THE APPLICATION OF FREE AMMONIA INHIBITION TOWARDS PARTIAL NITRITATION IN MAINSTREAM WASTEWATER TREATMENT

by

Yaqian Luo

Bachelor of Science, Renmin University of China, 2016

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF APPLIED SCIENCE

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

(Civil Engineering)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

April 2020

The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, the thesis entitled:

The application of free ammonia inhibition towards partial nitritation in mainstream wastewater treatment

submitted by Yaqian Luo in partial fulfillment of the requirements for

the degree of Master of Applied Science

in Civil Engineering

Examining Committee:

Ryan Ziels, Civil Engineering
Supervisor

Dean Shiskowski, Civil Engineering
Supervisory Committee Member

Abstract

Partial nitritation/anammox (PN/A) is a novel pathway for nitrogen removal in wastewater treatment that offers advantages of low oxygen and organic carbon demands as well as high potential for energy recovery. However, the partial nitritation process remains a key hurdle for the widespread implementation of the PN-A process in mainstream treatment due to the difficulty in washing out nitrite-oxidizing bacteria (NOB) from active sludge. Exposing biomass to high concentrations of free ammonia (FA) has been reported as an effective strategy to achieve partial nitritation. This study examined the effectiveness of treating 20% of return activated sludge with synthetic centrate containing FA at 200 mg N/L for 24 hours to promote partial nitritation in mainstream wastewater treatment. Experimental and control bioreactors were operated under two different conditions, with or without FA treatment, respectively, after reaching similar nitrification performance. Biokinetic parameters of ammonium-oxidizing bacteria (AOB) and NOB were estimated by performing respirometric batch tests with activated sludge biomass from the two bioreactors under different operational conditions, and calibrating a process model based on oxygen mass balance. The bioreactor performance showed that the FA treatment strategy promoted the PN process, with a maximum nitrite accumulation ratio (NAR) of 41.9 ± 2.1% after treating return sludge with high FA solution for 37 days. However, this nitrite accumulation was not stable, and the NAR decreased to 10.9 ± 6.0% after 33 days, indicating that NOB were able to acclimate to the temporary exposure to a high FA concentration. The biomass yield coefficient (Y) of AOB increased during FA treatment, while the maximum specific growth rate (μ_{max}) of AOB and NOB decreased under this condition. Microbial community analysis on activated sludge under FA treatment, and further investigations

on the optimization of the FA treatment strategy combined with other NOB out-selection strategies are required to better facilitate the application of PN-A to full-scale mainstream wastewater treatment.

Lay Summary

Partial nitritation-anammox is an innovative nitrogen removal pathway that saves energy in wastewater treatment plants. However, the application of this process to full-scale wastewater plants treating municipal wastewater is challenged by the difficulty of controlling the microbial community structure. This study attempts to promote partial nitritation in mainstream treatment systems by treating return activated sludge with a solution containing a high concentration of free ammonia (FA). This is first study to combine highly paralleled respirometry with process modelling to estimate biokinetics of bacteria involved in nitrogen removal processes, and to assess the impacts of this FA treatment on their metabolic activities.

Preface

I conducted all of the work presented in the thesis in the Environmental Engineering Lab at the University of British Columbia, Point Grey campus.

Dr. R. Ziels and I designed and installed the two bioreactors outlined in Section 2.1, and the process modelling development in Section 2.5. I was responsible for operating bioreactors, performing respirometric batch tests, conducting parameter estimation for nitrifier biokinetics using AQUASIM software, and analyzing data.

Table of Contents

Abstract ..... **iii**

Lay Summary ..... **v**

Preface ..... **vi**

Table of Contents ..... **vii**

List of Tables ..... **x**

List of Figures ..... **xi**

List of Symbols ..... **xii**

List of Abbreviations ..... **xiv**

Acknowledgements ..... **xv**

Dedication ..... **xvi**

1 Introduction **1**

1.1 Nitrogen pollutants in wastewater 1

1.1.1 Types of nitrogen pollutants present in wastewater 1

1.1.2 Rationale for removing nitrogen from wastewater 1

1.2 Conventional biological nitrogen removal in wastewater treatment 3

1.2.1 Pathways of conventional nitrogen removal 3

1.2.2 Disadvantages of conventional nitrogen removal pathway 5

1.3 The Partial Nitritation/Anammox (PN/A) pathway for nitrogen removal 6

1.3.1 The Anammox pathway 6

1.3.2 Partial nitritation/anammox (PN/A) process 7

1.3.3 Application of partial nitritation/anammox (PN/A) in wastewater treatment 8

1.3.4 Challenges of PN/A in mainstream wastewater treatment 10
1.3.5 Strategies to achieve partial nitritation in mainstream wastewater treatment..... 12
1.4 Research objectives..... 16
2 Materials and Methods.....19
2.1 Bioreactor operation and monitoring..... 19
2.1.1 Bioreactor operation..... 19
2.1.2 Monitoring 23
2.2 In-situ cycle monitoring experiments 23
2.2.1 3-hour SBR cycle monitoring 23
2.2.2 24-hour SBR effluent monitoring 24
2.3 Estimation biokinetics of nitrifying populations..... 25
2.3.1 Background and theory of modeling nitrification biokinetics 25
2.3.2 Respirometric batch tests 29
2.3.3 Process modeling development..... 32
2.4 Biomass determination..... 34
2.5 Analytical methods 35
2.6 Statistical inference..... 36
3 Results 39
3.1 Performance of SBRs during the Non-FA Phase..... 39
3.2 Performance of SBRs during the FA Phase 40
3.3 Biokinetic parameter estimation 45
4 Discussion.....48
5 Conclusions.....60

References **61**

Appendices **70**

Appendix A: Total suspended solids (TSS) and volatile suspended solids (VSS) of mixed liquor, and nitrogen removal performance in SBRs throughout the study 70

Appendix C: Adjusted respirograms, oxygen uptake rate (OUR) curves, cumulative oxygen uptake curves, and specific oxygen uptake (SOU) determination in respirometric batch tests 73

Appendix D: Total bacterial biomass determination 80

Appendix E: Biomass calculations for nitrifiers 83

Appendix F: Instructions of process modeling development in AQUASIM 84

Appendix G: P values of ANOVA on biomass yield coefficient (Y), nitrifiers biomass (X), maximum specific growth rate (μ_{max}), and half-saturation coefficient (K_S) between ER and CR in Non-FA and FA Phase 90

List of Tables

Table 1-1 Inhibitory effects of FA on AOB and NOB in literature 17

Table 2-1 Stoichiometric coefficients of substrate, oxygen and biomass in nitrification..... 27

Table 2-2 The stoichiometric coefficients between utilization or production rates of nitrogen species (ammonium and nitrite) and the growth rate of AOB and NOB 34

Table 2-3 Summary of analytical methods 37

Table 4-1 Biomass yield coefficient (Y), maximum specific growth rate (μ_{max}), and half-saturation coefficient (K_S) in this study and literature 57

List of Figures

Figure 1-1 Comparison of oxygen and carbon demand between (a) conventional nitrification/denitrification and (b) innovative partial nitritation/anammox (Robiglio 2018) 8

Figure 2-1 Schematic diagrams of SBRs during (a) Non-FA Phase and (b) FA Phase..... 22

Figure 2-2 Respirometric batch tests setup using SDR SensorDish® Reader: (a) batch tests setup; (b) SDR SensorDish® Reader setup 31

Figure 3-1 Influent ammonium and effluent nitrogen species in SBRs before and after the start of FA phase: (a) ER; (b) CR..... 40

Figure 3-2 Ammonium (NH_4^+-N), nitrite (NO_2^--N), and nitrate (NO_3^--N) concentrations of the 3-hour SBR cycle monitoring for Non-FA and FA Phase: (a) ER; (b) CR. The 3-hour SBR cycle monitoring was conducted at day -30 before and day 84 after the start of FA treatment for non-FA phase and FA phase respectively. Time of 0 min represents the start of feeding period of a SBR cycle. 42

Figure 3-3 Ammonium (NH_4^+-N), nitrite (NO_2^--N), and nitrate (NO_3^--N) concentrations in ER and CR during a 24-hour period. These 24-hour effluent monitoring were carried out at day (a) 37; (b) 75 of the FA Phase. 44

Figure 3-4 Nitrite accumulation ratio (NAR) in ER and CR after the start of FA phase. Error bars represent standard deviations 45

Figure 3-5 Estimated biokinetic parameters from respirometric batch tests: (a) bacterial yield coefficient (Y); (b) nitrifiers biomass (X); (c) maximum specific growth rate (μ_{max}); (d) half-saturation coefficient (K_S). Error bars represent standard deviations. 47

Figure 4-1 Comparison of observed and predicted OUR obtained by the respiromteric batch tests and AQUASIM process model respectively for different substrates applied in batch tests: (a) ammonium; (b) nitrite. The slope of linear regression represents similarity between observed and predicted OUR. 53

List of Symbols

b: endogenous decay coefficient, d^{-1}

$f_{S,NH}$: fraction of electrons used for AOB biomass synthesis

$f_{S,NO}$: fraction of electrons used for NOB biomass synthesis

K_S: half-saturation coefficient, mg N/L

$K_{S,AOB}$: half-saturation coefficient of AOB, mg N/L

$K_{S,NOB}$: half-saturation coefficient of NOB, mg N/L

OU_{NC}: total cumulative oxygen uptake in complete nitrification, mg O_2/L

OU_{NO}: total cumulative oxygen uptake in nitrite oxidation, mg O_2/L

r: substrate utilization rate, mg/(L·d)

S: substrate concentration, mg NH_4-N/L or mg NO_2-N/L in nitrification process

S_{NC}: concentration of ammonium consumed in complete nitrification, mg N/L

S_{NO}: concentration of nitrite consumed in nitrite oxidation, mg N/L

S_{ox}: concentration of substrate oxidized during reaction periods, mg NOD/L

$\overline{S_{in}}$: mean concentrations of substrate in feed within 24 hours during FA phase. mg NOD/L

$\overline{S_e}$: mean concentrations of substrate in effluent within 24 hours during FA phase. mg NOD/L

$\overline{S_{as}}$: mean concentrations of substrate used for assimilation within 24 hours during FA phase. mg NOD/L

SOU_{NC}: specific oxygen uptake for complete nitrificatioin, mg O_2/mg N

SOU_{NO}: specific oxygen uptake for nitrite oxidation, mg O_2/mg N

X: biomass concentration, mg COD/L

X_{AOB}: biomass concentration of AOB, mg COD/L

X_{NOB}: biomass concentration of NOB, mg COD/L

Y: biomass yield coefficient, mg COD/mg NOD

Y_{AOB}: biomass yield coefficient of AOB, mg COD/mg NOD

Y_{NOB}: biomass yield coefficient of NOB, mg COD/mg NOD

μ_{max}: maximum specific growth rate, d^{-1}

$\mu_{max,AOB}$: maximum specific growth rate of AOB, d^{-1}

$\mu_{max,NOB}$: maximum specific growth rate of NOB, d^{-1}

List of Abbreviations

WWTPs: wastewater treatment plants

PN/A: partial nitritation / anammox

FA: free ammonia

DO: dissolved oxygen, mg/L

NAR: nitrite accumulation ratio

SBR: sequencing batch reactor

ER: experimental reactor

CR: controlled reactor

SRT: solid retention time

TSS: total suspended solids

VSS: volatile suspended solids

AOB: ammonium-oxidizing bacteria

NOB: nitrite-oxidizing bacteria

OUR: oxygen uptake rate, mg/(L·min))

sCOD: soluble chemical oxygen demand (mg/L)

Acknowledgements

I would like to express my sincere gratitude to these people who have given me kind supports and help to let my thesis become a reality.

First, I would like to owe the deepest thanks to my supervisor, Dr. Ryan Ziels, who has expertly guided me throughout my graduate education at UBC and inspired me a lot in my research. His passion in the field of sustainable biological wastewater treatment motivated me to think more deeply and constantly engaged in my research. To my committee, Dr. Ryan Ziels and Dr. Dean Shiskowski, I am extremely grateful for their suggestions and assistance in the research.

I thank Natural Science and Engineering Research Council (NSERC) for giving financial support to my research and the University of British Columbia for providing graduate scholarship to me, which made life in Vancouver easier.

My sincere appreciation also goes to lab manager of Environmental Engineering Lab, Otman Abida and Felix Shuen, and staff in UBC Bioimaging Facility, Kevin Hodgson and Miki Fujita. Their professional lab skill made my research go smoothly. In addition, a thank you to my fellow students, Julie Pierce, Richard Kuo, Breno Chan, Holli Ye, for helping me during my research.

Finally yet importantly, I would like to express special thanks to my parents who have continuously given me both moral and finical support throughout my years of education.

Dedication

I dedicate this thesis to all the people fighting on the front-line of the war against COVID-19 in China and other places around the world, for their unremitting efforts to save life and control the global spread of COVID-19.

1 Introduction

1.1 Nitrogen pollutants in wastewater

1.1.1 Types of nitrogen pollutants present in wastewater

Water is the basis of life. Our society uses clean water to support our domestic life, as well as industrial and agricultural activities. Meanwhile, we produce used water containing a variety of contaminants at relatively high concentrations compared with their natural levels. Among these contaminants of public concern are nitrogenous compounds, including organic nitrogen, ammonia (NH_3), ammonium ion (NH_4^+), nitrite (NO_2^-), and nitrate (NO_3^-) (Water Environment Federation 2005). The total concentration of organic and ammonia nitrogen in a typical municipal wastewater is 25-45 mg N/L (Tchobanoglous et al. 2003). Municipal wastewater treatment plants (WWTPs) were the third biggest source of anthropogenic nitrogen pollutants in Canada, contributing to 13.4% of nitrogen loading to natural waters (Chambers et al. 2001). The largest source of nitrogen in municipal wastewater originated from human waste, followed by industrial discharge (Manuel 2014). There is very little nitrite and nitrate present in raw wastewater, but can be present in relatively high levels in WWTP effluent due to the nitrification process (see Section 1.2) (Tchobanoglous et al. 2003; Water Environment Federation 2005).

1.1.2 Rationale for removing nitrogen from wastewater

The reasons to remove nitrogenous matters from wastewater before its discharge into natural waters are: (1) to prevent eutrophication in aquatic environments; (2) to eliminate toxicity of free ammonia on aquatic life; and (3) to protect aquatic animals and human beings from nitrogen-related diseases like methemoglobinemia.

Eutrophication is a significant concern when wastewater containing large amounts of nitrogen and phosphorus is discharged into a receiving water (Water Environment Federation 2005). Harmful algal blooms occur in natural water system due to excessive nutrient inputs, threatening aquatic life and ecological balance by producing toxins, blocking light, and/or depleting dissolved oxygen, and may also bring indirect health hazards to humans via food sources (Donald et al. 2002; Schindler 2006). Therefore, as one of key limiting macronutrients in natural waters, nitrogen in wastewater should be effectively removed to prevent eutrophication of water bodies.

Another concern of untreated wastewater is the toxicity to aquatic animals caused by high ammonia levels (Water Environment Federation 2005). Numerous studies have shown that, compared with ammonium (NH_4^+), un-ionized ammonia (NH_3), also termed as *free ammonia (FA)*, has a higher toxicity to aquatic organisms, especially fish (Augspurger et al. 2003; Camargo and Alonso 2006). Based on substantial research on this topic, Environment Canada has established a level of 0.019 mg/L NH_3-N as a water quality guideline (Canadian Council of Ministers of the Environment 2010). The relationship between ammonium and FA is governed by temperature and pH, due to the nature of the acid-base equilibrium. Assuming that the total ammonia ($NH_3 + NH_4^+$) concentration in typical wastewater is 30 mg N/L, and the pH and water temperature are 7.0 and 20 °C respectively, the resulting concentration of NH_3 would be 0.14 mg/L, around 7.5 times higher than the guideline (Canadian Council of Ministers of the Environment 2010). As a result, the total ammonia level in wastewater must be reduced to maintain the health of many sensitive aquatic ecosystems.

Although raw sewage seldom contains nitrite and nitrate (Tchobanoglous et al. 2003; Water Environment Federation 2005), its direct discharge may elevate nitrite and nitrate concentrations in receiving waters via biological nitrification activity (see Section 1.2.1), and thus can escalate toxicity to aquatic species and human health (Camargo and Alonso 2006; Canadian Council of Ministers of the Environment 2012). Nitrite and nitrate can cause toxicity by reducing the oxygen-carrying capacity of blood, damaging electrolyte balance and disease tolerance, and potentially causing cancer (Scott and Crunkilton 2000; Jensen 2003; Camargo and Alonso 2006). In order to protect aquatic life and human health, 3 mg N/L and 45 mg N/L of nitrate have been set by Environment Canada as water quality guidelines for long-term exposure in freshwater and seawater respectively (Canadian Council of Ministers of the Environment 2012). Hence, nitrogen removal from wastewater before discharge is extremely important to protect aquatic life as well as human health and safety.

1.2 Conventional biological nitrogen removal in wastewater treatment

1.2.1 Pathways of conventional nitrogen removal

The most commonly-used biological approach for nitrogen removal in WWTPs involves three processes: hydrolysis and ammonification, nitrification, and denitrification (Tchobanoglous et al. 2003). Hydrolysis and ammonification involve the breakdown of organic nitrogen into soluble forms, accompanied by the release of ammonia nitrogen into wastewater (Water Environment Federation 2005). Since hydrolysis and ammonification processes are not the rate-limiting steps in nitrogen removal, most of the previous research on biological nitrogen removal has focused on the subsequent steps of nitrification and denitrification (Katipoglu-Yazan et al. 2012).

Nitrification is a two-step biological oxidation process conducted by two groups of autotrophic bacteria. The first step is oxidation of ammonium (NH_4^+) to nitrite (NO_2^-) by ammonia-oxidizing bacteria (AOB) (Eq. 1-1), followed by the oxidation of nitrite to nitrate (NO_3^-) by nitrite-oxidizing bacteria (NOB) (Eq. 1-2) (Tchobanoglous et al. 2003; Peng and Zhu 2006). Both of these two steps use oxygen as the electron acceptor, and thus aerobic conditions are required for nitrification to occur.

(Eq. 1-1) $$NH_4^+ + 1.5\ O_2 \rightarrow NO_2^- + 2\ H^+ + H_2O$$

(Eq. 1-2) $$NO_2^- + 0.5\ O_2 \rightarrow NO_3^-$$

Denitrification occurs following nitrification to complete nitrogen removal from wastewater. This process involves reduction of nitrate and nitrite to nitric oxide (NO) and nitrous oxide (N_2O), and finally nitrogen gas (N_2) which is released into the atmosphere (Lu et al. 2014). Many denitrifying bacteria are heterotrophic and are able to use oxygen, nitrite or nitrate as electron acceptors and biodegradable organic matter as an electron donor. Due to the lower growth efficiency of denitrifiers when respiring nitrite or nitrate in comparison to oxygen, these organisms utilize these oxidized nitrogen sources as electron acceptors only when little or no oxygen is available (Water Environment Federation 2005). This redox condition is referred to *anoxic* (Tchobanoglous et al. 2003). The stoichiometric equation for denitrification using methanol as electron donor is shown as Eq. 1-3 (Water Environment Federation 2005).

(Eq. 1-3) $$6\ NO_3^- + 5\ CH_3OH + H_2CO_3 \rightarrow 3\ N_2 + 8\ H_2O + 6\ HCO_3^-$$

1.2.2 Disadvantages of conventional nitrogen removal pathway

Although the conventional nitrogen removal pathway (nitrification/denitrification) has already demonstrated high nitrogen removal efficiencies in many full-scale WWTPs worldwide, the process has disadvantages that can increase treatment costs and decrease the sustainability of wastewater treatment. Some of its main drawbacks include the high energy consumption caused by the large oxygen demand for nitrification, as well as the need for exogenous organic carbon for denitrification (McCarty 2018; Maktabifard et al. 2018). According to the stoichiometric ratio of ammonium to oxygen shown in Eq. 1-1 and Eq. 1-2, around 4.6 kg of oxygen is needed to oxidize 1 kg N of ammonium to nitrate using the nitrification process. In a traditional WWTP, aeration is the largest energy-consuming component, and accounts for 55-70% of the total energy demand (Gandiglio et al. 2017). Assuming that a typical medium-strength wastewater contains 190 mg/L of biochemical oxygen demand (BOD_5) and 40 mg N/L of nitrogenous compounds (major components are organic nitrogen and ammonium), approximately half amount of supplemental oxygen would be utilized for nitrification and would consume 25-35% of the total energy demand (Water Environment Federation 2005).

Moreover, the demand for organic carbon by heterotrophic denitrification reduces the potential for energy production from wastewater. A minimum C/N ratio of 8 is suggested to support denitrification (Sun et al. 2010; McCarty 2018). Biodegradable organic matter in wastewater and endogenous decay products are two major sources of organics for denitrification (Tchobanoglous et al. 2003). When the total amount of organic carbon from these two sources is not sufficient, external carbon source like methanol or acetate must be added to the denitrification tank, which raises treatment costs. Meanwhile, the large organic carbon demand reduces its potential use for

energy recovery via biogas (methane) production from anaerobic sludge digestion (Tchobanoglous et al. 2003; McCarty 2018). Therefore, alternative nitrogen removal pathways with low energy requirements are desired for WWTPs to become both energy- and cost-efficient. Among various new technologies, partial nitritation-anammox is considered as an innovative and promising solution to replace conventional nitrogen removal design and convert WWTPs from energy-consuming facilities to energy-neutral or even energy-positive (Peng and Zhu 2006; Cao et al. 2017; McCarty 2018; Maktabifard et al. 2018).

1.3 The Partial Nitritation/Anammox (PN/A) pathway for nitrogen removal

1.3.1 The Anammox pathway

ANaerobic AMMonium OXidation (Anammox) was first detected by van de Graaf in a lab-scale fluidized bed reactor utilizing ammonium for nitrogen gas production in 1995 (van de Graaf et al. 1995; Kuenen 2008). According to Eq. 1-4, ammonium can be oxidized to gaseous N_2 by autotrophic anammox bacteria using nitrite (NO_2^-) as the electron acceptor and CO_2 as the carbon source under anoxic conditions (Strous et al. 1998).

(Eq. 1-4)
$$\mathrm{NH_4^+ + 1.32\ NO_2^- + 0.066\ HCO_3^- + 0.13\ H^+ \rightarrow}$$
$$\mathrm{1.02\ N_2 + 0.26\ NO_3^- + 0.066\ CH_2O_{0.5}N_{0.15} + 2.03H_2O}$$

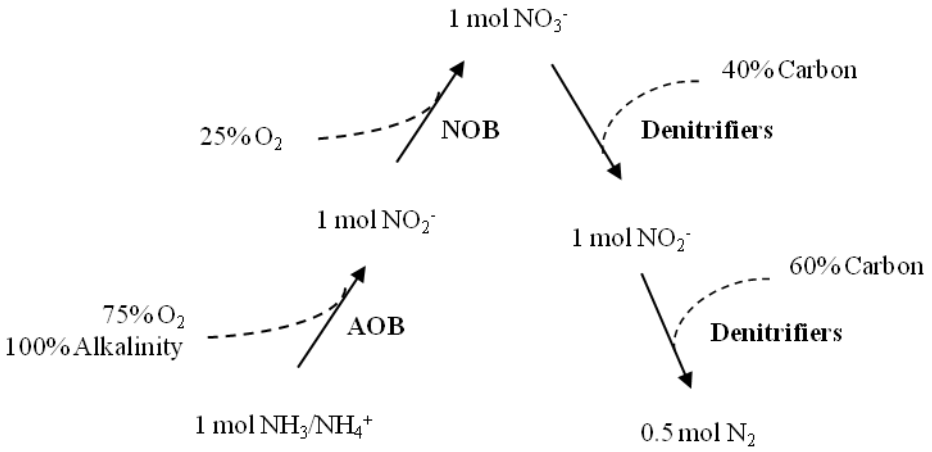
In this somewhat recently discovered process, aeration and organic carbon are not required. Thus, for the same nitrogen removal efficiency, anammox offers the advantages of less energy consumption and higher potential energy recovery than conventional nitrification/denitrification processes (He et al. 2015). Meanwhile, the low growth yields of anammox bacteria results in less

sludge production, and thus saves operational costs associated with sludge handling and treatment (Strous et al. 1998; Hu et al. 2013). The application of anammox in wastewater treatment plants can therefore enhance energy efficiency significantly. As a result, a new process — partial nitritation/anammox (PN/A) — for nitrogen removal has been proposed to build more sustainable WWTPs (Verstraete and Philips 1998; Peng and Zhu 2006; Kuenen 2008; McCarty 2018).

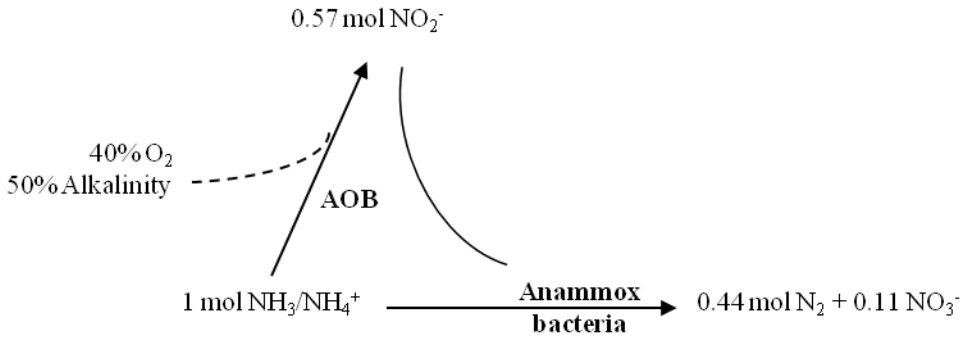
1.3.2 Partial nitritation/anammox (PN/A) process

Partial nitritation must be realized as an accompanying step for nitrogen removal via the anammox process to provide a mixture of ammonium and nitrite at ratio of 1:1.32 (as shown in Eq. 1-4). In partial nitritation, about half of the influent ammonium is oxidized to nitrite by AOB, generating equal concentrations of ammonium and nitrite that are subsequently consumed by anammox bacteria for nitrogen gas production (Figure 1-1 b) (Peng and Zhu 2006; Kuenen 2008). Compared with the conventional nitrogen removal pathway, PN/A has following advantages in terms of energy saving (Ma et al. 2016; Cao et al. 2017; Maktabifard et al. 2018):

1) 60% reduction of oxygen demand due to partial oxidation of ammonium to nitrite;
2) Almost 100% elimination of organic carbon dependency because both of AOB and anammox bacteria are autotrophic microorganisms;
3) 80% reduction of excess sludge;
4) Approximately 60% increase of methane potential from wastewater due to the possibility of re-directing organic carbon to methanogenic process.



