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**ROLE OF FREE NITROUS ACID
IN WASTEWATER TREATMENT PROCESSES:
ACTION AND INHIBITION**

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2014**

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IN WASTEWATER TREATMENT PROCESSES:
ACTION AND INHIBITION**

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS.....	I
TABLE OF CONTENTS	III
SUMMARY	VI
LIST OF PUBLICATIONS	VIII
LIST OF TABLES	IX
LIST OF FIGURES	X
ABBREVIATIONS	XIII
CHAPTER 1 – INTRODUCTION	1
1.1 Background	1
1.2 Objectives and Scope	5
CHAPTER 2 – LITERATURE REVIEW	6
2.1 Biological Nitrogen Removal	6
2.1.1 Conventional biological nitrogen removal.....	6
2.1.2 Nitrogen removal via nitrite pathway	8
2.1.3 N ₂ O emissions from biological nitrogen removal processes	9
2.2 Biological Phosphorus Removal	11
2.2.1 Enhanced biological phosphorus removal	11
2.2.2 Denitrifying PAO	12
2.2.3 Metabolisms of PAO.....	13
2.2.4 Glycogen accumulating organism (GAO)	17
2.2.5 Metabolism of GAO	18
2.3 SND and SNDPR.....	20
2.4 Free Nitrous Acid Inhibition.....	21
2.5 Impact of Coexistence of Electron Donor and Electron Acceptor on EBPR	22
CHAPTER 3 – MATERIALS AND METHODS.....	25
3.1 Biomass Concentration.....	25
3.2 Chemical Analysis.....	25
3.2.1 NH ₄ ⁺ -N, NO ₂ ⁻ -N, NO ₃ ⁻ -N, PO ₄ ³⁻ -P, and VFA	25
3.2.2 N ₂ O and NO	26

3.2.3 Glycogen and PHA	26
3.2.4 Protein peptide	28
3.3 Chemical Staining	28
3.3.1 PHA staining	28
3.3.2 Poly-P staining	29
3.4 Microbial Analysis	29
3.4.1 FISH	29
3.4.2 PCR-DGGE-Sequencing	33
3.5 ATP Measurement	34
3.6 Electron Transport Activity/Respiratory Assay	34
CHAPTER 4 – STUDY ON THE RESPONSE OF GAO TO FNA	
INHIBITION AND REVERSIBILITY OF THE INHIBITION	35
4.1 Summary	35
4.2 Methodology	35
4.2.1 Reactor set-up and operation for GAO culture	35
4.2.2 Media	36
4.2.3 Batch tests	36
4.3 Results and discussions	38
4.3.1 Performance of the parent reactor	38
4.3.2 Effects of FNA on the anaerobic metabolic processes of GAO	41
4.3.3 Effect of FNA on aerobic metabolism and anaerobic carbon uptake recovery	43
4.4 Conclusions	50
CHAPTER 5 – STUDIES ON THE RESPONSE OF PAO TO FNA	
INHIBITION AND INHIBITION REVERSIBILITY	51
5.1 Summary	51
5.2 Methodology	51
5.3 Results and discussions	55
5.3.1 Sludge characterization	55
5.3.2 Demonstration of strong inhibition on DPAO's anoxic metabolism	57
5.3.3 Hypotheses on the mechanisms of FNA inhibition	62
5.3.4 Continued inhibitory effects in the subsequent anaerobic phase	63

5.3.5 Comparison of PAO response to FNA under aerobic and anoxic conditions	64
5.4 Conclusions.....	70
CHAPTER 6 – USING FNA AS A SELECTION FACTOR TO ACHIEVE EBPR PERFORMANCE	71
6.1 Summary.....	71
6.2 Methodology	71
6.2.1 Reactor set-up and operation for the SNDPR reactor.....	71
6.2.2 Media for SNDPR culture.....	72
6.2.3 Kinetic tests.....	73
6.3 Results and discussions.....	74
6.3.1 Strategy to achieve EBPR through partial nitrification	74
6.3.2 Correlation between nitrite shortcut and EBPR development	74
6.3.3 Effect of nitrite accumulation on EBPR performance and community ...	80
6.3.4 Comparison with the conventional system	83
6.4 Conclusions.....	84
CHAPTER 7 – METABOLIC BEHAVIOR OF DENITRIFYING P ACCUMULATING ORGANISMS IN THE PRESENCE OF COEXISTING FREE NITROUS ACID AND CARBON SUBSTRATE.....	85
7.1 Summary.....	85
7.2 Methodology	85
7.3 Results and Discussions	89
7.3.1 Overview of batch test performance	89
7.3.2 Detrimental effects of FNA on the ‘supposed’ anaerobic metabolism of DPAO.....	93
7.3.3 N ₂ O accumulation from denitrification pathway of DPAOs in the presence of extracellular carbon source	97
7.4. Conclusions.....	100
CHAPTER 8 – CONCLUSIONS AND RECOMMENDATION	102
8.1 Conclusions.....	102
8.2 Recommendations.....	103
REFERENCES.....	105

SUMMARY

In current state-of-the-art, the biological process is typically considered the most economical and sustainable method for removal of key pollutants in municipal wastewater, i.e. organic carbon (C), nitrogen (N) and phosphorus (P). A process combining enhanced biological phosphate removal (EBPR) and N removal, called simultaneous nitrification, denitrification and phosphorus removal (SNDPR), has recently been developed and its application is potentially attractive because of its reduced organic carbon requirement (50%) and energy consumption (30%), as well as its reduced alkalinity/chemical requirements. The demands for carbon and energy could further be reduced by implementing partial nitrification in the SNDPR process. However, nitrite/free nitrous acid (FNA) could accumulate in the system and the accumulation would possibly cause deterioration of carbon, nitrogen and/or phosphate removal, and trigger N₂O production. A better understanding of the underlying mechanisms in the process is therefore necessary to improve the nutrients removal performance in wastewater treatment plant (WWTP) using the method, enabling such systems to meet the discharge standards with minimum associated costs and harmful impacts on the environment. This thesis is focused on the inhibitory effects as well as the advantageous exploitation of FNA on SNDPR systems.

The study involved the operation of two lab-scale sequencing batch reactors (SBR) to selectively enrich the two key organisms in an EBPR process, namely polyphosphate accumulating organisms (PAO) and glycogen accumulating organisms (GAO). The main contributions of this thesis are as follows:

- The non-FNA-adapted GAO culture investigated in a sub-study demonstrated its inability to denitrify nitrite. This was in contrast with the non-FNA-adapted PAO culture which was reported to exhibit the nitrite-denitrifying capability. The response of GAOs to FNA inhibition was examined, focusing on the continued FNA inhibition as well as the recovery from inhibition after FNA was removed. In order to better understand FNA effects on PAO, another sub-study also looked into the response of the FNA-adapted PAO population to FNA

inhibition and recovery. Several hypotheses on the mechanisms of inhibition and detoxification were also proposed. The sub-studies concluded that the different response of PAO and GAO to FNA, wherein GAO was unable to utilize nitrite as electron acceptor as opposed to PAO, signified that FNA could be used as a strategic tool to alter the microbial communities towards PAO enrichment to favor the EBPR performance.

- Based on the above findings on the potential role of FNA in PAO enrichment as well as the inhibitory effects of FNA at higher concentrations, the feasibility of partial nitrification to facilitate the achievement of EBPR process was evaluated. An anaerobic/aerobic/anoxic system was implemented. Partial nitrification of ammonia during the aerobic phase allowed an *in-situ* generation of nitrite/nitrous acid at a continuous low-rate feeding mode to ensure minimum FNA inhibition effects while the NO_2^- -anoxic environment applied after the aerobic phase provided a competitive advantage for PAO to prevent the proliferation of GAO, leading to the achievement of excellent EBPR performance.
- The coexistence of FNA and organic carbon (HAc) as a result of NO_2^- leakage into the ‘supposed’ anaerobic condition of the P removal system had been detrimental to the metabolism of denitrifying PAO (DPAO), and therefore deteriorating COD and P removal. Competition for electrons took place between the two key metabolic pathways, namely PHA synthesis and TCA cycle, and between nitrite and nitrous oxide reductases. In the latter case, the competition occurred even when carbon supply was not limiting and resulted in N_2O accumulation.

This study demonstrated that FNA, despite its inhibitory nature, could play a positive role in achieving the EBPR process by manipulating the microbial community structure towards the PAO-enriched system. In view of sustainability, this FNA-based strategy can be beneficially applied in WWTPs to achieve the simultaneous nutrients removal from wastewaters with reduced energy and carbon footprints.

LIST OF PUBLICATIONS

Technology Disclosure

- **Ganda, L.**, Zhou, Y., Liu, Y., Ng, W.J., 2013. Free nitrous acid (FNA) treatment to reduce EPS content and improve sludge dewatering properties (NIEO Ref: TD/148/13; NTU Ref: PAT/148/13/13/US PRV; Application no. 61/509,528 on 27 Nov 2013).
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Journal publications

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- **Ganda, L.**, Zhou, Y., Liu, Y., Ng, W.J. Response of glycogen accumulating organisms to free nitrous acid inhibition. *Submitted to Bioresource Technology*.
- Zhou, Y., **Ganda, L.**, Lim, M., Yuan, Z., Ng, W.J., 2012. Response of poly-phosphate accumulating organisms to free nitrous acid inhibition under anoxic and aerobic conditions. *Bioresource Technology* 116, 340-347.
- Zhou, Y., **Ganda, L.**, Lim, M., Yuan, Z., Kjelleberg, S., Ng, W.J., 2010. Free nitrous acid (FNA) inhibition on denitrifying poly-phosphate accumulating organisms (DPAOs). *Applied Microbiology and Biotechnology* 88, 359-369.

Conference publications

- **Ganda, L.**, Zhou, Y., Tan, C.H., Jiang, X., Liu, Y., Ng, W.J. Effects of Coexistence of FNA and HAc on Simultaneous Nitrification-Denitrification and Phosphorus Removal (SNDPR). Minamata International SympoSIum ON Environment and Energy Technology 2011 (MISSION 2011), 6-8 December 2011, Kumamoto City and Minamata City, Japan, pp. 90-92.
- **Ganda, L.**, Zhou, Y., Yuan, Z., Ng, W.J. Free Nitrous Acid (FNA) Inhibition on Aerobic and Anoxic Phosphate Uptake and Denitrification by Denitrifying Poly-phosphate Accumulating Organisms: A Comparative Study. WEF-IWA Nutrient Recovery and Management 2011, 9-12 January 2011, Miami, Florida, USA, pp. 102-106.

LIST OF TABLES

Table 2.1. Comparison of PAO anaerobic stoichiometry for uptake of 1 mol acetate	17
Table 2.2. Comparison of GAO anaerobic stoichiometry for uptake of 1 mol acetate	20
Table 3.1. FISH probes used in this study	32
Table 4.1. Experimental conditions applied in batch experiments.....	37
Table 4.2. Carbon transformation by GAO in anaerobic phase.....	39
Table 4.3. Comparison of direct FNA inhibition on aerobic metabolisms of non-nitrite/FNA-adapted PAOs (Pijuan et al. 2010) and GAOs (Ye et al. 2010).....	46
Table 5.1. Experimental conditions applied in the batch experiments	53
Table 5.2. Experimental conditions of the batch tests	54
Table 5.3. Anoxic control tests	55
Table 5.4. Calculation of electron balance for aerobic tests	69
Table 5.5. Calculation of ATP balance	70
Table 6.1. Summary of literature on long-term effect of nitrite on EBPR	79
Table 6.2. Comparison of process kinetics at various stages during SNDPR SBR operation	80
Table 6.3. Comparison between the amplified 16S rDNA nucleotide sequences and GenBank database.....	83
Table 6.4. Comparison between the SNDPR (this study) and conventional systems	84
Table 7.1. Experimental condition applied in batch tests	87
Table 7.2. Summary of metabolic activities of DPAOs in the presence of coexisting FNA & HAc as compared to the control test (HAc (+), FNA(-)).....	90
Table 7.3. Time dependent metabolic kinetics observed in Anoxic Test	92
Table 7.4. Comparison of metabolic activities of PAOs across different studies....	94
Table 7.5. Calculation of electron balance between the measured amounts of electron acceptors coupled with the consumed electron donors in Sets 2 & 3	99
Table 7.6. N ₂ O generation and reduction rates from denitrification by DPAO sludge at different conditions	100

LIST OF FIGURES

Figure 1.1. Conceptual design of a state-of-the-art biological wastewater treatment plant.....	1
Figure 2.1. Conceptual design of a continuous nitrification and denitrification plant Conceptual design of a continuous nitrification and denitrification plant (Rittmann and McCarty, 2001)..	8
Figure 2.2. Biological nitrification-denitrification via nitrite pathway (Peng and Zhu 2006)	9
Figure 2.3. Reaction pathway of N_2O production in the nitrification and the denitrification process (Hwang et al. 2006).....	11
Figure 2.4. Conceptual design of an EBPR plant (adapted from Rittmann and McCarty (2001), with modification).....	12
Figure 2.5. Schematic diagram of the metabolism of PAO (Zhou 2008).....	16
Figure 2.6. Schematic diagram of the metabolism of GAO.	18
Figure 4.1. Profiles of carbon transformation, ammonium and phosphate during a typical cycle study of the GAO enrichment reactor.....	39
Figure 4.2. Profiles of pH and DO during a typical cycle in the SBR operation....	40
Figure 4.3. Images of a FISH (blue – Competibacter (targeted by Cy5-labelled GAOMIX probes); red - all bacteria (targeted by FITC-labelled EUBMIX probes)) and b PHA staining of GAO sludge.....	41
Figure 4.4. Effects of FNA on acetate uptake of GAO in the anaerobic inhibition tests.....	42
Figure 4.5. FNA impact on GAO aerobic growth	43
Figure 4.6. FNA impact on GAO aerobic PHA consumption.....	44
Figure 4.7. FNA impact on GAO aerobic glycogen production.....	45
Figure 4.8. Effects of FNA on recovery of GAO anaerobic HAc uptake.....	47
Figure 4.9. HAc uptake rate in multiple cycles of anaerobic recovery	48
Figure 4.10. COD profiles in the initial, inhibition and recovery tests.....	49
Figure 5.1. FISH images of the SNDPR sludge.....	56
Figure 5.2. Images of PHA staining of the SNDPR sludge withdrawn from the SBR at the end of anaerobic period	57

Figure 5.3. Images of poly-P staining of the SNDPR sludge withdrawn from the SBR at the cycle end	57
Figure 5.4. FNA inhibition effects on (a) P uptake, (b) nitrite reduction, (c) PHA degradation, and (d) glycogen production of DPAO	58
Figure 5.5. Intracellular ATP level of biomass incubated at different FNA concentrations	60
Figure 5.6. FNA dependency of biomass intracellular ATP reduction rate.....	61
Figure 5.7. Profiles of DPAO respiratory activity	62
Figure 5.8. Effects of FNA inhibition in the subsequent FNA-free anaerobic phase	63
Figure 5.9. Comparison of aerobic and anoxic phosphate uptake under FNA inhibition.....	65
Figure 5.10. Comparison of aerobic and anoxic nitrite reduction under FNA inhibition.....	66
Figure 5.11. FNA impact on DPAO oxygen uptake rate.....	67
Figure 5.12. Comparison of FNA impact on aerobic and anoxic glycogen production	68
Figure 5.13. Comparison of FNA impact on aerobic and anoxic PHA degradation	69
Figure 6.1. Laboratory-scale SBR for the enrichment of SNDPR and GAO culture	73
Figure 6.2. pH, phosphate, nitrogen, PHA and oxygen concentration profiles during a cycle in the SNDPR reactor	75
Figure 6.3. Development of long-term partial nitrification performance and P-uptake activity (the three stages from left to right: initiation, steady state, and complete nitrification). The Pearson's correlation coefficient $r = 0.8213$	78
Figure 6.4. DGGE patterns of 16S rDNA of the SNDPR community.....	82
Figure 7.1. Experimental setup in N_2O batch study.....	88
Figure 7.2. Nutrient profiles of COD/N = 2.60 for Control A (a), Control B (b), and Anoxic Test (c)	91
Figure 7.3. Comparison of PHA accumulation rate in the presence (Anoxic Test) and absence (Control A) of FNA	95

Figure 7.4. Proteomics profiling displayed some changes in the level of several enzymes involved in DPAO metabolic pathways which could be attributed to FNA inhibition as well as explaining the possible mechanism of FNA inhibition	96
Figure 7.5. Percentages of N ₂ O accumulation (a) and N ₂ O generation (b), accumulation (c), reduction (d) rates at all tested COD/N ratios.....	98

ABBREVIATIONS

AOB	Ammonia oxidizing bacteria
AST	Advanced sludge treatment
ATP	Adenosine triphosphate
ATU	Allyl-N-thiourea
BNR	Biological nutrient removal
CLSM	Confocal laser scanning microscope
COD	Chemical oxygen demand
COD/N	Chemical oxygen demand-to-nitrogen
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
DPAO	Denitrifying poly-phosphate accumulating organism
EBPR	Enhanced biological phosphorus removal
ED	Entner Doudoroff
EMP	Embden – Meyerhoff – Parnas
EPS	Extracellular polymeric substance
FISH	Fluorescent <i>in situ</i> hybridization
FNA	Free nitrous acid
GAO	Glycogen accumulating organism
GC	Gas chromatography
HPLC	High performance liquid chromatography
MLSS	Mixed liquor suspended solids
MLVSS	Mixed liquor volatile suspended solids
N	Nitrogen
N ₂ O	Nitrous oxide
NO	Nitric oxide
NADH ₂	Reduced form of nicotinamide adenine dinucleotide
NOB	Nitrite oxidizing bacteria
OD	Optical density
P	Phosphorus

PAO	Poly-phosphate accumulating organism
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PHA	Poly- β -hydroxyalkanoate
PHB	Poly- β -hydroxybutyrate
PHV	Poly- β -hydroxybutyrate
PH2MV	Poly- β -hydroxy-2-methylvalerate
PLC	Programmable logic controller
pmf	proton motive force
RAS	Recycled activated sludge
SBR	Sequencing batch reactor
SND	Simultaneous nitrification and denitrification
SNDPR	Simultaneous nitrification, denitrification and phosphorus removal
SRT	Sludge retention time
TCA	Tricarboxylic acid
TSS	Total suspended solid
VFA	Volatile fatty acid
WAS	Waste activated sludge
WWTP	Wastewater treatment plant

Chapter 1 – INTRODUCTION

1.1 Background

Lower source water quality and global warming are two global environmental issues that have received increasing attention in the recent decades and can be associated with pollutants removal inadequacies. Often, water quality deterioration is caused by inadequate nutrients removal prior to discharge into aquatic systems. Nutrients, which include phosphorus (P) and nitrogen (N), must be removed from wastewaters to very low concentrations in order to reduce risk of eutrophication. Eutrophication is the condition of a water body whereby there is excessive algae and other aquatic plant growth. The accelerated growth and overcrowding of plants and algae is unfavorable to other aquatic life as they compete for dissolved oxygen (DO). When the algae and plants die off, their decomposition further exerts oxygen demand, hence depleting DO levels in the waterbodies and not infrequently leading to fish kills. In attempts to control eutrophication, biological nutrients removal (BNR) has been accepted as the most economical and sustainable method for the removal of N and P from wastewaters. BNR is typically incorporated in the biological (secondary) process of wastewater treatment train in WWTPs, as illustrated in Fig.1.1 below.

Figure 1.1. Conceptual design of a state-of-the-art biological wastewater treatment plant.

The influent wastewater first goes through primary clarifier, where the heavy organic solids settle and are subsequently pumped away as sludge (termed ‘primary

sludge'). The effluent (termed 'primary effluent') then flows into bioreactor where about 90% of the organic matter present in wastewater is removed. This thesis indeed focuses on the simultaneous removal of soluble organic matter, nitrogen and phosphorus in this biological treatment unit. Depending on the treatment design, the process may consist of a single unit or multiple coordinating units. In the following secondary clarifier, separation of biomass and the treated water takes place prior to discharge or tertiary treatment. Return flow (recycled activated sludge/RAS) from the clarifier is mixed with the primary effluent to re-enter the biological unit, while the accumulated sludge (i.e. primary sludge + waste activated sludge (WAS)) is thickened, digested anaerobically, and subsequently dewatered before the ultimate disposal. The supernatant of sludge thickener (GTO) recycles through the treatment facility.

Conventionally, biological nitrogen removal is achieved through sequential aerobic nitrification and anoxic denitrification while biological phosphate removal is carried out via enhanced biological phosphorus removal (EBPR) process. Typically, EBPR is achieved by recirculating poly-phosphate accumulating organisms (PAO), the key organisms responsible for luxury P uptake, under alternating anaerobic-aerobic conditions. It has been reported that at least some of the PAO communities, namely denitrifying poly-phosphate accumulating organisms (DPAO), are able to use nitrite or/and nitrate as electron acceptors. Besides PAO, another functional group of microorganisms, the so-called glycogen accumulating organisms (GAO) also co-exist in EBPR systems (Liu et al. 1996). The presence of GAO is undesirable as GAO take up the often limiting carbon substrate without performing P removal. Similar to the case of PAO, some of the GAO communities, called denitrifying GAO (DGAO) are also capable of performing denitrification (Zeng et al. 2003c).

Recent developments have shown that simultaneous nitrification, denitrification and phosphorus removal (SNDPR) can take place under alternating anaerobic-aerobic-anoxic conditions. The SNDPR process is realized by the co-performance of nitrifying bacteria and DPAO, which is benefited when chemical oxygen demand (COD) is limited for the BNR processes. This process has been demonstrated wherein COD can be accumulated as an internal carbon source in the anaerobic

phase to be used for both denitrification and phosphate uptake during the aerobic/anoxic phase. Hence, the COD will not be oxidized in the aerobic phase - which greatly reduces the oxygen requirement.

