กลยุทธ์ในการใช้เซลล์ตรึงเพื่อสร้างกระบวนการไนตริฟิเคชั่นอย่างไม่สมบูรณ์

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STRATEGIES FOR ACHIEVING PARTIAL NITRIFICATION BY IMMOBILIZED CELLS

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ไชยวัฒน์ รงค์สยามานนท์: กลยุทธ์ในการใช้เซลล์ตรึงเพื่อสร้างกระบวนการไนตริฟิเคชั่นอย่างไม่สมบูรณ์ (STRATEGIES FOR ACHIEVING PARTIAL NITRIFICATION BY IMMOBILIZED CELLS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ.ดร. เอกลักษณ์ คาน, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: อ.ดร.ตะวัน ลิมปิยากร, 192 หน้า.

การกำจัดไนโตรเจนทางชีวภาพแนวใหม่ที่ใช้ไนไตรท์เป็นตัวรับอิเล็กตรอนแทนการใช้ไนเตรทหรือ Shortcut Biological Nitrogen Removal สามารถบำบัดน้ำเสียที่มีปริมาณอินทรีย์คาร์บอนต่ำและ/หรือ มีปริมาณแอมโมเนียสูงได้อย่างประหยัดและมีประสิทธิภาพ หลักการของการกำจัดคือการสนับสนุนให้เกิดการไนตริฟิเคชั่นอย่างไม่สมบูรณ์ ซึ่งหมายถึงการสนับสนุนให้เกิดการออกซิไดซ์แอมโมเนียเป็นไนไตรท์หรือการไนไตรเตชั่นโดยแอมโมเนียออกซิไดซิ่งแบคทีเรียหรือเอไอบี แทนการสนับสนุนให้เกิดการออกซิไดซ์ไนไตรท์เป็นไนเตรทหรือการไนเตรเตชั่นโดยไนไตรท์ออกซิไดซิ่งแบคทีเรียหรือเอ็นไอบี การศึกษาครั้งนี้ประยุกต์ใช้เซลล์ที่ตรึงด้วยวิธี phosphorylated-polyvinly alcohol เพื่อยับยั้งแอคทิวิตีของเอ็นไอบีภายใต้สองยุทธศาสตร์พื้นฐาน คือการจำกัดปริมาณออกซิเจนละลายและสนับสนุนการสะสมของระดับแอมโมเนียอิสระในถังปฏิกริยา ผลการศึกษาจลนศาสตร์ของกระบวนการไนไตรเตชั่นและไนเตรเตชั่นในถังปฏิกริยาแบบแบทช์พบว่าทั้งสองยุทธศาสตร์สามารถถูกใช้เพื่อสร้างกระบวนการไนตริฟิเคชั่นอย่างไม่สมบูรณ์ได้แต่การใช้เพียงยุทธศาสตร์เดียวอาจไม่สามารถรักษาสภาพของกระบวนการไนตริฟิเคชั่นอย่างไม่สมบูรณ์ให้คงอยู่ได้อย่างมีประสิทธิภาพ สำหรับผลการศึกษาปัจจัยของหัวเชื้อเซลล์ตรึงที่แตกต่างกันต่อการเร่งให้เกิดกระบวนการไนตริฟิเคชั่นอย่างไม่สมบูรณ์ในระหว่างการเริ่มเดินระบบในถังปฏิกริยาเซลล์ตรึงแบบไหลต่อเนื่อง พบว่าหัวเชื้อที่มีปริมาณเอ็นไอบีสูงและมีความสามารถในการสร้างกระบวนการไนตริฟิเคชั่นอย่างสมบูรณ์และไม่สมบูรณ์ที่แตกต่างกันจะสามารถสร้างกระบวนการไนตริฟิเคชั่นอย่างไม่สมบูรณ์ได้ในระดับที่ไม่แตกต่างกันและภายในระยะเวลาใกล้เคียงกัน (65 – 66% ของแอมโมเนียที่ถูกกำจัดถูกสะสมในรูปไนไตรท์ ภายใน 30 – 42 วัน ของการเริ่มเดินระบบ) ซึ่งน่าจะเป็นผลจากปัจจัยของระดับออกซิเจนภายในเซลล์ตรึงมีผลต่อประสิทธิภาพของกระบวนการฯ มากกว่าปัจจัยของหัวเชื้อ สำหรับผลการศึกษาปัจจัยของระดับออกซิเจนละลายและ/หรือระดับแอมโมเนียอิสระต่อระดับของการไนตริฟิเคชั่นอย่างไม่สมบูรณ์ในระหว่างการเดินระบบระยะยาวของถังปฏิกริยา ฯ พบว่าระดับของการไนตริฟิเคชั่นอย่างไม่สมบูรณ์จะสูงกว่าภายใต้สภาวะที่ออกซิเจนละลายต่ำกว่า 2 มก./ล. และระดับแอมโมเนียอิสระสูงกว่า 0.6 – 0.9 มก./ล. นอกจากนี้พบว่าระดับของการยับยั้งกระบวนการไนเตรเตชั่นด้วยแอมโมเนียอิสระจะขึ้นกับระดับของออกซิเจนละลายในถังปฏิกริยา เป็นผลทำให้อัตราส่วนของออกซิเจนละลายต่อแอมโมเนียอิสระคือพารามิเตอร์ที่เหมาะสมในการควบคุมระดับของการไนตริฟิเคชั่นอย่างไม่สมบูรณ์มากกว่าการใช้ระดับออกซิเจนละลายหรือระดับแอมโมเนียอิสระแต่เพียงอย่างเดียว สำหรับผลการศึกษาปัจจัยของเสเทอโรทรอฟต่อแอคทิวิตีของเอไอบีในเซลล์ตรึงภายใต้สภาวะของการรับภาระบรรทุกสูงของสารพิษต้นแบบพาราไนโตรฟีนอลอย่างทันทีทันใด (shock load) พบว่าการลดลงของแอคทิวิตีของเอไอบีที่อาจนำไปสู่ความล้มเหลวของกระบวนการไนตริฟิเคชั่นอย่างไม่สมบูรณ์สามารถป้องกันได้ด้วยการใช้เซลล์ตรึงแทนการใช้เซลล์แขวนลอยซึ่งอาจเป็นผลเนื่องจากการลดการแพร่ของสารพาราไนโตรฟีนอลด้วยชั้นของเซลล์เสเทอโรทรอฟที่อยู่บริเวณขอบนอกของเซลล์ตรึง จึงทำให้ความอ่อนไหวต่อสารพิษของเอไอบีที่อยู่บริเวณชั้นที่ลึกกว่าลดลง