(a)



(b)

Figure 1-1 Comparison of oxygen and carbon demand between (a) conventional nitrification/denitrification and (b) innovative partial nitritation/anammox (Robiglio 2018)

1.3.3 Application of partial nitritation/anammox (PN/A) in wastewater treatment

Several cost-efficient biological nitrogen removal systems have been developed based on the PN/A process concept. The SHARON®/Anammox® (Single reactor system for High activity Ammonium Removal Over Nitrite/Anammox) process is a two-stage system separating partial

nitritation and anammox processes in two different reactors, and is able to achieve nitrogen removal efficiencies as high as 80% (van Dongen et al. 2001). Although the two-stage system is easy to optimize because AOB and anammox bacteria have different requirements for growth, the costs for infrastructure and operation are elevated in comparison to single-stage systems (Cao et al. 2017). As a result, many single-stage systems have been developed and are more widely used in full-scale installations, including the CANON (Completely Autotrophic Nitrogen removal Over Nitrite) process, OLAND (Oxygen-Limited Autotrophic Nitrification-Denitrification) process, SNAP (Single-stage Nitrogen removal using Anammox and Partial nitritation) process, DEMON® (DEamMONification) process, and ANITAMox™ process (Langone 2013; Lackner et al. 2014; Gonzalez-Martinez et al. 2018).

The CANON process consists of a single reactor where AOB coexist with anammox bacteria so that partial nitritation and anammox reactions take place simultaneously (Dijkman and Strous 1999; Jetten et al. 2002). To enhance bacterial activity, the OLAND process controls the dissolved oxygen at a low level (<0.5 mg/L) to remove oxygen-inhibition of anammox and prevent growth of NOB from competing with anammox bacteria for nitrite (Kuai and Verstraete 1998; Seviour and Nielsen 2010). Instead of using suspended biomass, the SNAP process utilized a biofilm reactor which creates anoxic environment inside of the attached biomass for the growth of anammox bacteria (Furukawa et al. 2006). In the DEMON® system, anammox bacteria grow in granular sludge, while AOB and other bacteria are kept in flocs (Innerebner et al. 2007; Gonzalez-Martinez et al. 2015). ANITAMox™ system is a one-stage moving-bed biofilm reactor (MBBR), where AOB and anammox bacteria are retained in biofilm fixed on suspended carriers (Veuillet et al. 2014; Chan et al. 2014).

With the rapid development of PN/A systems, over 100 full-scale installations using this innovative technology were built around the world by 2014 (Lackner et al. 2014). The survey by Lackner et al. (2014) on these implementations demonstrated that the PN/A process has been successfully carried out for nitrogen removal from wastewater with high-strength ammonium (500-1500 mg/L) and low C/N ratio (<2), like sidestream wastewater (e.g. anaerobic digester centrate), industrial wastewater, and landfill leachate. The energy demand of 6 examined full-scale wastewater plants was in the range of 0.8-2 kWh/kg-N, saving more than 50% energy consumed in comparison to conventional nitrification/denitrification processes (Wett et al. 2010; Lackner et al. 2014). In municipal WWTPs, however, sidestream wastewater from sludge dewatering only typically contributes 15-30% of the overall nitrogen load because of its small flow rate, and the remaining 70-85% of the nitrogen load originates from influent wastewater to the mainstream treatment process, which has low ammonium level (25-45 mg N/L) but a large flow rate (Constantine 2006). Hence, the implementation of PN/A process for mainstream nitrogen removal is currently lacking, but if achieved, could make significant contributions to the establishment of energy-neutral or energy-positive WWTPs.

1.3.4 Challenges of PN/A in mainstream wastewater treatment

In spite of successful applications of the PN/A process in full-scale operations, especially for sidestream treatment, this technology has not been scaled up for the treatment of low-ammonium wastewater with a high C/N ratio, like mainstream municipal wastewater. The major reason for this technology limitation is the difficulty in maintaining the activity of AOB and anammox bacteria while simultaneously inhibiting NOB that compete with anammox bacteria for nitrite. High C/N ratios favor heterotrophic bacteria, resulting in a decline in population and activities of

AOB and anammox bacteria, and thus promote low nitrogen removal rates (Ma et al. 2016; Cao et al. 2017). In addition, anammox bacteria are sensitive to low temperature, as their activity can decrease up to ten times when the temperature is reduced from 30^{o}C to 10^{o}C (Hu et al. 2013; Lotti et al. 2015).

The suppression of NOB activity is another important factor that affects the performance of the PN/A process in mainstream treatment. Different from the high temperatures (> 30^{o}C) commonly observed in sidestream treatment systems, mainstream treatment is conducted at lower ambient temperatures, with seasonal variation from 30^{o}C to below 0^{o}C in temperate regions. The low temperature range and wide seasonal variation challenges the implementation of the PN/A process. AOB were reported to have higher growth rates at high temperature (>25^{o}C), but NOB grew faster at temperatures below 15^{o}C, leading to large amounts of nitrate accumulation in PN/A reactors (Hellinga et al. 1999; Peng and Zhu 2006; Cao et al. 2017).

In addition, the high ammonium concentrations of sidestream wastewater (500-1500 mg/L) generate high levels of free ammonia (NH_3, FA) that are over the inhibitory threshold values of NOB, which enables the washout of NOB from PN/A systems (Peng and Zhu 2006; Lackner et al. 2014). The ammonium concentration in mainstream wastewater, however, is much lower than in sidestream wastewater, and thus produces low FA level making it difficult to selectively suppress NOB in mainstream PN/A process (Tchobanoglous et al. 2003; Cao et al. 2017).

Another major concern of PN/A application is the emission of nitrous oxide (N_2O), a greenhouse gas that has global warming potential of 298 (Stocker et al. 2014). Municipal wastewater treatment plants have been estimated to contribute approximately 1.6% of the total worldwide N_2O emission, and biological nitrogen removal process is considered to be a major source (Ahn

et al. 2010; Massara et al. 2017). Although PN/A process has potential advantages for energy savings, it does not show promise to mitigate N_2O emissions in comparison with conventional nitrification-denitrification process. Many studies have shown that partial nitritation is the primary N_2O hotspot in PN/A process, since high N_2O production is related to high ammonium removal rates and high nitrite concentrations through hydroxylamine (NH_2OH) oxidation and the nitrifier denitrification pathway (Kampschreur et al. 2007; Castro-Barros et al. 2015; Massara et al. 2017; Blum et al. 2018).

1.3.5 Strategies to achieve partial nitritation in mainstream wastewater treatment

Achieving and maintaining stable partial nitritation for wastewaters having low ammonium nitrogen strength and high C/N ratios is crucial for successful implementation of the PN/A process for mainstream wastewater treatment. Although the adoption of carbon pretreatment processes can reduce the influent C/N ratio to below 3 and suppress the growth of heterotrophic bacteria in the downstream PN/A reactor, the most effective methods of out-selecting NOB from autotrophic nitrifying communities under mainstream conditions have not yet been identified (Cao et al. 2017). Furthermore, the discovery of complete ammonium oxidizers (comammox) that are capable of oxidizing ammonium directly to nitrate, makes the inhibition of nitrate production via nitrification potentially even more difficult than expected (Xia et al. 2018). In order to achieve stable partial nitritation in mainstream wastewater treatment, previous research has mainly focused on exploiting the different growth and metabolism patterns of AOB and NOB under specific circumstances to develop control strategies for selective retention of AOB over NOB. These strategies include short solids retention time (SRT), dissolved oxygen (DO) control, and inhibitors like free ammonia (FA) and free nitrous acid (FNA).

1.3.5.1 Short solids retention time (SRT)

Implementing a short process solids retention time (SRT) is a practical method to selectively suppress NOB in a nitrifying community. At temperatures higher than 15^{o}C, AOB have a minimum sludge age that is lower than that of NOB, and the difference increase when temperature increased up to 30^{o}C (Hellinga et al. 1998). As a result, the process SRT can be controlled within a range that is shorter than the minimum sludge age of NOB but longer than that of AOB to prevent nitrite oxidation (Xu et al. 2015). The City of St. Petersburg (Florida) Southwest Water Reclamation Facility successfully removed nitrogen from wastewater by minimizing NOB activity at an SRT of 3.9 days and temperature of 29^{o}C (Jimenez et al. 2014). However, the effectiveness of short SRT strategy can be reduced at temperatures below 15^{o}C. Even though the growth rate of both AOB and NOB decrease with lower temperature, NOB can grow faster than AOB at low temperatures, making it difficult to determine a reliable minimum SRT for NOB inhibition (Hellinga et al. 1998). In this case, dissolved oxygen control becomes an alternative approach for achieving partial nitritation.

1.3.5.2 Dissolved oxygen (DO) control

Dissolved oxygen (DO) control strategies include low DO, intermittent aeration, and real-time DO control (Ma et al. 2016; Cao et al. 2017). Taking advantage of higher oxygen affinities of AOB over that of NOB, maintaining a low DO is regarded as an effective strategy to promote partial nitritation (Xu et al. 2015; Ma et al. 2016). Multiple studies have shown that nitrite oxidation can be efficiently suppressed at a DO below 1 mg/L (Tokutomi 2004; Ma et al. 2011; Gilbert et al. 2014). Nevertheless, recent research has shown that oxygen-limiting conditions can favor the growth of K-strategist NOB species like *Nitrospira* that have higher oxygen affinities

and lower specific growth rates (Liu and Wang 2013; Cao et al. 2017). However, low DO has been reported to be a potential factor that can increase N_2O emissions. N_2O emission in a pilot-scale continuous granular nitritation reactor could decrease from 6% to 2.2% of oxidized nitrogen when DO increased from 1 to 4.5 mg/L (Pijuan et al. 2014). Maintaining DO at a minimum of 1-1.5 mg/L may help to limit N_2O emission via nitrifier denitrification (Wang et al. 2014a; Frison et al. 2015).

Intermittent aeration is another promising DO control method for NOB out-selection. Prior research has suggested that NOB can have a longer lag time in their metabolic activity than AOB when redox conditions are switched from anoxic to aerobic. This is thought to be caused by the absence of substrates (nitrite and oxygen) at the start of the aerobic period, as well as NOB metabolic inactivation during the transition (Kornaros et al. 2010; Gilbert et al. 2014; Ma et al. 2016). Therefore, a controlled aerobic period shorter than the lag time of NOB but longer than lag time of AOB could help to suppress NOB activity (Ma et al. 2016). Real-time DO control has been developed to wash out NOB by linking aeration time to nitrogen species concentrations, like the ratio of NH_4^+-N to NO_x-N (Regmi et al. 2014; Cao et al. 2017). However this strategy has high requirement on the reliability of monitoring equipment, which may hinder the widespread application of this strategy in PN/A processes (Cao et al. 2017).

1.3.5.3 Free ammonia (FA) and free nitrous acid (FNA) inhibition

Exposure of biomass to free ammonia (FA, NH_3) and free nitrous acid (FNA, HNO_2) have been reported to be alternative methods to achieve partial nitritation, since NOB are more sensitive to inhibitory effects of these two chemicals than AOB (Anthonisen et al. 1976; Chung et al. 2006; Wang et al. 2014b). FNA was found to have a stronger biocidal effect on NOB than AOB at a

range of 0.24-1.35 mg HNO_2-N/L (Wang et al. 2014b). In terms of FA inhibition, it has been proposed that FA is able to cause cell inactivation or lysis by causing proton imbalance inside cells, directly inhibiting enzymes (like extracellular polymeric substances (EPS)), or disrupting microbial community structures (Liu et al. 2019). However, the exact mechanisms of toxicity remain unclear, and need to be further investigated (Liu et al. 2019). Table 1-1 summarizes suppressive effects of FA on AOB and NOB activities from literature. NOB activity can be inhibited significantly at FA below 9 mg N/L, while AOB activity starts to decrease at FA levels of 10-300 mg N/L. Due to the different FA tolerance of AOB and NOB, it is relatively straightforward to inhibit NOB and obtain partial nitritation in wastewater with high FA concentrations, like sidestream anaerobic digester centrate wastewater that contains 30-560 mg N/L of FA (Wang et al. 2017).

Based on this concept, Wang et al. (2017) proposed a new approach to achieve partial nitritation in mainstream treatment by treating a portion of return sludge with FNA or FA at the concentration adequate to completely suppress NOB activity while only partially inhibiting AOB activity. FA treatment has a major advantage of easy access to adequate FA directly from sidestream centrate wastewater produced from anaerobic digestion, (Wang et al. 2017), whereas an additional reactor would be required to generate FNA for use in treating the sludge. FA concentrations in centrate can be controlled via the total ammonium concentration ($NH_3 + NH_4^+$), temperature and pH. The novel FA treatment approach was also shown to be economically and environmentally favorable compared with the conventional nitrogen removal pathway (Wang et al. 2017). Wang et al. (2017) has shown the feasibility of the new FA approach to achieve over 90% of nitrite accumulation at low DO of 1.5 mg/L. However there are still many remaining

questions that need to be investigated before full-scale application of the new FA treatment approach in WWTPs. For instance, it is not known how the nitrifying bacterial activity and community structure respond to FA-treatment approach. Additionally, it remains unknown how to optimize operational conditions like FA concentration and treatment frequency to obtain the desired ratio of ammonium and nitrite for the anammox reaction.

1.4 Research objectives

This research aimed to examine the effectiveness of FA treatment of return activated sludge towards promoting stable partial nitritation in mainstream wastewater treatment without DO control, and to estimate changes in nitrifier biokinetics following an FA treatment approach. Two individual lab-scale sequencing batch reactors (SBRs) were operated as experimental and control reactors. The operational conditions of these two SBRs were the same in Phase I (non-FA phase) without FA treatment, so as to achieve the same nitrification performance prior to the following Phase II (FA phase) in which FA treatment was applied. In the FA phase, the experimental reactor had return sludge treated by synthetic centrate with high level of FA, while return sludge from the control reactor was treated by an identical solution but without FA. Meanwhile, biokinetics of AOB and NOB from the two SBRs was assessed by conducting respirometric batch tests with activated sludge biomass throughout the different phases, and by calibrating a process model for the different microbial populations.

Table 1-1 Inhibitory effects of FA on AOB and NOB in literature

Nitrifying bacteria	FA inhibition threshold (mg N/L)	Remarks	References
AOB			
AOB	> 210	Return sludge was treated by FA; AOB activity decreased by 9.4%	Wang et al. (2017)
Nitrosomonas	10-150	Activated sludge	Anthonisen et al. (1976)
Nitrosomonas	25	SBR system; AOB activity was decreased by 40%	Balmelle et al. (1992)
AOB	70	A SHARON reactor; FA inhibition on ammonium oxidation was started	Hellinga et al. (1999)
AOB	300	A SHARON reactor; FA inhibition on ammonium oxidation was started	van Hulle et al. (2007)
Nitrosomonas	>16	An SBR reactor	Vadivelu et al. (2006)
AOB	0.44-0.84	Submerged biofilters; ammonium oxidation was inhibited by about 60%	Villaverde et al. (2000)
AOB	58-62	Ammonium oxidation rate was reduced by 86.3%	Wang and Yang (2004)
AOB	24.7	AOB activities was inhibited	Li et al. (2012)
AOB	10	AOB activity started to decrease	Chung et al. (2006)

Nitrifying bacteria	FA inhibition threshold (mg N/L)	Remarks	References
NOB			
Nitrobacter	0.1-1.0	Activated sludge	Anthonisen et al. (1976)
NOB	1-5	A two-stage anaerobic-aerobic treatment	Abeling and Seyfried (1992)
Nitrobacter	<1.0 - 9	Growth of Nitrobacter was completely inhibited at FA > 6.0 mg N/L; Metabolism of Nitrobacter was inhibited by 12% at FA = 6.0-9.0 mg N/L	Vadivelu et al. (2007)
NOB	0.44-0.84	Submerged biofilters; NAR > 80%	Villaverde et al. (2000)
NOB	3.7	Acontinuous flow aerobic-anoxic reactor; NAR = 77.4%	Bae et al. (2001)
NOB	23	Acontinuous-flow hybrid reactors; NAR = 90%	Chung et al. (2007)
NOB	0.7	NOB activity decreased by more than 50%	Kim et al. (2008)
NOB	4.12-8.24	NOB activity was inhibited and partial nitritation was achieved	Li et al. (2012)
NOB	5	NOB activity was inhibited significantly	Chung et al. (2006)

2 Materials and Methods

2.1 Bioreactor operation and monitoring

2.1.1 Bioreactor operation

Two identical lab-scale sequencing batch reactors (SBRs, experimental reactor (ER) and controlled reactor (CR)) with working volumes of 4.28 L were seeded with blended granular sludge from a pilot-scale SBR at the University of Washington. Flocculated activated sludge was formed after seeding for a week. A typical SBR cycle lasted for 3 hours, including 2-min aerobic feeding, 148-min aerobic reaction, 20-min settling, 5-min decanting, and 5-min idle periods. The SBR cycle timing was controlled with ChronTrol XT timers (ChronTrol Coporation, California), and mixing was provided with overhead mixers. Temperature was maintained at $20 \pm 1^{o}C$ using an environmental chamber. The target solid retention time (SRT) was kept at 10 days for both reactors throughout the study, by wasting a determined amount of biomass depending on daily measurements of mixed liquor and effluent total suspended solids (TSS) and volatile suspended solids (VSS) (see Section 2.1.2).

The bioreactors were fed with a synthetic wastewater containing ammonium chloride (NH_4Cl) as the nitrogen source and sodium acetate (CH_3COONa) and propionic acid (CH_3CH_2COOH) as organic carbon sources, leading to 24.5 ± 2 mg NH_4-N/L and 100 ± 37 mg/L of chemical oxygen demand (COD) respectively. Additionally, the synthetic wastewater contained macro elements (162.4 mg/L of $MgCl_2$, 128.65 mg/L of $CaCl_2 \cdot 2H_2O$, 79.1 mg/L of KCl), trace elements (1.03

mg/L of $FeSO_4 \cdot 7H_2O$, 0.32 mg/L of $ZnSO_4 \cdot 7H_2O$, 0.055 mg/L of $CuSO_4 \cdot 5H_2O$, 0.0562 mg/L of $CoCl_2 \cdot 6H_2O$, 0.032 mg/L of $Na_2MoO_4 \cdot 2H_2O$, 0.05 mg/L of H_3BO_3, 0.05 mg/L of KI, 0.022 mg/L of $NiCl \cdot 6H_2O$, 0.14 mg/L of $Al_2(SO_4)_3 \cdot 18H_2O$, 0.283 mg/L of $MnCl_2 \cdot 4H_2O$, 6.87 mg/L of EDTA), phosphate (51.3 mg/L of $Na_2HPO_4 \cdot 7H_2O$, 16.7 mg/L of KH_2PO_4), alkalinity (298.3 mg/L of $NaHCO_3$), and yeast (20 mg/L).

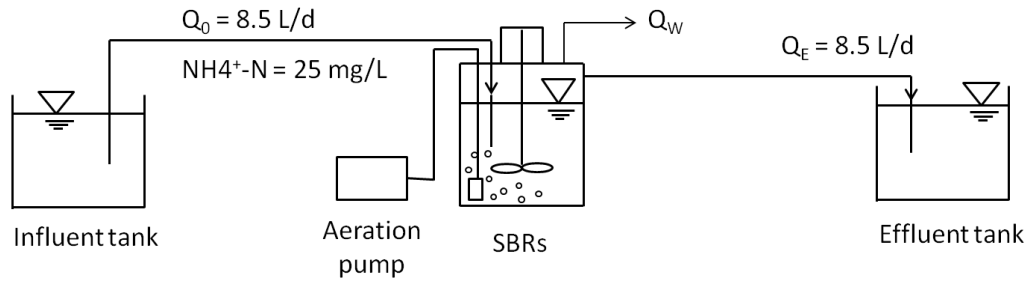
Two phases were performed throughout this study: Non-FA Phase and FA Phase. The first phase was Non-FA Phase, in which the two bioreactors were operated under the same aerobic conditions without FA treatment of sludge to obtain similar nitrification performance (Figure 2-1 a). During the Non-FA Phase, the SBRs were fed with 1.07 L of synthetic wastewater using peristaltic pumps (LabF1/YZ1515, Shenchen, China) for each SBR cycle, resulting in a hydraulic retention time (HRT) of 12 hours. pH was not controlled but measured within the range of 6.7-7.5. DO was provided by an air pump and increased from 3 to 8 mg/L in a typical SBR cycle. TSS and VSS of mixed liquor in both ER and CR decreased over the first 20 days in the FA Phase due to the addition of sludge treatment, but solid concentrations recovered to the initial level after day 60 (Figure A-1, Appendix A). TSS and VSS were on average of 637 ± 43 and 593 ± 39 mg/L in ER, and 703 ± 66 and 652 ± 60 mg/L in CR. The VSS/TSS ratio in the two reactors averaged 0.93. The Non-FA Phase lasted 274 operational days.

The FA Phase was conducted after stable complete nitrification was maintained in the two reactors, and lasted 90 operational days. The operational condition of the FA Phase was similar to those in the Non-FA Phase except for the following differences. In the FA Phase, 800mL of

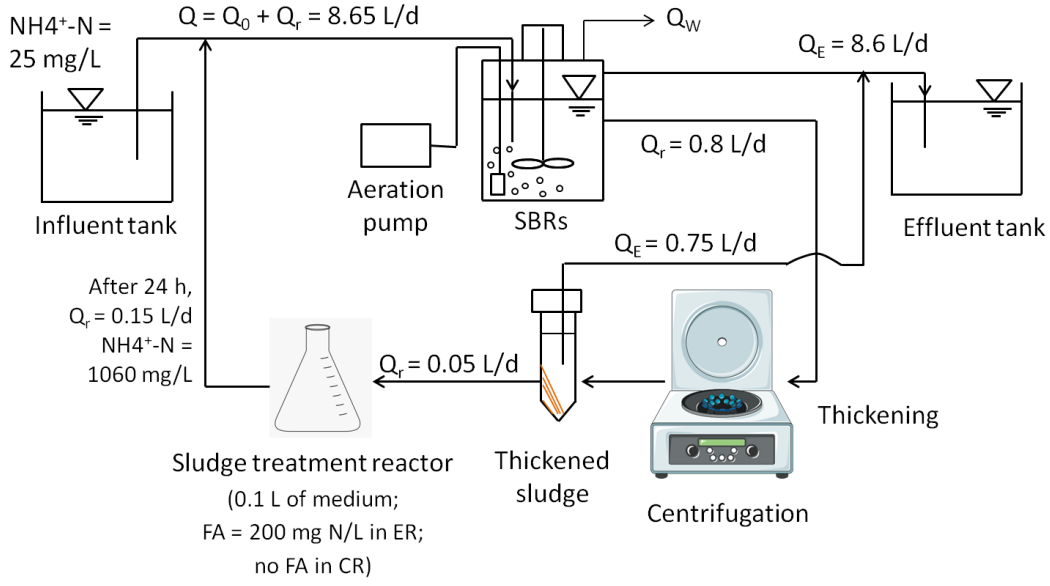
mixed liquor was removed from each reactor at the end of the reaction period of a cycle every 24 hours and thickened to 50 mL by settling and centrifugation (Thermo IEC Multi RF Centrifuge, Thermo IEC, USA). Supernatant produced in this process was collected for daily effluent nutrient measurements, while the thickened sludge was incubated in a 200-mL beaker containing 100 mL of media with the same compositions of macro and trace elements, phosphate and alkalinity as the synthetic feed, but with no COD. For the thickened sludge from ER, 0.406 g of ammonium chloride (NH_4Cl) was added into the 100-mL medium solution to simulate centrate containing FA at a high concentration, and the pH was adjusted to 9 with sodium hydroxide (NaOH). Using the following equation (Eq. 2-1 and 2-2) (K_b and K_w are ionization constant of ammonia equilibrium and water respectively), the concentration of FA in the incubation medium was 300 mg N/L at pH of 9 and temperature of 20^{o}C, and became 200 mg N/L after mixing with 50-mL thickened sludge (Wang et al. 2017). As a control condition, thickened sludge from CR was incubated in the same medium at a pH of 9 but without ammonium addition. After 24 hours of anaerobic incubation, the 150 mL of treated sludge was recycled back to the respective reactors at the start of a SBR cycle (e.g. during the 5-min aerobic feeding period) (Wang et al. 2017). To keep the same nitrogen load as ER, 0.406 g of NH_4Cl was added into CR simultaneously with the retreated return sludge. During the FA Phase, ER had 533$\pm$75 mg/L of TSS and 498$\pm$66 mg/L of VSS, while CR had 637$\pm$60 mg/L of TSS and 601$\pm$55 mg/L of VSS (Figure A-1, Appendix A). TSS/VSS ratio in the two reactors was 0.94 in the FA Phase. Figure 2-1 b shows operation of the two SBRs in FA Phase.

(Eq. 2-1)
$$NH_3 = (NH_3 + NH_4^+) \times \frac{10^{pH}}{\frac{K_b}{K_w} + 10^{pH}}$$

(Eq. 2-2)
$$\frac{K_b}{K_w} = e^{\frac{6344}{273+T}}$$



(a)



(b)

Figure 2-1 Schematic diagrams of SBRs during (a) Non-FA Phase and (b) FA Phase

2.1.2 Monitoring

TSS and VSS of mixed liquor and effluents were measured daily. Waste (Q_W) was calculated by Eq. 2-3 based on estimated TSS in mixed liquor and effluent to maintain 10-day SRT (Tchobanoglous et al. 2003). Nitrogen species (ammonium, nitrite, and nitrate), orthophosphate, and soluble COD (sCOD) in effluent samples were monitored three to four times per week. During the FA Phase, effluent samples were taken just before the addition of return sludge. pH and DO were measured at least three times per week. All relevant analytical methods are described in Section 2.4.

