Microbial synergies and dynamics in biological nutrient removal processes

Yang, Qin

2019

Yang, Q. (2019). Microbial synergies and dynamics in biological nutrient removal processes.
Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/83255>

<https://doi.org/10.32657/10220/48000>

Microbial Synergies and Dynamics in Biological Nutrient
Removal Processes

YANG QIN

INTERDISCIPLINARY GRADUATE SCHOOL

2019

Microbial Synergies and Dynamics in Biological Nutrient Removal Processes

Yang Qin

INTERDISCIPLINARY GRADUATE SCHOOL

A thesis submitted to the Nanyang Technological University
in partial fulfilment of the requirement for the degree of
Doctor of Philosophy

2019

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

5 Apr 2019

.....

Date

A handwritten signature in black ink, consisting of stylized Chinese characters '杨琴' (Yang Qin) followed by a period.

.....

Yang Qin

Supervisor Declaration Statement

I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

5 Apr 2019

.....

Date

.....

Prof Liu Yu

Authorship Attribution Statement

This thesis contains material from 1 paper published in the following peer-reviewed journal in which I am listed as an author.

Chapter 3 is published as Qin Yang, Nan Shen, Zarraz M.-P. Lee, Guangjing Xu, Yeshe Cao, Beehong Kwok, Winson Lay, Yu Liu, Yan Zhou; Simultaneous nitrification, denitrification and phosphorus removal (SNDPR) in a full-scale water reclamation plant located in warm climate. Water Sci Technol 20 July 2016; 74 (2): 448–456. doi: <https://doi.org/10.2166/wst.2016.214>

The contributions of the co-authors are as follows:

- Prof Liu provided the initial project direction and edited the manuscript drafts.
- I prepared the manuscript drafts.
- The manuscript was reviewed by Dr Xu, Dr Cao, Mrs. Kwok, and Dr Lay.
- I co-designed the study with Prof Liu and Asst/Prof Zhou and performed majority of the sampling work at Changi Water Reclamation Plant and laboratory work at NEWRI AEBC. I also analyzed the data.
- Dr Shen conducted one batch experiment and Dr Lee assisted the microbial analysis.

5 Apr 2019

.....
Date

.....
Yang Qin

Acknowledgements

I would like to express I would like to express my special thanks and gratitude to my PhD supervisor, Professor Liu Yu. He has so many inspiring ideas and brilliant thoughts that we couldn't learn enough. He also taught us a lot of life lessons in addition to academic knowledges and we benefited a lot.

Besides my supervisor, I would like to thank the rest of my thesis committee: Prof. Xu Rong, Dr. Winson Lay, and Prof Ng Wun Jern, for their insightful comments, patience and encouragement.

I would also like to extend my gratitude to Dr. Zarraz, Lee May Ping and Dr Xu Guangjing who always shares their experience and knowledge when I encounter problems which guidance have been helpful in my research.

Besides, I wish to thank the responsible staff and laboratory executives in Advanced Environmental Biotechnology Centre (AEBC), Mr. Ricky, Lim Kee Chuan, Ms. Emily Mar'atusalihat, and Ms. Ong Qian Mei, for their valuable assistance in the administration and instrumentation.

I also wish to acknowledge the help provided by Dr Gu Jun who offers me great help when some physical work was required. He is also a great friend and learning partner.

Last but not least, I am grateful for the strong support from my husband and parents whose understanding and comforting during gave me the courage and confidence to complete my study.

The author is supported by the Interdisciplinary Graduate School Scholarship. I appreciate IGS to provide us the opportunity and support to pursue our graduate study.

Table of Contents

Acknowledgements	i
Table of Contents	iii
Summary	viii
List of publications	x
List of tables	xi
List of figures	xii
List of abbreviations	xv
CHAPTER 1 INTRODUCTION	1
1.1 Background	1
1.2 Problem statement	3
1.3 Objectives	3
1.4 Organization of the thesis	4
CHAPTER 2 LITERATURE REVIEW	7
2.1 Biological Nutrient Removal from Wastewater	7
2.1.1 Biological Nitrogen Removal (BNR)	7
2.1.2 Enhanced Biological Phosphorous Removal (EBPR)	10

2.2	Microbial Interactions	12
2.2.1	Interactions among Functions Groups in Wastewater Treatment	13
2.2.2	Unstudied Area	14
2.3	Bacterial Motility and Chemotaxis	15
2.4	Knowledge Gap	19
CHAPTER 3 SIMULTANEOUS NITRIFICATION, DENITRIFICATION AND PHOSPHOROUS REMOVAL (SNDPR) IN A FULL-SCALE WRP UNDER TROPIC CLIMATE CONDITION		20
3.1	Introduction	20
3.2	Materials and Methods	22
3.2.1	Plant Configuration and Sampling	22
3.2.2	Batch experiment	23
3.2.3	Glycogen and PHA determination	26
3.2.4	Chemical analysis	26
3.2.5	qPCR	27
3.2.6	Floc size determination	27
3.3	Results and discussion	29
3.3.1	SNDPR performance in full-scale WRP	29
3.3.2	Confirmation of SNDPR potential in batch experiments	35

3.3.3	Microbial analysis and the relationship between relative abundance and activities	39
3.4	Conclusions	44
CHAPTER 4 SYNERGY AND DYNAMICS OF CO-CULTURED AOB AND NOB AT DIFFERENT INITIAL AOB/NOB RATIOS		46
4.1	Introduction	46
4.2	Material and methods	47
4.2.1	Bacterial strains	47
4.2.2	Chemostat reactors	48
4.2.3	Chemical analysis	50
4.2.4	FISH and image analysis	50
4.2.5	Field emission scanning electron microscopy (FESEM)	51
4.3	Results and discussion	51
4.3.1	Concentration profiles of nitrogenous compounds	51
4.3.2	AOB and NOB abundances	53
4.3.3	Distribution of AOB and NOB in cell cluster and flocs	56
4.4	Conclusions	63
CHAPTER 5 EFFECT OF CHEMOTAXIS ON FLOC STRUCTURE AND METABOLIC ACTIVITY OF NITRIFYING BACTERIA		65
5.1	Introduction	65

5.2	Material and methods	66
5.2.1	Medium and chemicals	66
5.2.2	Bacterial strains and growth conditions	66
5.2.3	Batch assay	67
5.2.4	Chemotaxis capillary assay	67
5.2.5	Motility capillary assay	69
5.3	Results and discussion	69
5.3.1	Chemotaxis response of AOB	69
5.3.2	Chemotaxis response of NOB	76
5.3.3	Effect of chemotaxis on floc structure	83
5.3.4	Chemotaxis and metabolic activity	85
5.4	Conclusions	86
CHAPTER 6	CONCLUSIONS AND RECOMMENDATIONS	88
6.1	Major findings	88
6.1.1	Discovery of SNDPR in a full-scale WRP	88
6.1.2	A balanced AOB and NOB community achievable	89
6.1.3	AOB and NOB had chemotaxis response to NH_4^+ and NO_2^-	89
6.2	Conclusion and implications	90
6.3	Recommendations	91

REFERENCES	93
APPENDIX A	109

Summary

With rapid global urbanization and growing energy shortage, biological nutrient removal from municipal used water has been required to reach high effluent quality and energy efficiency with small footprint. One of the solutions is to integrate multiple biological processes together for better system performance. However, these integrate systems often have a nature of high process complexity, e.g. multiple-metabolic competitions on various substrates, dynamic microbial composition, inhibition by intermediate products, instability of the system performance etc.

Given such a situation, this study aimed to investigate the microbial synergy and dynamics in biological nutrient removal processes at both laboratory-scale and full-scale with mixed and pure cultures. The full-scale study for the first time revealed simultaneous nitrification-denitrification with phosphorus removal (SNDPR) in a local water reclamation plant operated under warm climate. The working mechanisms of SNDPR were further determined and validated by the laboratory-scale study, e.g. the SDRP could be achieved through collective actions of low dissolved oxygen concentration in aerobic zones, step-feeding and high operation temperature. In addition, microbial profiling also confirmed that phosphorus accumulating organisms (PAO) were dominant over glycogen accumulating organisms (GAO), while the dynamic fluctuation of nitrifiers was observed. Likely this was the first complete large-scale study on enhanced biological phosphorus removal at elevated temperature.

It should be realized that in-depth understanding of AOB, NOB and their interaction is essential towards stable SNDPR and anammox for more efficient biological nitrogen removal. Given their relatively high abundance in nitrifying community, pure culture *Nitrosomonas europaea* and *Nitrobacter winogradskyi* were chosen to represent ammonium oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) in the later lab-scale experiments. In order to study the interaction between AOB and NOB, nine chemostat reactors were designed and operated with the co-culture of AOB and NOB at

different initial inoculum conditions. Results showed that the AOB/NOB ratios tended to converge to similar values after 2 ~ 4 weeks' cultivation with AOB dominant in the full-nitrification reactor, indicating that the operation conditions appeared to determine nitrifying community structure, while the dominant abundance of AOB could not serve as a good indication for partial-nitrification. In addition, close proximity between AOB and NOB cells was observed in the co-cultured clusters with layered floc structure. Such observation was likely due to food supply-based active interaction between AOB and NOB. It was also shown that the potential chemotaxis of AOB and NOB which facilitated bacterial movement in response to chemical stimuli, might explain the observed layered structure of microbial floc.

In order to better understand the role of chemotaxis in the interaction of AOB and NOB, a series of capillary assays with *Nitrosomonas europaea* and *Nitrobacter winogradskyi* were performed. It was confirmed that AOB had positive chemotaxis response to ammonium and negative chemotaxis response to nitrite while the reversed situation was observed for NOB. In addition to nitrogen compound, it was also revealed that AOB cells were repelled at low pH, while NOB did not respond to pH change. Such findings indicated that chemotaxis might be involved in the development of microbial community structure of AOB and NOB in biological nitrogen removal process. In addition to metabolic activity, the chemotaxis of AOB and NOB for the first time sheds lights on potential novel research direction for biological nutrient removal.

List of publications

1. Xu G, Zhou Y, Yang Q, Lee Z-P, Gu J, Lay W, Cao Y, Liu Y (2015) The challenges of mainstream deammonification process for municipal used water treatment. *Appl Microbiol Biotechnology* 99(6):2485-2490 doi:10.1007/s00253-015-6423-6
2. Qin Yang, Nan Shen, Zarraz M.-P. Lee, Guangjing Xu, Yeshe Cao, Beehong Kwok, Winson Lay, Yu Liu, Yan Zhou; Simultaneous nitrification, denitrification and phosphorus removal (SNDPR) in a full-scale water reclamation plant located in warm climate. *Water Sci Technol* 20 July 2016; 74 (2): 448–456. doi: <https://doi.org/10.2166/wst.2016.214>
3. Xiao, K., Chen, Y., Jiang, X., Yang, Q., Seow, W.Y., Zhu, W. and Zhou, Y. (2017) Variations in physical, chemical and biological properties in relation to sludge dewaterability under Fe (II) – Oxone conditioning. *Water Research* 109, 13-23.
4. Gu, J., Yang, Q. and Liu, Y. (2018) Mainstream anammox in a novel A-2B process for energy-efficient municipal wastewater treatment with minimized sludge production. *Water Research* 138, 1-6.

List of tables

Table 2.1 List of discovered taxis.	17
Table 3.1 Batch experiment design for verification of SNDPR	25
Table 3.2 Primer Sequences for qPCR	28
Table 3.3 Influent and effluent characterizations during sampling period	29
Table 3.4 Abundances of denitrifiers and <i>Accumulibacter</i> PAO in anoxic zones from three different sample collections.	40

List of figures

Figure 2.1 Nitrogen cycle. Modified from (Ruscalleda <i>et al.</i> , 2011).	8
Figure 2.2 Schematic illustration of the two-stage reaction in EBPR (Henze <i>et al.</i> , 2008).	10
Figure 2.3 Structure of a flagellum in the gram-negative bacterium (Venkataraman and Kao, 1999)	16
Figure 3.1 Process configuration of the studied full-scale WRP.....	23
Figure 3.2 (a) Nutrient and (b) PHA and glycogen concentrations profiles	30
Figure 3.3 Nutrient production and consumption rates in (a) anoxic and (b) aerobic zones of five basins.....	31
Figure 3.4 Floc size distribution of mixed liquor in the studied full-scale WRP.....	33
Figure 3.5 Nutrient production and consumption rates from batch experiment to verify SND potential under different conditions.....	36
Figure 3.6 Concentration profiles of N and P in Experiment 4.....	38
Figure 3.7 Abundances of 16S rRNA genes of AOB and NOB.....	40
Figure 3.8 Plant data (a) and off-line batch experiment data (b) of nitrification rates during different sampling periods.....	42
Figure 3.9 Comparison between relative activities and abundances of AOB and NOB.	43
Figure 4.1 Experimental set-up of chemostat reactors.	49

Figure 4.2 Concentration profiles of nitrogen (a: NH_4^+ ; b: NO_2^- ; c: NO_3^-) of three-group cultures in the start-up period.	53
Figure 4.3 Respective AOB and NOB cell density profiles in Group A, B and C.	55
Figure 4.4 FISH images of Group A batch samples on Day 0, 1, 2, 3, 4, 7, 14, 22.	57
Figure 4.5 FISH images of Group B batch sample on Day 0, 1, 2, 3, 4, 7, 14, 22.	58
Figure 4.6 FISH images of Group B batch sample on Day 0, 1, 2, 3, 4, 7, 14, 22.	59
Figure 4.7 Layered floc structure during late steady state.	61
Figure 4.8 SEM image of AOB (a, c and e) and NOB (b, d and f) on membrane.....	62
Figure 5.1 Cell density of AOB in batch assays.	70
Figure 5.2 AOB metabolic activity at different substrate concentration after 2hr.....	71
Figure 5.3 Concentration-response of AOB to ammonium in chemotaxis assays.	72
Figure 5.4 NO_2^- chemotaxis response curve of AOB.	73
Figure 5.5 Chemotaxis response of AOB to different pH values.	74
Figure 5.6 Normalized AOB cell number in capillary plotted against FA (a), NH_4^+ (b) and pH (c).	75
Figure 5.7 Cell density of NOB during batch assay.	77
Figure 5.8 NOB metabolic activity at different initial NO_2^- concentrations.....	78
Figure 5.9 NOB motility at $\text{NO}_2^- = 0, 50, 100, 200, 500, 1000 \text{ mg N/L}$ and the chemotaxis response curve using the same batch of culture.	79
Figure 5.10 NH_4^+ chemotaxis response curve of NOB.....	80

Figure 5.11 Chemotaxis response of NOB to different pH values.....	81
Figure 5.12 NOB cell number in capillary against FNA (a), NO_2^- (b) and pH (c).....	82
Figure 5.13 Hypothesized structure of AOB and NOB co-culture flocs.....	84

List of abbreviations

Anammox:	Anaerobic ammonium oxidation
AOA:	Ammonia oxidizing archaea
AOB:	Ammonia oxidizing bacteria
ATP:	Adenosine-5'-triphosphate
BNR:	Biological nitrogen removal
Comammox:	Complete ammonia oxidation
DO:	Dissolved oxygen
EBPR:	Enhanced biological phosphorus removal
EPS:	Extracellular polymeric substances
FA:	Free ammonia
FESEM:	Field emission scanning electron microscopy
FNA:	Free nitrous acid
FST:	Final settling tank
GAO:	Glycogen accumulating organism
MLSS:	Mixed liquor suspended solids
MLVSS:	Mixed liquor volatile suspended solids
NBT:	<i>Nitrobacter</i>
NOB:	Nitrite oxidizing bacteria
NSR:	<i>Nitrospira</i>
OD:	Optical density
OHO:	Ordinary heterotroph organisms
PAO:	Phosphorus accumulating organism
PH2MV:	Poly- β -hydroxy-2-methylvalerate
PHA:	Polyhydroxyalkanoates
PHB:	Poly- β -hydroxybutyrate
PHV:	Poly- β -hydroxyvalerate
qPCR:	Quantitative polymerase chain reaction

SND:	Simultaneous nitrification-denitrification
SNDPR:	Simultaneous nitrification-denitrification with phosphorus removal
SRT:	Solid retention time
WRP:	Water reclamation plant

CHAPTER 1 INTRODUCTION

1.1 Background

Discharging of wastewater and effluent with nitrogen and phosphate into water bodies could lead to eutrophication which would cause decreased biodiversity, changes in species composition and dominance, and toxicity effects. Its occurrence in natural or man-made reservoirs will directly affect the quality of water supply in surrounding cities, therefore poses risks on human health.

Biological nutrient removal processes have been widely adopted for the treatment of various types of wastewater streams. The discharge standards of N and P have been increasingly strengthened in more and more countries. Together with rapid urbanization and global energy shortage, biological nutrient removal needs to meet a higher requirement of treatment and energy efficiency with small footprint.

Given such situation, considerable efforts had been made to optimize the existing processes, while develop new treatment processes. One of such attempts was to integrate multiple biological processes together to achieve the purposes of lower energy consumption, smaller footprint, and higher effluent quality. Examples include simultaneous nitrification and denitrification (SND), which enables the integration of the conventional aerobic and anoxic reactors (Münch *et al.*, 1996, Zhu *et al.*, 2007), nitrification-anammox, which requires less energy for aeration, increases carbon efficiency and generates less biomass for subsequent treatment (Gut *et al.*, 2006, Szatkowska *et al.*, 2007), integrated biological phosphorus and nitrogen removal, which combines the two major nutrient removal in the same reactor (Kermani *et al.*, 2009), and even simultaneous nitrification-denitrification and phosphorus removal (SNDPR) (Zeng *et al.*, 2003a). Such applications take advantages of one (aerobic/anoxic/anaerobic) phase for multiple treatment processes and therefore substantially reduce the energy consumption and reactor volume. In addition, the integration of various processes may shorten the reaction

pathway and/or reduce the usage of external carbon and/or alkalinity, which would have been applied, so that further increases the overall process efficiency.

Despite all the superior features of the integrated process design, there are enormous challenges presented. Three of the major challenges are 1) the competition between different microbial groups for the same substrate; 2) the inhibition from the metabolite of one microbial group on another; and 3) the system instability in response to fluctuation in substrate or operating conditions. A typical competition lied between denitrifiers, ordinary heterotrophs and phosphate accumulating organisms (PAOs), which all utilize organic carbon in non-oxygenated environment. The example for inhibition is that the nitrification product, NO_2^- and NO_3^- are inhibitory for PAO and may cause process failure if not effectively controlled (Guerrero *et al.*, 2012). Lack of system stability is another common issue in these biological systems, where changing of one condition would easily induce the change of microbial composition and thus lead to system failure. To resolve such problems, more fundamental knowledge about the interaction mechanisms of different microbial groups is required so that engineers would able to design the corresponding strategies to avoid or mitigate the problems.

Previous studies had presented some results about the microbial interactions between the common wastewater bacterial strains in biofilms in terms of adhesion properties (Andersson *et al.*, 2008), secretion of signaling molecules (Liébana *et al.*, 2016), and change of metabolic activities in different amount of biofilm (Andersson *et al.*, 2011). However, not only the studies of interactions within biofilm are yet to complete, but also the knowledge about the interactions beyond the biofilm structure is very limited. There was still a big knowledge gap between the macro process engineering and the microbial interaction at the cellular level.

In addition, the previous mentioned integrated engineering processes were mostly reported at lab-scale studies whereas data of full-scale applications were still inadequate

where usually more fluctuations in influent quality and operating conditions were encountered and therefore more dynamic microbial composition was observed.

1.2 Problem statement

To further increase the efficiency and robustness of wastewater treatment, more and more studies had integrated different biological nitrogen (N) and phosphorus (P) removal processes in the same reactor design so that lower aeration cost and smaller footprint would be realized. However, due to the complex interaction mechanisms between different microbial groups, the proliferation of one group may impair adverse effects on the other and even led to system failure. Engineers were still not able to fully predict and control such system deterioration due to the lack of fundamental knowledge about the microbial interactions. Besides, experience on the lab-scale studies could not be completely applied on full-scale applications since the latter involved higher phylogenetic richness, more complex fluid dynamics and larger variation on the influent quality and operation conditions.

1.3 Objectives

This research aimed to reveal the possible interaction mechanisms of the main functional groups of nutrient removal process, especially AOB and NOB so that new insights may be shed for better process control and optimization. Specifically, the objectives are to:

a) Determine the relationship between plant performance and functionalities of nutrient (N, P) removal microbial groups in a local (Singapore) full-scale wastewater reclamation plant (WRP);

- b) Explore the population dynamics of ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) in pure cultures with different initial conditions;
- c) Study the chemotaxis behaviors of *Nitrosomonas europaea* and *Nitrobacter winogradskyi* and their effects on nitrification and bioflocculation.

1.4 Organization of the thesis

This thesis includes the following chapters.

Chapter 2 - Literature Review

This chapter provided the background knowledge about the main processes for biological nitrogen and phosphorus removals and summarized the strategies for each process. Then the bottleneck of the integrated processes due to the interactions between different microbial groups were reviewed. Lastly emphasis was given to chemotaxis which was an important form of microbial interaction but lack of study in the wastewater treatment processes.