COD requirements for BNR can be further reduced through the “nitrite shortcut”, where nitrite rather than nitrate is the intermediate for nitrogen removal. The nitrite shortcut process will save energy consumption by 25%, carbon source addition by 40%, reduce the reactor volume by 30-40%, reduce sludge production by 35% and 55% in nitrification and denitrification, respectively (Turk and Mavinic 1986; Peng and Zhu 2006). However, the accumulation of nitrite has been found to inhibit the metabolism of various groups of bacteria such as AOB, NOB, denitrifiers, PAO and GAO. Recently, more detailed studies have revealed that in addition to nitrite, pH may also regulate the inhibition, suggesting that FNA, the protonated species of nitrite, is likely the real inhibitor. This implies that FNA may significantly deteriorate the performance of EBPR and nitrogen removal in wastewater treatment facilities (Meinhold et al. 1999; Saito et al. 2004).

Recently, Zhou et al. (2007) reported that FNA inhibited the anoxic metabolic processes performed by PAO, namely phosphate uptake and denitrification, while Pijuan et al. (2010) reported inhibition on the aerobic counterparts, namely growth, phosphate uptake, glycogen production, PHA degradation and denitrification. Since PAO cultures are sequentially exposed to aerobic and anoxic environments in the combined N and P removal systems, comparison of the impact of FNA inhibition on PAO under the two conditions would be useful for obtaining a better strategy in operation of BNR-based WWTP. Besides, there has been little information on the effect of nitrite/FNA resulting from partial nitrification on the EBPR to date. Zeng et al. (2011) reported that the accumulation of nitrite of below 10 mg NO_2^- -N/L did not inhibit P removal processes while EBPR deterioration was observed when nitrite concentration reached 20 mg NO_2^- -N/L. The study, however, only focused on the impact of nitrite on anaerobic P release. The effect of FNA accumulation caused by partial nitrification on aerobic/anoxic P uptake of PAO is thus one of the objectives in this study. In addition to inhibition in the aerobic phase, Pijuan et al.

(2010) also examined recovery in the subsequent FNA-free anaerobic phase. It was revealed in the third cycle of the anaerobic recovery phase, P uptake rate reached 65% of the initial rate for the non-FNA-adapted PAO culture, while the FNA-adapted PAO culture only required two cycles of the anaerobic recovery phase to return to its initial P uptake rate. On the other hand, Ye et al. (2010; 2013) reported the effects of FNA on the key anaerobic and aerobic metabolic processes of GAO. However, to date, there have been no reports on the recovery of GAO after FNA/nitrite inhibition. Investigation on the ability of GAO to recover their carbon uptake activity is essential, particularly in the assessment of the competitiveness of PAO and GAO in EBPR systems with FNA/nitrite accumulation, and this therefore is another objective of this study.

N₂O, a potent greenhouse gas, can be generated and emitted substantially during BNR processes. More research has therefore focused on the development and optimization of nutrient removal strategies towards minimizing greenhouse gas emissions. FNA/nitrite is also known to be an inhibitor of N₂O reduction during denitrification (Zhou et al. 2008b). High nitrite concentration has been reported to inhibit N₂O reductase, leading to emissions of N₂O as the end product of denitrification (Schulthess et al. 1995). Besides FNA/nitrite, type and loading rate of organic carbon has also been known to affect N₂O generation during denitrification. In denitrifying EBPR system where DPAO culture is dominant, denitrification is mainly driven by PHA. Literature has suggested that PHA utilization may lead to a higher N₂O generation as compared to denitrification driven by external organic carbon sources in denitrifying activated sludge systems (Kampschreur et al. 2009). As organic carbon is often limited in municipal wastewaters, understanding the effects of availability and utilization of PHA and external carbon source on N₂O reduction would be useful in the efforts to minimize the emission of N₂O from biological nutrient removal system.

1.2 Objectives and Scope

The overall objectives of this thesis are:

- (1) To understand the response of PAO and GAO, the two co-existing functional bacterial groups in the EBPR process, with respect to FNA. The investigations would include effect of inhibition on key metabolic activities such as biomass growth and carbon transformation (extracellular HAc, intracellular PHA and glycogen) under different electron acceptor(s) conditions, as well as their recovery responses in the subsequent FNA-free environment. The outcome of these investigations is elaborated in Chapter 4 and Chapter 5.
- (2) It would be discussed in the preceding chapters in the thesis that PAO and GAO have differing responses to FNA. This portion of the work would investigate the advantages PAO has against GAO using FNA as electron acceptor to reduce or eliminate GAO communities. The nitrite short-cut process shall be applied on an SNDPR system in order to accumulate FNA and in turn control the GAO population. The study would evaluate the effects of FNA on the P-removal process. The challenges in the study are expected to be: (a) development of partial nitrification using low-strength wastewater, and (b) the long-term effect of FNA on P removal performance as well as the microbial community. This part of the work shall be described in Chapter 6 of this thesis.
- (3) The third portion of the work was intended to scrutinize the metabolic behavior of DPAO in the presence of coexisting electron donor (organic carbon) and electron acceptor (FNA). In the combined partial nitrification-EBPR process, incidental leakage of nitrite from the aerobic/anoxic stage to the anaerobic stage is inevitable, causing simultaneous presence of both electron acceptor and electron donor. This part of the study included comparison between internal PHA- and external carbon-driven denitrification, the comparison of DPAO metabolic behavior under different chemical oxygen demand-to-nitrogen (COD/N) ratio, the assessment of electron balance, examination on N₂O emission, and protein peptide quantification (an assessment of intracellular enzyme level). The outcome of this portion of the study is presented in Chapter 7.

Chapter 2 – LITERATURE REVIEW

This chapter examines the principles of biological nitrogen and phosphate removal processes, describes the recent research and development efforts to enhance nutrient removal performance from wastewaters, particularly in terms of oxygen consumption and carbon footprint reductions, and highlights drawbacks of the process which may lead to environmental issues, such as compromised effluent water quality and N₂O emission. This review aims to underline some knowledge gaps which would then lead to this study.

2.1 Biological Nitrogen Removal

Nitrogen is present in both organic and inorganic forms. The main species of nitrogen are ammonium, nitrite, nitrate, organic matter, and gaseous nitrogen, where ammonium is usually the major form of nitrogen in wastewaters. The assimilation of nitrogenous compound into biomass is carried out by most microorganisms as nitrogen is an essential compound in nucleic acids and proteins in living cells. However, when the nitrogenous compounds are present in excess, their removal from wastewaters is necessary due to their toxicity to both macro- and microorganisms which become a major environmental concern (Rittmann and McCarty 2001; Zeng 2002; Vadivelu 2006).

2.1.1 Conventional biological nitrogen removal

In order to prevent eutrophication, nitrogen in wastewater is required to be removed to very low levels prior to discharge into water bodies. Conventional biological nitrogen removal is achieved through a two-stage treatment process: aerobic nitrification and anoxic denitrification (Metcalf&Eddy 1991). Fig.2.1 illustrates the schematic of such a nitrogen removal plant.

During the nitrification stage, AOB aerobically oxidize ammonium to nitrite (nitritation), which is subsequently oxidized to nitrate by NOB (nitritation). The

overall stoichiometry of nitrification (Eq.1) and nitrification (Eq.2) can be written as the following:

In wastewater treatment systems, nitrification is primarily contributed by autotrophic microorganisms, where inorganic nitrogen is utilized as the sole energy source and inorganic carbon is used as the sole carbon source for biomass synthesis (Vadivelu 2006).

Denitrification is dissimilatory reduction of nitrogen oxides to gaseous nitrogen compounds under anoxic (or suboxic) conditions, coupled with electron transport phosphorylation. Generally, denitrification processes are catalyzed by heterotrophic bacteria with organic carbon as electron donor in wastewater treatment processes. Complete denitrification proceeds in a stepwise manner in which nitrate is sequentially reduced to nitrite, nitric oxide, nitrous oxide and dinitrogen gas. The half-reactions as well as the catalyzing enzymes are as follows (Rittmann and McCarty 2001):

Figure 2.1. Conceptual design of a continuous nitrification and denitrification plant (Rittmann and McCarty, 2001). Conventional biological nitrogen removal can be achieved by a two-stage treatment: aerobic nitrification and anoxic denitrification. During the nitrification stage, autotrophic nitrifiers oxidize NH_4^+ to NO_2^- , then to NO_3^- , using O_2 as electron acceptor. During the denitrification stage, heterotrophic denitrifiers reduce NO_3^- to NO_2^- , then to N_2 gas in the absence of O_2 , using organic carbon substrates (COD) as electron donor. Denitrification tank is typically located prior to the nitrification tank for the following reasons: (1) COD present in the influent wastewater is utilized for denitrification; and (2) this arrangement prevents the oxidation of COD under aerobic conditions which would lead to insufficient amounts for denitrification. Recycle flow returns the produced NO_3^- to denitrification tank.

2.1.2 Nitrogen removal via nitrite pathway

Nitrogen removal via nitrite pathway or “nitrite shortcut” is performed through partial nitrification and denitrification, which can be achieved by the elimination or inhibition of NOB, thus restricting ammonia oxidation to nitrite rather than to nitrate (Fig.2.2). As compared to complete nitrification and denitrification, nitrogen removal via nitrite pathway reduces organic carbon and oxygen demand by 40% and 25%, respectively (Turk and Mavinic 1986; Fux et al. 2003). Partial nitrification to nitrite is conducted through appropriate control of dissolved oxygen (DO), sludge retention time (SRT), pH, temperature, substrate concentration and load, operational and aeration pattern, and inhibitor (Peng and Zhu 2006).

Figure 2.2. Biological nitrification-denitrification via nitrite pathway (Peng and Zhu 2006)

2.1.3 N_2O emissions from biological nitrogen removal processes

The production of N_2O , a potent greenhouse gas which becomes one source of environmental concern due to its high global warming potential, can be substantial during nitrogen removal in a WWTP. A literature analysis by Kampschreur et al. (2009) identified 3 most crucial parameters causing N_2O emission in biological nutrients removal WWTPs: (1) low DO concentrations during nitrification and denitrification, (2) increased nitrite concentrations in both nitrification and denitrification, and (3) low COD/N ratio in denitrification stage.

During nitrification, AOB are able to produce N_2O even though N_2O is not an intermediate in the main catabolic pathway, known as nitrifiers denitrification. Several studies reported that lower DO concentrations led to higher N_2O emission since in oxygen limiting condition AOB use nitrite as the terminal electron acceptor to save oxygen for the oxygenation of ammonia to hydroxylamine (NH_2OH) and in such condition N_2O is produced as byproduct (Zheng et al. 1994; Colliver and Stephenson 2000; Tallec et al. 2006) (see Fig.2.3). While during denitrification, the presence of oxygen is known to inhibit the synthesis and activity of N_2O reductase and hence causing higher N_2O emission (Kampschreur et al. 2009).

Nitrite is widely known as a trigger for N_2O and NO emission (Schulthess et al. 1995). A study by Itokawa et al. (2001) concluded that N_2O emission was attributed to the nitrite build-up in a denitrifying sludge system. In addition to nitrite, FNA has been shown to inhibit N_2O reduction by DPAO sludge (Zhou et al. 2008b).

Another important factor promoting N_2O emission from denitrification processes is the carbon source. Limited carbon source has been shown to increase N_2O emission during denitrification (Schulthess and Gujer 1996; Chung and Chung 2000). Often, efficiency of use of the carbon substrate for denitrification is expressed as the COD/N ratio. Hanaki et al. (1992) studied the impact of COD/N ratios of 1.5, 2.5, 3.5 and 4.5 on N_2O emission and found that the lower COD/N ratios favored N_2O production. In their study, NO_3^- -N was used as nitrogen source. The theoretical value of COD/ NO_3^- -N for achieving complete denitrification is 2.86, however actual denitrification requires COD/ NO_3^- -N of 3.5 since organic matter is also used for biosynthesis. Thus, it was suggested that the higher N_2O generation in the tests where COD/N ratio of 1.5 and 2.5 were applied was due to substrate limiting condition. In this research study, nitrite, instead of nitrate, would be used as the nitrified nitrogen source with the theoretical COD/ NO_2^- -N of 2.4 to achieve full denitrification and fulfill assimilation requirement.

Additionally, the type of carbon source has also been addressed as a factor influencing N_2O emission. In a recent study, Lu and Chandran (2010) investigated the effect of methanol and ethanol as denitrifying carbon sources, on N_2O emission. It was concluded that N_2O emission could vary as a function of specific electron donor. Besides externally available carbon source, PHA has also been suggested to promote N_2O generation from denitrifying activated sludge (Schalk-Otte et al. 2000). In contrast, Zhou et al. (2010b) had revealed that N_2O reduction rates were generally higher with PHA (internal carbon source) utilized for denitrification by DPAO as compared to the utilization of externally available carbon source, suggesting that external carbon source was competed for in different metabolic pathways of DPAO and by other denitrifiers.

Figure 2.3. Reaction pathway of N_2O production in the nitrification and the denitrification process (Hwang et al. 2006)

2.2 Biological Phosphorus Removal

2.2.1 Enhanced biological phosphorus removal

Biological phosphorus removal from wastewater can be obtained in two ways, namely assimilation for microbial growth and luxury uptake for enhanced intracellular storage as poly-P. The former is of secondary importance as bacteria only contain 1-2% (weight % of dry biomass) phosphorus, while the latter, which is also commonly called enhanced biological phosphorus removal (EBPR), plays a crucial role in removing phosphate from wastewaters and have been widely implemented in full-scale BNR systems.

EBPR is achieved by recycling polyphosphate accumulating organisms (PAO) under alternating anaerobic-aerobic conditions. In the anaerobic phase, PAO take up carbon substrates, mainly volatile fatty acids (VFA), and store them intracellularly as PHA. The main forms of PHA are poly- β -hydroxybutyrate (PHB) and poly- β -hydroxyvalerate (PHV) (Comeau et al. 1987). The required energy is primarily obtained from hydrolysis of intracellular poly-phosphate (poly-P), resulting in orthophosphate release to the bulk liquid. Glycogen is also consumed during the anaerobic phase to provide reducing power required for PHA synthesis (Arun et al. 1989; Smolders et al. 1994a). The consumption of glycogen also yields additional energy for the transport and conversion of VFA (Van Groenestijn et al. 1987; Van Groenestijn et al. 1989; Van Heen et al. 1993). In the subsequent aerobic phase, PAO oxidize PHA to generate energy for growth, orthophosphate uptake and glycogen replenishment. Phosphate is removed by withdrawing excess sludge at the

end of the aerobic/anoxic phase when the intracellular poly-P content is high. Fig.2.4 illustrates a schematic of the continuous activated sludge EBPR plant.

Figure 2.4. Conceptual design of an EBPR plant (adapted from Rittmann and McCarty (2001), with modification). A pre-fermenter receives the incoming wastewater and converts the readily biodegradable substrate to volatile fatty acids (represented by COD). In the anaerobic tank, COD is taken up by EBPR sludge and internally stored in the form of PHA, and phosphate is simultaneously released. In the following aerobic tank, PHA is oxidized to generate energy, which is used for microbial growth as well as phosphate uptake. Net phosphorus removal is achieved by wasting the excess sludge with high phosphate content after the aerobic period.

2.2.2 Denitrifying PAO

There are two different groups of PAO: PAO which can only utilize oxygen as the terminal electron acceptor and PAO that can use both oxygen and nitrate or/and nitrite. The former is often called “aerobic PAO” while the latter refers to the widely known DPAO (Kern-Jespersen and Henze 1993). Compared to aerobic PAO, DPAO has been hypothesized to be 40% less efficient in energy generation, leading to 20-30% lower cell yield (Kuba et al. 1994). In addition, based on their activity, DPAO could be classified into two groups: (1) nitrate DPAO, which can use both oxygen and nitrate as electron acceptor; (2) nitrite DPAO, which can use oxygen, and nitrite as electron acceptor (Hu et al. 2003). However, not all of the latter are able to use nitrate as electron acceptor, as reported by Carvalho et al. (2007) and Guisasola et al. (2009).

It was reported that DPAO had similar potential for phosphorus removal (Kuba et al. 1993) and a similar biological metabolism based on intracellular PHA and glycogen as aerobic PAO (Kuba et al. 1994; Kuba et al. 1996a; Ahn et al. 2001). A number of studies had attempted to determine if DPAO and PAO are the same organism. A study by Zeng et al. (2003b) showed that the PAO dominating the anaerobic – aerobic SBR sludge were the same organisms as the DPAO. Recently, Oehmen et al. (2010a) revealed that PAO and DPAO were phylogenetically different through quantitative fluorescence *in situ* hybridization (FISH). The study found that *Accumulibacter* Type I was likely to be able to reduce nitrate and *Accumulibacter* Type II was not likely to be able to reduce nitrate. They also proposed that the relative chemical activity of PAO and DPAO could be best estimated using the ratio of total aerobic/anoxic P uptake capacity instead of the ratio of initial P uptake rates.

2.2.3 Metabolisms of PAO

The transport and conversion of VFA to PHA take place for the purpose of sequestering the available electron donor and carbon in the form of insoluble intracellular solid since no electron acceptors are present under anaerobic condition. For the polymerization of PHA, the cells require an activated chemical form, acetyl-CoA. The synthesis from acetyl-CoA to PHA requires reducing power, NADH_2 . The energy demand for the formation of acetyl-CoA is derived from the hydrolysis of poly-P (energy storage material) (Rittmann and McCarty 2001). The ATP produced by poly-P hydrolysis is also used to reestablish the proton motive force (pmf) consumed by substrate transport. The degradation of poly-P produces metallic cations, like K^+ , Ca^{2+} and Mg^{2+} , and H_2PO_4^- anions which are released to the bulk liquid. The literature suggests two possible biochemical models to explain the source of the reducing equivalents (NADH_2), namely the adapted Mino Model and the Comeau-Wentzel model. Comeau et al. (1986) theorized that the required NADH_2 was generated through TCA (tricarboxylic acid) cycle. On the other hand, Mino et al. (1987) concluded that the degradation of the intracellular glycogen through glycolysis (Embden – Meyerhoff – Parnas (EMP) pathway) supplied the reducing power to reduce acetyl-CoA to PHB. While Maurer et al. (1997) found

that PAO use the Entner – Doudoroff (ED) pathway to hydrolyze glycogen, instead of EMP pathway. The difference between the EMP and ED pathways is the amount of ATP generated from glycogen degradation.

A complete PAO anaerobic model developed by Smolders et al. (1994a) using acetate as the sole carbon source is presented in the following reactions:

- Acetate uptake and storage as PHB

with α_{PAO} is the energy requirement to transport one C-mol acetate across the cell membrane.

- Polyphosphate degradation

- Glycogen hydrolysis via the EMP pathway:

With the assumption that no ATP or $NADH_2$ accumulates within the cells, the overall stoichiometric equation for PAO under anaerobic condition can be expressed as follows:

α_{PAO} can be calculated as follows:

$$\alpha_{PAO} = 0.19 pH_{out} - 1.18 \quad \text{Eq.11}$$

According to Maurer et al. (1997), 2 units of ATP are generated from the conversion of 1 unit of glycogen into 2 units of pyruvate via the ED pathway, instead of 3 units of ATP through the EMP pathway. For this reason, Eq.9 is changed to:

Incorporation of the ED pathway into the above model results in the following equation:

$$-CH_2O - \frac{1}{2}CH_{10}O_{\frac{5}{6}} - \left(\frac{1}{3} + \alpha_{PAO}\right)HPO_3 - \left(\alpha_{PAO} - \frac{1}{12}\right)H_2O + \left(\frac{1}{3} + \alpha_{PAO}\right)H_3PO_4 + \frac{4}{3}CH_{1.5}O_{0.5} + \frac{1}{6}CO_2 = 0 \quad \text{Eq.13}$$

The overall internal processes in the anaerobic phase at pH 7 can be summarized as:

$$(CH_3COOH)_{\frac{1}{2}} + 0.5(C_6H_{10}O_5)_{\frac{1}{6}} + 0.48HPO_3 + 0.023H_2O \rightarrow 1.33(C_4H_6O_2)_{\frac{1}{4}} + 0.17CO_2 + 0.48H_3PO_4 \quad \text{Eq.14}$$

Table 2.1 summarizes the anaerobic metabolic models of PAO culture with acetate as carbon source which have been developed by previous researchers. The models are based on a carbon-mol (Cmol) basis. ATP for the anaerobic maintenance is generated from phosphate release (Murnleitner et al. 1997) , as follows:

When PAOs are exposed to aerobic/anoxic conditions, they have an ample supply of electron acceptors. PHA is hydrolyzed to acetyl-CoA which is then oxidized in the TCA cycle. The released electrons are carried on NADH₂ and used for ATP synthesis. The produced ATP is used to synthesize poly P, replenish intracellular glycogen, and for cell growth (Rittmann and McCarty 2001). The overall schematic of metabolism of PAO is described in Fig.2.5.

The PAO aerobic metabolic model developed by Smolders et al. (1994b) can be described in the following reactions:

- Growth

$$-1.27CH_{1.5}O_{0.5} - 0.20NH_3 - 0.015H_3PO_4 - 0.42O_2 + CH_{2.09}O_{0.54}N_{0.20}P_{0.015} + 0.27CO_2 + 0.305H_2O = 0 \quad \text{Eq.16}$$

- Maintenance

$$-CH_{1.5}O_{0.5} - 1.125O_2 + CO_2 + 0.75H_2O = 0 \quad \text{Eq.17}$$

- Phosphate uptake

- Glycogen formation

Figure 2.5. Schematic diagram of the metabolism of PAO (Zhou 2008). Under anaerobic condition, PAO take up extracellular volatile fatty acids, mainly acetic acids (HAc), and store them as PHA. Simultaneously, intracellular glycogen and polyphosphate are degraded, while the latter leads to significant phosphate release. Under the subsequent aerobic/anoxic condition, PHA is oxidized by O_2/NO_x . The energy generated is used for biomass growth, phosphate uptake and glycogen replenishment.