สาขาวิชา การจัดการสิ่งแวดล้อม ลายมือชื่อนิสิต.....

ปีการศึกษา 2553 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

4989712920: MAJOR ENVIRONMENTAL MANAGEMENT
KEYWORDS : PARTIAL NITRIFICATION / ENTRAPPED CELL / OXYGEN LIMITATION/ FREE AMMONIA/ SPATIAL DISTRIBUTION

CHAIWAT RONGSAYAMANONT: STRATEGIES FOR ACHIEVING PARTIAL NITRIFICATION BY IMMOBILIZED CELLS. ADVISOR: PROFESSOR EAKALAK KHAN, Ph.D., CO-ADVISOR: TAWAN LIMPIYAKORN, Ph.D., 192 pp.

Shortcut biological nitrogen removal (SBNR) is a cost effective innovative process to treat low carbon or/and high nitrogen wastewater. Partial nitrification (PN) is believed to be the rate-limiting step of the overall SBNR and can be achieved by the oxidation of ammonia (NH_3) to nitrite (NO_2^-) (or nitritation) without further oxidation of NO_2^- to nitrate (or nitratation). The most two common strategies to promote activity of ammonia oxidizing bacteria (AOB) over nitrite oxidizing bacteria (NOB) under normal temperature condition are to maintain oxygen (O_2)-limiting or/and free ammonia (FA)-accumulating conditions in the systems. The study was divided into three main tasks. Task 1 is to examine whether and how the two most common strategies can be applied for entrapped cell system. Results from batch nitritation and nitratation kinetic study implied that FA inhibition or O_2 limitation can be used to maintain PN in entrapped cell but might not be effective strategy. Task 2 is to find out the strategies to achieve PN in continuous-flow entrapped cell nitritation reactors. This part of experiment was sectioned into two subtasks, the first subtask is to study the effect of different entrapped inoculums on accelerating PN during start-up periods. The results showed that high NOB entrapped cells inoculums, which has different ability to nitrifying and partial nitrifying, can achieve the stable PN at comparable level and timeframe (65 – 66% nitritation after 30 - 42 days of the start-up). This indicated that a step for preparing sludge which is readily for nitrifying or partial nitrifying, was not needed for entrapped cells. The control factor is expected to be the levels of O_2 in the gel beads under the presence of high NH_3 concentration. Therefore, cell entrapment can be an effective way to accelerate partial nitrification. The second subtask is to study effect of bulk dissolved oxygen (DO) or/and FA concentrations on PN during the long term operation period. Higher NO_2^- accumulation was found at the lower concentration of bulk DO and the higher concentration of FA. Because the accumulation of NO_2^- depended on both concentrations of bulk DO and FA, a relative ratio of both parameters (ratio of DO/effluent FA) rather than either one is recommended to use as a control parameter for PN. Task 3 is to study effect of heterotrophs on the activity of AOB in entrapped cell under condition simulated the shock load of a model toxic chemical, *p*-nitrophenol (PNP). Two sequentially tests, 1st and 2nd batch test, were used to investigate the PNP degradation and NH_3 oxidation under condition simulated the 1st and 2nd time of PNP shock load in batch reactor with nitritation entrapped cells which have a different amounts of heterotrophs in the gel beads. Results from task 3 (and a part of the results for suspended cells from task 1) implied that an inability to recover the AOB activity after experience with the toxic PNP shock can be partly prevented in the entrapped cell based-rather than the suspended cell based-reactor as a results from reducing the penetration of PNP by the outer layer of heterotrophs and subsequently reduce toxic sensitivity of AOB in the deeper part of the biofilm-like layer structure in the peripheral of the gel bead.

Field of Study : Environmental Management Student's Signature .....

Academic Year : 2010 Advisor's Signature .....

Co-advisor's Signature .....

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CONTENTS

Page

ABSTRACT (Thai)..... iv

ABSTRACT (English)……...…….....……...……. v

ACKNOWLEDGEMENTS……. vi

CONTENTS……..…… vii

LIST OF TABLES……..……...…….. x

LIST OF FIGURES……...……..……...…. xii

LIST OF ABBREVIATIONS……. xx

CHAPTER I INTRODUCTION……...……. 1

1.1 State of Problem.....……...……..... 1

1.2 Objectives…….…….……...…….. 6

1.3 Hypothesis…….……..... 7

1.4 Scope of study……..…….……..... 8

CHAPTER II BACKGROUND AND LITERATURE REVIEW.…….…. 9

2.1 Biological nitrogen removal……..……..... 9

2.2 Partial nitrification……..…….……..... 16

2.3 Microorganisms involved in nitrification……..…….…… 22

2.4 Current practical implementation of partial nitrification in SBNR system……..…….……..... 26

Page

2.5 Immobilized cell……..…….……..... 31

2.6 *p*-nitrophenol……..…….……..... 34

CHAPTER III EXPERIMENTAL FRAMEWORK…… 37

3.1 Experimental framework…… 37

CHAPTER IV RELATIONSHIP BETWEEN RESPIROMETRIC ACTIVITY AND COMMUNITY OF ENTRAPPED NITRIFYING BACTERIA: IMPLICATIONS FOR PARTIAL NITRIFICATION…… 40