Chapter 3 - Simultaneous Nitrification, Denitrification and Phosphorous Removal (SNDPR) in a Full-scale WRP under Tropic Climate Condition

A local full-scale WRP performance in nutrients removal was studied in 2014. It was verified by both plant data analysis and lab experiments that simultaneous nitrification, denitrification and phosphorous removal was occurred during the period. Microbial analysis confirmed the dominant growth of phosphorous accumulating organisms (PAOs) over with glycogen accumulating organisms (GAOs). However, the abundance of AOB and NOB had been through a very dynamic fluctuation with *Nitrospira* was extremely high in the last round. Relationship between the abundance and activities had been discussed.

Chapter 4 – Synergy and Dynamics of Co-cultured AOB and NOB at Different Initial AOB/NOB Ratios

In Chapter 3, it was observed that relatively stable total nitrogen removal despite the dynamic fluctuations of AOB-NOB abundance. Hence, pure AOB and NOB cultures were applied in the chemostat reactors with different initial ratio of AOB/NOB inoculums to further investigate the relationship between AOB and NOB interactions regarding their development in terms of abundance. The physical distribution of AOB and NOB in cell cluster was also examined using FISH. The results indicated that AOB and NOB population were able to achieve steady state at AOB/NOB ratio around 2 ~ 3 regardless of the initial inoculum condition. Besides, layered structure was observed in the coculture cell clusters and chemotactic effect was hypothesized to contributed to the distribution pattern.

Chapter 5 - Effect of Chemotaxis on Floc Structure and Metabolic Activity of Nitrifying Bacteria

Observing the close physical contact between AOB and NOB in the previous chapter (Chapter 4), their potential chemotaxis characteristics was explored with capillary assays. The results suggested that AOB and NOB had positive chemotaxis response to NH_4^+ and NO_2^- respectively and they had negative response to the two chemicals in reverse. These findings were closely related to the hypothesized floc formation mechanism. In addition, the independent chemotaxis movement of the two microbial groups may enhance or inhibit the metabolic activity and therefore affect the system efficiency.

Chapter 6 - Conclusions and Recommendations

This last chapter listed the major findings and drew conclusions from the results discussed in the previous chapters. With proper operating conditions, SNDPR was feasible even in tropical climate as in the studied WRP although the microbial groups went through complex interactions. Microbial abundance of AOB and NOB were able to reach a steady ratio when operating conditions remain stable and the chemotaxis of AOB and NOB enhanced the process of nutrient uptake in turbulent condition and directly

affect the floc structure during the floc formation stage. Besides these interactions, other perspectives of interaction between AOB and NOB, and other functional groups in N and P removal shall be further investigated to further elucidate the nutrient removal process and possible generation of strategies for process optimization in large scale applications

CHAPTER 2 LITERATURE REVIEW

2.1 Biological Nutrient Removal from Wastewater

With rapid urbanization and the growth of population, eutrophication due to the excess discharge of nutrients (e.g. N & P) to natural water bodies, is becoming a global environmental problem. It has been well known that water eutrophication can have serious negative impact on aquatic life, depletion of desirable flora and fauna, pollution of drinking water source for nearby residence, etc. Obviously, there is an growing need for removing soluble nutrients from wastewater in order to prevent eutrophication. As such, many different biological processes have been developed for the removal of soluble nutrients from wastewater.

2.1.1 Biological Nitrogen Removal (BNR)

Nitrogen exists in most domestic wastewater streams as ammonium (NH_4^+) and organic nitrogen (e.g. proteins, peptides, and amino acids) while organic nitrogen could be further biodegraded to NH_4^+ . Wastewater ammonium is ultimately converted to nitrogen gas in biological wastewater treatment process.

Conventional biological nitrogen removal involves nitrification and denitrification (Figure 2.1). The former includes nitritation and nitrataion performed by ammonia oxidizing bacteria (AOB) or ammonia oxidizing archaea (AOA) and nitrite oxidizing bacteria (NOB) respectively. Denitrification is performed mostly by heterotrophic denitrifiers, which consist of a large group of heterotrophic facultative anaerobic microorganisms. Conventional nitrification-denitrification process required large footprint for the successful occurrence of nitrification and denitrification in different reactors. Furthermore, the energy consumption and sludge production are usually of considerable amount.

The discovery of anaerobic ammonium oxidation (Anammox) in 1990 (Graaf *et al.*) and the subsequent identification of the first anammox bacteria in 2001 (Kuenen) had been a great surprise for the scientific community. Since then, anammox coupled with nitrification had been applied in many high strength wastewater treatment plants and proven to be an advanced process with superior features, e.g. cost reduction of up to 60% (Siegrist *et al.*, 2008) due to less aeration and external carbon requirement, less sludge production and lower CO₂ emission (Hu *et al.*, 2013). However, the long start-up period and its lack of applications in low strength wastewater still required further studies for process optimization (Qin *et al.*, 2017).

More recently, the presence of complete ammonia oxidiser (Comammox) was identified within *Nitrospira* species which was able to directly convert NH₄⁺ into NO₃⁻ within a single organism (Daims *et al.*, 2015). Study of Kits *et al.* (2017) reported that this complete nitrifier had slow growth rate, low ammonia oxidation rate and high yield. The discovery of comammox updated the knowledge of nitrogen cycle (Figure 2.1).

Figure 2.1 Nitrogen cycle. Modified from (Ruscalleda *et al.*, 2011).

Besides the new discovery of anammox and comammox, which leads to the potential novel nitrogen removal processes, ways to improve the conventional nitrification-denitrification have also been investigated. As illustrated in Figure 2.1, for both nitrification-denitrification and nitrification-anammox pathways, the additional step of $\text{NO}_2^- \rightarrow \text{NO}_3^- \rightarrow \text{NO}_2^-$ at the bottom of the nitrogen cycle is in fact a redundant step which requires additional 25% aeration, 40% carbon and 40% subsequent sludge treatment (Ruscalleda Beylier *et al.*, 2011). Therefore, in more and more research studies, constant efforts have been devoted to inhibiting NOB activity so that NO_3^- could be kept out of the reaction pathway. By shortening the biological nitrogen cycle, partial nitrification or short-cut nitrification yields faster reaction rate and larger treatment efficiency.

In real applications, however, the occurrence and maintenance of partial nitrification relies on various factors. Strategies for promoting AOB and arresting NOB included: maintaining short SRT to wash-out NOBs (Han *et al.*, 2016); maintaining low dissolved oxygen (DO) concentration in aerobic tanks which took advantage of the higher DO affinity of AOB than NOB (Waki *et al.*, 2018); treating high strength wastewater containing higher free ammonia (FA) which inhibited NOBs (Ren *et al.*, 2016); frequent transition of anoxic and aerobic conditions (Ge *et al.*, 2014, Hou *et al.*, 2017); etc.

For low strength municipal wastewater, it appears to be difficult to maintain stable partial nitrification, especially in continuous flow processes (Ge *et al.*, 2015). The fast start-up strategies of partial nitrification also need further exploration due to the slow growth rate of AOB. In addition, the enhanced biological phosphorus removal (EBPR) is hardly achieved in systems with partial nitrification due to the inhibition from accumulated NO_2^- and free nitrous acid (FNA) on phosphorus accumulating organisms (PAOs). Therefore, more fundamental studies about the interacting growth mode of AOB and NOB are required to better monitor and control the activity of the two groups especially in large scale applications which involves more fluctuation in influent quality and micro-

environment. Meanwhile, the balance between NO_2^- accumulation and the encouraged proliferation of PAO remains a challenge task in biological nutrient removal systems.

2.1.2 Enhanced Biological Phosphorous Removal (EBPR)

EBPR refers to the process in which intracellular polyphosphate is consumed and stored during the cyclic anaerobic and aerobic periods by a group of heterotrophic bacteria termed phosphate-accumulating organism (PAO). As illustrated in Figure 2.2, PAO stores simple organic carbon as polyhydroxyalkanoates (PHA) under anaerobic conditions, then oxidizes the stored PHA and uptakes excess phosphorus in the subsequent aerobic phase which is also known as luxury uptake. By wasting the sludge containing PAO, which accumulates the poly-P, phosphorus is removed from the system and low P level in the effluent water is achieved.

Figure 2.2 Schematic illustration of the two-stage reaction in EBPR (Henze *et al.*, 2008).

This process is economic and environmentally friendly and has been applied in wastewater treatment for decades. However, it is challenging to maintain system stability and the troubleshooting is often not effective due to limited understanding of the

responsible microorganism (He *et al.*, 2007). Sometimes the strategies based on lab-scale and full-scale studies are even contradictory (Gebremariam *et al.*, 2011).

According to the previous studies, PAOs are sensitive to many factors including fluctuation of substrate concentration, inhibition from NO_2^- (Saito *et al.*, 2004), FNA (Zhou *et al.*, 2010), metal ions (Kim *et al.*, 2010, Tsai *et al.*, 2016), dissolved oxygen, pH and temperature change (Lopez-Vazquez *et al.*, 2009). Besides these, a well-known competitor of PAO, GAO, which also consumed carbon as PAO but does not contribute to phosphorous removal is blamed primarily for the deterioration of EBPR process (Gebremariam *et al.*, 2011). Although the competition between PAO and GAO and its role in the impairment of phosphorus removal was not yet adequately elucidated, studies reported a number of factors which might determine the competition: influent carbon to phosphorus ratio (Muszyński and Miłobędzka, 2015), presence of ferric iron (Jobbagy *et al.*, 2006) and NO_2^- (Tayà *et al.*, 2013), dissolved oxygen level (Griffiths *et al.*, 2002, Dai *et al.*, 2007), temperature (Sayi-Ucar *et al.*, 2015), type of carbon source (Shen and Zhou, 2016) and sludge age (Whang and Park, 2006). Among these factors, temperature and type of carbon source are the most critical.

Studies had contradictory results regarding EBPR efficiency and stability at different temperature ranges, but it was widely agreed that low temperature (below 20°C) favored PAO against GAO in many lab-scale studies (Whang and Park, 2002, Panswad *et al.*, 2003, Whang and Park, 2006). Nevertheless, the reason for cold temperature favoring PAO was still under debate (Gebremariam *et al.*, 2011).

As to the effect of carbon availability and source types on process stability, numerous studies have reported various conclusions for different combinations of carbon sources. Focus has been given to COD- or VFA-to-phosphorus ratio (Schuler and Jenkins, 2003, Barnard and Abraham, 2006, Muszyński and Miłobędzka, 2015), influence of specific type of carbon source (acetate, propionate and glucose were mostly studied) (Chen *et al.*,

2015, Nittami *et al.*, 2017, Shen *et al.*, 2017), and the coverage of different types of carbon sources (Oehmen *et al.*, 2005b, Lu *et al.*, 2006, Gebremariam *et al.*, 2012).

In general, due to the phylogenetic and physiologic diversity of PAOs and GAOs, results of different lab-scale studies easily led to distinct or even contradictory findings. Besides, full-scale data often could not agree well with the theory drawn from lab-scale studies, suggesting that the PAO-GAO competition model might be too simple to explain the EBPR process performance. More in-depth investigations, including both culture-dependent and culture-independent approaches should be considered for each specific case.

2.2 Microbial Interactions

Microorganisms (e.g. viruses, bacteria, archaea and protists) can form complex ecologically interacting webs. Compared with individual isolations, mixed culture consortia can achieve more complicated functions and are more adaptable to environmental changes. New metabolite product which is not present in pure sample strains may be produced in mixed culture consortia (Andersson *et al.*, 2011). By dividing the multiple steps for a certain task to several different microbial groups, the imbalance caused by exogenous elements is reduced and each microbial population can be individually optimized (Brenner *et al.*, 2008). Activated sludge process as the most common biological processes in wastewater treatment is actually the application of engineered mixed culture consortium. All the microorganisms including the desirable functional groups and all the unwanted strains form an artificial ecological web within the sludge.

In natural habitat, different species tend to evolve to adapt to each other and generate series of mechanisms to cope with the ecological web. The interactions between microorganisms may have positive (win), negative (lose) or neutral impact (not affected)

on each component. Depending on the nature of these interactions, the relationships between any two interacting parties could be defined as being mutualistic, endosymbiotic, competitive, antagonistic, pathogenic and parasitic (Braga *et al.*, 2016). A common mutualistic paradigm among bacteria is biofilm formation that is jointly developed by different taxonomic groups to mitigate diverse stressful conditions (Faust and Raes, 2012).

Studying natural cell-cell interactions may highlight new pathways for re-engineering, establish models for monitoring microbial performance and allow better predictions of microbial community in response to environmental changes. Establishing synthetic interactions between species provides possibilities for new engineering systems. Besides, knowledge about the microbial interaction mechanism is of great important for developing specific agents that can alter the microbial communication for designing specific strategy to enhance or inhibit the target process.

2.2.1 Interactions among Functions Groups in Wastewater Treatment

In wastewater treatment process, the study on microbial interactions is still in its infancy due to the complexity of different community structures and the consequent difficulties in response identification of various functional groups. Andersson *et al.* (2008, 2011) contributed valuable knowledges about microbial interactions in biofilms of wastewater treatment systems. During the initial attempt, they compared the different potential for attachment and biofilm formation of 13 bacterial strains commonly found in wastewater systems as pure culture, dual culture and mixed culture (including all 13 strains). The different adhesion properties of the strains in different culture combinations confirmed that the interspecies microbial interaction altered the biofilm formation process and tendency (Andersson *et al.*, 2008). A few years later, they tried to link the biofilm formation with the nutrient removal activities. The results showed that denitrification activities within the biofilms generally increased with the amount of biofilm while

phosphorus removal depended on the bacterial growth rate (Andersson *et al.*, 2011). Synergistic effect was also observed between a denitrification and a phosphorus removal strain.

More recently, Pérez *et al.* (2015) attempted to establish interactions between AOB and NOB during nitrification process for the first time. The physiological adaptation of *Nitrosomonas europaea* and *Nitrobacter winogradskyi*, which were chosen as the model microorganisms for AOB and NOB respectively, was examined and compared between co-culture and their respective pure culture. It was found that with the same total nitrogen loading as substrate, population of the co-culture was over 50% higher than the sum of that in pure cultures while *N. europaea* benefited more (reached higher biomass concentration) from the synergetic growth mode. Transcriptome data analysis revealed that 30.2% of the genes in *N. europaea* and 11.8% of the genes in *N. winogradskyi* expressed at significantly different level in the co-culture compared with their respective pure culture. Specifically, the defense mechanism and cell motility of both strains decreased in the co-culture. *N. europaea* also had elevated expression for biogenesis and energy production while those two groups of genes expressed with opposite trend for *N. winogradskyi* in co-culture. These results greatly improved our knowledge on the nitrification-nitrification process and provided insights towards more effective process control strategies.

2.2.2 Unstudied Area

Despite the valuable previous studies of microbial interactions in wastewater treatment, there are still a lot more details of various ecological aspects about the key functional groups waiting for further exploitation, e.g. possible secondary metabolite exchange, metabolite conversion, signaling, chemotaxis, horizontal gene transfer, physical contact or proximity of relative groups, etc.

AOB and NOB, which are of great importance for the successful maintenance of partial nitrification, develop multiple forms of symbiotic relationship in the natural and engineered systems, i.e. mutualism, amensalism and competition. The complex interaction mechanisms between AOB and NOB might in turn affect their proliferation, nutrient degradation, biofilm formation, flocculation structure, virulence secretion, detoxification mechanism and so on. These features required further investigation and this study chose their chemotaxis features for detailed study and the subsequent flocculation and nutrient degradation were also discussed.

2.3 Bacterial Motility and Chemotaxis

Although once considered the result of Brownian Motion, bacterial motility was finally realized to be a self-propelled motion and defined themselves as living entities in the early observations (Mitchell and Kogure, 2006).

The bacterial flagellum is the best studied prokaryotic motility structure which is usually 12 ~ 30 nm in diameter and 3 ~ 12 μ m in length. The rigid structure consists of helical filaments, the hook structure and the basal body as shown in Figure 2.3. Driven by energy from a proton-motive force rather than directly from adenosine-5'-triphosphate (ATP) (Gabel and Berg, 2003), the flagella were able to rotate either clockwise or counterclockwise. With multiple flagella on each cell, bacteria switch between run (flagella aligning in a bundle and the cell swimming along a straight line) and tumble (flagella propelling in different directions and the cell punctuating with tumbles) paradigm, exhibiting a random walk trajectory in no-gradient environment.

Figure 2.3 Structure of a flagellum in the gram-negative bacterium (Venkataraman and Kao, 1999)

Many but not all bacterial species exhibit motility. A motile strain can switch between motile and non-motile mode according to the surrounding environment. It was reported that the proportion of motile bacteria at the Scripps pier had seasonal variation between 5% to 70% and many bacteria swam less than 20% of the time (Grossart *et al.*, 2001). Within the run time, the typical swimming speed of a cell is $10 \sim 30 \mu\text{m/s}$ (Rivero *et al.*, 1989) while the lower and upper limit range from 1 to $1000 \mu\text{m/s}$ with both ends represent the physical limit (Mitchell and Kogure, 2006).

When a gradient of any kind exists, a cell may move along or against the concentration gradient. This motion is termed “taxis”. Depending on the type of stimulus which causes the gradient, there are chemotaxis, phototaxis, magnetotaxis, osmotaxis, galvanotaxis and thermotaxis as summarized in Table 2.1. Among them, chemotaxis is the most widely observed and studied taxis in bacteria (Manson, 1992).

Table 2.1 List of discovered taxis.

Name of taxis	Type of stimuli	Representative species	References
Chemotaxis	Chemicals	<i>Escherichia coli</i>	(Tso and Adler, 1974)
Phototaxis	Intensity or wavelength of light	<i>Halobacterium halobium</i> , <i>Rhodospseudomonas</i> spp.	(Spudich and Bogomolni, 1984, Häder, 1987)
Magnetotaxis	Earth magnetic field	<i>Aquaspirillum magnetotacticum</i>	(Blakemore, 1982) (Frankel and Blakemore, 1989)
Osmotaxis	Osmotic pressure	<i>Escherichia coli</i> .	(Qi and Adler, 1989)
Galvanotaxis	Electric current	<i>Escherichia coli</i> , <i>Salmonella typhimurium</i>	(Adler and Shi, 1988)
Thermotaxis	Temperature	<i>Escherichia coli</i>	(Maeda <i>et al.</i> , 1976)

With chemotaxis, bacteria are able to move away from the virulence factors or towards the substrate. However, harmfulness was neither necessary nor sufficient to make a compound a repellent even though most of the repellents were harmful (Tso and Adler, 1974). Meanwhile, substrates not always play as chemoattractants for motile bacteria, e.g. the cholera bacteria in the intestine was highly motile, but not chemotactic (Merrell *et al.* (2002) .

Chemotaxis is initiated with the chemical-specific chemoreceptors located on the cell membrane which detect the chemoattractant or repellent without interfering the metabolic process. After receiving the chemotaxis signal from the chemoreceptors, the cells transmit the signal to the bacterial flagella with a series of signaling proteins and regulate the tumble frequency of the cell. When a cell moves along a certain direction, the concentration of chemical stimulus is constantly detected. If the attractants' concentration increases or the repellents' concentration decreases, the frequency of tumble will decrease, leading to a net move towards that direction; when the detected concentration of attractants decreases or that of repellents increases, the tumble frequency will increase, leading to a retarded motion and eventually away from the previous direction of motion.

Bacterial chemotaxis provided the significantly increased nutrient availability, hence chemotactic strains were usually able to grow faster than their counterparts (Singh and Olson, 2008). Kiørboe and Jackson (2001) reported that chemotaxis potentially increased the growth rate of bacteria in the chemical plume by 10 ~ 20 times. Similarly, Watteaux *et al.* (2015) also found that the strategy of chemotaxis could enhance bacterial nutrient uptake rate by 2.2 times compared to their non-chemotactic counterparts in the turbulent environment. In addition, motile and chemotactic bacteria exhibited enhanced colonization and aggregation, while non-motile or non-chemotaxis strains did not (Kiørboe *et al.*, 2002, Tamar *et al.*, 2016). Obviously, the chemotaxis-enhanced nutrient availability and colonization ability allow bacteria to survive better in biological process for wastewater treatment.

Traditionally, chemotaxis was studied in the medicine and clinical areas of microbiological research. Recently it has gained increasing interest in the field of environmental biotechnology for wastewater treatment since the chemotaxis can bring bacteria into closer proximity to pollutants, therefore is helpful for improving the remediation efficiency. It had been demonstrated that the chemotaxis of some bacteria played a role in the bacterial colonization on fixed or mobile surfaces, which was an

essential step for biofilm formation (Tamar *et al.*, 2016). On the other hand, as Wong-
Ng *et al.* (2018) mentioned that chemotaxis was found to be strongly associated with
bacterial metabolism and growth. Thus, there is an urgent need to explore the effect of
chemotaxis on the performance of the major functional groups in biological wastewater
treatment process in a systematic manner.

To the author's best knowledge, chemotaxis had been intensively studied for *E. coli* and
many pathogenetic bacteria, while little information is currently available for the major
groups of nutrient-removing bacteria in biological wastewater treatment process.

2.4 Knowledge Gap

Although biological nutrient removal processes have been extensively studied for years,
there are still many challenges to address, e.g. microbial competition, system stability,
long start-up period or recovery period after a process failure, etc. The novel biological
process integrating multiple functional microbial groups, e.g. anammox, has gained
increasing attention and interest (Xu *et al.*, 2015, Gu *et al.*, 2018). However, the design
and operation of such a complex biological process involving multiple microbial groups
need a sound understanding of the interactions and competitions among them. For
example, partial nitrification is an essential step towards anammox, in which nitrite-
oxidizer may over-compete anammox bacteria for nitrite. Therefore, the study of
microbial interactions between the major functional groups would be greatly beneficial
for developing novel biological nutrient removal process with high efficiency and
stability.