Murnleitner et al. (1997) elaborates the ATP-generating and -requiring processes in the aerobic/denitrifying phase, as follows:

- Polyphosphate formation

- Growth and maintenance

- Glycogen formation

- PHA degradation within the TCA cycle

- ATP production from NADH (with oxygen)

- ATP production from NADH (with nitrate)

Table 2.1. Comparison of PAO anaerobic stoichiometry for uptake of 1 mol acetate

Model	PHA/C	PHB/C	PHV/C	PH2MV/C	Gly/C	P/C
Comeau-Wentzel. (Comeau et al. 1986; Wentzel et al. 1986)	0.89	0.89	N/A	N/A	0	0.5
Mino et al. (1987)	1.33	1.33	N/A	N/A	0.5	N/A
Smolders et al. (1994a)	1.33	1.33	0	N/A	0.5	0.48
Filipe et al. (2001b)	1.33	1.33	0	N/A	0.5	0.57
Hesselmann et al. (2000)	1.40	1.11	0.29	N/A	0.61	0.37
Yagci et al. (2003)	1.23	1.08	0.12	0.03	0.48	0.48
Lu et al. (2006)	1.25	1.18	0.07	0	0.46	0.62

*Units shown are in C-mol or P-mol

2.2.4 Glycogen accumulating organism (GAO)

Besides PAO, another functional group of microorganisms, the so-called glycogen accumulating organism also exist in EBPR systems (Liu et al. 1996; Saunders et al. 2003). PAO and GAO compete for the carbon source since GAO also take up carbon anaerobically but without performing P removal, thus their presence may

lead to deterioration of EBPR performance (Oehmen et al. 2007). Under anaerobic condition, GAOs take up VFA and store this intracellularly as PHA. The required energy for this biotransformation is obtained from degradation of their intracellular glycogen. Under subsequent aerobic/anoxic condition, GAOs oxidize PHA for cell growth and glycogen replenishment. The overall schematic of GAO metabolism is described in Fig.2.6 below.

Figure 2.6. Schematic diagram of the metabolism of GAO. Under anaerobic condition, intracellular glycogen is degraded in order to supply the required ATP and NADH₂ to synthesize PHA from the organic carbon substrate (mainly HAc) taken up. Under aerobic/anoxic condition, GAOs oxidize the stored PHA for biomass growth and glycogen replenishment.

2.2.5 Metabolism of GAO

Three reactions occur under anaerobic conditions, namely VFA uptake, glycogen hydrolysis and PHA synthesis. The anaerobic metabolic model for GAO developed by Filipe et al. (2001a) can be detailed as follows:

- Acetate uptake

$$-acetate - (1 + 2\alpha_{GAO})ATP + acetylCoA = 0 \quad \text{Eq.20}$$

- Glycogen degradation through the EMP pathway

- Production of acetyl-CoA

- Production of propionyl-CoA from pyruvate through the propionate-succinate pathway

- Production of PHA from acetyl-CoA and propionyl-CoA

Based on the assumption that no ATP, $NADH_2$, acetyl-CoA and propionyl-CoA accumulate within the cells, the overall stoichiometry for anaerobic metabolism of GAO can be expressed as:

$$\begin{aligned} & -acetate - \left(\frac{21}{24} + \frac{3}{2}\alpha_{GAO} \right) glycogen + \frac{\left(\frac{35}{24} + \frac{1}{2}\alpha_{GAO} \right)^2}{\left(\frac{19}{12} + \alpha_{GAO} \right)} PHB + \\ & \frac{2.5 \left(\frac{35}{24} + \frac{1}{2}\alpha_{GAO} \right) \left(\frac{3}{24} + \frac{1}{2}\alpha_{GAO} \right)}{\left(\frac{19}{12} + \alpha_{GAO} \right)} PHV + \frac{1.5 \left(\frac{3}{24} + \frac{1}{2}\alpha_{GAO} \right)^2}{\left(\frac{19}{12} + \alpha_{GAO} \right)} PH2MV + \left(\frac{11}{48} + \frac{1}{4}\alpha_{GAO} \right) CO_2 = 0 \end{aligned} \quad \text{Eq.25}$$

α_{GAO} can be calculated as follows:

$$\alpha_{GAO} = 0.057 pH_{out} - 0.34 \quad \text{Eq.26}$$

In the above model, the succinate-propionate pathway is used to describe the production of propionyl-CoA, an intermediate product in the above-mentioned pathway. In this manner, significant errors in the stoichiometric coefficients exist as there is an unaccounted ATP consumption from succinate to succinyl-CoA as a result of stopping at propionyl-CoA instead of propionate. Zeng et al. (2002) modified the model, as follows:

$$\begin{aligned}
& -acetate - (1 + 2\alpha_{GAO})glycogen + \frac{\left(\frac{9}{6} + \frac{2}{3}\alpha_{GAO}\right)^2}{\left(\frac{5}{3} + \frac{4}{3}\alpha_{GAO}\right)} PHB + \\
& \frac{2.5\left(\frac{9}{6} + \frac{2}{3}\alpha_{GAO}\right)\left(\frac{1}{6} + \frac{2}{3}\alpha_{GAO}\right)}{\left(\frac{5}{3} + \frac{4}{3}\alpha_{GAO}\right)} PHV + \frac{1.5\left(\frac{1}{6} + \frac{2}{3}\alpha_{GAO}\right)^2}{\left(\frac{5}{3} + \frac{4}{3}\alpha_{GAO}\right)} PH2MV + \left(\frac{1}{4} + \frac{1}{4}\alpha_{GAO}\right) CO_2 = 0
\end{aligned}$$

Eq.27

At pH 7, the overall internal processes of GAO in anaerobic phase can be summarized as:

$$\begin{aligned}
& -acetate - 1.12glycogen + 1.358PHB + 0.456PHV + 0.0367PH2MV + \\
& 0.27CO_2 = 0
\end{aligned}$$

Eq.28

Table 2.2. Comparison of GAO anaerobic stoichiometry for uptake of 1 mol acetate

Model	PHA/C	PHB/C	PHV/C	PH2MV/C	Gly/C
Zeng et al. (2002)	1.85	1.358	0.456	0.0367	1.12
Filipe et al. (2001a)	1.721	1.348	0.351	0.0219	0.965
Liu et al. (1994)	1.76-1.91	1.167	0.676	0.063	1.105-1.25

2.3 SND and SNDPR

Simultaneous nitrification and denitrification (SND) for nitrogen removal depends on concurrent ammonium oxidation and denitrification under identical operating conditions (low DO concentration). The application of SND offers two advantages in wastewater treatment systems: (1) a reduction in capital and operational costs by eliminating the separate anoxic denitrification tank and recycle flows, and (2) simplification of the operating process by the removal of anoxic stage (Zeng 2002).

Lately, the efforts to integrate biological nitrogen and phosphate removals have been increasing, owing to the potential savings in energy and organic carbon. Earlier, Kuba et al. (1996b) reported that denitrifying dephosphatation, as an alternative to conventional activated sludge process, could decrease oxygen requirement and sludge production by about 30% and 50%, respectively. Recent developments have shown that SND and EBPR can take place concurrently under alternating anaerobic/aerobic/anoxic conditions (Zeng et al. 2003a). This integration

has received increasing attention as the soluble COD in domestic wastewaters is typically limiting. Denitrifying dephosphatation over nitrite can achieve SNDPR at even lower carbon and energy consumption.

2.4 Free Nitrous Acid Inhibition

Nitrite has generally been recognized as an inhibitor of metabolisms of various groups of bacteria such as AOB (Anthonisen et al. 1976; Vadivelu et al. 2006b), NOB (Boon and Laudelaout 1962), denitrifiers (Almeida et al. 1995) and PAOs (Meinhold et al. 1999; Saito et al. 2004; Yoshida et al. 2006; Zhang et al. 2010).

Recently, more detailed studies have revealed that in addition to nitrite, pH has also been observed to have significant impact on inhibition, suggesting that FNA, the protonated species of nitrite (HNO_2), is likely the real inhibitor (Prakasam and Loehr 1972; Vadivelu et al. 2006a; Zhou et al. 2007; Zhou et al. 2008b; Ye et al. 2010). FNA concentration is calculated using the following formula (Anthonisen et al. 1976):

$$\frac{S_{\text{NO}_2^- - \text{N}}}{K_a \times 10^{\text{pH}}} \quad \text{Eq.7}$$

$$K_a = e^{-2300/(273+T)} \quad \text{Eq.8}$$

where $S_{\text{NO}_2^- - \text{N}}$ and T represent nitrite concentration and temperature ($^{\circ}\text{C}$), respectively.

In previous studies, nitrite had been reported to inhibit both aerobic and anoxic phosphate uptake by PAOs in EBPR processes (Meinhold et al. 1999; Hu et al. 2003; Saito et al. 2004; Yoshida et al. 2006; Sin et al. 2008), with aerobic phosphate uptake being more sensitive to nitrite as compared to anoxic phosphate uptake (Saito et al. 2004). More recently, Pijuan et al. (2010) reported that aerobic metabolism of PAOs in terms of biomass growth, phosphate uptake, respiration, PHA degradation, and glycogen replenishment, were seriously affected by FNA. Since FNA-induced inhibition has been proposed to severely deteriorate BNR performance in full-scale WWTPs (Meinhold et al. 1999; Saito et al. 2004), a comprehensive understanding of the impact of FNA on EBPR system under various conditions is required.

2.5 Impact of Coexistence of Electron Donor and Electron Acceptor on EBPR

As mentioned in Section 2.2.3, a characteristic feature of the EBPR plant is recirculation of poly-phosphate accumulating organisms, the organisms responsible for the “luxury” P uptake, through alternating anaerobic and aerobic environments for the provision of physical/temporal separation between the electron donors (COD) and the terminal electron acceptor (O_2/NO_x). Utilization of NO_x as electron acceptor for the oxidation of intracellular carbonaceous compound in the anoxic tank by DPAO often led to an incidental transfer of NO_x to the anaerobic tank with RAS. Thus far, numerous reports in the literature have reported on the undesirable impact of nitrate introduction to the anaerobic phase of an EBPR system due to coexistence of both the electron donor and the terminal electron acceptor (Kuba et al. 1994; Barker and Dold 1996; Chuang et al. 1996; Yuan and Oleszkiewicz 2010). Such occurrence has been detrimental to the P removal performance as it suppressed P release and PHA formation under the “supposed” anaerobic condition, and consequently reduced the available PHA for oxidation in the aerobic/anoxic condition. Iwema and Meunier (1985) elaborated two possible mechanisms for inhibition of EBPR in the presence of nitrate, i.e. shift in redox potential of the “supposed” anaerobic environment and carbon source competition with denitrifying bacteria.

There are several other situations which also lead to the simultaneous presence of electron donors and final electron acceptors and thus inducing EBPR failure. For instance, heavy rainfall and after weekend conditions, and WWTPs with extended aeration operation, these often shortening the anaerobic phase and prolonging the aerobic phase, causing leakage of carbon substrate into the aerobic environment (Brdjanovic et al. 1998; Pijuan et al. 2006). Pijuan et al. (2006) investigated the behavior of PAO under continuous aerobic condition in the presence of carbon substrate which would often be encountered due to the temporarily shortened anaerobic hydraulic retention time. Their study concluded that exposure of PAO to excessive aeration deteriorated the P removal capacity of PAOs as well as changed the metabolic activities with respect to its intracellular biochemical transformations

but without significant changes in the microbial community. As a consequence of oxygen presence in the bulk liquid, P released per C-uptake was lower than that of the classical anaerobic/aerobic condition, which was in agreement with a study by Kuba et al. (1994) where both nitrate and carbon substrate were present in the bulk liquid. Guisasola et al. (2004) revealed that other heterotrophic organisms might be responsible for additional acetate uptake and PHA formation under aerobic conditions, which also supported the “carbon source competition” mechanism. It could therefore be deduced that the behavior of PAO did not vary significantly when COD as electron donor and oxygen or nitrate as electron acceptor were coupled.

To further improve the energy efficiency and reduce the overall carbon footprint, inclusion of partial nitrification in biological C/N/P removal systems has been considered and assessed in laboratory-scale studies (Meinhold et al. 1999; Saito et al. 2004; Lemaire et al. 2006; Zhou et al. 2008a; Coma et al. 2010) as well as in pilot-scale studies (Sin et al. 2008; Yuan and Oleszkiewicz 2011). However, inhibition of microbial activities due to FNA/nitrite accumulation in such systems has been reported in many cases (Meinhold et al. 1999; Saito et al. 2004), thus deterring potential application in full-scale WWTPs. FNA was shown to have a major impact on the P-uptake, NO_x reduction and several other metabolic activities of (D)PAO under aerobic and anoxic conditions (Zhou et al. 2008b; Ma et al. 2010; Zhou et al. 2010a) as well as carbon uptake, P-release and intracellular carbon transformations of PAO under anaerobic condition (Ye et al. 2011). However, to date, there has been no publication specifically reporting the metabolic behavior of DPAO in the simultaneous presence of carbon substrates and nitrite/FNA, a phenomenon which is likely to occur due to the FNA/nitrite transfer to anaerobic tank and/or the temporarily shortened anaerobic period. This trace leakage of nitrite/FNA may contribute to failure of the EBPR system operated in combination with nitrogen removal via nitrite-shortcut in the full-scale WWTP. In addition, N_2O rather than N_2 was in most cases the major denitrification end-product, which is undesirable, being an environmental concern (Zeng et al. 2004), as well possibly adversely compromising the advantages of the biological C/N/P removal process

(Lemaire et al. 2006). Investigation on N_2O emission from such denitrification-EBPR is thus gaining importance in relation to its future full-scale application. Such study is crucial as it could provide a deeper insight on the biochemical transformations of cellular carbonaceous compounds as well as the fate of phosphate and nitrogen species in the presence of coexisting FNA/nitrite and external substrate.

Chapter 3 – MATERIALS AND METHODS

Analytical Procedures

3.1 Biomass Concentration

MLSS and MLVSS were analyzed in accordance with Standard Methods (APHA 1995). Briefly, 10 ml of mixed liquor was filtered through the previously prepared filter paper (GF/C 47 mm, Whatman) using a vacuum pump. The biomass cake and the filter were subsequently dried at 103°C in an oven overnight to reach a constant weight, which was recorded to 0.1 mg accuracy. The total weight of the cake and the filter were measured and used to compute the MLSS value. For the determination of MLVSS, the MLSS filter was heated in a furnace at 550°C for 0.5 h. The filter cake was then reweighed after cooling in a desiccator. The MLVSS value was obtained from the difference in the two weights.

Preparation of GF/C filter paper was carried out as follows: (1) the filter paper was washed by filtering 30 mL milli-Q water using a vacuum pump; and (2) the wet filter paper was placed on aluminium foil and dried at 103°C overnight to constant weight.

3.2 Chemical Analysis

3.2.1 $\text{NH}_4^+\text{-N}$, $\text{NO}_2^-\text{-N}$, $\text{NO}_3^-\text{-N}$, $\text{PO}_4^{3-}\text{-P}$, and VFA

The ammonia ($\text{NH}_4^+\text{-N}$), nitrite ($\text{NO}_2^-\text{-N}$), nitrate ($\text{NO}_3^-\text{-N}$) and orthophosphate ($\text{PO}_4^{3-}\text{-P}$) concentrations were analyzed in accordance with Standard Methods (APHA 1995). Commercially available NH_4^+ , NO_2^- , NO_3^- and PO_4^{3-} standards (Hach, USA) are used for obtaining all calibration curves. Acetate and propionate were measured with the Agilent GC and DB – FFAP 15 m $\times$ 0.53 mm $\times$ 1.0 μm (length $\times$ ID $\times$ film) column. The injector and FID detector were operated at 250°C and 300°C, respectively. High purity helium was used as carrier gas at a constant pressure of 103 kPa. 0.9 mL of the filtered sample was transferred into a GC vial to which 0.1mL of formic acid was added. For the analysis, a volume of 1 μL of

sample was injected in splitless mode. Commercial VFA standard obtained from Supelco (USA) was used to construct the calibration curve.

3.2.2 N₂O and NO

Dissolved N₂O was determined online with a N₂O microsensor (N₂O25, Unisense A/S, Aarhus, Denmark). The sensor was calibrated using pure N₂O standard gas (Soxal, Singapore) prior to each measurement. The upper limit of microsensor calibration used in this work was 370.8 µmol N₂O/L (equivalent to 10.4 mg N₂O-N/L). For the extraction of dissolved gases from the liquid phase, the tubes containing the mixed liquor samples were shaken overnight in room temperature to reach the equilibrium of gas and liquid phases. The liquid phases were subsequently transferred to new vacuum tubes for extraction and analysis of any residual NO and N₂O. Gaseous NO and N₂O were determined using an Agilent GC 6890 coupled with GS Gaspro 60m×0.32mm×1.0µm capillary column. For the analysis, 100 µL of gas samples were manually injected into the GC completed with the micro-cell electron capture detector (µ-ECD). The oven and the µ-ECD were operated at 35°C and 340°C, respectively, while the inlet pressure was constantly maintained at 141.3 kPa. Helium was used as the carrier gas at a flowrate of 3 mL/min. The NO and N₂O detection limit of the GC was 0.1 ppm.

3.2.3 Glycogen and PHA

25 mL mixed liquor samples were taken at intervals and each immediately put into a centrifuge tube (Greiner Bio-One, Germany) with 1 mL 37% w/v formaldehyde (Merck, Germany). Biomass fixation with formaldehyde was carried out in order to stop all biological activities immediately. After 1 h incubation, the tubes were centrifuged at 5000 rpm for 8 min. The supernatant was discharged and the biomass pellet was washed with 25 mL PBS in order to remove any salts attached to the biomass. The biomass pellet was frozen at -20°C and then freeze-dried at 0.01 mbar vacuum and -45°C overnight.

Glycogen was extracted according to the methods described in Zeng et al. (2003c). A volume of 5 mL of 0.6 M HCl was added to the weighed lyophilized biomass in screw-topped glass tubes, and then digested at 105°C for 6 hours. After cooling and centrifugation, 40 µL of the supernatant was used in the carbohydrate assay for glucose analysis.

Determination of glucose concentrations was conducted by colorimetric method. For the assay, anthrone reagent (0.125% anthrone in 95% sulphuric acid) was freshly prepared and used within 2 hours. Glucose sample was prepared by diluting 40 µL of the sample to a volume of 0.4 mL using sterile milli-Q water (10×dilution) in 2 mL micro-centrifuge tube. 0.8 mL of anthrone reagent was then slowly added into the tube and mixed by inversion. The tube was heated to 100°C using dry bath for 14 min and then put in the 4°C fridge for 5 min cooling. The mixture was then transferred into individual cuvette and allowed to reach room temperature. After that, absorbance values were measured at 625 nm using spectrophotometer. A series of glucose standards (0-500 ppm) was also prepared and included in the above assay to construct the calibration curve.

PHA analysis was performed using the method of Oehmen et al. (2005a) to determine PHB, PHV, and PH2MV. Weighed freeze-dried biomass and PHB/V and PH2MV standards (Aldrich, USA) were put into screw-topped glass tubes. The standards used consist of 3-hydroxybutyric acid and 3-hydroxyvaleric acid (88% and 12% wt) and 2-hydroxycaproic acid (0-2.5 mg). The tubes were heated at 100°C for 20 hours after being suspended in 2 mL methanol acidified with 3% H₂SO₄ and 2 mL chloroform. After cooling, 1 mL Milli-Q water was added and the sample was mixed. When the phases were separated, around 1 mL of the bottom organic layer was transferred to the GC vials for analysis.

The quantification of PHA was performed by GC using column DB-5ms 30 m × 0.25 mm × 0.25 µm (length × ID × film). High purity helium was used as carrier gas at 138 kPa. 3 µL of sample volume was injected in split mode. Both injector port and FID detector were operated at 250°C.

3.2.4 Protein peptide

Relative protein peptides quantification was performed using the method of Zhang et al. (2008). Briefly, biomass were sampled and lysed in 150 μ L of lysis buffer (8M urea, 4% w/v CHAPS and 0.05% SDS w/v) on ice for 20 min with regular vortexing. Cell lysates were subsequently centrifuged at $15,000 \times g$ for 60 min at 4°C, the supernatant was harvested, followed by protein quantification using the 2-D Quant Kit (GE Healthcare, UK). The protein concentrations were quantitated with a standard curve constructed using BSA provided in the kit. A volume of 100 μ g was then precipitated by addition of four volumes of cold acetone at -20°C for 2h. After centrifugation at $15,000 \times g$ for 60 min at 4°C, supernatant was discarded, and the precipitates were dissolved in solution buffer, denatured, and cysteines were blocked following the iTRAQ protocol (Applied Biosystems, USA). Samples were then digested with 20 μ L of 0.25 μ g/ μ L sequence grade modified trypsin solution (Promega, USA) at 37°C overnight and labeled with iTRAQ tags. The labeled samples were subsequently pooled for LC-MS/MS analysis. Relative quantification of proteins was obtained from the ratio of the areas under the peaks at 114, 115, 116, and 117 Da, which were the masses of the tags corresponding to the iTRAQ reagents.