4.1 Introduction……..... 40

4.2 Materials and methods……....…… 42

4.3 Results and discussion……. 48

4.4 Conclusions…….. 60

CHAPTER V EFFECT OF INOCULUM AND DO/FA RATIO ON ACHIEVING PARTIAL NITRIFICATION USING GEL ENTRAPMENT…….. 62

5.1 Introduction……..... 62

5.2 Materials and methods……..... 65

5.3 Results and discussion……. 72

5.4 Conclusions.……..... 113

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

Page

CHAPTER VI EFFECT OF HETEROTROPHS ON ACTIVITY OF AOB IN GEL BEADS UNDER CONDITION OF *p*-NITROPHENOL INHIBITION…… 115

6.1 Introduction……..... 115

6.2 Materials and methods……..…..…… 117

6.3 Results and discussion……. 122

6.4 Conclusions.……..…… 135

CHAPTER VII CONCLUSIONS AND RECOMMENDATIONS…… 137

7.1 Conclusions.……..... 137

7.2 Recommendations.……... 141

REFERENCES……..... 143

APPENDICES…….…… 168

APPENDIX A…….....……. 169

APPENDIX B…… 175

BIOGRAPHY……..……. 192

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

LIST OF TABLES

Table		Page
2.1	Kinetic parameters for nitrification at 20^{o}C…….....	11
2.2	Concentrations of COD, BOD, and total nitrogen in high nitrogen-containing wastewater …….....	14
2.3	Comparison of stoichiometrical requirement for oxygen and organic compound (as COD) in various BNR scheme ……...	15
2.4	Half saturation constants for oxygen of AOB and NOB……	17
2.5	Previous evidence achieving nitrite accumulation using low-DO-level maintaining strategy…….....	17
2.6	FA and FNA inhibition on *Nitrosomonas* and *Nitrobacter* ……	21
2.7	Half saturation constants for ammonia of AOB pure cultures……..	23
2.8	Kinetic parameters for *Nitrospira* and *Nitrobacter*……	26
2.9	Operating condition and performance of partial nitrification in separate reactor system…….....	30
2.10	Physical and chemical properties of *p*-nitrophenols……...	35
4.1	Oligonucleotide probes used…….....	47
4.2	Summary of Monod kinetic parameters …….....	50
5.1	Setup and operation of laboratory-scale reactors for preparation of suspended inoculums.....	66
5.2	PCR primers used in this study.....	71
5.3	Labelled 16SrRNA oligonucleotide probes used in this study.....	72

Table Page

5.4	Operating conditions and steady-state performance of laboratory-scale reactors during preparation of suspended inocula.....	73
5.5	Operating conditions and steady-state performance of entrapped cell nitritation reactors (ENN, EN, EPNI, and EPNII reactors) during start up periods.	86
5.6	Summary of operating conditions and performance of ENN entrapped cell nitritation reactor.....	102
5.7	Summary of operating conditions and performance of EPNI entrapped cell nitritation reactor.....	103
5.8	Summary of operating conditions and performance of EPNII entrapped cell nitritation reactor.....	104
6.1	Labeled 16SrRNA oligonucleotide probes used in this study ……	122
6.2	Operating conditions and steady-state performance of entrapped cell nitritation reactors …….....	123

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

LIST OF FIGURES

Figure		Page
1.1	Shortcut biological nitrogen removal (SBNR)……..	1
3.1	Experimental framework of Task 1 (Chapter IV)	37
3.2	Experimental framework of Task 2 (Chapter V)	38
3.3	Experimental framework of Task 3 (Chapter VI)	39
4.1	SOUR of entrapped and suspended nitrifiers as a function of total ammonia concentration [(a) for SMH and EMH sludge, and (c) for SFF and EFF sludge] and nitrite concentration [(b) for SMH and EMH sludge, and (d) for SFF and EFF sludge]. The prefixes S and E prior to MH or FF represent suspended and entrapped cells, respectively…….	48
4.2	SOUR of entrapped and suspended nitrifiers as a function of DO concentration in nitritation [(a) for SMH and EMH sludge, and (c) for SFF and EFF sludge] and in nitratation [(b) for SMH and EMH sludge, and (d) for SFF and EFF sludge]. The prefixes S and E prior to MH or FF represent suspended and entrapped cells, respectively..	52
4.3	$SOUR/SOUR_{MAX}$ of entrapped and suspended nitrifiers as a function of free ammonia concentration in nitritation [(a) for SFF and EFF sludge, and (c) for SMH and EMH sludge] and in nitratation [(b) for SFF and EFF sludge, and (d) for SMH and EMH sludge]. The prefixes S and E prior to FF or MH represent suspended and entrapped cells, respectively.…….	54