CHAPTER 3 SIMULTANEOUS NITRIFICATION, DENITRIFICATION AND PHOSPHOROUS REMOVAL (SNDPR) IN A FULL-SCALE WRP UNDER TROPIC CLIMATE CONDITION

3.1 Introduction

Conventional biological nitrogen removal requires two reaction basins for aerobic nitrification and anoxic denitrification respectively. To achieve satisfactory removal efficiency, intensive aeration and dosage of external carbon are required. In the short-cut nitrification process, where NH_4^+ is only oxidized to NO_2^- rather than NO_3^- and the denitrification would directly start from NO_2^- reduction, cost for aeration and carbon source could be reduced then. With recent technological advances, simultaneous nitrification and denitrification (SND) was achieved and further cost-reduction for nitrogen removal was realized with low oxygen supply and smaller reactor footprint (Zhu *et al.*, 2007). The scientific rationality of SND lay on two points: the dissolved oxygen (DO) gradient within sludge flocs or biofilms, and the aerobic denitrification capability of certain microbial groups (Frette *et al.*, 1997).

Other than nitrogen removal, phosphorus removal is another major objective of wastewater treatment, especially domestic wastewater. The biological solution for P was termed enhanced biological phosphorus removal (EBPR) where phosphate accumulating organisms (PAOs) release and store polyphosphate in anaerobic and aerobic condition respectively, resulting a net P removal in the effluent.

The alternating DO requirement for EBPR is in fact also the suitable operating condition for biological nitrogen removal, which offers the possibility to integrate the superior nitrogen removal processes, e.g. short-cut nitrification-denitrification, SND with EBPR, for higher system functionality and efficiency. So far, efforts have been made to

demonstrate the feasibility of SNDPR in lab-scale systems at temperature of 18 ~ 25°C (Meyer *et al.*, 2005, Peng *et al.*, 2008). However, there was no report of SNDPR in full-scale continuous flow activated sludge system especially under warm climate. The high temperature was generally agreed to be unfavorable to PAO compared to its competitor GAO, which utilized similar substrate but did not contribute to P removal. Besides the challenge due to high temperature, integration of several functional microbial groups would definitely induce the competition for the limiting substrate such as oxygen competition among nitrifiers, aerobic ordinary heterotroph organisms (OHO) and PAO; carbon competition among denitrifiers, OHO and PAO. At the same time, metabolite of one microbial group may cause inhibition to the other. For example NO_2^- , product of AOB and substrate of NOB, is toxic to many other microbial groups including PAO (Saito *et al.*, 2004, Zhou *et al.*, 2010). Therefore, extensive knowledge, frequent sampling and precise operational control are required to maintain the balance among various biological processes in order to achieve a stable operating system.

In this study, the performance of a full-scale water reclamation plant (WRP) in tropical climate was investigated via multiple samplings over a period of more than half a year. Occurrence of SNDPR was discovered by plant data analysis and further verified off-line by a series of batch experiments with fresh sludge from the WRP. Molecular study was also conducted to support the investigation. The results indicated that alternative anoxic aerobic conditions with step-feeding influent and high percentage of return sludge provided feasible conditions for stable SNDPR at satisfactory effluent quality while the molecular data raised new concern about the relationship between microbial abundance and activities.

3.2 Materials and Methods

3.2.1 Plant Configuration and Sampling

The studied full-scale WRP locates in Singapore and has a treatment capacity of 800,000 m³ municipal wastewater per day. Each of the four identical treatment trains contains five basins with aerobic and anoxic zones in sequence, denoted by 1A to 5O as shown in Figure 3.1. The dissolved oxygen (DO) is maintained below 0.15 mg/L in anoxic zones and 1.4 ~ 1.8 mg/L in the aerobic zones by online sensor control system. Influent water (primary effluent or PE) is step-fed into the anoxic zones of five basins and the mixed liquor from the last basin (returned active sludge or RAS) is returned to the first basin at 50% of the influent flow rate, leading to a decreasing hydraulic retention time (HRT) of 1.65, 1.28, 1.05, 0.88 and 0.77 hours in basin 1 to 5 respectively. The mixed liquor suspended solid (MLSS) concentrations are gradually decreasing from 3700 mg/L in basin 1 to 2000 mg/L in basin 5. The solid retention time (SRT) of each treatment train is controlled at 5 days. Samples were taken from the influent, effluent, return sludge and at the outlet of each aerobic and anoxic zones in four rounds of collections during the second half year of 2014. Liquid samples were filtered using 0.45 µm syringe filters to remove all the biomass in the liquid samples and prevent further biological reaction during transportation. The filtration process was conducted immediately after the samples were taken and usually completed within 10 min. Both liquid and sludge samples were immediately put in an ice box and transported to the lab for subsequent analysis. MLSS and nutrient concentrations were measured within the same day of sampling to minimize the loss from cell lysis. Some mixed liquor samples were centrifuged to remove the top liquid portion and the bottom sludge pellets were stored in -80°C to preserve the DNA and RNA before any molecular analysis.

Figure 3.1 Process configuration of the studied full-scale WRP. The above figure showed the process of one train. All four rounds of samples were taken from the same train. PE: primary effluent; RAS: return activated sludge.

3.2.2 Batch experiment

Sludge samples were tested within 24 hours after sampling to preserve the activeness of the biomass. The batch experiments were designed to examine the SNDPR potential of the biomass in off-line condition. NaHCO_3 (1.25 g/L) was added to all testing medium to supply inorganic carbon source and buffer pH during the experiments. Trace elements solution of 1.25 ml/L were prepared with the composition referring to the study of Suneethi and Joseph (2011). Nitrogen removal batch experiment and EBPR batch experiment were conducted for 1.5 hours and 3 hours respectively. The latter included anaerobic and aerobic phases with 1.5 hours for each. Total four groups of batch experiments were conducted according to different process condition and substrate type as stated in Table 3.1.

In Experiment 1, 30 mg $\text{NH}_4^+\text{-N/L}$ which was similar to the influent concentration was dosed as substrate and both high (2 ~ 4 mg $\text{O}_2\text{/L}$) and moderately low (1 ~ 2 mg $\text{O}_2\text{/L}$) DO conditions were tested. The latter was denoted as “low DO” in the later discussion. Aeration was provided by purging filtered air to remove airborne microorganism. Low DO condition was further fallen into two sub-conditions: with (1c) and without (1b) organic carbon where sodium acetate at 100 mg COD/L was used as external carbon source. This was to verify the aerobic denitrification capability by ordinary denitrifiers. The COD level was provided at similar level as the influent wastewater and sufficient for aerobic denitrification. Experiment 2 had similar set-ups as Experiment 1 except that the substrate was substituted by 30 mg $\text{NO}_2^-\text{-N/L}$ so that nitrification rates by nitrite oxidizing bacteria (NOB) and possible denitrification via nitrite could be determined.

In order to compare aerobic and anoxic denitrification rates, Experiment 3 was carried out under anoxic conditions with 30 mg $\text{NO}_2^-\text{-N/L}$ and 300 mg COD/L in the form of sodium acetate. The COD level in Experiment 3 was provided at 10 times the stoichiometric ratio of nitrogen to ensure full denitrification. N_2 gas was purged into the mixed liquor in Experiment 3 to ensure that no oxygen from air diffused into the experiment medium and the DO was below detection limit (0.01 mg $\text{O}_2\text{/L}$) throughout the experiment period. Sludge from 5 basins of the same MLSS concentration as the plant data were run in parallel batches to serve as the replicates in Experiment 1 ~ 3.

Experiment 4 examined the EBPR potential using the biomass from the return sludge line and the real wastewater as batch experiment medium since synthetic wastewater could not provide sufficient types of organic carbon for a satisfactory P removal rate (data using synthetic wastewater not shown). The aerobic phase in Experiment 4 referred to a low DO condition (1 ~ 2 mg $\text{O}_2\text{/L}$).

Table 3.1 Batch experiment design for verification of SNDPR

Test conditions	Nitrification						Denitrification	EBPR	
	Experiment 1			Experiment 2			Experiment 3	Experiment 4	
	(a)	(b)	(c)	(a)	(b)	(c)			
DO Level	High	Low	Low	High	Low	Low	Anoxic	Anaerobic	Low
Organic Carbon	-	-	100 mg/L as COD	-	-	100 mg/L as COD	300 mg/L as COD	Influent wastewater	
Nutrient	30 mg/L NH_4^+ -N			30 mg/L NO_2^- -N			30 mg/L NO_2^- -N		

3.2.3 Glycogen and PHA determination

Glycogen extraction method of Zeng *et al.* (2003b) was adopted. Briefly, sludge sample was firstly freeze-dried and weighted then added with 5 ml 0.6 M HCl and heated at 105°C for 6 hours. The glucose concentration in the supernatant was analyzed using Agilent 1200 series HPLC system (Agilent Technologies, Inc., Germany). The PHA was extracted according to the method of Oehmen *et al.* (2005a). Again, lyophilization of sludge sample was conducted first. The dry pellets were then re-suspended with 3% H₂SO₄ acidified methanol and chloroform mixture and heated for 20 hours at 105°C. After cooled down, the sample was added with deionized water to remove impurities and debris and the aqueous phase was discarded. The organic portion was measured with Agilent 7890A GC system (Agilent Technologies, Inc., USA). In this study, PHA was represented by the sum of poly-β-hydroxybutyrate (PHB), poly-β-hydroxyvalerate (PHV) and poly-β-hydroxy-2-methylvalerate (PH2MV).

3.2.4 Chemical analysis

Nitrogenous compounds (NH₄⁺-N, NO₂⁻-N and NO₃⁻-N) were determined by colorimetric method using UV-1800 spectrophotometer (Shimadzu Co., Ltd., Kyoto, Japan). Hach Nessler reagent set was used to measure the NH₄⁺-N concentration based on USEPA Nessler method. NO₂⁻-N, NO₃⁻-N, PO₄³⁻-P, MLSS and mixed liquor volatile suspended solid (MLVSS) concentration were determined according to standard methods (null, 1998).

3.2.5 qPCR

DNA of the sludge was extracted according to the improved method of Griffiths (Towe *et al.*, 2011). SYBR Green based qPCR with the primers in Table 3.2 was conducted to quantify the relative abundance of the target microbial communities.

3.2.6 Floc size determination

Mixed liquor floc size distribution was measured by Shimadzu SALD-3101 Laser Diffraction Particle Size Analyzer (Shimadzu Co., Ltd., Kyoto, Japan) with a measurement range from 50 nm to 3000 μm .

Table 3.2 Primer Sequences for qPCR

Primer	Gene	Target	Sequence	Annealing Temp. (°C)	Amplification Efficiency (%)
CTO189f^a	16S	β-Subdivision AOB		55	99
CTO654r	rRNA		5'-CTAGCYTTGTAGTTTCAAACGC-3'		
FGPS872	16S	Nitrobacter	5'-CTAAAACTCAAAGGAATTGA-3'	55	91
FGPS1269	rRNA		5'-TTTTTTGAGATTGCTAG-3'		
NSR1113f	16S	Nitrospira	5'-CCTGCTTTCAGTTGCTACCG-3'	55	98
NSR1264r	rRNA		5'-GTTTGCAGCGCTTTGTACCG-3'		
nosZF	<i>nosZ</i>	Denitrifiers	5'-CGYTGTTCMTCGACAGCCAG-3'	55	100
nosZ1622R			5'- CGSACCTTSTTGCCSTYGCG-3'		
518f	16S	Accumulibacter	5'- CCAGCAGCCGCGGTAAT-3'	61	95.7
PAO846r	rRNA		5'- GTTAGCTACGGCACTAAAAGG-3'		

^aMix CTO189fA-B (50- GGAGRAAAGCAGGGGATCG-30) and CTO189fC (50-GGAGGAAAGTAGGGGATCG-30) at ratio 2:1.

3.3 Results and discussion

3.3.1 SNDPR performance in full-scale WRP

During the period of study from Jun 2014 to Nov 2014, the temperature of the mixed liquor in the treatment train was relatively stable in the range of 28 ~ 30°C, while the environment temperature fluctuated between 26 ~ 32°C. The influent flow contained 30 ~ 35 mg $\text{NH}_4^+\text{-N/L}$ and 3 ~ 7 mg $\text{PO}_4^{3-}\text{-P/L}$ with negligible nitrite⁻ and nitrite (Table 3.3). The soluble COD (sCOD) in the influent ranged between 80 ~ 150 mg COD/L. The average removal efficiency during the period in the WRP reached over 70%, 91% and 76% for sCOD, total nitrogen and phosphorus respectively. These results suggested excellent biological nutrient removal performance in full-scale WRPs.

Table 3.3 Influent and effluent characterizations during sampling period

	$\text{NH}_4^+\text{-N}$	$\text{NO}_2^-\text{-N}$	$\text{NO}_3^-\text{-N}$	sCOD	$\text{PO}_4^{3-}\text{-P}$
Influent (mg/L)	30 ~ 35	< 0.5	< 0.5	80 ~ 150	3 ~ 7
Effluent (mg/L)	0.5 ~ 3	0 ~ 0.6	0 ~ 1	15 ~ 35	1 ~ 2

The concentration profile of individual basins was shown in Figure 3.2. Only PHB and PHB were plotted to represent PHA in Figure 3.2(b) since PH2MV was not detected. Data in Figure 3.2(a) was the average of four samples collected from the outlet of each anoxic and aerobic basins. It appeared that the nutrient concentration decreased from Basin 1 to 5 as an overall trend. Cyclic NH_4^+ increasing in anoxic zones was due to the step feed while cyclic NH_4^+ removal and NO_x (NO_2^- and NO_3^-) production in aerobic basins attributed to the biological nitrogen removal. The existence of NO_2^- accumulation in aerobic zones marked the occurrence of partial nitrification. The little residue of NO_2^- and NO_3^- at the outlet of anoxic zones indicated the efficient denitrification without external carbon dosage. PO_4^{3-} release and PHA synthesis in anoxic zones, PO_4^{3-} uptake and PHA consumption in aerobic zones with net PO_4^{3-} removal indicated a typical EBPR

process driven by PAO. It was notable that glycogen, whose changes reflected the activities of GAO, the PAO's competitor, did not display a significant variation.

Figure 3.2 (a) Nutrient and (b) PHA and glycogen concentrations profiles at the outlet of each anoxic and aerobic zone in five basins (1A ~ 5O) and return sludge (RS).

After further analyzing the data in Figure 3.2, the reaction rate of nutrients in each basin was plotted in Figure 3.3, which better represented the cyclic biological changing pattern that was discussed above. The NH₄⁺ production in the first and second anoxic zones was likely from the hydrolysis of particulate matters in the influent. In the aerobic zones,

NH_4^+ removal was higher than the production of NO_2^- and NO_3^- , or NO_x . After considering the NH_4^+ uptake for cell synthesis, the NH_4^+ removal was still higher than NO_x production. This suggested that NO_x produced by nitrification could be partially removed by denitrification, i.e. simultaneous nitrification and denitrification (SND) occurred in aerobic zones. According to Equation 3.1 by Third *et al.* (2003) which defined the SND efficiency in aerobic conditions, an average of 40% nitrogen was removed through simultaneous denitrification in the aerobic zones over the sampling period.

$$\text{SND efficiency} = \left(1 - \frac{(\text{NO}_{2,e}^- - \text{NO}_{2,i}^-) + (\text{NO}_{3,e}^- - \text{NO}_{3,i}^-)}{\text{NH}_{4,i}^+ - \text{NH}_{4,e}^+} \right) \times 100\% \quad (3.1)$$

where $\text{NH}_{4,i}^+$ is the NH_4^+ -N concentration at the outlet of anaerobic zone, in the unit of mg NH_4^+ -N/L; $\text{NH}_{4,e}^+$ is the NH_4^+ -N concentration at the outlet of aerobic zone, mg NH_4^+ -N/L, and likewise the annotation for NO_2^- and NO_3^- .

Figure 3.3 Nutrient production and consumption rates in (a) anoxic and (b) aerobic zones of five basins. Negative values indicate net removal and positive values indicate net accumulation. Error bars represent standard error of data from four sampling dates. (NO_x: sum of NO_2^- and NO_3^-).

It was hypothesised that the low DO condition (0.7 ~ 1.1 mg/L) greatly contributed to the occurrence of SND in the studied WRP. This was supported by the knowledge that a DO concentration of 0.5 ~ 1.5 mg/L was favorable to SND (Ruscalleda Beylier *et al.*, 2011). In fact, Zhu *et al.* (2007) found a linear relationship between DO and the ratio of NO_x production and NH₄⁺ removal, i.e. lower DO led to higher SND efficiency in aerobic conditions. Besides DO, another important factor affecting SND efficiency was the floc size. One explanation for SND was that flocs of enough size would develop the multiple-layer structure due to diffusion of dissolved oxygen inside the floc, i.e. an anoxic inner core for denitrification, and an aerobic outer layer for nitrification (Gogina and Gulshin, 2016). The median diameter of sludge flocs in the studied WRP was 76 µm (Figure 3.4), which was in the lower range of that in conventional activated sludge plants (median 70 ~ 300 µm) (Zhang *et al.*, 1997). Pochana and Keller (1999) had reported that SND efficiency from 52% at median floc size of 80 µm reduced to only 20% at floc size of 40 µm, indicating that sufficient floc size was required for simultaneous nitrification and denitrification. In the study of Gómez-Silván *et al.* (2014), it was also reported that the transcription of *nosZ*, the key functional gene of denitrifiers, was at similar level in the aerobic conditions compared to that in anoxic conditions, which implied the capability of aerobic denitrification.

Median D	75.716			Mean V	80.174			
Modal D	63.792			Std Dev	0.366			
90%D(μ m)	50%D(μ m)	10%D(μ m)	0%D(μ m)	0%D(μ m)	0%D(μ m)	0%D(μ m)	0%D(μ m)	0%D(μ m)
250.413	75.716	28.666	0.000	0.000	0.000	0.000	0.000	0.000

Figure 3.4 Floc size distribution of mixed liquor in the studied full-scale WRP.

In the first basin, maximum average P release rate of 6.8 ± 1.2 mg P/g VSS·hr occurred in the anoxic zone which was significantly higher than that of other basins (less than 1.5 mg P/g VSS·hr). It was believed that the return sludge, which was the recycled sludge from final settling tank (FST) at 50% of the influent flow rate, greatly contributed to this high value. In the return sludge, the PHA storage in biomass and P concentration in the liquid phase was significantly higher than those in the last reactor zone (5O) at 6.5 Vs 3.5 mg PHA/g VSS and 8.9 vs 1.4 mg P/L respectively. The prolonged anaerobic phase from FST (HRT = 4.3 hours) to Basin 1 anoxic zone provided the condition for fermentation and/or hydrolysis of sludge from which carbon was generated. The occurrence of hydrolysis was partially confirmed by the NH_4^+ release in Basin 1A and the batch experiments in the later discussion. Due to the absence of NO_x , most of the carbon source could be consumed by PAO rather than denitrifiers so that maximum PHA uptake and P release occurred in Basin 1A. Since PHA was detected at very low concentration in all anoxic basins and return sludge, it was proposed that in the relatively long anaerobic/anoxic phase compared to other WRP, cell hydrolysis happened, and the carbon released from it was immediately consumed by PAO. On the other hand, a low carbon environment was always maintained, which was considered to encourage the growth of PAO than GAO as the latter could not survive well in low carbon environment (Tu, 2012). A recent study also reported that PAO could be favored against GAO by the side-stream sludge hydrolysis or a longer anaerobic period (Wang *et al.*, 2018), which agreed the previous work of Stockholm-Bjerregaard *et al.* (2015). Further study would be required to investigate on the competition between PAO and GAO with continuous low carbon supply under warm climate.

In addition, it was observed that the decrease of P-uptake rate (from 4.1 to 1.5 mg P/g VSS·hr in basin 1O to 5O) corresponded to the loss of cell internal carbon in PAO (PHA reduced from 10.4 to 3.8 mg PHA/g VSS). This was considered the result of carbon competition between PAO and denitrifiers and the decreased P availability from Basin 1 to 5. Although having decreasing trend along different basins, the net P-uptake in aerobic zones was larger than net P release in anoxic zones, leading to a net P removal. The

concurrent occurrence of nitrification, denitrification and phosphorus removal in the studied WRP indicated a typical SNDPR performance.

3.3.2 Confirmation of SNDPR potential in batch experiments

The SND potential of the sludge from the studied full-scale WRP was verified with a series of batch experiment in the lab (Figure 3.5). The experiment conditions were specified in Table 3.1. When sufficient DO was supplied (Experiment 1a), NH_4^+ was completely converted to NO_2^- and NO_3^- with NO_x production rate slightly higher than NH_4^+ consumption rate (4.0 mg N/g VSS·hr v.s. 3.3 mg N/g VSS·hr). The excess NO_x production may come from the NH_4^+ release due to cell lysis during the experiment period when no organic carbon was provided. At this DO level (2 ~ 4 mg O_2/L), oxygen was able to fully penetrate the flocs in the studied mixed liquor (median 76 μm or smaller due to washing steps) and therefore denitrification was inhibited to a great extent. At low DO condition (Experiment 1b and 1c), nitrification rate was reduced to $84 \pm 2\%$ and $79 \pm 10\%$ of that in high DO condition while the NO_x accumulation was reduced to $34 \pm 2\%$ and $17 \pm 6\%$ only. The loss of NO_x accumulation verified the occurrence of denitrification in aerobic condition. The reduction of nitrification rate in Experiment 1(c) compared to 1(b) may come from the organic carbon inhibition which promoted the growth of heterotrophs and competed with AOB for O_2 and NH_4^+ source.