(Eq. 2-3)
$$Q_W = \left(\frac{X}{X - X_e}\frac{V}{SRT} - \frac{X_e}{X - X_e}Q\right) \times 1000$$

Where Q_W is waste sludge flow rate (mL/d); X is TSS in mixed liquor (mg/L); X_e is TSS in effluent (mg/L); V is reactor volume (L); Q is influent flow rate (L/d).

2.2 In-situ cycle monitoring experiments

2.2.1 3-hour SBR cycle monitoring

In order to evaluate ammonium removal performance, experiments were conducted over 3-hour SBR cycle after reactors reached steady state in Non-FA and FA Phases. This 3-hour SBR cycle monitoring was conducted only once in each phase. A spike of ammonium solution was added into the two SBRs during the 2-min feeding period to obtain an initial ammonium level at around 10 mg N/L. Mixed liquor samples were taken every 15 to 20 min during the 3-hour SBR cycle,

and were pretreated (see Section 2.4) and stored at 4°C for further measurement of nitrogen species and COD. DO was measured to be above 3 mg/L in the two reactors during all cycle tests, and pH varied within 6.5-7.5 and 7.4-7.9 in non-FA and FA Phase, respectively (Figure B-1, Appendix B).

2.2.2 24-hour SBR effluent monitoring

During the FA Phase, treated sludge was returned back to the SBRs every 24 hours, and effluent nitrogen results showed that the concentrations of nitrogen species changed throughout the day. Therefore, 24-hour monitoring experiments were performed approximately every 10 days to monitor ammonium removal performance over 8 of the 3-hour SBR cycles within 24 hours. Mixed liquor samples were sampled just after the addition of treated sludge during the feeding period, and effluent samples were obtained during the settling period of the 1st, 2nd, 3rd, 4th, and 8th cycles after recycling treated sludge into reactors. Samples were pretreated and stored at 4°C for further nitrogen species measurements. The nitrite accumulation ratio (NAR) of each cycle was calculated based on Eq. 2-4 (S_{NO_2} and S_{NO_3} are nitrite and nitrate concentrations in effluent, respectively).

(Eq. 2-4)
$$\mathrm{NAR} = \frac{\mathrm{S_{NO_2}}}{\mathrm{S_{NO_2}} + \mathrm{S_{NO_3}}}$$

2.3 Estimation biokinetics of nitrifying populations

2.3.1 Background and theory of modeling nitrification biokinetics

The Monod equation has been widely used to describe the relationship between the substrate utilization rate (r, mg/(L·d)), the substrate concentration (S, mg NH_4-N/L or mg NO_2-N/L in nitrification process), and biomass concentration (X, mg COD/L) as shown in Eq. 2-5 shown (Tchobanoglous et al. 2003). There are three significant parameters included in the Monod equation: maximum specific growth rate (μ_{max}, d^{-1}), biomass yield coefficient (Y, mg COD/mg NOD), and half-saturation coefficient (K_S, mg NH_4-N/L or mg NO_2-N/L in nitrification process). μ_{max} indicates the maximum growth rate of a microorganism when substrate is being consumed at the maximum rate, while Y describes the ratio of the amount of biomass synthesized to the amount of substrate consumed (Tchobanoglous et al. 2003). K_S is related to the affinity of the microorganism to the substrate of interest. A low K_S value represents a high substrate affinity, which means that the metabolic activity is greater at low substrate concentrations relative to that with a higher K_S value. All three of these parameters reflect the interaction between bacteria and substrate under specific circumstances, and are independent of substrate and biomass concentrations.

An oxygen mass balance describing electrons transfer processes in biological substrate oxidation is established as Eq. 2-7, in which electrons donated by oxidized substrate ($\frac{dS}{dt}$) are utilized by

oxygen (-OUR) and biomass synthesized ($\frac{dX}{dt}$). Eq. 2-6 is the Monod equation for biomass synthesis ($\frac{dX}{dt}$), neglecting biomass decay during the short duration of the batch test.

(Eq. 2-5) $$r = \frac{dS}{dt} = \frac{\mu_{max} X\, S}{Y\,(K_S + S)}$$

(Eq. 2-6) $$\frac{dX}{dt} = \frac{\mu_{max} X\, S}{(K_S + S)}$$

(Eq. 2-7) $$OUR = \frac{d\,DO}{dt} = \frac{dX}{dt} - \frac{dS}{dt} = \left(1 - \frac{1}{Y}\right)\frac{\mu_{max} X\, S}{(K_S + S)}$$

A process model based on the oxygen mass balance (Eq. 2-7) was developed in the study to estimate growth kinetic characteristics of nitrifiers (AOB and NOB). In this model, the biomass yield (*Y*) was determined by respriometric batch tests using ammonium or nitrite (see Section 2.3.2). Respirometric batch tests are an effective method to quantify the relationship among biomass yield, substrate consumption, and oxygen uptake (Liu and Wang 2012). The respirometric method applied in this research was adapted from the approach described in Liu and Wang 2012. The stoichiometry of ammonium oxidation and nitrite oxidation are described as Eq. 2-8 and Eq. 2-9 respectively, and Eq. 2-10 for complete nitrification (Liu and Wang 2012). The stoichiometric links between substrate (NH_4^+ or NO_2^-), oxygen (O_2), and biomass ($C_5H_7O_2N$) are summarized in Table 2-1. $f_{S,NH}$ and $f_{S,NO}$ are defined as the fraction of electrons used for AOB and NOB synthesis respectively.

(Eq. 2-8)

$$NH_4^+ + \frac{3}{10} f_{s,NH}\ NH_4^+ + \frac{3-3f_{s,NH}}{2}\ O_2 + \frac{6}{5} f_{s,NH} CO_2 + \frac{3}{10} f_{s,NH}\ HCO_3^- = \frac{3}{10} f_{s,NH} C_5H_7O_2N + NO_2^- + 2H^+ + (1 - \frac{3}{10} f_{s,NH}) H_2O$$

(Eq. 2-9)

$$NO_2^- + \frac{1}{10} f_{s,NO}\ NH_4^+ + \frac{1-f_{s,NO}}{2}\ O_2 + \frac{2}{5} f_{s,NO} CO_2 + \frac{1}{10} f_{s,NO}\ HCO_3^- + \frac{1}{10} f_{s,NO}\ H_2O = \frac{1}{10} f_{s,NO} C_5H_7O_2N + NO_3^-$$

(Eq. 2-10)

$$NH_4^+ + \frac{3f_{s,NH} + f_{s,NO}}{10}\ NH_4^+ + (2 - \frac{3f_{s,NH} + f_{s,NO}}{2})\ O_2 + \frac{6f_{s,NH} + 2f_{s,NO}}{5}\ CO_2 + \frac{3f_{s,NH} + f_{s,NO}}{10}\ HCO_3^- = \frac{3f_{s,NH} + f_{s,NO}}{10}\ C_5H_7O_2N + NO_3^- + 2H^+ + (1 - \frac{3\ f_{s,NH} + f_{s,NO}}{10}) H_2O$$

Table 2-1 Stoichiometric coefficients of substrate, oxygen and biomass in nitrification

Biological reaction	**Substrate (NH_4^+ or NO_2^-)**	**Oxygen (O_2)**	**Biomass ($C_5H_7O_2N$)**
Ammonium oxidation	$1 + \frac{3}{10} f_{s,NH}$	$\frac{3-3f_{s,NH}}{2}$	$\frac{3}{10} f_{s,NH}$
Nitrite oxidation	1	$\frac{1-f_{s,NO}}{2}$	$\frac{1}{10} f_{s,NO}$
Complete nitrification	$1 + \frac{3f_{s,NH} + f_{s,NO}}{10}$	$2 - \frac{3f_{s,NH} + f_{s,NO}}{2}$	$\frac{3f_{s,NH} + f_{s,NO}}{10}$

Based on the stoichiometric links in Table 2-1, $f_{S,NH}$ and $f_{S,NO}$ can be determined when the amount of substrate consumed and oxygen uptake are known. For nitrite oxidation, Eq. 2-11 can

be used to calculate $f_{S,NO}$ using a measured specific oxygen uptake (SOU_{NO}, mg O_2/mg N) with Eq. 2-12 expressing the determination of SOU_{NO}. OU_{NO} and S_{NO} are total cumulative oxygen uptake (mg O_2/L) and corresponding concentration of nitrite consumed (mg N/L) for nitrite oxidation.

(Eq. 2-11) $$f_{s,NO} = \frac{1.14 - SOU_{NO}}{1.14}$$

(Eq. 2-12) $$SOU_{NO} = \frac{OU_{NO}}{S_{NO}}$$

$f_{S,NH}$ can be obtained by another respirometric batch test in which ammonium is the only substrate and complete nitrification takes place. The calculation of $f_{S,NH}$ is expressed as Eq. 2-13, and the specific oxygen uptake (SOU_{NC}, mg O_2/mg N) is defined in Eq. 2-14. OU_{NC} and S_{NH} are total cumulative oxygen uptake (mg O_2/L) and the corresponding concentration of ammonium consumed (mg N/L) respectively during the respirometric batch test for complete nitrification.

(Eq. 2-13) $$f_{s,NH} = \frac{4.57 - SOU_{NC} - 1.14f_{s,NO} - 0.1f_{s,NO}SOU_{NC}}{3.43 + 0.3SOU_{NC}}$$

(Eq. 2-14) $$SOU_{NC} = \frac{OU_{NC}}{S_{NH}}$$

The biomass yield coefficient (Y) is commonly defined as the amount of biomass produced per amount of nitrogen oxidized (mg biomass/mg N-oxidized). Since nitrification is a redox biological reaction, the substrate (ammonium or nitrite) and biomass concentration are expressed

in terms of oxygen demand to calculate and track all state variables in batch tests in similar units (Chandran and Smets 2000). Nitrogen species are defined in terms of nitrogenous oxygen demand (NOD), and carbonaceous oxygen demand (COD) is used for the unit of biomass (X). As a result, 1 mg N of NH_4^+ or NO_2^- have 3.43 mg or 1.14 mg of NOD in ammonium and nitrite oxidation, respectively, while 1 mg of biomass ($C_5H_7O_2N$) has 1.42 mg of COD. The unit of Y can be expressed as mg COD/ mg NOD, which makes Y for AOB and NOB (Y_{AOB} and Y_{NOB}) equal to $f_{s,NH}$ and $f_{s,NO}$ respectively as shown in Eq. 2-15 and Eq. 2-16.

(Eq. 2-15) $$Y_{AOB} = f_{s,NH}$$

(Eq. 2-16) $$Y_{NOB} = f_{s,NO}$$

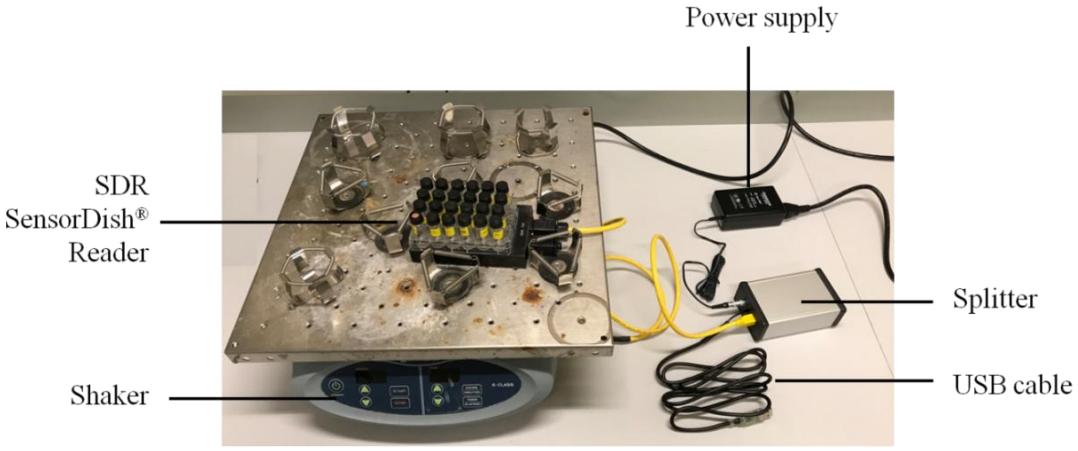
2.3.2 Respirometric batch tests

Respirometric batch tests were performed by providing a certain amount of substrate (NH_4^+ or NO_2^-) to activated sludge biomass to promote complete nitrification or nitrite oxidation respectively. Sufficient oxygen was supplied to ensure all the substrate would be consumed during the test. The DO within the batch vials was then measured over time. Total oxygen uptake and the oxygen uptake rate (OUR) were then calculated for determination of biomass yield, as well as for biokinetic parameter estimation from the process model.

The respirometric batch tests were conducted once the two SBRs became stable, which occurred by day -20 in the Non-FA Phase and by day 70 after the start of FA Phase (Figure A-2, Appendix A). For the batch tests, 100 mL of mixed liquor was taken at the end of the reaction period, and

washed by the medium containing same compositions of macro and trace elements, phosphate and alkalinity as the synthetic feed to remove ammonium, nitrite and organic carbon. The washed sludge was then resuspended in 200 mL of feed medium without ammonium and organic carbon. Samples collected from the resuspended mixed liquor revealed that no ammonium, nitrite, nitrate, nor COD remained (data not shown). After that, the mixed liquor was aerated by an air diffuser connected to an oxygen tank to increase the DO to over 22 mg/L. A 24-channel reader for non-invasive detection of oxygen in multiple vials (SDR SensorDish® Reader, PreSens Precision Sensing, Regensburg, Germany), was employed in the batch tests. The SDR SensorDish® Reader is equipped with a 24-position deep well plate, each containing a 5-mL vials with a colorimetric optode on the inside bottom surfaces, which makes it possible to perform respirometric monitoring of shaken cultures and simultaneously test 8 different initial substrate concentrations in triplicate (Figure 2-2 b). After the DO reached 22 mg/L, 4.75 mL of the aerated mixed liquor and 0.25 mL of ammonium chloride (NH_4Cl) or sodium nitrite ($NaNO_2$) stock solutions were immediately added into each SDR vial. In order to estimate maximum specific growth rate (μ_{max}) and half-saturation coefficient (K_S) at the same time, the respirometric batch tests were carried out with initial substrate concentrations below or equal to 3 mg N/L. The initial ammonium concentrations employed in the batch tests for complete nitrification were 0, 0.25, 0.5, 1, 1.5, 1.75, 2, and 2.5 mg N/L, while initial nitrite concentrations in the batch tests for nitrite oxidation were 0, 0.25, 0.5, 1, 1.5, 2, 2.5, and 3 mg N/L. The 24 vials were sealed with caps and placed onto the SDR SensorDish® Reader, which was configured to measure DO every 30 seconds. The SDR SensorDish® Reader was placed on a shaker (Lab-Line SHKE2000 MaxQ 2000, Barnstead) with a speed of 275 rpm. Biomass mixing was further promoted by including a

small glass bead with a diameter of 4 mm in each vial. Temperature was controlled at 20°C. DO levels were recorded by a laptop computer connected to the SDR SensorDish® Reader. The SDR reader stopped recording after 5 hours, or when the DO was fully depleted. One respirometric batch test was carried out with each substrate (ammonium or nitrite) for each SBR biomass. Therefore, in order to estimate biokinectis of both AOB and NOB in the two SBRs biomasses, four respirometric batch tests were required in total for each operational phase.



(a)

(b)

Figure 2-2 Respirometric batch tests setup using SDR SensorDish® Reader: (a) batch tests setup; (b) SDR SensorDish® Reader setup

Raw respirograms, which recorded DO with time, were saved in the laptop computer. Adjusted respirograms (Figure C-1, Appendix C) were generated by recalculating DO values with calibration curves made with each vial before the batch tests. DO values below 1 mg/L were removed to avoid impacts of oxygen limitation on the biological reaction rate constants (Ma et al., 2011). Oxygen uptake rate (OUR) curves (Figure C-2, Appendix C) were produced by calculating the slope of DO within a moving window of 5 min. The decay rate of sludge was assumed to be the same in vials with different initial substrate concentrations, and was accounted for by subtracting the OUR respirograms of the blank vials (0 mg N/L of substrate). Cumulative oxygen uptake for each time point (Figure C-3, Appendix C) was calculated by integrating OUR from beginning of the batch tests. Maximum cumulative oxygen uptake (OU_{NO} and OU_{NC}) and specific oxygen uptake (SOU_{NO} and SOU_{NC}) for different initial substrate concentrations were obtained from the cumulative oxygen uptake curves (Figure C-4, Appendix C). Finally, the biomass yield coefficient (Y) for AOB (Y_{AOB}) and NOB (Y_{NOB}) was determined by equations Eq. 2-11 to Eq. 2-16. All of the above calculations were carried out using in R version 3.4.3 (R. Core Team 2014).

2.3.3 Process modeling development

To estimate maximum specific growth rate (μ_{max}) and half-saturation coefficient (K_S) for AOB and NOB, a process model was implemented in AQUASIM (Reichert 1994). The process model constructed in AQUASIM was based on the oxygen mass balance (Eq. 2-7) and Monod equation for biomass synthesis (Eq. 2-6) described above. Since ammonium or nitrite was the only substrate for growth and the bacterial decay rate was removed via subtraction of blanks, it was

assumed that only complete nitrification or nitrite oxidation occurred during batch tests containing ammonium or nitrite respectively. Hence, the oxygen consumption in excess of endogenous respiration was assumed be via the activity of AOB and NOB, and the oxygen mass balance can thus be expressed as Eq. 2-24. Concentrations of ammonium and nitrite (S_{NH4} and S_{NO2}) at each time point were stimulated by relating the substrate consumption with growth rates of AOB and NOB with the stoichiometric coefficients in Table 2-2 and Eq. 2-21 to 2-23. μ_{max} and K_S were estimated by fitting Eq. 2-24 to measured OUR series (Figure C-2, Appendix C) using the 'Parameter Estimation' function in AQUASIM. More details about the process modeling refer to Appendix F.

(Eq. 2-21) $$\mathrm{OUR} = \frac{\mathrm{d\ DO}}{\mathrm{dt}} = \left(1 - \frac{1}{\mathrm{Y_{AOB}}}\right)\frac{\mathrm{dX_{AOB}}}{\mathrm{dt}} + \left(1 - \frac{1}{\mathrm{Y_{NOB}}}\right)\frac{\mathrm{dX_{NOB}}}{\mathrm{dt}}$$

(Eq. 2-22) $$\frac{\mathrm{dX_{AOB}}}{\mathrm{dt}} = \frac{\mu_{\mathrm{max,AOB}}\mathrm{X_{AOB}}\mathrm{S_{NH}}}{(\mathrm{K_{S,NH}} + \mathrm{S_{NH}})}$$

(Eq. 2-23) $$\frac{\mathrm{dX_{NOB}}}{\mathrm{dt}} = \frac{\mu_{\mathrm{max,NOB}}\mathrm{X_{NOB}}\mathrm{S_{NO}}}{(\mathrm{K_{S.NO}} + \mathrm{S_{NO}})}$$

(Eq. 2-24) $$\mathrm{OUR} = \frac{\mathrm{d\ DO}}{\mathrm{dt}} = \left(1 - \frac{1}{\mathrm{Y_{AOB}}}\right)\frac{\mu_{\mathrm{max,AOB}}\mathrm{X_{AOB}}\mathrm{S_{NH}}}{(\mathrm{K_{S,NH}} + \mathrm{S_{NH}})} + \left(1 - \frac{1}{\mathrm{Y_{NOB}}}\right)\frac{\mu_{\mathrm{max,NOB}}\mathrm{X_{NOB}}\mathrm{S_{NO}}}{(\mathrm{K_{S.NO}} + \mathrm{S_{NO}})}$$

Table 2-2 The stoichiometric coefficients between utilization or production rates of nitrogen species (ammonium and nitrite) and the growth rate of AOB and NOB

Nitrogen species	Growth rate of AOB	Growth rate of NOB
Ammonium (NH_4^+)	$-1/Y_{AOB} - 0.3$[a]	-0.1[a]
Nitrite (NO_2^-)	$1/(3\ Y_{AOB})$	$-1/Y_{NOB}$

a. 0.3 and 0.1 are nitrogen content of AOB and NOB biomass (mg NOD/mg COD) respectively (Chandran and Smets 2000)

2.4 Biomass determination

Acquiring accurate values of biomass concentration (X) for AOB (X_{AOB}) and NOB (X_{NOB}) is important for estimating biokinetic parameters, as the value of X can affect the result of μ_{max} as shown in the Monod equation (Eq. 2-5). However, it is very difficult to measure X_{AOB} and X_{NOB} directly in a mixed culture like activated sludge, which contains numerous bacterial species that are either active or not (Ellis et al. 1996). Some previous studies have used the total volatile suspended solids (VSS) or total biomass concentration to represent nitrifier biomass, which not only introduces large errors into final result but makes it hard to compare nitrifiers' performance among studies (Chandran and Smets 2000; Dytczak et al. 2008; Lotti et al. 2015). Therefore, an alternative method was used in this study to determine the biomass of nitrifiers.

When the SBR performance reached steady state, X_{AOB} and X_{NOB} in the reactors were calculated based on Eq. 2-17 (Tchobanoglous et al. 2003). The endogenous decay coefficient (b, d^{-1}) was

assumed to be 0.15 d^{-1} for both AOB and NOB during Non-FA and FA Phases (Manser et al. 2006). S_{ox} (mg NOD/L) is the amount of substrate oxidized during reaction periods and calculated by Eq. 2-18 and Eq. 2-19 for AOB and NOB respectively. $\overline{S_{in}}$, $\overline{S_e}$, and $\overline{S_{as}}$ are mean concentrations of substrate (mg NOD/L) in feed, effluent and used for assimilation respectively over 10 days (1 SRT). $\overline{S_{as}}$ is determined by Eq. 2-20. NAR is nitrite accumulation ratio in effluent defined as Eq. 2-4. More details on biomass calculation for nitrifiers refer to Appendix E.

(Eq. 2-17) $$X = \frac{SRT}{HRT} \frac{Y \times S_{ox}}{(1 + b \times SRT)}$$

(Eq. 2-18) $$S_{ox-NH} = \overline{S_{in-NH_4}} - \overline{S_{e-NH_4}} - \overline{S_{as-NH_4}}$$

(Eq. 2-19) $$S_{ox-NO} = \left(\overline{S_{in-NO_2}} - \overline{S_{e-NO_2}} - \overline{S_{as-NO_2}}\right) \times (1 - NAR)$$

(Eq. 2-20) $$\overline{S_{as}} = \overline{S_{in-NH_4}} - \overline{S_{e-NO_3}}$$

In this study, total bacterial biomass was also measured by epifluorescent microscopy based on staining of intercellular DNA with specific fluorescent dyes, and determining biovolume via imaging software analysis (Appendix D).

2.5 Analytical methods

MLSS, MLVSS, ammonium, nitrite, nitrate, and orthophosphate were analyzed according to Standard Methods (APHA 2012). Volatile fatty acids, which comprised the added soluble COD (sCOD), were determined by a gas chromatography (HP 6890 Series GC system, Hewlett

Packard (Agilent)) equipped with a flame ionization detector. pH and DO were measured by a Beckman 40 pH Meter (Beckman Coulter, Massachusetts, USA) and a HQ30D Portable DO Meter (Hach, Colorado, USA), respectively. Table 2-3 summarizes analytical methods applied in the research.

2.6 Statistical inference

The ANOVA test was applied for comparing biokinetic parameters between the two SBRs and the two operational phases. Two parameters with P value smaller than 0.05 were considered to be significantly different.

Table 2-3 Summary of analytical methods

Analyte	Method	Sample Pretreatment	Instrument
TSS	Standard Method 2540 D. Total Suspended Solids Dried at 103–105°C	Filtered onto glass microfiber filters (1.5-µm pore size, Whatman, Pittsburgh, USA), and dried in oven for 12 hours	Fisher Scientific Isotemp 737F Oven for dyring; Ohaus Adventurer AR2140 Analytical Balance (Ohaus, Parsippany, NJ, USA) for weighting
VSS	Standard Method 2540 E. Fixed and Volatile Solids Ignited at 550°C	Ignite residue from TSS measurement in furnace for 30 min	Thermolyne 30400 Furnace (Thermo Fisher Scientific) for burning; Ohaus Adventurer AR2140 Analytical Balance (Ohaus, Parsippany, NJ, USA) for weighting
Ammonia (NH_3-N)	Standard Method 4500-NH_3 H. Flow Injection Analysis	Centrifuged at 5000 rpm for 5 min, filtered through glass filters (1.2-µm pore size, Fisherbrand™), and stored at 4 ^oC before measurement	Thermo Iec Multi Rf Centrifuge (Thermo IEC, USA); Lachat QuikChem® 8000 Series (Lachat Instrument, Wisconsin)
Nitrite (NO_2^--N)	Standard Method 4500-NO_2^- I. Cadmium Reduction Flow Injection Method	Centrifuged at 5000 rpm for 5 min, filtered through glass filters (1.2-µm pore size, Fisherbrand™), and stored at 4 ^oC before measurement	Thermo Iec Multi Rf Centrifuge (Thermo IEC, USA); Lachat QuikChem® 8000 Series (Lachat Instrument, Wisconsin)
Nitrate (NO_3^--N)	Standard Method 4500-NO_3^- I. Cadmium Reduction Flow Injection Method	Centrifuged at 5000 rpm for 5 min, filtered through glass filters (1.2-µm pore size, Fisherbrand™), and stored at 4 ^oC before measurement	Thermo Iec Multi Rf Centrifuge (Thermo IEC, USA); Lachat QuikChem® 8000 Series (Lachat Instrument, Wisconsin)
Orthophosphate (PO_4^{3-}-P)	Standard Method 4500-P G. Flow Injection Analysis for Orthophosphate	Centrifuged at 5000 rpm for 5 min, filtered through glass filters (1.2-µm pore size, Fisherbrand™), and stored at 4 ^oC before measurement	Thermo Iec Multi Rf Centrifuge (Thermo IEC, USA); Lachat QuikChem® 8000 Series (Lachat Instrument, Wisconsin)

Analyte	**Method**	**Sample Pretreatment**	**Instrument**
sCOD	N/A	Centrifuged at 5000 rpm for 5 min, filterd through glass filters (1.2-µm pore size, Fisherbrand™), acidified to pH 2 by H_2SO_4, and stored at 4 ^{o}C before measurement	Thermo Iec Multi Rf Centrifuge (Thermo IEC, USA); HP 6890 Series GC system (Hewlett Packard (Agilent))
pH	N/A	Take less than 10 mL of mixed liquor from SBRs into beakers	Beckman 40 pH Meter (Beckman Coulter, Massachusetts, USA)
DO	N/A	N/A	HQ30D Portable DO Meter (Hach, Colorado, USA)

3 Results

3.1 Performance of SBRs during the Non-FA Phase

Complete nitrification was achieved in the two SBRs before the start of the FA Phase. Less than 1.0 mg N/L of ammonium (NH_4^+) and no nitrite (NO_2^-) was present in the effluent of the two SBRs during the last 50 days of the Non-FA Phase (Figure 3-1). Moreover, the effluent nitrogen species were similar in the two SBRs by the end of the Non-FA Phase, indicating that their identical operation selected for similar metabolic pathways. The effluent nitrate (NO_3^-) level was slightly lower than the influent ammonium level in both of two SBRs, which was likely due to assimilation of ammonium for biomass production (Chandran and Smets 2000; Liu and Wang 2012). The 3-hour cycle monitoring for the Non-FA Phase was carried out when the reactors had reached steady-state at day -35 in the Non-FA Phase. During a single SBR cycle, ammonium was fully depleted and less than 1.2 mg N/L of nitrite was present in the two SBRs at the end of the reaction period, indicating the achievement of complete nitrification in ER and CR (Figure 3-2).