3.3 Chemical Staining

3.3.1 PHA staining

The presence of PHA as intracellular granules was confirmed by staining the cells with Sudan black-B. Thin smear of the sludge was applied on a clean glass slide and was heat-fixed. The slide was then immersed in 0.3% (w/v) Sudan black-B solution (in 70% ethanol) for 10 min. Finally, the microscopic slide was counter-stained for 10 s with aqueous safranin (0.5% w/v). The slide was then rinsed with tap water, blot dried and examined under light microscope.

3.3.2 Poly-P staining

Neisser staining method was used to confirm the presence of polyphosphates stored within the cells. The following solutions were required: (A) 0.1 g methylene blue, 5 mL glacial acetic acid, 5 mL 96% ethanol, and 100 mL distilled water, (B) 3.3 mL 10% crystal violet in 96% ethanol, 6.7 mL 96% ethanol, and 100 mL distilled water, and (C) 33.3 mL 1% chrysoidin Y and 100 mL distilled water. Firstly, a fixed smear was prepared. A mixture of solutions A and B with ratio of 2:1 was freshly prepared and applied on the slide for a contact period of 10-15 s. The excess dye was allowed to run off the slide afterwards. Subsequently, solution C was placed onto the slide for a contact period of approximately 45 s. The slide was then rinsed with tap water with the flow against the back of the slide. Finally, the slide was allowed to dry prior to the viewing with a 100X bright field objective.

3.4 Microbial Analysis

3.4.1 FISH

Fluorescent *in situ* hybridization (FISH) was performed as previously described in Amann (1995). FISH involves the following steps: sample fixation and storage, oligonucleotide probe hybridization, washing and biomass visualization and quantification.

Sample Fixation

Sample fixation was carried out by using 4% paraformaldehyde (PFA). PFA fixative and sludge sample were added at a volumetric ratio of 3 and held at 4°C for 1 to 3 hours. The mixture was then spun down for 5 minutes to remove the fixative. The cells were subsequently washed with 1 × PBS. After washing, the samples were resuspended in 1:1 of 1 × PBS and 100% ethanol solution and stored at -20 °C. The PFA and PBS preparation procedures are described below.

4% PFA solution was prepared as follows: (1) heat 65 mL of autoclaved milli-Q water to 60°C, (2) add 4 g PFA, (3) add 2 drops of 2 M NaOH and stir rapidly to dissolve the PFA, (4) cool down to room temperature, (5) add 33 mL of 3×PBS, (6)

adjust to pH 7.2 with 1 M HCl, (7) remove any undissolved crystals by filtering through 0.2 μm filter unit, (8) immediately cool to 4°C and store at this temperature.

10 $\times$ PBS (0.1 M) was prepared by mixing 25.8 g of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 4.4 g of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, and 75.4 g NaCl to 1 L milli-Q. The mixture was then autoclaved and stored at room temperature.

10 μL of fixed cell suspension was applied to the glass slide and air dried on the bench for several hours. The sample was then coated with 0.5% solution of DNA grade agarose in order to prevent sample loss during hybridization procedure. The slide was dehydrated in an ethanol series (3 min in each): 50%, 80% and 98% ethanol. The dehydrated slide could then be stored for a long term at -20°C.

Oligonucleotide Probe Hybridization

Oligonucleotide probes used in this study are summarized in Table 3.1. For the hybridization procedure, the following items were required: (1) 50 mL screw-top tube, (2) tissues, (3) hybridization buffer (composition: 360 μL of 5 M NaCl, 40 μL of 1 M Tris/HCl, 700 μL of formamide, 898 μL of Milli-Q water, 2 μL of 10% SDS) prepared in 2 mL sterile microcentrifuge tubes on the day of use, (4) hybridization oven at 46°C, and (5) probes.

8 μL hybridization buffer was added to each well on the slide and the remainder was used to keep the chamber in 50 mL tube moist. 1 μL of the probe from a stock concentration of 25 ng/ μL was then added and carefully mixed. The slide was then placed in 50 mL tube containing moistened tissue. The tube was put into hybridization oven at 46°C for 1.5 hours.

Equal amounts of probes PAO462, PAO651, and PAO846, each of which targeted most “*Candidatus Accumulibacter phosphatis*” or *Accumulibacter*, were mixed to form PAOMIX probes which were labeled with Cy3. For PAO quantification, Cy5-labelled EUBMIX probes (comprising equal amounts of probes EUB338, EUB338-II, EUB338-III which targeted most bacteria) were used.

“*Candidatus* Competibacter phosphatis” or *Competibacter* was detected using Cy5-labelled GAOMIX probes (consisting of equal amounts of probes GAOQ431 and GAOQ989). Cy5-labelled DF1MIX comprising equal amounts of probes DEF218 and DEF618 were used to target *Defluviicoccus vanus* cluster 1-related bacteria or α -GAO. Cy5-labelled DF2MIX, comprising equal amount of probes DF988 and DF1020 plus helper probes H966 and H1038, was used for detecting *Defluviicoccus vanus* cluster 2-related GAOs. All of GAOMIX, DF1MIX and DF2MIX probes were against FITC-labeled EUBMIX probes.

Washing

Composition of the wash buffer is as follows: (1) 700 μ L of 5 M NaCl, (2) 1 mL of 1 M Tris/HCl, (3) 500 μ L of 0.5 M EDTA, (4) 47.75 mL milli-Q water, and (5) 50 μ L of 10% SDS. The wash buffer was warmed to 48°C using water bath during hybridization. After hybridization, the slide was removed from the tube and rinsed immediately with wash buffer by pipetting a small amount of the wash buffer gently over the slide. Slide was then placed into the washing buffer tube and into the water bath at 48°C for 10-15 min. Rapid transfer of slides prevents cooling which could lead to non specific probe binding. After the wash, the slide was removed, and gently immersed in the cold water (4°C) for approximately 2-3 seconds, to remove the salt, which was highly autofluorescent. The slide was immediately air dried using compressed air.

Biomass Visualization and Quantification

Prior to visualization, anti-fading mountant solution was applied to the samples. Confocal laser scanning microscope (LSM 510 Meta, Zeiss) with Plan-Apochromat $\times$ 63 oil (NA1.4) objective was used to visualize the FISH-probe hybridized biomass. Thirty images were taken from each sample for quantification. All the images were 8-bit, 512 $\times$ 512 pixel with 0.1 μ m pixel size. The area containing cells targeted by the Cy3- or Cy5-labelled specific probes (PAOMIX, GAOMIX, or α -GAO) was quantified as a percentage of the area of Cy5 or FITC labeled bacteria probe (EUBMIX) within each image using a pixel counting program. The final quantification result was expressed as a mean percentage obtained from 30 images

analyzed along with a standard error. The standard error of the mean (SE_{mean}) was calculated as the standard deviation of the percentage area divided by the square root of the number of images analyzed.

Table 3.1. FISH probes used in this study

Probe	Sequence	Specificity	Reference
PAO462	CCGTCATCTACWCAGG GTATTAAC	<i>Accumulibacter</i>	Crocetti et al. (2000)
PAO651	CCCTCTGCCAAACTCC AG	<i>Accumulibacter</i>	Crocetti et al. (2000)
PAO846	GTTAGCTACGGCACTA AAAGG	<i>Accumulibacter</i>	Crocetti et al. (2000)
GAOQ431	TCCCCGCCTAAAGGGC TT	<i>Competibacter</i>	Crocetti et al. (2002)
GAOQ989	TTCCCCGGATGTCAAG GC	<i>Competibacter</i>	Crocetti et al. (2002)
DEF218	GAAGCCTTTGCCCCTC AG	<i>Defluviicoccus vanus</i>	Wong et al. (2004)
DEF618	GCCTCACTTGTCTAAC CG	<i>Defluviicoccus vanus</i>	Wong et al. (2004)
DF988	GATACGACGCCCATGT CAAGGC	<i>Defluviicoccus vanus</i>	Meyer et al. (2006)
DF1020	CCGGCCGAACCGACTC CC	<i>Defluviicoccus vanus</i>	Meyer et al. (2006)
EUB338	GCTGCCTCCCGTAGGA GT	Most bacteria	Amann (1995)
EUB338-II	GCAGCCACCCGTAGGT GT	Most bacteria	Daims et al. (1999)
EUB338-III	GCTGCCACCCGTAGGT GT	Most bacteria	Daims et al. (1999)

3.4.2 PCR-DGGE-Sequencing

Sludge samples were periodically collected and preserved for community analysis. Total genomic DNA was extracted from the sludge samples using an automated nucleic acid extractor (MagNA Pure, Roche Diagnostics GmbH) with a commercial DNA extraction kit (MagNA Pure LC DNA Isolation Kit I, Roche Applied Science). PCR was performed to amplify the variable V3 region of the bacterial 16S rRNA using universal primers, 341F-GC and 518R. Amplification was conducted with the C1000TM Thermal Cycler (Bio-Rad, Hercules, CA, USA) with same settings as those used for bacteria-specific amplification. DGGE was performed with the DCodeTM universal mutation detection system (Bio-Rad, Hercules, CA, USA) to separate the PCR products. The PCR product was loaded onto 8% polyacrylamide gels with a 40 to 70% linear denaturing gradient in TAE buffer. The electrophoresis was performed at 70V and 60°C for 16 hours. After electrophoresis, the gels were stained with ethidium bromide and photographed using Gel Doc XR systems (Bio-Rad, Hercules, CA, USA).

Dark bands on the gel were selected for cloning and sequencing. Bands were carefully cut from the gel and dissolved in TE buffer overnight at 4°C. PCR was performed again with the extracted products using 341F and 518R primers without the GC clamps. Electrophoresis with agarose gel was run to check the size of PCR products. The targeted bands were excised from the agarose gel and extracted with a commercial kit (GeneAll, USA). The extracted products were cloned into *Escherichia coli* JM109 using the pGEM-T vector system (Promega, Madison, WI). Blue/ white screening was done to select colonies containing required plasmids. Amplicons were sequenced by AITbiotech Pte Ltd (Singapore). The resultant sequences were compared with data deposited in GenBank database using the BLAST program.

3.5 ATP Measurement

ATP measurement assay was performed using an ATP determination kit (BacTiter-Glo™, Promega). The assay is based on the requirement for ATP by mono-oxygenation of luciferin that is catalyzed by luciferase in producing light (emission maximum ~560 nm) from the following reaction:

Sludge samples were diluted 50 times to 100 µl with autoclaved 1×PBS. Diluted samples were transferred to an opaque-walled 96-well plate, following the addition of 100 µL of ATP reaction solution. The final reaction volume was 200 µL. After 5 minutes incubation on an orbital shaker, luminescence was measured by a plate reader. Luminescence readings were standardized against cell concentration that was determined by optical density at a wavelength of 600 nm.

3.6 Electron Transport Activity/Respiratory Assay

CTC is a redox dye which produces a fluorescent formazan when it is being reduced and thus is used as an indicator of electron transport activity. For the assay, 100 µL sludge sample was stained with 0.5 mM CTC for 4 h at 28°C. Before incubation with CTC, sludge was washed twice with 1×PBS to remove remaining substrates. OD of each sample was measured at a wavelength of 600 nm. Incubation tubes were then centrifuged at 6,000 rpm to remove excess CTC and the stained cells were harvested. Absorbed stain was eluted from cells with 95% ethanol by incubating for 15 min. Immediately after solubilization of stain with ethanol, the supernatant was transferred to a black-side and clear-bottom microplate, and the light absorbance was read at 450 nm. A greater absorbance indicates greater concentration of stain. Duplicate samples without ethanol elution were prepared to be viewed under CLSM (confocal laser scanning microscope).

Chapter 4 – Study on the response of GAO to FNA inhibition and reversibility of the inhibition

4.1 Summary

The competition between PAOs and GAOs in an EBPR system is based on the capability of GAOs to uptake the organic carbon substrate without performing P uptake, and therefore possibly compromising EBPR performance. In a combined nitrite-pathway and EBPR system, the accumulation of FNA is inevitable. As little is known about FNA impacts on the carbon uptake performed by GAO in the subsequent FNA-free anaerobic condition, more in-depth investigation on this topic could be useful and therefore became the objective of this portion of the study. The information on recovery capacity of GAO to uptake VFA after being exposed to FNA in the previous phase would be beneficial to further evaluate the competitiveness of PAO and GAO in EBPR systems with FNA/nitrite accumulation. It was demonstrated that GAOs failed to denitrify nitrite in the presence of FNA, and a 60-70% decrease in VFA uptake rate of GAO was observed when FNA was increased. The inhibition was found to be reversible with GAO being able to achieve approximately 70% of the initial VFA uptake rate (the uptake rate prior to FNA exposure) at the third cycle of the recovery phase. These findings indicated the high tolerance of GAO with respect to FNA inhibition, which is undesirable for a successful and stable EBPR process, and thus underlined the necessity to suppress GAO proliferation.

4.2 Methodology

4.2.1 Reactor set-up and operation for GAO culture

The GAO was cultivated in a lab-scale SBR (10 × 76.4 cm, diameter × height) operating under alternating anaerobic-aerobic conditions. The population was fed with synthetic wastewater with acetate as the sole carbon source, ammonium, phosphate and microelements. Since GAO are able to proliferate under alternating anaerobic and aerobic conditions without performing P release and uptake, the

wastewater with lower P level was used. It should be noted, however, that the P concentration must not be limiting in order to ensure nutrient sufficiency for the culture. Detailed composition of the synthetic wastewater is given in Section 4.2.2. Activated sludge from a conventional nutrient (C and N) removal WWTP with biomass concentration of 3500 mg VSS/L was used as inoculum. The reactor's operation was managed with a PLC and a cyclic time of 4 h consisting of 70 min anaerobic, 160 min aerobic, and 10 min settling and decanting periods. One litre of wastewater was fed into the reactor in the first 10 min of the anaerobic phase, resulting in a HRT of 16 h. SRT was maintained at 8 d through mixed liquor withdrawal at the end of each cycle. Allylthiourea was applied to inhibit nitrification. The reactor was operated in a temperature – controlled room at 24 ± 1 °C. Additionally, GAO can be enriched by adopting pH range which is more desirable for GAO (~7) than for PAO (~8) (Oehmen et al. 2005b). Therefore, the pH was controlled during both the anaerobic and aerobic phases at a range of 7.0 – 7.4 by dosing 0.5 M HCl and 0.5 M NaOH as required.

4.2.2 Media

For the enrichment of GAO, synthetic wastewater with the following composition was used: 200 mg COD/L acetate, 5 mg/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ -Mg, 3 mg/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ -Ca, 5 mg/L NH_4Cl -N, 1 mg/L $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ -P, 1 mg/L KH_2PO_4 -P, 0.5 mg/L yeast extract, 0.5 mg/L peptone, and trace elements. Trace elements solution was also prepared in accordance with Smolders et al. (1994a). All the chemicals used in this study were of analytical grade.

4.2.3 Batch tests

A series of batch tests were carried out with GAO sludge to investigate the impact of FNA on the anaerobic and aerobic metabolisms of GAO as well as reversibility of the inhibition in the subsequent FNA-free anaerobic phase(s). The GAO culture used in this study had never been exposed to nitrite during the acclimation period. The sludge was withdrawn from the reactor at the cycle end and subsequently washed with phosphate buffer to remove any residual nitrite. The washed sludge

was then distributed into batch reactors and N_2 gas was sparged through the headspace to ensure anaerobic conditions. Acetate and nitrite stock solutions were then injected. The resulting initial acetate concentration was approximately 150 mg COD/L while the initial nitrite concentration as shown in Table 4.1.

Table 4.1. Experimental conditions applied in batch experiments

No.	pH	NO_2^- (mg NO_2^- -N/L)	$FNA \times 10^3$ (mg HNO_2 -N/L)
1	8	2	0.05
2	8	13	0.34
3	8	37	1.00
4	8	67	1.77
5	7	0	Control
6	7	2.3	0.53
7	7	5.6	1.30
8	7	10.6	2.44
9	7	15.8	3.65
10	7	22.0	5.08
11	7	33.3	7.69
12	7	41.7	9.63
13	7	53.8	12.41
14	7	63.9	14.75
15	7	71.6	16.53
16	7	91.5	21.11
17	7	105.5	24.35
18	7.5	2.2	0.16
19	7.5	26.8	1.95
20	7.5	58.0	4.24
21	7.5	86.9	6.34
22	6.5	31.8	23.23
23	6.5	62.4	45.54
24	6.5	89.6	65.39
25	6.5	113.0	82.49

Each anaerobic test was designed for a 2-h duration. Mixed liquor samples were taken at intervals using a syringe and immediately filtered through disposable Millipore filter units (0.45 μm pore size) for analyses of acetate and nitrite. The rates of VFA uptake were determined from the measured acetate profiles using linear regression.

At the end of the anaerobic inhibition period, the sludge was washed with phosphate buffer to remove the remaining nitrite. An hour of aerobic phase was then initiated by the injection of ammonium, phosphate and ATU at the concentrations of 10 mg NH_4^+ -N/L, 2 mg PO_4^{3-} -P/L and 5 mg ATU/L, respectively. A continuous air supply was provided to ensure the aerobic condition. Liquid samples were taken for the analysis of ammonia, which was used to quantify the bacterial growth. For the analyses of PHA and glycogen, solid samples were fixed with formaldehyde and subsequently washed with PBS. Subsequently, an anaerobic recovery phase was applied in all tests for 2 h with the injection of acetate at 150 mg COD/L. During tests 7, 10, 16 and 17 (refer to Table 4.1), three consecutive cycles of anaerobic recovery periods were applied in order to examine the VFA uptake recoverability of GAO.

4.3 Results and discussions

4.3.1 Performance of the parent reactor

A typical GAO phenotype obtained after the SBR reached steady-state condition is shown in Fig.4.1, which displays the consumption of acetate in the anaerobic phase with concurrent PHA production and glycogen consumption. In the following aerobic period, PHA was degraded and glycogen replenished. Due to the application of ATU which resulted in the absence of nitrification, the consumption of ammonium was attributed to bacterial growth. The carbon transformation, as summarized in Table 4.2, was in agreement with Liu et al. (1994). The level of PH2MV detected was insignificant. Fig.4.2 shows the typical pH and DO profiles obtained during the SBR operation.

Table 4.2. Carbon transformation by GAO in anaerobic phase

Test	PHB/C	PHV/C	Gly/C
This study	1.09	0.73	1.07
Liu et al. (1994)	1.17	0.68	1.10-1.25

Figure 4.1. Profiles of carbon transformation, ammonium and phosphate during a typical cycle study of the GAO enrichment reactor

Figure 4.2. Profiles of pH and DO during a typical cycle in the SBR operation

FISH quantification showed that *Competibacter* was at an abundance of $\pm 90\%$ (Fig.4.3). The abundances of *Defluviicoccus vanus*-cluster 1 and cluster 2 groups were $\leq 5\%$ and $\leq 1\%$, respectively, while the *Accumulibacter* PAO was undetected. Fig.4.3 also shows the image of PHA staining of GAO sludge which was withdrawn from the SBR at the cycle end.

Figure 4.3. Images of **a** FISH (blue – *Competibacter* (targeted by Cy5-labelled GAOMIX probes); red - all bacteria (targeted by FITC-labelled EUBMIX probes)) and **b** PHA staining of GAO sludge

4.3.2 Effects of FNA on the anaerobic metabolic processes of GAO

The impact of FNA exposure on acetate uptake by GAO is shown in Figure 4.4. The inhibition level is denoted as the percentage of the biomass-normalized rate obtained in the control reactor, in which the maximum rate of each metabolic activity was obtained. In all tests, acetate was taken up with concurrent PHA production and glycogen consumption despite the FNA/nitrite presence. Contrary to the literature on nitrite denitrifying ability of non-FNA-adapted PAO (Zhou et al. 2007), the non-FNA-adapted GAO used in this study was unable to reduce nitrite. The biomass-normalized rate of acetate uptake decreased as the applied initial FNA concentration increased. The uptake of acetate decreased sharply in the FNA concentration range of 0.00005-0.02 mg $\text{HNO}_2\text{-N/L}$. However, the acetate uptake was not completely inhibited even at a higher FNA concentration range (0.02-0.08 mg $\text{HNO}_2\text{-N/L}$) and the activity remained at 30% of the control test. Similar findings on the impact of FNA exposure on acetate uptake of GAO were reported recently by Ye et al. (2013), with a 50% inhibition (with respect to the biomass-normalized rate) at 0.025 mg $\text{HNO}_2\text{-N/L}$ (the highest concentration tested). The difference in the inhibition threshold between the two studies could probably be attributed to the environmental conditions applied in the control reactor(s), such as

pH and temperature. pH and temperature have indeed been reported to affect the energy requirement for acetate uptake of PAO and GAO (Smolders et al. 1994a). At higher pH, the amount of glycogen degradation and PHV accumulation in the GAO cells increased, suggesting the increase in energy requirement for transporting acetate across cellular membrane. pH 7.0 was uniformly applied in the control reactor (without nitrite addition) throughout this study (see Table 4.1) as this condition was previously revealed to allow maximum acetate uptake rate and minimum energy requirement for the uptake by enriched GAO population (Filipe et al. 2001a). While, several pH values were applied for the control tests in the study of Ye et al. (2013), thus explaining the different inhibition thresholds obtained in the two studies.

Figure 4.4. Effects of FNA on acetate uptake of GAO in the anaerobic inhibition tests

4.3.3 Effect of FNA on aerobic metabolism and anaerobic carbon uptake recovery

At the end of the anaerobic inhibition tests, FNA/nitrite was removed from the bulk liquid by washing the sludge with phosphate buffer. An aerobic phase was then applied to assess the aerobic metabolism of GAO after FNA exposure. In the absence of nitrification due to ATU dosing, ammonium consumption was attributed to bacterial growth (Pijuan et al. 2010). Figs.4.5, 4.6 and 4.7 showed the continued effects of FNA on the aerobic growth, PHA degradation and glycogen replenishment, respectively. Bacterial growth was inhibited by 50% at FNA concentration of approximately 0.01 mg $\text{HNO}_2\text{-N/L}$ (equivalent to 43 mg $\text{NO}_2^- \text{-N/L}$ at pH 7) and completely stopped at FNA concentrations above 0.025 mg $\text{HNO}_2\text{-N/L}$. The accumulation of nitrite at 43 mg $\text{NO}_2^- \text{-N/L}$ is possible in the wastewater treatment plant treating medium- to high-strength wastewater.