Figure 3.5 Nutrient production and consumption rates from batch experiment to verify SND potential under different conditions. Experiment 1 at high DO (a), low DO (b) and low DO with carbon (c); Experiment 2 at high DO (a), low DO (b) and low DO with carbon (c); Experiment 3 anoxic denitrification with carbon. Error bars represent standard error of five replicates for Experiment 1& 2 and ten replicates for Experiment 3.

Modified from Equation 3.1 by Third *et al.* (2003), the SND efficiency for batch experiment was defined as Equation 3.2. SND efficiency of Experiment 1(b) and 1(c) was therefore calculated at $46 \pm 6\%$ and $72 \pm 3\%$ respectively. The efficiency of Experiment 1(b) was comparable with that of the full-scale WRP in which no external carbon source was available and denitrifiers were supposed to utilize the cell internal carbon storage and the release of organic matter from cell lysis. In Experiment 1(c), sodium acetate was added as external carbon to investigate the full aerobic denitrification potential. The reduction of nitrification rate in Experiment 1(c) compared to 1(b) indicated that organic carbon slightly inhibited the activity of AOB by promoting the growth of heterotrophs (average denitrification rate was enhanced by 36.3%) which competed with

AOB for O₂ and NH₄⁺ source and impaired AOB's affinity of NH₄⁺ as reported by other studies (Van Benthum *et al.*, 1997, Mousavi and Ibrahim, 2014).

$$\text{SND efficiency} = \left(1 - \frac{\text{NO}_x \text{ production rate}}{\text{NH}_4^+ \text{ reduction rate}}\right) \times 100\% \quad (3.2)$$

To further verify the aerobic denitrification potential, Experiment 2 was conducted at the same three conditions as Experiment 1 except that the nitrogen source was changed to 30mg N/L NO₂⁻ instead of NH₄⁺. In Experiment 2(a) which was high DO condition, the NO₃⁻ production was 79% higher than observed NO₂⁻ consumption rate (Figure 3.5). This agreed with our previous assumption that during experiment period NH₄⁺ had been continuously released into the mixed liquor due to cell lysis or endogenous metabolism and re-utilized by AOB. In Experiment 2(a), NO₃⁻ production rate rather than observed NO₂⁻ uptake rate would be considered as a more accurate representation of nitrataion rate. Under low DO condition, observed NO₂⁻ reduction rate in Experiment 2(b) and 2(c) was 13% and 116% higher than the NO₃⁻ production rate or the nitrataion rate in Experiment 2(a), and the observed NO₃⁻ production was 58% and 93% lower. The more significant loss of NO₂⁻ and NO₃⁻ with addition of organic carbon strongly indicated the presence of aerobic denitrification coexisting with nitrataion.

With continuous purging of N₂ gas, experiment 3 explored the maximum denitrification potential of the mixed liquor under completely anoxic condition. The average NO₂⁻ reduction rate at 10.9 ± 0.3 mg N/g VSS·hr was 2 times of that in aerobic condition which included both nitrataion and denitrification (Experiment 2c). The results indicated that anoxic denitrification was still more efficient. Interestingly, the denitrification potential of sludge from anoxic zones was only 7 ± 3% higher than that of sludge from anoxic zones (data not shown). The similar denitrification potential showed that denitrifiers did not experience significant adverse effect by periodic aerobic exposure.

In the last batch experiment, where raw influent wastewater was utilized, a typical EBPR paradigm was observed as in Figure 3.6, where phosphorus was released during

anaerobic phase and removed during aerobic phase with a net P removal. After a fast P release stage in the first 2min, the average P-release rate in the anaerobic phase was 5.7 mg P/g VSS·hr. The average P-uptake rate in the aerobic phase at 14.3 mg P/g VSS·hr was higher than the plant data in all basins.

Figure 3.6 Concentration profiles of N and P in Experiment 4.

It was noteworthy that in the aerobic phase, low DO (1 ~ 2 mg O₂/L) was applied, i.e. limited O₂ was available for different microbial groups. In the first 20 min of aeration, PO₄³⁻ underwent a steep drop while NH₄⁺ reduced slower compared to the next 70 min. Literature study showed that nitrifiers had higher oxygen half saturation coefficient (0.2 ~ 1.1 mg O₂/L) (Rongsayamanont *et al.*, 2010) than PAO (0.002 ~ 0.32 mg O₂/L) (Carvalho *et al.*, 2014), which suggested PAO had higher affinity to oxygen than nitrifiers. Therefore, it was likely that during the initial period of aerobic phase when nutrient substrate was sufficient for both nitrifiers and PAO, the PAO was able to outcompete the former, and reached the maximum nutrient removal rate first. When the PHA storage of PAO reduced to a relatively low level, the oxygen demand of PAO was reduced and nitrifiers reached their maximum reaction rate. The delay of nitrification as a result of competition of oxygen between PAO and nitrifiers was also reported in another SNDPR system in SBR mode (Meyer *et al.*, 2005). Through the 90min's aerobic phase, 19.0 mg/L NH₄⁺ was removed while only 13.8 mg/L NO_x was produced, which indicated the occurrence of simultaneous denitrification such that in Experiment 4 the SNDPR

potential of the sludge from the studied WRP was fully verified.

3.3.3 Microbial analysis and the relationship between relative abundance and activities

The relative abundance of key microbial groups responsible for N and P removal in the studied full-scale WRP was quantified using quantitative PCR (qPCR) with the primers described in Table 3.2. 16sRNA gene was used to quantify the relative abundance of AOB, NOB (including *Nitrobacter* and *Nitrospira*), and “*Candidatus Accumulibacter phosphatis*”, which was the major species of PAO (Oehmen *et al.*, 2007). Denitrifiers were targeted by the number of genes encoding the functional enzyme NorZ due to the fact that a variety of species were able to carry out denitrification. The results in Figure 3.7 specified the relative abundance of AOB and NOB. PAO and denitrifiers were quantified in different sampling periods and the abundance data were shown in Table 3.4. The PAO and denitrifiers had relatively stable abundance corresponding to a relative stable performance of EBPR and denitrification. GAO was rarely detected in the studied WRP (data not shown) which agreed with the plant performance.

Figure 3.7 Abundances of 16S rRNA genes of AOB and NOB. NBT: *Nitrobacter*; NSR: *Nitrospira*.

Table 3.4 Abundances of denitrifiers and *Accumulibacter* PAO in anoxic zones from three different sample collections. All values are reported as 10^{10} copies/g VSS. Each value represents the average of five basins

Sample	Denitrifiers (<i>nosZ</i>)	All <i>Accumulibacter</i> (16S)
Sample 1	22.0 ± 12.5	5.6 ± 1.3
Sample 2	9.5 ± 2.8	1.6 ± 0.6
Sample 3	18.1 ± 10.9	8.4 ± 2.0

Very different from the stable abundance of PAO and denitrifiers, the population of AOB and NOB changed in a highly dynamic manner during the sampling period (Figure 3.7) while the overall nitrogen removal rate remained stable. While AOB abundance changed only one order of magnitude ($2.6 \times 10^8 \sim 5.7 \times 10^9$ copies/g VSS), NOB abundance varied from 2.9×10^9 to 4.1×10^{11} copies/g VSS, i.e. NOB increased more than 142 times from

the lowest in Jun to the highest in Nov. Within NOB group, the relative abundance of *Nitrobacter* and *Nitrospira* also had dramatical change with *Nitrospira* finally gained the dominance in Nov. Previous studies showed that *Nitrospira* was a K strategist, i.e. it had higher affinity to NO_2^- (Nowka *et al.*, 2015) and DO (Huang *et al.*, 2010) than *Nitrobacter*. In other words, *Nitrospira* was able to outcompete *Nitrobacter* in low DO and low NO_2^- condition. In a recent study, one strain of *Nitrospira* species was even reported to accomplish complete ammonia oxidation (Comammox) (Daims *et al.*, 2015). Since the average DO in aerobic zones showed minor increase (~ 0.3 ppm) after the cleaning process in Nov 2013 (data not shown), it was possible that the decreased efficiency of oxygen diffusors after long time of operation led to the DO drop which contributed to the build-up of *Nitrospira*. Besides, seasonal variation may also introduce change on the substrate structure which could be affected by the amount of rainfall and the temperature. Besides the hypothetical contributing factors discussed above, another possible explanation for the dynamic AOB-NOB abundance could be the chaotic theory where oscillating microbial composition naturally occurred in the complex microbial food web (Graham *et al.*, 2007). The abundance of the species in the food web may fluctuated over several order of magnitude (Benincà *et al.*, 2008). The underlined mechanism for the dynamic nitrifier community requires further investigation.

It should also be noted that although NOB constituted most of the nitrifying community during the last three samplings, NO_2^- accumulation still occurred i.e. overall AOB activity was higher than NOB activity for all four samplings both in the plant data and in the batch experiments whose condition was the same as experiment 1(a) (Figure 3.8). The activity of NOB was equal or lower than those of AOB even though the abundance of the former was up to 588 times the abundance of the latter. This indicated that the advantage in abundance of a microbial group did not necessarily lead to its enhanced performance. At the same time, the microbial group being minority may still have a significant metabolic activity. In other words, unit cell activity can be very different among different microbial groups. In this sense, when engineers and researchers devoted

efforts to enhance AOB and inhibit NOB growth to achieve short-cut nitrification, focus should be put on the control of the in-situ activity rather than their relative abundance.

Figure 3.8 Plant data (a) and off-line batch experiment data (b) of nitrification rates during different sampling periods.. Error bar represent standard error of samples from 5 basins.

Along the sampling period, the amount of NO₂⁻ accumulation decreased with time in both plant data and batch data (Figure 3.8), i.e. NOB activity increased. This was consistent with the microbial result that the abundance of NOB increased with time (Figure 3.7). The increase in activity and abundance, however, was not in proportion. Specifically, although the total activities of NOB became higher, the activity per cell became lower, i.e. individual cell turned to be more inactive in NO₂⁻ oxidation. Literature study also reported that data for abundance (rDNA) and activity (rRNA) were not coupled for some populations (Hunt *et al.*, 2013). Therefore, microenvironment of individual cell should be considered to understand the development of unit cell activity. In addition, it cannot be ignored that there was possibility that NOB might find alternate substrate other than NO₂⁻ for cell propagation, e.g. comammox pathway. This would require further investigation. In future studies, microbial study may be considered as a routine method for data acquisition and individual cell performance should be taken into consideration for a better understanding of the overall operation evaluation.

Considering the significant difference between the abundance of AOB and that of NOB in this study, data from other studies were also analyzed and plotted in Figure 3.9(b). With short-cut nitrification or full nitrification as in this study ($q_{\text{AOB}}/q_{\text{NOB}} \geq 1$), the ratio of abundance of AOB and NOB ranged from 0.1 to 13 (Liu and Wang, 2013, Ge *et al.*, 2014, Regmi *et al.*, 2014) or even higher (Sun *et al.*, 2014) (higher data not shown in Figure 3.9). This implied that activity and abundance data of the same microbial groups were not comparable across different operation conditions. The unit cell activity largely depended on the specific microenvironment rather than their intrinsic maximum growth rate. In the study of Laanbroek and Gerards (1993), it was even reported that the unit cell activity of NOB changed more than 400 times from 0.13 to 58 $\text{fmol NO}_2^-/\text{cell/hr}$ while that of AOB was hardly affected at 1.28 $\text{fmol NH}_4^+/\text{cell/hr}$ when HRT and oxygen tension were manipulated between 1.5 ~ 8.3 days and 0 ~ 6.7 mg/L respectively which covered the common operation conditions of most wastewater treatment processes.

Figure 3.9 Comparison between relative activities and abundances of AOB and NOB. a): This study; b): Other references.

In summary, overall performance cannot be predicted with abundance data and vice versa since the unit cell activity of different species are not comparable under different environment and different total population. As pointed by Agrawal *et al.* (2017), reactor operation had significant impact on the overall community composition. However, by

keeping microbial analysis as part of routine measurement, the abundance data coupled with kinetic data in time series might better explain the situation which an individual cell was experiencing. Having a more comprehensive knowledge about each microbial group, more accurate control strategies might be developed so that better and more stable process performance could be anticipated.

3.4 Conclusions

A municipal full-scale WRP under tropical climate was investigated through multiple rounds of sampling during the second half year of 2014. Plant nutrient data analysis, microbial analysis and batch experiments were conducted to characterize the performance of the plant. SNDPR with short-cut nitrification and effective nitrogen and phosphorus removal were observed in the plant data analysis. Batch experiments further validated the potential of the sludge to carry SNDPR with partial nitrification.

Being the first full-scale plant achieving successful SNDPR with good effluent quality, the studied WRP could be benefited by the following operation conditions: low DO in the aerobic zones which favored aerobic denitrification, step-feeding process which increased the efficiency of carbon usage and high temperature which induced additional carbon release from cell hydrolysis were hypothesized to be the three major contributors of SNDPR in the studied WRP. From the microbial analysis, PAO was found to have comparable abundance as denitrifiers while GAO was hardly detected. This may be partly attributed to the additional anaerobic phase in FST where small amount of sCOD was released and favored PAO growth during its competition with GAO.

The abundance of nitrifiers had been through a highly dynamic change during the sampling period while the overall nitrogen removal rate was relatively stable and satisfying. Being inter-related, activity or abundance cannot be used to predict the other. However, keeping monitoring the abundance of major microbial group in a routine

manner would enhance the understanding of the development of overall plant performance which may aid the accurate control of plant operation strategy.

In addition, the dynamic AOB-NOB abundance during the sampling period may due to some minor operational change, seasonal variation of influent or the chaotic behavior of the nitrifiers. The latter had been demonstrated in previous study (Graham *et al.*, 2007) in three parallel mixed liquor chemostats where various influences from species other than AOB and NOB could not be ignored. Considering the complexity of mixed liquor environment and diverse influence from the change of various operation conditions, the interactions between pure AOB and NOB in a controlled condition should be studied and detailed investigation was presented in Chapter 4.

CHAPTER 4 SYNERGY AND DYNAMICS OF CO-CULTURED AOB AND NOB AT DIFFERENT INITIAL AOB/NOB RATIOS

4.1 Introduction

With increasing demand on energy self-sufficiency of used water reclamation, attention has been gradually turning to deammonification which is a combination of short-cut nitrification and anammox, in which short-cut nitrification is a prerequisite. It has been thought that the interaction between AOB and NOB indeed not only determines SNDPR as presented in Chapter 3, but also play a decisive role in anammox process. In order to establish stable short-cut nitrification, understanding the interaction between AOB and NOB is of essential.

With advanced molecular biotechnology, abundances of AOB and NOB have been quantified in many studies for developing the operational strategy towards NOB suppression against AOB in nitrification process. Theoretically, the biomass yield of AOB is about two times of that of NOB, i.e. the theoretical AOB/NOB abundance ratio should be around 2 for complete nitrification (Winkler *et al.*, 2012). In real BNR systems, however, this ratio ranged from 0.068 to over 10,000 for various cases with different operation conditions and microbial compositions (Kindaichi *et al.*, 2006, Li *et al.*, 2013, Regmi *et al.*, 2014, Sun *et al.*, 2014). Such wide range of AOB/NOB ratio brings attention to the possibility of chaos in the microbial dynamics of AOB and NOB. Chaos in ecological models was defined as bounded aperiodic fluctuations with sensitive dependence on initial conditions (Turchin, 2003). Previous study had shown the fluctuated population of a multispecies system in a period of several weeks due to the continuous interspecies interactions (Becks *et al.*, 2005). However, a fundamental question of whether chaos exists in the population dynamics of pure AOB and NOB cultures remains unknown. The understanding of population dynamics of AOB and NOB

is crucial to maintain a stable SND, short-cut processes or nitrification-anammox process. Such interspecies interactions have not been investigated nor reported extensively so far.

In this study, pure culture of AOB and NOB were cocultured in chemostat reactors to exclude the potential interaction from other microbial groups. The inoculum contained different initial combinations so that the effects of initial inoculum on population dynamics of AOB and NOB would be verified on top of the possible chaos status. These reactors were operated up to 30 days with typical operation parameters in BNR systems to better benchmark the real situation. Throughout the period, the population and floc structure of AOB and NOB were monitored to reveal the possible interaction pattern of two groups in terms of population dynamics and physical interaction.

4.2 Material and methods

4.2.1 Bacterial strains

AOB strain was isolated from a lab-scale short-cut nitrification reactor using 1% agar plate in a moist chamber with low concentration of NH_3 evaporated from NH_4Cl solution. The nutrient composition of the agar plates was modified from ATCC medium 2265 for *Nitrosomonas europaea*, i.e. 100 mg N/L $(\text{NH}_4)_2\text{SO}_4$, 0.41 g/L KH_2PO_4 , 123 mg/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 15 mg/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 2 mg/L FeSO_4 , 2 mg/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 5.44 g/L KH_2PO_4 , 0.48 g/L NaH_2PO_4 , 0.4 g/L Na_2CO_3 and pH was adjusted to 7.8. The isolated strain was analyzed by Sanger sequencing, and the results confirmed the isolated culture belonged to *Nitrosomonas europaea*. Detailed sequencing data was presented in Appendix A. The isolated AOB was maintained in liquid medium with the same composition as the agar plate except that 25 mM of $(\text{NH}_4)_2\text{SO}_4$ instead of 3.57 mM was used. The commercially available NOB strain of *Nitrobacter winogradskyi* (DSM 10237) was purchased from DSMZ. The medium developed by Sayavedra-Soto *et al.* (2015) was used for NOB cultivation, which contained 840 mg N/L NaNO_2 , 123 mg/L MgSO_4 ,

15 mg/L CaCl_2 , 0.71 mg/L K_2HPO_4 , 0.4 g/L Na_2CO_3 , 2 mg/L FeSO_4 , 2 mg/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, and trace metals: 0.14 mg/L FeCl_3 , 0.15 mg/L Na_2MoO_4 , 0.31 mg/L MnCl_2 , 0.14 mg/L CoCl_2 and 0.03 mg/L $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (pH 7.5). The cell concentration was estimated with optical density at 420 nm using an Infinite Pro 200 microplate reader (Tecan, Männedorf, Switzerland). AOB and NOB were cultivated to their exponential phases in a shaking incubator at 30°C before use. The exponential phase was indicated by a linear increase in recorded OD on a log scale plot.

4.2.2 Chemostat reactors

Nine one-liter glass bottles placed in a 30°C water bath were operated as chemostats (Fig. 4.1). The working volume of each reactor was set to be 800 ml. Aeration with filtered air was provided for both mixing and oxygen supply. Fresh AOB and NOB cultures in exponential phase were first filtered through sterilized mixed cellulose esters membranes with the diameter of 47 mm and the pore size of 0.45 μm (Millipore, Darmstadt, Germany). The harvested cells were resuspended in blank medium with no NH_4^+ or NO_2^- . After quantifying the culture density, a total number of 4×10^8 cells with AOB/NOB ratio being 10:1, 1:1, and 1:10 were inoculated into the nine reactors to start the experiment. Each AOB/NOB ratio were inoculated in three replicate reactors and later referred to as Group A, B and C respectively. The pre-autoclaved medium in the reactor bottles contained 70 mg N/L $(\text{NH}_4)_2\text{SO}_4$, 123 mg/L MgSO_4 , 15 mg/L CaCl_2 , 2 mg/L FeSO_4 , 2 mg/L CuSO_4 , 5.44 g/L KH_2PO_4 , 0.48 g/L NaH_2PO_4 , and 0.4 g/L Na_2CO_3 at pH = 7.8. In the first three days, the influent was zero and the cultures grew in batch mode for faster start-up. On Day 3, influent flow containing 100 mg N/L NH_4^+ and the rest the same as the initial medium was started. The influent flow was around 147 ml/d, having an SRT of 5 ~ 5.5 days. The influent flow was driven by a peristaltic pump that had maximum 12 channels running at the same speed. The effluent flow was blown out with the excess air due to the pressure in the headspace. pH was controlled by the buffer in

the medium so that the influent pH of 7.8 dropped to around 7.5 in the effluent. All parts of reactor, tubes, connectors and the medium were autoclaved for sterilization before use. Growth and kinetics of the cultures in the nine reactors were monitored routinely by taking culture samples from the reactor bottles. Immediately after sampling, the optical density was measured using Infinite Pro 200 microplate reader (Tecan, Männedorf, Switzerland) to track the growth condition. Each sample (1.8 ml) was stored in 3.7% formaldehyde in 2 ml microtube for 16 hours at 4°C. Then 0.5 ml of the fixed sample was filter through a sterilized white polycarbonate membrane (diameter 25 mm, pore size 0.2 μm ; type GTTP 2500; Millipore, Darmstadt, Germany) and air dried. The membrane samples were then stored at -20°C before subsequent staining process.

Figure 4.1 Experimental set-up of chemostat reactors.

