitrification. It was reported in a pilot scale study using different aeration patterns and control, that the lowest COD/TIN removal ratio was 13.6 ± 3.0 in a fully aerobic CSTR operated in intermittent aeration pattern using AvN control which resulted in a TIN removal of 54.4 ± 13.7% (Klaus and Bott, 2020). Moreover, the COD/TIN removal ratio for the CSTR with continuous aeration controlled by Ammonia based aeration control (ABAC) following a Modified Ludzack Ettinger (MLE) configuration was 12 and led to a TIN removal of 80 ± 0.7%. As can be seen from the previous results, the COD/TIN removal ratio achieved in this study was much lower than that achieved in the previous studies. A possible explanation is that the DO concentrations in the SBR was 0.2 mg/L which was lower than the DO in the CSTR of 1.5 mg/L for intermittent aeration and 0.54 mg/L for continuous aeration. Such lower DO concentrations might have provided heterotrophic denitrifies an advantage to compete with the aerobic heterotrophs for COD which resulted in higher SND rates. In fact, the SND in the MLE continuously aerated CSTR was only 3.8 mg/L and represented 14% of the total TIN removal while the SND in phase I in this study was 16.8 mg N/L and represented 55% of the total TIN removed. Furthermore, the lower DO concentrations and the previous adaptation of the nitrifying community in this study resulted in a high NAR of 87.2% unlike the pilot scale study where it was reported that no NOB inhibition was observed. Such high NAR resulted in lower COD requirements for TIN removal since the influent COD was utilized mostly to reduce nitrite to nitrogen gas which corresponded to lower COD/TIN removal. Moreover, another factor that contributed to the lower COD/TIN removal ratio in this phase is the lower influent C:N ratio of 3 which is close to the theoretical value for complete nitrogen removal compared to the C:N in the pilot study of 10-14. It was suggested in the literature that maintaining an influent C:N ratio at optimum level is important to achieve a high COD utilization efficiency since COD that is not used for NO_x reduction is oxidized aerobically (Regmi et al., 2014). Thus, a higher abundance of COD concentrations would be expected to result in higher COD oxidation which would result in lower COD utilization efficiency.

The previous conclusion was further demonstrated in Phase II when the feed C:N was increased from 3 to 6. Such an increase resulted in an increase in COD/TIN removal from 3.6 ± 0.6 to 7 ± 0.5 which indicates that lower portion of the influent COD was utilized for denitrification/denitritation compared the total influent COD consumption. Despite the lower COD utilization efficiency, TIN removal in phase II increased to 86.3 ± 5.9% from 73.4 ± 2.7% at phase

I which can be attributed to the increase in the influent COD concentrations. To develop a better understanding about the mechanism of TIN removal and COD utilization, the variation in nitrogen compounds and COD during a typical SBR cycle are shown in **Figure 3.4b**. As illustrated in the figure, the increased TIN removal resulted in lower remaining nitrite and nitrate concentrations in the pre-anoxic phase compared to the last phase. Thus, COD utilization for the reduction of the remaining oxidized compounds in the pre-anoxic phase decreased by almost half compared to the previous phase. This decrease in pre-anoxic COD utilization combined with the increase in the influent C:N resulted in higher sCOD concentrations of around 140 mg/L compared to 70 mg/L in phase I at the beginning of the aerobic phase which indicates that more COD was available for aerobic oxidation. Such high COD concentrations (mainly rbCOD) explain the higher COD/TIN removal. On the other hand, the increase in TIN removal in this phase was driven by the high SND which was equal to 22.3 mg N/L compared to 16.3 mg N/L in the last phase. In agreement, it was reported in a study performed in an oxidation ditch without a pre-anoxic zone at influent C:N ratio of 3-11, that high C:N enhanced the occurrence of SND and resulted in higher nitrogen removal. Moreover, it was suggested in the literature that the amount of nitrogen denitrified in the pre-anoxic zone correlates negatively with the amount denitrified aerobically through SND which was the case in the study where lower nitrogen removal in the pre-anoxic phase in phase II compared to phase I resulted in higher nitrogen removal in the aerobic phase (Klaus and Bott, 2020). Overall, it can be concluded that the increase in C:N ratio should result in higher TIN removal, however lower COD utilization efficiency can be expected unless high nitrate and nitrogen concentrations were made available in the pre-anoxic phase (internal recycle) to reduce COD concentrations before the aerobic zone.

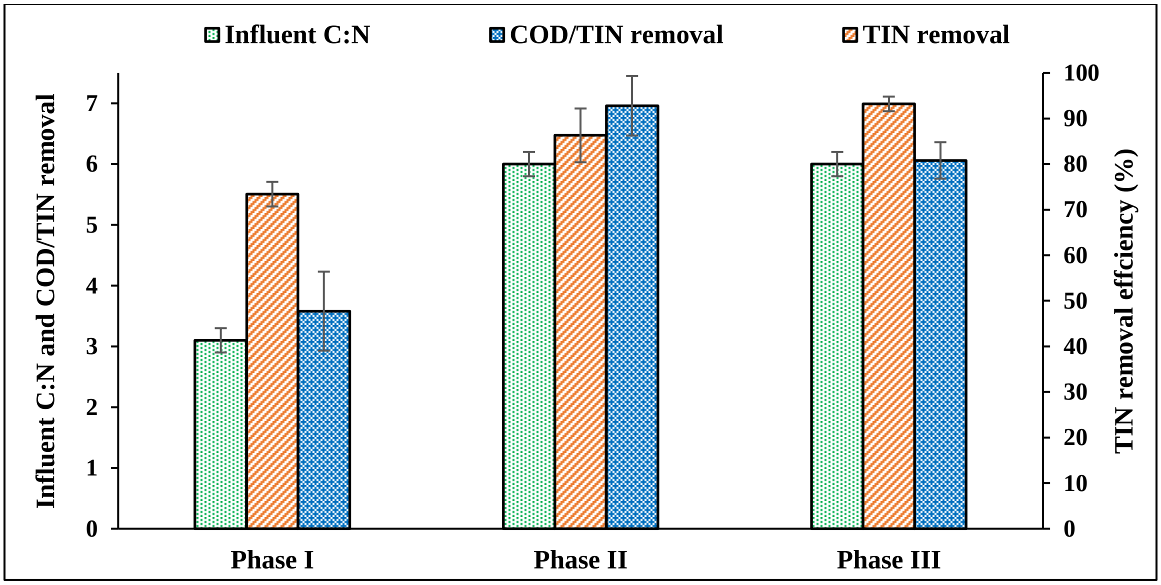

Figure 3.5: Comparison between Influent C:N, TIN removal efficiency, and COD/TIN removal during different phases of operation (Error bars represent standard deviation)

In Phase III, the introduction of pCOD in the feed resulted in higher TIN removal of 93.2 ± 1.6% compared to 86.3 ± 5.9% despite the fact that the same C:N ratio was maintained. Such an increase can be explained by the lower COD/TIN removal of 6.1 ± 0.3 achieved in this phase compared to 7 ± 0.5 in the last phase. Thus, it can be concluded that influent COD was utilized more efficiently, and less COD oxidation took place in this phase. It is hypothesized that sCOD present in the feed was utilized first in the anoxic zone through denitrification and perhaps for internal storage which is combined with the fact that pCOD needs prior hydrolysis before its oxidation, led to less COD available at the beginning of the aerobic phase for aerobic heterotrophs. In the meantime, pCOD hydrolysis in the aerobic phase might have allowed more time for ammonia oxidation which provided an electron donor source for the denitrification of the oxidized nitrogen compounds through SND further down towards the end of the aerobic phase. This hypothesis was demonstrated by the increase in SND in phase III by 10% compared to Phase II. Similar observations have been reported in the literature where an increase in pCOD fraction in the feed resulted in higher TIN removal (Klaus and Bott, 2020; Klaus et al., 2020). It was suggested that the amount of denitrification occurring in the aerobic zone depends on the influent COD fractionation where higher pCOD fraction could lead to higher SND.

3.3.4. Effect of C:N ratio and COD fractionation on NOB out-selection

In order to investigate the effect of C:N ratio and COD fractionation on NOB out-selection, effluent NAR and NOB/AOB rates ratio will be compared between the different phases of operation. The average effluent NAR were 87.2 ± 1.3, 87.7 ± 1.8. and 89.9 ± 2.1%, for phase I, II, and III, respectively. Whereas the average NOB/AOB rates 0.2 ± 0.02, 0.19 ± 0.01, and 0.16 ± 0.02, respectively. The previous results indicate that the increase in C:N ratio from 3.1 ± 0.2 in phase I to 6.0 ± 0.2 in phase II did not result in a significant difference in NOB out-selection. In agreement, it was reported in a B-stage study performed using two different configurations at different C:N ratios and COD fractionations that no correlation was found between sCOD concentrations and NAR (Klaus et al., 2020). Nonetheless, the increase in C:N ratio resulted in a decrease in both AOB and NOB maximum activity from 14.7 ± 0.5 to 12.7 ± 0.2 mg N/g VSS/h and 3.0 ± 0.3 to 2.4 ± 0.1 mg N/g VSS/h, respectively, as shown in **Figure 3.3b**. This decrease in activity might be referred to the increase in VSS concentrations from 1294 ± 359 mg/L in phase I to 1517 ± 336 mg/L in phase II indicating the enrichment of heterotrophic bacteria following the higher abundance of influent COD.

On the other hand, the presence of pCOD in the influent of Phase III resulted in a slight increase in NAR and a decrease in NOB/AOB rates implying higher NOB activity suppression. This can be explained by the longer competition between the heterotrophic denitrifiers and NOB for nitrite provided by the hydrolyzed pCOD further down in the aerobic phase. To elaborate more, the availability of only sCOD in the beginning of the aeration zone provides an advantage for aerobic heterotrophs to utilize it for oxidation. Consequently, when approaching the end of the aeration phase, there might be a lack of available COD for denitrification which provides NOB with an advantage to use the produced nitrite as substrate. However, in the case of pCOD prior hydrolysis is required in the aerobic phase before it can be used as a substrate, thus it can elongate the length of the competition between heterotrophs and NOB which provides NOB less time to consume the nitrite produced, as shown in **Figure 3.4**. The previous hypothesized was further demonstrated by the decrease in the average NOB maximum activity between phase II and III from 2.4 ± 0.1 to 2.1 ± 0.2 mg N/g VSS/h despite maintaining the same C:N ratio. Whereas no such decrease was observed for the average maximum activity of AOB which slightly increased from 12.7 ± 0.2 to 12.8 ± 0.4 mg N/g VSS/h. The previous observation was in accordance to the reported positive correlation between influent pCOD and NAR in a B-stage pilot scale fed with A-stage effluent (Klaus et al., 2020).

3.3.5. Denitrification rates

In order to monitor the activity of heterotrophic denitrification bacteria during the different phases of operation, ex-situ batch activity tests were performed every 2 weeks. As shown in **Figure 3.6a,** NO_2-N denitrification rates were higher than NO_3-N denitrification rates during all phases of operation. This observation indicates that denitritation rates were faster than denitratation rates which can be explained by the abundance of nitrite in the SBR. This hypothesis was further reinforced by the observation of the increase in the denitritation/denitratation ratio following the increase in NAR and vice versa. This was in agreement with the reported inverse relationship between the (NO_2-N denitrification rate)/(NO_3-N denitrification rate) ratio and the NOB/AOB rate ratio (Klaus et al., 2020). Overall, the average ratio of denitritation/denitratation rates during the entire study period was 1.8 ± 0.1. Similar ratios have been reported in mainstream nitrite shunt studies achieving high NARs. In fact, Jimenez et al., 2020 reported a denitritation/denitratation ratio of 1.4 in the Southwest Water Reclamation Facility (WRF) while the average NAR in the plant was 60 ± 22% (Jimenez et al., 2020). Moreover, it was reported in a partial nitrification-denitrifying phosphorus SBR that the denitritation/denitratation ratio was 2.0 when the NAR varied between 75-85% (Zaman et al., 2021). Moreover, the average NO_3-N and NO_2-N denitrification rates in this study were 8.4 ± 0.6 and 15.1 ± 1.3 mg N/g VSS/h, respectively. These rates were higher than the reported range in the previous 2 studies of 3.3-3.6 mg N/g VSS/h and 4.7-7.6 for NO_3-N and NO_2-N, respectively, which can explain the high TIN removal and SND rates achieved in this study.

Another important observation during the batch tests was that NO_2-N denitrification rates were much higher than NO_2 oxidation rates during all the different phases of operation as illustrated in **Figure 6b.** Such results indicate that heterotrophic denitrifiers activity rates in the SBR were greater than NOB activity rates which might have provided them an advantage over NOBs in their competition for nitrite and consequently resulted in NOB out-selection. In agreement, Dold et al., 2015 suggested that the key to achieve nitrite shunt is removing nitrite produced by AOB through denitritation before NOB can oxidize it to nitrate and by doing so less NOB would be present in the system (Dold et al., 2015). Similar conclusion has been reported in other studies in the literature (Ge et al., 2014; Klaus et al., 2020; Lemaire et al., 2008).

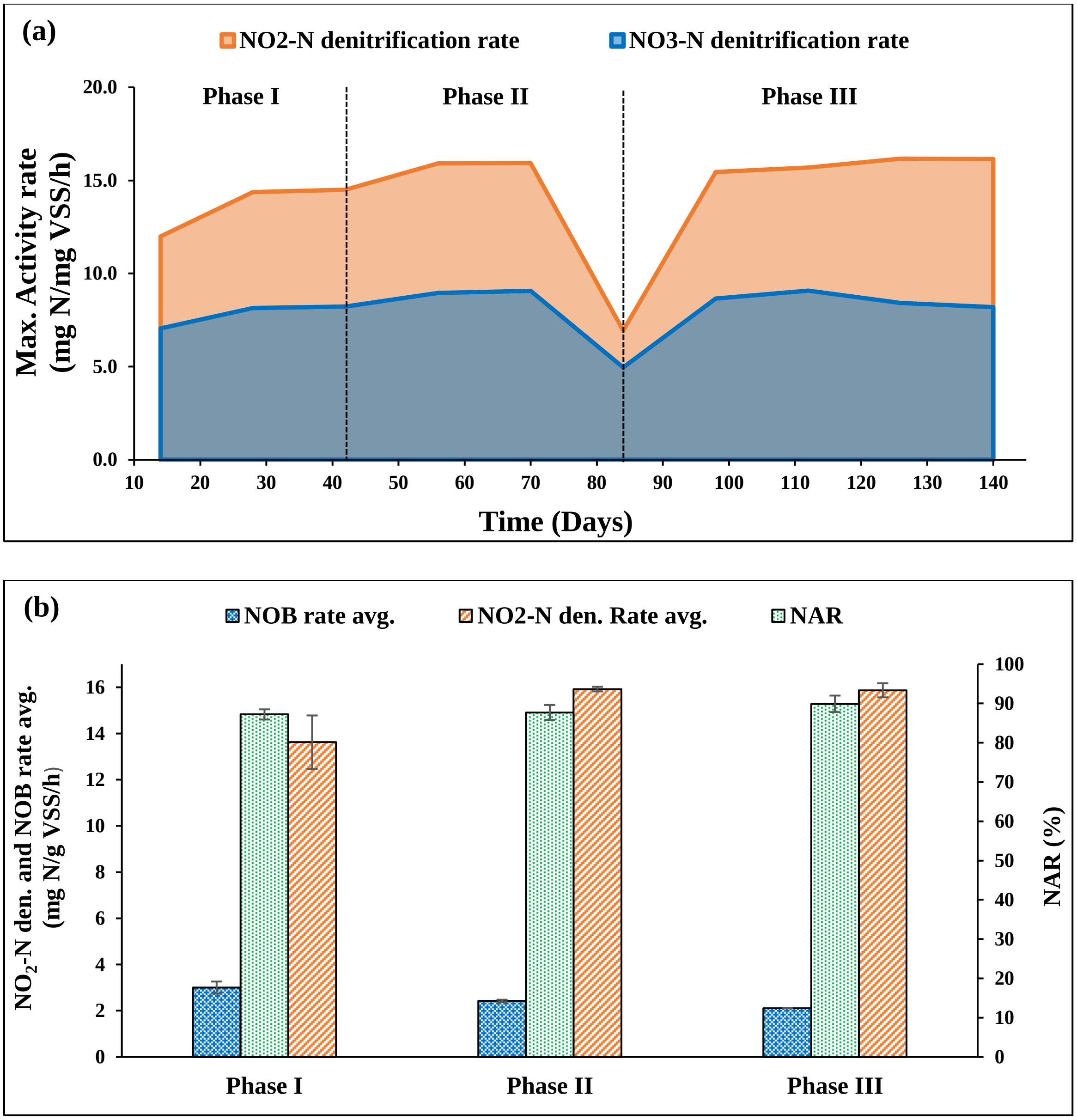

Figure 3.6: (a) variations in NO_2-N and NO_3-N denitrifiers maximum activities, and (b) Comparison between the average NO_2-N heterotrophic denitrifiers maximum activity rate, the average NOB maximum activity rate, and the average nitrite accumulation rates (NAR) during the different phases of operation (Error bars represent standard deviation)

3.3.6. Microbial community dynamics

16S rRNA gene amplicon sequencing was performed with samples for each phase of operation to gain further insights on the microbial population responsible for nitrification and denitrification in the SBR under the low DO concentrations as shown in **Figure 3.7**. *Dechloromonas* and *Thauera* which are known for the denitrification ability were the most abundant genus in all the different phases of operation (Ni et al., 2017). Several *Thauera* species have been reported to reduce nitrate and nitrite even in the presence of oxygen and thus are considered as aerobic denitrifiers (Scholten et al., 1999; Takaya et al., 2003). Interestingly, in an AO system, it was found that *Thauera* relative abundance was higher in the aerobic zones than in the anoxic zones. Such an ability might explain the high SND during the aerobic phase achieved in this study. Moreover, It has been reported that *Thauera* reduces nitrite faster than nitrate and in the case of the presence of both nitrate and nitrite, nitrate reduction rate is significantly lower which might have contributed to the higher NO_2-N denitrification rates compared to those of NO_3-N reported in this study (Ren et al., 2021). Furthermore, it has been reported that *Thauera* might have an advantage over other denitrifiers in low C:N ratio. Such a hypothesis can explain the reduction in the relative abundance of *Thauera* from 14.5% in Phase I to 8.8% in Phase II when the C:N ratio was increased from 3.1 ± 0.2 to 6.0 ± 0.2. Similar observation was reported in an SBR in which *Thauera* relative abundance increased from 11.3 to 19.5% following a decrease in C:N ratio from 10 to 5 (Zhang et al., 2018).