Figure 4.5. FNA impact on GAO aerobic growth

Unlike growth, PHA utilization and glycogen production were not completely stopped at higher FNA concentrations. PHA utilization decreased more slowly, reaching approximately 60% of the initial rate at the highest concentration applied, while in the case of glycogen replenishment, a sharp decrease took place when FNA was increased from 0.00005-0.02 mg $\text{HNO}_2\text{-N/L}$, and followed by a slower decrease, to approximately 40% at the highest FNA concentration studied. Several previous studies have indeed revealed the stronger impact of FNA inhibition or FNA-induced inhibition on the energy-requiring processes or anabolisms than on the energy-generating processes or catabolisms by various groups of microorganisms (Vadivelu et al. 2006b; Pijuan et al. 2010; Zhou et al. 2010a).

Figure 4.6. FNA impact on GAO aerobic PHA consumption

It has often been reported that FNA depletes the intracellular energy pool of bacteria (Zhou et al. 2010a), which would lead to lower anabolic (energy-consuming) activities. The reduced energy reserve is possibly due to the following mechanisms: (1) additional energy requirement to carry out detoxification process by expelling protons out of the cells; and (2) increase in energy requirement for

cellular maintenance. These thus explained the more sensitive anabolic response to the continued impact of FNA exposure in comparison with the catabolic counterpart. This study would be the first to report the FNA impact on metabolic recovery by GAOs, which also includes a detailed comparison of PAO's and GAO's robustness under FNA-induced inhibition.

Figure 4.7. FNA impact on GAO aerobic glycogen production

Table 4.3 summarizes the comparison of PAO and GAO response to FNA inhibition under aerobic condition obtained by Pijuan et al. (2010) and Ye et al. (2010), respectively. Neither population was adapted to FNA during the acclimation. However, the PAO culture used in Pijuan et al. (2010) was able to consume nitrite aerobically without any lag phase, while the GAO culture employed in Ye et al. (2010) was unable to denitrify nitrite. Similar observation was also obtained by Zhou et al. (2007) where the non-nitrite/FNA-adapted PAO population was able to denitrify nitrite upon exposure to FNA. From Table 4.3, it can be seen that (1) all aerobic metabolic activities (bacterial growth, PHA degradation and

glycogen production) of PAOs were more severely affected by direct exposure to FNA as compared to GAOs, and (2) more notable differences between PAO and GAO response were obtained from the anabolic activities (i.e. bacterial growth and glycogen production) than the corresponding catabolic activities (i.e. PHA degradation). The latter may be due to the difference in electron acceptor(s) consumed under aerobic condition, i.e. oxygen and/or nitrite in the study by Pijuan et al. (2010) and oxygen as the sole electron acceptor in the study by Ye et al. (2010). Degradation of the intracellular PHA using nitrite is energetically less efficient than that by oxygen (Murnleitner et al. 1997), thus leading to a much lower anabolic metabolism of PAOs than that of GAOs whereas the difference in the catabolic metabolism of PAOs and GAOs being less notable.

Table 4.3. Comparison of direct FNA inhibition on aerobic metabolisms of non-nitrite/FNA-adapted PAOs (Pijuan et al., 2010) and GAOs (Ye et al., 2010)

Parameter	Culture	FNA Inhibition (%)	FNA $\times 10^3$ (mg HNO ₂ -N/L)
Growth	PAO	50	0.5
		100	6
	GAO	50	1.5
		100	7.1
PHA oxidation	PAO	60	17
		100	Not reached
	GAO	60	24
		100	Not reached
Glycogen production	PAO	50	0.5
		100	2
	GAO	40	7.1
		70	24
		100	Not reached

In fact, Oehmen et al. (2010b) has reported that the two subgroups of *Accumulibacter* PAO, namely *Accumulibacter* PAO I and *Accumulibacter* PAO II, have the capacity to denitrify nitrite, while most *Competibacter* GAO subgroups do not have the capacity to denitrify nitrite (except *Competibacter* GAO subgroup 6). Besides, Zhou et al. (2012a) had suggested that the utilization of nitrite by PAOs was intended to generate extra energy to perform detoxification processes by expelling protons out of the cells. These might therefore suggest that different responses of PAOs and GAOs to FNA exposure are likely attributable to the denitrifying capability of respective groups.

Subsequently, the sludge was incubated under anaerobic condition which was initiated by the introduction of 150 mg COD/L. The relationship between FNA and the carbon uptake recovery is shown in Figs.4.8 and 4.9. It could be seen from Fig.4.8 that the effects of FNA-induced inhibition still continued in the subsequent anaerobic recovery period. The rates of HAc uptake were negatively correlated with the FNA concentration applied in the previous anaerobic period. The inhibition even continued till the third cycle of the anaerobic recovery periods.

Figure 4.8. Effects of FNA on recovery of GAO anaerobic HAc uptake

The extent of recovery, however, improved as the number of recovery cycles increased (see Fig.4.9). Fig.4.10 showed the profiles of COD as obtained in test 10. At the third cycle of recovery, the acetate uptake reached approximately 60% of its initial rate. A 70% recovery was obtained in test 7. While for tests 16 and 17, the acetate uptake reached approximately 50% of the initial rate at the third recovery cycle. This result showed that FNA inhibition was indeed reversible, as also reported in a study by Pijuan et al. (2010) using non-FNA-adapted PAO. Pijuan's study showed that at an initial FNA concentration of 9.5×10^{-3} mg $\text{HNO}_2\text{-N/L}$, P uptake reached approximately 65% of its initial rate at the third cycle of recovery period, therefore demonstrating a higher recovery kinetic than GAOs (60% acetate uptake recovery for 5.08×10^{-3} mg $\text{HNO}_2\text{-N/L}$). This indicated the stronger FNA-induced effects on GAO metabolisms than on PAOs during recovery periods.

Figure 4.9. HAc uptake rate in multiple cycles of anaerobic recovery

A hypothesis could be made that after FNA had been removed, expulsion of protons out of the cells would still continue during the following periods to gradually restore the transmembrane proton gradient which was previously disrupted by FNA diffusion into the cells. This would then enable adenosine triphosphate (ATP) synthase to synthesize ATP through oxidative phosphorylation, and therefore allowing bioprocesses to progressively recover under the FNA-free condition. However, the higher recovery capacity of PAOs as compared to that of GAOs could possibly be linked to the nitrite denitrifying capability of PAOs and GAOs. The capacity of PAOs to reduce nitrite in the presence of FNA would possibly provide some competitive advantages over GAOs in the subsequent phases once FNA had depleted or removed from the system. As a result of nitrite reduction, the intracellular nitrite concentration would decrease and it is likely that the inhibition would be subsequently subsided.

Figure 4.10. COD profiles in the initial, inhibition and recovery tests

4.4 Conclusions

Based on the findings, several conclusions can be drawn, as follows:

- (1) The non-FNA-adapted GAO used in this study was unable to denitrify nitrite. This was contrary to findings reported in the literature on the capability of non-FNA-adapted PAO to perform nitrite reduction. As nitrite utilization energetically yield lower ATP, different response of PAO and GAO to FNA may be attributed to different denitrifying capacity of the corresponding bacterial groups.
- (2) The relative inability of GAO could hypothetically lead to their competitive disadvantage over PAOs post FNA-exposure. Nitrite reduction activity by PAOs might alleviate the inhibitory effects in the following period after FNA has been removed due to decreased level of intracellular nitrite concentrations.

Chapter 5 – Studies on the response of PAO to FNA inhibition and inhibition reversibility

5.1 Summary

FNA has been widely known as an inhibitor on a variety of microorganisms in wastewater treatment. Literature has reported FNA inhibition on non-FNA-adapted PAO. In order to better understand PAO response to FNA, FNA-induced inhibitions on FNA-adapted PAO (or DPAO) metabolism under anoxic conditions as well as its comparison with aerobic conditions were discussed in this chapter. The findings revealed that FNA had a much stronger inhibitory effect on the anabolic (energy-consuming: P uptake and glycogen production) processes than the catabolic (energy-generating: NO_2^- reduction and PHA degradation) processes. Furthermore, FNA also significantly inhibited ATP generation in the biomass, resulting in positive correlation between the decreasing intracellular ATP level and increasing FNA concentration. A similar trend was displayed by the electron transport activity of DPAO. In the subsequent anaerobic FNA-free conditions, DPAO metabolism was not completely recovered. The recovery rate was negatively correlated with the FNA concentration applied in the previous anoxic phase. Results also revealed that anoxic P uptake was more severely affected by FNA as compared to the aerobic counterparts. The aerobic nitrite reduction rate increased with the applied FNA concentration with concurrent decrease in the oxygen consumption rate, indicating that electrons were transferred from oxygen utilization to nitrite reduction under high FNA concentration incubation. By contrast, the anoxic nitrite reduction decreased with increasing FNA concentration. These findings as well as the investigation of electron and energy balances suggested the role of nitrite utilization as FNA detoxification in an aerobic denitrifying EBPR process.

5.2 Methodology

Anoxic batch inhibition and anaerobic recovery tests

Batch tests were carried out using PAO sludge which was adapted to nitrite/FNA during the cultivation. Sludge was withdrawn at the cycle end and subsequently

washed with phosphate buffer solution. An-hour of anaerobic period was then initiated by the addition of sodium acetate with an initial concentration of 150 mg COD/L. The application of anaerobic period was intended for the acetate uptake and storage as PHA to drive the metabolism in the following anoxic phase. At the end of the anaerobic condition, the biomass was washed with phosphate buffer to remove any residual acetate and subsequently sparged with N₂ gas to ensure an oxygen-free condition. At the start of anoxic condition, ammonium, phosphate and nitrite solutions were injected. The resulting initial concentrations were 5 mg N/L and 20 mg P/L for ammonium and phosphate, respectively, while the initial concentrations for nitrite varied between 20-100 mg N/L (see Table 5.1). The FNA concentration was calculated using $\frac{S_{NO_2^- - N}}{K_a \times 10^{pH}}$, with the K_a value obtained using $K_a = e^{-2300/(273+T)}$ for any given temperature T (°C) (Anthonisen et al. 1976). Table 5.1 shows that various FNA concentrations (0.0007-0.073 mg HNO₂-N/L) obtained by varying the pH and nitrite levels were tested in 15 batch reactors. The pH level was maintained constant at the pre-set points by manually dosing 0.5 M HCl and NaOH solutions.

The anoxic inhibition tests lasted for 2 hours. For the analyses of ammonia, phosphate, and nitrite, mixed liquor samples were withdrawn at a 30-min interval and immediately filtered through Millipore filter units with 0.45 µm pore size. Whereas for the analyses of PHA and glycogen, solid samples were taken at the same sampling points, immediately fixed with formaldehyde and subsequently washed with PBS. In the tests 5, 6, 11, and 14 (see Table 5.1), the intracellular ATP of biomass was determined using an ATP determination kit (BacTiter-Glo™, Promega). While in the tests 5 and 11, the electron transport activity of biomass was quantified using the respiratory dye 5-cyano-2,3-ditolyltetrazolium chloride (CTC). Details on the analytical methods can be found in Chapter 3.

At the end of the anoxic inhibition period, the sludge was washed three times with phosphate buffer to ensure the complete removal of nitrite, as confirmed with nitrite test strips (Merck). Another 2-h anaerobic condition was then applied in order to investigate the recovery capability of DPAO population in the subsequent FNA-free

environment. pH was adjusted to 7.5 and kept at 7.5 ± 0.1 throughout the recovery phase through manually dosing 0.5 M HCl and NaOH. The anaerobic condition was then initiated by the addition of 150 mg COD/L acetate. Liquid, mixed liquor and solid samples were taken every 30 min.

Table 5.1. Experimental conditions applied in the batch experiments (for anoxic only)

Test	pH	NO ₂ ⁻ (mg N/L)	FNA $\times 10^3$ (mg HNO ₂ -N/L)
1	8	30	0.7
2	8	50	1.1
3	7.5	30	2.2
4	7.5	50	3.6
5	7	30	6.9
6	7	50	11.5
7	7	60	13.8
8	6.8	80	29.3
9	6.5	30	21.9
10	6.5	50	36.5
11	6.5	60	43.8
12	6.5	80	58.4
13	6.5	88	64.2
14	6.5	100	73.0
15	6	20	46.1

Aerobic and anoxic batch inhibition tests

Similarly, FNA-adapted PAO biomass was withdrawn from the SBR at the cycle end and subsequently incubated under anaerobic condition after the addition of sodium acetate at 150 mg COD/L. At the end of the 1-h anaerobic phase, the biomass was washed with phosphate buffer solution to ensure a complete removal of any residual COD. The mixed liquor was subsequently distributed to 27 batch reactors with a working volume of 100 mL each. ATU, ammonia and phosphate were then added at concentrations of 5 mg/L, 10 mg NH₄⁺-N/L and 20 mg PO₄³⁻-

P/L, respectively. Various FNA concentrations were obtained by varying pH and the initial nitrite concentrations as summarized in Table 5.2. The reference test for the aerobic experiment (FNA concentration at zero) was conducted in the absence of nitrite, while the reference test for the anoxic experiment was performed through continuous nitrite feeding at various rates in parallel (see Table 5.3). The continuous method for the anoxic control test was selected to prevent a non-limiting nitrite concentration in the bulk liquid as it could induce FNA inhibition. In the reference test, the maximum rate of each metabolic process was obtained. The oxygen uptake rate was determined as the net oxygen consumption rate from each aerobic batch test. The maximum rate of nitrite reduction rate was determined by subtracting the nitrite accumulation rate from NO_2^- loading rate under very low nitrite concentration conditions

Table 5.2. Experimental conditions of the batch tests

Aerobic Test				Anoxic Test			
No.	pH	NO_2^- (mg NO_2^- -N)	FNA $\times 10^3$ (mg HNO_2 -N/L)	No.	pH	NO_2^- (mg NO_2^- -N)	FNA $\times 10^3$ (mg HNO_2 -N/L)
1	8	0	0	15	8	10.7	0.25
2	8	11.0	0.25	16	7.5	10.0	0.73
3	7.5	10.6	0.77	17	7.5	30.4	2.22
4	7.5	32.2	2.35	18	7.5	40.7	2.97
5	7.5	42.0	3.06	19	7	26.3	6.07
6	7	27.4	6.32	20	7	39.0	9.00
7	7	43.5	10.04	21	6.5	29.9	21.82
8	6.5	31.6	23.06	22	6.5	39.0	28.46
9	6.5	40.0	29.19	23	6.5	46.0	33.57
10	6.5	49.0	35.76	24	6.5	52.1	38.02
11	6.5	51.3	37.44	25	6.5	53.3	38.90
12	6.5	55.1	40.21	26	6.5	55.4	40.43
13	6.5	61.9	45.18	27	6.5	64.9	47.37
14	6.5	67.3	49.12				

Each batch experiment lasted for 1 h. pH was maintained constant throughout the test with the addition of 0.1 M HCl or NaOH solutions. Mixed liquor samples were withdrawn every 10 min whereas solids samples were taken every 20 min. For the analyses of ammonium, nitrite and phosphate, the mixed liquor samples were immediately filtered using disposable filters (0.45 μm pore size). The solids samples for the analyses of PHA and glycogen were fixed with formaldehyde. In the aerobic test, air was sparged intermittently using an on-off controller at DO levels between 2-6 mg O_2/L . In the anoxic test, nitrogen gas was sparged continuously through the headspace of the batch reactor.

Table 5.3. Anoxic control tests

Tests	Feeding rate (mg N/min.g VSS)	Final NO_2^- (mg N/L)	NO_2^- stock solution (mg N/L)
1	0.044067	1.02	8.34
2	0.063549	0.61	10
3	0.073137	1.69	11.66
4	0.075653	2.34	13.34
5	0.132959	2.81	20
6	0.146438	3.31	23.34

5.3 Results and discussions

5.3.1 Sludge characterization

FISH analysis showed that *Accumulibacter* was at an abundance of 39.8% ($\pm 5.1\%$) of all bacteria bound to the EUBMIX probes at the time of most experiments. GAO belonging to the *Competibacter* was less than 1%. The *Defluviicoccus vanus*-cluster 1 and cluster 2 groups were not detected (Fig.5.1). The images of PHA and poly-P staining of the SNDPR sludge are shown in Figs.5.2 and 5.3, respectively. As can be seen, the intracellular PHA and poly-P were at abundance at the end of anaerobic period and the end of cycle, respectively.

Figure 5.1. FISH images of the SNDPR sludge. **a** Magenta - *Accumulibacter* (targeted by Cy3-labelled PAOMIX probes); blue - all bacteria (targeted by Cy5-labelled EUBMIX probes). **b** Magenta - *Competibacter* (targeted by Cy5-labelled GAOMIX probes); blue - all bacteria (targeted by FITC-labelled EUBMIX probes). **c** Blue - all bacteria (targeted by FITC-labelled EUBMIX probes); *D.vanus* cluster 1-related bacteria (targeted by Cy5-DF1mix probes) was not detected. **d** Blue - all bacteria (targeted by FITC-labelled EUBMIX probes); *D.vanus* cluster 1-related bacteria (targeted by Cy5-DF1mix probes) was not detected (The images have been reported in Zhou et al. (2010a).)

Figure 5.2. Images of PHA staining of the SNDPR sludge withdrawn from the SBR at the end of anaerobic period

Figure 5.3. Images of poly-P staining of the SNDPR sludge withdrawn from the SBR at the cycle end

5.3.2 Demonstration of strong inhibition on DPAO's anoxic metabolism

Fig.5.4 shows the dependence of P uptake, glycogen production, PHA degradation and nitrite reduction on FNA concentrations. The bioactivity was presented as a percentage of the maximum biomass-normalized rate measured in this study. It can be seen from Fig.5.4 that P uptake significantly decreased in the FNA concentration range of <0.01 mg $\text{HNO}_2\text{-N/L}$. P uptake rate was 50% inhibited at an FNA concentration of approximately 0.01 mg $\text{HNO}_2\text{-N/L}$ and completely stopped at 0.037 mg $\text{HNO}_2\text{-N/L}$. At FNA concentration range >0.037 mg $\text{HNO}_2\text{-N/L}$, P release

occurred, with increasing rate of release as FNA concentration increased. Nitrite reduction, however, was not completely inhibited and varied in the range of 30-40% of the measured maximum rate at the highest FNA concentration range in this study. PHA oxidation rate was less significantly inhibited by FNA as it remained at approximately 60% of the measured maximum rate for the highest range of FNA concentrations tested in this study.

Figure 5.4. FNA inhibition effects on (a) P uptake, (b) nitrite reduction, (c) PHA degradation, and (d) glycogen production of DPAO

The relatively consistent PHA degradation activity suggested that the observed P release was less likely caused by cell lysis. Meanwhile, glycogen production was severely deteriorated by FNA inhibition. The glycogen production was 60% inhibited at an FNA concentration of 0.02 mg HNO₂-N/L, and completely inhibited at 0.046 mg HNO₂-N/L. Consequently, glycogen degradation occurred at FNA

concentrations above 0.046 mg HNO₂-N/L. Similar to the argument for P release, the relatively constant glycogen consumption might rule out the possibility of cell lysis occurrence.

Freitas et al. (2009) has indeed reported that FNA concentration as low as 0.05×10^{-3} mg HNO₂-N/L could affect P uptake activity. Such a concentration is indeed the lowest reported value to date (nitrite accumulation of 0.2 mg NO₂⁻-N/L at pH 7). While in this study, it was found that the anoxic metabolisms were seriously inhibited by FNA at 0.01 mg HNO₂-N/L, therefore suggesting that the accumulation of 50 mg NO₂⁻-N/L at pH 7 in WWTP could severely inhibit P uptake as well as other key metabolic processes. At the FNA concentration range >0.044 mg HNO₂-N/L, instead of P uptake and glycogen production, P release and glycogen consumption occurred. P uptake and glycogen production belong to the anabolic processes (the energy-consuming processes), while P release and glycogen degradation belong to the catabolic processes (the energy-generating processes). Results of this study thus demonstrated that FNA inhibition on the energy-consuming activities of DPAO was more severe than the energy-generating counterparts. This outcome is in agreement with Vadivelu et al. (2006b; 2006c) which reported stronger FNA inhibition effects on the growth of *Nitrosomonas* and *Nitrobacter* as compared to their energy-generating activities.

Fig.5.5 shows the profile of intracellular ATP level of biomass incubated under different FNA concentrations. The transition rates of ATP were expressed as relative light unit (RLU) against optical density and time (RLU/(h.OD)) (refer to Fig.5.6). After the addition of FNA, the cellular ATP generally increased and the extent of increase positively correlated with FNA concentrations. The intracellular ATP levels subsequently reduced after the initial peak and the reduction rates were increasing with FNA concentrations. In the tests 5 and 6, the reduction trend reversed in the last 30 min when nitrite in the bulk liquid had been depleted. Greater ATP reduction rates at higher FNA concentrations might indicate the ATP deficiency for microbial growth and maintenance. While, the increase in the intracellular ATP contents at FNA concentrations of 0.012, 0.044 and 0.073 mg

HNO₂-N/L in the first 15 min may have been caused by insufficient energy generation for maintenance and the need for anaerobic energy generating processes (P-release and glycogen degradation) to produce ATP. This may also have caused the slow-down or termination of the energy-consuming process. The replenishment of the ATP content in the following FNA-free condition suggests that the ATP generating system had not been permanently damaged by FNA inhibition and could be recovered once the inhibitory factor was removed.