4.2.3 Chemical analysis

The culture sample (3 ml) was filtered through a 0.45 μm nylon syringe filter to obtain the liquid sample which was stored in 4°C before analysis of nutrient concentrations within 8 hours. Concentration of nitrogenous compounds ($\text{NH}_4^+\text{-N}$, $\text{NO}_2^-\text{-N}$ and $\text{NO}_3^-\text{-N}$) was determined by colorimetric method using UV-1800 spectrophotometer (Shimadzu Co., Ltd., Kyoto, Japan). Hach Nessler reagent set was used to measure the $\text{NH}_4^+\text{-N}$ concentration based on USEPA Nessler method. $\text{NO}_2^-\text{-N}$ and $\text{NO}_3^-\text{-N}$ concentration were determined according to standard methods.

4.2.4 FISH and image analysis

The relative quantity of AOB and NOB was determined by fluorescence in situ hybridization (FISH) on membrane samples (Glöckner *et al.*, 1996) and the subsequent image analysis. Briefly, the membrane samples were dehydrated with 50%, 70% and 95% ethanol in series then air dried. Mixture of probe and hybridization buffer, which was prepared according to the probe type, was applied on the membrane sample on a piece of glass slide. The whole slide with membrane sample was then fitted in a chamber moisturized with hybridization buffer and incubated at 46°C for 2 hours. The sample was then soaked in the washing buffer at 48°C for 20 min and rinsed with cold deionized water. After a final step of air drying, the samples were visualized with Zeiss LSM 880 confocal laser scanning microscope (Carl Zeiss, Jena, Germany) and processed with Imaris (Bitplane, Zurich, Switzerland). At least 20 images for each sample were analyzed to obtain a representative quantitative result. The probes used to stains AOB and NOB were NEU 5'-CCCCTCTGCTGCACTCTA-3' (Wagner *et al.*, 1995) and NIT3 5'-CCTGTGCTCCATGCTCCG-3' (Wagner *et al.*, 1996) respectively. Probe NEU targets most halophilic and halotolerant *Nitrosomonas spp.* and probe NIT3 targets *Nitrobacter spp.* It was tested that these two probes were able to stain the cultivated AOB and NOB species with good resolution.

4.2.5 Field emission scanning electron microscopy (FESEM)

The culture for FESEM (or SEM in later context) was collected on a sterilized white polycarbonate membrane (diameter 25 mm, pore size 0.2 μm ; type GTTP 2500; Millipore, Eschborn, Germany) and fixed with 2% glutaraldehyde for 2 hours. The membrane samples were then washed three times with 0.1 M sodium cacodylate buffer for 20 min/each time. After a graded series of ethanol dehydration, the samples were dried in a Labconco freeze dryer overnight. Sputter coating with Au-Pd was done before observation with FESEM (Jeol JSM-7600F, Japan).

4.3 Results and discussion

4.3.1 Concentration profiles of nitrogenous compounds

AOB and NOB with a total cell number of about 4×10^8 cells were inoculated into sterilized medium of 70 mg NH_4^+ -N/L at three different AOB/NOB ratios. After the first day of batch culture, NH_4^+ removal as an indication of AOB activity was observed for Group A at the AOB/NOB ratio of 10:1 (Figure 4.2a), i.e. a relatively fast start-up could be achieved under this condition. For Group B and C at the respective AOB/NOB ratios of 1:1 and 1:10, cell lysis likely occurred in the starting period with insufficient nitrite produced for NOB and subsequently the accumulation of NH_4^+ . Group C with the lowest AOB/NOB ratio, i.e. the NOB number, even displayed a net NH_4^+ increase of 1.46 ± 0.64 mg N/L on Day 1. On Day 2, NH_4^+ was removed significantly compared to the previous day, with the production of NO_3^- which indicated NOB activity. The observed lag phase for NOB was probably due to the lack of substrate during the starting period. On Day 3 the cultures were switched to continuous mode when the NH_4^+ concentration was reduced to below 3 mg N/L in Groups A and B. It was found that the effluent NH_4^+ concentration was quickly stabilized at 2.5 ~ 3.5 mg N/L for all the batches from Day 4

onwards. On the other hands, it was also observed that the NO_2^- concentrations in Group A and B batches started to decrease rapidly on Day 3, indicating a significant NOB growth and activity. The NO_2^- concentration in Group C, however, continued to accumulate to its peak on Day 4 (Figure 4.2b), showing the NOB activity was significantly retarded compared to Groups A and B. Interestingly, the highest NOB activity (Figure 4.2c) and the fastest depletion of accumulated NO_2^- (Figure 4.2b) were firstly achieved in Group B. This indicated that the initial inoculum composition would play a vital role in the subsequent operation and performance of a biological process. After 6 days, all the chemostat reactors achieved the stable full nitrification with NO_2^- undetectable in the effluent.

Figure 4.2 Concentration profiles of nitrogen (a: NH_4^+ ; b: NO_2^- ; c: NO_3^-) of three-group cultures in the start-up period. Vertical red line marked the boundary between batch and chemostat. Error bar represented the standard error of three replicates.

4.3.2 AOB and NOB abundances

The individual cell density of AOB and NOB were estimated according to the FISH image analysis. It is reasonable to consider that the total optical density of mixed culture was the sum of partial optical density contributed by AOB and NOB cells in this study. The partial optical density of AOB and NOB were estimated with the empirical formula derived from pure culture:

$$\text{Cell density for AOB (cell/ml)} = 344400 \times \text{OD}_{420} \quad (4.1)$$

$$\text{Cell density for NOB (cell/ml)} = 1822200 \times \text{OD}_{420} \quad (4.2)$$

The cell density profiles for the three groups with different initial AOB/NOB ratios were shown in Figure 4.3. The abundance of AOB reached 6×10^6 cell/ml on Day 3 while NOB remained at below 6×10^5 cell/ml in all three groups, which was more than 10 times lower than AOB. These observations indeed were consistent with the nutrient availability. Although the initial AOB and NOB were inoculated at three different ratios (i.e. 10:1, 1:1 and 1:10), their relative abundance was found to be converged to 2.0:1 after 7 days' cultivation for all three groups. On Day 22, the AOB/NOB ratios at the steady-state lied in between 2.6 to 3.1 with the cell density of AOB at $(1.09 \pm 0.02) \times 10^7$ cell/ml and NOB at $(3.89 \pm 0.19) \times 10^6$ cell/ml. Such comparable ultimate AOB/NOB ratios once again seemed to suggest that the initial inoculum structure might not be a decisive factor for microbial composition at the steady state, but it would be significantly related to the length of lag phase.

Figure 4.3 Respective AOB and NOB cell density profiles in Group A, B and C. Vertical red line marked the boundary between batch and chemostat. ●: AOB; ▲: NOB.

In this study, at the steady state, the AOB/NOB ratio in all three groups reached 2.82 ± 0.17 . This suggested that every 2 ~ 3 AOB cells were able to support the growth of one NOB cell as nitrite produced by AOB is the food for NOB. This, to a great extent, may explain why it is difficult to control NOB against AOB in partial nitrification which is a prerequisite for anammox. This study also revealed that the strategies aiming to reduce NOB abundance may not be effective for partial nitrification. Laanbroek and Gerards (1993) reported that the growth yield of *N. europaea* was relatively stable under different conditions, however that of NOB highly depended on the culture environment, e.g. oxygen supply, HRT, etc. For example, the growth yield of *N. winogradskyi* varied in a large range from 200% to 3% of that of *N. europaea*. These suggested that NOB was more flexible than AOB in surviving in different environments. As such, it would be extremely difficult to completely get rid of NOB in any biological processes for wastewater reclamation, including anammox.

4.3.3 Distribution of AOB and NOB in cell cluster and flocs

The filtered cell samples from the 9 chemostat reactors were stained with FISH probes to identify AOB and NOB cells as illustrated in Figure 4.4 to Figure 4.6 for the batch cultures of Group A, B and C respectively. For all three groups, AOB grew significantly from Day 2 regardless of the initial AOB/ NOB ratio. With the increase in abundance, AOB cells began to form small clusters of 2 ~ 12 cells (based on the analysis of at least 20 images for each sample) on Day 1 in Figure 4.4, Day 2 in Figure 4.5 and Day 3 in Figure 4.6 for the three groups respectively. Diplococci, tetrads and staphylococci (grape-like) structures were commonly observed, while streptococci (line structure) was rare probably due to the aeration-associated shear force.

Figure 4.4 FISH images of Group A batch samples on Day 0, 1, 2, 3, 4, 7, 14, 22. AOB was stained with probe NEU and shown in red. NOB was stained with probe NIT3 and shown in green. Probe EUB which stained all bacteria was shown in blue. One image was shown for each sample as illustration. At least 20 images were analyzed for data acquisition.

Figure 4.5 FISH images of Group B batch sample on Day 0, 1, 2, 3, 4, 7, 14, 22. AOB was stained with probe NEU and shown in red. NOB was stained with probe NIT3 and shown in green. Probe EUB which stained all bacteria was shown in blue. One image was shown for each sample as illustration. At least 20 images were analyzed for data acquisition.

Figure 4.6 FISH images of Group B batch sample on Day 0, 1, 2, 3, 4, 7, 14, 22. AOB was stained with probe NEU and shown in red. NOB was stained with probe NIT3 and shown in green. Probe EUB which stained all bacteria was shown in blue. One image was shown for each sample as illustration. At least 20 images were analyzed for data acquisition.

With the build-up of NOB abundance, NOB cells tended to attach to AOB cell clusters from the edge, and sometimes penetrated into the inner area (Figure 4.4 ~ 4.6). It should be noted that pure NOB cell clusters were seldom observed. In the AOB-NOB dual-species clusters formed in the period of day 3 to day 14, such a single cluster only contained less than 50 cells in total, while it appeared that the compositions of AOB and NOB in such clusters were significantly different, with the NOB content of 1 ~ 2 cells per cluster to over 50% of total cell number.

After 14-days cultivation, the larger flocs of more than 100 cells were observed with a clear layered structure (Figure 4.7). In these flocs, AOB remained at the outer layer, while NOB cells formed a compact inner core. The NOB cells in the center core were so cohesive that the confocal microscope was not able to determine their cell edges clearly. These loose and compact floc structure of AOB and NOB were consistent with their pure culture flocs observed in Figure 4.8. The flocs of pure AOB culture were well arranged in a grape-like structure (Figure 4.8a). The individual AOB cells of 0.8 ~ 1.5 μm in size appeared to be round or oval in shape (Figure 4.8 c and e). The NOB flocs, however, were much more cohesive with more cell debris and/or extracellular polymeric substances (EPS) (Figure 4.8 b and d) than AOB (Figure 4.8 a and c). The individual AOB and NOB cells showed a pleomorphic shape instead of the regular coccoid or rod shape (Figure 4.8 d and f).

Figure 4.7 Layered floc structure during late steady state. AOB was stained with probe NEU and shown in red. The three images were representations from Group A, B and C from left to right respectively. NOB was stained with probe NIT3 and shown in green.

Probe EUB which stained all bacteria was shown in blue.

Figure 4.8 SEM image of AOB (a, c and e) and NOB (b, d and f) on membrane at ×5k (a and b), ×20k (c and d) and ×50k (e and f) magnification respectively.

In the AOB and NOB co-culture, AOB abundance increased quickly with the formation of cell clusters that could protect themselves from stressful environment, e.g. high NO_2^- and O_2 concentrations etc. (Vejmelkova *et al.*, 2012). The gradually increased NOB population tended to stay close with their food provider (i.e. AOB cells), facilitating the formation of mixed AOB-NOB cell clusters. In fact, previous study had demonstrated that in AOB-NOB co-culture, their gene expression for cell motility and defense mechanism decreased compared with their respective pure cultures (Pérez *et al.*, 2015), indicating that the formation of the mixed culture clusters was a more preferred way of survival compared with planktonic growth mode.

Interestingly, for the large dual-culture clusters, the special layered pattern of loose AOB outer layer surrounding compact NOB inner core was commonly observed which was presumed to favor the survival and development of both species. The observed AOB-NOB structure in a certain arrangement likely resulted from some active interaction. In this sense, chemotaxis was probably the driven force for such structural development. So far, little has been known about the role of chemotaxis in the development of nitrifying community.

4.4 Conclusions

AOB which was represented by *Nitrosomonas europaea* and NOB represented by *Nitrobacter winogradskyi* were inoculated into nine parallel chemostat reactors at three different initial AOB/NOB ratios. The group of AOB/NOB ratio = 1:1 firstly reached the steady state when NO_2^- accumulation disappeared and stable full nitrification was maintained, indicating that balanced initial AOB-NOB abundance yielded fastest start-up. The final AOB/NOB ratio of the three groups converged to similar values (2.82 ± 0.17), showing that unlike the chaotic behavior in mixed liquor reactors in the study of Graham *et al.* (2007), initial inoculum composition did not affect the steady state condition of pure AOB-NOB co-culture. This implied that operation conditions and

substrate availability were the key parameters in shaping the microbial composition of the two groups studied. The results also revealed the difficulty in biological processes to achieve stable partial nitrification by control of NOB abundance only.

In addition, AOB and NOB cells were found to form joint clusters in which NOB tended to colonize onto small AOB clusters at the very beginning. In the later stage, a layered structure with AOB at outer layer and NOB being the compact inner core was formed. Likely such spatial arrangement of AOB and NOB in the clusters would be driven by chemotaxis of nitrifiers.

CHAPTER 5 EFFECT OF CHEMOTAXIS ON FLOC STRUCTURE AND METABOLIC ACTIVITY OF NITRIFYING BACTERIA

5.1 Introduction

Biological nitrogen removal has been widely used in wastewater treatment process. Compared to conventional nitrification-denitrification in which NH_4^+ is fully oxidized to NO_3^- then reduced to N_2 in aerobic and anoxic condition respectively, partial nitrification-denitrification and partial nitrification-anammox have many advantages, such as less aeration, less alkalinity consumed, and less organic C source dosage are required. Obviously, in these novel biological nitrogen removal processes, partial nitrification is essential. As revealed in Chapter 4, the biggest challenge is how to create an environment where NOB could be effectively suppressed over AOB. For this purpose, many operation strategies have been proposed, including shorter SRT (Hellenga *et al.*, 1998), low DO (Blackburne *et al.*, 2008), inhibition by FA and FNA (Sun *et al.*, 2010), frequent transition of anoxic and aerobic conditions (Ge *et al.*, 2014) etc. However, there is a clear lack of fundamental understanding of interaction between AOB and NOB at cellular level.

Therefore, this chapter aimed to provide new insights into the chemotaxis responses of AOB and NOB, which would be helpful for better understand the interaction between AOB and NOB in terms of cell allocation and the subsequent formation of microbial clusters or flocs. The information generated may offer an alternative option for achieving stable partial nitrification which is essential for mainstream anammox.

5.2 Material and methods

5.2.1 Medium and chemicals

For liquid growth medium, recipe from American Type Culture Collection (ATCC) 2265 (ATCC, Manassas, VA) was used to prepare AOB growth medium which contained 700 mg N/L $(\text{NH}_4)_2\text{SO}_4$, 0.41 g/L KH_2PO_4 , 123 mg/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 15 mg/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 2 mg/L FeSO_4 , 2 mg/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 5.44 g/L KH_2PO_4 , 0.48 g/L NaH_2PO_4 , 0.4 g/L Na_2CO_3 . Pure NOB culture was grown in medium with 840 mg N/L NaNO_2 , 123 mg/L MgSO_4 , 15 mg/L CaCl_2 , 0.71 mg/L K_2HPO_4 , 0.4 g/L Na_2CO_3 , 2 mg/L FeSO_4 , 2 mg/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, and trace metals: 0.14 mg/L FeCl_3 , 0.15 mg/L Na_2MoO_4 , 0.31 mg/L MnCl_2 , 0.14 mg/L CoCl_2 and 0.03 mg/L $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (pH 7.5).

5.2.2 Bacterial strains and growth conditions

The AOB strain used was the same as Chapter 4 which was isolated from a lab reactor and the strain belonged to *Nitrosomonas europaea* by sequencing analysis. The NOB strain used in the experiment was commercially purchased *Nitrobacter winogradskyi* (DSM 10237).

AOB or NOB culture was inoculated 1:10 into fresh medium and incubated to exponential phase and $\text{OD} > 0.3$ (4 ~ 6 days) in 30°C in a shaking incubator before the next inoculum.

Agar plates contains 1% agar and 100 mg $\text{NH}_4^+\text{N/L}$ or 500 mg $\text{NO}_2^-\text{N/L}$ with the rest component following the growth medium recipe of AOB and NOB respectively were prepared before capillary assay for cell counting.

5.2.3 Batch assay

The batch assay simulated the experiment conditions in the capillary tubes to indirectly monitor the maximum chemical change and biomass growth during capillary assay. Specifically, the substrate was provided at 0, 50, 100, 200, 500, 1000 mg N/L as NH_4^+ or NO_2^- in 100 ml medium during AOB and NOB batch assays respectively with pH buffered at 7.8 for all tests. The biomass concentration was adjusted to the same density as the pond culture in capillary assays. The cultures were incubated at 30°C and sampled every 15 min for optical density (OD) and chemical measurement.

Chemical analysis

NO_2^- -N was determined by colorimetric method using Infinite Pro 200 microplate reader (Tecan, Mannedorf, Switzerland) according to the modified standard methods. NO_3^- -N was measured with HACH reaction kit, Nitrate TNTplus, LR (0.2 ~ 13.5 mg N/L) and HR (5 ~ 35 mg N/L).

5.2.4 Chemotaxis capillary assay

Blank medium which contained no nitrogen source, i.e. 13.2 g/L K_2HPO_4 , 0.4 g/L Na_2CO_3 , 123 mg/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 15 mg/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and the same trace metals as in NOB medium was used in the subsequent capillary essays. The pH of blank medium was adjusted to 7.8 unless stated otherwise. AOB or NOB culture in exponential phase was filtered through a 0.2 μm polycarbonate membrane (Millipore, Ireland) to remove any potential attractants, repellents or inhibitory metabolite products, then resuspended in blank medium and adjusted to target cell density. NaNO_2 or $(\text{NH}_4)_2\text{SO}_4$ was added in blank medium with no bacteria at 0, 50, 100, 200, 500 and 1000 mg N/L to be used as either attractant or repellent solutions later.

a. Chemical in capillary method

To investigate the potential chemotaxis, modification of J. Adler's method (1973) was adopted, i.e. capillary tubes with radius of 0.2 mm and length of > 2 cm having one end sealed in flame were heated and plunged into prepared attractant/repellent solution of different concentrations in a sterile 96-well plate at the open end. As the capillary tubes cooled down, attractant/repellent solution was drawn in about 1 cm or more. The tubes were then transferred to a second 96-well plate containing AOB or NOB culture suspension (the "pond" culture) at the target cell density (AOB: 1.0×10^7 cell/ml, NOB: 5.4×10^7 cell/ml) and incubate at 30°C. Each attractant/repellent concentration had five replicates. After two hours, the capillary tubes were removed from the pond culture and rinsed with a stream of deionized water for at least 5 seconds. The sealed end was then broken off and the liquid inside was squirted into 100 μ l blank medium pre-dripped on the agar plates just before plating. The 100 μ l solution with capillary content was then spread onto the whole plate with an L-shape spreader. The AOB agar plates were incubated with an open bottle of 10 g N/L NH_4Cl as NH_3 source while NOB agar plates were incubated with an open bottle of deionized water, both in the closed containers. AOB plates were incubated for 10 days and NOB plates were incubated for 14 days for easy recognition of the bacterial colonies before counting.

b. Chemical in pond method

Chemical in capillary method may not distinguish the negative chemotaxis clearly and the chemical in pond method (Tso and Adler, 1974) is a more suitable practice. Unlike the previous chemical in capillary method, possible chemotaxis repellent was added to the culture suspension at the above specified concentrations. During the two-hour incubation, motile cells may escape into the capillary tubes containing blank medium

free of the repellent. The liquid in the capillary was then collected and spreading on the agar plates in the same way as capillary method to be incubated for any growth of bacterial colonies.

5.2.5 Motility capillary assay

In motility assay, the same concentration of attractant/repellent was applied in both the pond culture and the capillary solution. Cells in the pond culture would swim into the capillary tube by random motility. Different concentration levels were examined to explore the effect of the testing chemicals on the random motility.

5.3 Results and discussion

5.3.1 Chemotaxis response of AOB

a. AOB Batch assay

Before the capillary assays, the batch assay under the same condition as in the capillary assay was conducted to determine the nutrient change over 2 hours. Figure 5.1 showed the cell density profile of AOB according to which the cell density was found to vary less than 5% and remained at 1.0×10^7 cell/ml in all the six AOB batch experiments.

Figure 5.1 Cell density of AOB in batch assays.

The NO_2^- production was measured in this test to quantify the metabolic activity of AOB. The maximum NO_2^- production occurred at an initial NH_4^+ concentration of 500 mg N/L (Figure 5.2), while it was comparable in the batch cultures with 200 mg NH_4^+ -N/L and 500 mg NH_4^+ -N/L. At the initial NH_4^+ concentration of 1000 mg N/L, the ammonium uptake rate was only 5% lower than its maximum value. As only less than 9% of ammonium was removed, the concentration gradient between the pond culture and the solution in capillary tubes would be considered unaffected.