Figure Page

4.4 Bacterial community structures of suspended and entrapped MH and FF sludge determined by FISH. The percentages of AOB, *Nitrospira* genus, and *Nitrobacter* spp., in sludge were determined from the relative areas of NSO190, Ntspa662 and Nit3 probes to the EUB338 probe (all bacteria), respectively. The fraction of heterotrophs was estimated from the remaining relative area other than those of AOB and NOB. The average relative area and standard deviation were determined by evaluating at least nine representative microscopic fields…… 56

4.5 Example of FISH-CLSM images of entrapped nitrifiers in Moorhead sludge, hybridized with Nse1472, specific for the detection of *Nitrosomonas europaea* (in green) and Nit3, specific for the detection of *nitrobacter* spp. (in red) …… 57

4.6 Example of FISH-CLSM images of nitrifiers in Moorhead sludge: (a) entrapped nitrifiers, hybridized with NSO190 (in red) and EUB338, specific for the detection of all bacteria (in green). The target cells complimentary to both Rhodamine Red-labeled probe (in red) and Oregon Green 488-labeled probe (in green), are in yellow. The observed bacteria in green, which stayed around the peripheral layer of the bead, were heterotrophs and (b) suspended nitrifiers, hybridized with NSO190, specific for the detection of beta-proteobacteria AOBs (in red) and Nse1472, specific for the detection of *Nitrosomonas europaea* (in green). …… 58

Figure Page

5.1 Abundance of ammonia oxidizing bacteria (AOB), ammonia oxidizing archaea (AOA), *Nitrobacter* (NOB) and *Nitrospira* (NOB) in suspended inocula at the end of the preparation periods. Limit of detection (LOD) for the bacterial and archaeal *amoA* genes was 6.0×10^{1} and 6.9×10^{3} cells $mgVSS^{-1}$, respectively. And, the LOD for *Nitrobacter* and *Nitrospira* 16S rRNA genes was 1.7×10^{3} and 5.1×10^{3} cells $mgVSS^{-1}$, respectively..... 74

5.2 FISH image showing nitrifying microorganisms in suspended inocula: NN sludge (a, b), N sludge (c, d), PNI sludge (e, f), and PNII sludge (g, h). Left-side (a, c, e, g) are suspended inocula, hybridized with NSO190 (beta-proteobacterial AOB; green) and EUB338 (all bacteria; red). Right-side is suspended inocula, hybridized with Ntspa662 (*Nitrospira;* red) (b) and NIT3 (*Nitrobacter;* red) (d, f, h). The target cells complimentary to both Cy3-labeled probe (red) and Alexa Flour 488-labeled probe (green), are shown in orange-yellow. NOTE: Non bright green and red signals were suggested to be non-active target cells, autofluorescence of cell lysate, exopolymeric substance or/and and precipitated salts..... 75

5.3 Abundance of AOB, AOA, *Nitrobacter* (NOB) and *Nitrospira* (NOB) in entrapped inocula (before starting up nitritation reactors). The LOD for the AOB and AOA *amoA* genes were 1.45×10^{2} and 1.01×10^{2} cells unit volume of $bead^{-1}$, respectively. And, the LOD for *Nitrobacter* and *Nitrospira* 16S rRNA genes were 5.16×10^{2} and 1.54×10^{3} cells unit volume of $bead^{-1}$, respectively..... 82

Figure Page

5.4 FISH image showing nitrifying microorganisms in entrapped inocula: ENN entrapped cell (a, b), EN entrapped cell (c, d), EPNI entrapped cell (e, f), and EPNII entrapped cell (g, h). Left-side images are entrapped cells, hybridized with NSO190 (beta-proteobacterial AOB; green) and EUB338 (all bacteria; red). Right-side images are entrapped cells, hybridized with Ntspa662 (*Nitrospira*; red) (b) and hybridized with NIT3 (*Nitrobacter*; red) (d, f, h). The target cells complimentary to both Cy3-labeled probe (red) and Alexa Flour 488-labeled probe (green), are shown in orange-yellow. NOTE: Non bright green and red signals were suggested to be non-active target cells, autofluorescence of cell lysate, exopolymeric substance or/and and precipitated salts. 83