The generation of ATP is also closely related to the electron transport activity. During the tests 5 and 11, the electron transport was quantified using CTC staining method. The absorbance profiles were shown in Fig.5.7. Results showed that the bacterial respiratory activity demonstrated a similar trend as the intracellular ATP contents. At an FNA concentration of 0.044 mg HNO₂-N/L, following the addition of FNA, an increase in electron activity was observed, resulting in a peak within the first 30 min of FNA exposure. A sharp decrease took place thereafter, with concurrent observation of ATP reduction.

Figure 5.5. Intracellular ATP level of biomass incubated at different FNA concentrations

Figure 5.6. FNA dependency of biomass intracellular ATP reduction rate

Results on the anabolic activities, ATP and electron transport quantifications indeed indicated that the ATP generation of DPAO was disturbed. Mortensen et al. (2008) reported that intracellular pH decreased instantly after the addition of acidified nitrite and thereafter maintained constant for more than 6 h, suggesting that FNA inhibition instantly reached a saturated state and remained constant throughout the incubation period. Nevertheless, the reduced ATP content coupled to reduced anabolic processes, in all the tests performed here, suggests that the ATP generation of DPAOs was affected. The reduction rate of ATP, which is an energy reserve for microorganisms, increased with increasing FNA concentrations

Figure 5.7. Profiles of DPAO respiratory activity

5.3.3 Hypotheses on the mechanisms of FNA inhibition

Hinze and Holzer (1985) reported that FNA inhibition on the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GADP) resulted in a severe decrease in the intracellular ATP content of the yeast *Saccharomyces cerevisiae*. The GADP enzyme catalyzes both the glycolysis and its reversed pathway. Therefore, the inhibition of FNA on the enzyme affects the replenishment of the intracellular glycogen through pyruvate in the reverse glycolysis pathway in the anoxic environment. As can be seen in Fig.5.4, glycogen production was completely inhibited at high FNA concentrations. However, the glycogen consumption still occurred, indicating that glycolysis was not halted.

Additionally, FNA has widely been recognized as a chemical uncoupler (uncoupling growth from the energy generation). This study demonstrated that the ATP production system was disturbed by FNA inhibition. Sijbesma et al. (1996) has indeed reported that FNA was able to cross the membrane and to shuttle protons between the two sides without generating energy, thereby leading to the ATP synthesis inhibition. Furthermore, Hinze and Holzer (1985) reported that FNA was

able to passively diffuse across the cell membrane and be trapped inside the cell in its anionic form as a consequence of the neutral intracellular pH. Brul and Coote (1999) reported that in order to counteract the effect of acidification, the cell would pump out protons, thus leading to the uncoupling of energy generation from growth. While in this study, it was found that P uptake and glycogen production (the energy-consuming processes) were more sensitive to FNA inhibition than nitrite reduction and PHA degradation (the energy-producing processes), suggesting that the uncoupling effect may not be the sole inhibition mechanism.

5.3.4 Continued inhibitory effects in the subsequent anaerobic phase

Fig.5.8 showed the continued effects of FNA inhibition in the anaerobic period after FNA had been removed. It was found that P release, acetate uptake, PHA production and glycogen degradation had decreased proportionately with increasing FNA concentration.

Figure 5.8. Effects of FNA inhibition in the subsequent FNA-free anaerobic phase

The results demonstrated that DPAO did not completely recover from FNA inhibition in the following FNA-free anaerobic phase and the extent of recovery was dependent on the applied FNA concentration in the previous anoxic environment. Lower recovery in metabolic processes was evident in the DPAO biomass which had previously been incubated at higher FNA concentrations. Additionally, the recovery of VFA uptake and glycogen degradation activities was greater than that of P release and PHA accumulation activities. This could be due to the partial damage of the ATP generation system.

5.3.5 Comparison of PAO response to FNA under aerobic and anoxic conditions

Results of the aerobic control test demonstrated that the maximum phosphate uptake rate was 22.9 mg P/h.g VSS, while results of the anoxic counterparts showed that the values for phosphate uptake and N reduction were 10.8 mg P/h.g VSS and 5.5 mg N/h.g VSS, respectively. The comparison between the aerobic and the anoxic P uptake of PAO is shown in Fig.5.9. It was found that the P uptake activity using nitrite as a sole electron acceptor (anoxic test) was lower than that using both nitrite and oxygen (aerobic test). In the anoxic condition, P uptake was completely inhibited at 0.005 mg $\text{HNO}_2\text{-N/L}$ while aerobic P uptake stopped at 0.01 mg $\text{HNO}_2\text{-N/L}$. The results indicated anoxic P uptake was more severely affected by FNA inhibition, which agreed with the findings by Meinhold et al. (1999). P release, instead of P uptake, was observed at high FNA concentrations in both aerobic and anoxic conditions.

Several hypotheses on the greater sensitivity (1-6 times) of anoxic metabolism of PAO as compared to the aerobic counterpart are as follows: (1) nitrite was the sole electron acceptor in the anoxic experiment, and therefore the cells had less flexibility in the respiratory pathway; and (2) the oxygen respiratory pathway has higher energy potential and thus higher P uptake rate was observed under aerobic condition.

Figure 5.9. Comparison of aerobic and anoxic phosphate uptake under FNA inhibition

Fig.5.10 displays aerobic and anoxic nitrite reduction rates under different FNA concentrations. In the FNA concentration range of 0-0.01 mg HNO₂-N/L both rates increased with the increasing FNA concentrations, while it was found that anoxic NO₂⁻ reduction rates decreased sharply when FNA concentration exceeded 0.01 mg HNO₂-N/L. In contrast, aerobic NO₂⁻ reduction rates were not inhibited by the increasing FNA. It is worth pointing out that when FNA concentration exceeded 0.03 mg HNO₂-N/L, DPAOs' aerobic denitrification rates were higher than that of anoxic denitrification rates, indicating that the anoxic nitrite reduction was inhibited at higher FNA concentrations. Similar results were reported by Pijuan et al. (2010) that the aerobic denitrification rates displayed an increasing trend with the increasing FNA concentrations (range of FNA concentrations tested: 0-0.017 mg HNO₂-N/L).

Figure 5.10. Comparison of aerobic and anoxic nitrite reduction under FNA inhibition

Inhibitory effects on oxygen consumption followed a similar trend as that for aerobic phosphate uptake (Fig.5.11). The reduced OUR activity showed that oxygen requirement was reduced. This suggested the DPAOs could have switched the metabolic pathway from utilizing O₂ as the main electron acceptor to NO₂⁻. A hypothesis could be that inhibition from FNA forced the electron flow to switch from the oxygen respiratory pathway to nitrite reductase and other enzymes of denitrification (Robertson and Kuenen 1984).

Figure 5.11. FNA impact on DPAO oxygen uptake rate

A similar pattern in the glycogen replenishment rate was also observed in both the conditions (see Fig.5.12). At FNA concentrations >0.01 mg $\text{HNO}_2\text{-N/L}$, glycogen degradation, which is part of anaerobic metabolism, was observed without the presence of external organic carbon sources. While, complete inhibition of PHA degradation was not reached even at the highest FNA concentration tested (see Fig.5.13). The activity of aerobic PHA degradation was shown to be relatively higher than the anoxic counterpart.

Figure 5.12. Comparison of FNA impact on aerobic and anoxic glycogen production

Nitrite reduction under aerobic conditions might be for the purposes of detoxification instead rather than growth. Calculation of electron and ATP balance (see Tables 5.4 and 5.5) was carried out to verify this hypothesis, in which data in the first 20 min was used. Several assumptions were made for the calculation, as follows: (1) sufficient electron donor in the first 20 min of the inhibition test; (2) the model of Smolders et al. (1995) was used for the stoichiometric determination of PHA utilization to generate energy for P uptake, glycogen production, biomass growth and cellular maintenance. The required O_2 as stated in Table 5.5 indicates the amount of O_2 required if all the consumed PHA was oxidized by O_2 . It can be seen that as the FNA concentration increased, the measured O_2 consumption was lower than the required amount and therefore nitrite was consumed to further facilitate the PHA oxidation. However, the actual nitrite consumption was found to be higher than the required amount for the microbial metabolism. It was therefore suggested that the nitrite utilization at higher FNA concentration was carried out as a means of FNA detoxification.

Figure 5.13. Comparison of FNA impact on aerobic and anoxic PHA degradation

Table 5.4. Calculation of electron balance for aerobic tests

FNA ($\times 10^3$ mg HNO ₂ -N/L)	O ₂ consumption (mmol O ₂ /g VSS)	Required O ₂ (mmol O ₂ /g VSS)	Required NO ₂ ⁻ (mmol N/g VSS)	NO ₂ ⁻ consumption (mmol N/g VSS)
0	0.385	0.400	-	-
0.25	0.329	0.332	0.004	0.016
10.04	0.192	0.256	0.085	0.083
29.19	0.072	0.126	0.072	0.091
45.18	0.054	0.080	0.034	0.130

Table 5.5. Calculation of ATP balance

FNA ($\times 10^3$ mg HNO ₂ -N/L)	PHA oxidation (C-mmol/g VSS)		ATP from PHA oxidation (mmol ATP/g VSS)		ATP source (mmol ATP/g VSS)	
	O ₂	NO ₂ ⁻	O ₂	NO ₂ ⁻	P release	Gly degradation
29.19	0.064	0.048	0.314	-	0.102	0.193
45.18	0.048	0.023	0.231	-	0.099	0.266

For the ATP balance, the energy from PHA degradation was calculated based on Murnleitner et al. (1997). For the first 20-min period, the ATP requirement for PAO maintenance is equivalent to 0.244 mmol ATP/g VSS. At an FNA concentration of 29.19×10^{-3} mg HNO₂-N/L, the required energy for cellular maintenance could be solely obtained from the PHA oxidation. It was therefore hypothesized that the additional ATP from P release and glycogen degradation was used to carry out the detoxification process, such as to pump proton across the cellular membrane to counteract the effects of FNA acidification.

5.4 Conclusions

The inhibitory effects of FNA on the aerobic and anoxic metabolisms of DPAO were demonstrated in this study. Results indicated that (1) FNA inhibition on the anabolic activities of DPAO was more severe than on the catabolic counterparts; (2) FNA disturbed the ATP synthesis and electron transport chain of DPAO; (3) The mechanisms of FNA inhibition could be described by the inhibition of certain metabolic enzymes and/or the collapse of the proton gradients as a result of proton shuttling activity of FNA; and (4) FNA inhibition on DPAO in the anoxic condition was found to be 1-6 times more severe than in the aerobic condition. This could be attributed to the opportunity to carry out detoxification aerobically, as suggested from the investigation on electron and ATP balances. This detoxification would enhance the role of FNA in favoring PAO enrichment over GAO.

Chapter 6 – Using FNA as a selection factor to achieve EBPR performance

6.1 Summary

In the previous chapters, it was shown that unlike PAO, GAO did not have the capability to denitrify nitrite. This chapter discusses the “positive” role of FNA as a selection factor to achieve EBPR performance. FNA was shown to alter the microbial community structure in favor of PAO while preventing the proliferation of GAO. This strategy is in contrast to the popular belief that FNA/nitrite severely deteriorated the EBPR process and demonstrated that partial nitrification could serve as an effective control of FNA concentration as well as an adaptation for microbial enrichment method. Excellent P removal was obtained when nitrite-pathway nitrification had successfully been developed using activated sludge from a local WWTP performing complete nitrification as the seed sludge. The process developed would normally be termed SNDPR process. P removal had started to deteriorate and PAO was washed out once partial nitrification was shifted back to complete nitrification as a result of a longer aeration period, confirming that nitrite was the preferred electron acceptor for the enriched PAO. This strategy also underlined the synergy between the two processes, in which partial nitrification promoted enrichment of PAO while denitrifying P-uptake would lead to self-buffering capacity in the system thus yielding significant savings in chemical requirements for the nitrification process. This work is likely among the first to report achievement of partial nitrification in low-strength wastewater treatment.

6.2 Methodology

6.2.1 Reactor set-up and operation for the SNDPR reactor

The microbial population was cultivated in an anaerobic-aerobic-anoxic sequencing batch reactor with a working volume of 4 L. The dimensions of the SBR were same as that for GAO culture. Feed for the PAO culture was synthetic wastewater containing acetate and propionate as carbon sources with a ratio of 3:1. Details of wastewater composition can be found in Section 6.2.2. Sludge from a conventional

nutrient removal activated sludge wastewater treatment plant (4000 mg VSS/L) was used as inoculum. At an initial stage, the reactor had a cycle period of 6 h comprising 71 min anaerobic, 120 min aerobic, and 45 min anoxic phases, followed by a second phase of 40 min anaerobic, 40 min aerobic and 30 min anoxic periods, and 14 min settling and decanting period. At steady state, the reactor was operated with a cycle time of 4.8 h consisting of 64 min anaerobic, 71 min aerobic, and 41 min anoxic phases, followed by a second phase of 38 min anaerobic, 23 min aerobic and 35 min anoxic periods, and 14 min settling and decanting period. In each cycle, one litre of synthetic wastewater is fed to the reactor in the first 8 min and 3 min of the two subsequent anaerobic phases, resulting in a hydraulic retention time of 24 h and 19 h at the initial stage and steady state, respectively. The reactor was operated in a temperature – controlled room at 24 ± 1 °C. pH in the reactor was monitored online and controlled below 8.2 by a one-way controller through dosing 0.5 M HCl using PLC (programmable logic controller). During anaerobic and anoxic periods, nitrogen gas was sparged through the mixed liquor at a flow rate of 1.0 L/min through the reactor. While during the aerobic period, DO concentrations were controlled within a range of 0.8 – 1.0 mg O₂/L by supplying air at a flow rate of 2.0 L/min. Cyclic study was conducted on a weekly basis to monitor nutrient removal performance. A photograph of the SBR system used for PAO and GAO (Chapter 4) culture is as shown in Fig.6.1.

6.2.2 Media for SNDPR culture

Synthetic wastewater with the following composition was used: 150 mg COD/L acetate, 50 mg COD/L propionic acid, 10 mg/L MgSO₄·7H₂O-Mg, 3 mg/L CaCl₂·2H₂O-Ca, 20 mg/L NH₄Cl-N, 5 mg/L K₂HPO₄·3H₂O-P, 5 mg/L KH₂PO₄-P, 0.5 mg/L yeast extract, 0.5 mg/L peptone, and trace elements. Trace elements solution was prepared according to Smolders et al. (1994a), comprising of 0.1 mg/L FeCl₃·6H₂O, 0.01 mg/L H₃BO₃, 0.002 mg/L CuSO₄·5H₂O, 0.014 mg/L KI, 0.009 mg/L MnCl₂·4H₂O, 0.0045 mg/L Na₂MoO₄·2H₂O, 0.009 mg/L ZnSO₄·7H₂O, 0.01 mg/L CoCl₂·6H₂O, and 0.75 mg/L EDTA. The medium was autoclaved in order to prevent bacterial activity in the feed bottles.

Figure 6.1. Laboratory-scale SBR for the enrichment of SNDPR and GAO culture

6.2.3 Kinetic tests

Several batch tests were performed to determine the kinetics of phosphate uptake and denitrification using only nitrite and nitrate as electron acceptor. Sludge samples were withdrawn from the SBR at cycle end and washed to remove any residual NO_x . Nitrite/nitrate stock solution was injected at the beginning of the 1-h reaction period at a concentration of 10 mg N/L. During each test, mixed liquor samples were taken every 10 min and immediately filtered through disposable Millipore filters (0.45 μm pore size) for the analysis of ammonium, nitrite, nitrate and phosphate. Mixed liquor samples for total suspended solids (TSS) and volatile suspended solids (VSS) determination were taken at the beginning and end of each test. pH was maintained at 7.5 using 0.25 M HCl.

6.3 Results and discussions

6.3.1 Strategy to achieve EBPR through partial nitrification

As previously mentioned, the strategy was developed using a laboratory-scale SBR. The SBR was selected as it provided flexibility to supply nitrite/FNA in the desired concentration range more readily. An alternating anaerobic/aerobic/anoxic conditions was employed to promote the EBPR process which was facilitated by partial nitrification-denitrification. In order to improve the removal efficiency, each cycle of the SBR operation was divided into two sub-cycles of anaerobic-aerobic-anoxic phases. During the anaerobic period, VFA in the mixed liquor was taken up and converted to intracellular carbonaceous compound. In the following aerobic treatment, the oxidation of ammonia allows an *in-situ* generation of nitrite/nitrous acid, an electron acceptor favored enrichment of *Accumulibacter* PAO as suggested by Martin et al. (2006) who had reported *Accumulibacter* did not have the functional gene for encoding the respiratory nitrate reductase. The continuous generation of nitrite during aerobic period followed by its reduction minimized the inhibition effects arising from FNA/nitrite accumulation/shock loading. The subsequent anoxic period then served as a polishing step for both nitrite reduction and P uptake. This arrangement was purposefully designed in order to optimize the P removal capacity of PAO by first prompting the aerobic P-uptake instead of the anoxic counterpart so that reliance on anoxic P-uptake would be reduced as the preliminary study (details can be found in section 5.3) had revealed P-uptake in the anoxic condition was 1-6 times more sensitive to nitrite/FNA as compared to that in aerobic condition. Typical performance of the SNDPR reactor operated with this strategy is shown in Fig.4.1.

6.3.2 Correlation between nitrite shortcut and EBPR development

The first part of the development was aimed at achieving partial nitrification from complete nitrification during the aerobic period. Partial nitrification served as a source of nitrite, a strategic tool to favor *Accumulibacter* enrichment while preventing *Competibacter* proliferation. The critical point in achieving such a process is inhibition or wash-out of NOB (nitrite-oxidizing bacteria) while retaining

AOB (ammonia-oxidizing bacteria). It is widely known, however, that partial nitrification is difficult to achieve in a system treating wastewater with low nitrogen loading due to the close proximity between the specific growth of AOB and NOB ($1.05 \text{ d}^{-1} < \mu_{\text{max,AOB}} < 1.4 \text{ d}^{-1}$ and $0.91 \text{ d}^{-1} < \mu_{\text{max,NOB}} < 1.31 \text{ d}^{-1}$ at 20°C (Munz et al. 2011). In this study's system, the aeration pattern and duration had been employed as the primary keys to establishing shortcut nitrification with low nitrogen loading.

Figure 6.2. pH, phosphate, nitrogen, PHA and oxygen concentration profiles during a cycle in the SNDPR reactor

The SBR was operated for a 73-week period with excellent partial nitrification performance achieved after 20 weeks. As shown in Fig.6.3, the overall SBR operation can be divided into three periods, the initiation, steady-state, and complete-nitrification periods. During the initiation period, partial nitrification was gradually developed from the full nitrification. Under aerobic conditions (i.e. the second period), the control strategy utilized pH and dissolved oxygen (DO) as the

on-line control parameters to achieve steady partial nitrification through the appropriate control of the system's aeration pattern and duration. Aeration pattern was determined with automatic DO control, which was preset within a range of 0.8-1.0 mg O₂/L by an on/off aeration supply at a flow rate of 2.0 L/min. Such a low range of oxygen level was applied to inhibit growth of NOB while favoring AOB (Peng and Zhu 2006) as DO half-saturation coefficients of the AOB and NOB are 0.2-0.4 mg O₂/L and 1.2-1.5 mg O₂/L, respectively (Wiesmann 1994). DO and pH signals were also used to determine duration of the aerobic condition. pH decreased due to reduction in alkalinity and acid production during nitrification and then started to increase once nitrification was completed. The inflection point is often termed the “ammonia valley” as it indicated the completion of ammonium oxidation. Aeration was then halted as soon as the “ammonia valley” was deduced from the pH signals. Similarly, aeration was also cut once a distinct reduction in the oxygen uptake rate (OUR) was detected from the DO profile. In this manner, stable nitrite/FNA accumulation was obtained.

Achievement of partial nitrification was exhibited as nitrite accumulation was observed with the maximum level at approximately 50% of the consumed ammonia at the end of aerobic phase, suggesting partial simultaneous nitrification-denitrification in the aerobic condition. The maximum level of FNA, however, was less than 1.20×10^{-3} mg HNO₂-N/L and thus still tolerated by the nitrite-DPAO population. This could be attributed to the DPAO capacity to perform denitrification under aerobic condition, and therefore regulating the FNA concentration in the system to below the inhibition threshold. Excellent P removal was obtained when the nitrite-pathway nitrification had successfully been developed. The steady-state condition was maintained in the following 40-week period, where nitrite accumulation remained stable. During the steady-state stage, COD, N, and P removal efficiencies were within the ranges of 98-99%, 90-99% and 95-99%, respectively.

A longer aeration period to upset the previously-developed partial nitrification was then imposed on the SBR in order to assess robustness of the process established. During the last 18-week period, P removal performance deteriorated as partial

nitrification became full nitrification. Deterioration in P removal in the presence of full nitrification suggested that nitrate production (over nitrite) had been detrimental as the then-selected nitrite-DPAO could not utilize nitrate to oxidize their internal carbon to drive P uptake. Fig.6.1 shows the correlation between phosphate uptake activity and achievement of the nitrite shortcut over the 73-week period. Results indicated the importance of nitrite/FNA (product of partial nitrification) at low concentrations ($< 1.20 \times 10^{-3}$ mg $\text{HNO}_2\text{-N/L}$) as electron acceptor to drive the P uptake activity by the nitrite-DPAO population, and therefore favoring selection of PAO in the system. On the other hand, both complete and partial nitrification processes resulted in comparatively good nitrogen removal efficiencies, suggesting the specific role of free nitrous acid/nitrite in DPAO metabolism.