On the other hand, *Dechloromonas* has been associated with phosphorus removal and assumed to be a denitrifying phosphate accumulating organisms (DPAO) with high denitrification and phosphorus accumulating ability (Liu et al., 2016; Stokholm-Bjerregaard et al., 2017). *Dechloromonas* has been found with high relative abundance in several mainstream nitrite shunt studies (Feng et al., 2021; Klaus and Bott, 2020; Zaman et al., 2021). It has been reported that

Dechloromonas has the ability to perform simultaneous denitrification and phosphorus removal at low and moderate DO concentrations (Kong et al., 2004; J. B. Park et al., 2002; Terashima et al., 2016; Zaman et al., 2021). It has been suggested that *Dechloromonas* can drive denitrification in aerobic conditions by utilizing internally stored carbon synthesized in a previous anaerobic zone (Feng et al., 2021; Zhang et al., 2018). As shown in **Figure 7**, *Dechloromonas* relative abundance increased from 8.6% in Phase I to 15.8% in Phase II. This increase can be attributed to the increase in the influent COD in the form of acetate (VFA) which is the main substrate for *Dechloromonas*.

In terms of nitrifiers, the genus *Nitrosomonas* and *Nitrospira* were the most abundant AOB and NOB, respectively, during the entire study. The relative abundance for *Nitrosomonas* and *Nitrospira* were 2.5, 2.1, and 2.4%, and 0.41, 0.34, and 0.25% in Phase I, II, and III, respectively. Such results elucidate the successful NOB out-selection during the different phases of operation and explain the high NAR achieved. In a nitrite shunt plug flow system achieving a NAR of 89.3%, it was reported that *Nitrosomonas* and *Nitrospira* were the dominant AOB and NOB species and had relative abundances of 0.23 and 0.2%, respectively (Feng et al., 2021). In another study SBR study performing SND and achieving an NAR of 75-85%, it was found that AOB genus had a total relative abundance of 0.88% while NOB relative abundance was 0.23% (Zaman et al., 2021). In a study investigating the effect of low DO concentrations on nitrogen removal, it was reported that *Nitrosomonas* the most abundant AOB genus had a relative abundance of 1.0 ± 0.36% and 0.79 ± 0.33% in a pilot-scale and a full-scale plant, respectively, while *Nitrospira* the dominant NOB genus had a relative abundance of 1.3 ± 1.2% and 0.25 ± 0.17%. Thus, it can be concluded from the previous result that AOB was present in this study in higher relative abundance which demonstrates the high ammonia oxidation rate achieved despite the low DO concentrations.

As shown in **Figure 3.7**, other genus that were present in relatively high abundance were *Flavobacterium, Acinetobacter, Pseudomonas,* and *Paracoccus* which represented about 6-12% of the overall microbial population during the different phases of operation. Several species of these genus have been previously isolated from wastewater and identified as aerobic denitrifiers (Rajta et al., 2020). Moreover, *Acinetobacter* and *Pseudomonas* have been reported to prefer nitrite reduction over nitrate reduction when both substrates are available. Additionally, their nitrite reduction rate is higher than nitrate reduction rate (Martienssen and Schöps, 1997). Moreover, *Flavobacterium* which possess phenotypes of t denitrification pathways were reported to specifically grow on nitrite (Baideme et al., 2022). Therefore, the abundance of these genus can be attributed to the abundance of nitrite in the SBR and justifies the higher NO_2-N denitrification rates compared to those of NO_3-N.

A key observation from the microbial community composition was the absence of glycogen accumulating organisms (GAO) and denitrifying GAOs which were not detected during all the different phases of operation. However, this can be explained by the low DO concentrations in the SBR. It has been suggested that PAO have an advantage over GAO at low DO since they have a higher oxygen affinity (Zeng et al., 2003b). Moreover, DGAO are characterized by their high nitrate reduction ability (Carvalheira et al., 2014). Hence, the low nitrate availability compared to nitrite in the SBR due to the high NAR might have provided DPAO a kinetic advantage which resulted in their proliferation. Similar observation have been reported in other mainstream nitrite shunt studies achieving high NAR at low DO concentrations (Jimenez et al., 2020; Zaman et al., 2021)

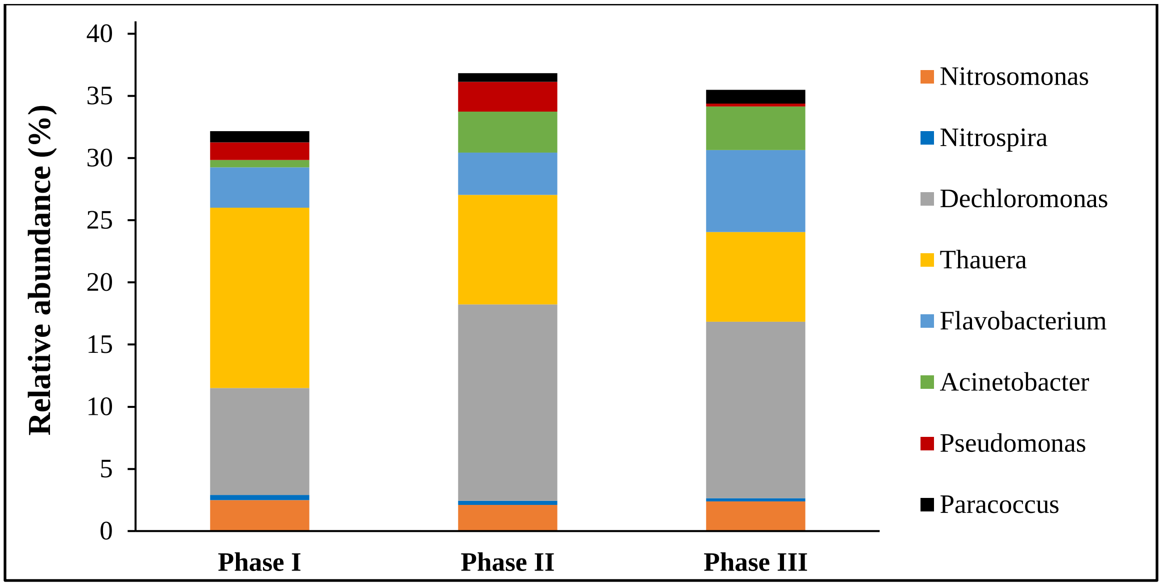

Figure 3.7: 16S rRNA gene amplicon sequencing results for the dominant genus during the different phases of operation

3.4. Conclusion

The implementation of the nitrite shunt process as a B-stage has been hindered by the challenges facing NOB out-selection at mainstream conditions. The inability of NOB suppression means that nitrogen removal will be performed via nitrate pathways rather than nitrite pathways which results in higher aeration and COD requirements. Commonly, the COD required for complete denitrification is higher than the COD available in the A-stage effluent which requires external COD addition and increases the process cost. Therefore, in this study, the achievement of nitrite shunt via nitrite pathways was targeted in an SBR operated at mainstream conditions. The SBR was operated at ambient temperature and with an HRT, total SRT, and aerobic SRT of 6 h, 6.3 ± 0.7 d, and 3.7 ± 0.4 d, respectively. Under these operational conditions, the SBR was able to maintain an ARE, COD and TIN removal efficiencies of 99.2 ± 0.7, 94.0 ± 0.1 and 93.2 ± 1.6% at a C:N ratio as low as 6.0 ± 0.2. The influent COD constituted of a mixture of sCOD and pCOD at a ratio of around 1:1. Such influent characteristics were in the same range reported for A-stage effluent quality which indicates that no external COD addition is required. The total inorganic nitrogen concentrations in the effluent were less than 3 mg N/L which should be able to meet the stringent discharge limits. The combination of low DO concentrations, short SRT, competition between heterotrophic denitrifiers and NOB for nitrite as well as the previous adaptation of the nitrifying community to low DO played a pivotal role in NOB out-selection which resulted in high TIN removal at low C:N ratio. Amplicon sequencing revealed that NOB genus had a total relative abundance of 0.25-0.41% during different phases of operation compared to 2.1-2.5% for AOB. Moreover, the effect of C:N ratio and COD fractionation on TIN removal and NOB out-selection was studied. It was demonstrated that higher C:N ratios results in higher TIN removal but also higher COD/ TIN removal ratios. Thus, maintaining an adequate C:N ratio that balances between TIN removal and COD/TIN removal is a key to achieving high COD utilization efficiency. The increase in C:N ratio was also shown to not exhibit a significant effect on NOB out-selection. On the other hand, it was demonstrated that pCOD correlated positively with both TIN removal and NOB out-selection. Hence, these results suggest that controlling the A-stage effluent COD fractionation can help achieve a more efficient B-stage system.

3.5. References

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Chapter 4: Evaluation of the nitrite shunt process as a B-stage in the A/B stage scheme for low carbon to nitrogen (C:N) wastewater using intermittent aeration at different DO concentrations

ABSTRACT

The successful implementation of mainstream nitrite shunt as a B-stage depends upon maximizing nitrogen removal while minimizing its COD utilization so more carbon can be captured and diverted in the A-stage. To that end, intermittent aeration has been suggested as a suitable aeration strategy for efficient COD utilization through the dedicated anoxic zones it provides for the denitritation step. However, there is no consensus on the level of the DO concentrations in the aerobic zones of intermittent aeration. Therefore, in this study, two SBRs were operated at similar operational conditions but at two different DO levels, (i) DO concentrations of 1.5 mg/L (high DO SBR), and (ii) DO concentrations of 0.2 mg/L (low DO SBR).

At mainstream conditions, the low DO SBR was able to maintain an average ammonia removal efficiency (ARE), total inorganic nitrogen (TIN) removal and COD removal of 99.0 ± 1.6, 95.8 ± 0.9, and 94.7 ± 1.0%, respectively, at a C:N of 5.9 ± 0.3. It was found that the previous adaptation of the nitrifying sludge to low DO played an important role in maintaining high AOB activity rates and consequently achieving high ARE at low DO. The high TIN removal achieved was driven by successful NOB out-selection which resulted in high nitrite accumulation rate (NAR) of 88.9 ± 2.2%. Low DO concentrations, short aerobic SRT, and the competition between heterotrophic denitrifiers and NOB for nitrite in the aerobic phases were the driving force for NOB out-selection. Whereas the high DO SBR maintained an average ARE, TIN removal and COD removal of 99.8 ± 0.2, 73.8 ± 1.7, and 95.7 ± 0.7%, respectively, at a C:N of 6.1 ± 0.2. It was found that NOB was not washed out from the system but rather its activity was suppressed which resulted in lower NARs of 60.1 ± 3.5%, and by consequence less TIN removal. NOB suppression was driven by transient anoxia induced by intermittent aeration as well as the reduced nitrite abundance due to its removal by heterotrophic denitrifiers in the anoxic phases.

4.1. Introduction

Nitrite Shunt has been proposed as a suitable B-stage process in the A/B scheme. However, its implementation in full-scale plants has been hindered by two main challenges: (i) NOB out-selection in mainstream conditions and (ii) the efficient utilization of influent COD for denitritation at low carbon to nitrogen (C:N) ratio wastewater to eliminate the need of external carbon addition. To overcome such challenges, intermittent aeration (cycling anoxic/oxic conditions) has been proposed. Even though intermittent aeration requires more complex aeration control compared to continuous aeration, it provides the advantage of dedicated anoxic zones for the denitritation step. Meanwhile continuous aeration relies on simultaneous nitrification/denitrification (SND) for denitrification in the aerated zones which promotes higher COD oxidation and consequently less COD is available for denitrification. Thus, intermittent aeration can be an efficient tool towards improving the influent COD utilization for denitrification and lower the C:N requirements for complete nitrogen removal. Moreover, intermittent aeration provides an additional advantage over continuous aeration since it was reported that NOB might exhibit a period of lag phase following anoxic conditions which results in its reduced growth (Gilbert et al., 2014; Kornaros et al., 2010). Hence, intermittent aeration allows for an added pressure on NOB growth which can help tackle the challenge of NOB out-selection at mainstream conditions.

Several aeration strategies have been proposed to control the aeration pattern in intermittent aeration including (i) DO set point control, (ii) Ammonia based aeration control (ABAC), (iii) Ammonia vs NO_x (AvN) control. The first aeration strategy is the simplest method of aeration control as it does not require any additional nitrogen compounds online probes which can significantly add to the process cost and operation complexity. In this method, the oxic zones of intermittent aeration are controlled at a specific DO set point and aerobic and anoxic duration are selected at constant intervals to maintain a specific aerobic fraction. The total SRT in the system is controlled according to the aerobic fraction to maintain an aerobic SRT within the required range.

Whereas in the ABAC control strategy, the aerobic duration is controlled based on the desired effluent ammonia concentration (Regmi et al., 2014). In this method, two effluent ammonia set points are defined, a high and a low set point and ammonia is continuously monitored by an online ammonia probe connected to a programmable logic controller (PLC). When the effluent ammonia concentration is greater than the high set point, the controller increases the aerobic duration whereas when effluent ammonia is below the lower set point the controller decreases the aerobic duration. In the case of the effluent ammonia between the two set points, the aerobic duration remains unchanged. By this manner, the ABAC ensures that the aerobic duration always allows AOB enough time to nitrify the required amount of ammonia and at the same time does not provide NOBs more time to further oxidize the nitrite produce and consequently the reactor is always operated at the minimum SRT. However, in this method the effluent always contains residual ammonia concentration which might exceed the required effluent standards concentrations.

On the other hand, in the AvN control strategy, the aerobic duration is controlled based on maintaining an effluent NH_4 to NO_x (NO_2 + NO_3) ratio equal to 1 to be suitable for a following anammox polishing step to remove any residual ammonia. In AvN, ammonia, nitrate and nitrite through online probes connected to a PLC and in the same manner as in ABAC, when the NH_4/NOx ratio is higher than 1, the controller increase the aerobic duration and when it is lower than 1, the aerobic duration is decreased. Compared to ABAC, AvN offers an additional advantage of oxidizing only the amount of ammonia that can be subsequently reduced using the available influent COD which can help improve the COD utilization efficiency. However, this method requires an online ammonia, nitrate, nitrite probe which results in additional cost requirements. Moreover, if the NH_4 to NO_x ratio is set to 1, an additional polishing step is commonly required which requires additional resources.

In addition to the aeration control strategy, a crucial design factor in intermittent aeration nitrite shunt is the DO concentration levels in the oxic zones of the intermittent aeration. There have been two approaches for operating the DO levels in intermittent aeration, (i) at low DO concentrations of 0.2-0.8 mg/L (Feng et al., 2018; Hu et al., 2021; Xu et al., 2020; Zhou et al., 2020) and (ii) at moderate/high levels of 1.0-2.0 mg/L (Chen et al., 2020; Cui et al., 2020; Gu et al., 2018; Regmi et al., 2014). The first approach adopts operating the aerobic zones of the intermittent aeration at low DO concentrations in order to minimize the aeration-associated energy consumption and consequently maximize the energy gain from the carbon stream. This approach relies on the widely reported observation that that the oxygen half-saturation coefficient for AOB ($K_{o,AOB}$) is lower than that of NOB ($K_{o,NOB}$) (Blackburne et al., 2008; Guisasola et al., 2005; Hunik, 1993; Nowak et al., 1995; Z. Wang et al., 2021; Wiesmann, 1994; Yu et al., 2020). Thus, it can be implied that AOBs are more prone to outcompete NOBs at low DO concentrations. In agreement, it was reported in an intermittently aerated SBR operated that low DO concentrations of 0.1-0.7 mg/L effectively inhibited NOBs and a nitrite accumulation rate (NAR) of 96.45% was successfully maintained (Hu et al., 2021). However, here it is worth mentioning that the SBR was operated at a 21h HRT and at a SRT of 13 days which are considered high for mainstream application. Similarly, a high NAR of 82.49% was successfully achieved at DO concentrations below 1 mg/L in an SBR operated using intermittent aeration. It was reported that the key towards achieving NOB suppression was that AOB had a lower oxygen half saturation coefficient of 0.2-1.5 mg/L compared to NOB which had a value of 1.2-1.5 mg/L. However, in this study, pH was controlled at high values of 7.7-8.4 which resulted in a free ammonia (FA) concentrations of 1.96-4.92 mg/L. Such high FA concentrations is within the range reported for NOB inhibition of 0.1-1.0 mg/L which might have contributed to NOB inhibition (Soliman and Eldyasti, 2018). Moreover, in this study, it was stated that the SRT was not controlled and the actual SRT values were not reported. Hence, it might be that high AOB activity was maintained through operating at high SRTs.

On the other hand, the second approach of operating the aerobic zones of intermittent aeration at moderate/high DO concentrations is motivated by the reports of the dominance of *Nitrospira sp.* which has higher oxygen affinity compared to *Nitrobacter sp*. in mainstream conditions (Al-Omari et al., 2015; Regmi et al., 2014). It was suggested that such a dominance might limit AOB advantage for oxygen at low DO concentrations, in contrast it would result in lower AOB activity rate and consequently deteriorate the nitrification rates (Keene et al., 2017). Thus, it was recommended to control the DO at higher concentrations to ensure that high AOB rates are maintained and consequently lower high nitrogen removal rates. In terms of NOB out-selection, this approach relies on the combination of transient anoxia, short SRT, heterotrophic denitrifiers competition with NOB for nitrite to provide unfavorable condition for NOB growth (Regmi et al., 2014). In fact, it was reported in an intermittently aerated CSTR operated at DO concentrations of 1.6 mg/L that a NAR of 60 ± 22% was successfully maintained at a short aerobic SRT of 3.2 days (Regmi et al., 2014). Even higher NARs of up to 97% were reported in an SBR operated as a B-stage following an anaerobic fixed bed reactor (AFBR) A-stage system. In this study, the DO in the aerobic zones of intermittent aeration was controlled at 1.5 mg/L and total SRT was controlled at 4-6 days. Moreover, it was reported that a decrease in the C/N ratio resulted in a decrease in NAR to 28% which implies that the pressure added by heterotrophic denitrifiers on NOB growth played an important role in NOB suppression in this study. However, it is worth noting that the temperature in the SBR was kept at 30 ºC which presumably contributed to the high NAR achieved. Such high temperature is impractical to be maintained in mainstream lines.

Therefore, it can be concluded from the previous literature survey that there is a lack of consensus of the optimal DO level in the aerobic zones of intermittent aeration. Moreover, a critical factor that has been overlooked in intermittent aeration studies is the reported adaptation of AOBs to low DO concentrations (Keene et al., 2017; Regmi et al., 2022; Yu et al., 2020). A major barrier in achieving stable partial nitrification at low dissolved concentrations is lower AOB removal rates at limited DO conditions. Hence, most of the low DO studies relied on elongating the SRT to ensure that high ammonia removal rates are achieved. However, it has been recently reported that if an acclimation period is provided to AOB to adapt to low DO, high ammonia oxidation rates can be achieved at lower SRTs (Kirim et al., 2022; Klaus and Bott, 2020; Regmi et al., 2022).