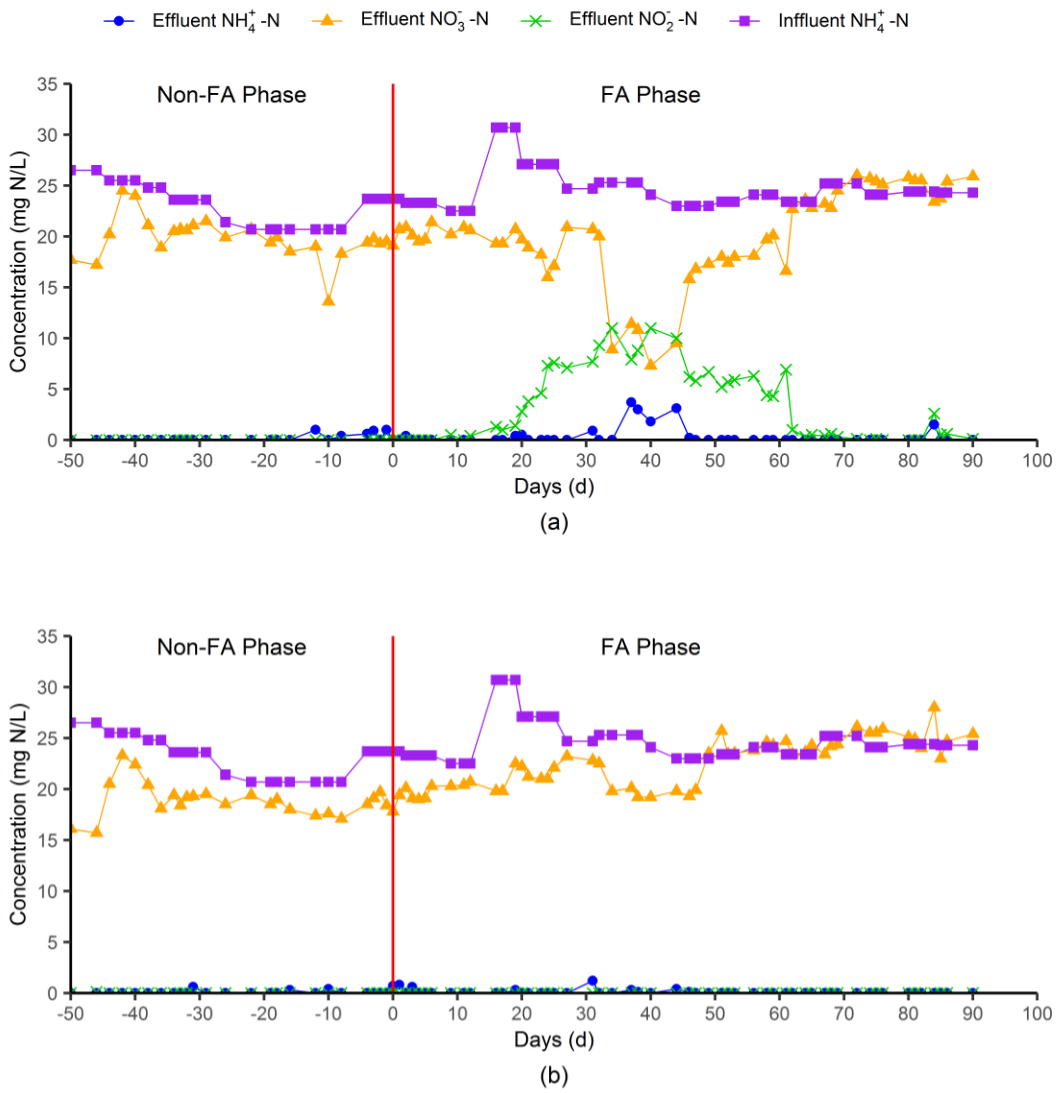


Figure 3-1 Influent ammonium and effluent nitrogen species in SBRs before and after the start of FA phase: (a) ER; (b) CR

3.2 Performance of SBRs during the FA Phase

FA treatment of return sludge helped to promote nitrite accumulation in the ER. After treating a fraction of the return sludge from ER with synthetic centrate containing 200 mg N/L as FA continuously, nitrite started to accumulate after 16 days in the effluent of ER, and reached its

peak of 11 mg N/L at day 34 of the FA Phase, and further maintained at around 10 mg N/L for 10 days (Figure 3-1a). During the same period of time, no nitrite was observed in the effluent from CR. This change in effluent nitrite concentration within ER reflects that NOB activity decreased, and became slower than AOB when return sludge was treated by the high-FA solution (Cao et al. 2017). However, an average of 3 mg N/L of ammonium remained in effluent samples from ER when the effluent nitrite level was at its highest (Figure 3-1a), revealing that AOB activity was also inhibited by FA treatment, but was less sensitive than NOB activity. Meanwhile, the accumulation of nitrite and ammonium resulted in a significant reduction of nitrate from 19 mg N/L to 10 mg N/L in the ER effluent. It is important to note that these effluent nutrient values were obtained at the end of a single SBR cycle daily, and thus do not represent cumulative daily averages of nutrient values over all SBR cycles. For that reason, effluent nutrient values were also monitored throughout select days to determine how AOB and NOB activities changed diurnally.

The 24-hour SBR effluent monitoring of ER (solid curves in Figure 3-3a) was conducted on day 37 of the FA Phase when effluent nitrite from ER reached its plateau, and demonstrated stable effluent nitrite accumulation up to 8.3 ± 0.3 mg N/L within 24 hours in Figure 3-3a. Based on the effluent nitrogen levels monitored over the 24-hour period on day 37, the average NAR was 41.9 $\pm$ 2.1% in ER (Figure 3-4). In contrast, CR had its return sludge treated by a solution that did not contain FA, and had maintained NAR values below 10% throughout the entire FA Phase (Figure 3-4). The observed NAR in CR was significantly lower than that of ER over the FA Phase ($P < 0.05$). Therefore, the ER performance described above demonstrates that 200 mg N/L

of FA can help to accumulate nitrite and promote the partial nitritation pathway by inhibiting NOB activity more than AOB.

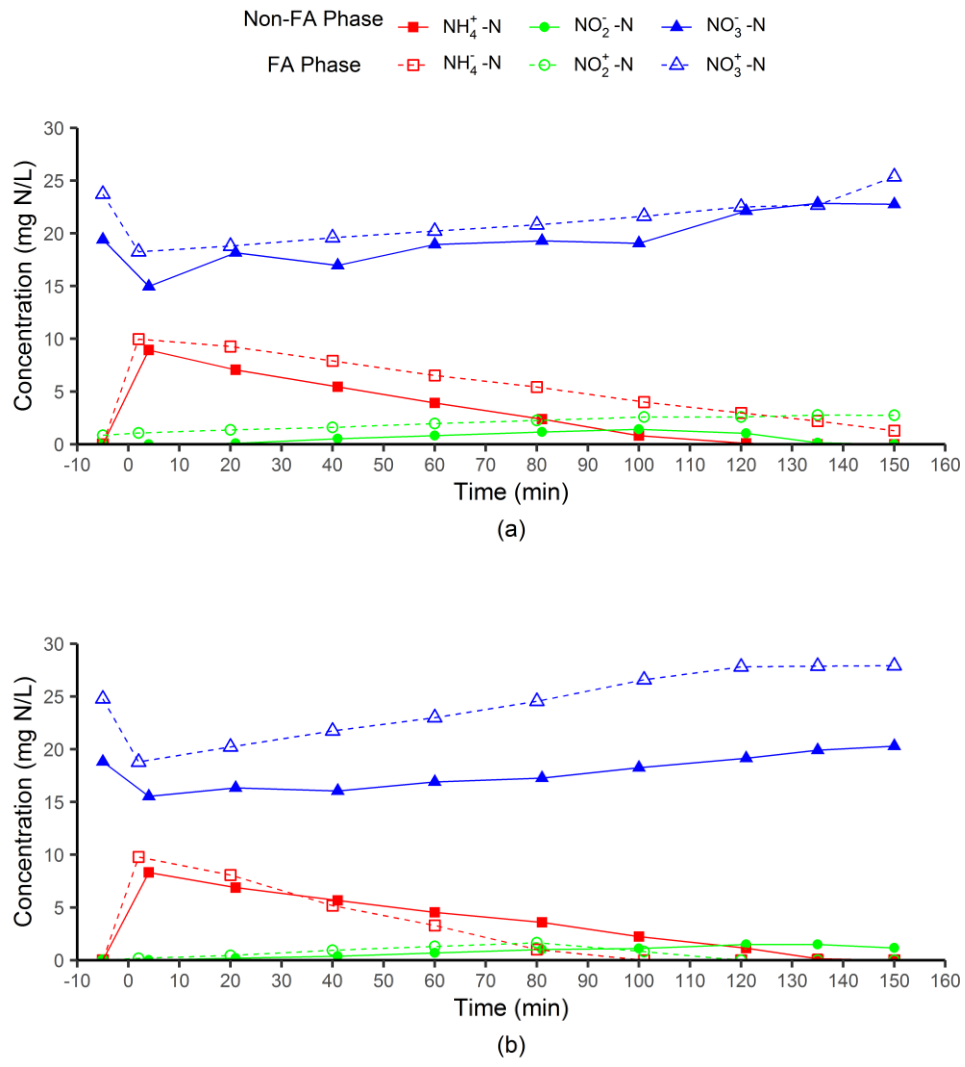


Figure 3-2 Ammonium (NH_4^+-N), nitrite (NO_2^--N), and nitrate (NO_3^--N) concentrations of the 3-hour SBR cycle monitoring for Non-FA and FA Phase: (a) ER; (b) CR. The 3-hour SBR cycle monitoring was conducted at day -30 before and day 84 after the start of FA treatment for non-FA phase and FA phase respectively. Time of 0 min represents the start of feeding period of a SBR cycle.

However, the nitrite accumulation caused by FA treatment of return sludge was not stable in ER. After remaining at its peak value for 10 days, the effluent nitrite declined to less than 1 mg N/L after day 64 of FA treatment (Figure 3-1a). At the same time, no ammonium was detected in ER effluent. These changes reflects that NOB and AOB in ER were able to acclimate to the exposure to high FA levels in sludge treatment reactor (Villaverde et al. 2000; Wu et al. 2016), so that by day 60 the nitrification performance in ER returned back to similar condition as the beginning of the FA Phase. The acclimation of the ER biomass was further revealed by the 24-hour effluent monitoring of ER on day 75 of the FA Phase, in which nitrite transiently accumulated within the first four SBR cycles and was subsequently consumed by the 8^{th} SBR cycle (Figure 3-3b), leading to a low NAR of $10.9 \pm 6.0\%$ (Figure 3-4).

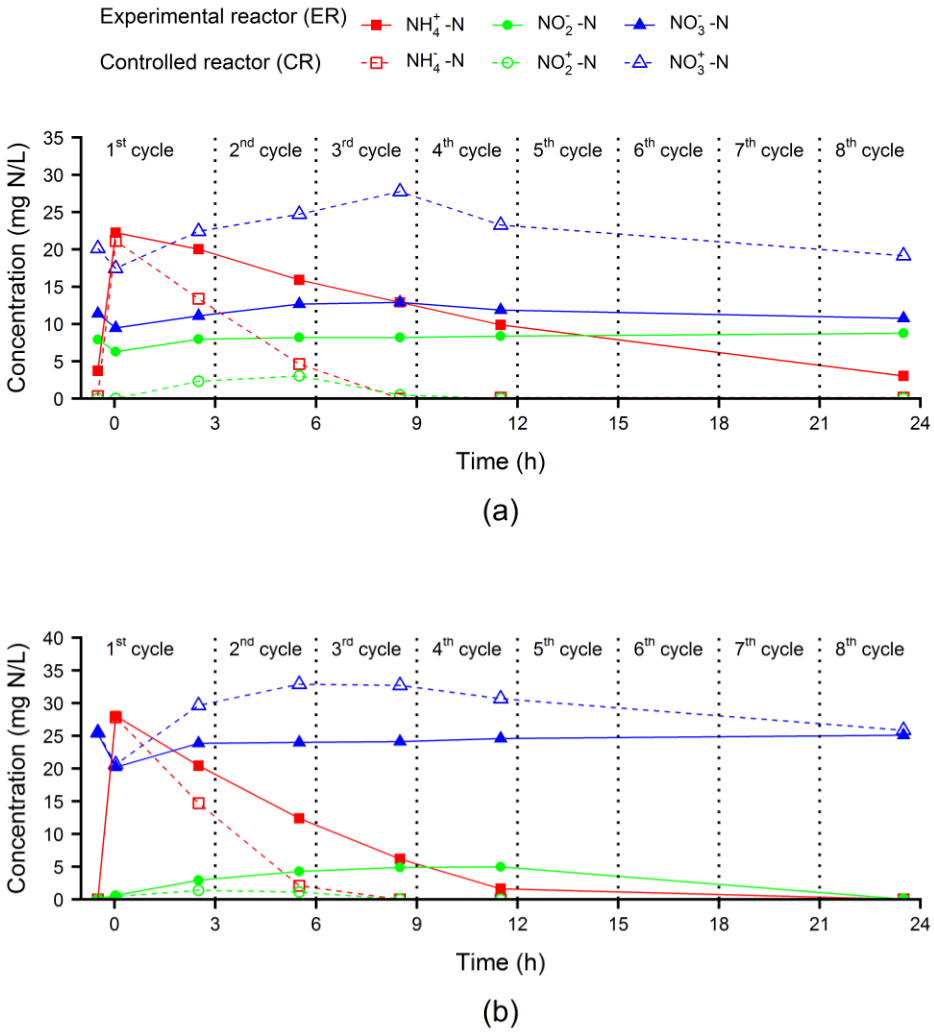


Figure 3-3 Ammonium (NH_4^+-N), nitrite (NO_2^--N), and nitrate (NO_3^--N) concentrations in ER and CR during a 24-hour period. These 24-hour effluent monitoring were carried out at day (a) 37; (b) 75 of the FA Phase.

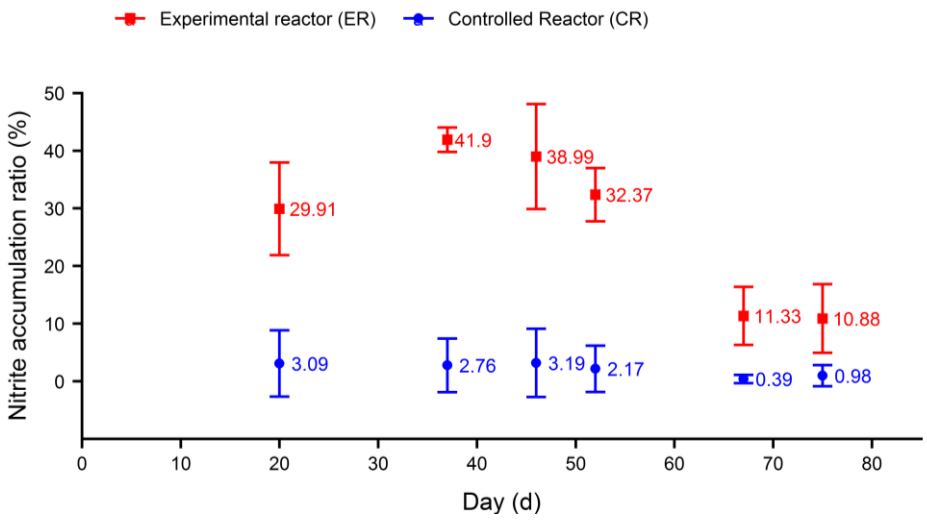


Figure 3-4 Nitrite accumulation ratio (NAR) in ER and CR after the start of FA phase. Error bars represent standard deviations

3.3 Biokinetic parameter estimation

Biokinetic parameter estimation, including respirometric batch tests and biomass determination, was carried out after day -35 of the Non-FA Phase and day 65 of the FA Phase for FA phase, which were periods when SBR performance was stable (Figure 3-1). Except for the yield coefficient of NOB (Y_{NOB}), the yield coefficient (Y) and biomass concentration (X) of AOB and NOB during the Non-FA Phase had no significant differences between ER and CR ($P>0.05$, Table G-1, Appendix G), indicating that the two SBRs had similar substrate utilization for cell production at the initial steady-state period of replicated operation. When the SBR performance became stable in the FA Phase, Y and X for AOB and NOB in the two SBRs were increased relative to the Non-FA Phase (Figure 3-5).

However, the increasing trends of Y and X after starting FA treatment were not of the same magnitude in ER and CR. Y_{AOB} in ER increased by 397% during the FA Phase, which was much greater than that of Y_{NOB} (15%) (Figure 3-5a). Y_{AOB} and Y_{NOB} in CR, on the contrary, increased at similar ratios so that the value of Y_{NOB} was always 2-3 times higher than Y_{AOB} (Figure 3-5a). X_{AOB} in ER at steady state of the FA Phase was about 7 times higher than that of the Non-FA Phase. However, statistical analysis shows that no significant increase (Table G-2, Appendix G) was found in X_{NOB} from ER when operation was switched from the Non-FA Phase to the FA Phase (Figure 3-5c).

The maximum growth rates (μ_{max}) of both AOB and NOB were inhibited by temporary FA treatment at 200 mg N/L, but NOB was more sensitive to FA inhibition throughout operation. The estimated μ_{max} of AOB and NOB from CR increased during the FA Phase, while that of ER decreased (Figure 3-5c). Compared with a 27% reduction of $\mu_{max,AOB}$, the μ_{max} of NOB in ER decreased by 65%, which was 2.4 times higher than that of AOB, indicating a higher sensitivity of NOB to FA inhibition. Both ER and CR had increased $K_{S,AOB}$ at steady state of the FA Phase, while $K_{S,NOB}$ declined in ER and increased in CR.

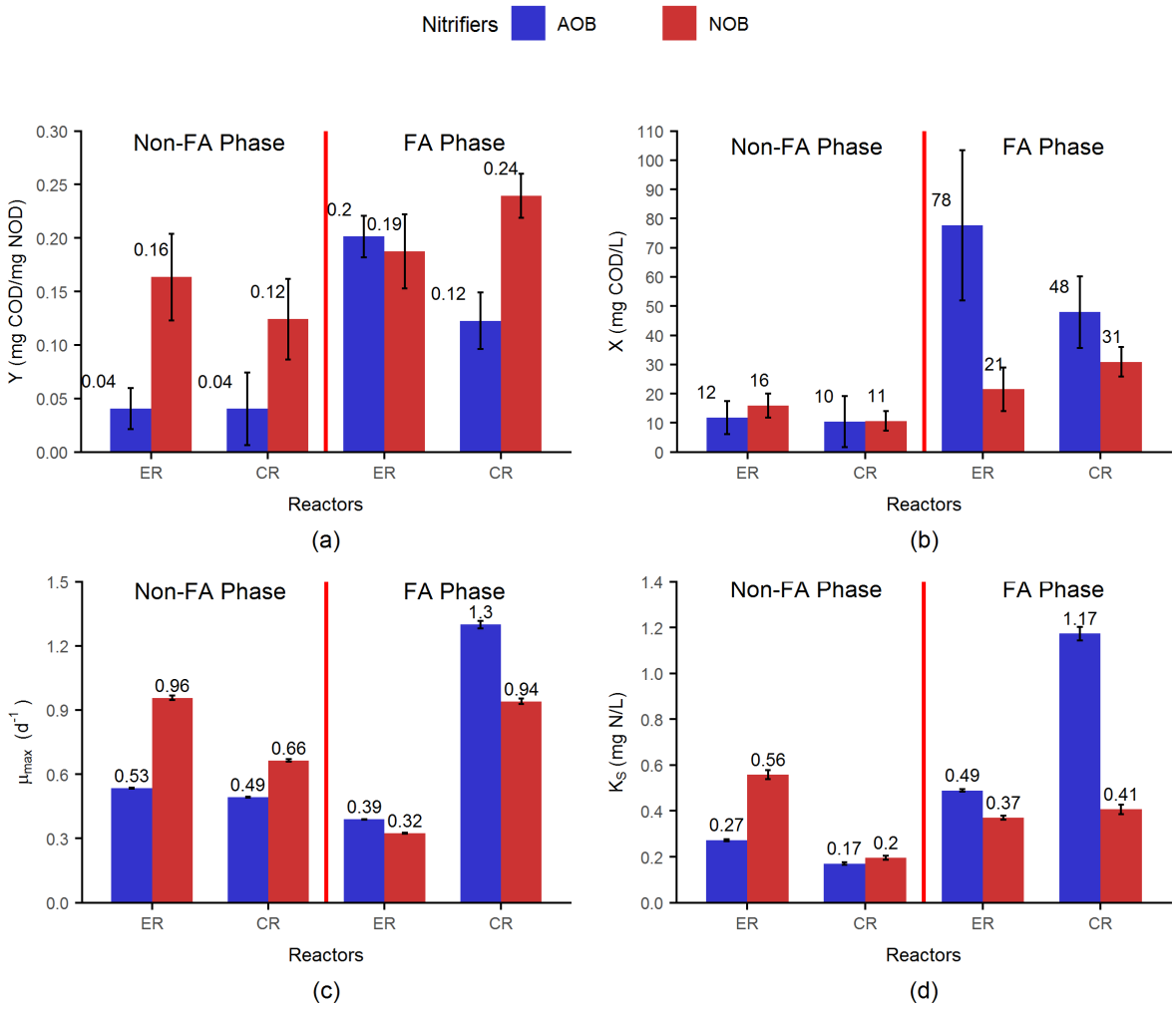


Figure 3-5 Estimated biokinetic parameters from respirometric batch tests: (a) bacterial yield coefficient (Y); (b) nitrifiers biomass (X); (c) maximum specific growth rate (μ_{max}); (d) half-saturation coefficient (K_S). Error bars represent standard deviations.

4 Discussion

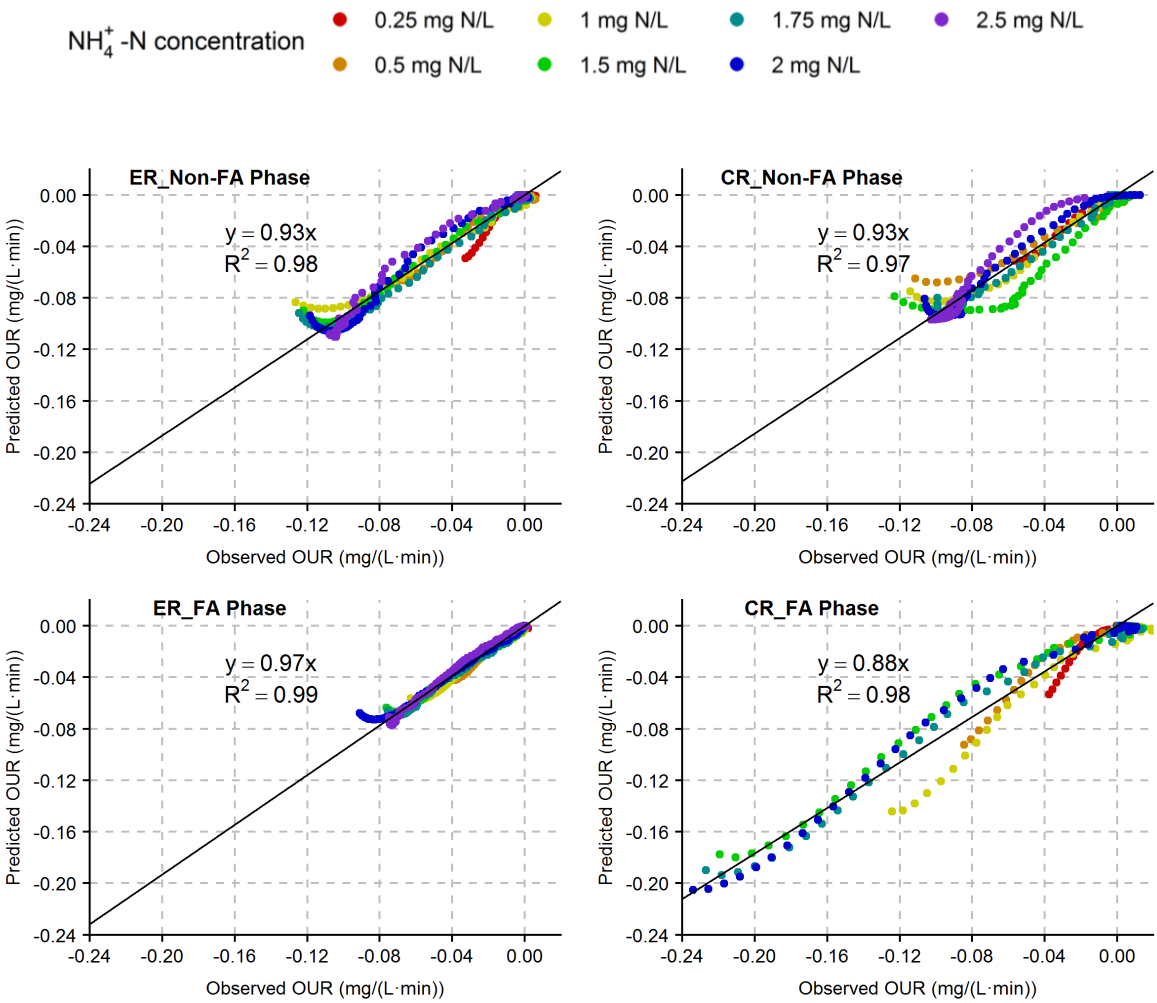
The observed loss of nitrite accumulation and the decrease in NAR in the ER during the latter part of the FA Phase indicates that NOB were able to acclimate to operational conditions that involved temporary exposure to a high FA concentration (200 mg N/L). This acclimation might be the result of elevated resistance of NOB to the high FA concentration. Villaverde et al. (2000) observed that the threshold inhibitory FA concentration of NOB increased from 0.44-0.84 to 1.50-2.87 mg N /L within 4 months. Ruiz et al. (2003) also reported temporal nitrite accumulation and biomass adaptation to FA as high as 124 mg N/L. It was also suggested that the level of acclimation might be greater for a smaller NOB concentration in comparison to AOB, which is not beneficial for the application of FA treatment into full-scale WWTPs to achieve stable partial nitritation (Villaverde et al. 2000).