Indeed, the role of nitrite in the selection of PAO over GAO has recently been reported by Taya et al. (2013). However, the system used in their study was solely EBPR and did not involve nitrogen removal, thus the source of nitrite came from the external addition, instead of inherent nitrite production (partial nitrification) as investigated in this study. In fact, the phenomenon of close correlation between achievement of partial nitrification and excellent EBPR performance could also be explained with the findings by Martin et al. (2006) which revealed that *Accumulibacter* did not have the functional gene for encoding the respiratory nitrate reductase, and therefore disabling reduction from nitrate to nitrite. This report is in agreement with a study by Guisasola et al. (2009) which reported the failure of nitrite-DPAO to respire nitrate.

Another recent study by Zheng et al. (2013) reported the long-term effect of nitrite on granule-based EBPR system and its reversibility. The study, however, showed contradictory findings. Nitrite was found to favor GAO proliferation and caused PAO washout from the system, thus deteriorating EBPR performance. These contradictory findings could be due to different strains of *Accumulibacter* and *Competibacter* which were present in the system. Recent literature indeed reported that most types of *Competibacter*, but one, cannot denitrify nitrite (Oehmen et al.

2010b). Literature on the long-term effect of nitrite on EBPR is summarized in Table 6.1.

It is also important to highlight that FNA concentration higher than 1.2×10^{-3} mg $\text{HNO}_2\text{-N/L}$ led to significant (>25%) inhibition on P uptake of PAO (Pijuan et al. 2010; Zhou et al. 2010a; Zhou et al. 2012a). Zhou et al. (2010a) reported that 50% inhibition occurred at FNA concentration of approximately 0.01 mg $\text{HNO}_2\text{-N/L}$ and complete inhibition took place at FNA concentration of 0.037 mg $\text{HNO}_2\text{-N/L}$ for nitrite-adapted PAO. While, Pijuan et al. (2010) reported that P uptake of non-nitrite-adapted PAO stopped at 0.01 mg $\text{HNO}_2\text{-N/L}$.

Figure 6.3. Development of long-term partial nitrification performance and P-uptake activity (the three stages from left to right: initiation, steady state, and complete nitrification). The Pearson's correlation coefficient $r = 0.8213$.

The FNA concentration range required for PAO enrichment ($< 1.20 \times 10^{-3}$ mg $\text{HNO}_2\text{-N/L}$) noted in this study is attributable to competition with GAO in EBPR

system. The FNA inhibition threshold for the P uptake activity of PAO was lower than that for glycogen production of GAO. Intracellular polyphosphate and glycogen are the primary source of energy reserve for PAO and GAO, respectively. Ye et al. (2010) reported that at FNA concentration below 1.5×10^{-3} mg $\text{HNO}_2\text{-N/L}$, glycogen production of non-nitrite-adapted *Competibacter* GAO did not change significantly and the activity reached 30% of its maximum at FNA concentration of 0.024 mg $\text{HNO}_2\text{-N/L}$. The GAO population investigated in their study was not able to denitrify nitrite, and therefore the production of intracellular glycogen as well as other anabolic activity, such as microbial growth, could only take place in the presence of oxygen. The above-mentioned findings suggested that the inhibition threshold of P uptake activity of PAO ($< 1.20 \times 10^{-3}$ mg $\text{HNO}_2\text{-N/L}$) was the bottleneck in determining the achievement of excellent EBPR performance in an SNDPR system via the FNA/nitrite strategy.

Table 6.1. Summary of literature on long-term effect of nitrite on EBPR

No	Study	Source	Effect	Description
1	This study	Nitrification	+	FNA: $< 1.20 \times 10^{-3}$ mg N/L (17 mg NO_2^- -N/L at pH 7.5)
2	Taya et al. (2013)	External	+	FNA: 1.42×10^{-3} - 2.13×10^{-3} mg N/L (20-30 mg NO_2^- -N/L at pH 7.5)
3	Zheng (2013)	External	-	FNA: 0.36×10^{-3} - 2.13×10^{-3} mg N/L (5-30 mg NO_2^- -N/L at pH 7.5)
4	Zeng et al. (2011)	Nitrification	+	So long as the supplied C is sufficient
5	Coma et al. (2010)	Nitrification	+	FNA: $< 0.71 \times 10^{-3}$ mg N/L (< 10 mg NO_2^- -N/L at pH 7.5)
6	Guisasola et al. (2009)	External	+	FNA: 2.13×10^{-3} - 2.84×10^{-3} mg N/L (30-40 mg NO_2^- -N/L at pH 7.5)

6.3.3 Effect of nitrite accumulation on EBPR performance and community

To further investigate the development of nitrite-DPAO, batch kinetic studies using different electron acceptors (nitrite and nitrate), as elaborated in Section 6.2.3, were carried out and the data obtained are as shown in Table 6.2. It can be seen that the outcomes were in agreement with the above-mentioned phenomena, as follows: (1) in the initiation stage where full nitrification took place, denitrifying PAO were acclimatized with greater capacity to utilize nitrate as compared to nitrite; (2) in the steady-state condition where partial nitrification had been developed, higher nitrite reduction and phosphate uptake rates were obtained as nitrite was the substance that DPAO biomass was acclimated to (the DPAO population can henceforth be called “nitrite-DPAO”); and (3) in the complete-nitrification stage where partial nitrification was forced to shift to full-nitrification, nitrite-DPAO had started to wash out from the system, as suggested by the significant drop in P uptake rate as well as that of nitrite reduction. The fact that nitrate reduction still occurred when the sludge from stages 2 and 3 was exposed to nitrate in the batch reactor tests suggested that the reduction from nitrate to nitrite was likely performed by the other heterotrophic bacteria, such as DGAO, existing in the system.

Table 6.2. Comparison of process kinetics at various stages during SNDPR SBR operation

Stage	Time zone	NO ₂ ⁻ reduction rate (mg N/h.g VSS)	NO ₃ ⁻ reduction rate (mg N/h.g VSS)	P uptake rate (mg P/h.g VSS)
1	Initiation	2.1	6.0	9.4
2	Steady state	4.7	2.6	14.8
3	Complete nitrification	2.9	6.0	4.2

The SNDPR microbial community was characterized using PCR-DGGE-sequencing methods. The results confirmed the correlation between achievement of partial nitrification and EBPR performance. Fig.6.4 shows the DGGE patterns of 16S rDNA fragments of the SNDPR sludge. An obvious shift in microbial community was observed from stage 1 to stage 2 due to the development of partial nitrification and EBPR, as follows: (1) the intensity of bands A1 and A3 weakened, while bands A5 and A8 strengthened, and (2) the disappearance of bands A2 and A4, and the appearance of bands A6 and A7. From stage 2 to stage 3, the following shift was noticed: (1) the intensity of bands A5, A6 and A8 weakened, while band A1 strengthened, and (2) the disappearance of bands A7, and the appearance of bands A9 and A10. However, the population diversity, particularly in terms of the number of bands, was relatively similar from L1-L3. Ten bands in the DGGE gel were excised and sequenced in order to obtain the identities of the major bacterial populations. Table 6.3 summarizes the results of comparison between the amplified 16S rDNA sequence and the sequence data of GenBank database. The results demonstrated that band A8 showed 98% similarity to *Candidatus Accumulibacter phosphatis*, the key organism responsible for the enhanced P uptake. It can be seen that its intensity in the three different lanes was in agreement with the P removal performance during SBR operation. The band intensity increased from L1 to L2 and decreased from L2 to L3 with increasing rate of P uptake from the initiation stage to the steady state and the decreasing rate from the steady state to complete-nitrification stage, respectively.

Figure 6.4. DGGE patterns of 16S rDNA of the SNDPR community. L1, L2, and L3 are the DGGE profiles from sludge samples drawn at week 8, 36 and 65 i.e. within stages 1, 2, and 3, respectively.

Table 6.3. Comparison between the amplified 16S rDNA nucleotide sequences and GenBank database

Bands	Closest relatives	Accession	Identity (%)
A1	Uncultured bacterium clone Q7703-ASSA	JN391613	100
A2	Uncultured bacterium clone AOA-158	AB479754	100
A3	Uncultured bacterium clone SBRFL126	HQ158757	100
A4	Uncultured bacterium clone Pohang_WWTP_December.2006_2924	HQ512865	100
A5	Uncultured bacterium clone Pohang_WWTP_October.2006_2102	HQ505792	99
A6	Uncultured Bacteroidetes bacterium clone MEf05b11G6	FJ828154	98
A7	Uncultured bacterium clone F5K2Q4C04IQBU7	GU912899	97
A8	Uncultured Candidatus Accumulibacter sp. clone 4.6h22	JN679123	98
A9	Uncultured Bacteroidetes bacterium clone AS38	EU283366	99
A10	Uncultured alpha proteobacterium clone Ebpr11	AF255640	100

6.3.4 Comparison with the conventional system

In order to assess the competitive advantage of the combined partial nitrification-EBPR (this study) over the conventional activated sludge technology, a comparative summary of the process kinetics is presented in Table 6.4. Faster kinetics of N and P removal (>100 % and >300%, respectively) were obtained from this combined EBPR and N removal system as compared to the conventional system. To date, shortcut nitrogen removal has not fully been applied in the mainstream of the wastewater treatment and most pilot- or full-scale partial nitrification plants have been focusing on the treatment of reject water or industrial wastewaters. The much higher rates obtained in SNDPR system could be attributed to the synthetic wastewater used in the lab-scale study as opposed to the real wastewaters used in the full-scale plant. However, such comparison is still important as it serves as the baseline information for the development of the combined partial nitrification-EBPR process. Additionally, the studied SNDPR system yielded significant savings in chemical requirements due to its inherent alkalinity production.

Table 6.4. Comparison between the SNDPR (this study) and conventional systems

Parameter	This study	Conventional system	Reference
NO ₂ ⁻ reduction rate (mg N/h.g VSS)	2.1-4.7 Partial nitrification	N.A.	N.A.
Phosphate uptake rate (mg P/h.g VSS)	14.8 Steady-state	2.2-3.6 Full-scale	Rohrbacher et al. (2007)

6.4 Conclusions

FNA accumulation resulting from partial nitrification was found to provide a novel strategy which favored the enrichment of *Accumulibacter* PAO, leading to the achievement of excellent P removal efficiency in the system. The partial nitrification also served as an effective control of residual nitrite concentration in the reactor's liquor. Failure to maintain the partial nitrification would result in severe EBPR deterioration and washout of *Accumulibacter* from the system.

Chapter 7 – Metabolic behavior of denitrifying P accumulating organisms in the presence of coexisting free nitrous acid and carbon substrate

7.1 Summary

The response of denitrifying poly-phosphate accumulating organisms (DPAO) with respect to their metabolic activities following exposure to the coexisting electron donor (acetate/HAc) and electron acceptor (FNA/nitrite) was investigated. Anoxic batch experiments were carried out at four chemical oxygen demand (COD) to nitrite-nitrogen ($\text{COD}/\text{NO}_2^- \text{-N}$) ratios – i.e. 1.75, 2.60, 3.50, and 4.35 respectively. The coexistence of FNA and HAc was found to be detrimental to the metabolism of DPAO. As compared to the control test, HAc uptake was reduced by about 15-30%, while anoxic phosphorus release, instead of uptake, was observed with P/C ratios of 150-200%. A 40-80% decrease in glycogen degradation could be explained through proteomic analysis which revealed that FNA inhibited secretion of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase by DPAO. PHA/C ratio and evaluation of electron balance confirmed that carbon consumption for PHA formation was dominant over denitrification via TCA cycle. The electron balance also suggested electron competition between nitrite and nitrous oxide reductases even when carbon supply was not limiting, leading to N_2O accumulation. For DPAO culture, the electron competition was less affected by source of the organic carbon (intracellular storage compound or external source).

7.2 Methodology

Batch tests

Batch tests were carried out under three different combinations of electron donor (HAc) and electron acceptor (FNA), namely Control A, Control B, and Anoxic Test, as detailed in Table 7.1. Four chemical oxygen demand-to-nitrite-nitrogen ($\text{COD}/\text{NO}_2^- \text{-N}$) ratios (1.75, 2.60, 3.50, 4.35) were tested in anoxic batch experiments using DPAO sludge withdrawn from the SNDPR reactor. Zhang and Wang (2009) reported that the optimal $\text{COD}/\text{NO}_2^- \text{-N}$ ratio for complete nitrite

reduction is 2.7 – 4.3. Since influent wastewaters are often notably deficient in carbon supply, COD/NO₂⁻-N ratios of lower than and within the reported range were therefore chosen for this study, representing the carbon-limiting and non-carbon-limiting conditions. For each set, the mixed liquor was washed twice with phosphate buffer solution in order to remove any residual nitrite, and subsequently sparged with nitrogen gas to remove any residual DO. The Control A batch experiments consisted of 2-h anaerobic incubation of mixed liquor with synthetic wastewater containing various pre-set COD concentrations (see Table 7.1) prepared by supplementation of different volumes of sodium acetate stock solution (41.7 mmol/L CH₃COONa.3H₂O). The Control B batch experiments were operated under anaerobic pretreatment and anoxic reaction configuration. The mixed liquor was firstly incubated for 2 h under anaerobic condition which was initiated by acetate addition, followed by a 2-h anoxic phase which was initiated by the injection of 34.5 mg NO₂⁻-N/L, 20 mg PO₄³⁻-P/L, and 10 mg NH₄⁺-N/L. In the Anoxic Test, the biomass was simultaneously exposed to acetate and FNA/nitrite under similar conditions. Different carbon loading in the Control A and Anoxic Test were obtained by varying the initial acetate concentration (in a range of 60-150 mg COD/L). Particularly for the Control B tests, the COD/N ratio was determined by the amount of intracellular PHA at the start of anoxic phase, as PHA rather than external carbon substrate served as carbon source in the tested condition. pH of the mixed liquor was maintained at 7.5 throughout the course of the batch assay, resulting in an initial FNA concentration of approximately 2.5×10⁻³ mg HNO₂-N/L. Such low concentrations of FNA were chosen to mimic the real situation in full-scale WWTPs where the sludge return is a relatively small fraction of the influent flow, thus the FNA returned would be of a small ratio of the total FNA from the aeration stage. Five mg/L of ATU was added to inhibit nitrification activity in the batch reactor. To further evaluate the effect of FNA on the expression of key enzyme in DPAO metabolism, 50 mL mixed liquor was incubated with 150 mg COD/L and 34.5 mg NO₂⁻-N/L over 24-h in a similar working condition as the Anoxic Test. The reference test was carried out in the absence of FNA/nitrite. Each test was repeated in three replicates.

Table 7.1. Experimental condition applied in batch tests

Batch	HAc (mg COD/L)	pH	NO ₂ ⁻ (mg NO ₂ -N/L)	FNA (μg HNO ₂ -N/L)	COD/NO ₂ ⁻ -N* (mg/mg)
CA1	60	7.5	-	-	-
CA2	90	7.5	-	-	-
CA3	120	7.5	-	-	-
CA4	150	7.5	-	-	-
CB1	-	7.5	34.5	2.5	1.75
CB2	-	7.5	34.5	2.5	2.60
CB3	-	7.5	34.5	2.5	3.50
CB4	-	7.5	34.5	2.5	4.35
AN1	60	7.5	34.5	2.5	1.75
AN2	90	7.5	34.5	2.5	2.60
AN3	120	7.5	34.5	2.5	3.50
AN4	150	7.5	34.5	2.5	4.35

Reactor operation and sampling protocol

Experiments were carried out in an air tight cylindrical 100mm×150mm (ID×IH) batch reactor with a working volume of 1.2 L (see Fig.7.1) at 24°C. The reactor was sealed at the top and the lid had five ports for the mounting of an N₂O microsensor, two 50 mL mixed-liquor reservoirs and two pH-adjustment solutions (0.5 M HCl and 0.5 M NaOH). The N₂O microsensor (N₂O25, Unisense A/S, Aarhus, Denmark) was installed at the center of the lid for on-line N₂O measurement while a pH probe (Thermo Scientific, US) was inserted to a port at the reactor side for on-line pH measurement. The batch reactor was mixed with a magnetic stirrer at a speed of 250 rpm and was completely filled in order to avoid N₂O exchange between liquid and gas phases. During each test, mixed liquor samples were taken every 30 mins and divided into two portions for liquid and solid samples. For the analysis of liquid samples, mixed liquor was immediately filtered through 0.45 μm disposable Millipore filter unit and directly kept in 4°C, while solid samples for PHA and glycogen analysis were fixed with formaldehyde and subsequently washed with phosphate buffer saline (PBS). Total suspended solids (TSS) and volatile suspended solids (VSS) were determined from the mixed liquor samples withdrawn at the beginning and end of each batch assay. Mixed liquor samples for NO analysis were

collected in vacuum tubes (BD Diagnostic, USA). During each sampling, same volume of mixed liquor was injected from the reservoirs to replace the withdrawn samples in order to maintain the completely filled condition of the batch reactor.

Figure 7.1. Experimental setup in N_2O batch study

Calculations

Due to the absence of nitrification, the ammonia consumption could be fully attributed to biomass growth. Several crucial parameters investigated include (1) the phosphorus-to-carbon (P/C) ratio, (2) the polyhydroxyalkanoate-to-carbon (PHA/C) ratio, (3) the glycogen-to-carbon (Gly/C) ratio. As NO , the other denitrification intermediate, was not detected in all tests, the biomass-specific nitrite reduction rate represented N_2O generation rate. N_2O reduction rate was determined by subtracting N_2O accumulation rate from N_2O generation rate.

7.3 Results and Discussions

7.3.1 Overview of batch test performance

The main processes involved in DPAO metabolism were examined, namely the biochemical carbon transformations of HAc, PHA and glycogen, and transitions of phosphate, nitrite, and N_2O . The graphical presentations of the abovementioned compounds for COD/ NO_2^- -N ratio 2.60 are shown in Fig.7.2. The biomass expressed an ideal anaerobic phenotype of PAO in Control A (Fig.7.2a) and a typical anoxic P-removal characteristic of DPAO in Control B (Fig.7.2b), while in the Anoxic Test (Fig.7.2c), DPAO exhibited a transient anaerobic phenotype coupled with denitrifying activity. In the absence of an electron acceptor in the Control A test, an increase of the initial COD concentrations from 60-150 mg COD/L resulted in sharp increases of biomass-specific HAc uptake, P release, PHA accumulation and glycogen degradation as reported in Table 7.2. As a result, subtle dynamics of P/C (0.40-0.47), PHA/C (0.93-1.24) and Gly/C (0.54-0.59) ratios were obtained accordingly, which were in close proximity with the anaerobic metabolic model of PAOs developed by Smolders et al. (1994a). Whereas in the absence of external electron donor in Control B experiment, the level of cellular PHA dropped concomitantly with the decrease in extracellular ortho-P level, with PHA degradation accounted for 15-30% of the initial available PHA at the end of the 2-h period. This indicated that carbon source was not limiting and therefore suggesting that PHA degradation, which was coupled with FNA/nitrite respiration, was a rate-limiting step not only in the metabolism of heterotrophic bacteria as frequently reported in literature (Beun et al. 2000; Schalk-Otte et al. 2000), but also in DPAO metabolism.

Glycogen degradation and poly-P accumulation were observed in response to the initial supplementation of nitrite which was not completely consumed by the end of the anoxic reaction in all tests. The extracellular inorganic phosphate, however, had not fully been taken up by the end of the anoxic test. The occurrence of glycogen degradation, instead of replenishment varying from the typical aerobic/anoxic environments, was a result of FNA inhibition, as was also reported by Pijuan et al. (2010) and Zhou et al. (2010a) under aerobic and anoxic conditions, respectively.

Biomass growth, represented by ammonium consumption due to the absence of nitrification, was not observed. N_2O accumulated linearly throughout the test and ranged from 45-85% of denitrified nitrogen with increasing $\text{COD}/\text{NO}_2^- \text{-N}$ ratio led to higher N_2O reduction and lower N_2O accumulation rates. NO_x was never detected as in this study.

Table 7.2. Summary of metabolic activities of DPAOs in the presence of coexisting FNA & HAc as compared to the control test (HAc (+), FNA(-))

COD/ $\text{NO}_2^- \text{-N}$ (mg/mg)	Set 1 (Control A)				Set 3 (Anoxic Test)			
	HAc consumed (Cmmol/ gBiomass)	PO_4 release (mmol P/ gBiomass)	PHA formation (Cmmol/ gBiomass)	Glycogen degradation (Cmmol/ gBiomass)	HAc consumed (Cmmol/ gBiomass)	PO_4 release (mmol P/ gBiomass)	PHA formation (Cmmol/ gBiomass)	Glycogen degradation (Cmmol/ gBiomass)
1.75	1.51	0.71	1.88	0.89	1.30	0.96	1.52	0.55
2.60	2.52	1.09	2.90	1.48	1.74	1.34	1.67	0.37
3.50	2.80	1.12	3.13	1.58	2.10	1.65	2.06	0.42
4.35	3.45	1.41	3.76	1.86	2.50	1.81	2.42	0.40

Figure 7.2. Nutrient profiles of COD/N = 2.60 for Control A (a), Control B (b), and Anoxic Test (c)

The Anoxic Test expressed a non-ideal anaerobic condition due to the presence of FNA/nitrite. Fig.7.2c shows that there were two different zones during the experimental course where in the first 30min-1h, PHA formation, PO_4 release as well as N_2O accumulation took place at relatively higher rates. The comparison of the kinetics of DPAO metabolism in the first and the second 1 h during the Anoxic Test is presented in Table 7.3. Particularly for the lowest COD/NO_2^- -N test, the HAc was completely taken up in the first 1 h. While for the remaining tests, HAc uptake occurred at higher rates in the first 1 h and was not completely taken up at the end of 2-h period, indicating that the carbon supply was not limiting. Higher rates of PHA accumulation coupled with higher N_2O accumulation rates indicated that the two biochemical pathways competed for electrons which were solely provided by HAc. In parallel, the trends observed for P release were similar to that of HAc uptake, signifying the role of intracellular poly-P as the source of energy for carbon uptake under the nitrite-anoxic environment. On the other hand, glycogen degradation and nitrite consumption exhibited comparatively consistent rates throughout the test. Similar to the outcome of Control B experiments, biomass growth was not observed and N_2O was a significant end product of denitrification with its accumulation accounting for 40-90% of denitrified N with N_2O reduction rate increasing as COD/NO_2^- -N ratio increased. The above-mentioned phenomena led to the following hypotheses: (1) carbon loading was positively correlated to N_2O reduction; (2) the electron competition between nitrite and N_2O reductases took place even when carbon supply was not limiting; and (3) such competition occurred regardless of the type of carbon source (intracellular storage compound or external source).