Hence, the objective of this study is to study the effect of different DO levels in the aerobic zones of intermittent aeration on partial nitrification and nitrite shunt performance. Two SBRs will be operated at high and low DO concentrations of 1.5 and 0.2 mg/L to perform mainstream nitrite shunt towards its implementation as a B-stage system in the A/B scheme. Intermittent aeration will be controlled through DO set point control in order to eliminate the high costs of the online sensors required for other DO control strategies. Aerobic and anoxic duration will be controlled to maintain an aerobic fraction between 0.4-0.5 which have been reported to achieve a minimum effluent total inorganic nitrogen (TIN) (Klaus and Bott, 2020). The two systems will be seeded using nitrifying sludge that was previously adapted to low DO concentrations to address the challenge of inhibiting NOB while maintaining a high nitrogen removal rate. The effect of high and low DO levels on total inorganic nitrogen (TIN) removal, COD removal, nitrite accumulation rates (NAR), and AOB and NOB activities will be studied. Moreover, the effect of influent C:N ratio and COD fractionation on total inorganic nitrogen (TIN) removal at different DO concentrations will be evaluated through operating the SBR at 3 different phases. In each phase, batch tests will be performed to monitor AOB, NOB, denitritation and denitritation rates in the two SBRs at different operational conditions. Lastly, the microbial population dynamics following the operational conditions change will be quantified using 16S rRNA gene amplicon sequencing.

4.2. Materials and Methods

4.2.1. Reactors configuration

Two sequencing batch reactors (SBR) with a working volume of 1.5 L were used to perform nitrite shunt. The schematic diagram for the typical SBR is depicted in **Figure 4.1a.** Both were equipped with an air pump for aeration and a stirrer for mixing during reaction operation. The cycle duration of the two SBRs was 4 h, which included 5 min feeding, 200 mins reaction, 30 min settling, and 5 min decanting. The influent was delivered to the bottom of the bioreactor through a fixed tube during the feeding period. The effluent was discharged out of the bioreactor during the decanting time through another fixed tube at the level of 500 mL. Excess sludge was wasted during the settling period from an outlet installed at the bottom part of the SBR to control the SRT at the targeted range. Influent, effluent, and wastage flow rates were controlled using peristaltic pumps with a maximum flow rate of 380 mL/min. Air was delivered using to the system through air diffusers located at the lowest possible point of the SBRs. A DO probe was installed in each SBR for online monitoring of DO concentrations. The air pump and DO probes were connected to a control processing device. The control device was set up to switch the air pump on and off during the reaction time at 15 mins intervals for the low DO SBR and 25 mins off and 15 mins on for the high DO SBR, as shown in **Figure 4.1b.** During the aerobic period, the control device was set up to control the DO at the desired set point through changing the air flow rate according to the DO probe online readings.

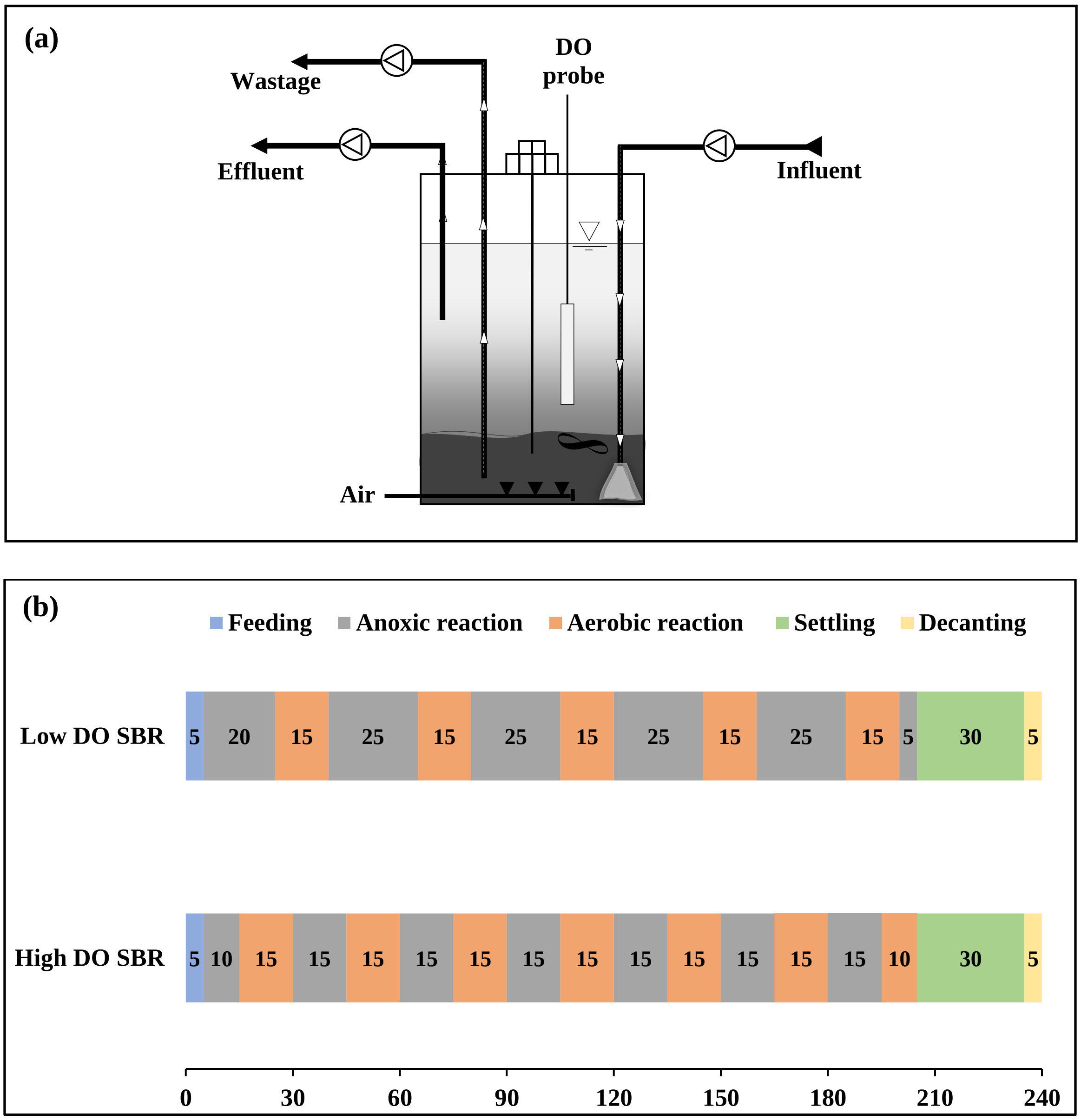

Figure 4.1: (a) Schematic diagram of the typical SBR configuration and (b) the SBR's cycle duration during different phases of operation for the low DO SBR and High DO SBR

4.2.2. Experimental setup

The two SBRs were operated for 140 days, and the experimental process was divided into three phases with different operational conditions as shown in **Table 4.1**. The DO concentrations in the aerobic phase were controlled at 0.2 mg/L for the first SBR and 1.5 mg/L for the second SBR. The hydraulic retention time (HRT) was maintained constant in both SBRs at 6 h for all the three phases. During the entire study, the two SBR were operated at ambient temperature 20 ± 2 ºC and a pH of 7.2-7.6.

Table 4.1: Detailed operational conditions in the two SBRs during the different phases of operation

Phase	Reactor	Aerobic Phase DO (mg/L)	Total SRT (Days)	Aerobic SRT (Days)	VSS (mg/L)	TSS (mg/L)	VSS/TSS (g/g)
I	SBR 1	0.2	5.9 ± 0.4	2.4 ± 0.2	886 ± 309	1016 ± 332	0.87 ± 0.02
	SBR 2	1.5	6.3 ± 0.6	2.0 ± 0.2	822 ± 242	978 ± 305	0.84 ± 0.03
II	SBR 1	0.2	7.2 ± 0.8	3.0 ± 0.3	1623 ± 247	1774 ± 251	0.91 ± 0.01
	SBR 2	1.5	7.3 ± 0.4	2.3 ± 0.1	1518 ± 226	1706 ± 249	0.89 ± 0.02
III	SBR 1	0.2	7.3 ± 0.5	3.1 ± 0.2	2317 ± 163	2529 ± 183	0.92 ± 0.01
	SBR 2	1.5	7.6 ± 0.5	2.5 ± 0.2	2266 ± 148	2516 ± 169	0.90 ± 0.01

4.2.3. Seed sludge and synthetic feed

The two SBRs were inoculated with a previously adapted nitrifying sludge to low DO. The seed sludge was enriched in a lab-scale mainstream nitrite shunt SBR which was operated at low DO concentrations (0.2 mg/L) and short aerobic SRT of 3.7 days. The SBR was successfully maintaining a NAR of 89.9% using continuous low DO aeration. The total suspended solids and volatile suspended solids of the seeding sludge were 2048 and 2228 mg/L, respectively.

The two SBRs were fed with synthetic wastewater. The feeding solutions were prepared in a daily basis using deionized water combined with concentrated stock solutions of ammonium chloride as the source of ammonia, sodium carbonate for alkalinity, sodium acetate as the source of sCOD, and milk powder, starch, and yeast as the source of pCOD. The trace concentrated stock solutions (1mL/1000mL) contained 990 mg $MnCl_2 \cdot 4H_2O$/L, 500 mg $FeSo_4 \cdot 7H_2O$/L, 430 mg $ZnSo_4 \cdot 7H_2O$/L and the mineral salt stock solution contained 190 mg $NiCl_2 \cdot 6H_2O$/L, 220 mg $Na_2MoO_4 \cdot 2H_2O$/L, 250 mg $CuSO_4 \cdot 5H_2O$/L, 240 mg $CoCl_2 \cdot 6H_2O$/L, 210 mg $MnCl_2 \cdot 4H_2O$/L, 19 mg $H_3BO_4 \cdot 7H_2O$/L, and 5 g EDTA/L. A detailed description of the synthetic wastewater composition is listed in **Table 4.2**.

Table 4.2: The characteristics of the synthetic wastewater in the two SBRs during the 3 phases of operation

Phase	Reactor	Time (Days)	Ammonia conc. (mg N/L)	sCOD (mg/L)	TCOD (mg/L)	C:N (-)	sCOD/TCOD (%)
I	SBR 1	1-42	49.4 ± 1.5	-	-	-	-
	SBR 2	1-42	49.2 ± 1.4	-	-	-	-
II	SBR 1	43-84	39.6 ± 1.6	119 ± 5	119 ± 5	3.0 ± 0.2	100
	SBR 2	43-84	39.7 ± 1.2	120 ± 4	120 ± 4	3.0 ± 0.1	100
III	SBR 1	85-140	39.4 ± 0.8	115 ± 6	233 ± 12	5.9 ± 0.3	49.4 ± 1.6
	SBR 2	85-140	40.1 ± 1.0	121 ± 5	246 ± 9	6.1 ± 0.2	49.2 ± 1.6

4.2.4. AOB and NOB activity measurements

In order to monitor AOB and NOB maximum activities rate in the two SBRs, two specific nitrification rate (SNR) batch tests were performed every 2 weeks with samples taken from each reactor. The test was conducted by collecting a 2 L sample from the SBR during the aeration phase. The sample was then washed two times with synthetic wastewater and centrifuged. The supernatant was discharged, and the settled sludge was transferred to a 2L batch reactor. The batch reactor was then aerated for 30 mins to ensure all excess COD (if available) has been oxidized. Afterwards, a synthetic stock solution was added to the reactor to reach an initial ammonia concentration of 30-40 mg NH_4-N/L and an initial nitrite concentration of 15-20 mg NO_2-N/L. DO concentrations were maintained at concentrations above 4 mg/L using an air pump and a diffuser to ensure that no oxygen limitation is inhibiting AOB and NOB activity. DO concentrations were monitored using a DO probe (Hach LDO, CO). The test was conducted until all the ammonia was oxidized and samples were taken every 15 mins and analyzed for ammonia, nitrite, nitrate, TSS, and VSS. The slopes of the linear responses of NO_x-N (NO_3-N + NO_2-N) production and of NO_3-N production were calculated. The AOB rates (mg N/g VSS/h) were then calculated by dividing the NO_x-N production slope by the batch test average VSS concentrations, and the NOB rates (mg N/g VSS/h) were calculated by dividing the NO_3-N production slope by the batch test average VSS concentrations.

4.2.5. Denitrification rates measurements

In order to monitor denitrification rates during the different phases of operation, two batch tests were performed every 2 weeks with samples taken from each reactor. The test was conducted by collecting a 2 L sample from the SBR during the anoxic phase. The sample was then washed two times with synthetic wastewater and centrifuged. The supernatant was discharged, and the settled sludge was transferred to a 2L anoxic, sealed batch reactor. Prior to the start of the test, N_2 gas was purged in the batch reactor until the reactor became anoxic as measured by the DO probe. Afterwards, a synthetic stock solution was added to the reactor to reach an initial nitrate concentration of 20-30 mg NO_3-N/L and an initial nitrite concentration of 15-20 mg NO_2-N/L. Acetate was added in excess to the synthetic solution to ensure that no COD limitation is inhibiting denitrification. The test was conducted for 2 hours, and samples were taken every 15 mins and analyzed for sCOD, ammonia nitrite, nitrate, TSS, and VSS. The slopes of the linear responses of NO_x-N (NO_3-N + NO_2-N) consumption and of NO_3-N consumption were calculated. The NO_2-N denitrification rates which indicate the reduction rate from nitrite to nitrogen gas (mg N/g VSS/h) were then calculated by dividing the NO_x-N consumption slope by the batch test average VSS concentrations. Whereas the NO_3-N denitrification rates which indicates the reduction rate from nitrate to nitrite (mg N/g VSS/h) were calculated by dividing the NO_3-N consumption slope by the batch test average VSS.

4.2.6. DNA extraction and amplicon sequencing

DNA was extracted using the DNeasy PowerSoil Pro Kit (Qiagen). The Illumina MiSeq sequencer (Illumina Incorporated, San Diego CA) was used for amplicon sequencing of the 16S rRNA gene. The Illumina adapted primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 806R (5'-GGACTACNVGGGTWTCTAAT-3') were used to amplify the V3-V4 regions of the 16S rRNA genes, through polymerase chain reaction (PCR).

Raw reads were processed using DADA2 (Callahan et al., 2016). The DNA sequence reads were filtered and trimmed based on the quality of the reads, error rates were learned, and sequence variants were determined by DADA2. Bimeras were removed and taxonomy was assigned using the RDP classifier against the SILVA database version 1.3.8. Additional information can be found in **section 3.3.6.**

4.2.7. Analytical methods

Influent and effluent samples were collected from both SBRs 3 days a week, and analyzed for ammonia, nitrate, and nitrite using HACH TNTplus kits and a HACH DR3900 spectrophotometer (HACH Loveland, CO). Total and soluble COD were measured using HACH testing kits, a HACH

DRB200 digital digester, and a HACH DR3900 spectrophotometer (HACH, Loveland, CO, USA). Samples from inside the reactor and from the effluent were collected twice a week and analyzed for total and volatile suspended solids (TSS and VSS) using standard methods 2540D and 2540E, respectively (APHA, 2012). Nutrient and sCOD samples were filtered through 0.45 μm and 1.5 μm filters, respectively. Temperature, pH, and DO were monitored using online sensors equipped in the SBR.

4.2.8. Calculations

The ammonia removal efficiency (ARE) and nitrite accumulation rate (NAR) were calculated according to Eq. (4.1) and Eq. (4.2) as follows:

$$\boldsymbol{ARE}\ (\%) = \frac{(NH_3-N)_{inf} - (NH_3-N)_{eff}}{(NH_3-N)_{inf}}\ \boldsymbol{X\ 100} \tag{4.1}$$

$$\boldsymbol{NAR}\ (\%) = \frac{(NO_2-N)_{eff}}{(NO_2-N)_{eff} + (NO_3-N)_{eff}}\ \boldsymbol{X\ 100} \tag{4.2}$$

The TIN removal efficiency (%) and COD/TIN removal ratio (g/g) were calculated according to Eq. (4.3) and Eq. (4.4) as follows:

$$\boldsymbol{TIN\ removal\ efficiency}\ (\%) = \frac{(NH_4-N)_{inf} - [(NH_4-N)_{eff} + (NO_3-N)_{eff} + (NO_2-N)_{eff}]}{(NH_4-N)_{inf}}\ \boldsymbol{x\ 100} \tag{4.3}$$

$$\boldsymbol{COD/TIN\ removal\ ratio}\ \left(\frac{g}{g}\right) = \frac{(TCOD)_{inf} - (TCOD)_{eff}}{(NH_4-N)_{inf} - [(NH_4-N)_{eff} + (NO_3-N)_{eff} + (NO_2-N)_{eff}]} \tag{4.4}$$

The Simultaneous Nitrification Denitrification (SND) rate (mg N/L) was calculated as the total inorganic nitrogen (TIN) removed across the aerobic phase in the typical SBR cycle as shown in Eq. (4.5)

$$\boldsymbol{SND}\ \left(\frac{mg\ N}{L}\right) = \boldsymbol{TIN_{end\ of\ anoxic\ phase} - TIN_{end\ of\ aerbic\ phase}} \tag{4.5}$$

4.3. Results and Discussion

The objective of this study is to examine the effect of DO concentrations in the aerobic zones of intermittent aeration on partial nitrification and nitrite sunt at mainstream conditions. Thus, two SBRs were operated with same influent characteristics and operational conditions that mimic mainstream conditions except for the DO set point in the aeration phases. The first SBR was operated at a low DO set point of 0.2 mg/L while the second SBR was operated at a higher set point of 1.5 mg/L. In this section, the first SBR will be referred to as the low DO SBR while the second SBR will be referred to as the high DO SBR. Both systems were operated for three phases, each with different influent characteristics. The first phase was operated with no organic carbon source in the feed to study the effect of DO on the nitrifying community (AOB and NOB) while eliminating the effect of heterotrophic bacteria. In Phase I, the ammonia removal efficiency (ARE), nitrite accumulation rate (NAR), AOB and NOB maximum activity, oxygen half saturation coefficients (K_o) for AOB and NOB, and the relative abundance of AOB and NOB were determined to compare the effect of DO on partial nitrification. Whereas the objective of Phases II and III was to study the effect of DO concentrations on nitrite shunt. Hence, COD was added in the feed to start the denitrification step and complete the nitrite shunt process. In phase II, the C:N ratio was controlled at 3 with sCOD (acetate) as the sole source of organic carbon to monitor the effect of sCOD and low C:N ratio on nitrite shunt at different DO concentrations. While, in Phase III, pCOD was introduced in the feed to explore its effect and the effect of higher C:N ratio on nitrite shunt at different DO concentrations. In phase III, the C:N ratio was increased to 6 with sCOD fraction of 50% of the total COD which mimics the composition of the A-stage effluent directed to the B-stage (Klaus and Bott, 2020; Rahman et al., 2019; Regmi et al., 2014). In phase

II and III, ARE, COD removal, TIN removal, NAR, nitrification rates, denitrification rates and amplicon sequencing were used to study the effect of DO on nitrite shunt.