In addition, the treatment of return activated sludge with high FA could lead to a shift in microbial community structure, in which nitrifying bacteria that grow faster under high FA environments are favored, and thus the ammonium and nitrite oxidation rate would increase after a transient period of nitrite accumulation. *Nitrospira* and *Nitrobacter* are two different NOB genera commonly observed in WWTPs (Cao et al. 2017). *Nitrospira* are regarded as K strategists that have a low K_S and low μ_{max}, while *Nitrobacter* are typical r-strategists possessing a high K_S and high μ_{max} (Xu et al. 2015; Cao et al. 2017). The activity of both of these two NOB groups can be suppressed by FA (Blackburne et al. 2007; Cao et al. 2017). However, *Nitrospira* were reported to be more sensitive to FA than *Nitrobacter* when FA increased from 0.08 to 150 mg N/L, which indicates that *Nitrobacter* would be more likely to dominate under a high FA environment, and tend to have higher K_S and μ_{max} (Blackburne et al. 2007). However, the observed changes of $\mu_{max,NOB}$ and $K_{S,NOB}$

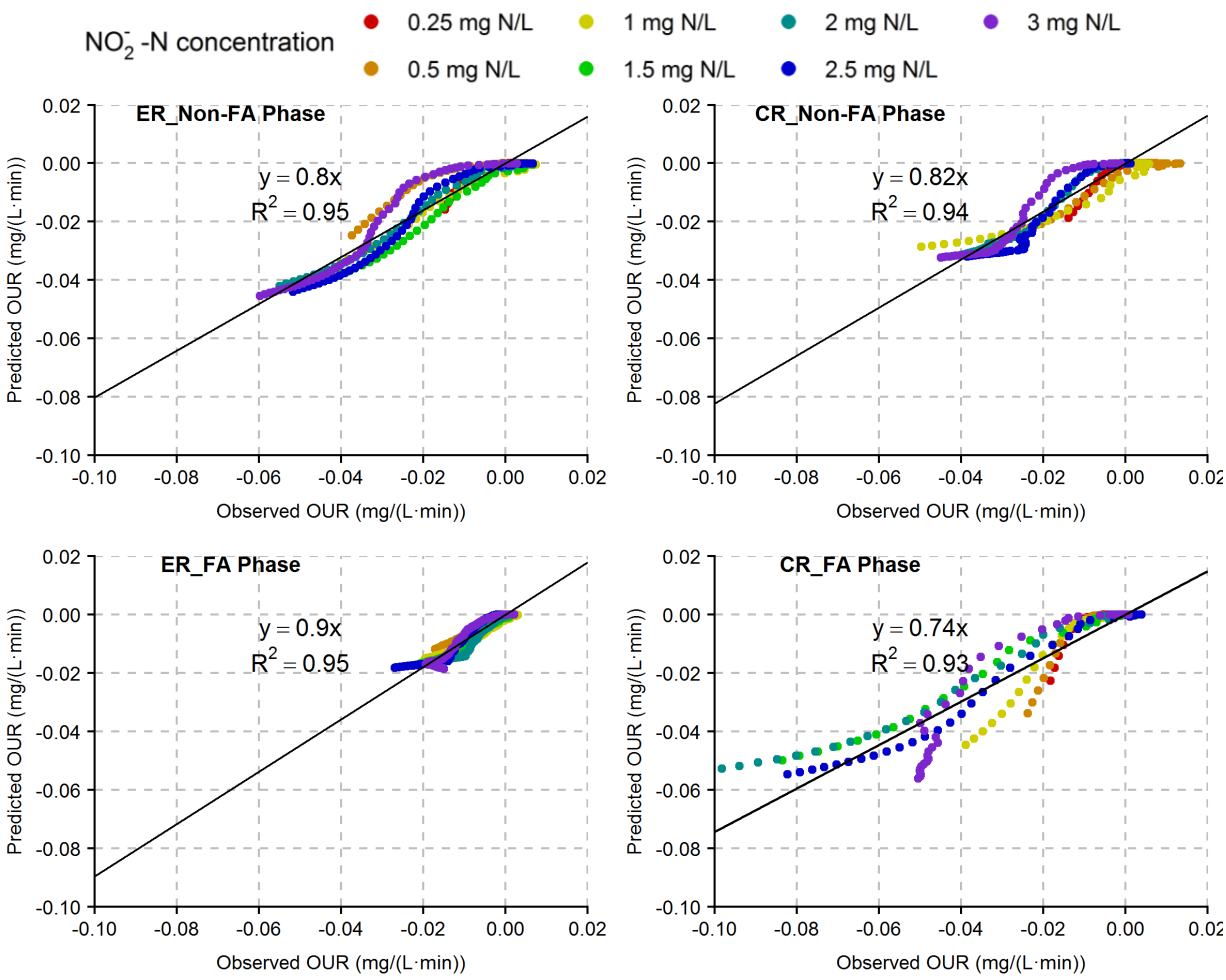
in the ER in this study trended in the opposite way, in that both $\mu_{max,NOB}$ and $K_{S,NOB}$ declined simultaneously after FA sludge treatment. Thus, *Nitrobacter* and *Nitrospira* might have similar activities at an FA above 150 mg N/L (Blackburne et al. 2007), and the observed changes in NOB biokinetics in this study may have occurred at the species or strain level. There are also two genera of AOB, *Nitrosomonas* (r-strategists) and *Nitrosospira* (K-strategist), that are commonly found in activated sludge (Dytczak et al. 2008; Cao et al. 2017). Compared with *Nitrosomonas*, *Nitrosospira* were washed out more easily under a high FA environment at around 60 mg N/L in a partial nitritation reactor (Yao et al. 2017). Consequently, the elevated $K_{S,AOB}$ for ER in the FA phase might be attributed to increasing number of *Nitrosomonas* in the microbial community. Another possible reason for the short period of nitrite accumulation was proposed in Simm (2004) based on the analysis of RNA in a lab-scale SBR, that an initial perturbation (like increasing pH, FA, etc) might be able to induce nitrite accumulation, while the length of the shunt period may depend on the ratio of NOB to AOB and the operational conditions after perturbation. Meanwhile, the discovery of complete ammonia oxidation (comammox) may complicate the interpretation of biokinetic parameters and different community physiologies, since FA inhibition of comammox bacteria has not been studied (Xia et al. 2018). Nonetheless, it is difficult to determine the major reason of transient nitrite accumulation achieved by the strategy of FA treatment on return sludge, based only on results obtained in this study. In order to better understand the mechanisms of nitrifier acclimation towards FA treatment, regular batch tests for biokinetic parameter estimation coupled with microbial community analysis using molecular biological tools, like 16S rRNA sequencing and/or metagenomics, are suggested to obtain more details about the nitrifying community structure and its changes during operation.

This study represents a first attempt to use highly paralleled respirometry, with 8 initial concentrations in triplicate, to routinely characterize nitrifier biokinetics. The measured OUR data points (Figure C-2, Appendix C) obtained from the respirometric batch tests were used as input to the process model in AQUASIM software for the estimation of μ_{max} and K_S, allowing for predicted OUR to be calculated based on the estimated biokinetic parameter values. Linear regression of observed versus predicted OUR shows that predicted OUR for complete nitrification was 93±4% of measured OUR, while predicted OUR for nitrite oxidation was 82±7% of corresponding observed OUR (Figure 4-1). Deviations between observed and predicted OUR may have been introduced from instrument noise during periods of small changes in DO, which was the case during low initial substrate concentrations (e.g. 0.25 and 0.5 mg N/L). Such inaccurate DO recordings could also explain the large deviation between predicted and observed OUR for nitrite oxidation, as this process has a smaller oxygen demand per g N than complete nitrification. On the other hand, a low DO environment can limit nitrifier activity, and thus could be a source of error in the biokinetic parameter estimation. Similar to K_S for nitrogenous substrates, the nitrifier affinity to oxygen is defined as K_O (Tchobanoglous et al. 2003). The metabolic activity of AOB and NOB will be decreased when the DO concentration is close to, or smaller than, the K_O value. The K_O for AOB and NOB has been reported to be within 0.24-1.22 mg/L and 0.43-1 mg/L respectively (Pambrun et al. 2006; Blackburne et al. 2007; Cao et al. 2017). Although when DO was lower than 1 mg/L OUR values were disregarded from respirograms, oxygen limitation of nitrification might have occurred when DO declined to below 3 mg/L, resulting in a decreased similarity between observed and predicted OUR values. In spite of deviations produced during batch tests, SDR SensorDish® Reader employed in the batch tests allowed for more rapid characterization of multiple initial nitrogen concentrations in replicate in comparison with other respirometric setups consisting of large DO

probes and BOD bottles, which typically are not conducted in replicate and thus lack information for robust parameter estimation (Liu and Wang 2012).


NH_4^+ -N concentration
0.25 mg N/L
0.5 mg N/L
1 mg N/L
1.5 mg N/L
1.75 mg N/L
2 mg N/L
2.5 mg N/L
ER_Non-FA Phase
y = 0.93x
R2 = 0.98
CR_Non-FA Phase
y = 0.93x
R2 = 0.97
ER_FA Phase
y = 0.97x
R2 = 0.99
CR_FA Phase
y = 0.88x
R2 = 0.98
Predicted OUR (mg/(L·min))
Observed OUR (mg/(L·min))
0.00
-0.04
-0.08
-0.12
-0.16
-0.20
-0.24

(a)



(b)

Figure 4-1 Comparison of observed and predicted OUR obtained by the respiromteric batch tests and AQUASIM process model respectively for different substrates applied in batch tests: (a) ammonium; (b) nitrite. The slope of linear regression represents similarity between observed and predicted OUR.

During the steady state of the FA Phase, the biomass yield coefficient (*Y*) of both AOB and NOB increased, attributed to a decrease in the specific oxygen uptake (*SOU*) (Figure C-4, Appendix C). In the nitrification process, electrons donated by oxidized nitrogen species (ammonium or nitrite) are utilized for biomass generation or consumed via the respiration of oxygen (Chandran and Smets 2000). Thus, lower *SOU* values suggest that fewer electrons are used for respiration, and more electrons applied to cell production, resulting in a higher yield coefficient. The increase of *Y* in the two SBRs during the FA Phase indicates that the addition of the sludge treatment reactor might have impact on metabolic efficiency and/or energy partitioning. However the mechanism behind such as impact is not clear, and further investigations using molecular expression profiles, such as transcriptomics or proteomics, in the sludge reactor would be required. Despite the increase in biomass yield coefficient, the increase in ER was a different magnitude as CR. The significant increase in Y_{AOB} in ER during the FA Phase reveals that AOB maintained their ammonium oxidation ability by increasing cell production when faced with the new condition of sludge treatment with a high concentration of FA.

Before the observed increase in *Y* in the FA Phase, the biomass yield coefficient (*Y*) determined in the Non-FA Phase are consistent with values reported in literature (Table 4-1). Assuming a nitrifier biomass molecular composition of $C_5H_7O_2N$, which requires 1.42 g of COD to fully oxidize 1 g of cell, Y_{AOB} in the two SBRs are both converted to 0.098 mg cell/mg N, falling within the range of 0.05-0.29 mg cell/mg N reported in literature (Liu and Wang 2012). For NOB, *Y* values in literature range from 0.06 to 0.18 mg COD/mg NOD, depending on the NOB species (Park et al. 2017). As K strategists, *Nitrospira*-NOB are reported to have a higher *Y* of 0.11-0.18 mg COD/mg NOD, compared with 0.06-0.09 mg COD/mg NOD for *Nitrobacter*-NOB (Blackburne et al. 2007; Park et al. 2017). No matter what kind of operational condition was

applied, Y_{NOB} in this study were always larger than 0.11 mg COD/mg NOD in ER and CR, suggesting that *Nitrospira* may be the dominant NOB in the activated sludge. Again, microbial community analysis like metagenomics or 16S rRNA sequencing is necessary to identify dominant bacterial species in the sludge (Xia et al. 2018).

Quantification of specific biomass concentration (X) for nitrifiers is of importance to the estimation of the maximum specific growth rate (μ_{max}), as μ_{max} is inversely proportional to X in the Monod equation (Eq. 2-5) (Ahn et al. 2008). X_{AOB} and X_{NOB} in this study were calculated through substrate mass balance during steady state. The increase in estimated X_{AOB} and X_{NOB} concentrations in CR and X_{AOB} in ER during the FA Phase was mainly due to the increase in biomass yields and large nitrogen loading, which increased from 205 mg N/d to 300 mg N/d due to the addition of synthetic centrate return during FA treatment. In the mass balance calculation, the value of the decay rate (b) of nitrifiers was taken from literature, and was assumed to be a constant of 0.15 d^{-1} throughout the whole experiment. However, this parameter may change under different operational conditions, and introduce error into X and μ_{max} estimation (Manser et al. 2006). Ahn et al. (2008) reported b to be 0.15 ± 0.06 d^{-1} and 1.7 ± 1.9 d^{-1} for AOB and NOB, respectively, from a partial nitritation bioreactor with nitrite accumulation higher than 66%. Liu et al. (2017) applied an aerobic starvation strategy to achieve nitritation at temperature of 29°C and determined b of AOB and NOB as 0.24 ± 0.02 d^{-1} and 0.35 ± 0.02 d^{-1}, respectively. Reported numbers of b for nitrifiers are different from system to system, and so far specific b values under FA inhibition have not been reported yet. Therefore, additional tests to estimate the decay rate may be required to obtain accurate biomass concentrations and avoid erroneous estimates on μ_{max} when using the substrate mass balance method (Ahn et al. 2008). Additionally, X_{AOB} and X_{NOB} could be quantified through molecular tools like quantitative PCR (qPCR) and Fluorescence In

Situ Hybridization (FISH) combined with epifluorescent microscopy. Ahn et al. (2008) applied qPCR to measure functional DNA concentrations and used average cell mass and genomic DNA content value to calculate X_{AOB} and X_{NOB}. Manser et al. (2005) developed a method using FISH to label AOB and NOB with specific fluorescent probes and measure the biovolume of nitrifier aggregates with epifluorescence microscopy. However, an average cell density must be assumed to convert gene/cell counts and/or biovolume into cell mass if qPCR or FISH-epifluorescence microscopy is applied, which is not well known for AOB and NOB (Andreottola et al. 2002; Foladori et al. 2010).

The values of μ_{max} and K_S for nitrifiers estimated in this study correspond to values reported in literature (Table 4-1), and their observed changes in ER after the start of FA phase showed that 200 mg N/L of FA treatment on return sludge has different effects on biokinetics for AOB and NOB. The decline of $\mu_{max,AOB}$ and $\mu_{max,NOB}$ in ER indicates an inhibitory effect of FA at 200 mg N/L on growth and activity of both AOB and NOB, but AOB appeared to be more tolerant to the suppression from FA than NOB, which has also been reported previously (Anthonisen et al. 1976; Chung et al. 2006; Pambrun et al. 2006; van Hulle et al. 2007; Li et al. 2012). The value of $K_{S,AOB}$ for ER biomass was increased during the FA Phase, while $K_{S,NOB}$ decreased, which differs from the results of Pambrun et al. (2006), in which an increasing ammonia concentration led simultaneously to a decreasing in $\mu_{max,NOB}$ and an increasing in $K_{S,NOB}$. For CR, the reason for the significant increase of μ_{max} for AOB and NOB during the FA Phase is not clear, which need to be estimated in the future.

Table 4-1 Biomass yield coefficient (Y), maximum specific growth rate (μ_{max}), and half-saturation coefficient (K_S) in this study and literature

Nitrifiers	Y (g cell/g N oxidized)	μ_{max} (d^{-1})	K_S (mg N/L)	Remarks	Literature
AOB					
AOB	0.098	0.49-0.53	0.17-0.27	20°C, non-FA phase	This study
AOB	0.49±0.047	0.39±0.0014	0.49±0.0054	20°C, FA phase, ER	This study
AOB	0.30±0.064	1.3±0.018	1.2±0.029	20°C, FA phase, CR	This study
AOB	0.18±0.03	NA	NA	20°C, activated sludge, complete nitrification	Liu and Wang (2012)
Nitrosomonas	0.05-0.29	0.03-1.08	0.063-2.0	Activated sludge	Beccari et al. (1979)
AOB	0.22±0.24	1.08±1.03	NA	Room temperature, partial nitritation	Ahn et al. (2008)
AOB	0.16-0.18	0.44-0.67	NA	30°C, activated sludge, complete nitrification	Sepehri and Sarrafzadeh (2019)
AOB	0.15	1.96	0.5	30°C, partial nitritation	Pambrun et al. (2006)
Nitrosomonas	NA	1.77±0.98	23.25	28°C, aerobic sludge	Cho et al. (2013)
Nitrosospira	NA	NA	0.56	30°C, mixed culture	Schramm et al. (1999)
Nitrosospira	NA	0.79–0.84	NA	NA	Siripong and Rittmann (2007)
NOB					
NOB	0.10-0.13	0.66-0.96	0.20-0.56	20°C, non-FA phase	This study
NOB	0.15±0.028	0.32±0.0021	0.37±0.0094	20°C, FA phase, ER	This study

Nitrifiers	Y (g cell/g N oxidized)	μ_{max} (d^{-1})	K_S (mg N/L)	Remarks	Literature
NOB					
NOB	0.19±0.017	0.94±0.013	0.40±0.021	20°C, FA phase, CR	This study
NOB	0.06 ±0.02	NA	NA	20°C, activated sludge, complete nitrification	Liu and Wang (2012)
Nitrobacter	0.02-0.084	0.14-1.44	0.22-1.77	Activated sludge	Beccari et al. (1979)
Nitrospira	0.099±0.014	0.69±0.10	0.52±0.14	22°C, enriched culture	Park et al. (2017)
Nitrospira	0.12-0.2	NA	0.9-1.1	22°C, enriched culture	Blackburne et al. (2007)
Nitrobacter	NA	NA	1.2-1.3	22°C, enriched culture	Blackburne et al. (2007)
Nitrospira	NA	0.5-1.2	0.4-1.2	28-37°C, pure culture	Nowka et al. (2015)
Nitrobacter	NA	1.9-4.0	1.7-13	28°C, pure culture	Nowka et al. (2015)
NOB	0.028±0.014	2.6±2.05	NA	Room temperature, partial nitritation	Ahn et al. (2008)
NOB	0.062-0.072	0.63-0.71	NA	30°C, activated sludge, complete nitrification	Sepehri and Sarrafzadeh (2019)
NOB	0.021	0.67	1.62	30°C, partial nitritation	Pambrun et al. (2006)

Overall, this study justifies the effectiveness of FA treatment on return activated sludge at 200 mg N/L to select for partial nitritation in mainstream wastewater treatment. This was demonstrated with significantly higher NAR values with FA treatment of return sludge in comparison to the control reactor without FA treatment. However, the eventual decrease in nitrite accumulation reveals that the FA treatment strategy implemented in the study does not promote stable partial nitritation treatment performance, likely due to microbial acclimation. It is also important to note that an increase in NOB activity was observed throughout a daily period following FA treatment, and thus continuous recirculation of FA-treated sludge is recommended rather than the batch mode used in this study. Therefore, the FA treatment strategy should be further optimized by varying the amount of treated sludge, treatment period, and FA concentration before its application at full-scale. Besides, the 90-day operational period of FA Phase is a short duration to evaluate the long-term impacts of FA treatment in microbial community. Thereby a relatively longer operational period (e.g. 1 year or longer) may be necessary for future study. On the other hand, combining the FA treatment with other strategies like low DO control below 1.5 mg O_2/L may help to achieve stable partial nitritation (Yao et al. 2017; Wang et al. 2017). Economic analysis, such as energy and construction cost, and global warming impact (e.g. estimation of greenhouse gas emission) also need to be estimated when applying the FA treatment strategy to a full-scale mainstream treatment system.

5 Conclusions

The objective of this study was to examine the effectiveness of the FA treatment strategy to promote partial nitritation in mainstream wastewater treatment and to estimate changes in the nitrifier biokinetics during the inhibition strategy. A synthetic centrate containing FA at 200 mg N/L was used to treat 20% of return activated sludge for 24 hours before recycling the sludge back into the bioreactor, which was also fed with a low-strength ammonium wastewater typical of municipal wastewater. The bioreactor performance and biokinetic parameter results showed that:

- The FA treatment strategy could help to promote nitrite accumulation in the bioreactors, reaching a maximum NAR of 41.9±2.1% after treating return sludge with high FA solution continuously for 37 days;
- Nitrite accumulation achieved by FA treatment, however, was not stable and NAR decreased to 10.9±6.0% after day 70 of the FA Phase;
- The application of FA treatment on return sludge resulted in larger values of Y_{AOB} and decreasing $\mu_{max,AOB}$ and $\mu_{max,NOB}$, while $\mu_{max,NOB}$ had a decrease 2.4 times higher than $\mu_{max,AOB}$.

The adaptation of NOB to treatment with FA hinders its application to full-scale mainstream systems. Therefore, further investigations are needed to optimize FA treatment in combination with other NOB out-selection strategies to suppress bacterial acclimation. In addition, microbial community analysis on activated sludge is also suggested to better understand the response of nitrifiers populations to the operation of FA treatment.

References

Abeling U, Seyfried CF (1992) Anaerobic-Aerobic Treatment of High-Strength Ammonium Wastewater - Nitrogen Removal via Nitrite. Water Science and Technology 26:1007–1015. https://doi.org/10.2166/wst.1992.0542

Ahn JH, Kim S, Park H, et al (2010) N2O Emissions from Activated Sludge Processes, 2008−2009: Results of a National Monitoring Survey in the United States. Environmental Science & Technology 44:4505–4511. https://doi.org/10.1021/es903845y

Ahn JH, Yu R, Chandran K (2008) Distinctive microbial ecology and biokinetics of autotrophic ammonia and nitrite oxidation in a partial nitrification bioreactor. Biotechnology and Bioengineering 100:1078–1087. https://doi.org/10.1002/bit.21863

Andreottola G, Foladori P, Gelmini A, Ziglio G (2002) Biomass active fraction evaluated by a direct method and respirometric techniques. Water science and technology : a journal of the International Association on Water Pollution Research 46:371–379. https://doi.org/10.2166/wst.2002.0504

Anthonisen AC, Loehr RC, Prakasam TBS, Srinath EG (1976) Inhibition of Nitrification by Ammonia and Nitrous Acid. Journal (Water Pollution Control Federation) 48:835–852

APHA (2012) Standard methods for the examination of water and wastewater. American Water Works Association/American Public Works Association/Water Environment Federation.