Table 7.3. Metabolic kinetics observed in Anoxic Test

COD/ NO_2^- -N (mg/mg)	HAc uptake rate (Cmmol/h.gBiomass)		PHA accumulation rate (Cmmol/h.gBiomass)		PO_4 release rate (mmol P/h.gBiomass)		N_2O accumulation rate ($\mu\text{mol N/h.gBiomass}$)	
	1 st 1 hour	2 nd 1 hour	1 st 1 hour	2 nd 1 hour	1 st 1 hour	2 nd 1 hour	1 st 1 hour	2 nd 1 hour
1.75	1.28	-	1.29	0.23	0.77	0.18	222	99
2.60	1.56	0.18	1.51	0.16	1.05	0.29	242	92
3.50	1.86	0.24	1.96	0.10	1.33	0.32	161	62
4.35	2.08	0.42	2.19	0.23	1.49	0.32	163	69

7.3.2 Detrimental effects of FNA on the ‘supposed’ anaerobic metabolism of DPAO

Several studies have suggested that nitrite in the form of FNA inhibited the metabolism of PAO under anaerobic, aerobic and anoxic conditions (Zhou et al. 2007; Pijuan et al. 2010; Ye et al. 2013). In this study, the key metabolic activities of DPAO under different carbon loading in the presence and absence of FNA were assessed. The observed behavior for P release, glycogen degradation and PHA accumulation under purely anaerobic condition (in the absence of FNA) in Control A test was in agreement with the Mino model. In the Anoxic Test, the biomass-specific HAc consumption and P release were well correlated with the COD/NO₂⁻-N ratio, with increasing COD/NO₂⁻-N ratio (1.75-4.35) leading to 1.3-2.0 times increase in both activities (see Table 7.2). On the other hand, there was no significant correlation between the ratio of the Anoxic Test to the corresponding control test (in the range of 15-30% lower and 1.2-1.5 times higher for HAc consumption and P release, respectively) and the COD/NO₂⁻-N ratio. The decreased HAc consumption suggested that FNA inhibited the anaerobic HAc uptake by DPAO while the coupling of lower HAc uptake and greater P release indicate that FNA might have inhibited the energy generation mechanism in DPAO anaerobic metabolism at a concentration as low as 2.5×10^{-3} mg HNO₂-N/L. It had been revealed that FNA inhibited the adenosine triphosphate (ATP) generation of DPAO under anoxic incubation (with the absence of external electron donor), therefore prompting the need of anaerobic energy-releasing activities like P release to generate more ATP (Zhou et al. 2010a). In other recent studies (Zhou et al. 2012a; Ye et al. 2013), FNA has been proposed to cause bacteria to spend more energy for detoxification and maintenance purposes under aerobic condition. Table 7.4 summarizes the comparison of different parameters of (D)PAO metabolic activities obtained in this study and several published studies performed under the coexistence of electron donor and acceptor. As can be seen, P/C results of this study are in agreement with that by Ye et al. (2013) where FNA was used as the electron acceptor, and opposed to those by Kuba et al. (1994) and Pijuan et al. (2005) where NO₃⁻ and O₂ were used, respectively. The fact that more phosphate release was required to supply energy for the same amount of HAc uptake implied that the

downstream EBPR cycles would be negatively affected, as higher P release would demand even higher P uptake at the subsequent phase in order to achieve net-positive P removal from the system. A substantially higher P uptake would mean that higher PHA oxidation is required. Contrastively, the actual biomass-specific PHA formation was 20-50% lower (see Table 7.2), denoting that P removal performance had been compromised. Fig.7.3 illustrated the kinetic comparison of PHA accumulation between the Control A and Anoxic Test. The lower PHA formation was a result of electron competition which will be elaborated in the next discussion. Moreover, it can also be concluded that under the coexistence of electron acceptor and electron donor, PHA accumulation has been the predominant pathway for electron donor utilization by DPAO.

Table 7.4. Comparison of metabolic activities of PAOs across different studies

Parameter	Anoxic Test (HAc(+),FNA(+))	Ye et al. (2013) * (HAc(+),FNA(+))	Kuba et al. (1994) ** (HAc(+),NO ₃ (+))	Pijuan et al. (2005) (HAc(+),O ₂ (+))
%HAc uptake (Cmmol/gBiomass)	70-85%	~50%	110-150%	-
%P/C (mmol P/Cmmol)	150-200%	~125-175%	~50-60%	14-18%
%PHA/C (Cmmol/Cmmol)	80-95%	~80-90%	~90%	77-78%
%Gly/C (Cmmol/Cmmol)	30-70%	~60%	~160%	24-48%

* Values presented based on graphical interpretation at approximately 2.5×10^{-3} mg HNO₂-N/L

** Mino model was used as reference

Figure 7.3. Comparison of PHA accumulation rate in the presence (Anoxic Test) and absence (Control A) of FNA

The trend for glycogen degradation was found to be similar to the PHA formation. In the absolute anaerobic condition, glycogen degradation is another energy generation process secondary to the poly-P hydrolysis as well as the main source of reducing power. As suggested by the Mino model, the up-taken acetate was utilized for PHA synthesis and accumulation by the cells with reducing equivalents to reduce acetyl-CoA to PHA provided primarily through the degradation of the intracellular glycogen through glycolysis (Embden – Meyerhoff – Parnas pathway). According to the model, 0.5 mol glycogen need to be degraded to supply the required reducing equivalents for PHA formation for each mol of acetate uptake. In the Anoxic Test, however, glycogen degradation which was observed at 40-80% lower with respect to the control tests, was less than the requirement for the actual PHA formation, suggesting that the TCA cycle had also played an important role in supplying the reducing power. In fact, the more pronounced role of TCA cycle was not surprising as it was functional in the nitrite reduction process, through which the complete oxidation of acetyl CoA was accomplished. The proteomic analysis results (see Fig.7.4) indeed supported this finding as the level of glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase in the presence of coexisting FNA and HAc was at 74.29% as compared to the control test in the absence of FNA. This outcome showed that FNA inhibited the secretion of glyceraldehyde-3-phosphate

dehydrogenase enzyme. Several studies have indeed suggested that nitrite in the form of FNA could freely cross the cell membrane and inhibit the intracellular enzymes (Oleary and Solberg 1976; Rowe et al. 1979). Hinze and Holzer (1985) revealed that FNA inhibited the glyceraldehyde-3-phosphate dehydrogenase enzyme in the yeast *Saccharomyces cerevisiae* and consequently decreased the ATP content. Two mechanisms are thus proposed to explain the decrease in glycogen degradation in the Anoxic Test: (1) FNA inhibition on the secretion of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase, and (2) greater role of the TCA cycle in the supply of the reducing power for PHA synthesis. Additionally, results on the acetyl CoA synthase are in agreement with a reduction in HAc uptake obtained in Anoxic Test.

Figure 7.4. Proteomics profiling displayed some changes in the level of several enzymes involved in DPAO metabolic pathways which could be attributed to FNA inhibition as well as explaining the possible mechanism of FNA inhibition

7.3.3 N₂O accumulation from denitrification pathway of DPAOs in the presence of extracellular carbon source

Fig.7.2c displays that nitrite reduction occurred instantly in the Anoxic Test, signifying that denitrification was driven by the oxidation of HAc as the carbon source, instead of the intracellular PHA. However, as elaborated earlier, in the lowest COD/N tested, the external organic carbon finished in the first 1 h while denitrification occurred throughout the 2-h period, suggesting that PHA was utilized as the internal carbon source in the last 1-h period. It has been suggested in the literature that PHA utilization may lead to a higher N₂O generation as compared to denitrification driven by external organic carbon sources (Kampschreur et al. 2009). In all the tested batch reactors, N₂O was always accumulated with higher accumulation rates in the first 1 h (see Table 7.3) and increasing rates as COD/NO₂⁻-N increased. The higher N₂O accumulation rates took place concurrently with higher PHA formation in the first 1-h period, suggesting that N₂O accumulation could be attributed to the competition of electron donor. Five mechanisms, which may occur simultaneously, could be used to explain the N₂O accumulation in the simultaneous presence of FNA and carbon source from the studied system: (1) competition of electrons for PHA synthesis by DPAO and nitrite reduction by denitrifiers; (2) competition of electrons for different biochemical pathways of DPAO, namely PHA synthesis and nitrite reduction; (3) competition of electron between different denitrification enzymes, i.e. nitrite and N₂O reductases; (4) FNA/nitrite inhibition on nitrous oxide reductase; and (5) kinetics of electron supply from PHA degradation being the bottleneck for electron utilization. In this study, the presence of denitrifiers was suppressed due to zero external carbon in the aerobic and anoxic periods of culture acclimation in the SBR, and thus ruling out the possibility of first mechanism. While, results showed that the other mechanisms (2-5) would be attributable to N₂O accumulation in the system.

Fig.7.5 presents the profiles of N₂O generation, accumulation and reduction in the Anoxic Test as well as in the reference test (Control B) with respect to the COD/NO₂⁻-N ratio applied. It could be seen that there has been insignificant difference in the rates of N₂O generation (nitrite reduction) as well as N₂O

reduction between the PHA-driven and HAc-driven denitrifications, leading to relatively similar N_2O accumulation in both tests. Both N_2O accumulation and reduction in the Anoxic Test and Control B were well correlated to the COD/NO_2^- -N ratio. Table 7.5 summarizes the results of electron balance calculations performed between the measured amount of electron acceptors and the consumed electron donors in the Control B and Anoxic Test. In the highest COD/NO_2^- -N test for Control B experiment, the electrons from PHA degradation were available in excess, and therefore it was hypothetically sufficient to drive the complete denitrification (with N_2 as final product). However, N_2O still constituted 45% of the total denitrified NO_2^- -N in the system, indicating that the kinetics of electron supply from PHA degradation, which comprises carbon oxidation and electron transfer, could be the bottleneck for electron utilization.

Figure 7.5. Percentages of N_2O accumulation (a) and N_2O generation (b), accumulation (c), reduction (d) rates at all tested COD/N ratios.

PHA utilization has indeed been reported to be the rate limiting step and to cause N_2O accumulation due to the electron competition among different denitrification enzymes. The “apparent” unutilized electrons according to the electron balance were mostly attributed to the limiting kinetics of PHA metabolism and the short-term nature of this study. While in the remaining COD/NO_2^- -N tests for Control B experiment, the amount of PHA degradation was insufficient to drive complete denitrification regardless of the abundance of PHA pool. Similarly, in the highest COD/NO_2^- -N ratio of the Anoxic Test, N_2O accumulation formed 42.8% of the total denitrified NO_2^- -N although the amount of consumed HAc corresponded to denitrification was more than sufficient for the full denitrification. This showed that electron competition among nitrite reductase and N_2O reductase took place even when carbon source was available in excess. N_2O accumulation happened as a result of the lower affinity of N_2O reductase for electrons as compared to that of nitrite reductase.

Table 7.5. Calculation of electron balance between the measured amounts of electron acceptors coupled with the consumed electron donors in Sets 2 & 3

COD/ NO_2^- -N (mg/mg)	Set 2 (Control B)					
	NO_2^- Reduced (mmol N/ gBiomass)	$\text{NO}_2^- > \text{N}_2\text{O}$ (meq/ gBiomass)	$\text{NO}_2^- > \text{N}_2$ (meq/ gBiomass)	Total theoretical e^- (meq/gBiomass)	Req e^- for full DN (meq/gBiomass)	e^- from measured PHA (meq/ gBiomass)
1.75	0.53	0.91	0.23	1.14	1.60	1.26
2.60	0.62	0.73	0.78	1.51	1.86	1.48
3.50	0.64	0.65	0.95	1.60	1.92	1.72
4.35	0.64	0.57	1.06	1.63	1.91	1.98
COD/ NO_2^- -N (mg/mg)	Set 3 (Anoxic Test)					
	NO_2^- Reduced (mmol N/ gBiomass)	$\text{NO}_2^- > \text{N}_2\text{O}$ (meq/ gBiomass)	$\text{NO}_2^- > \text{N}_2$ (meq/ gBiomass)	Total theoretical e^- (meq/ gBiomass)	Req e^- for full DN (meq/gBiomass)	HAc > DN (meq/ gBiomass)
1.75	0.48	0.82	0.20	1.02	1.43	1.32
2.60	0.60	0.83	0.56	1.39	1.81	1.71
3.50	0.63	0.55	1.08	1.63	1.91	1.85
4.35	0.64	0.55	1.10	1.65	1.92	1.88

Table 7.6 summarizes the literature comparison with other relevant studies with respect to N_2O generation and reduction rates of DPAO. Results showed that nitrite/FNA respiration by DPAO generally yielded significant amount of N_2O and the extent of N_2O accumulation varied with respect to the applied $\text{COD}/\text{NO}_2^- \text{-N}$.

Table 7.6. N_2O generation and reduction rates from denitrification by DPAO sludge at different conditions

Study	$\text{COD}/\text{NO}_2^- \text{-N}$ (mg/mg)	Carbon source for denitrification	Experimental conditions	N_2O production rate ($\mu\text{mol N/h.gBiomass}$)	N_2O reduction rate ($\mu\text{mol N/h.gBiomass}$)
This study	1.75-4.35	PHA	FNA: 2.5×10^{-3} mg HNO ₂ -N/L	266.2-320.7	38.4-175.9
	1.75-4.35	HAc	FNA: 2.5×10^{-3} mg HNO ₂ -N/L	237.7-319.8	32.7-182.8
Zhou et al. (2012b)	3.75-5.15*	PHA	FNA: 2.7×10^{-3} mg HNO ₂ -N/L	275.4-348.4	152.5-213.2
	0.625-2.50	HAc	FNA: 2.7×10^{-3} mg HNO ₂ -N/L	157.7-343.1	8.3-211.2
Lemaire et al. (2006)	**	PHA	NO ₂ ⁻ : 7.5 mg NO ₂ ⁻ - N/L	187.3-215.7	86.1-95.5
	**	PHA	NO ₃ ⁻ : 7.5 mg NO ₃ ⁻ - N/L	**	81.2-92.2

*The reported COD/N values in Zhou et al. (2012b) were 0.625-2.50, which were based on HAc-COD/ $\text{NO}_2^- \text{-N}$ applied in the pretreatment step. In this table, PHA-COD/ $\text{NO}_2^- \text{-N}$ values are presented.

**Not specified

7.4. Conclusions

This chapter presented a study on the impact of the coexistence of FNA/nitrite and carbon substrate (HAc), a phenomenon which is distinctly different from the ideal EBPR operational conditions, on the metabolic biochemical behavior of DPAO. Two primary focuses of the study were the effects on EBPR performance and N_2O accumulation. It was found that the concurrent exposure of DPAO sludge to FNA and external carbon substrate induced EBPR failure. Firstly, the utilization of HAc for denitrification via the TCA cycle reduced the amount of HAc available for sequestration, thus reducing the efficiency of P-removal performance. Secondly, FNA inhibited the energy generation mechanisms as well as the secretion of

glycolytic enzyme by DPAO. This study also concluded that the electron competition and the kinetics of electron supply were the two main reasons for N_2O accumulation from DPAO denitrification, while the type of carbon source (intracellular storage compound vs external source) was less of a factor. The adverse impact on COD and phosphate removal performance indicated necessity to limit concentration of FNA entering the anaerobic zone through several methods: (1) appropriate control measure in the aerobic zone and the secondary settling tank for minimizing FNA accumulation and its subsequent recirculation to the anaerobic tank, respectively; (2) anoxic treatment of mixed liquor prior to its transfer to the anaerobic zone.

Chapter 8 – Conclusions and Recommendation

8.1 Conclusions

This thesis focused on the inhibitory effects as well as the positive role of FNA on denitrifying EBPR systems in a nutrient removal WWTP. The system used in this study was the SNDPR via nitrite shortcut for municipal wastewater treatment process. The driving force for application of such a process is the reduction in energy requirement and carbon footprint, which is particularly useful for WWTPs treating low- and medium-strength wastewaters where organic carbon is often limited.

Objective 1: Studies on the response of PAO and GAO to FNA

Different behavior of PAO and GAO in their response to FNA was found in this study. Unlike PAO, GAO was unable to denitrify nitrite. This study also found that under high FNA concentration inhibition, PAO would consume nitrite for FNA detoxification purpose. The consumption of nitrite would yield lower ATP and therefore the anabolic activities of PAO in the presence of FNA would be lower than those of GAO. In contrast, PAO's activities were higher than GAO's during recovery period. This could suggest that nitrite denitrifying ability of PAO might have played an important role in alleviating nitrite/FNA toxicity effects by lowering its intracellular level. In addition, inhibition of FNA on PAO and GAO was reversible.

Objective 2: Using FNA as a selection factor to achieve EBPR performance

FNA generated from partial nitrification could be used as a selection factor for the enrichment of PAO over GAO, leading to the development of excellent phosphate removal performance. Partial nitrification of ammonia allowed an *in-situ* generation of nitrite/nitrous acid, an electron acceptor favored for altering the microbial community structure towards the PAO-enriched system. Additionally, partial nitrification also served as an adaptation method as well as an effective control of FNA concentration. Future work may look into the implementation of this strategy in pilot- or full-scale plants.

Objective 3: Examination of the effects of coexistence of FNA and carbon substrate (HAc) on DPAO metabolism and N₂O emission from denitrifying phosphorus removal process

The coexistence of FNA and organic carbon (HAc) as a result of NO₂⁻ leakage in the ‘supposed’ anaerobic condition of the P removal system was found to be detrimental for the metabolism of DPAO and deteriorating COD and P removal. Competition for electrons took place between the two key metabolic pathways, namely PHA synthesis and TCA cycle, and between nitrite and nitrous oxide reductases. In the latter case, the competition occurred even when carbon supply was not limiting and resulting in N₂O accumulation. Results suggested that a proper control measure to prevent NO₂⁻ leakage to the anaerobic phase has to be implemented in order to prevent performance deterioration and N₂O generation from the nutrient removal system. Future study may look deeper into the mechanism of FNA inhibition on the TCA cycle.

This thesis concluded that FNA could be potentially advantageous or detrimental on the biological nutrients removal in WWTP and not necessarily always detrimental. FNA accumulation caused by partial nitrification could play an important role in controlling the microbial consortia in the EBPR system in favor of PAO enrichment, leading to the achievement of excellent EBPR process with minimum inhibition impacts.

8.2 Recommendations

Based on the findings of this thesis, several research questions arise and may offer perspectives on future research directions, which are outlined as follows:

- **Comparison of FNA inhibition on DPAOs and DGAOs as well as the recoverability**

In Chapter 4, the continued inhibitory effects of FNA on GAOs were found to be more detrimental than those on PAOs. Both populations of PAOs and GAOs were not adapted to nitrite/FNA. However, the PAO culture was able to denitrify nitrite upon exposure to FNA while the GAO culture was unable to do so. The findings thus lead to a hypothesis that nitrite denitrifying ability plays a significant role in

FNA detoxification process. In relation to these findings, it would be interesting to compare FNA inhibitory effects on FNA-adapted PAOs and GAOs as well as their recoverability. Therefore, studies on enriched DPAO and DGAO cultures are relevant to address these issues.

- **Using FNA as a selection factor to favor PAOs' proliferation over GAOs'**

Chapter 6 demonstrated that the presence of FNA favored the enrichment of PAOs over GAOs during long-term SBR operation, indicating that FNA can be used as a selection tool to achieve EBPR performance. This strategy was carried out using a C/N-removing biomass as the seed sludge through acclimatization under alternating anaerobic/aerobic/anoxic phases with partial nitrification as the source of FNA. In order to validate the FNA selection strategy, experimental confirmation using different approach is still required. For instance, it will be interesting to see if the long-term presence of FNA can suppress GAOs' proliferation and cause their wash out, and in turn lead to PAOs' enrichment in the system. This new approach may employ partial nitrification on a GAO-dominant biomass at the start. Subsequently, whether or not FNA from the partial nitrification would affect the population shift can be assessed in the long-term reactor operation. Further investigation using real wastewater as source of substrate would add value.

- **How will the concurrent presence of FNA and carbon substrate affect DGAO metabolisms?**

Chapter 7 showed that the concurrent presence of FNA and carbon substrate was detrimental to DPAO metabolism. Competition for electron donor occurred between PHA formation and denitrification. This study could be extended with an investigation on the effects of concurrent presence of FNA and carbon substrate to DGAO metabolism. However, as proposed by Oehmen et al. (2010), most GAOs (most sub-groups of *Competibacter*, except *Competibacter* sub-group 6, and both clusters of *Defluviicoccus vanus*) are unable to denitrify nitrite. This might indicate a potential difficulty to obtain an enriched DGAO sludge to achieve this research objective.

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