4.3.1. The effect of DO concentrations on mainstream partial nitrification (Phase I)

The objective of Phase I was to study the effect of DO concentrations in the aerobic zones of intermittent aeration on partial nitrification under carbon starvation conditions. The influent ammonia concentrations in this phase were 49.4 ± 1.2 and 49.2 ± 1.4 mg N/L for the low DO SBR and the high DO SBR, respectively.

As shown, in **Figure 4.1**, almost all ammonia was removed in both reactors and effluent ammonia concentrations were 0.14 ± 0.07 and 0.05 ± 0.04 mg N/L which corresponded to an ARE of 99.7 ± 0.1 and 99.9 ± 0.1%, for the low DO SBR and the high DO SBR, respectively. Such a high ARE is expected in the high DO SBR since no oxygen limitations conditions are present to inhibit AOB activity. On the other hand, for the low DO SBR, some reports in the literature have suggested that low DO concentrations can result in lower ammonia removal rates and high SRTs would be required to achieve higher ammonia oxidation (Al-Omari et al., 2015; Regmi et al., 2014). However, the high ARE achieved at low DO concentrations in this study can be explained by the previous adaptation of the sludge to the low DO concentrations which allowed for a similar ammonia removal efficiency at similar operational conditions. This was in agreement with the studies suggesting that higher ammonia oxidation rates can be achieved at low DO concentrations through the adaptation of the nitrifying bacteria population (Giraldo et al., 2012; Keene et al., 2017; Klaus and Bott, 2020; H. D. Park et al., 2002). Moreover, the absence of organic carbon in the feed in this phase resulted in the absence of competition between heterotrophs and AOB for oxygen which provides AOBs with an advantage to maintain its activity. These hypotheses were further supported by the ex-situ AOB activity tests.

As shown in **Figure 4.2.**, the average AOB maximum activity in the low DO SBR during this phase was 52.0 ± 8.7 mg N/g VSS/h which was slightly lower than that of the high DO SBR of 61.0 ± 6.7 mg N/g VSS/h despite the difference in the DO set point between the two reactors. Such results confirm that AOB activity was not majorly affected by the low DO concentrations which is attributed to the sludge pre-adaptation and absence of heterotrophs competition.

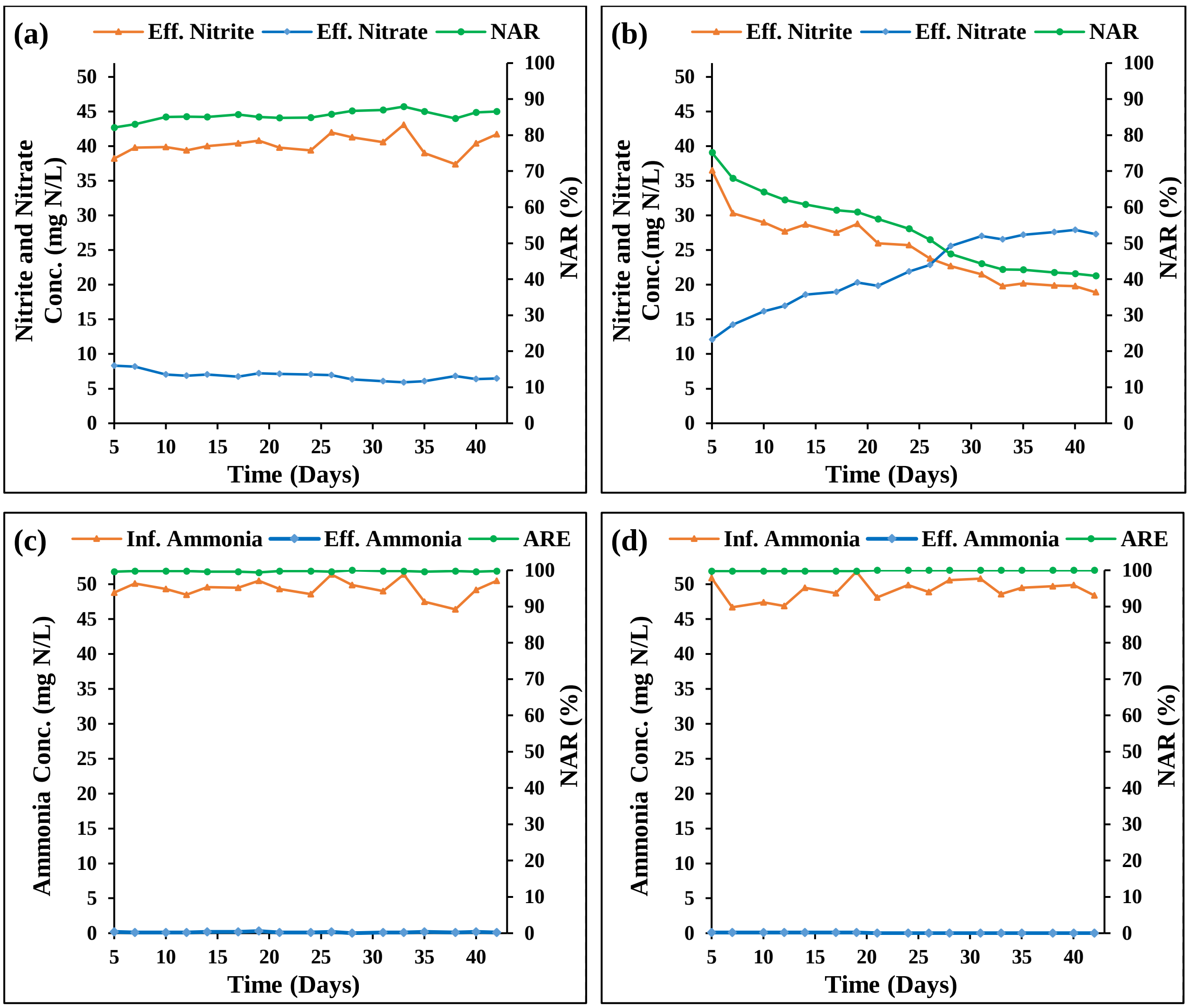

Figure 4.2: SBRs performance during Phase I: (a) effluent nitrite and nitrate concentrations and nitrite accumulation rates (NAR) in the Low DO SBR, (b) effluent nitrite and nitrate concentrations and NAR in the High DO SBR, (c) Influent and effluent ammonia concentrations and ammonia removal efficiencies (ARE) in the low DO SBR, and (d) Influent and effluent ammonia concentrations and ARE in the High DO SBR

In contrast to the similar ARE, the two SBRs showed a different NAR performance. As illustrated in **Figure 4.1**, the low DO SBR was able to maintain a high NAR of 85.4 ± 1.4% over the 42 days of operation during this phase. On the other hand, the NAR kept decreasing in the high DO SBR from 75.1% at the beginning of the phase to 40.9% at the end of the phase. The average NAR in the high DO SBR during phase I was 53.6 ± 10.3% which was significantly less than that obtained in the low DO SBR. These results were further demonstrated by the NOB maximum activity tests measured for both reactors during phase I. As shown in **Figure 4.2**, the average NOB maximum activity in the high DO SBR was 31.2 ± 6.1 mg N/g VSS/h which was much higher than that in the low DO SBR of 10.9 ± 1.6 mg N/g VSS/h.

In order to develop a better understanding about AOB and NOB rates behavior, the ratio between NOB/AOB rates was calculated for both reactors as shown in **Figure 4.2b**. The theoretical NOB/AOB ratio to achieve complete nitrification is 0.78, and it was suggested that a ratio in the range of 0.2-0.3 need to be maintained to achieve successful partial nitrification (Dold et al., 2015; Jimenez et al., 2020). The average NOB/AOB ratio during phase I in the low DO SBR was 0.21 ± 0.01 which is within the range reported for partial nitrification explaining the high NAR achieved in this SBR. Whereas in the high DO SBR, the average NOB/AOB ratio was 0.51 ± 0.05 which is closer to the value of complete nitrification and thus, lower NAR were achieved. Moreover, another key observation in the NOB maximum activity tests was that in the low DO SBR, despite increasing in the first couple of weeks which can be attributed to the washout of heterotrophic bacteria, NOB activity was decreasing towards the end of the phase. Such a decrease indicates that NOB was not able to adapt to the low DO concentrations and reach higher activity rates. On the other hand, in the high DO SBR, NOB activity rate kept increasing from the beginning of the phase till the end of it. Such an increase indicates that NOB was gradually restoring its activity which resulted in the gradual decrease in NAR over the operation of the first phase. This observation was also reflected in the NOB/AOB ratio decreasing from 0.22 in the beginning of the phase to 0.2 at the end of the phase in the low DO SBR. Whereas NOB/AOB ratio increased from 0.44 to 0.55 during the same period in the high DO SBR.

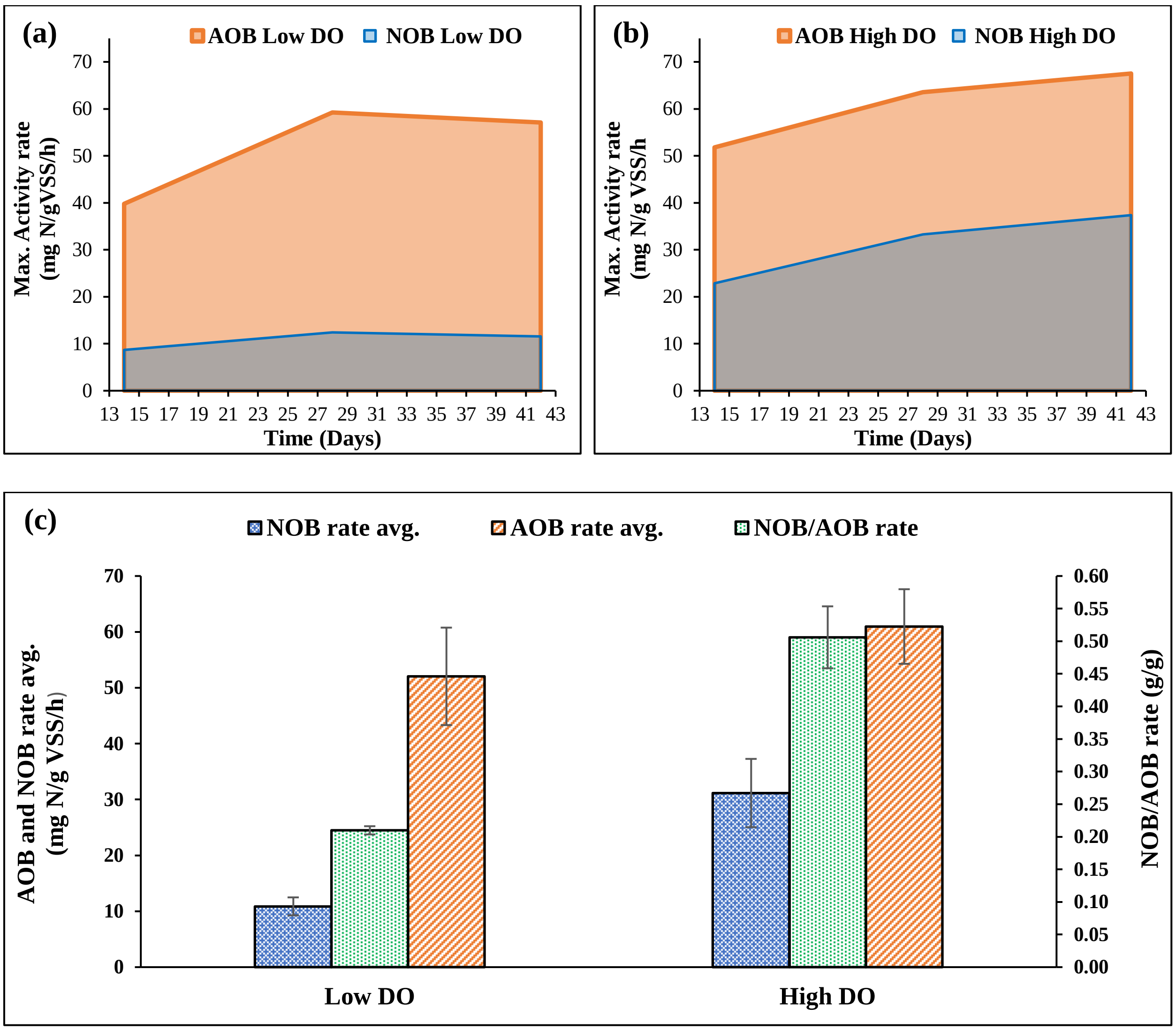

Figure 4.3: Variations in AOB and NOB maximum activities during Phase I, (a) in the Low DO SBR, (b) in the High DO SBR, and (c) Comparison between the average NOB maximum activity rate, the average AOB maximum activity rate, and the average ratio between NOB/AOB for the Low DO SBR and the High DO SBR (Error bars represent standard deviation)

As previously discussed, the hypothesis for operating the aerobic zone of the intermittent aeration at low DO concentrations was that the system can benefit from the reported kinetic advantage of AOB over NOB at limited DO conditions. This advantage stems from the reports that AOBs have lower oxygen half saturation coefficient than NOBs and thus can maintain higher activity at lower DO concentrations (Blackburne et al., 2008; Guisasola et al., 2005; Hunik, 1993; Trotsenko and Torgonskaya, 2009; Z. Wang et al., 2021) However, this hypothesis was challenged by the fact that K_o are not fixed constants, and their values could be affected by different factors, including different species of AOB and NOB. This claim was further supported by the studies reporting higher K_o for NOB than that of AOB (Cui et al., 2020; Jimenez et al., 2014a; Regmi et al., 2014). Thus, it was suggested that the aerobic zones should be operated at moderate DO concentrations to eliminate any inhibition on AOB activity at limited oxygen concentrations and by consequence maintaining higher ammonia removal rates.

To test both theories, 6 batch tests were conducted at different DO concentrations for each SBR at the end of Phase I to determine AOB and NOB oxygen half saturation coefficients. As shown in **Figure 4.3**, in the low DO SBR, $K_{o,AOB}$ was 0.15 mg/L which was much lower than $K_{o,NOB}$ of 0.3 mg/L. In particular, $K_{o,AOB}$ in the low DO SBR was lower than the DO concentrations operated in the aerobic phases of 0.2 mg/L contrarily to $K_{o,NOB}$ which was higher than the operated DO level. Thus, it can be concluded that a critical factor that contributed to NOB out-selection and by consequence achieving high NARs was operating the SBR at a DO level higher than $K_{o,AOB}$ but lower $K_{o,NOB}$. It was previously hypothesized that the adaptation of AOB to low DO concentration might result in lower $K_{o,AOB}$ values (Keene et al., 2017; Regmi et al., 2022a; Yu et al., 2020). The results obtained in this study confirm this hypothesis and demonstrate that AOB adaptation can play a pivotal role in NOB out-selection. Moreover, NOB out-selection was confirmed by the amplicon sequencing data. As shown in **Figure 4.4**, the relative abundance of *Nitrosomonas* (the dominant AOB genus) was 26% which was much higher than the relative abundance of *Nitrospira* (the dominant NOB genus) which was 3.4%. These results elucidate the success of the low DO intermittent aeration in out-selecting NOBs and consequently achieving mainstream partial nitrification.

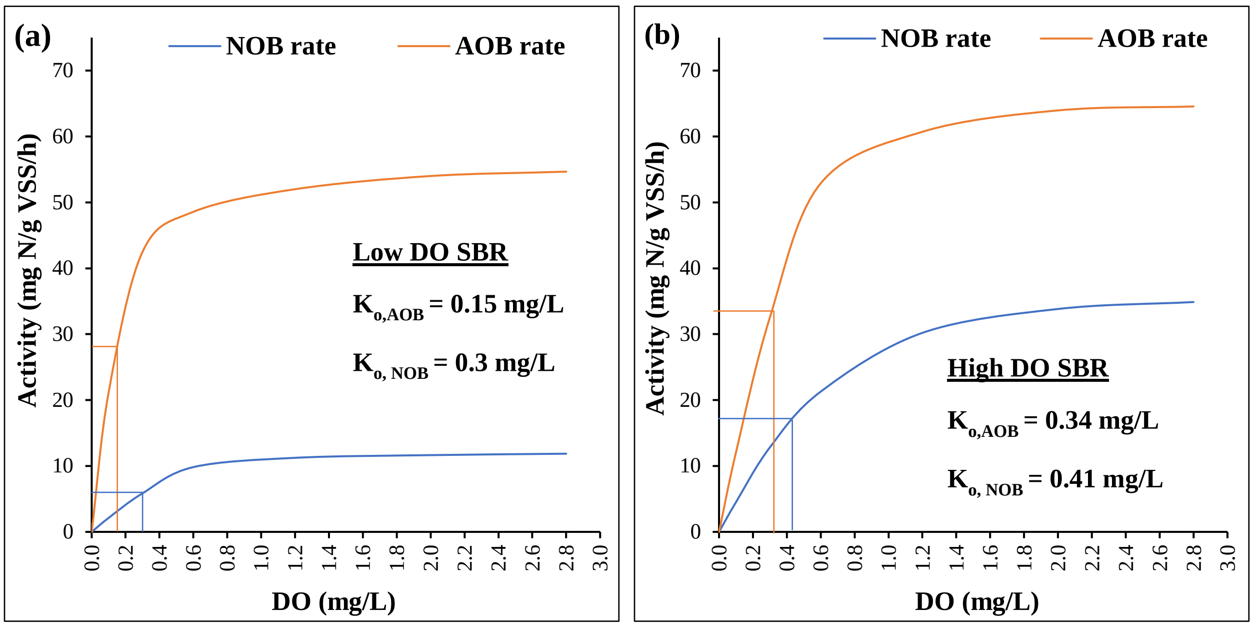

Figure 4.4: Oxygen half-saturation coefficients of AOB and NOB (K_O,AOB and K_O,NOB) in Phase I, (a) in the Low DO SBR and (B) in the high DO SBR

On the other hand, in the high DO SBR, higher values for both $K_{o,AOB,}$ and $K_{o,NOB}$ were observed which were 0.34 and 0.41 mg/L, respectively. However, since the DO concentrations in the SBR were much higher than both values, it can be concluded that the high DO did not contribute to NOB suppression which might explain the lower NAR achieved compared to the low DO SBR. Nonetheless, the NAR achieved in the high DO SBR can be attributed to the reported transient anoxia phenomena. In particular, it was reported that NOB exhibits a lag phase following anoxic periods which might result in reduced growth rates (Gilbert et al., 2014; Kornaros et al., 2010). Hence, it is hypothesized that the NOB enzymatic lag induced by the intermittent aeration pattern was responsible for the nitrite accumulation in the high DO SBR rather than NOB washout like in the low DO SBR. This hypothesis was further demonstrated by the amplicon sequencing which revealed that *Nitrospira* genus relative abundance in the high DO SBR was 20.4% higher than that of *Nitrosomonas* of 12.2%, as elucidated in **Figure 4.4**.