Augspurger T, Keller AE, Black MC, et al (2003) Water quality guidance for protection of freshwater mussels (Unionidae) from ammonia exposure. Environmental Toxicology and Chemistry 22:2569–2575. https://doi.org/10.1897/02-339

Bae W, Baek S, Chung J, Lee Y (2001) Optimal operational factors for nitrite accumulation in batch reactors. Biodegradation 12:359–366. https://doi.org/10.1023/A:1014308229656

Balmelle B, Nguyen KM, Capdeville B, et al (1992) Study of Factors Controlling Nitrite Build-Up in Biological Processes for Water Nitrification. Water Science and Technology 26:1017–1025. https://doi.org/10.2166/wst.1992.0543

Beccari M, Marani D, Ramadori R (1979) A critical analysis of nitrification alternatives

Blackburne R, Vadivelu VM, Yuan Z, Keller J (2007) Kinetic characterisation of an enriched Nitrospira culture with comparison to Nitrobacter. Water Research 41:3033–3042. https://doi.org/10.1016/j.watres.2007.01.043

Blum J-M, Jensen MM, Smets BF (2018) Nitrous oxide production in intermittently aerated Partial Nitritation-Anammox reactor: oxic N2O production dominates and relates with ammonia removal rate. Chemical Engineering Journal 335:458–466. https://doi.org/10.1016/j.cej.2017.10.146

Brown MR, Hands CL, Coello-Garcia T, et al (2019) A flow cytometry method for bacterial quantification and biomass estimates in activated sludge. Journal of Microbiological Methods 160:73–83. https://doi.org/10.1016/j.mimet.2019.03.022

Camargo JA, Alonso Á (2006) Ecological and toxicological effects of inorganic nitrogen pollution in aquatic ecosystems: A global assessment. Environment International 32:831–849.

https://doi.org/10.1016/j.envint.2006.05.002

Canadian Council of Ministers of the Environment (2010) Canadian water quality guidelines for the protection of aquatic life: Ammonia. In: Canadian environmental quality guidelines, 1999. Canadian Council of Ministers of the Environment, Winnipeg，Canada

Canadian Council of Ministers of the Environment (2012) Canadian water quality guidelines for the protection of aquatic life: Nitrate. In: Canadian environmental quality guidelines. Canadian Council of Ministers of the Environment, Winnipeg，Canada

Cao Y, Loosdrecht M van, Daigger G (2017) Mainstream partial nitritation–anammox in municipal wastewater treatment: status, bottlenecks, and further studies. Applied Microbiology and Biotechnology 101:1365–1383. https://doi.org/10.1007/s00253-016-8058-7

Castro-Barros CM, Daelman MRJ, Mampaey KE, et al (2015) Effect of aeration regime on N2O emission from partial nitritation-anammox in a full-scale granular sludge reactor. Water Research 68:793–803. https://doi.org/10.1016/j.watres.2014.10.056

Chambers PA, Guy M, Roberts ES, et al (2001) Nutrients and their impact on the Canadian environment. Agriculture and AgriFood Canada, Environment Canada, Fisheries and Oceans Canada, Health Canada and Natural Resources Canada

Chan AA, Johansson N, Christensson M (2014) Increased nitrogen removal in existing volumes at Sundet wastewater treatment plant, Växjö. Water Practice and Technology 9:215–224. https://doi.org/10.2166/wpt.2014.025

Chandran K, Smets BF (2000) Single‐step nitrification models erroneously describe batch ammonia oxidation profiles when nitrite oxidation becomes rate limiting. Biotechnology and Bioengineering 68:396–406. https://doi.org/10.1002/(SICI)1097-0290(20000520)68:4<396::AID-BIT5>3.0.CO;2-S

Cho K, Nguyen D, Lee S, Hwang S (2013) Use of real-time QPCR in biokinetics and modeling of two different ammonia-oxidizing bacteria growing simultaneously. Journal of Industrial Microbiology & Biotechnology 40:1015–1022. https://doi.org/10.1007/s10295-013-1297-z

Chung J, Bae W, Lee Y-W, Rittmann BE (2007) Shortcut biological nitrogen removal in hybrid biofilm/suspended growth reactors. Process Biochemistry 42:320–328. https://doi.org/10.1016/j.procbio.2006.09.002

Chung J, Chung J, Shim H, et al (2006) Optimization of free ammonia concentration for nitrite accumulation in shortcut biological nitrogen removal process. Bioprocess and Biosystems Engineering 28:275–282. https://doi.org/10.1007/s00449-005-0035-y

Constantine TA (2006) North American Experience with Centrate Treatment Technologies for Ammonia and Nitrogen Removal. Proceedings of the Water Environment Federation 2006:5271–5281. https://doi.org/10.2175/193864706783763291

Daims H, Stoecker K, Wagner M (2004) Fluorescence in situ hybridization for the detection of prokaryotes. In: Smith C, Osborn M (eds) 1st edn. Taylor & Francis, London, pp 208–228

Dijkman H, Strous M (1999)

Donald MA, Patricia MG, JoAnn MB (2002) Harmful Algal Blooms and Eutrophication: Nutrient Sources, Composition, and Consequences. Estuaries 25:704–726.

https://doi.org/10.1007/BF02804901

Dytczak MA, Londry KL, Oleszkiewicz JA (2008) Activated sludge operational regime has significant impact on the type of nitrifying community and its nitrification rates. Water Research 42:2320–2328. https://doi.org/10.1016/j.watres.2007.12.018

Ellis TG, Barbeau DS, Smets BF, Grady CPL (1996) Respirometric technique for determination of extant kinetic parameters describing biodegradation. Water Environment Research 68:917–926. https://doi.org/10.2175/106143096X127929

Foladori P, Bruni L, Tamburini S, Ziglio G (2010) Direct quantification of bacterial biomass in influent, effluent and activated sludge of wastewater treatment plants by using flow cytometry. Water Research 44:3807–3818. https://doi.org/10.1016/j.watres.2010.04.027

Frison N, Chiumenti A, Katsou E, et al (2015) Mitigating off-gas emissions in the biological nitrogen removal via nitrite process treating anaerobic effluents. Journal of Cleaner Production 93:126–133. https://doi.org/10.1016/j.jclepro.2015.01.017

Frossard A, Hammes F, Gessner MO (2016) Flow Cytometric Assessment of Bacterial Abundance in Soils, Sediments and Sludge. Frontiers in microbiology 7:903. https://doi.org/10.3389/fmicb.2016.00903

Furukawa K, Lieu PK, Tokitoh H, Fujii T (2006) Development of single-stage nitrogen removal using anammox and partial nitritation (SNAP) and its treatment performances. Water science and technology : a journal of the International Association on Water Pollution Research 53:83–90. https://doi.org/10.2166/wst.2006.175

Gandiglio M, Lanzini A, Soto A, et al (2017) Enhancing the Energy Efficiency of Wastewater Treatment Plants through Co-digestion and Fuel Cell Systems. Frontiers in Environmental Science 5:. https://doi.org/10.3389/fenvs.2017.00070

Gilbert EM, Agrawal S, Brunner F, et al (2014) Response of Different Nitrospira Species To Anoxic Periods Depends on Operational DO. Environmental Science & Technology 48:2934–2941. https://doi.org/10.1021/es404992g

Gonzalez-Martinez A, Morillo JA, Garcia-Ruiz MJ, et al (2015) Archaeal populations in full-scale autotrophic nitrogen removal bioreactors operated with different technologies: CANON, DEMON and partial nitritation/anammox. Chemical Engineering Journal 277:194–201. https://doi.org/10.1016/j.cej.2015.04.137

Gonzalez-Martinez A, Muñoz-Palazon B, Rodriguez-Sanchez A, Gonzalez-Lopez J (2018) New concepts in anammox processes for wastewater nitrogen removal: recent advances and future prospects. FEMS microbiology letters 365:. https://doi.org/10.1093/femsle/fny031

He S, Niu Q, Ma H, et al (2015) The Treatment Performance and the Bacteria Preservation of Anammox: A Review. Water, Air, & Soil Pollution 226:1–16. https://doi.org/10.1007/s11270-015-2394-6

Hellinga C, Loosdrecht MCM van, Heijnen JJ (1999) Model Based Design of a Novel Process for Nitrogen Removal from Concentrated Flows. Mathematical and Computer Modelling of Dynamical Systems 5:351–371. https://doi.org/10.1076/mcmd.5.4.351.3678

Hellinga C, Schellen AAJC, Mulder JW, et al (1998) The sharon process: An innovative method for nitrogen removal from ammonium-rich waste water. Water Science and Technology 37:135.

https://doi.org/10.1016/S0273-1223(98)00281-9

Hu Z, Lotti T, Kreuk M de, et al (2013) Nitrogen Removal by a Nitritation-Anammox Bioreactor at Low Temperature. Applied and Environmental Microbiology 79:2807–2812. https://doi.org/10.1128/AEM.03987-12

Innerebner G, Insam H, Franke-Whittle IH, Wett B (2007) Identification of anammox bacteria in a full-scale deammonification plant making use of anaerobic ammonia oxidation. Systematic and Applied Microbiology 30:408–412. https://doi.org/10.1016/j.syapm.2007.02.001

Jensen FB (2003) Nitrite disrupts multiple physiological functions in aquatic animals. Comparative biochemistry and physiology Part A, Molecular & integrative physiology 135:9–24

Jetten MSM, Schmid M, Schmidt I, et al (2002) Improved nitrogen removal by application of new nitrogen-cycle bacteria (Review). Reviews in Environmental Science and Biotechnology 1:51–63. https://doi.org/10.1023/A:1015191724542

Jimenez J, Wise G, Burger G, et al (2014) Mainstream Nitrite-Shunt with Biological Phosphorus Removal at the City of St. Petersburg Southwest WRF. Proceedings of the Water Environment Federation 2014:696–711. https://doi.org/10.2175/193864714815942116

Kampschreur MJ, Star WRL van der, Wielders HA, et al (2007) Dynamics of nitric oxide and nitrous oxide emission during full-scale reject water treatment. Water Research 42:812–826

Katipoglu-Yazan T, Cokgor EU, Insel G, Orhon D (2012) Is ammonification the rate limiting step for nitrification kinetics? Bioresource Technology 114:117–125. https://doi.org/10.1016/j.biortech.2012.03.017

Kim J-H, Guo X, Park H-S (2008) Comparison study of the effects of temperature and free ammonia concentration on nitrification and nitrite accumulation. Process Biochemistry 43:154–160. https://doi.org/10.1016/j.procbio.2007.11.005

Kornaros M, Dokianakis SN, Lyberatos G (2010) Partial Nitrification/Denitrification Can Be Attributed to the Slow Response of Nitrite Oxidizing Bacteria to Periodic Anoxic Disturbances. Environmental Science & Technology 44:7245–7253. https://doi.org/10.1021/es100564j

Kuai L, Verstraete W (1998) Ammonium Removal by the Oxygen-Limited Autotrophic Nitrification-Denitrification System. Applied and Environmental Microbiology 64:4500–4506

Kuenen JG (2008) Anammox bacteria: from discovery to application. Nature Reviews Microbiology 6:320–326. https://doi.org/10.1038/nrmicro1857

Lackner S, Gilbert EM, Vlaeminck SE, et al (2014) Full-scale partial nitritation/anammox experiences – An application survey. Water Research 55:292–303. https://doi.org/10.1016/j.watres.2014.02.032

Langone M (2013) Simultaneous partial Nitritation, Anammox and Denitrification (SNAD) process for treating ammonium-rich wastewaters. PhD Thesis

Lebaron P, Parthuisot N, Catala P (1998) Comparison of Blue Nucleic Acid Dyes for Flow Cytometric Enumeration of Bacteria in Aquatic Systems. Applied and Environmental Microbiology 64:1725–1730. https://doi.org/10.1128/AEM.64.5.1725-1730.1998

Li S, Chen Y-P, Li C, et al (2012) Influence of Free Ammonia on Completely Autotrophic Nitrogen Removal over Nitrite (CANON) Process. Applied Biochemistry and Biotechnology

167:694–704. https://doi.org/10.1007/s12010-012-9726-4

Liu G, Wang J (2013) Long-Term Low DO Enriches and Shifts Nitrifier Community in Activated Sludge. Environmental Science & Technology 47:5109–5117. https://doi.org/10.1021/es304647y

Liu G, Wang J (2012) Probing the stoichiometry of the nitrification process using the respirometric approach. Water Research 46:5954–5962. https://doi.org/10.1016/j.watres.2012.08.006

Liu W, Yang Q, Ma B, et al (2017) Rapid Achievement of Nitritation Using Aerobic Starvation. Environmental Science & Technology 51:4001–4008. https://doi.org/10.1021/acs.est.6b04598

Liu Y, Ngo HH, Guo W, et al (2019) The roles of free ammonia (FA) in biological wastewater treatment processes: A review. Environment International 123:10–19. https://doi.org/10.1016/j.envint.2018.11.039

Lotti T, Kleerebezem R, Hu Z, et al (2015) Pilot-scale evaluation of anammox-based mainstream nitrogen removal from municipal wastewater. Environmental Technology 36:1167–1177. https://doi.org/10.1080/09593330.2014.982722

Lu H, Chandran K, Stensel D (2014) Microbial ecology of denitrification in biological wastewater treatment. Water Research 64:237–254. https://doi.org/10.1016/j.watres.2014.06.042

Ma B, Wang S, Cao S, et al (2016) Biological nitrogen removal from sewage via anammox: Recent advances. Bioresource Technology 200:981–990. https://doi.org/10.1016/j.biortech.2015.10.074

Ma B, Zhang L, Zhang S, et al (2011) The feasibility of using a two-stage autotrophic nitrogen removal process to treat sewage. Bioresource Technology 102:8331–8334. https://doi.org/10.1016/j.biortech.2011.06.017

Maktabifard M, Zaborowska E, Makinia J (2018) Achieving energy neutrality in wastewater treatment plants through energy savings and enhancing renewable energy production. Reviews in Environmental Science and Bio/Technology 17:655–689. https://doi.org/10.1007/s11157-018-9478-x

Manser R, Gujer W, Siegrist H (2006) Decay processes of nitrifying bacteria in biological wastewater treatment systems. Water Research 40:2416–2426. https://doi.org/10.1016/j.watres.2006.04.019

Manser R, Muche K, Gujer W, Siegrist H (2005) A rapid method to quantify nitrifiers in activated sludge. Water Research 39:1585–1593. https://doi.org/10.1016/j.watres.2004.12.040

Manuel J (2014) Nutrient Pollution: A Persistent Threat to Waterways. Environmental health perspectives 122:A304–A309. https://doi.org/10.1289/ehp.122-A304

Massara TM, Malamis S, Guisasola A, et al (2017) A review on nitrous oxide (N2O) emissions during biological nutrient removal from municipal wastewater and sludge reject water. Science of the Total Environment 596–597:106–123. https://doi.org/10.1016/j.scitotenv.2017.03.191

McCarty PL (2018) What is the Best Biological Process for Nitrogen Removal: When and Why? Environmental Science & Technology 52:3835–3841. https://doi.org/10.1021/acs.est.7b05832

Nowka B, Daims H, Spieck E (2015) Comparison of Oxidation Kinetics of Nitrite-Oxidizing

Bacteria: Nitrite Availability as a Key Factor in Niche Differentiation. Applied and environmental microbiology 81:745–753. https://doi.org/10.1128/AEM.02734-14

Pambrun V, Paul E, Spérandio M (2006) Modeling the partial nitrification in sequencing batch reactor for biomass adapted to high ammonia concentrations. Biotechnology and Bioengineering 95:120–131. https://doi.org/10.1002/bit.21008

Park M-R, Park H, Chandran K (2017) Molecular and Kinetic Characterization of Planktonic Nitrospira spp. Selectively Enriched from Activated Sludge. Environmental Science & Technology 51:2720–2728. https://doi.org/10.1021/acs.est.6b05184

Patel A, Nobel RT, Steele JA, et al (2007) Virus and prokaryote enumeration from planktonic aquatic environments by epifluorescence microscopy with SYBR Green I. Nature Protocols 2:269–276. https://doi.org/10.1038/nprot.2007.6

Peng Y, Zhu G (2006) Biological nitrogen removal with nitrification and denitrification via nitrite pathway. Applied Microbiology and Biotechnology 73:15–26. https://doi.org/10.1007/s00253-006-0534-z

Pijuan M, Torà J, Rodríguez-Caballero A, et al (2014) Effect of process parameters and operational mode on nitrous oxide emissions from a nitritation reactor treating reject wastewater. Water research 49:23–33. https://doi.org/10.1016/j.watres.2013.11.009

R. Core Team (2014) R Foundation for Statistical Computing

Regmi P, Miller MW, Holgate B, et al (2014) Control of aeration, aerobic SRT and COD input for mainstream nitritation/denitritation. Water Research 57:162–171. https://doi.org/10.1016/j.watres.2014.03.035

Reichert P (1994) AQUASIM – A TOOL FOR SIMULATION AND DATA ANALYSIS OF AQUATIC SYSTEMS. Water Science and Technology 30:21–30. https://doi.org/10.2166/wst.1994.0025

Robiglio A (2018) Nitrogen removal from municipal wastewater by mainstream Partial Nitritation/Anammox process

Ruiz G, Jeison D, Chamy R (2003) Nitrification with high nitrite accumulation for the treatment of wastewater with high ammonia concentration. Water Research 37:1371–1377. https://doi.org/10.1016/S0043-1354(02)00475-X

Saccà A (2017) Methods for the estimation of the biovolume of microorganisms: A critical review. Limnology and Oceanography: Methods 15:337–348. https://doi.org/10.1002/lom3.10162

Schindler DW (2006) Recent Advances in the Understanding and Management of Eutrophication. Limnology and Oceanography 51:356–363. https://doi.org/10.4319/lo.2006.51.1_part_2.0356

Schramm A, Beer D de, van den Heuvel JC, et al (1999) Microscale distribution of populations and activities of Nitrosospira and Nitrospira spp. along a macroscale gradient in a nitrifying bioreactor: quantification by in situ hybridization and the use of microsensors. Applied and environmental microbiology 65:3690–3696

Scott G, Crunkilton RL (2000) Acute and chronic toxicity of nitrate to fathead minnows (Pimephales promelas), ceriodaphnia dubia, and Daphnia magna. Environmental Toxicology and

Chemistry 19:2918–2922. https://doi.org/10.1002/etc.5620191211

Sepehri A, Sarrafzadeh M-H (2019) Activity enhancement of ammonia-oxidizing bacteria and nitrite-oxidizing bacteria in activated sludge process: metabolite reduction and CO2 mitigation intensification process. Applied Water Science 9:1–12. https://doi.org/10.1007/s13201-019-1017-6

Seviour R, Nielsen PH (2010) Microbial ecology of activated sludge. IWA Publ, London [u.a.]

Simm R (2004) A conceptual model for apparent free ammonia inhibition in wastewater systems. PhD Thesis

Siripong S, Rittmann BE (2007) Diversity study of nitrifying bacteria in full-scale municipal wastewater treatment plants. Water Research 41:1110–1120. https://doi.org/10.1016/j.watres.2006.11.050

Stocker TF, D Qin G-KP, Stocker TF, Midgley PM (2014) Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change

Strous M, Heijnen JJ, Kuenen JG, Jetten MSM (1998) The sequencing batch reactor as a powerful tool for the study of slowly growing anaerobic ammonium-oxidizing microorganisms. Applied Microbiology and Biotechnology 50:589–596. https://doi.org/10.1007/s002530051340

Sun S-P, Nàcher CP i, Merkey B, et al (2010) Effective Biological Nitrogen Removal Treatment Processes for Domestic Wastewaters with Low C/N Ratios: A Review. Environmental Engineering Science 27:111–126. https://doi.org/10.1089/ees.2009.0100

Tchobanoglous G, Burton FL, Stensel HD (2003) Fundamentals of Biological Treatment. In: Wastewater Engineering: treatment and reuse, 4th edn. The McGraw-Hill Companies, Inc., Boston, USA, pp 545–658

Tokutomi T (2004) Operation of a nitrite-type airlift reactor at low DO concentration. Water science and technology : a journal of the International Association on Water Pollution Research 49:81–88. https://doi.org/10.2166/wst.2004.0740

Vadivelu VM, Keller J, Yuan Z (2006) Effect of free ammonia and free nitrous acid concentration on the anabolic and catabolic processes of an enriched Nitrosomonas culture. Biotechnology and Bioengineering 95:830–839. https://doi.org/10.1002/bit.21018

Vadivelu VM, Keller J, Yuan Z (2007) Effect of free ammonia on the respiration and growth processes of an enriched Nitrobacter culture. Water Research 41:826–834. https://doi.org/10.1016/j.watres.2006.11.030

van de Graaf AA, Mulder A, Bruijn P de, et al (1995) Anaerobic oxidation of ammonium is a biologically mediated process. Applied and Environmental Microbiology 61:1246–1251. https://doi.org/10.1128/AEM.61.4.1246-1251.1995

van Dongen U, Jetten MS, Loosdrecht MC van (2001) The SHARON®-Anammox® process for treatment of ammonium rich wastewater. Water science and technology : a journal of the International Association on Water Pollution Research 44:153–160. https://doi.org/10.2166/wst.2001.0037

van Hulle SW, Volcke EI, Teruel JL, et al (2007) Influence of temperature and pH on the

kinetics of the Sharon nitritation process. Journal of Chemical Technology & Biotechnology 82:471–480. https://doi.org/10.1002/jctb.1692

Verstraete W, Philips S (1998) Nitrification-denitrification processes and technologies in new contexts. Environmental Pollution 102:717–726. https://doi.org/10.1016/S0269-7491(98)80104-8

Veuillet F, Lacroix S, Bausseron A, et al (2014) Integrated fixed-film activated sludge ANITA^{TM}Mox process – a new perspective for advanced nitrogen removal. Water science and technology : a journal of the International Association on Water Pollution Research 69:915–922. https://doi.org/10.2166/wst.2013.786

Villaverde S, Fdz-Polanco F, García PA (2000) Nitrifying biofilm acclimation to free ammonia in submerged biofilters. Start-up influence. Water Research 34:602–610. https://doi.org/10.1016/S0043-1354(99)00175-X

Wang Q, Duan H, Wei W, et al (2017) Achieving Stable Mainstream Nitrogen Removal via the Nitrite Pathway by Sludge Treatment Using Free Ammonia. Environmental Science & Technology 51:9800–9807. https://doi.org/10.1021/acs.est.7b02776

Wang Q, Jiang G, Ye L, et al (2014a) Heterotrophic denitrification plays an important role in N2O production from nitritation reactors treating anaerobic sludge digestion liquor. Water Research 62:202–210. https://doi.org/10.1016/j.watres.2014.06.003

Wang Q, Ye L, Jiang G, et al (2014b) Side-stream sludge treatment using free nitrous acid selectively eliminates nitrite oxidizing bacteria and achieves the nitrite pathway. Water Research 55:245–255. https://doi.org/10.1016/j.watres.2014.02.029

Wang W, Yang N (2004) Partial nitrification under limited dissolved oxygen conditions. Process Biochemistry 39:1223–1229. https://doi.org/10.1016/S0032-9592(03)00249-8

Water Environment Federation (2005) Biological Nutrient Removal (BNR) Operation in Wastewater Treatment Plants. McGraw-Hill Professional Publishing, Blacklick, USA

Wett B, Hell M, Nyhuis G, et al (2010) Syntrophy of aerobic and anaerobic ammonia oxidisers. Water science and technology : a journal of the International Association on Water Pollution Research 61:1915–1922. https://doi.org/10.2166/wst.2010.969

Wu J, He C, Loosdrecht MCM van, Pérez J (2016) Selection of ammonium oxidizing bacteria (AOB) over nitrite oxidizing bacteria (NOB) based on conversion rates. Chemical Engineering Journal 304:953–961. https://doi.org/10.1016/j.cej.2016.07.019

Xia Y, Wen X, Zhang B, Yang Y (2018) Diversity and assembly patterns of activated sludge microbial communities: A review. Biotechnology Advances 36:1038–1047. https://doi.org/10.1016/j.biotechadv.2018.03.005

Xu G, Zhou Y, Yang Q, et al (2015) The challenges of mainstream deammonification process for municipal used water treatment. Applied Microbiology and Biotechnology 99:2485–2490. https://doi.org/10.1007/s00253-015-6423-6

Yao Q, Peng D, Wang B, et al (2017) Effect of free ammonium and free nitrous acid on the activity, aggregate morphology and extracellular polymeric substance distribution of ammonium oxidizing bacteria in partial nitrification. Journal of Bioscience and Bioengineering 124:319–326. https://doi.org/10.1016/j.jbiosc.2017.03.015

Zeder M, Kohler E, Zeder L, Pernthaler J (2011) A Novel Algorithm for the Determination of Bacterial Cell Volumes That is Unbiased by Cell Morphology. Microscopy and Microanalysis 17:799–809. https://doi.org/10.1017/S1431927611012104

Appendices

Appendix A: Total suspended solids (TSS) and volatile suspended solids (VSS) of mixed liquor, and nitrogen removal performance in SBRs throughout the study

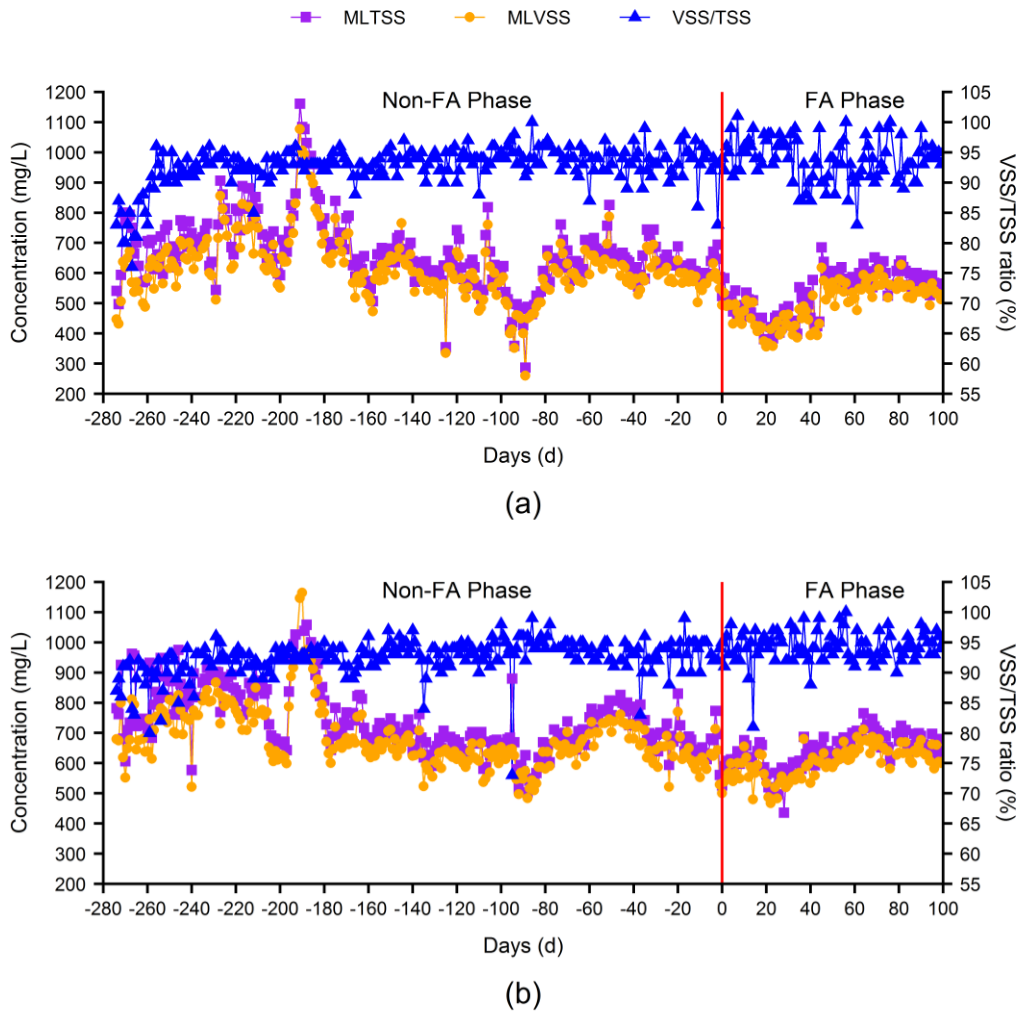


Figure A-1 Total suspended solids (TSS), volatile suspended solids (VSS), and VSS/TSS ratio of mixed liquor in SBRs: (a) ER; (b) CR