Figure 5.2 AOB metabolic activity at different substrate concentration after 2hr.

b. Chemotaxis response of AOB to NH_4^+

In the AOB chemotaxis assay for NH_4^+ , AOB cells swimming into the capillary tubes were collected and spread on the agar plates to form visible colonies. The average colony numbers from 5 replicates were reported in Figure 5.3. Cells from the pond culture with no nitrogenous migrated into the capillary tube by two driving forces, i.e. random motility and chemotaxis attraction. Since NH_4^+ is the substrate for AOB, diffusion of NH_4^+ from the capillary tube into the pond culture may slightly increase the activity of AOB cells near the tube opening and therefore increase the random motility of nearby cells. To exclude the effect of random motility, the motility assay using the same batch of culture was conducted with NH_4^+ in both capillary tube and pond culture at the same concentration. The AOB chemotaxis and motility responses to different NH_4^+ concentrations were shown in Figure 5.3.

Figure 5.3 Concentration-response of AOB to ammonium in chemotaxis assays. Error bar represents the standard error of five measurements.

The number of cells entering the capillary tube with no nutrient in the pond or tube medium was taken as the baseline, and the results were then normalized to the baseline value. An obvious increasing trend was observed in the chemotaxis response curve, while the motility curve showed an increasing trend from 0 to 500 mg N/L and then decreased afterwards. At 1000 mg N/L, the number of cells in the capillary tube in the chemotaxis assay was over 3 times more than that in the motility assay, indicating a real attractive response of AOB to NH_4^+ . The motility of AOB at 1000 mg N/L, however, may be inhibited by the high concentration of FA and hence shown a decreasing trend although the value was still higher than the baseline. It was noted that in the chemotaxis assay, no substrate was available in the pond culture so that the random motility of the cells should be much lower than that in the motility assay. These results demonstrated that AOB had a positive chemotaxis response to NH_4^+ up to 1000 mg NH_4^+ -N/L although at which the substrate inhibition to metabolic activity and motility might occur. The observed independent chemotaxis response apart from the metabolic activity suggested that AOB cells could move through chemotaxis into the environment with high NH_4^+ concentration, where their metabolic activity was probably affected.

c. Chemotaxis response of AOB to NO_2^-

In the AOB chemotaxis assay for NO_2^- , no NO_2^- in the capillary was taken as the baseline condition and the results obtained in the presence of NO_2^- were then normalized to the baseline value. The number of cells swimming into the capillary tube displayed a decreasing trend as the concentration of NO_2^- increased (Figure 5.4). Such a decrease of cell number could be attributed to the chemotaxis repulsive effect of NO_2^- , to whose derivative HNO_3 was reported to inhibit AOB's metabolic activity (Claros *et al.*, 2013). The few cells swimming into the capillary tube at high NO_2^- concentration could be considered as non-chemotaxis mutant.

Figure 5.4 NO_2^- chemotaxis response curve of AOB. Error bar represents the standard error of five measurements.

d. Chemotaxis response of AOB to pH and FA

To distinguish the chemotaxis effect between NH_4^+ and free NH_3 on AOB, different pH conditions of the blank medium with no nitrogen presence were examined in the capillary assays, i.e. pH = 6, 6.5, 7, 7.5 and 8 (Figure 5.5).

Figure 5.5 Chemotaxis response of AOB to different pH values. Blank medium of pH = 6, 6.5, 7, 7.5 and 8 was in the capillary tube.

Since the pH of pond culture remained at 7.8, cell number in the capillary tube at pH = 8 or $\text{H}^+ = 10^{-8}$ M was used as the baseline value (Figure 5.5). It was clearly shown that the number of AOB cells swimming into the capillary tube decreased by 37% at pH = 7.5 and 14% at pH = 7. The cell number in the capillary tubes at pH 6.5 and 6 remained at similarly low levels as that at pH 7. These suggested that pH below 7 would keep AOB cells apart although previous study demonstrated that high pH had stronger inhibition on the metabolic activity of AOB than low pH (Claros *et al.*, 2013). The opposite influence between chemotaxis effect and metabolic activity confirmed that the two functions were regulated through different mechanisms. At higher H^+ concentration, the number of cells entering capillary tube of pH = 6 and 6.5 further decreased compared with pH = 7. It is reasonable to consider that some AOB might be non-chemotactic mutant and would not response to H^+ in this case. Since AOB produce H^+ in the oxidation of ammonium to

nitrite, AOB would tend to swim away from their metabolic product and seek for available food source in natural or engineering systems. Knowing that free NH_3 (FA) rather than NH_4^+ was the true substrate of AOB (Claros *et al.*, 2010), three NH_4^+ concentrations at five different pH values were applied to identify the true chemotaxis attractant in a set of capillary assays (Figure 5.6).

Figure 5.6 Normalized AOB cell number in capillary plotted against FA (a), NH_4^+ (b) and pH (c). Three NH_4^+ concentration (0, 50 and 500 mg N/L) was applied at five pH values (6, 6.5, 7, 7.5 and 8). Cell number in capillary at $\text{NH}_4^+ = 0$ mg/L and pH = 8 was taken as baseline value. Error bar represents the standard error of five measurements.

●: 0 mg NH_4^+ -N/L; ▲: 50 mg NH_4^+ -N/L; ◆: 500 NH_4^+ -N/L.

Compared with FA (Figure 5.6a), NH_4^+ had a better correlation with the number of cells entering capillary tubes (Figure 5.6b). Unlike AOB's metabolic activity, NH_3 was not recognized as the true functional group by the chemotactic receptor on the cell membrane of AOB. Instead, NH_4^+ played the major role of chemotaxis attraction. Compared with the chemotaxis attraction of NH_4^+ , repulsive effect of H^+ was insignificant as shown in Figure 5.6c. The upper 5 and middle 5 points represented the results obtained at the ammonium concentrations of 500 mg N/L and 50 mg N/L NH_4^+ respectively, while the bottom 5 data points for the assays without NH_4^+ dosage. Although the repulsive effect of H^+ was obvious within the group of equal total NH_4^+ (bottom 5 dots showed similar pattern as Figure 5.5 if smaller scale was used), when H^+ repulsion and NH_4^+ attraction was combined, e.g. from 50 mg N/L at pH = 7 to 500 mg N/L at pH = 6 (as the arrow

indicated in Figure 5.6c), both the NH_4^+ concentration and H^+ increased 10 times, the number of cells entering capillary tube still increased, showing a net attractive chemotaxis effect. Hence, it is a reasonable consideration that the sensitivity of AOB to NH_4^+ was higher than H^+ . It was noteworthy that at 500 mg NH_4^+ -N/L, cells entering capillary tube with higher pH (i.e. lower H^+) slightly decreased, which was inconsistent with the other data at 0 and 50 mg NH_4^+ -N/L where the cell number at pH = 8 ($\text{H}^+ = 10^{-8}$ M) reached the highest. This may due to the inhibition of free NH_3 whose concentration was increased from 5.0 mg N/L to 46.8 mg N/L when pH jumped from 7 to 8. High-concentration FA in the capillary tube may diffuse to the adjacent area of the tube opening and therefore lower the motility of the nearby cells, which was consistent with our hypothesis in the motility assay of AOB (Figure 5.3) that high FA would decrease cell motility.

5.3.2 Chemotaxis response of NOB

a. NOB Batch assay

Similar to AOB, a series of batch assays was conducted for NOB to determine the experimental conditions for the subsequent capillary assays. Based on the cell density profile in Figure 5.7, the culture density ($5.44 \pm 0.02 \times 10^7$ cells/ml) changed less than 2.2% after two hours with initial NO_2^- concentration ranging from 0 to 1000 mg N/L.

Figure 5.7 Cell density of NOB during batch assay.

In NOB batch assay, the NO_3^- production was measured to indicate its metabolic activity. The maximum uptake of 13.8 mg N/L was observed in the batch with 200 mg NO_2^- -N/L, corresponding to an initial FNA concentration of 0.021 mg N/L. Since the maximum NO_2^- uptake was less than 13% of the initial value of all the batches, concentration gradient between the capillary tube and the pond culture was still considered maintained at the six designed levels.

Interestingly, unlike AOB whose substrate uptake rate changed in a relatively narrow range at different NH_4^+ concentrations (e.g. 50 mg N/L reached 75% of maximum while 1000 mg/L only caused 5% inhibition), the substrate uptake by NOB at 50 mg nitrite-N/L was about 47% of the maximum, while 23% drop was found at 1000 mg NO_2^- -N/L, i.e. the metabolic activity of NOB was more sensitive to the substrate concentration than AOB.

Figure 5.8 NOB metabolic activity at different initial NO_2^- concentrations.

b. Chemotaxis response of NOB to NO_2^-

Similar to AOB, the chemotaxis effect of NO_2^- on NOB was investigated by comparing the result of chemotaxis assay and motility assay using the same batch of culture as in Figure 5.9. Again, the results were normalized to the baseline value where no nutrient was available in either pond or tube medium. The curve representing chemotaxis assay showed an increasing curve as the NO_2^- concentration increased from 0 to 1000 mg/L. The motility curve, however, after experiencing an increasing trend from 0 to 200 mg N/L, dropped below the baseline value at 500 and 1000 mg N/L. The decrease of motility was likely associated with the inhibition effect of NO_2^- or HNO_2 and consistent with the result of batch assay where inhibition of metabolic activity occurred at 500 mg/L and 1000 mg/L. The number of cells entering the capillary tube in the chemotaxis assay continuously increased regardless of the decreasing motility, implying that NO_2^- was indeed a chemotaxis attractant of NOB and the chemotaxis response was regulated by a separate control mechanism independent of metabolic activity and motility.

Figure 5.9 NOB motility at $\text{NO}_2^- = 0, 50, 100, 200, 500, 1000$ mg N/L and the chemotaxis response curve using the same batch of culture. Tube media contained no bacterial and NO_2^- at 0, 50, 100, 200, 500, 1000 mg N/L. Pond culture of 5.4×10^7 cell/ml contained no nitrogen in the chemotaxis assay and the same NO_2^- concentration as the tube media in motility assay. Error bar represented the standard deviation of five measurements.

c. Chemotaxis response of NOB to NH_4^+

Although NH_4^+ is neither a substrate nor a metabolite product of NOB, it is more ubiquitous in the natural environment and engineering applications than NO_2^- or NO_3^- . By investigating NOB's chemotaxis respond to NH_4^+ , better knowledge about the surviving strategy of NOB might be acquired. For this purpose, 50 mg NO_2^- -N/L was added to in both pond culture and tube solution to maintain the cell activity, while NH_4^+ was provided in the capillary tube medium at 0 to 1000 mg N/L (Figure 5.10a). The data with NH_4^+ dosing was all below the baseline value where NH_4^+ was absent, which indicated a probable repulsive effect of NH_4^+ . Since the number of cells entering the capillary tube fluctuated with the NH_4^+ dosage in the range of 50 to 1000 mg/L, NH_4^+ was dosed in the pond culture at different concentration while the tube medium had no testing chemical (chemical in pond method) to confirm that the reduced cell number was

associated with chemotaxis repulsion rather a random bias. The result was shown in Figure 5.10b. When cells experienced the chemotaxis repellent, their tumble frequency increased so that they tended to swim to the area with less repellent. In this case, cells would be forced into the capillary tubes if the pond culture contained chemotaxis repellent. The result in Figure 5.10b showed that the capillary tube contained more cells when NH_4^+ was added in the pond culture, which confirmed the above hypothesis. The data, however, did not display a smooth curve like the chemical in capillary method either. The possible explanation for the fluctuation was that the chemotaxis receptor for NH_4^+ was already saturated at 50 mg/L of ammonium-N or even below. Further increase of NH_4^+ dosage could not induce additional repulsive effect so that the data obtained was just a reflection of random variation at the similar level of repulsion.

Figure 5.10 NH_4^+ chemotaxis response curve of NOB. Chemical in tube (a) and chemical in pond (b) method were conducted at $\text{NH}_4^+ = 0, 50, 100, 200, 500$ and 1000 mg N/L to double confirm the repulsive effect. Error bar represented the standard error of five measurements.

d. Chemotaxis responses of NOB to pH, and FNA

In contrast with AOB which experienced more severe inhibition at high pH, NOB was more affected by low pH when metabolic activity was concerned. It was reported that the activity of NOB was totally inhibited at pH of 6.5 while remained unaffected at pH as high as 9.95 (Jiménez *et al.*, 2011). Similar to the pH capillary assay for AOB, NOB pond culture of $5.44 \pm 0.02 \times 10^7$ cells/ml was inserted with capillary tube containing blank medium at pH = 6 ~ 8. The result at pH = 8 was again used as the baseline value (Figure 5.11). Unlike AOB, NOB cells did not respond to different pH gradients. Even at $\text{pH} \leq 6.5$ where NOB's metabolism was reported to be totally inhibited, NOB cells still swam into the capillary tubes at the same level as other pH values. The numbers of cells in the capillary tube at the five pH values were not statistically different, indicating that NOB had no chemotaxis respond to H^+ or OH^- in the pH range of 6 ~ 8.

Figure 5.11 Chemotaxis response of NOB to different pH values. Data at pH = 8 was taken as the baseline value. Error bar represented the standard error of five measurements.

While NO_2^- was reported to be the true substrate for NOB (Jiménez *et al.*, 2011), its derivative, FNA, had severe inhibition on NOB growth (Vadivelu *et al.*, 2006). Since the previous section of this study demonstrated that NO_2^-/FNA had positive chemotaxis effect on NOB and H^+/OH^- did not attract or repel NOB cells, another set of capillary

assays were conducted to identify which one between NO_2^- and FNA the functional chemotaxis attractant for NOB was. Similar as that for AOB, three NO_2^- concentrations at the five pH values were adopted in this assay. Cell number in capillary tubes was normalized to the average of baseline line condition at zero NO_2^- dosage and pH = 8 (Figure 5.12).

Figure 5.12 NOB cell number in capillary against FNA (a), NO_2^- (b) and pH (c). Cell number in capillary at $\text{NO}_2^- = 0$ mg/L and pH = 8 was taken as baseline value. ●: 0 mg NO_2^- -N/L; ▲: 50 mg NO_2^- -N/L; ◆: 500 NO_2^- -N/L.

The results demonstrated that the cell number in the capillary tubes was better correlated with the NO_2^- concentration than the FNA concentration (Figure 5.12 a and b). In other words, NO_2^- or both NO_2^- and HNO_2 were the chemotaxis attractant of NOB with NO_2^- playing the main role. Yet high FNA concentration still affected the observations, e.g. at the same level of total NO_2^- -N, the cell number in the capillary tubes decreased as FNA increased (Figure 28a) or H^+ increased (Figure 5.12c). This could be due to the inhibition of FNA which lowers the random motility of NOB cells as discussed in the preceding section (Figure 5.10) and therefore lowered the number of cells entering the capillary tube.

5.3.3 Effect of chemotaxis on floc structure

The previous discussions can be summarized as that AOB was attracted by NH_4^+ and repelled by NO_2^- and H^+ , while NOB was attracted by NO_2^- and repelled by NH_4^+ . Based on these findings, when AOB and NOB coexisted in the natural environment or engineering processes where NH_4^+ was the primary nitrogen source, there would be a promising trend that free AOB and NOB cells or clusters would swim toward each other driven by the chemotactic forces, specifically, the AOBs were driven by the chemotactic repulsion from high to low NO_2^- area to NOB surrounding which the local NO_2^- concentration was lower, while NOBs were driven by the chemotactic attraction to higher NO_2^- area and chemotactic repulsion to lower NH_4^+ area towards AOB surrounding which the local NO_2^- concentration was higher and NH_4^+ concentration was lower. At the same time, the AOB-NOB dual clusters tended to swim towards the influent outlet for higher NH_4^+ driven by the chemotactic attraction of AOB.

When the two groups of cells reached close enough, cell cluster or floc structure was formed. It was reasonable to hypothesis the following floc structure based on their chemotaxis characteristics: AOB preferred to stay at the outer layer and NOB formed the inner core as illustrated in Figure 5.13 (i.e. the areas did not represent the relative amount of AOB and NOB). Following this pattern, AOB stayed in contact with the bulk liquid and had easy access to NH_4^+ ; by inhabiting inside AOB clusters, the NOB was “protected” from NH_4^+ and able to receive continuous supply of NO_2^- . Besides chemotaxis driving force, NOB’s more compact floc adhesion than AOB flocs as discussed in Section 4.3.3 also contributed to the hypothesized structure which had a more compact inner core than cover layer. This hypothesized structure was indeed observed in the AOB-NOB co-culture in the preceding chapter after one- to two-weeks’ cultivation (Figure 4.7).

Figure 5.13 Hypothesized structure of AOB and NOB co-culture flocs.

In fact, the stratification of AOB and NOB had been reported by previous studies in granular sludge from an anammox upflow reactor (Vlaeminck *et al.*, 2010) and biofilm from nitrifying trickling filter systems (Almstrand *et al.*, 2013) where AOB stayed in the outermost layer followed by NOB inhabited the middle layer, anammox, denitrifiers and/or other anoxic/anaerobic groups occupied the inner core or directly attached to the surface of carriers. However, cells in these structures were completely immobilized within the EPS matrix with a thickness of larger than 50 μm . Previous studies proposed that the stratification was the result of the competition between different microbial groups for oxygen and/or nutrients which determined the different growth capacity of microorganisms with different affinity. Usually at least a few months was required to form such growth pattern. The findings of this study, however, proposed that the stratified structure was formed by the active movement of bacterial cells rather than the passive selection by the surrounding environment, i.e. AOB or NOB chose to stay at the certain position of a floc in response to their chemoattractant/chemorepellent rather than randomly attached to a surface and then gained the dominance or became minor within a certain area as a result of the microbial competition. The size of the observed stratified flocs ranged between 10 ~ 30 μm . In real applications or natural systems, the active selection by bacteria and passive selection by environment probably coexisted while the former was much faster (within days or even hours depending on the cell density) and possibly contributed more to the initial stage of floc/biofilm formation.

5.3.4 Chemotaxis and metabolic activity

As mentioned earlier, chemotaxis and metabolic activity were regulated by different control pathways. With different evolutionary development of different species, chemotaxis response may or may not work toward a favorable way for the bacteria's metabolism or growth, e.g. the chemotaxis response of *E. coli* to amino acids correlates well with their utilization (attracted by nutrient amino acids and repelled by inhibiting or non-utilizable ones) while *B. subtilis* had no such strong correlation between chemotaxis response and nutrient-uptake ability (Yang *et al.*, 2015).

For AOB and NOB in this study, their response to the chemicals generally correlated well with their utilization. It was worth mentioned that the response of *N. europaea* and *N. winogradskyi* to NH_4^+ and NO_2^- respectively at high concentration were very similar to the response of *E. coli* to serine and cysteine which were both growth inhibitors (but still utilizable) (Harris, 1981, Hama *et al.*, 1990) and chemotaxis attractants of *E. coli* (Yang *et al.*, 2015). Unlike these two amino acids, another two amino acids, valine and leucine, which also have inhibitory effect at 1 mM but are not utilizable, are chemotaxis repellent of *E. coli*. For the two types of growth inhibitors, physical avoidance was chosen for the non-utilizable inhibitors while approaching and detoxification become the preferred strategy for nutritionally valuable ones. Likewise, *N. europaea* and *N. winogradskyi* also follow the latter strategy and move towards NH_4^+ and NO_2^- respectively even at inhibitory concentrations. In other words, nutritional value is a determinant criterion for chemotaxis response of AOB and NOB instead of the effect on their metabolic activity.

In this sense, maintaining a concentration gradient in space in engineering systems where the maximum concentration was lower than the inhibition threshold could possibly gather active biomass to the nutrient-rich zone and enhance the system efficiency since AOB and NOB had higher metabolic activity at higher nutrient concentrations as

demonstrated in the batch assays. On the other hand, high strength wastewater or other bioremediation applications with high NH_4^+ or NO_2^- concentration required special attention on the effectiveness of mixing systems so that the high concentration pollution streams could be diluted sufficiently, and active biomass would not be “trapped” in the concentrated zones.

In addition, the benefit and risk of the flocculation of AOB and NOB mixed consortia would be another issue that required more study to justify, i.e. the physical proximity of AOB and NOB in cell clusters could reduce the diffusion limit and increase the metabolic efficiency given the nutrient exchange between the floc and the bulk liquid was sufficient. When the floc grew larger and more compact, however, chemical exchange between the inner and outer part of the floc and the bulk liquid would be impaired. The overall efficiency could be reduced due to the continuous flocculation.

5.4 Conclusions

AOB and NOB played the key role in biological nitrogen removal while their chemotaxis characteristics had not been systematically studied after decades of studies. In this study *Nitrosomonas europaea* and *Nitrobacter winogradskyi* were used as the model strains of AOB and NOB respectively and the capillary assays were adopted to investigate their chemotaxis response to NH_4^+ , NO_2^- and pH. The Results showed that *N. europaea* was attracted by NH_4^+ rather than NH_3 although the latter was known to be the true substrate of AOB. The attractive response was observed at the NH_4^+ concentration as high as 1000 mg N/L despite that such high concentration already induced inhibition on their metabolic activity and motility. Considering this feature, it was possible that in systems with insufficient mixing power and high influent NH_4^+ concentration, cells of *N. europaea* tended to gather towards the high- NH_4^+ zone although their metabolic activity could be inhibited and therefore lower the overall system efficiency. As the metabolites of *N. europaea*, NO_2^- and H^+ effectively repelled their producer, suggesting that *N.*

europaea would actively search for fresh substrate and escape from the crowded environment as long as they were still in a motile state.