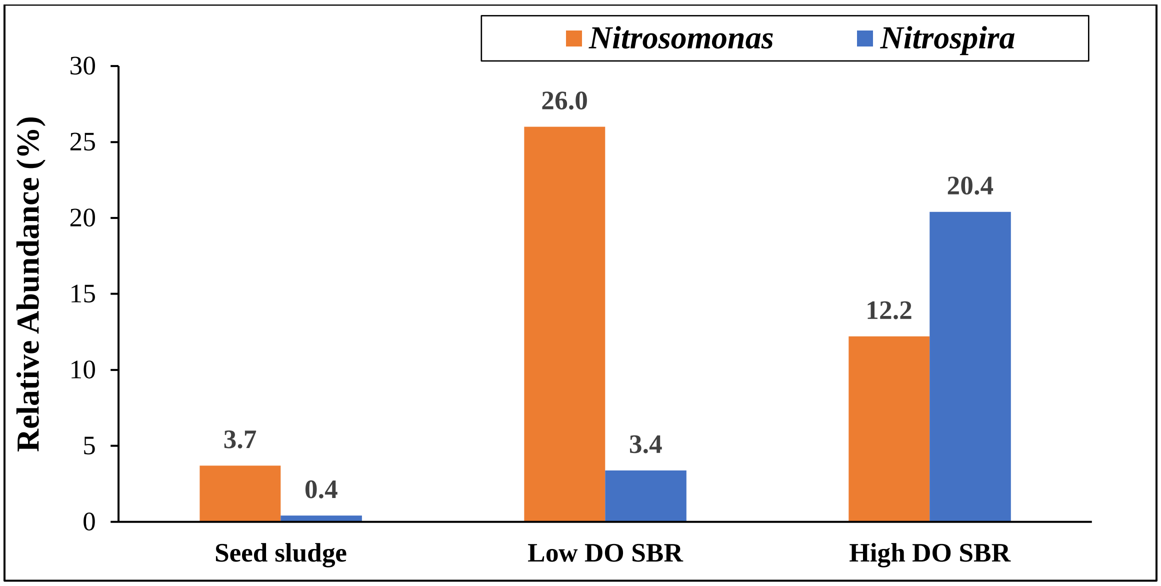

Figure 4.5: Change in relative abundances of the dominant AOB and NOB species in Phase I between the seed sludge, Low DO SBR, and high DO SBR

All in all, it was demonstrated in Phase I, that AOB activity was not significantly affected by the low DO concentrations in the aerobic phase of intermittent aeration due to its previous adaptation to the low DO. Therefore, high ammonia removal rates were maintained successfully in both the low and high DO SBRs at short aerobic SRTs. Moreover, it was proven that the combination of low DO and short SRT resulted in NOB out-selection. The mechanism of this out-selection was explained by the oxygen half saturation test which revealed that the DO operated in the low DO SBR was higher than $K_{o,AOB}$ but lower than $K_{o,NOB}$. NOB out-selection was further confirmed by the amplicon sequencing which showed that AOB genus relative abundance was almost 8 times higher than that of NOB genus. NOB out-selection led to achieving an average NAR of 85.4 ±

1.4% in the low DO SBR implying successful partial nitrification. On the other hand, it was demonstrated that NOB out-selection was not achieved in the high DO SBR, and NOB genus relative abundance was higher than that of AOB genus. Nonetheless, the transient anoxia phenomena induced by intermittent aeration was most likely responsible for the slightly lower NOB activity rates and resulted in an average NAR of 53.6 ± 10.3%.

4.3.2. The effect of DO concentrations on mainstream nitrite shunt (Phase II and III)

As explained above in Phase I, no organic carbon source was provided in the feed in Phase I to study the effect of DO concentrations on partial nitrification. However, starting from phase II, COD was introduced in the feed to provide an electron donor for the denitrification step in order to study the effect of DO concentrations on the nitrite shunt process. Phase II was operated at a target of C:N ratio of 3 and using sCOD in the form of acetate as the sole source of organic carbon. Whereas Phase III was operated at a target C:N ratio of 6 and the feed COD was composed of a mixture of sCOD in the form acetate and pCOD in the form of milk powder, starch, and yeast to mimic the composition of an A-stage effluent.

4.3.2.1. The effect of DO on the overall SBRs performance at a C:N ratio of 3 (Phase II)

In Phase II, organic carbon in the form of acetate (sCOD) was introduced in the feed for both SBRs and no pCOD source was included in the feed preparation. The average influent sCOD and ammonia concentrations in the low DO SBR were 119 ± 5 mg/L and 39.6 ± 1.6 mg N/L, respectively, which corresponded to a C:N ratio of 3.0 ± 0.2. Whereas in the high DO SBR, the average influent sCOD and ammonia concentrations were 120 ± 4 mg/L and 39.7 ± 1.2 mg N/L, respectively, which corresponded to a C:N ratio of 3.0 ± 0.1.

The introduction of influent COD resulted in an increase in the TSS and VSS in both reactors. After 2 weeks of operation, VSS and TSS in the low DO SBR increased from 692 mg VSS/L and 804 mg TSS/L to 1647 mg VSS/L and 1785 mg TSS/L, respectively. A similar pattern was observed in the high DO SBR where TSS and VSS increased from 608 mg VSS/L and 737 mg TSS/L to 1523 mg VSS/L and 1765 mg TSS/L after 2 weeks of operation, respectively. Such an increase is attributed to the enrichment of heterotrophic bacteria in the SBRs following the introduction of organic carbon in the feed in phase II unlike phase I which was operated in the absence of any organic carbon source. The presence of heterotrophs was further confirmed by the improvement in COD and TIN removal in both SBRs which increased from 24.6 and 34.3% to 83.6 and 75.7% in the low DO SBR, and from 46.1 and 22.6% to 91.3 and 62.0% in the high DO SBR, respectively, after 2 weeks of operation as shown in **Figure 4.6**. Moreover, the introduction of influent COD resulted in a decrease in ARE in the low DO SBR which dropped from 99.8% by the end of Phase I to 87.2% after 2 weeks of operation. Such a decrease can be explained by the competition between AOB and the newly enriched heterotrophs for oxygen which have affected AOB activity especially at the low DO concentrations operated in the SBR. However, afterwards, a gradual increase in ARE was observed, and after 3 weeks of operation, high ARE of up to 99.8% was restored despite the fact that no change in the operational conditions was induced in the SBR which implies AOB adaptability. The adaptation of AOB at low DO and the increase in its activity at the same the operational conditions have been previously reported in several studies in the literature (Blackburne et al., 2008; Keene et al., 2017). On the other hand, no drop in ARE was observed in the high DO SBR which indicates that the enrichment of heterotrophic bacteria did not affect AOB activity when the SBR was operated at high DO concentrations. Such results reinforces the reports suggesting that AOB might be sensitive to the change in the operational conditions when the system is operated at low DO concentrations (Regmi et al., 2022). Thus, such sensitivity can be considered as one of the drawbacks that need to be taken into account when operating the process at low DO. Despite the temporary drop in ARE, the low DO SBR was able to achieve an average ARE of 97.0 ± 4.1% during Phase II which was slightly lower than that achieved in the high DO SBR of 99.6 ± 0.6%. Nonetheless, the average effluent ammonia concentration in the low DO SBR was as low as 1.2 ± 1.5 mg N/L which indicates that AOB sensitivity did not affect the overall long term ammonia removal performance. In terms of COD removal, both SBRs were able to achieve high removal efficiency in Phase II. The average COD removal was 92.5 ± 4.8% and 94.4 ± 2.2% for the low DO SBR and the high DO SBR, respectively. Such results indicate that aerobic heterotrophic bacteria activity was not inhibited by the low DO concentrations which is attributed to their low oxygen half saturation coefficients (Arnaldos et al., 2015).

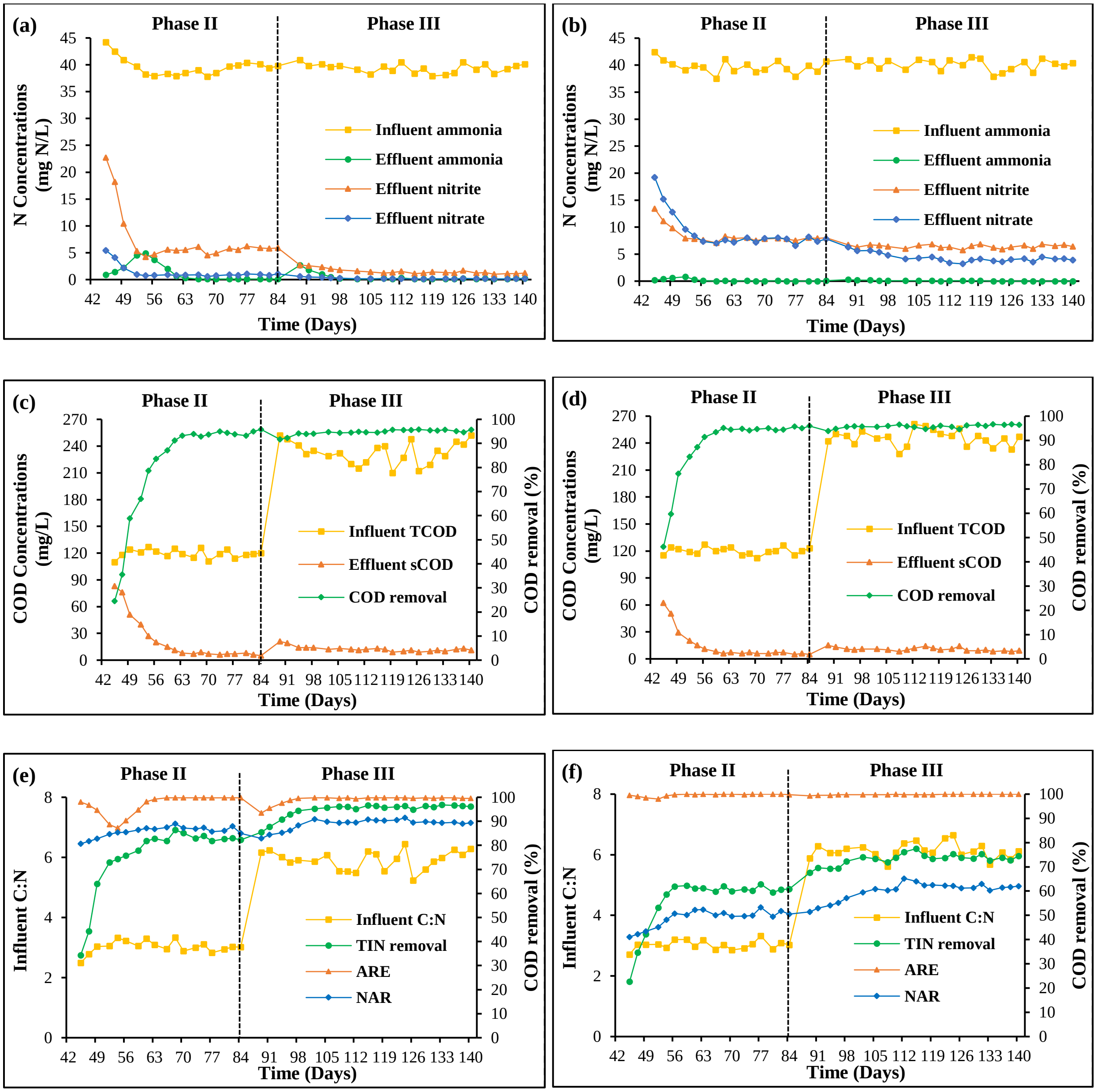

Figure 4.6: SBR performance during Phase II and III: Influent ammonia, effluent ammonia, effluent nitrite and effluent nitrate concentrations (a) in the Low DO SBR and (b) in the high DO SBR, Influent TCOD and effluent sCOD concentrations and COD removal efficiencies (c) in the Low DO SBR and (d) in the high DO, and Influent C:N, TIN removal efficiency, ammonia removal efficiency (ARE), and nitrite accumulation rate (NAR) (e) in the Low DO SBR and (f) in the high DO

On the other hand, the low DO SBR was able to achieve much higher TIN removal efficiencies than the high DO SBR despite having a similar influent C:N ratio. The average TIN removal efficiency in Phase II was 81 ± 3.8% in the low DO SBR compared to 60.3 ± 2.2% in the high DO SBR which corresponded to an average effluent TIN of 7.4 ± 1.5 mg N/L and 15.7 ± 1.0 mg N/L, respectively. The first factor that contributed to the difference in TIN removal performance between the two SBRs is the difference in nitrite accumulation rate. As shown in **Figure 4.6**, the average NAR in the low DO SBR was 85.7 ± 2.1% compared to 50.3 ± 1.9% in the high DO SBR. Since the theoretical COD requirements for denitritation (nitrite denitrification) is 1.71 g COD/g N which is less that of complete denitrification (nitrate denitrification) of 2.82 g COD/g N, it can be concluded that the higher NAR in the low SBR contributed to the higher TIN removal performance.

Another factor which can contribute to the difference in TIN removal performance is the possibility of the occurrence of simultaneous nitrification/denitrification (SND). SND occurrence can lower COD oxidation by utilizing a portion of the COD for denitrification in the aerobic phase which eventually results in higher COD utilization efficiency. SND was reported to take place in the aerobic zones at low DO levels (0.2-0.7 mg/L) (Jimenez et al., 2011). Hence, to monitor the occurrence of SND and better understand the mechanisms of TIN removal, samples were taken from both SBRs at the beginning and end of each anoxic and aerobic cycle during a typical SBR cycle. The samples were analyzed for ammonia, nitrite, nitrate, sCOD, TSS and VSS, as illustrated in **Figure 4.7.**

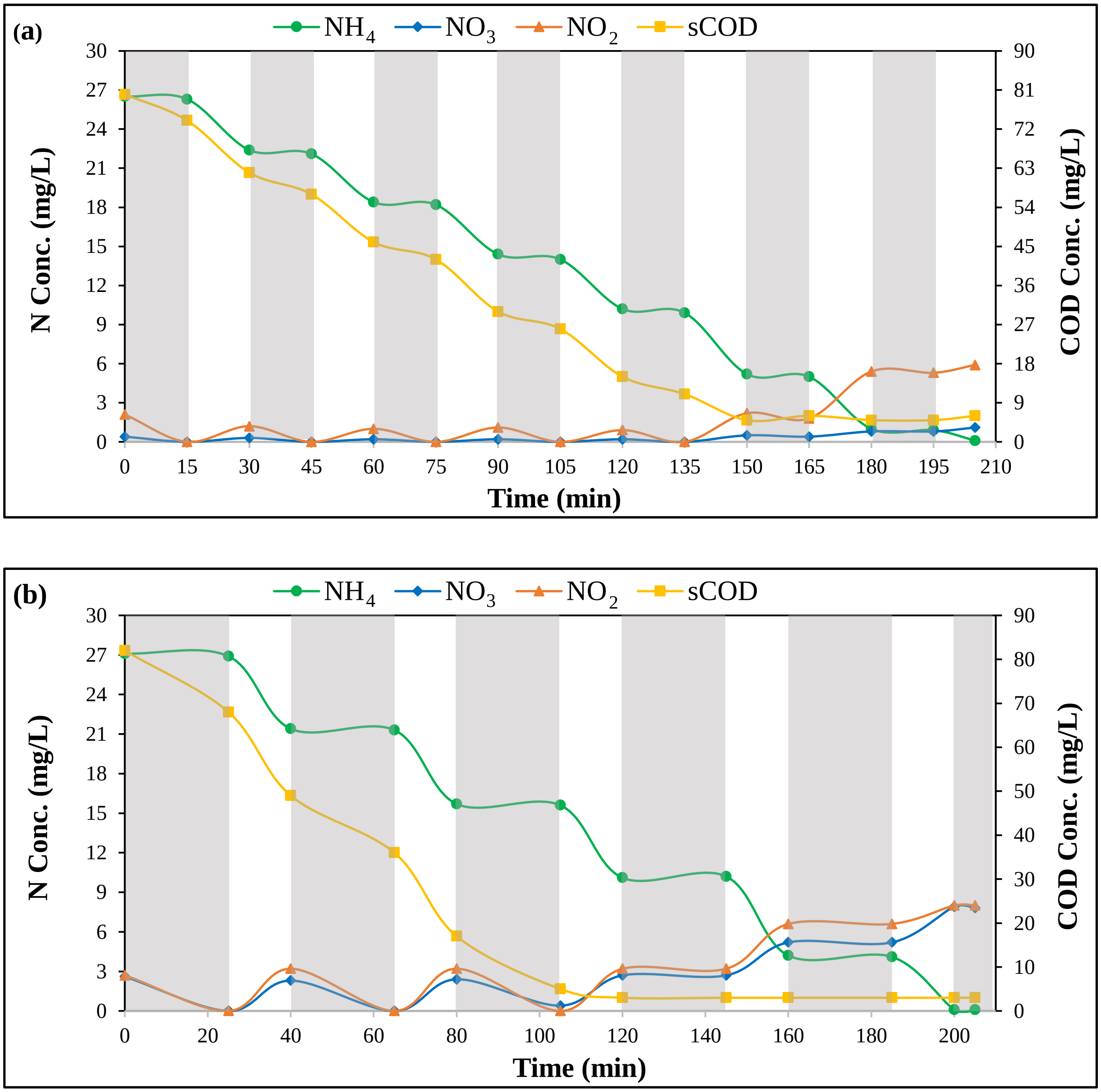

Figure 4.7: Variations of sCOD, ammonia, nitrate, and nitrite concentrations during a typical SBR cycle in Phase II, (a) in the Low DO SBR and (b) in the high DO SBR, where the shaded gray areas represent the anoxic cycles and white aeras represents the aerobic cycles

As shown in **Figure 4.7**, in the pre-anoxic phase, all the oxidized nitrogen from the previous cycle was completely denitrified by heterotrophic denitrifies using the influent sCOD as an electron donor in both SBRs. Moreover, at the start of the following aerobic phase, a big portion of the influent COD was oxidized by aerobic heterotrophic bacteria in both SBRs since aerobic heterotrophs have a clear advantage for soluble substrate which was the sole source of carbon in this phase. However, in the low SBR DO, a TIN loss of 2.4 mg/L was observed in the first aerobic cycle whereas in the high DO SBR no TIN loss was observed. Such TIN loss is attributed to the occurrence of SND which allowed for a portion of the influent COD to be used for the denitrification of the oxidized nitrogen compounds. Similar pattern was observed in the following aerobic cycles in both SBRs. Overall, during the entire cycle, 12.1 mg N/L of total inorganic nitrogen were removed during the aerobic reaction phases in the low DO SBR which indicates that SND contributed to around 30% of the total TIN removal. Whereas no TIN removal was observed in the aerobic reaction phases in the high DO SBR which implies that SND did not take place which is expected given the high DO concentrations of 1.5 mg/L (not within the reported DO for SND occurrence). The previous observation indicates that, in the high DO, SBR denitrification was only carried out in the anoxic reaction zones and that COD removal in the aerobic phases was solely through oxidation.