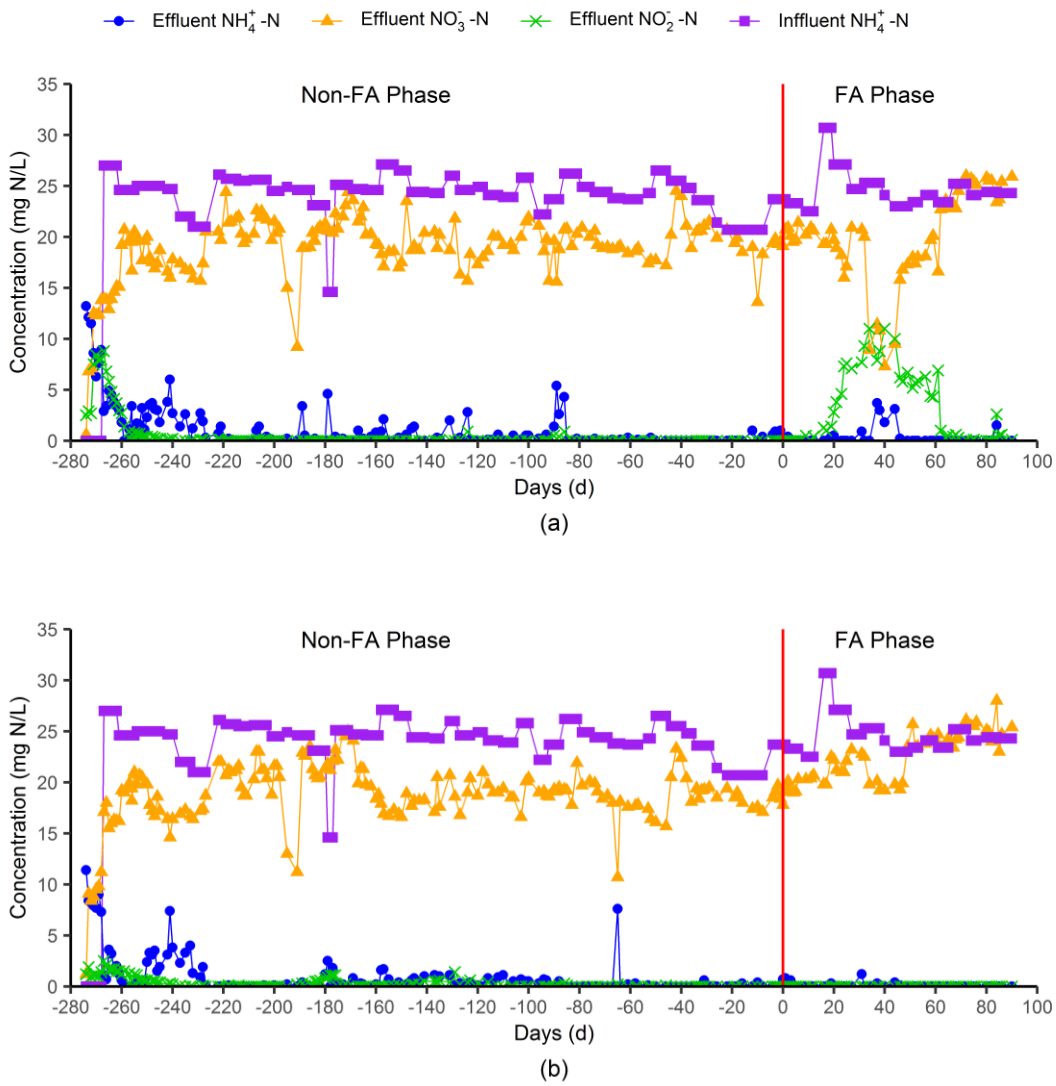


Figure A-2 Influent ammonium and effluent nitrogen species in SBRs throughout the experiment: (a) ER; (b) CR

Appendix B: pH and DO in 3-hour SBR cycle monitoring

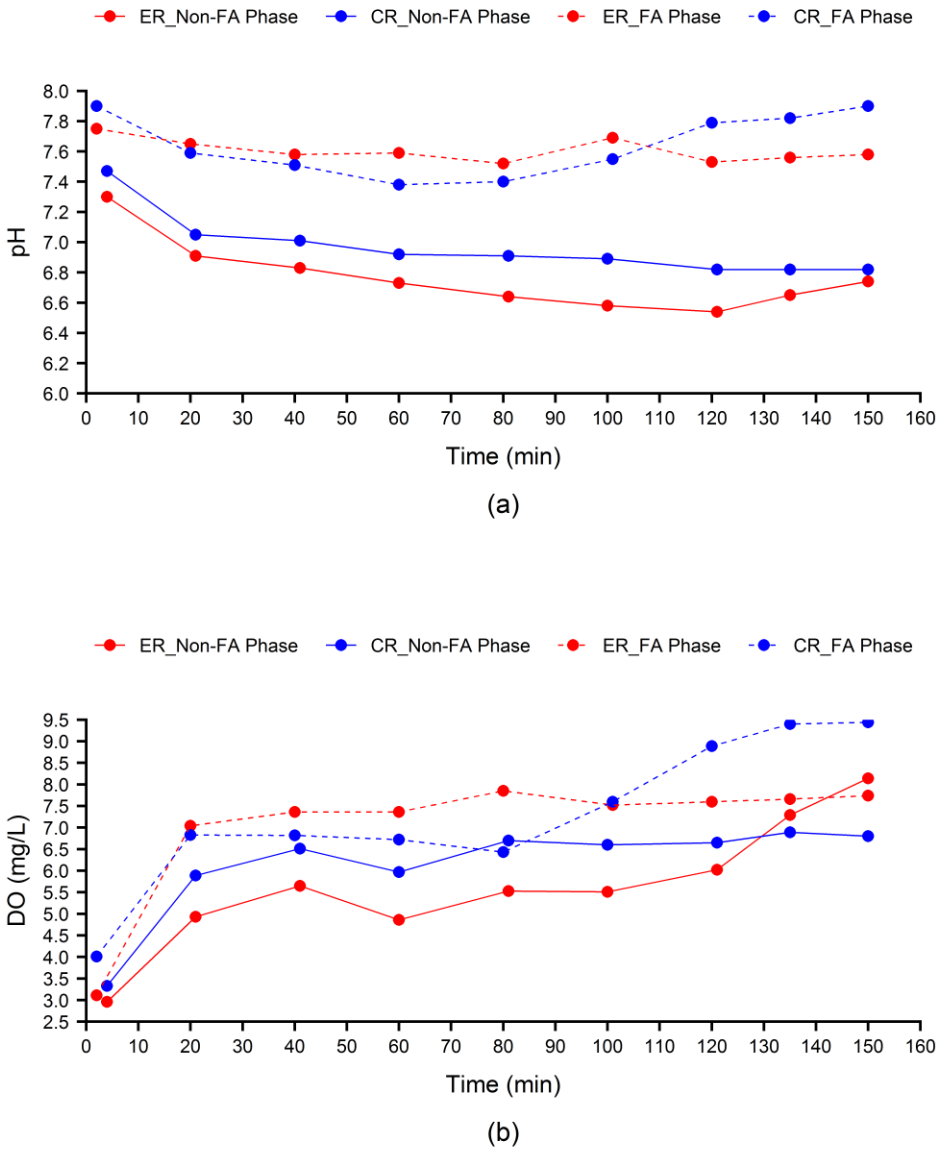
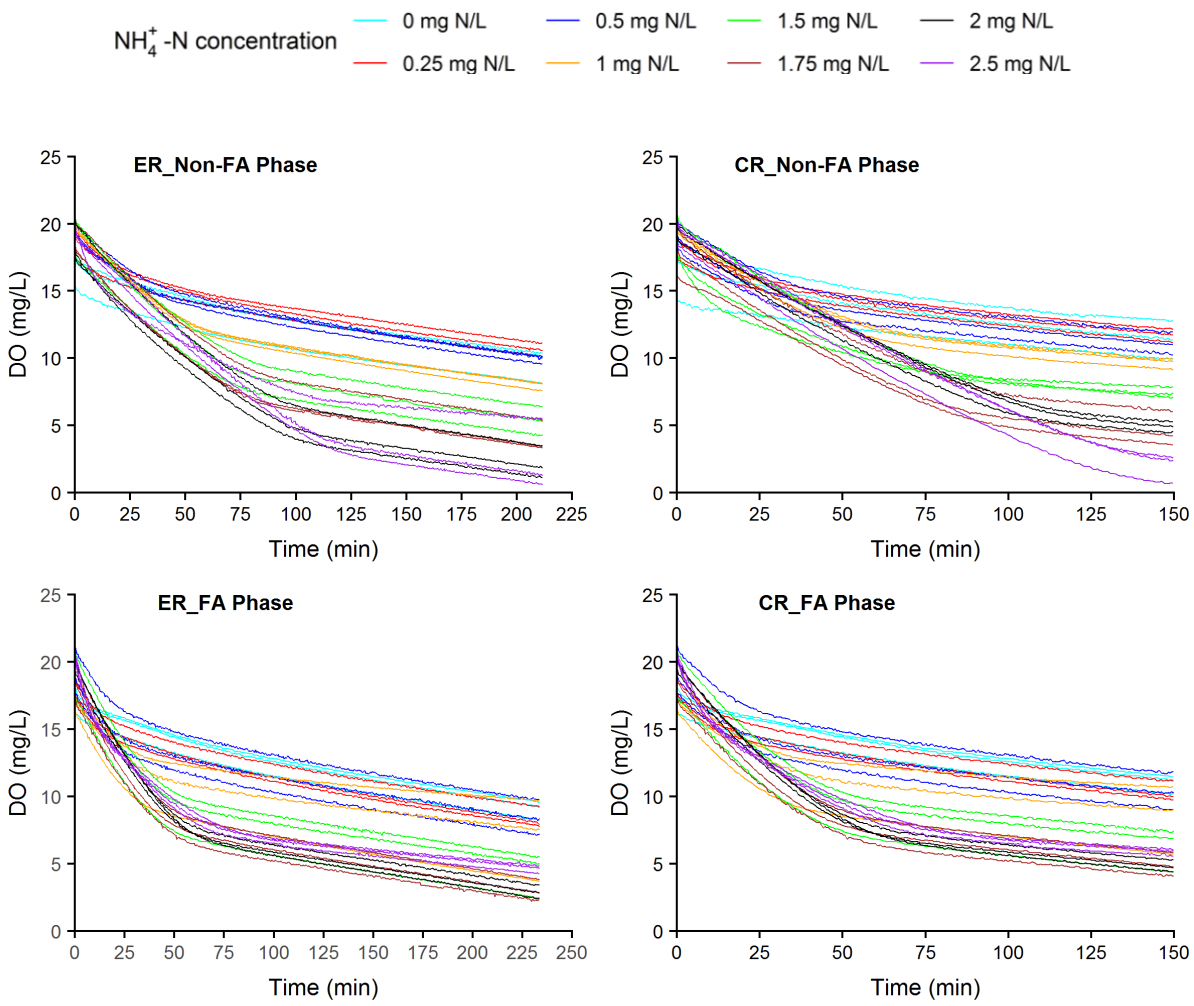
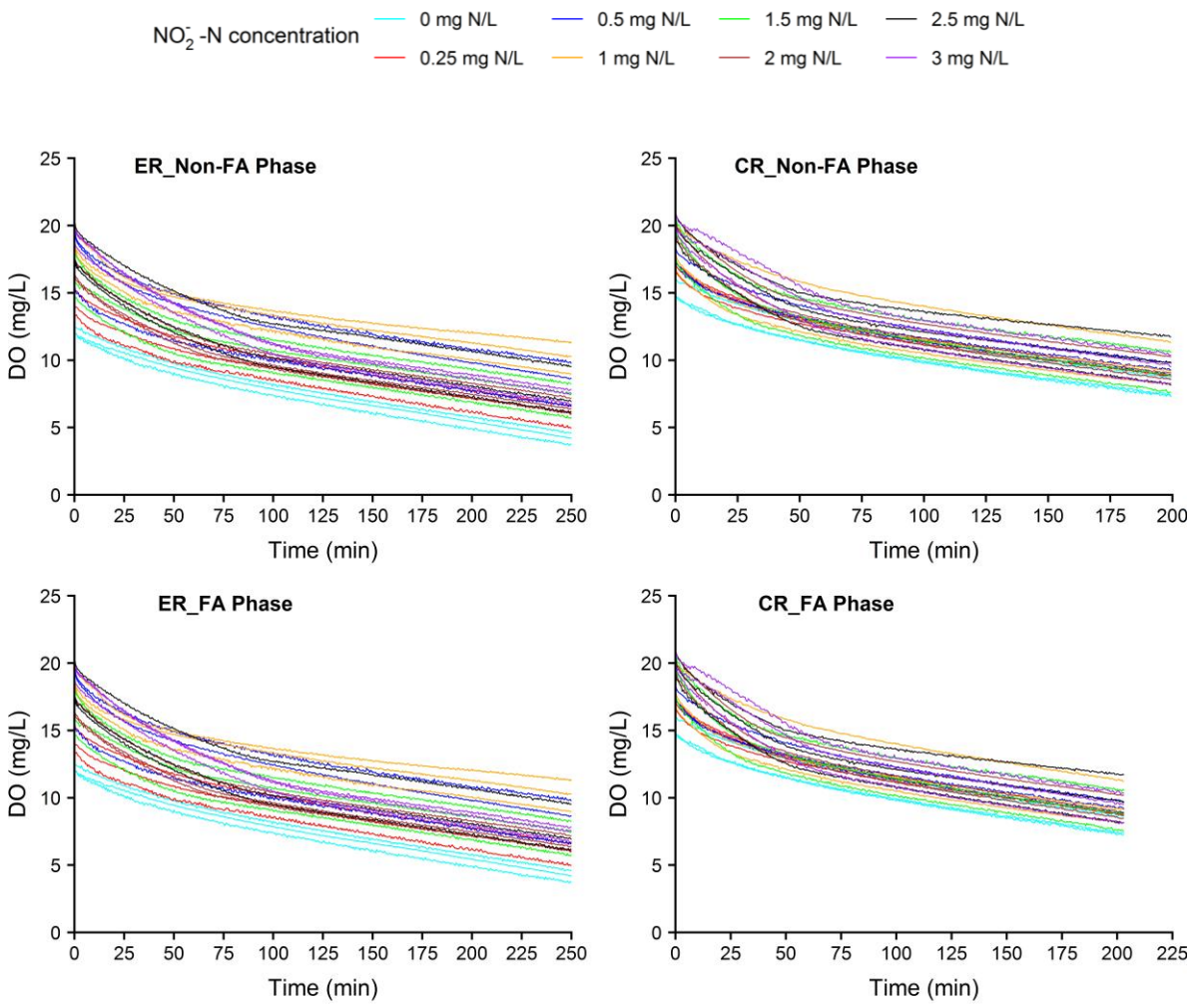


Figure B-1 pH and DO in ER and CR during 3-hour SBR cycle monitoring: (a) pH; (b) DO. 3-hour SBR cycle monitoring was conducted at day -30 before and day 84 after the start of FA treatment for Non-FA Phase and FA Phase respectively. Time of 0 min represents the start of feeding period of a SBR cycle.

Appendix C: Adjusted respirograms, oxygen uptake rate (OUR) curves, cumulative oxygen uptake curves, and specific oxygen uptake (SOU) determination in respirometric batch tests

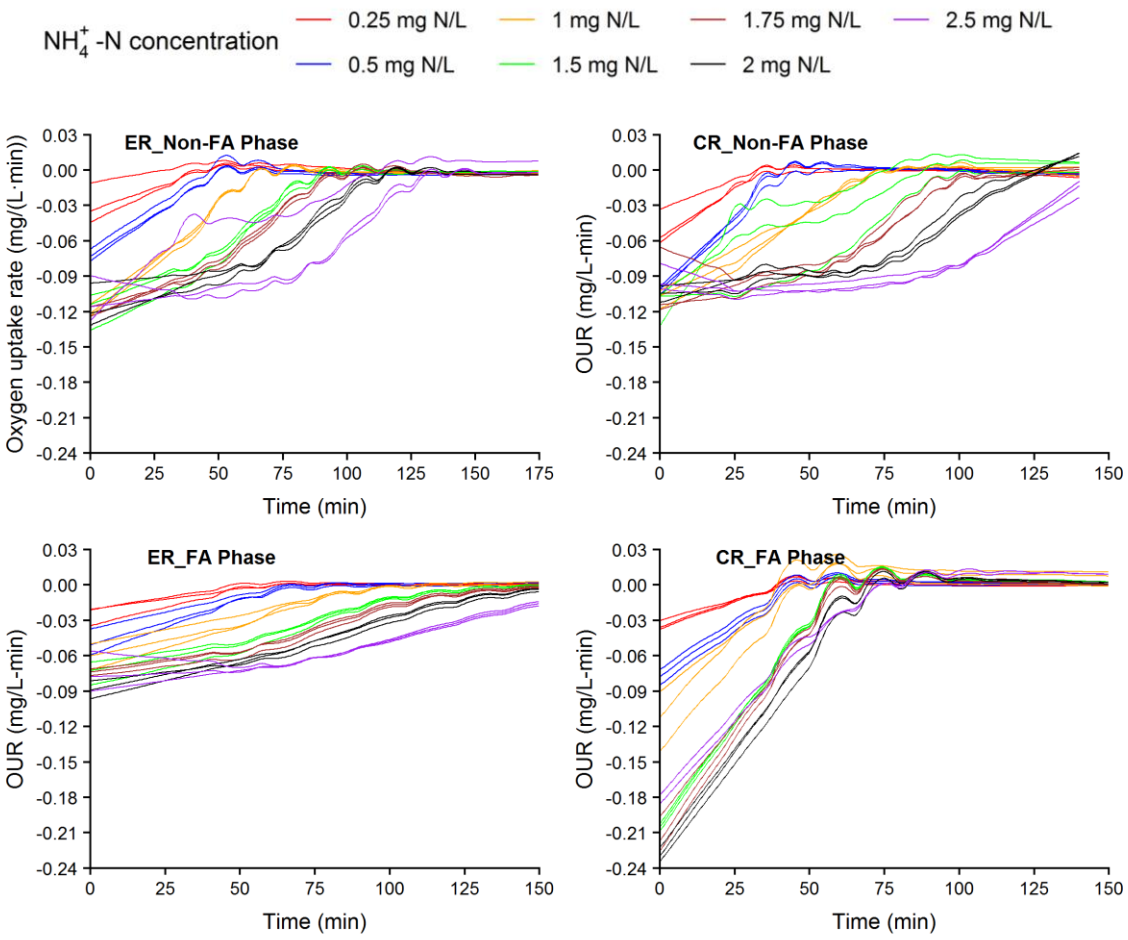


(a)



(b)

Figure C-1 Adjusted respirograms for ER and CR using different nitrogenous substrates in respirometric batch tests: (a) ammonium; (b) nitrite


NH_4^+-N concentration
0.25 mg N/L
0.5 mg N/L
1 mg N/L
1.5 mg N/L
1.75 mg N/L
2 mg N/L
2.5 mg N/L
ER_Non-FA Phase
CR_Non-FA Phase
ER_FA Phase
CR_FA Phase
Oxygen uptake rate (mg/(L·min))
OUR (mg/L-min)
Time (min)

(a)

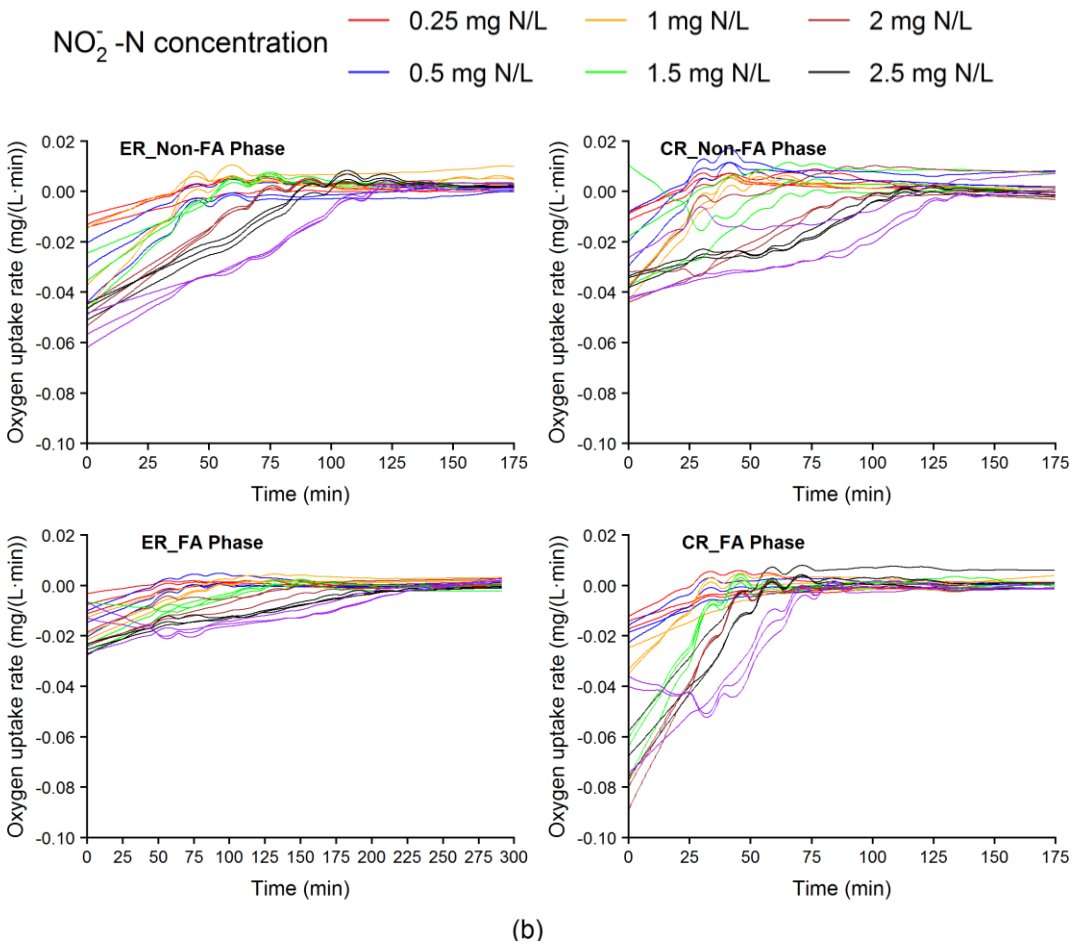
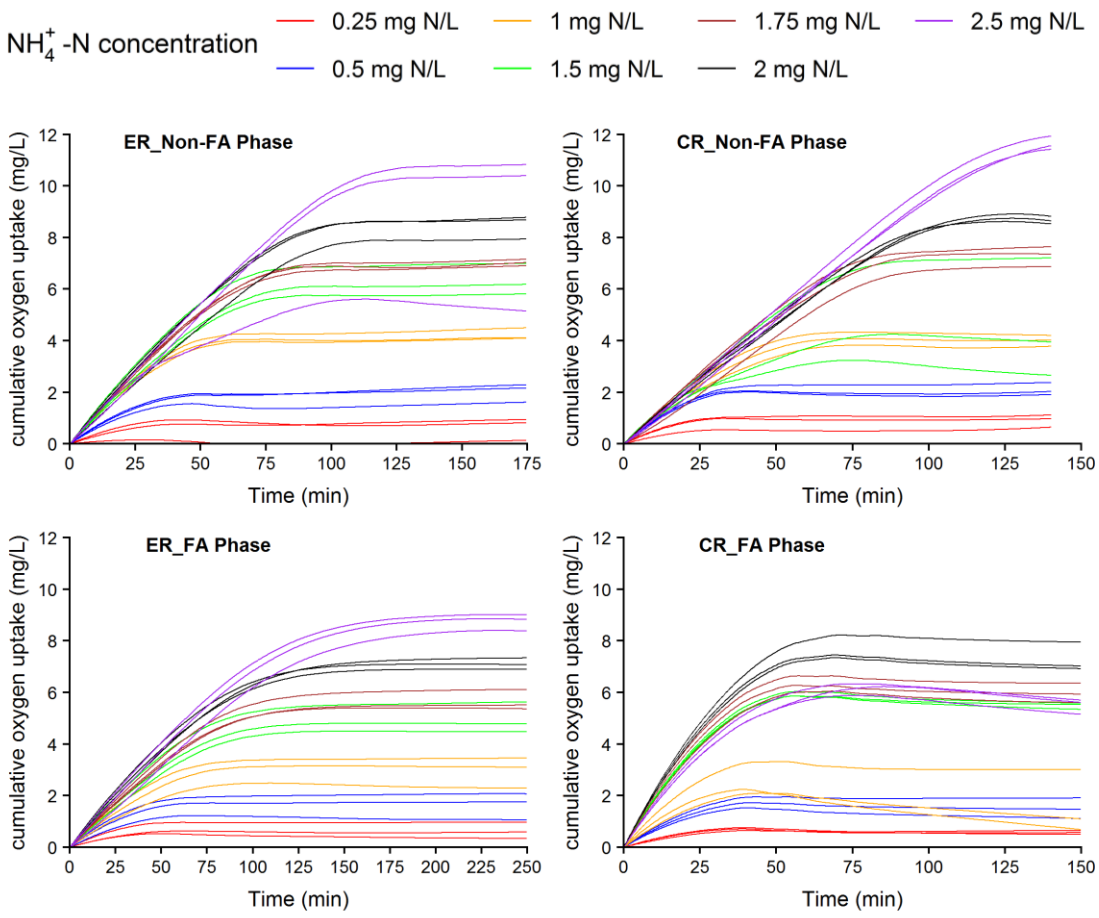
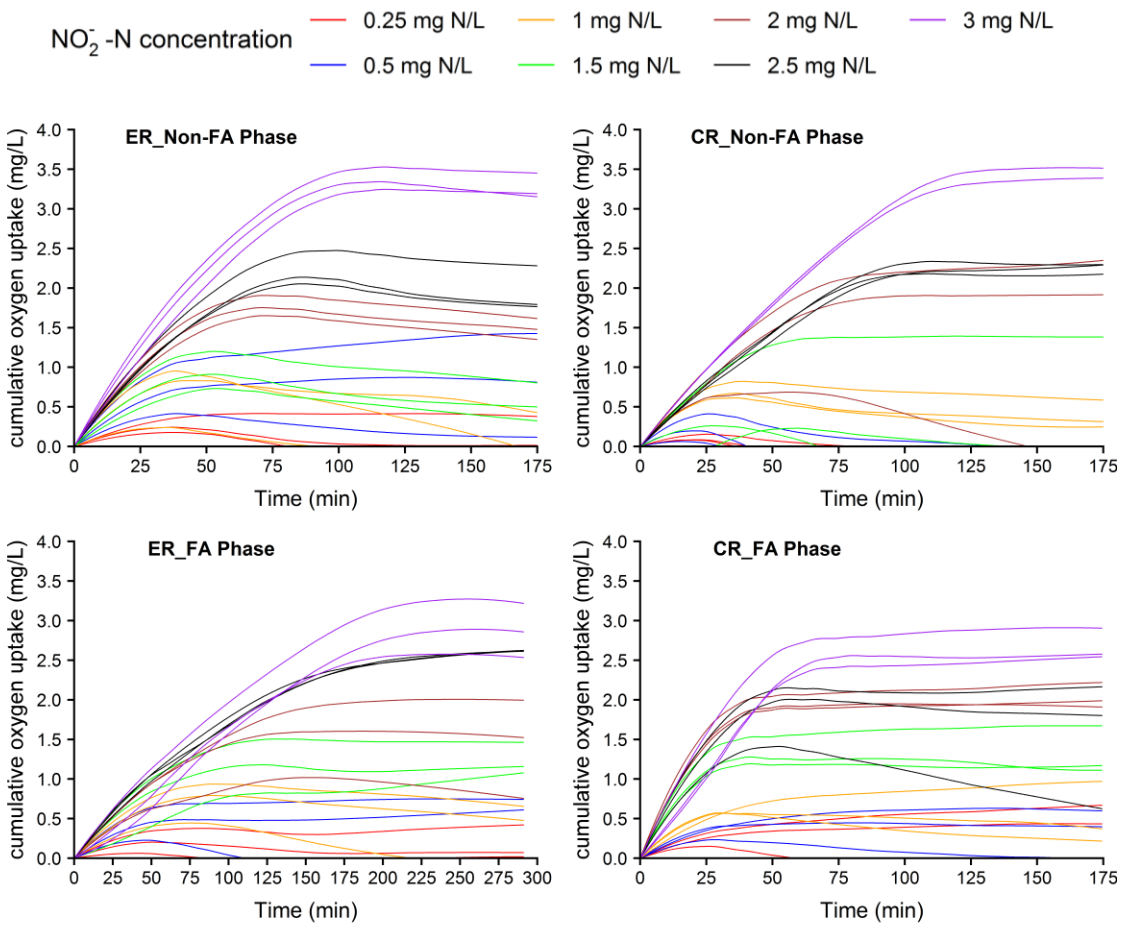