Similarly, *N. winogradskyi* was attracted by NO_2^- up to 1000 mg N/L even though the cells' metabolic activity and motility was significantly inhibited due to the high FNA. NH_4^+ , which was not involved the metabolic pathway of NOB but known to inhibit its growth, repelled *N. winogradskyi* from the lowest testing concentration at 50 mg N/L. However, *N. winogradskyi* did not show chemotaxis response to the change of pH between 6 to 8, indicating that *N. winogradskyi* might lack the corresponding chemotaxis receptor for H^+ or OH^- .

This study also discussed the stratified floc structure based on AOB and NOB's chemotaxis characteristics and proposed the active formation hypothesis compared with the traditional passive selection process during the initial stage of floc formation.

With the chemotaxis movement, AOB and NOB might undergo self-regulation in the micro-scale environment for the more efficient distribution pattern. Further studies were required to better exploit this feature for higher efficiency in nitrogen removal applications.

CHAPTER 6 CONCLUSIONS AND RECOMMENDATIONS

6.1 Major findings

6.1.1 Discovery of SNDPR in a full-scale WRP

By analyzing the plant nutrient data at different locations of reactor basins and conducting off-line batch experiments with samples from multiple rounds of sampling, it was confirmed that stable SNDPR had been achieved in the studied WRP. It was the first reported full-scale SNDPR under tropical climate. The hypothesized contributing factors included the low DO in aerobic zones which favored denitrification in aerobic conditions, step-feeding process which ensured that the carbon was utilized by the nutrient (N, P) removers in addition to the ordinary heterotrophs, and the high temperature which was traditionally considered adverse to the growth of PAO but here induced supplementary carbon released from cell lysis in the anaerobic phases including settling period and the return flow.

Besides the nutrient data analysis, the microbial data supported that PAO had outcompeted GAO with the former having consistently high abundance in all rounds of samplings and the latter barely detected. At the same time, the abundance of the nitrifiers, i.e. AOB and NOB, was highly dynamic during the sampling period while overall nitrogen removal remained stable. The uncoupled activity and abundance data suggested that there might be still lack of knowledge on the functionality of microbial groups so that the change of activity or abundance could not be simply interpreted as the precursor or outcome of the other. Nevertheless, keeping monitoring the abundance of the major microbial groups in a routine manner would enhance the understanding of the development of overall plant performance which could aid the development of plant operation strategy.

6.1.2 A balanced AOB and NOB community achievable

Giving the wide range of AOB/NOB ratio across different studies, *Nitrosomonas europaea* and *Nitrobacter winogradskyi* which represented AOB and NOB respectively were cocultured in chemostat reactors with three different AOB/NOB ratios in the inoculum. After cultivation of only one week, three groups achieved full nitrification at similar AOB/NOB ratio and the value remained stable at around 2 ~ 3 during the next few weeks, which was close to the theoretical value of 2. The above results revealed that stable substrate and operating conditions were sufficient to determine a stable microbial composition for pure culture AOB and NOB. The initial or previous conditions did not affect the steady state composition. At the same time, higher AOB content at full nitrification indicated that NOB was able to generate two times or higher activity/cell than AOB so that it was difficult to maintain partial nitrification by targeting low NOB abundance. Besides, the observed AOB-outer layer and NOB-inner core structure further increased the difficulty to remove NOB in engineering applications.

6.1.3 AOB and NOB had chemotaxis response to NH_4^+ and NO_2^-

Knowing that the two-layer structure was observed in *Nitrosomonas europaea* and *Nitrobacter winogradskyi* cell clusters, the potential chemotaxis effect between the two groups and $\text{NH}_4^+/\text{NO}_2^-$ were examined with a series of capillary assays. Results showed that AOB had positive chemotaxis response to NH_4^+ and negative chemotaxis response to NO_2^- while NOB had the reverse responses to the two chemicals as AOB. In addition, AOB was repelled by low pH or H^+ while NOB did not respond to pH change. It was noteworthy that the chemotaxis responses were independent of metabolic activity and their intrinsic motility, e.g. NH_3 rather than NH_4^+ was known to be the true substrate of AOB but the chemotaxis receptor did not distinguish between NH_4^+ and NH_3 ; at high total ammonium nitrogen concentrations ($\geq 500 \text{ mg N/L}$), both the metabolic activity and

motility of AOB were inhibited but the chemotaxis attraction was even stronger than low concentrations, likewise for NOB.

The above finding about AOB and NOB's chemotaxis characteristics well supported the two-layer structure of AOB and NOB flocs so that a probable hypothesis on the initial stage of floc formation process of aerobic flocs was provided.

6.2 Conclusion and implications

This study started with the investigation of a full-scale local WRP and revealed the successful SNDPR with dynamic nitrifier abundance. With high temperature generally considered to be disadvantageous to the proliferation of PAO than GAO, the studied WRP still accomplished PAO enrichment with up to 76% P removal, indicating that with proper process design and control, EBPR was still achievable even at high temperature (around 30°C) which also might be an important contributor due to the hydrolysis in settling tank and return flow at high temperature. In addition, the integration of SND and EBPR would bring further saving of energy and reactor footprint.

At the same time, the dynamic nitrifier community raised an interesting topic about the relationship between abundance and activity. Conventionally, the performance of microbial groups was widely expressed as nutrient removed per unit of biomass which included all the microorganisms in the studied activated sludge. However, abundance of different microbial groups varied dramatically, and the microbial composition of different studies also had a large variation, therefore both abundance data and activity data were suggested to be monitored concurrently for more accurate and comprehensive presentation of the microbial performance for both lab-scale and full-scale processes.

Following the interest of abundance and activity especially for AOB and NOB which were the two key groups for many novel processes in Chapter 3, the next chapter studied the relative abundance of pure culture AOB and NOB with more details. Although

previous studies have demonstrated chaotic behavior of the abundance of nitrifiers in mixed liquor chemostats (Graham *et al.*, 2007), it was demonstrated in this study that the steady state AOB/NOB ratio was higher than the theoretical value of 2 at approximately 2.8 regardless of the initial inoculum combination. In other words, pure AOB and NOB co-culture was able to achieve steady state condition and the dynamic abundance should be attributed to the influence of other species, variation of influent quality or change of operation conditions. In addition, when nitrogen was the only energy source, higher AOB abundance than NOB would be the expected outcome rather than an indication for partial nitrification. The abnormal high abundance of NOB therefore had a high possibility to associate with other food source.

Finally, the chemotaxis of AOB and NOB was discovered and demonstrated with the capillary assays for the first time in Chapter 5. The different chemotaxis response of AOB and NOB to NH_4^+ and NO_2^- properly explained the two-layer structure of AOB-NOB flocs and provided the theoretical principle for the formation of such unique floc structure in the initial phase. Meanwhile, being independent of metabolic activity, the chemotaxis response of microbial groups may be either advantageous or disadvantageous for their growth. The existence of chemotaxis response of AOB and NOB provided a new perspective when their metabolic performance and physical movement were analyzed, which may assist the optimization of partial nitrification and the related processes.

6.3 Recommendations

In the AOB-NOB co-culture study (Chapter 4), AOB/NOB ratio with N as the only energy source had been demonstrated to be around 2 ~ 3. Considering the value was as low as 0.001 in the studied full-scale WRP (Chapter 3), possible alternative energy source of NOB other than NO_2^- , especially of *Nitrospira* which finally gained predominance in the competition with *Nitrobacter*, may contribute to the unusual NOB

abundance. Previous study had suggested that the mixotrophic pathway of *Nitrobacter* allowed the growth of NOB to uncouple from the NO_2^- supply of AOB (Winkler *et al.*, 2015). In the case of the studied WRP, however, this might be the case for the period in Oct 2014 when *Nitrobacter* outcompeted *Nitrospira*, but the data in Nov was definitely caused by other reasons when the abundance of *Nitrospira* was more than 30 times higher than that of *Nitrobacter*. Although the species of *Nitrospira* in this study was found to be different from the one which was reported to possess comammox capability (Daims *et al.*, 2015, Kits *et al.*, 2017), there was still possibility that the *Nitrospira* species in this study had alternating energy source which required further investigation.

In Chapter 5, this study has demonstrated that *N. europaea* and *N. winogradskyi* have chemotaxis responses to NH_4^+ , NO_2^- and pH, which plays a potentially important role in the flocculation initiation and metabolic activity. However, the denitrifiers and anammox bacteria have not been studied yet for any chemotaxis features, e.g. whether they have active physical movement against the concentration gradient of nutrient, inhibiting chemicals, pH, and any signalling molecules from its own or other groups. The potential results may provide new insights on the granulation, enrichment, start-up boosting, system control, and process optimization of nitrification-anammox process. Even PAOs and GAOs may be further studied to extend the microbial synergy story for better understanding and control of the biological nutrient removal processes. Besides, the chemotaxis feature of AOB and NOB was studied on the wild-type strains which may contain certain proportion of non-chemotaxis mutant. The proportion was not quantified in the current study and may vary according to different process designs, e.g. lab-scale completely mixed reactors and full-scale plug flow reactors. Whether the different process design has any selection effect on the chemotaxis strains may verify the effect of chemotaxis on microbial growth in process engineering context.

REFERENCES

- Adler, J. (1973) A method for measuring chemotaxis and use of the method to determine optimum conditions for chemotaxis by *Escherichia coli*. *Microbiology* 74(1), 77-91.
- Adler, J. and Shi, W. (1988) *Galvanotaxis in bacteria*, pp. 23-25, Cold Spring Harbor Laboratory Press.
- Agrawal, S., Karst, S.M., Gilbert, E.M., Horn, H., Nielsen, P.H. and Lackner, S. (2017) The role of inoculum and reactor configuration for microbial community composition and dynamics in mainstream partial nitrification anammox reactors. *MicrobiologyOpen* 6(4), e00456.
- Almstrand, R., Daims, H., Persson, F., Sörensson, F. and Hermansson, M. (2013) New methods for analysis of spatial distribution and coaggregation of microbial populations in complex biofilms. *Applied and Environmental Microbiology* 79(19), 5978-5987.
- Andersson, S., Dalhammar, G. and Rajarao, G.K. (2011) Influence of microbial interactions and EPS/polysaccharide composition on nutrient removal activity in biofilms formed by strains found in wastewater treatment systems. *Microbiological research* 166(6), 449-457.
- Andersson, S., Kuttuva Rajarao, G., Land, C.J. and Dalhammar, G. (2008) Biofilm formation and interactions of bacterial strains found in wastewater treatment systems. *FEMS microbiology letters* 283(1), 83-90.
- Barnard, J. and Abraham, K. (2006) Key features of successful BNR operation. *Water Science and Technology* 53(12), 1-9.
- Becks, L., Hilker, F.M., Malchow, H., Jürgens, K. and Arndt, H. (2005) Experimental demonstration of chaos in a microbial food web. *Nature* 435, 1226.

Benincà, E., Huisman, J., Heerkloss, R., Jöhnk, K.D., Branco, P., Van Nes, E.H., Scheffer, M. and Ellner, S.P. (2008) Chaos in a long-term experiment with a plankton community. *Nature* 451, 822.

Blackburne, R., Yuan, Z. and Keller, J. (2008) Partial nitrification to nitrite using low dissolved oxygen concentration as the main selection factor. *Biodegradation* 19(2), 303-312.

Blakemore, R.P. (1982) Magnetotactic Bacteria. *Annual Review of Microbiology* 36(1), 217-238.

Braga, R.M., Dourado, M.N. and Araújo, W.L. (2016) Microbial interactions: ecology in a molecular perspective. *Brazilian Journal of Microbiology* 47(Suppl 1), 86-98.

Brenner, K., You, L. and Arnold, F.H. (2008) Engineering microbial consortia: a new frontier in synthetic biology. *Trends in Biotechnology* 26(9), 483-489.

Carvalho, M., Oehmen, A., Carvalho, G., Eusébio, M. and Reis, M.A. (2014) The impact of aeration on the competition between polyphosphate accumulating organisms and glycogen accumulating organisms. *Water Research* 66, 296-307.

Chen, H.-b., Wang, D.-b., Li, X.-m., Yang, Q. and Zeng, G.-m. (2015) Enhancement of post-anoxic denitrification for biological nutrient removal: effect of different carbon sources. *Environmental Science and Pollution Research* 22(8), 5887-5894.

Claros, J., Jiménez, E., Aguado, D., Ferrer, J., Seco, A. and Serralta, J. (2013) Effect of pH and HNO₂ concentration on the activity of ammonia-oxidizing bacteria in a partial nitrification reactor. *Water Science and Technology* 67(11), 2587-2594.

Claros, J., Jiménez, E., Borrás, L., Aguado, D., Seco, A., Ferrer, J. and Serralta, J. (2010) Short-term effect of ammonia concentration and salinity on activity of ammonia oxidizing bacteria. *Water Science and Technology* 61(12), 3008-3016.

Dai, Y., Yuan, Z., Wang, X., Oehmen, A. and Keller, J. (2007) Anaerobic metabolism of *Dechlorococcus* vanus related glycogen accumulating organisms (GAOs) with acetate and propionate as carbon sources. *Water Research* 41(9), 1885-1896.

Daims, H., Lebedeva, E.V., Pjevac, P., Han, P., Herbold, C., Albertsen, M., Jehmlich, N., Palatinszky, M., Vierheilig, J. and Bulaev, A. (2015) Complete nitrification by *Nitrospira* bacteria. *Nature* 528(7583), 504-509.

Faust, K. and Raes, J. (2012) Microbial interactions: from networks to models. *Nature Reviews Microbiology* 10(8), 538.

Frankel, R.B. and Blakemore, R.P. (1989) Magnetite and magnetotaxis in microorganisms. *Bioelectromagnetics* 10(3), 223-237.

Frette, L., Gejlsbjerg, B. and Westermann, P. (1997) Aerobic denitrifiers isolated from an alternating activated sludge system. *FEMS microbiology ecology* 24(4), 363-370.

Gabel, C.V. and Berg, H.C. (2003) The speed of the flagellar rotary motor of *Escherichia coli* varies linearly with protonmotive force. *Proceedings of the National Academy of Sciences of the United States of America* 100(15), 8748-8751.

Ge, S., Peng, Y., Qiu, S., Zhu, A. and Ren, N. (2014) Complete nitrogen removal from municipal wastewater via partial nitrification by appropriately alternating anoxic/aerobic conditions in a continuous plug-flow step feed process. *Water Research* 55(0), 95-105.

Ge, S., Wang, S., Yang, X., Qiu, S., Li, B. and Peng, Y. (2015) Detection of nitrifiers and evaluation of partial nitrification for wastewater treatment: A review. *Chemosphere* 140, 85-98.

Gebremariam, S.Y., Beutel, M.W., Christian, D. and Hess, T.F. (2011) Research advances and challenges in the microbiology of enhanced biological phosphorus removal—a critical review. *Water Environment Research* 83(3), 195-219.

Gebremariam, S.Y., Beutel, M.W., Christian, D. and Hess, T.F. (2012) Effects of glucose on the performance of enhanced biological phosphorus removal activated sludge enriched with acetate. *Bioresource technology* 121, 19-24.

Glöckner, F.O., Amann, R., Alfreider, A., Pernthaler, J., Psenner, R., Trebesius, K. and Schleifer, K.-H. (1996) An In Situ Hybridization Protocol for Detection and Identification of Planktonic Bacteria. *Systematic and Applied Microbiology* 19(3), 403-406.

Gogina, E. and Gulshin, I. (2016) Simultaneous Nitrification and Denitrification with Low Dissolved Oxygen Level and C/N ratio. *Procedia Engineering* 153, 189-194.

Gómez-Silván, C., Vílchez-Vargas, R., Arévalo, J., Gómez, M.A., González-López, J., Pieper, D.H. and Rodelas, B. (2014) Quantitative response of nitrifying and denitrifying communities to environmental variables in a full-scale membrane bioreactor. *Bioresource technology* 169(0), 126-133.

Graaf, A.A.V.D., Mulder, A., Slijkhuys, H., Robertson, L.A. and Kuenen, J.G. (1990) Anoxic ammonium oxidation. *Anoxic Ammonium Oxidation Tu Delft Institutional Repository* (1990).

Graham, D.W., Knapp, C.W., Van Vleck, E.S., Bloor, K., Lane, T.B. and Graham, C.E. (2007) Experimental demonstration of chaotic instability in biological nitrification. *The Isme Journal* 1, 385.

Griffiths, P., Stratton, H. and Seviour, R. (2002) Environmental factors contributing to the “G bacteria” population in full-scale EBPR plants. *Water Science and Technology* 46(4-5), 185-192.

Grossart, H.-P., Riemann, L. and Azam, F. (2001) Bacterial motility in the sea and its ecological implications. *Aquatic Microbial Ecology* 25(3), 247-258.

Gu, J., Yang, Q. and Liu, Y. (2018) Mainstream anammox in a novel A-2B process for energy-efficient municipal wastewater treatment with minimized sludge production. *Water Research* 138, 1-6.

Guerrero, J., Tayà, C., Guisasola, A. and Baeza, J.A. (2012) Understanding the detrimental effect of nitrate presence on EBPR systems: effect of the plant configuration. *Journal of Chemical Technology and Biotechnology* 87(10), 1508-1511.

Gut, L., Płaza, E., Trela, J., Hultman, B. and Bosander, J. (2006) Combined partial nitrification/Anammox system for treatment of digester supernatant. *Water science and technology* 53(12), 149-159.

Häder, D.P. (1987) Photosensory behavior in procaryotes. *Microbiological reviews* 51(1), 1-21.

Hama, H., Sumita, Y., Kakutani, Y., Tsuda, M. and Tsuchiya, T. (1990) Target of serine inhibition in *Escherichia coli*. *Biochemical and biophysical research communications* 168(3), 1211-1216.

Han, M., De Clippeleir, H., Al-Omari, A., Wett, B., Vlaeminck, S.E., Bott, C. and Murthy, S. (2016) Impact of carbon to nitrogen ratio and aeration regime on mainstream deammonification. *Water Science and Technology* 74(2), 375-384.

Harris, C.L. (1981) Cysteine and growth inhibition of *Escherichia coli*: threonine deaminase as the target enzyme. *Journal of Bacteriology* 145(2), 1031-1035.

He, S., Gall, D.L. and McMahon, K.D. (2007) "Candidatus *Accumulibacter*" population structure in enhanced biological phosphorus removal sludges as revealed by polyphosphate kinase genes. *Applied and Environmental Microbiology* 73(18), 5865-5874.

Hellinga, C., Schellen, A., Mulder, J., Van Loosdrecht, M. and Heijnen, J. (1998) The SHARON process: an innovative method for nitrogen removal from ammonium-rich waste water. *Water science and technology* 37(9), 135-142.

Henze, M., van Loosdrecht, M.C., Ekama, G.A. and Brdjanovic, D. (2008) *Biological wastewater treatment*, IWA publishing.

Hou, J., Xia, L., Ma, T., Zhang, Y., Zhou, Y. and He, X. (2017) Achieving short-cut nitrification and denitrification in modified intermittently aerated constructed wetland. *Bioresource technology* 232, 10-17.

Hu, Z., Lotti, T., de Kreuk, M., Kleerebezem, R., van Loosdrecht, M., Kruit, J., Jetten, M.S. and Kartal, B. (2013) Nitrogen removal by a nitrification-anammox bioreactor at low temperature. *Applied and Environmental Microbiology* 79(8), 2807-2812.

Huang, Z., Gedalanga, P.B. and Olson, B.H. (2010) Distribution of nitrobacter and nitrospira communities in an aerobic activated sludge bioreactor and their contributions to nitrite oxidation. *Proceedings of the Water Environment Federation* 2010(15), 2390-2403.

Hunt, D.E., Lin, Y., Church, M.J., Karl, D.M., Tringe, S.G., Izzo, L.K. and Johnson, Z.I. (2013) Relationship between abundance and specific activity of bacterioplankton in open ocean surface waters. *Applied and environmental microbiology* 79(1), 177-184.

Jiménez, E., Giménez, J., Ruano, M., Ferrer, J. and Serralta, J. (2011) Effect of pH and nitrite concentration on nitrite oxidation rate. *Bioresource technology* 102(19), 8741-8747.

Jobbagy, A., Literathy, B., Wong, M.-T., Tardy, G. and Liu, W.-T. (2006) Proliferation of glycogen accumulating organisms induced by Fe (III) dosing in a domestic wastewater treatment plant. *Water Science and Technology* 54(1), 101-109.

Kermani, M., Bina, B., Movahedian, H., Amin, M.M. and Nikaeen, M. (2009) Biological phosphorus and nitrogen removal from wastewater using moving bed biofilm process. *Iranian journal of biotechnology* 7(1), 19-27.

Kim, H.-J., Yoo, R.-B., Han, S.-S., Woo, S.-H., Lee, M.-S., Baek, K.-T. and Chung, K.-Y. (2010) Effect of the various heavy metals on the growth and phosphorus (P) Removal capacity of the phosphorus accumulating microorganism (*Pseudomonas* sp.). *Korean Journal of Environmental Agriculture* 29(2), 189-196.

Kindaichi, T., Kawano, Y., Ito, T., Satoh, H. and Okabe, S. (2006) Population dynamics and in situ kinetics of nitrifying bacteria in autotrophic nitrifying biofilms as determined by real-time quantitative PCR. *Biotechnology and bioengineering* 94(6), 1111-1121.

Kjørboe, T., Grossart, H.-P., Ploug, H. and Tang, K. (2002) Mechanisms and rates of bacterial colonization of sinking aggregates. *Applied and Environmental Microbiology* 68(8), 3996-4006.