Therefore, it can be concluded that the higher NARs achieved in the low DO SBR compared to the high DO SBR combined with the occurrence of SND in its aerobic reaction phases led to the better TIN removal performance. To further confirm this conclusion, the ratio of COD/TIN removal was computed to compare the efficiency of the influent COD utilization for TIN removal between the two SBRs as illustrated in **Figure 4.8**. The lower COD/TIN removal ratio indicates that a bigger portion of the influent COD was used for denitrification/denitritation, and that a lower portion of the COD was oxidized. As shown in **Figure 4.8,** the average COD/TIN removal in the low DO SBR was 3.2 ± 0.5 which was lower than that achieved in the high DO SBR of 4.8 ± 0.3. Such lower COD/TIN removal demonstrates that the influent COD was more efficiently used in the low DO SBR which led to the higher TIN removal performance.

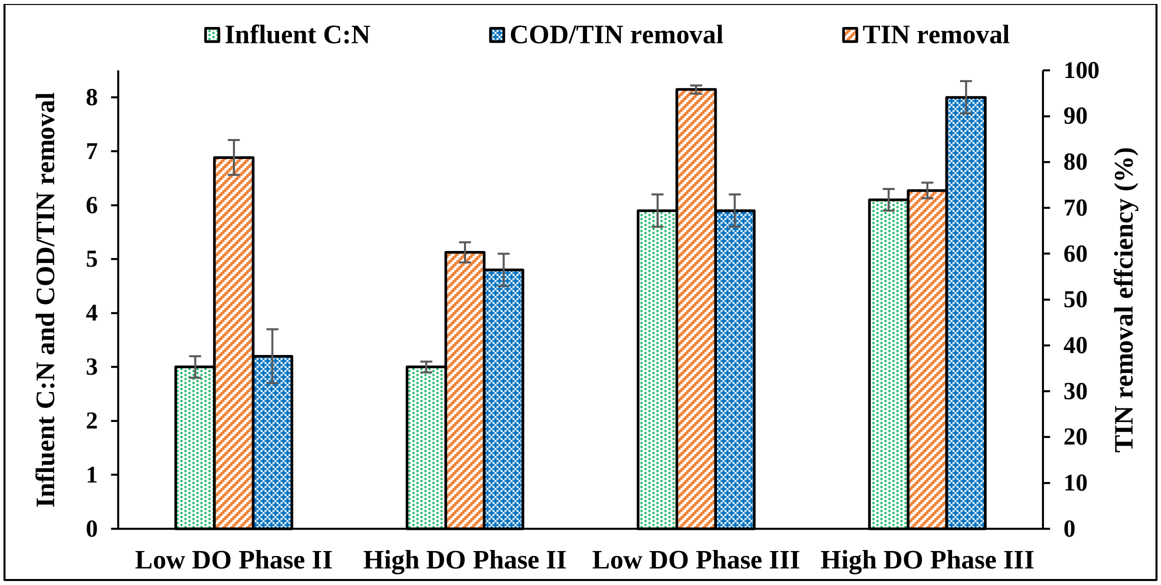

Figure 4.8: Comparison between Influent C:N, TIN removal efficiency, and COD/TIN removal in Phase II and III in the low DO SBR and the high DO SBR
(Error bars represent standard deviation)

4.3.2.2. The effect of DO on the overall SBRs performance at a C:N ratio of 6 (Phase III)

In Phase III, the influent COD was increased in both SBRs to reach a C:N of 6 and pCOD in the form of milk powder, starch, and yeast was introduced in the feed to investigate its effect on the performance. The average influent TCOD and ammonia concentrations in the low DO SBR were 233 ± 12 mg/L and 39.4 ± 0.8 mg N/L, respectively, which corresponded to a C:N ratio of 5.9 ± 0.3. The average sCOD concentrations in the low DO SBR were 115 ± 6 mg/L which represented 49.4 ± 1.6% of the total influent COD. Whereas in the high DO SBR, the average influent TCOD and ammonia concentrations were 246 ± 9 mg/L and 40.1 ± 1.0 mg N/L, respectively, which corresponded to a C:N ratio of 6.1 ± 0.2. The average sCOD concentrations in the high DO SBR were 121 ± 4 mg/L which represented 49.2 ± 1.6% of the total influent COD. The influent characteristics in Phase III are close to the reported A-stage effluent characteristics (Klaus and Bott, 2020; Rahman et al., 2019; Regmi et al., 2014).

Similar to the last phase, the increase in influent COD resulted in a slight increase in VSS and TSS concentrations in the low DO SBR from an average of 1623 ± 247 mg VSS/L and 1774 ± 251 mg TSS/L to 2317 ± 163 mg/L and 2529 ± 183 mg TSS/L, respectively. The increase in solids was accompanied by a drop in the ARE during the first couple days of operation. However, after a period of operation, AOB was able to restore its activity and improved ARE was observed until it reached back 99%. On the other hand, the high DO SBR experienced a similar increase in VSS and TSS concentrations from an average of 1518 ± 226 mg VSS/L and 1706 ± 249 mg TSS/L to 2266 ± 148 mg/L and 2516 ± 169 mg TSS/L, respectively. However, in the high DO SBR, the increase in the solids concentrations did not result in a decline in the ARE. Such results reinforce the previous conclusion of AOB sensitivity to operational conditions changes at low DO concentrations.

Furthermore, both SBRs were able to maintain high COD removal efficiencies despite the increase in influent COD concentrations. In Phase III, the average COD removal was 94.7 ± 1% and 95.7 ± 0.7% for the low DO SBR and the high DO SBR, respectively. Such results confirms the previous observation that aerobic heterotrophic bacteria activity was not inhibited by the low DO concentrations due to their low oxygen half saturation coefficients (Arnaldos et al., 2015).

In terms of TIN removal, despite the fact that the influent C:N ratio was doubled compared to the last phase, only an around 20% increase in the TIN removal efficiencies was observed for both SBRs. It is hypothesized that the higher abundance of COD concentrations resulted in higher COD oxidation. This hypothesis is in agreement with reports in the literature suggesting that higher C:N ratios might lead to lower COD utilization efficiency since any COD that is not consumed for denitrification is oxidized aerobically (Regmi et al., 2014). Nonetheless, the low DO SBR was able to achieve higher TIN removal efficiencies of 95.8 ± 0.9% compared 73.8 ± 1.7% in the high DO

SBR despite the similar influent C:N ratio in both reactors. Similar to the last phase, the average NAR in the low DO SBR (88.9 ± 2.2%) was higher than that of the high DO SBR (60.1 ± 3.5%). High NAR indicates that lower influent COD is required for nitrogen removal since denitritation requires less COD than denitratation which contributed to the higher TIN removal efficiencies achieved in the low DO SBR. Moreover, in order to quantify the contribution of SND in the TIN removal in both SBRs, samples were taken at the beginning and end of each anoxic and aerobic cycle during a typical SBR cycle in Phase III. The samples were analyzed for ammonia, nitrite, nitrate, sCOD, TSS and VSS. Similar to the last phase, all the oxidized nitrogen from the previous cycle was completely denitrified by heterotrophic denitrifies in the pre-anoxic phase using the influent sCOD as an electron donor in both SBRs, as shown in **Figure 4.9.** Over the entire cycle period, 14.9 mg N/L of the total inorganic were removed during the aerobic phases in the low DO SBR which can be attributed to SND. Therefore, it can be concluded that SND in Phase III contributed to around 40% of the total TIN removal in the low DO SBR compared to 30% in the last phase. Such an increase in SND removal can be referred to the increase in influent COD concentrations as well as the presence of pCOD. It has been previously reported that pCOD is more beneficial for denitrifiers than sCOD in the aerobic zones resulting in an increased TIN removal efficiencies (Klaus et al., 2020). In fact, pCOD is not readily available for aerobic heterotrophs consumption at the beginning of the aeration phase since prior hydrolysis is required. Such hydrolysis would allow more time for ammonia oxidation to nitrite/nitrate and consequently provide denitrifiers electron acceptors for denitrification using the hydrolyzed pCOD towards the end of the reaction phase. This hypothesis was further confirmed by the typical cycle samples. As shown in **Figure 4.9a,** total inorganic nitrogen loss was observed until the last aerobic cycle unlike in Phase II where no nitrogen loss was observed in the last two aerobic cycles as shown in **Figure**

4.8.a. Such results indicate that the presence of pCOD was an important factor in increasing SND contribution which led to the higher TIN removal in phase III compared to phase II. On the other hand, no TIN removal was observed in the high DO SBR which confirms the previous observation of absence of SND at high DO concentrations.

Moreover, the higher TIN removal in low DO SBR than the high DO SBR was reflected in the ratio of COD/TIN which averaged at 5.9 ± 0.4 and 8.0 ± 0.3, as shown in **Figure 4.8**. The lower COD/TIN achieved in the low DO SBR indicates that influent COD was used more efficiently for nitrite shunt and less COD was lost through oxidation compared to the high DO SBR. As previously discussed, the higher NAR and higher SND rates were the main contributor for the efficient utilization of influent COD for TIN removal at low DO concentrations. Nonetheless, compared to Phase II, the COD/TIN ratios achieved in this phase in both SBRs were much higher. In the low DO SBR, the COD/TIN increased from 3.2 ± 0.5 to 5.9 ± 0.4 while in the high DO SBR it increased from 4.8 ± 0.3 to 8.0 ± 0.3 which implies lower COD utilization efficiency in Phase III. Such lower efficiency can be explained by the fact that the higher influent COD concentrations in Phase III allowed for more COD oxidation in the aerobic zones and consequently less COD became available for denitrification.

Overall, in Stage III, at influent wastewater characteristics close to the reported effluent quality of A-stage systems, the low DO SBR was able to maintain an average ARE, TIN removal and COD removal of 99.0 ± 1.6, 95.8 ± 0.9, and 94.7 ± 1.0%, respectively. The effluent ammonia, nitrite, and nitrate concentrations were as low as 0.4 ± 1.0, 1.5 ± 0.5, 0.2 ± 0.1 mg N/L, respectively corresponding to a TIN concentrations of 2.1 ± 1.2 mg N/L which is an acceptable value according to the stringent discharge limits. Whereas the high DO SBR at the same influent wastewater characteristics was able to maintain an average ARE, TIN removal and COD removal of 99.8 ±

0.2, 73.8 ± 1.7, and 95.7 ± 0.7%, respectively. The effluent ammonia, nitrite, and nitrate concentrations were 0.1 ± 0.1, 6.4 ± 0.3, 4.3 ± 0.8 mg N/L corresponding to a TIN concentrations of 10.8 ± 1.0 mg N/L which is still considered high for some of the stringent discharge limits. The two SBRs were operated at low HRT of 6 h, ambient temperature of 20 ± 2ºC, short aerobic SRT of 3.1 ± 0.2 and 2.4 ± 0.3, for the low DO SBR and high DO SBR, respectively. Such operational conditions are suitable for mainstream B-stage application.

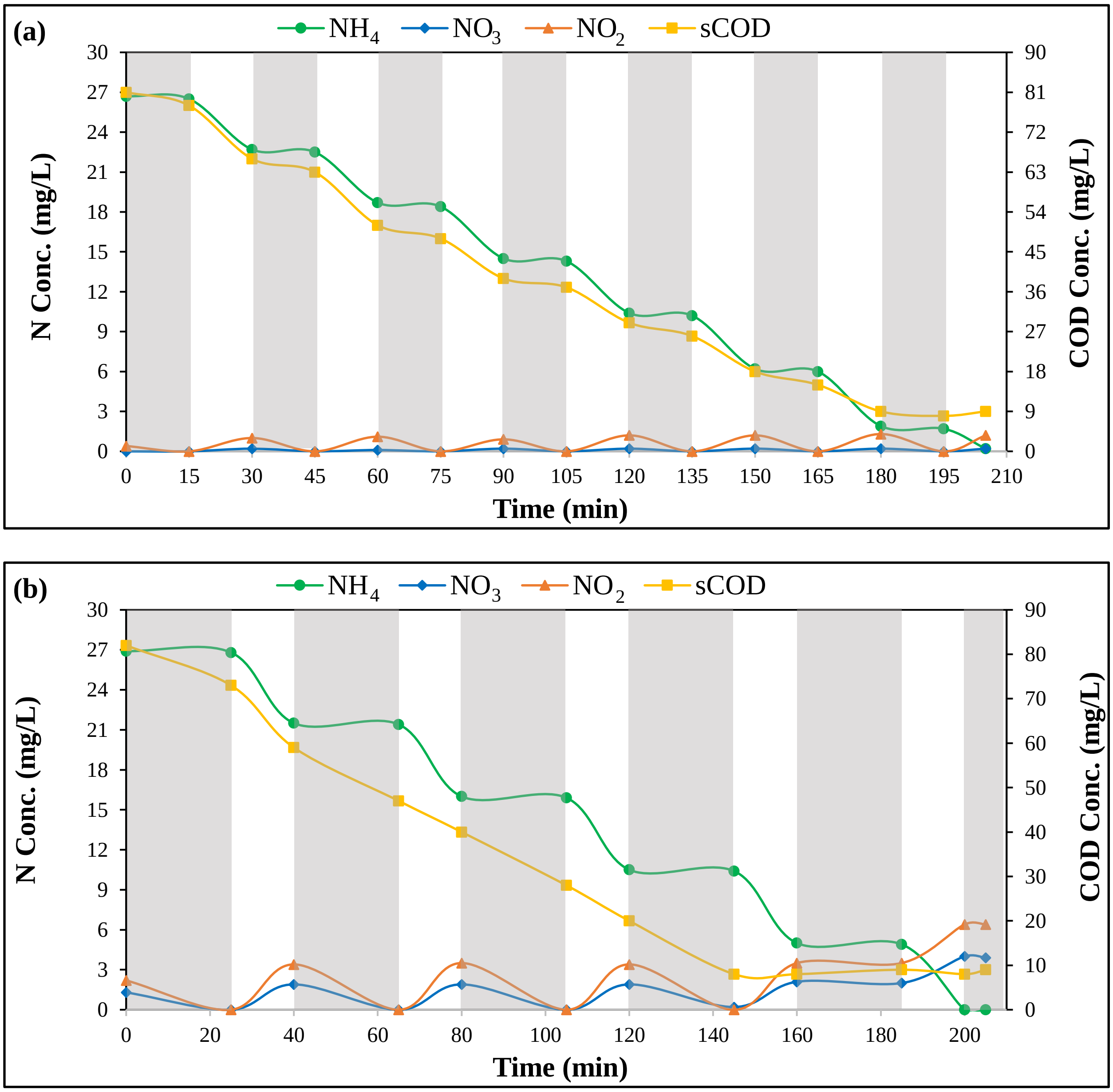

Figure 4.9: Variations of sCOD, ammonia, nitrate, and nitrite concentrations during a typical SBR cycle in Phase III, (a) in the Low DO SBR and (b) in the high DO SBR where the shaded gray areas represent the anoxic cycles and white aeras represents the aerobic cycles

4.3.3.3. The effect of DO on NOB out-selection

In this study, nitrite accumulation rate in the effluent (NAR) and ex-situ AOB and NOB maximum activity tests were used as indicators for NOB out-selection. As explained in the materials and methods section, every 2 weeks a batch test was performed to monitor AOB and NOB maximum activity in the low DO SBR **(Figure 4.10a)** and high DO SBR **(Figure 4.10b**). It was reported in the literature that the ratio between NOB maximum activity to AOB maximum activity (NOB/AOB rate) for a conventional nitrification/denitrification system (no NOB inhibition) is around 0.78 (Dold et al., 2015). Whereas lower NOB/AOB ratio (0.2-0.3) should indicate that NOB activity is suppressed and nitrite shunt is taking place in the system (Jimenez et al., 2020). Thus, the average NOB/AOB rate was calculated in Phase II and III for both SBRs, as illustrated in **Figure 4.10c**.