Figure C-2 OUR curves for ER and CR using different nitrogenous substrates in respirometric batch tests: (a) ammonium; (b) nitrite


NH_4^+ -N concentration
0.25 mg N/L
0.5 mg N/L
1 mg N/L
1.5 mg N/L
1.75 mg N/L
2 mg N/L
2.5 mg N/L
ER_Non-FA Phase
CR_Non-FA Phase
ER_FA Phase
CR_FA Phase
cumulative oxygen uptake (mg/L)
Time (min)

(a)



(b)

Figure C-3 Cumulative oxygen uptake curves for ER and CR using different nitrogenous substrates in respirometric batch tests: (a) ammonium; (b) nitrite

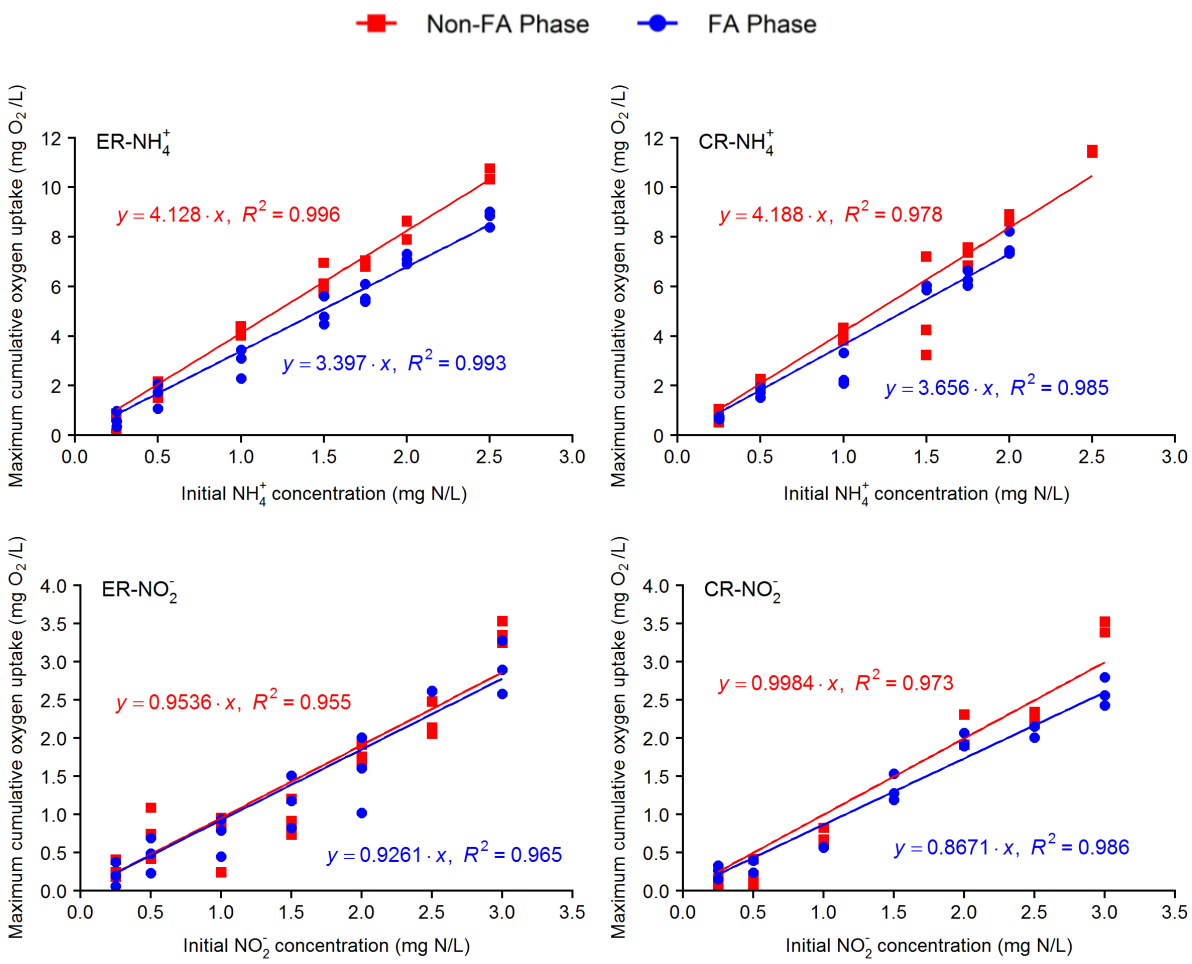


Figure C-4 Determination of specific oxygen uptake (SOU_{NO} and SOU_{NC}) for ER and CR. The slope of linear regression equation on maximum cumulative oxygen uptake versus initial substrate concentration is SOU.

Appendix D: Total bacterial biomass determination

D.1 Materials and methods

Epifluorescence microscopy has been used as a reliable method to estimate total bacterial counts (Manser et al. 2005; Saccà 2017; Brown et al. 2019). Bacterial cells, including viable and dead cells, can be stained by fluorescent dyes like SYBR Green II or SYTO 9, and identified under epifluorescence microscope (Frossard et al. 2016; Brown et al. 2019). In this study, SYBR Green-II (λ_{ex} = 497 nm, λ_{em} = 520 nm, SYBR™ Green II RNA Gel Stain, 10,000X concentrate in DMSO, Molecular Probes Inc., OR, USA) was used for sludge samples collected during Non-FA Phase, while sludge samples collected during FA Phase were stained by SYTO 9 (λ_{ex} = 485 nm, λ_{em} = 498 nm, SYTO™ 9 Green Fluorescent Nucleic Acid Stain, 5 mM, Life Technologies, Carlsbad, CA, USA) due to malfunction of SYBR Green-II. A correlation coefficient of 0.956 was used for comparison of total bacterial biomass stained by the two different dyes. (Lebaron et al. 1998).

Sludge samples were collected from washed sludge in respirometric batch tests and from SBRs during 3-hour SBR cycle monitoring. The sludge samples were fixed with 4% paraformaldyhe (PFA) solution, and stored in Phosphate-Buffered-Saline (PBS)-ethanol solution (50%/50%, v/v) at -20 ^{o}C for future use (Daims et al. 2004). Before staining with fluorescent dyes, sludge samples were disintegrated by a PT 10-35 Blade-type Homogenizer (Brinkmann (Polytron) Instruments) for 3 min in ice, and mixed with 5% v/v of dispersants Tween 20 (Brown et al. 2019). The mixed samples were diluted 100 times with autoclaved PBS solution (2.45 g Na_2HPO_4, 1g KH_2PO_4 and 8.5 g NaCl/L; pH = 7.2) and dispersed again in a sonication bath

(Aquasonic model 50T, VWR Scientific Products) for 20 min. After that, 0.25 mL of treated samples were added into 4.75 mL of autoclaved Tris buffer (10 mM, pH = 8.0) with additional 50 µL of 100X SYBR Green-I stock solution (1:100 v/v dilution of 10000X SYBR™ Green II in DMSO) or 50 µL of 250-µM SYTO 9 stock solution (1:20 v/v dilution of 5-mM SYTO™ 9 Green), and incubated for 15 min in dark at room temperature (Patel et al. 2007; Brown et al. 2019). 2mL of stained solutions were then filtered onto black filters (0.2-µm pore size, 25-mm diameter, Cyclopore Track Etched, Whatman), and mounted onto microscope slides. Filters were observed at 400× magnification using an Olympus BX53 light fluorescent microscope (Olympus, Japan), equipped with a YFP filter (excitation filter: 490-510 nm, barrier filter: 520-550 nm). 40 random fields of view were captured for each filter by a DP80 Dual Chip Color and Monochrome Camera (Olympus, Japan), and analyzed by YABBA which is able to process microscope images, count bacterial cells in images, and calculate bacterial cell volume (Zeder et al. 2011).

Total bacterial concentration (N (cells/L)) is determined using Eq. D-1. N_{40} (cells) is total bacterial cell counts within 40 random fields of view. A_{40} and A_F are the areas of the 40 fields of view and black filters respectively. D is dilution factor as 2121, and V_S is sample volume as 2 mL for all sludge samples. Carbon content per unit of cell volume (M_C) is assumed to be 310 µg C/mm^3 (Andreottola et al. 2002; Foladori et al. 2010). Total bacterial biomass (X_{total}, mg COD/L) is calculated by Eq. D-2. V (µm3/cell) is the mean volume of a bacterial cell obtained by YABBA.

(Eq. D-1)
$$N = \frac{N_{40} \times A_{40}}{A_F} \times \frac{D}{V_S} \times 1000 \left(\frac{mL}{L}\right)$$

(Eq. D-2) $$X_{total} = N \times V \times M_C \times 1.88 \frac{\text{mg VSS}}{\text{mg C}} \times 1.42 \frac{\text{mg } O_2}{\text{mg VSS}} \times 10^{-12}$$

D.2 Total bacterial biomass results

Table D-1 Total bacterial biomass results obtained by epifluorescent microscopy

Phase	Reactor	Activity	Total bacterial biomass (mg COD/L)
Non-FA Phase	ER	Batch test using ammonium as substrate	105.42±10.73
		Batch test using nitrite as substrate	89.45±9.11
		3-hour SBR cycle monitoring	168.83±26.99
	CR	Batch test using ammonium as substrate	71.69±7.30
		Batch test using nitrite as substrate	89.62±5.89
		3-hour SBR cycle monitoring	162.43±52.41
FA Phase	ER	Batch test using ammonium as substrate	19.60±7.07
		Batch test using nitrite as substrate	31.19±14.72
		3-hour SBR cycle monitoring	42.10±12.70
	CR	Batch test using ammonium as substrate	16.81±6.32
		Batch test using nitrite as substrate	22.91±17.31
		3-hour SBR cycle monitoring	50.34±13.72

Appendix E: Biomass calculations for nitrifiers

Table E-1 Biomass calculations for nitrifiers using the method of substrate mass balance

Phase	Reactors	Nitrifiers	SRT (d)	HRT (d)	*Y* (mg COD/mg NOD)	*Sox* (mg NOD/L)	*b* (d^{-1})	NAR	*X* (mg COD/L)
Non-FA Phase	ER	AOB	10.43±0.79	0.50±0.16	0.04±0.02	71.53±1.42	0.15	0	11.82±5.63
		NOB	10.43±0.79	0.50±0.16	0.16±0.04	23.84±0.47	0.15	0	15.9±4.08
	CR	AOB	10.00±0.89	0.51±0.11	0.04±0.03	65.68±1.56	0.15	0	10.36±8.72
		NOB	10.00±0.89	0.51±0.11	0.12±0.04	21.89±0.52	0.15	0	10.61±3.31
FA Phase	ER	AOB	10.95±3.59	0.50±0.17	0.20±0.02	92.36±1.79	0.15	0	77.60±25.74
		NOB	10.95±3.59	0.50±0.17	0.19±0.03	26.48±0.60	0.15	0.11±0.06	21.47±7.48
	CR	AOB	9.56±0.98	0.49±0.11	0.12±0.03	97.18±9.28	0.15	0	47.93±12.28
		NOB	9.56±0.98	0.49±0.11	0.24±0.02	31.98±3.09	0.15	0.01±0.02	30.90±5.01

Appendix F: Instructions of process modeling development in AQUASIM

1. Start the window interface version of AQUASIM or click the command ***File → New*** from the main menu bar.

b. Definition of variables

State variables

2. Define two state variables:

C_NH for concentration of NH_4 with unit of mg NOD/L;

C_NO for concentration of NO_2 with unit of mg NOD/L.

Program variables

3. Define a program variable **t** referring to time.

4. Define a program variable **calcnum** referring to calculation number.

Constant variables

5. Define two constant variables **K_AOB, K_NOB** for half saturation constants (K_S) of AOB and NOB, with unit of mg NOD/L, values of 1.5 mg NOD/L, a minimum of 0, maximum of 10, and standard deviations of 0.2 mg/L. Click "active for sensitivity analysis" and "active for parameter estimation".

6. Define two constant variables **u_AOB** and **u_NOB** for maximum growth rates (μ_{max}) of AOB and NOB, with unit of min^{-1}, values of 0.000118 min^{-1}, a minimum of 0, maximum of 1, and standard deviations of 0.00002. Click "active for sensitivity analysis" and "active for parameter estimation".

7. Define two constant variables **Y_AOB** and **Y_NOB** for biomass yield coefficients (Y) of AOB and NOB with unit of mg COD/mg NOD and corresponding values in Figure 3-5.

Formula variables

8. Define two formula variables **X_AOB** and **X_NOB** for biomass concentrations (X) of AOB and NOB equal to values in Table E-1, Appendix E with unit of mg COD/L.

9. Define two formula variables **i_AOB** and **i_NOB** for nitrogen content of AOB and NOB biomass, equal to 0.3 and 0.1 mg NOD/mg COD respectively.

10. Define a formula variable **r_AOB** for the growth rate of AOB, expressed as u_AOB*C_NH*X_AOB/ (K_AOB + C_ NH), with unit of mg COD/(L• min).

11. Define a formula variable **r_NOB** for the growth rate of NOB, expressed as u_NOB*C_NO*X_NOB/ (K_NOB + C_ NO), with unit of mg COD/(L• min).

12. Define a formula variable **r_O2** for the predicted OUR, expressed as (1-1/Y_AOB)*r_AOB + (1 - 1/Y_NOB)*r_NOB), with unit of mg O2/(L•min).

Real-list variables

13. Define a real-list variable **our_meas1** with argument of t and unit of (mg O2/L-min), a minimum of -1e+009 and maximum of 1e+009. Input OUR data points of the first experimental vial (A4) using ammonium as substrate stored in a tab-separated file.

14. Duplicate our_meas1 and rename as a new real-list variable **our_meas2**. Change the inputted data to read OUR data points of the second experimental vial (A5) using ammonium as substrate.

15. Repeat step 14 for all 42 experimental vials using ammonium or nitrite as substrate. (our_meas1 to our_meas21 are real-list variables for vials using ammonium as substrate, and

our_meas22 to our_meas42 are real-list variables for vials using nitrite as substrate)

16. Define a real list variable, **C_NH_ini**, with argument of calcnum and unit of mg NOD/L. Input a list of argument value pairs, so that the calcnum corresponds to the vial number (e.g. calcnum 1 = via A4) and the value corresponds to the initial NH4 concentration in that vial as mg NOD/L.

17. Define a real-list variable **C_NO_ini** with argument of calcnum and unit of mg NOD/L. Input a list of argument value pairs, so that the calcnum corresponds to the vial number (e.g. calcnum 1 = via A4) and the value corresponds to the initial NO2 concentration in that vial as mg NOD/L.

18. Save the file.

c. Definition of processes

19. Define a dynamic process **growth_NOB** with rate = r_NOB:

    the stoichiometric coefficient for C_NO is: -1/Y_NOB;

    the stoichiometric coefficient for C_NH is: -i_NOB;

    The stoichiometric coefficient for X_NOB is: +1.

20. Define a dynamic process **growth_AOB** with rate = r_AOB:

    the stoichiometric coefficient for C_NH is: -1/Y_AOB - i_AOB;

    the stoichiometric coefficient for C_NO is: 1/(3*Y_AOB);

    the stoichiometric coefficient for X_AOB is: +1.

21. Save the file.

d. Definition of compartment

22. Define a mixed reactor compartment called **batch**, with a volume of 0.005 L. In this compartment activate state variables *C_NO* and *C_NH*, and processes *growth_AOB* and *growth_NOB*. Finally, specify *C_NO_ini* and *C_NH_ini* to be the initial concentrations of *C_NO* and *C_NH*, respectively.

23. Save the file.

e. Definition of plots

24. Click the command ***View → Results*** , and then *New* to create a new plot. Name it as **OUR1_NH4**, for OUR in vials with initial ammonium concentration of 0.25 mg N/L. The title should be the substrate type and initial concentration (i.e. NH4 – 0.25 mg N/L). The abscissa label *Time (min)*, and the ordinate label *OUR (mg O2/L/min).* Click *Add* and leave Type as *value*, change the variable to ***our_meas1***. Set the calculation number as 1. Deactivate the *Line*, and activate the *Marker* (black circle with size 3). Click *OK*. Click *Add* again to add variables ***our_meas2*** and ***our_meas3*** as *our_meas1*. Click *Add* again, and change variable to ***r_O2.*** Set calculation number as 1. Change line color to red. Click *OK*.

25. Duplicate plot **OUR1_NH4** and name it as **OUR2_NH4** for OUR in vials with initial ammonium concentration of 0.5 mg N/L. Repeat step 24 for ***our_meas4*** to ***our_meas6*** and ***r_O2.***

26. Repeat step 25 for all 14 different initial substrate concentrations.

27. Save the file.

f. Definition of calculations

28. Click the command ***Calc → Simulation*** from the main menu bar and define a new

calculation of with 350 steps of size 0.5 min. Change calculation number to 1. Name this calculation as **calc1**. Select this calculation to be "active for simulation" as well as "active for sensitivity analysis".

29. Duplicate calc1, and rename it as **calc2**. Change the calculation number to 2. Repeat this step for all 42 vials.

30. Click the command ***Calc → Parameter Estimation*** from the main menu bar and click the button New in the Parameter Estimation box. Name this fit as **fit1**. Change calculation number to 1. Have the initial state be given, made consistent. Click *Status* "active for parameter estimation". Click the *Add* button, and select ***our_meas1*** as Data and ***r_O2*** as *Variable*. Select Batch as Compartment.

31. Duplicate **fit1** and rename the new fit as **fit2**. Change the calculation number to 2 and change *Data* to ***our_meas2*** and *Variable* to ***r_O2***.

32. Repeat step 31 for all 42 vials.

33. Save the file

g. Execution of the simulation and presentation of results

34. In the dialog box of ***Parameter Estimation***, active parameters *K_NOB* and *u_NOB* and select Method to be secant. Select *fit 22* to *fit 42* to be active in calculations box. Click button *Start* to execute parameter estimation for NOB. Restart the parameter estimation to improve results until the value of final *Chi^2* does not change.

35. Deactive parameters *K_NOB* and *u_NOB* and active *K_AOB* and *u_AOB*. Select *fit 1* to *fit 21* to be active in calculations box. Click button *Start* to execute parameter estimation for AOB.

Restart the parameter estimation to improve results until the value of final *Chi^2* does not change.

36. Click the command ***Calc → Simulation*** from the main menu bar. Active *calc 1* to *calc 42* in the dialog box of *Simulation*. Click button Start/Continue to calculate OUR based on estimated parameters (μmax and KS).

37. Click the command ***View → Results***. Select *OUR1_NH4* and click button *Plot to Screen* to plot measured and calculated OUR in a figure. Click button *List to File* to save data points of measured and calculated OUR in a txt. file for further process.

38. Repeat step 36 for all 14 plots.

39. Save the file.

Appendix G: P values of ANOVA on biomass yield coefficient (Y), nitrifiers biomass (X), maximum specific growth rate (μ_{max}), and half-saturation coefficient (K_S) between ER and CR in Non-FA and FA Phase

Table G-1 P values of ANOVA on biomass yield coefficient (Y) of nitrifiers: left- AOB; right-NOB

Y_{AOB}		Non-FA Phase		FA Phase	
		ER	**CR**	**ER**	**CR**
Non-FA Phase	ER	1	**9.89E-01**	1.14E-26	4.40E-13
	CR	-	1	3.88E-21	7.97E-10
FA Phase	ER	-	-	1	7.39E-13
	CR	-	-	-	1

Y_{NOB}		Non-FA Phase		FA Phase	
		ER	**CR**	**ER**	**CR**
Non-FA Phase	ER	1	4.75E-03	4.45E-02	4.38E-09
	CR	-	1	6.21E-06	1.97E-13
FA Phase	ER	-	-	1	1.05E-06
	CR	-	-	-	1

Table G-2 P values of ANOVA on biomass (X) of nitrifiers: left- AOB; right-NOB

X_{AOB}		Non-FA Phase		FA Phase	
		ER	**CR**	**ER**	**CR**
Non-FA Phase	ER	1	**7.60E-01**	1.74E-04	7.65E-05
	CR	-	1	1.69E-04	9.92E-05
FA Phase	ER	-	-	1	1.07E-02
	CR	-	-	-	1

X_{NOB}		Non-FA Phase		FA Phase	
		ER	**CR**	**ER**	**CR**
Non-FA Phase	ER	1	**5.44E-02**	**1.58E-01**	1.58E-04
	CR	-	1	1.15E-02	6.80E-06
FA Phase	ER	-	-	1	1.02E-02
	CR	-	-	-	1

Table G-3 P values of ANOVA on maximum specific growth rate (μ_{max}) of nitrifiers: left- AOB; right-NOB

$\mu_{max,\,AOB}$		Non-FA Phase		FA Phase	
		ER	**CR**	**ER**	**CR**
Non-FA Phase	ER	1	2.45E-37	9.31863E-64	5.74E-56
	CR	-	1	1.7773E-56	9.57E-57
FA Phase	ER	-	-	1	1.54E-60
	CR	-	-	-	1

$\mu_{max,\,NOB}$		Non-FA Phase		FA Phase	
		ER	**CR**	**ER**	**CR**
Non-FA Phase	ER	1	4.19E-44	8.91E-66	5.96E-05
	CR	-	1	3.22E-60	2.87E-40
FA Phase	ER	-	-	1	1.68E-61
	CR	-	-	-	1

Table G-4 P values of ANOVA on half-saturation coefficient (K_S) of nitrifiers: left- AOB; right-NOB

$K_{S,\,AOB}$		Non-FA Phase		FA Phase	
		ER	**CR**	**ER**	**CR**
Non-FA Phase	ER	1	1.55E-40	1.10E-53	1.11E-50
	CR	-	1	9.46E-60	2.48E-52
sfsdf	ER	-	-	1	9.55E-48
	CR	-	-	-	1

$K_{S\,,\,NOB}$		Non-FA Phase		FA Phase	
		ER	**CR**	**ER**	**CR**
Non-FA Phase	ER	1	1.31E-38	2.56E-33	1.19E-24
	CR	-	1	1.87E-36	2.08E-29
FA Phase	ER	-	-	1	6.78E-09
	CR	-	-	-	1