Kjørboe, T. and Jackson, G.A. (2001) Marine snow, organic solute plumes, and optimal chemosensory behavior of bacteria. *Limnology and Oceanography* 46(6), 1309-1318.

Kits, K.D., Sedlacek, C.J., Lebedeva, E.V., Han, P., Bulaev, A., Pjevac, P., Daebeler, A., Romano, S., Albertsen, M. and Stein, L.Y. (2017) Kinetic analysis of a complete nitrifier reveals an oligotrophic lifestyle. *Nature* 549(7671), 269-272.

Kuenen, J.G. (2001) Extraordinary Anaerobic Ammonium-oxidizing Bacteria. *Asm News* 67(9), 456-463.

Laanbroek, H.J. and Gerards, S. (1993) Competition for limiting amounts of oxygen between *Nitrosomonas europaea* and *Nitrobacter winogradskyi* grown in mixed continuous cultures. *Archives of Microbiology* 159(5), 453-459.

Li, Q., Sun, S., Guo, T., Yang, C., Song, C., Geng, W., Zhang, W., Feng, J. and Wang, S. (2013) Short-cut nitrification in biological aerated filters with modified zeolite and nitrifying sludge. *Bioresource technology* 136(0), 148-154.

Liébana, R., Arregui, L., Santos, A., Murciano, A., Marquina, D. and Serrano, S. (2016) Unravelling the interactions among microbial populations found in activated sludge during biofilm formation. *FEMS microbiology ecology* 92(9), fiw134-fiw134.

Liu, G. and Wang, J. (2013) Role of solids retention time on complete nitrification: mechanistic understanding and modeling. *Journal of Environmental Engineering* 140(1), 48-56.

Lopez-Vazquez, C.M., Oehmen, A., Hooijmans, C.M., Brdjanovic, D., Gijzen, H.J., Yuan, Z. and van Loosdrecht, M.C. (2009) Modeling the PAO–GAO competition: effects of carbon source, pH and temperature. *Water Research* 43(2), 450-462.

Lu, H., Oehmen, A., Viridis, B., Keller, J. and Yuan, Z. (2006) Obtaining highly enriched cultures of *Candidatus Accumulibacter phosphatus* through alternating carbon sources. *Water Research* 40(20), 3838-3848.

Maeda, K., Imae, Y., Shioi, J.I. and Oosawa, F. (1976) Effect of temperature on motility and chemotaxis of *Escherichia coli*. *Journal of Bacteriology* 127(3), 1039-1046.

Manson, M.D. (1992) Bacterial Motility and Chemotaxis. *Advances in Microbial Physiology* 33, 277-346.

Merrell, D.S., Butler, S.M., Qadri, F., Dolganov, N.A., Alam, A., Cohen, M.B., Calderwood, S.B., Schoolnik, G.K. and Camilli, A. (2002) Host-induced epidemic spread of the cholera bacterium. *Nature* 417(6889), 642.

Meyer, R.L., Zeng, R.J., Giugliano, V. and Blackall, L.L. (2005) Challenges for simultaneous nitrification, denitrification, and phosphorus removal in microbial

aggregates: mass transfer limitation and nitrous oxide production. *FEMS microbiology ecology* 52(3), 329-338.

Mitchell, J.G. and Kogure, K. (2006) Bacterial motility: links to the environment and a driving force for microbial physics. *FEMS microbiology ecology* 55(1), 3-16.

Mousavi, S.A. and Ibrahim, S. (2014) Effect of COD/N Ratio on Growth and Adaptation of Nitrifying Bacteria. *American Journal of Oil and Chemical Technologies: Volume* 2(6).

Münch, E.V., Lant, P. and Keller, J. (1996) Simultaneous nitrification and denitrification in bench-scale sequencing batch reactors. *Water Research* 30(2), 277-284.

Muszyński, A. and Miłobędzka, A. (2015) The effects of carbon/phosphorus ratio on polyphosphate-and glycogen-accumulating organisms in aerobic granular sludge. *International journal of environmental science and technology* 12(9), 3053-3060.

Nittami, T., Mukai, M., Uematsu, K., Yoon, L.W., Schroeder, S., Chua, A.S.M., Fukuda, J., Fujita, M. and Seviour, R.J. (2017) Effects of different carbon sources on enhanced biological phosphorus removal and “*Candidatus Accumulibacter*” community composition under continuous aerobic condition. *Applied Microbiology and Biotechnology* 101(23-24), 8607-8619.

Nowka, B., Daims, H. and 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(2), 745-753.

null, n. (1998) *Standard Methods for the Examination of Water and Wastewater*.

Oehmen, A., Keller-Lehmann, B., Zeng, R.J., Yuan, Z. and Keller, J. (2005a) Optimisation of poly- β -hydroxyalkanoate analysis using gas chromatography for enhanced biological phosphorus removal systems. *Journal of Chromatography A* 1070(1), 131-136.

Oehmen, A., Lemos, P.C., Carvalho, G., Yuan, Z., Keller, J., Blackall, L.L. and Reis, M.A.M. (2007) Advances in enhanced biological phosphorus removal: From micro to macro scale. *Water Research* 41(11), 2271-2300.

Oehmen, A., Yuan, Z., Blackall, L.L. and Keller, J. (2005b) Comparison of acetate and propionate uptake by polyphosphate accumulating organisms and glycogen accumulating organisms. *Biotechnology and bioengineering* 91(2), 162-168.

Panswad, T., Doungchai, A. and Anotai, J. (2003) Temperature effect on microbial community of enhanced biological phosphorus removal system. *Water Research* 37(2), 409-415.

Peng, Y., Hou, H., Wang, S., Cui, Y. and Zhiguo, Y. (2008) Nitrogen and phosphorus removal in pilot-scale anaerobic-anoxic oxidation ditch system. *Journal of Environmental Sciences* 20(4), 398-403.

Pérez, J., Buchanan, A., Mellbye, B., Ferrell, R., Chang, J.H., Chaplen, F., Bottomley, P.J., Arp, D.J. and Sayavedra-Soto, L.A. (2015) Interactions of *Nitrosomonas europaea* and *Nitrobacter winogradskyi* grown in co-culture. *Archives of Microbiology* 197(1), 79-89.

Pochana, K. and Keller, J. (1999) Study of factors affecting simultaneous nitrification and denitrification (SND). *Water science and technology* 39(6), 61-68.

Qi, Y.L. and Adler, J. (1989) Salt taxis in *Escherichia coli* bacteria and its lack in mutants. *Proceedings of the National Academy of Sciences of the United States of America* 86(21), 8358-8362.

Qin, Y., Han, B., Cao, Y. and Wang, T. (2017) Impact of substrate concentration on anammox-UBF reactors start-up. *Bioresource technology* 239, 422-429.

Regmi, P., Miller, M.W., Holgate, B., Bunce, R., Park, H., Chandran, K., Wett, B., Murthy, S. and Bott, C.B. (2014) Control of aeration, aerobic SRT and COD input for mainstream nitrification/denitrification. *Water Research* 57, 162-171.

Ren, Y., Yan, L., Hao, G., Zhang, X., Wen, Y., Guo, Y. and Chen, Z. (2016) Shortcut Nitrification Treatment of High Strength Ammonia Nitrogen Wastewater by Aerobic Granular Sludge. *Journal of the Chemical Society of Pakistan* 38(6).

Rivero, M.A., Tranquillo, R.T., Buettner, H.M. and Lauffenburger, D.A. (1989) Transport models for chemotactic cell populations based on individual cell behavior. *Chemical engineering science* 44(12), 2881-2897.

Rongsayamanont, C., Limpiyakorn, T., Law, B. and Khan, E. (2010) Relationship between respirometric activity and community of entrapped nitrifying bacteria: Implications for partial nitrification. *Enzyme and Microbial Technology* 46(3-4), 229-236.

Ruscalleda Beylier, M., Balaguer, M.D., Colprim, J., Pellicer-Nàcher, C., Ni, B.J., Smets, B.F., Sun, S.P. and Wang, R.C. (2011) *Comprehensive Biotechnology (Second Edition)*. Moo-Young, M. (ed), pp. 329-340, Academic Press, Burlington.

Ruscalleda, M., Balaguer, M., Colprim, J., Pellicer-Nàcher, C., Ni, B.-J., Smets, B., Sun, S.P. and Wang, R. (2011), pp. 329-340.

Saito, T., Brdjanovic, D. and Van Loosdrecht, M. (2004) Effect of nitrite on phosphate uptake by phosphate accumulating organisms. *Water Research* 38(17), 3760-3768.

Sayavedra-Soto, L., Ferrell, R., Dobie, M., Mellbye, B., Chaplen, F., Buchanan, A., Chang, J., Bottomley, P. and Arp, D. (2015) *Nitrobacter winogradskyi* transcriptomic response to low and high ammonium concentrations. *FEMS Microbiol Lett* 362, 1-7.

Sayi-Ucar, N., Sarioglu, M., Insel, G., Cokgor, E., Orhon, D. and van Loosdrecht, M. (2015) Long-term study on the impact of temperature on enhanced biological phosphorus and nitrogen removal in membrane bioreactor. *Water Research* 84, 8-17.

Schuler, A.J. and Jenkins, D. (2003) Enhanced biological phosphorus removal from wastewater by biomass with different phosphorus contents, part I: experimental results and comparison with metabolic models. *Water Environment Research* 75(6), 485-498.

Shen, N., Chen, Y. and Zhou, Y. (2017) Multi-cycle operation of enhanced biological phosphorus removal (EBPR) with different carbon sources under high temperature. *Water Research* 114, 308-315.

Shen, N. and Zhou, Y. (2016) Enhanced biological phosphorus removal with different carbon sources. *Applied Microbiology and Biotechnology* 100(11), 4735-4745.

Siegrist, H., Salzgeber, D., Eugster, J. and Joss, A. (2008) Anammox brings WWTP closer to energy autarky due to increased biogas production and reduced aeration energy for N-removal. *Water Science and Technology* 57(3), 383-388.

Singh, R. and Olson, M.S. (2008) *Emerging Environmental Technologies*. Shah, V. (ed), pp. 149-172, Springer Netherlands, Dordrecht.

Spudich, J.L. and Bogomolni, R.A. (1984) Mechanism of colour discrimination by a bacterial sensory rhodopsin. *Nature* 312, 509.

Stokholm-Bjerregaard, M., Albertsen, M., Nguyen, H.T.T., Saunders, A.M., Vollertsen, J., Borregaard, V.R. and Nielsen, P.H. (2015) Can return-sludge-sidestream hydrolysis control GAOs in full-scale EBPR-plants?, pp. 363-371, Gdańsk, Poland.

Sun, F., Sun, B., Li, Q., Deng, X., Hu, J. and Wu, W. (2014) Pilot-scale nitrogen removal from leachate by ex situ nitrification and in situ denitrification in a landfill bioreactor. *Chemosphere* 101, 77-85.

- Sun, S.-P., Nàcher, C.P.i., Merkey, B., Zhou, Q., Xia, S.-Q., Yang, D.-H., Sun, J.-H. and Smets, B.F. (2010) Effective biological nitrogen removal treatment processes for domestic wastewaters with low C/N ratios: a review. *Environmental Engineering Science* 27(2), 111-126.
- Suneethi, S. and Joseph, K. (2011) ANAMMOX process start up and stabilization with an anaerobic seed in anaerobic membrane bioreactor (AnMBR). *Bioresource technology* 102(19), 8860-8867.
- Szatkowska, B., Cema, G., Plaza, E., Trela, J. and Hultman, B. (2007) A one-stage system with partial nitrification and Anammox processes in the moving-bed biofilm reactor. *Water science and technology* 55(8-9), 19-26.
- Tamar, E., Koler, M. and Vaknin, A. (2016) The role of motility and chemotaxis in the bacterial colonization of protected surfaces. *Scientific Reports* 6, 19616.
- Tayà, C., Garlapati, V.K., Guisasola, A. and Baeza, J.A. (2013) The selective role of nitrite in the PAO/GAO competition. *Chemosphere* 93(4), 612-618.
- Towe, S., Wallisch, S., Bannert, A., Fischer, D., Hai, B., Haesler, F., Kleinedam, K. and Schlöter, M. (2011) Improved protocol for the simultaneous extraction and column-based separation of DNA and RNA from different soils. *J Microbiol Methods* 84(3), 406-412.
- Tsai, Y., Lu, M., Lin, J., Chou, C. and Hu, L. (2016) Temperature Effects on Zn (II) Toxicity to Metabolisms of Polyphosphate Accumulating Organisms in Aerobic and Anaerobic Conditions. *J Environ Anal Chem* 3(187), 2.
- Tso, W.-W. and Adler, J. (1974) Negative chemotaxis in *Escherichia coli*. *Journal of Bacteriology* 118(2), 560-576.

Tu, Y. (2012) The effects of acetate concentrations on competition between microbial populations in enhanced biological phosphorus removal from wastewater, The University of New Mexico.

Turchin, P. (2003) Complex population dynamics: a theoretical/empirical synthesis, Princeton university press.

Vadivelu, V.M., Yuan, Z., Fux, C. and Keller, J. (2006) The inhibitory effects of free nitrous acid on the energy generation and growth processes of an enriched *Nitrobacter* culture. *Environmental Science & Technology* 40(14), 4442-4448.

Van Benthum, W., Van Loosdrecht, M. and Heijnen, J. (1997) Control of heterotrophic layer formation on nitrifying biofilms in a biofilm airlift suspension reactor. *Biotechnology and bioengineering* 53(4), 397-405.

Vejmelkova, D., Sorokin, D.Y., Abbas, B., Kovaleva, O.L., Kleerebezem, R., Kampschreur, M.J., Muyzer, G. and van Loosdrecht, M.C. (2012) Analysis of ammonia-oxidizing bacteria dominating in lab-scale bioreactors with high ammonium bicarbonate loading. *Applied Microbiology and Biotechnology* 93(1), 401-410.

Venkataraman, C. and Kao, A.S. (1999) Comparison of particle lung doses from the fine and coarse fractions of urban PM-10 aerosols. *Inhalation Toxicology* 11(2), 151-169.

Vlaeminck, S.E., Terada, A., Smets, B.F., De Clippeleir, H., Schaubroeck, T., Bolca, S., Demeestere, L., Mast, J., Boon, N. and Carballa, M. (2010) Aggregate size and architecture determine microbial activity balance for one-stage partial nitrification and anammox. *Applied and Environmental Microbiology* 76(3), 900-909.

Wagner, M., Rath, G., Amann, R., Koops, H.-P. and Schleifer, K.-H. (1995) In situ Identification of Ammonia-oxidizing Bacteria. *Systematic and Applied Microbiology* 18(2), 251-264.

Wagner, M., Rath, G., Koops, H.-P., Flood, J. and Amann, R. (1996) In situ analysis of nitrifying bacteria in sewage treatment plants. *Water science and technology* 34(1), 237-244.

Waki, M., Yasuda, T., Fukumoto, Y., Béline, F. and Magrí, A. (2018) Treatment of swine wastewater in continuous activated sludge systems under different dissolved oxygen conditions: Reactor operation and evaluation using modelling. *Bioresource technology* 250, 574-582.

Wang, D., Tooker, N.B., Srinivasan, V., Li, G., Schauer, P., Menniti, A., Maher, C., Bott, C.B., Dombrowski, P. and Barnard, J.L. (2018) A Full-Scale Comparative Study of Conventional and Side-Stream Enhanced Biological Phosphorus Removal Processes.

Watteaux, R., Stocker, R. and Taylor, J.R. (2015) Sensitivity of the rate of nutrient uptake by chemotactic bacteria to physical and biological parameters in a turbulent environment. *Journal of theoretical biology* 387, 120-135.

Whang, L.-M. and Park, J. (2002) Competition between polyphosphate-and glycogen-accumulating organisms in biological phosphorus removal systems—effect of temperature. *Water Science and Technology* 46(1-2), 191-194.

Whang, L.-M. and Park, J.K. (2006) Competition between polyphosphate-and glycogen-accumulating organisms in enhanced-biological-phosphorus-removal systems: effect of temperature and sludge age. *Water Environment Research* 78(1), 4-11.

Winkler, M.-K.H., Le, Q.H. and Volcke, E.I.P. (2015) Influence of Partial Denitrification and Mixotrophic Growth of NOB on Microbial Distribution in Aerobic Granular Sludge. *Environmental Science & Technology* 49(18), 11003-11010.

Winkler, M.K.H., Bassin, J.P., Kleerebezem, R., Sorokin, D.Y. and van Loosdrecht, M.C.M. (2012) Unravelling the reasons for disproportion in the ratio of AOB and NOB in aerobic granular sludge. *Applied microbiology and biotechnology* 94(6), 1657-1666.

Wong-Ng, J., Celani, A. and Vergassola, M. (2018) Exploring the function of bacterial chemotaxis. *Current Opinion in Microbiology* 45, 16-21.

Xu, G., Zhou, Y., Yang, Q., Lee, Z.M.-P., Gu, J., Lay, W., Cao, Y. and Liu, Y. (2015) The challenges of mainstream deammonification process for municipal used water treatment. *Applied Microbiology and Biotechnology* 99(6), 2485-2490.

Yang, Y., M. Pollard, A., Höfler, C., Poschet, G., Wirtz, M., Hell, R. and Sourjik, V. (2015) Relation between chemotaxis and consumption of amino acids in bacteria. *Molecular microbiology* 96(6), 1272-1282.

Zeng, R.J., Lemaire, R., Yuan, Z. and Keller, J. (2003a) Simultaneous nitrification, denitrification, and phosphorus removal in a lab-scale sequencing batch reactor. *Biotechnology and bioengineering* 84(2), 170-178.

Zeng, R.J., van Loosdrecht, M., Yuan, Z. and Keller, J. (2003b) Metabolic model for glycogen-accumulating organisms in anaerobic/aerobic activated sludge systems. *Biotechnology and bioengineering* 81(1), 92-105.

Zhang, B., Yamamoto, K., Ohgaki, S. and Kamiko, N. (1997) Floc size distribution and bacterial activities in membrane separation activated sludge processes for small-scale wastewater treatment/reclamation. *Water science and technology* 35(6), 37-44.

Zhou, Y., Ganda, L., Lim, M., Yuan, Z., Kjelleberg, S. and Ng, W.J. (2010) Free nitrous acid (FNA) inhibition on denitrifying poly-phosphate accumulating organisms (DPAOs). *Applied Microbiology and Biotechnology* 88(1), 359-369.

Zhu, G.-b., Peng, Y.-z., Wu, S.-y., Wang, S.-y. and Xu, S.-w. (2007) Simultaneous nitrification and denitrification in step feeding biological nitrogen removal process. *Journal of Environmental Sciences* 19(9), 1043-1048.

APPENDIX A

AOB isolate (Trimmed condition: the terminal 20 bases with quality value higher than 30, effective length:1361, closest to *Nitrosomonas europaea* strain ATCC 25978, no gap, 1360 of 1361 bps matched, risk level 1)

CTTCGGCCTGCCGGCGAGTGGCGAACGGGTGAGTAATACATCGGAACGTGT
CCTTAAGTGGGGAATAACGCATCGAAAGATGTGCTAATACCGCATATCTCT
GAGGAGAAAAGCAGGGGATCGCAAGACCTTGCGCTAAAGGAGCGGCCGAT
GTCTGATTAGCTAGTTGGTGGGGTAAAGGCTTACCAAGGCAACGATCAGTA
GTTGGTCTGAGAGGACGGCCAACCACACTGGGACTGAGACACGGCCCAGA
CTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGAAAGCCTG
ATCCAGCCATGCCGCGTGAGTGAAGAAGGCCTTCGGGTGTAAAGCTCTTT
TAGTCGGAAAGAAAGAGTTGCAATGAATAATTGTGATTTATGACGGTACCG
ACAGAAAAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAG
GGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGGGTGCGCAGGCGGTCT
TGCAAGTCAGATGTGAAAGCCCCGGGCTTAACCTGGGAATTGCGTTTGAAA
CTACAAGGCTAGAGTGCAGCAGAGGGGAGTGGAATTCCATGTGTAGCAGT
GAAATGCGTAGAGATGTGGAAGAACACCGATGGCGAAGGCAGCTCCCTGG
GTTGACACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGA
TACCCTGGTAGTCCACGCCCTAAACGATGTCAACTAGTTGTTCGGATCTAATT
AAGGATTTGGTAACGTAGCTAACGCGTGAAGTTGACCGCCTGGGGAGTACG
GTCGCAAGATTAAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTG
GATTATGTGGATTAATTCGATGCAACGCGAAAAACCTTACCTACCCTTGAC
ATGCTTGGAATCTAATGGAGACATAAGAGTGCCCGAAAGGGAGCCAAGAC
ACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTAAAG
TCCCGCAACGAGCGCAACCCTTGTCATAATTGCTATCATTTTTAATGAGCA
CTTTAGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCA
AGTCCTCATGGCCCTTATGGGTAGGGCTTCACACGTAATACAATGGCGTGT
ACAGAGGGTTGCCAACCCGCGAGGGGGAGCCAATCTCAGAAAGCACGTCG

TAGTCCGGATCGGAGTCTGCAACTCGACTCCGTGAAGTCGGAATCGCTAGT
AATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGTCTTGTACACAC
CGCCCGTCACACCATGGGAGTGGTTTTTCACCAGAAGCAGGTAGC