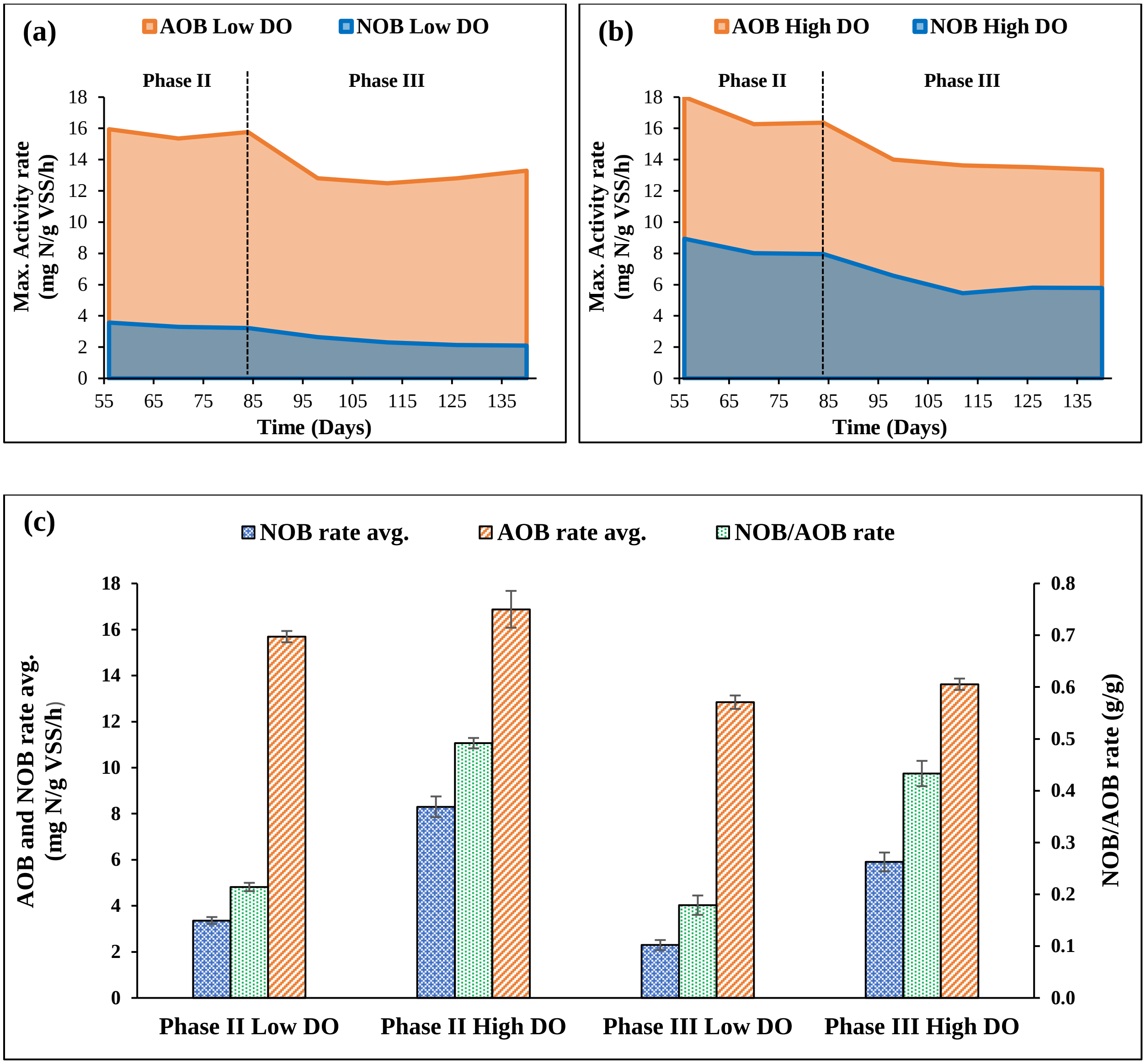

Figure 4.10: Variations in AOB and NOB maximum activities during Phase II and III, (a) in the Low DO SBR, (b) in the High DO SBR, and (c) Comparison between the average NOB maximum activity rate, the average AOB maximum activity rate, and the average ratio between NOB/AOB for the Low DO SBR and the High DO SBR (Error bars represent standard deviation)

In Phase II, the average NAR in the low DO SBR was 85.7 ± 2.1% which was higher than the average NAR in the high DO SBR of 48.9 ± 3.5%. Such results indicate that low DO SBR was more successful in suppressing NOB activity. NOB out-selection in the low DO SBR can be referred to the fact that it was demonstrated in the previous phase that $K_{o,AOB}$ in the SBR was lower than the operating DO unlike $K_{o,NOB}$ which was higher than the operating DO. The previous observation indicates that AOB are prone to maintain their activity at low DO levels, in contrast NOB is expected to experience reduced growth rates. Such reduced growth combined with the short SRT would result in NOB washout. The previous hypothesis was further demonstrated by the ex-situ activity tests which showed that NOB activity was much lower than that of AOB in the low DO SBR. As shown in **Figure 4.10,** the average NOB maximum activity rate in the low DO SBR during Phase II was 3.3 ± 0.15 mg N/g VSS/h compared to 15.7 ± 0.25 mg N/g VSS/h for AOB. Thus, the corresponding average NOB/AOB ratio was 0.21 ± 0.01 which is within the range suggested for nitrite shunt. Another factor that might have contributed to NOB suppression is the competition between denitrifying heterotrophs and NOB for nitrite. In fact, the low DO in the aerobic phase would allow denitrifying heterotrophs to use the nitrite produced by AOB as an electron acceptor for denitrification through SND. Such competition for NOB substrate would add another pressure over NOB growth in the SBR. Thus, in order to monitor heterotrophic denitrification bacteria activity, ex-situ batch activity tests were performed every 2 weeks. As shown in **Figure 4.11b**, NO_2-N denitrification rates were much higher than NO_2 oxidation rates which indicates that heterotrophic denitrifiers activity rates in the SBR were greater than NOB activity rates. Such higher rates might have provided heterotrophic denitrifiers with an advantage over NOBs in their competition for nitrite and consequently contributed to NOB out-selection. In agreement, Dold et al., 2015 suggested that the key to achieve nitrite shunt is removing nitrite produced by AOB through denitritation before NOB can oxidize it to nitrate and by doing so, less NOB would be present in the system (Dold et al., 2015).

Nonetheless, one of the main challenges of operating the system at low DO is the ability to maintain a high AOB activity in the presence of elevated organic carbon levels. Such a challenge stems from the competition between AOB and aerobic heterotrophs, which are characterized by their high oxygen affinity, over the available oxygen mostly at short SRTs. Therefore, to overcome this challenge the SBR was seeded with a nitrifying sludge previously adapted to low DO. The success of the previous strategy was demonstrated by the high AOB maximum activity rate of 15.7 ± 0.25 mg N/g VSS/h achieved in the present study. In comparison, it was reported in a pilot scale operated at low DO concentrations (0.2-0.3 mg/L) that the average AOB maximum activity was 3.5 ± 0.25 mg N/g VSS/h (Klaus and Bott, 2020). In another study operated at a DO of 0.37 ± 0.27 mg/L, the average AOB maximum activity reported of 4.1 mg N/g VSS/h was slightly higher than the previous study but still much lower than the one achieved in the present study (Regmi et al., 2022). Moreover, in a full-scale study operated at DO concentrations of 0.1-0.4 mg/L, the AOB maximum activity was as low as 0.94 mg N/g VSS/h (Jimenez et al., 2020). Therefore, it can be concluded that the previous adaptation of the nitrifying sludge allowed the system to maintain high AOB activity at low DO levels which is combined with the short aerobic SRT and the competition between heterotrophic denitrifiers and NOB for nitrite led to NOB out-selection in the low DO SBR.

On the other hand, in the high DO SBR, lower NAR were achieved in Phase II. Ex-situ batch tests showed that the average NOB maximum activity rate was 8.3 ± 0.45 mg N/g VSS/h compared to 16.9 ± 0.80 mg N/g VSS/h for AOB. Compared to the low DO SBR, AOB maximum activity in the high DO was 8% higher, however NOB maximum activity was 2.5 times higher which resulted in the lower NARs. The corresponding average NOB/AOB ratio in the high DO SBR was 0.49 ± 0.01 which is between the suggested range for efficient nitrite shunt of 0.2 and the theoretical range for full nitrification/denitrification of 0.78 which explains the 49% average NAR achieved. Such higher NOB rates can be explained by the absence of limited DO inhibition for NOBs in the high DO SBR. As demonstrated in the last phase, the DO concentrations of 1.5 mg/L were much higher than both $K_{o,AOB}$ and $K_{o,NOB}$. As discussed previously, the rationale behind operating at high DO levels was maintaining high AOB activity rates through eliminating any inhibition effect on AOB due to the limited DO conditions. However, in this study, the previous adaptation of the nitrifying sludge to low DO was proven successful in reducing the inhibition effect of limited oxygen conditions which was demonstrated through the slight difference between AOB maximum activity in the high and Low DO SBRs. Nonetheless, despite the higher NOB activities in the high DO SBR, AOB activity rates were almost double those of NOB which demonstrates that NOB activity was suppressed to lower extents. Such suppression can be explained by the previously reported NOB lag phase following anoxic periods, i.e., transient anoxia (Gilbert et al., 2014; Kornaros et al., 2010). Thus, it can be argued that cyclic anoxic/aerobic provided by the intermittent aeration regime has resulted in reduced NOB growth rates. Moreover, as shown **Figure 4.7b**, nitrite produced by AOB in the aerobic phases was consumed by heterotrophic denitrifiers in the following anoxic zones using the influent COD. The consumption of nitrite by denitrifiers resulted in less substrate available for NOBs in the following aerobic cycle which contributed to the lower NOB rates. Thus, it can be concluded that in the high DO SBR, transient anoxia, short aerobic SRT and nitrite consumption by heterotrophic denitrifiers were the driving force for NOB suppression. Similar conclusion was suggested in a mainstream CSTR study achieving 60 ± 22% NAR using intermittent aeration operated at a DO concentrations of 1.6 mg/L (Regmi et al., 2014).

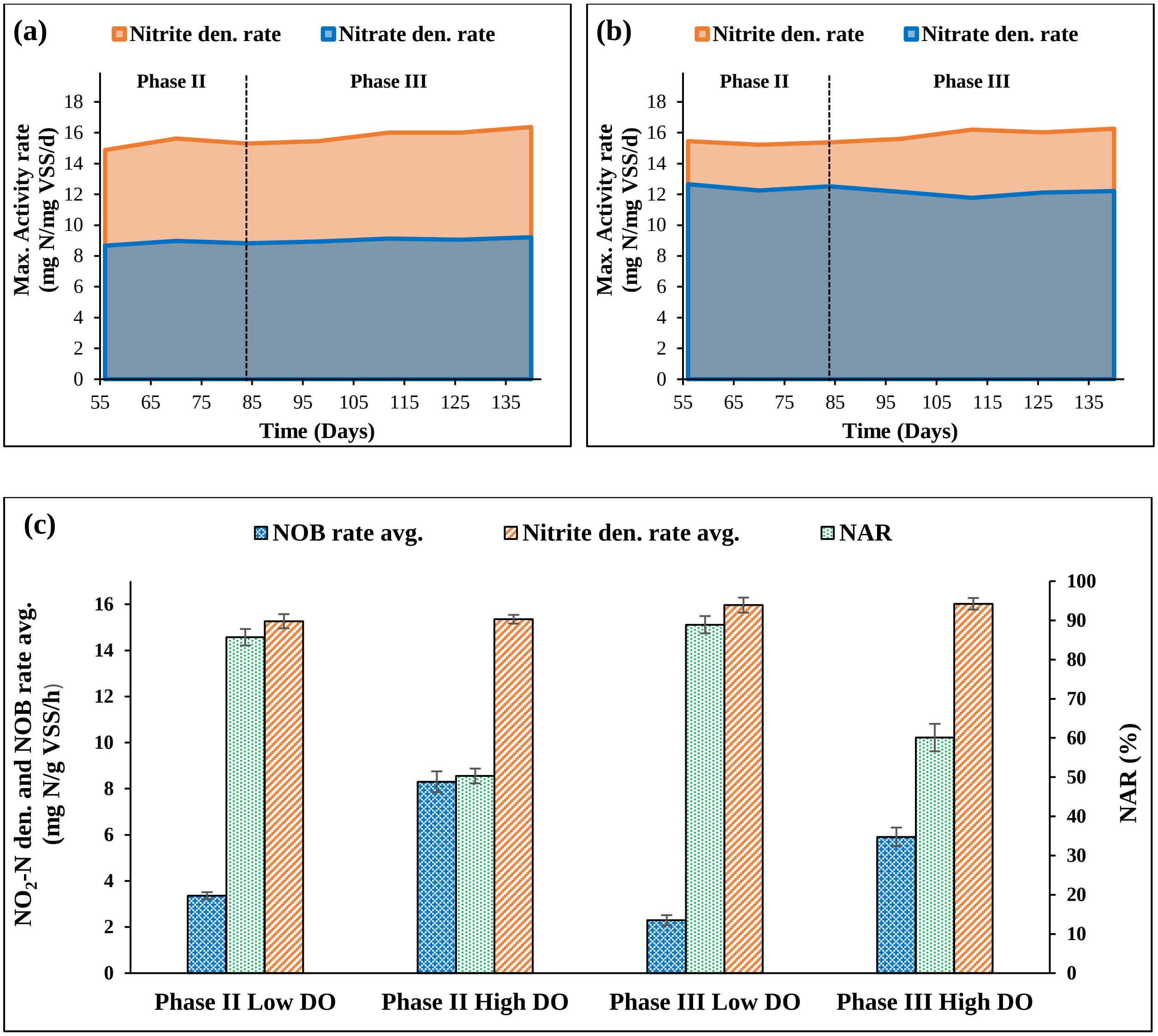

Figure 4.11: Variations in nitrite and nitrate denitrifiers maximum activities during Phase II and III, (a) in the Low DO SBR, (b) in the High DO SBR, and (c) Comparison between the average NOB maximum activity rate, the average nitrite heterotrophic denitrifiers maximum activity rate, and the average nitrite accumulation rates (NAR) for the Low DO SBR and the High DO SBR (Error bars represent standard deviation)

In Phase III, the increase in C:N ratio from 3 to 6 and introduction of pCOD resulted in an increase in NARs in both SBRs. The NAR increased by 5% in Phase III compared to Phase II in the low DO SBR while it increased by 20% in the high DO SBR. As shown in **Figure 4.10a**, the average AOB maximum activity in the low DO SBR decreased by 22%. This decrease in activity might be referred to the increase in VSS concentrations from 1623 ± 247 mg/L in phase II to 2317 ± 163 mg/L in phase III indicating the enrichment of heterotrophic bacteria following the higher abundance of influent COD. However, the average NOB maximum activity experienced a more significant drop of 26%. The average NOB/AOB ratio in this phase decreased to 0.18 compared to 0.21 in phase II which explains the jump in NARs from 85.7 ± 2.1% to 88.9 ± 2.2%. Whereas in the high DO SBR, a 14% decrease in the average AOB maximum activity was observed accompanied by an increase in VSS concentrations from 1518 ± 226 mg/L in phase II to 2266 ± 148 mg/L following the increase in C:N ratio. While the average NOB maximum activity dropped by 30% which resulted in a decrease in the average NOB/AOB ratio from 0.49 in phase II to 0.43 in phase III, as shown in **Figure 4.10b**. Such results imply that higher NOB activity suppression was achieved in Phase III compared to Phase II in both SBRs. It is hypothesized that this higher suppression was driven by the presence of pCOD in the feed. To elaborate more, pCOD requires pre-hydrolysis before it can be consumed by either aerobic heterotrophs for COD oxidation or heterotrophic denitrifiers for nitrite/nitrate reduction. In contrast, acetate, which was provided in the feed as the main source of sCOD in this study, is the simplest form of COD. Thus, it is consumed at fast rates during the first couple anoxic and aerobic cycles resulting in the absence of COD in the last anoxic and aerobic cycles. Such absence of COD affects the NAR performance in both the low and high DO SBRs. In the high DO SBR, the lack of available COD in the anoxic phase prevents the denitrification of the produced nitrite which results in higher substrate concentrations for NOB in the following aerobic cycle, as shown in **Figure 4.7b.** In addition to the abundance of NOB substrate, the lack of COD in the low DO SBR eliminates the competition between heterotrophic denitrifiers and NOB for the nitrite produced by AOB in the aerobic phase through SND, as shown in **Figure 4.7a**. However, in the case of pCOD presence, the required prior hydrolysis helps provide denitrifiers with an electron donor further down towards the end of the reaction phase. Hence, as shown in **Figure 4.9b**, less nitrite was available in the last aerobic cycles in phase III compared to phase II which resulted in the lower NOB rates and consequently higher NARs. Similarly, it can be observed in **Figure 4.9a** that SND took place in the last aerobic cycles unlike in phase when no COD was available for SND to occur. Therefore, it can be concluded that the presence of pCOD can elongate the length of the competition between heterotrophs and NOB for nitrite resulting in lower NOB rates. The previous conclusion was in agreement with the reported positive correlation between influent pCOD and NAR in a B-stage pilot scale fed with A-stage effluent (Klaus et al., 2020).

4.4. Conclusion

In this study, two SBRs were operated at similar operational conditions but at two different DO levels in the aerobic phases of intermittent aeration, (i) DO concentrations of 1.5 mg/L (high DO SBR), and (ii) DO concentrations of 0.2 mg/L (low DO SBR). In the first phase, the effect of DO concentrations on partial nitrification was studied in the absence of any feed organic carbon. It was demonstrated that the previous adaptation of the nitrifying sludge to low DO resulted in comparable AOB rates in the two SBRs. Consequently, almost complete ammonia oxidation was achieved in the two SBRs at short aerobic SRT. Such results suggest that previous adaptation can play an important role in maintaining high AOB rates in low DO concentrations. Moreover, at low DO concentrations, NOB out-selection was achieved which was confirmed by the amplicon sequence data revealing that the relative abundance of the dominant NOB genus was 3.4% compared to 26% for that of AOB. As such, the low DO SBR was able to maintain an average NAR of 85.4 ± 1.4%. It was concluded that operating the reactor at DO levels of 0.2 mg/L higher than $K_{o,AOB}$ of 0.15 mg/L but lower than $K_{o,NOB}$ of 0.3 mg/L resulted in low NOB rates which combined with the short aerobic SRT led to NOB out-selection. On the other hand, high DO levels did not result in NOB washout, since the relative abundance of its dominant genus was 20.4% which was higher than that of AOB of 12.2%. However, the enzymatic lag induced by the intermittent aeration pattern resulted in lower NOB rates compared to AOB rates in the high DO SBR. As such, the high DO SBR was able to maintain an average NAR of 53.6 ± 10.3%.

In the following phases, organic carbon was provided in the feed which resulted in high inorganic nitrogen removal in both SBRs. Overall, the low DO SBR was able to maintain an average ARE, TIN removal and COD removal of 99.0 ± 1.6, 95.8 ± 0.9, and 94.7 ± 1.0%, respectively, at a C:N of 5.9 ± 0.3. Whereas the high DO SBR was able to maintain an average ARE, TIN removal and COD removal of 99.8 ± 0.2, 73.8 ± 1.7, and 95.7 ± 0.7%, respectively, at a C:N of 6.1 ± 0.2. The two SBRs were operated at low HRT, ambient temperature, short aerobic SRT which are suitable for mainstream application. The higher NARs and SND rates achieved in the low DO SBR were the driving force for the higher TIN removal efficiencies. The average effluent TIN concentrations in the low DO SBR were 2.1 ± 1.2 mg N/L, which is an acceptable value according to the stringent discharge limits, compared to 10.8 ± 1.0 mg N/L in the high DO SBR. Hence, these results suggest that nitrite shunt using intermittent aeration at low DO concentrations can be a suitable B-stage process.

4.5. References

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Chapter 5: Conclusions and Future recommendations

5.1. Conclusions and key findings

To facilitate the shift from energy intensive wastewater treatment plants (WWTPs) to efficient water resources recovery facilities (WRRFs), the adsorption/bio-oxidation (A/B) configuration has been proposed for carbon and nutrients removal. The A/B scheme is compatible with the existing infrastructure which indicates that no major changes are required for its implementation in the next generation wastewater facilities. However, such an implementation is still hindered by two major challenges facing B-stage, (i) NOB out-selection at mainstream conditions and (ii) the efficient utilization of influent COD for denitritation for mainstream nitrite shunt process.

Therefore, in this dissertation a novel kinetic adaptation-based strategy to engineer the microbial community has been proposed to address the above-mentioned challenges. The kinetic-adaptation strategy adopted lowering the DO concentration in a stepwise fashion and transitioning gradually from side stream to mainstream conditions allowing the biomass sufficient acclimation period. It was hypothesized that such an acclimation period would allow AOB enough time to adapt to each change of operational conditions and consequently can maintain its activity in unfavorable growth conditions. While the induced unfavorable growth conditions, i.e., low DO concentrations and low substrate concentrations would result in reduced NOB growth which is combined with the short aerobic SRT can lead to NOB washout. The implementation of the kinetic-adaptation strategy and its underlying mechanisms was demonstrated and investigated for more than 400 days. In result, an ammonia removal efficiency of 99.4 ± 0.4% and nitrite accumulation rate of 87.4 ± 0.6% under low DO levels of 0.1–0.2 mg/L was reached. Successful NOB out-selection was further confirmed by amplicon data revealing that the relative abundance of NOB genus in the system at mainstream conditions was 3.7% compared to 40% for the AOB genus.

Afterwards, the potential of using the kinetically engineered microbial community to perform mainstream nitrite shunt as energy and resources efficient B-stage was investigated using different aeration strategies. As such, three systems were operated using different influent carbon to nitrogen (C:N) ratio and different influent organic carbon fractionation, (i) continuous low DO aeration system, (ii) low DO intermittent aeration system, and (iii) high DO intermittent aeration system. As shown, in **Table 5.1,** all three systems were able to achieve high total inorganic nitrogen (TIN) removal at low C:N ratios. Investigations revealed that previous adaptation of the kinetically engineered nitrifying community to low DO played a key role in maintaining high ammonia oxidation rates at short aerobic SRT in the low DO continuous and intermittent aeration systems. Moreover, the combination of adaptation strategy, high denitrifiers activity rates, and short aerobic SRT (below 4 days) was successful in maintaining NOB suppression in mainstream conditions. NOB out-selection/activity-suppression resulted in lower ammonia oxidation to nitrate and hence, lower organic carbon was required for nitrogen removal which led to the high TIN removal at low C:N ratio. Comparing the three systems, the low DO intermittent aeration system was able to achieve the highest TIN removal which was slightly higher than the continuous aeration low DO system. However, here it is worth noting that continuous aeration is considered easier and more practical to operate compared to intermittent aeration due to the limitations of aeration blowers. On the other hand, the high DO intermittent aeration system, in addition to its lower TIN removal performance, would require higher energy requirements for its full-scale implementation. Thus, the results of this dissertation suggest that employing the developed kinetic-adaptation strategy at low DO concentrations either through continuous or intermittent aeration can be a powerful tool to overcome the major bottleneck of NOB out-selection towards the full-scale implementation of nitrite shunt as a B-stage process.

Table 5.1: Comparison between the mainstream nitrite shunt performance achieved in this dissertation using different aeration strategies and literature studies

Reference	**(Regmi et al., 2022)**	**(Klaus and Bott, 2020)**	**(Klaus and Bott, 2020)**	**This Dissertation**	**This Dissertation**	**This Dissertation**
Aeration strategy	Continuous	Intermittent	Intermittent	Continuous	Intermittent	Intermittent
DO (mg/L)	0.37 ± 0.27	0.3	1.5	0.2	0.2	1.5
Aerobic SRT (Days)	7.7 ± 1.4	8.8 ± 1.0	7.3 ± 0.6	3.7 ± 0.4	3.1 ± 0.2	2.5 ± 0.2
Influent C:N (-)	6.0 ± 1.6	12.6	13.6 ± 0.9	6.1 ± 0.3	6.1 ± 0.2	5.9 ± 0.3
TIN removal (%)	76	86.0 ± 2.9	89.4 ± 2.1	93.2 ± 1.6	95.8 ± 0.9	73.8 ± 1.7
ARE (%)	95	> 97	> 97	99.2 ± 0.7	99.0 ± 1.6	99.8 ± 0.2
NAR (%)	-	<5	<5	89.9 ± 2.1	88.9 ± 2.2	60.1 ± 3.5
Effluent TIN (mg/L)	11.7	5	3.7	2.8 ± 0.9	2.1 ± 1.2	10.8 ± 1.0

In Chapter 1, four objectives have been developed to tackle the identified research gaps. Thus, in the following sections, the specific conclusion and key lessons learned in the pursuit of these objectives will be discussed.

5.1.1. Developing a novel strategy to suppress NOB activity at mainstream conditions

To address this objective, a novel kinetic adaptation strategy was presented in Chapter 2. This study is up to our knowledge the first to present a robust strategy to maintain NOB out-selection at mainstream conditions. NOB out-selection at mainstream conditions is a major bottleneck hindering the implementation of the shortcut BNR systems in the mainstream lines of full-scale WWTPs. The key findings of this study are summarized as follows:

- The developed novel kinetic-adaptation strategy allowed the SBR to maintain an ammonia removal efficiency of 99.4 ± 0.4% and a stable nitrite accumulation ratio of 87.4 ± 0.6% at low DO concentrations of 0.1-0.2 mg/L at mainstream conditions.
- It was observed that AOB exhibited sensitivity to the change in the operational conditions. In fact, each drop in DO and nitrogen loading rate (NLR) was followed by a decrease in

AOB activity. However, after a period of operation under the same conditions, AOB was able to adapt to the induced change and restore back its high activity. Thus, the kinetic adaptation strategy allowed AOB an acclimation period after each DO and NLR change to adapt to the new conditions.

- The kinetic-adaptation strategy provided AOB an advantage over NOB which resulted in higher AOB rates than NOB during all the different partial nitrification phases. At the mainstream phase, AOB maximum activity was five times higher than that of NOB which resulted in the high NAR.
- It was demonstrated that operating the SBR at DO concentrations higher than the oxygen half saturation coefficient of AOB ($K_{o,AOB}$) but lower than $K_{o,NOB}$ played a pivotal role in suppressing NOB activity. The acclimation period after each DO change provided by the adaptation strategy resulted in a decrease in $K_{o,AOB}$ and by the last phase (mainstream phase), $K_{o,AOB}$ was 0.16 mg/L compared to 0.44 mg/L in the first phase. Whereas NOB was not able to exhibit the same adaptation as AOB and its Ko in the mainstream phase was 0.3 mg/L which was higher than the DO inside the SBR of 0.1-0.2 mg/L which resulted in its reduced activity.
- The kinetic adaptation strategy combined with the short SRT resulted in successful NOB washout. Amplicon sequencing data revealed that the relative abundance of *Nitrospira* (the dominant NOB genus in the SBR) in the mainstream phase was 3.7% compared to 9.9% in the complete nitrification phase. While *Nitrosomonas'* relative abundance in the mainstream phase (the dominant NOB genus in the SBR) was 40% compared to 6.8% in the complete nitrification phase. Such results confirm the success of the adaptation strategy to out-select NOB at mainstream conditions.

5.1.2. Evaluating the nitrite shunt process as a B-stage in the A/B stage scheme for low C:N wastewater using continuous aeration at low DO concentrations

Chapter 3 presented the results of operating a mainstream nitrite shunt SBR as a B-stage treating a low carbon to nitrogen (C:N) wastewater to mimic the effluent of an A-stage system. The SBR was operated for 140 days using continuous aeration at low DO concentrations (0.2 mg/L). The key findings of this chapter are summarized as follows:

- The SBR was able to maintain a total inorganic nitrogen (TIN) removal of 93.2 ± 1.6%, an ammonia removal efficiency (ARE) of 99.2 ± 0.7%, and a COD removal of 94 ± 0.1% at a C:N ratio as low as 6.1 ± 0.3 under low DO concentrations of 0.2 mg/L. The influent TCOD in the SBR was 238 ± 11 mg/L and the sCOD fraction represented 49.5 ± 2.8% of the TCOD which is within the range reported for A-stage effluent characteristics. The total SRT, aerobic SRT, HRT, and temperature were 6.3 ± 0.7 days, 3.7 ± 0.4 days, 6 h, and 20 ± 2 ºC, respectively, which are suitable operational conditions for application in mainstream lines.
- The SBR was able to maintain an effluent ammonia, nitrite, nitrate, and COD of 0.3 ± 0.1 mg N/L, 0.26 ± 0.1 mg N/L, 2.28 ± 0.6 mg N/L, and 14 ± 3 mg/L, respectively. Such effluent characteristics are below the stringent discharge limits.
- It was concluded that the previous adaptation of the nitrifying community was a crucial factor to maintain high AOB activity at low DO concentrations and by consequence achieving high ammonia oxidation rates at such oxygen limited conditions.
- It was demonstrated that the low DO concentrations allowed for the occurrence of simultaneous nitrification/denitrification (SND) in the aerobic phase. SND provided heterotrophic denitrifiers the chance to outcompete NOB for the nitrite generated through

the oxidation of ammonia by AOB. In fact, microbial activity test revealed that heterotrophs nitrite denitrification rates were on average 7 times higher than NOB nitrite oxidation rates during all the different phases of operations.

- The competition between heterotrophic denitrifiers and NOB for nitrite resulted in reduced NOB growth which is combined with the short aerobic SRT led to NOB washout. In fact, the amplicon sequencing data revealed that relative abundance of *Nitrospira* (the dominant NOB genus) ranged between 0.25-0.41% during the different phases of operation.
- The success in out-selecting NOBs resulted in achieving high NAR of 88.4 ± 3.44%. Such high NAR led to less required COD for nitrogen removal and consequently high TIN removal was achieved at low C:N ratio. In fact, the ratio between the COD removal to TIN removal was as low as 6.1 ± 0.3 which indicates high COD utilization efficiency especially since the SBR was operated at continuous aeration and no internal mixing regime was applied.
- It was demonstrated that SND was the main route for oxidized nitrogen compounds removal rather than denitrification in the pre-anoxic phase due to the limited nitrite and nitrate concentrations in the pre-anoxic phase.

5.1.3. Studying the effect of C:N ratio and COD fractionation on the nitrite shunt process

In order to address this object, the nitrite shunt SBR in Chapter 3 was operated at three phases with different influent C:N ratios and COD fractionation. The results obtained should help control the A-stage effluent to provide an optimum B-stage influent towards efficient carbon utilization. The key findings are summarized below:

- The increase in C:N ratio from 3 to 6 resulted in an increase in TIN removal. However, such an increase was accompanied by an increase in the COD/TIN removal which indicates lower influent COD utilization efficiency. Thus, it was concluded that maintaining an adequate influent C:N ratio to balance between TIN removal and COD utilization efficiency is a key to achieve efficient nitrite shunt process.

- The change in C:N ratio did not result in a significant effect on NOB out-selection and consequently no correlation between C:N and NAR was observed.
- The increase in the pCOD fraction in the influent COD resulted in an increase in TIN removal as well as an increase in the COD utilization efficiency. It was concluded that the pre-required pCOD hydrolysis led to less available substrate for aerobic heterotrophs (rbCOD) at the beginning of the aerobic phase which resulted in less influent COD oxidation. Furthermore, the hydrolysis of pCOD provided an electron donor for heterotrophic denitrifiers to reduce the oxidized nitrogen compounds further down in the aerobic phase.
- The influent pCOD correlated positively with NAR. It was concluded that the time required for pCOD hydrolysis in the aerobic phase elongated the length of the competition between heterotrophic denitrifiers and NOB which resulted in less nitrite oxidation by NOB at the end of the aerobic phase.

5.1.4. Evaluating the nitrite shunt process as a B-stage in the A/B stage scheme for low carbon to nitrogen (C:N) wastewater using intermittent aeration at different DO concentrations

To tackle this objective, two SBRs were operated using intermittent aeration as the aeration strategy at two DO levels in the aerobic phase, (i) DO levels of 0.2 mg/L (Low DO SBR) and (ii) DO levels of 1.5 mg/L (High DO SBR). The two systems were operated for 140 days at different C:N ratios and COD fractionation. The key findings are summarized below:

- The two SBRs were able to achieve high ammonia during all the different phases of operation. In the low DO SBR, the average ARE ranged between 97-99.7% compared to 99.6-99.9% in the high DO SBR. Such results demonstrate that AOB was not inhibited by the low DO concentrations and was able to compete with heterotrophic bacteria despite the limited oxygen conditions. This was further confirmed by ex-situ tests which revealed that AOB maximum activity rates in the low DO SBR were close to that in the high DO SBR. Such high AOB rates were referred to the previous adaptation of the nitrifying community to the low DO concentrations provided by the kinetic adaptation strategy.
- It was demonstrated that AOB was more sensitive to the change in operational conditions when the system is operated at low DO concentrations. This conclusion was driven by the observation of a drop in ammonia removal rates in the first couple of days following a change in the operational conditions in the low DO SBR. However, after a period of operation, AOB was able to adapt to the new conditions and restore its high ammonia oxidation rates. Such a drop in the ammonia removal rates was not observed in the high DO SBR which reinforces the conclusion of AOB sensitivity at low DO.
- The two SBRs were able to maintain high COD removal efficiencies during the different phases of operation. The average COD removal in the low DO SBR ranged between 92.5-94.7% compared to 94.4-95.7% in the high DO SBR. Such results demonstrate that aerobic heterotrophs activity was not inhibited by the low DO which can be referred to their reported high oxygen affinity.
- The low DO SBR was able to achieve higher NARs than the high DO SBR during the different phases of operation. The average NARs in the low DO SBR ranged between 85.4-88.9% compared to 50.3-60.1% in the high DO SBR. The difference in the achieved NARs

in the two systems was referred to the difference in NOB inhibition mechanisms. It was demonstrated that in the low DO the combination of low DO concentrations, SND in the aerobic phases, nitrite reduction in the anoxic phases, short aerobic SRT and transient anoxia resulted in NOB washout. Whereas in the high DO SBR, amplicon sequencing data revealed that the relative abundance of NOB genus was higher than that of AOB which indicated that NOB was not washed out. Such dominance of NOB in the system was referred to the observation that the DO set point in the high DO SBR was higher than $K_{o,NOB}$ which combined with the absence of competition for the nitrite in the aerobic phases resulted in higher NOB growth rates. However, it was demonstrated that the combination of nitrite reduction in the anoxic phases, short aerobic SRT and transient anoxia resulted in NOB activity suppression which led to lower nitrite accumulation

- The low DO SBR was able to achieve higher TIN removal rates than the high DO SBR at different influent characteristics. At a C:N ratio of 3, the low DO SBR was able to maintain an average TIN removal of 81 ± 3.8% compared to 60.3 ± 2.2% in the high DO SBR. Moreover, the increase in the influent C:N ratio to 6 which is close to the reported A-stage effluent ratio resulted in increase in TIN removal to 95.8 ± 0.9% and 73.8 ± 1.7%. The higher TIN removal achieved in the low DO SBR was referred to the higher NARs and the occurrence of SND in the aerobic phases which resulted in lower COD oxidation.

5.2. Future work direction recommendations

Considering the potential of the implementation of nitrite shunt process in the A/B scheme, several future research points are suggested to continue the investigations of the current work:

- This dissertation focused on investigating and optimizing the nitrogen removal performance of the nitrite shunt process. However, nitrite shunt has also shown prominent performance for phosphorus removal (Jimenez et al., 2020; Regmi et al., 2022; Roots et al., 2020, 2019). In **Chapter 3 and 4**, amplicon sequence data revealed that the dominant genus in the system was *Dechloromonas* which has been associated with phosphorus removal and assumed to be a denitrifying phosphate accumulating organisms (DPAO) with high denitrification and phosphorus accumulating ability (Liu et al., 2016; Stokholm-Bjerregaard et al., 2017). Such an observation suggests that the operational conditions applied in the systems can be suitable for the co-enrichment of phosphorus removal organisms. However, further studies should be performed to investigate and optimize the comibined nitrogen and phosphorus removal performance of the developped systems.
- In **Chapter 3,** the SBR was operated in an anoxic-aerobic configuration, however, recent studies have reported post-anoxic denitrification occuring above the expected endogenous rates despite the absence of any organic carbon source (Anne Klaus et al., 2019; Printz et al., 2019; Regmi, 2022; Zhang et al., 2020). However, the mechanisms driving this post-anoxic denitrification are not understood yet as well as which are the reposnible microorganims. In **Chapter 3**, the typical cylces tests showed the occurrence of denitrification despite no the lack of available electron donor. Morever, the amount of sCOD removed in the pre-anoxic phases was higher than the theoretical denitrfication equivalents which can be attributed to internal storage. Such observation might be in

support to the post-anoxic denitrification studies, however no deep investigation was carried out in this disseratation. Thus, it is suggested that future studies can investigate the addition of a post anoxic zone to the developed system in Chapter 3 and monitor the potential of improved performance at lower C:N ratios.

- There has always been concern regarding the effect of low DO on the sludge settleabilty. It has been suggested that the low DO might results in the proliferation of filamentous bacteria especially at low SRTs which can lead to poor settleability (Henze et al., 2008). In the studies performed in this dissertation, good sludge settleability was observed except for a short period in Phase II in the low DO continuous SBR (**Chapter 3**). In this phase, the C:N ratio was increased from 3 to 6 with sCOD being the sole organic carbon source. After a period of operation, poor settleability was observed which led to a deterioration in the system performance. Further investigations showed that the sludge had a significantly high SVI_{30} values of 577 ± 21 mL/g. However, here it is worth mentioning that at the same period, an error in sludge wastage calculations resulted in significantly low aerobic SRT (below 2 d). However, it was not clear whether the poor settleability was due to the drop in SRT or due to the presence of such high soluble COD concentrations. Therefore, further investigation should be performed to study the effect of low DO on sludge settleability at different operational conditions and influent characteristics.
- As discussed in **Chapter 4**, several aeration strategies have been proposed to control the aeration pattern in intermittent aeration including (i) DO set point control, (ii) Ammonia based aeration control (ABAC), (iii) Ammonia vs NOx (AVN) control. In the study performed in **Chapter 4**, DO set point control since its the simplest method of control and does not require any additional nitrogen compounds online probes. However, further

studies should investigate the effect of applying a more advanced and complex aeration control on the system performance.

- In the study performed in **Chapter 4**, anoxic and aerobic intervals were set at 15 mins interval to maintain a constant pressure on NOB growth due to transient anoxia. However, it was suggested that these aeration intervals could be extended while maintaining NOB suppression (Roots, 2020). Extended aeration intervals can help simplify the oepration of intemittent aeration in full-scale implementation. Hence further research should be conducted to investigate the effect of the extended aeration intervals on NOB activity and nitrite shunt performance.

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