5.5 Percent NH3 removal during start up of entrapped cell nitritation reactors using ENN, EN, EPNI and EPNII entrapped inocula..... 87

5.6 Nitritation level (ratio of NO_2^- produced per NH_3 removed) during start up of entrapped cell nitritation reactors using ENN, EN, EPNI and EPNII entrapped inocula..... 87

5.7 Abundance of AOB, AOA, *Nitrobacter* (NOB) and *Nitrospira* (NOB) of entrapped cells before and after (at the end of phase II) start up of nitritation reactor. The LOD for the bacterial and archaeal *amoA* genes were 1.45×10^2 and 1.01×10^2 cells unit volume of bead^{-1}, respectively. And, the LOD for *Nitrobacter* and *Nitrospira* 16S rRNA genes were 5.16×10^2 and 1.54×10^3 cells unit volume of bead^{-1}, respectively..... 88

Figure Page

5.8 FISH images showing nitrifying microorganisms in entrapped harvested after phase I: ENN entrapped cell (a, b), EN entrapped cell (c, d), EPNI entrapped cell (e, f), and EPNII entrapped cell (g, h). Left-side images are entrapped cells, hybridized with NSO190 (beta-proteobacterial AOB; green) and EUB338 (all bacteria*;* red), Right-side images (b, d, f, h) are entrapped cells, hybridized with NIT3 (*Nitrobacter*; red). The target cells complimentary to both Cy3-labeled probe (red) and Alexa Flour 488-labeled probe (green), are shown in orange-yellow. NOTE: Non bright green and red signals were suggested to be non-active target cells, autofluorescence of cell lysate, exopolymeric substance or/and and precipitated salts. 89

5.9 FISH image showing nitrifying microorganisms in an inner part of ENN entrapped cells harvested after phase I. Entrapped cell was hybridized with NSO190 (beta-proteobacterial AOB; green) and EUB338 (all bacteria; red). The target cells complimentary to both Cy3-labeled probe (red) and Alexa Flour 488-labeled probe (green), are shown in orange-yellow. NOTE: Non bright green and red signals were suggested to be non-active target cells, autofluorescence of cell lysate, exopolymeric substance or/and and precipitated salts. 90

Figure Page

5.10 FISH images showing nitrifying microorganisms in entrapped cells harvested after phase II: ENN entrapped cell (a, b), EN entrapped cell (c, d), EPNI entrapped cell (e, f), and EPNII entrapped cell (g, h). Left-side images are entrapped cells, hybridized with NSO190 (beta-proteobacterial AOB; green) and EUB338 (all bacteria; red), Right-side images (b, d, f, h) are entrapped cells, hybridized with NIT3 (*Nitrobacter*; red). The target cells complimentary to both Cy3-labeled probe (red) and Alexa Flour 488-labeled probe (green), are shown in orange-yellow. NOTE: Non bright green and red signals were suggested to be non-active target cells, autofluorescence of cell lysate, exopolymeric substance or/and and precipitated salts. 91

5.11 Percentage of NH_3 removal during steady-state conditions at different concentrations of bulk DO and pH of a) ENN, b) EPNI, and c) EPNII entrapped cell nitritation reactors…….. 105

5.12 Nitrite accumulation indicated by percentage of effluent NO_2^- concentration per NH_3 removed during steady-state conditions at various concentrations of bulk DO and effluent FA concentration of a) ENN, b) EPNI, and c) EPNII entrapped cell nitritation reactors… 108

5.13 Nitrite accumulation indicated by percentage of effluent NO_2^- concentration per NH_3 removed during steady-state conditions at various ratios of bulk DO and effluent FA concentration..... 111

Figure Page

6.1 FISH images showing spatial distributions of AOB, NOB and other bacteria in nitritation entrapped cells from C/N0 (a, b), C/N0.4 (c, d) and C/N0.8 (e, f) reactors. Images in the left column (a, c, e) are entrapped cells, hybridized with NSO190 (beta-proteobacterial AOB; green) and EUB338 (all bacteria; red). Images in the right column (b, d, f) are entrapped cells, hybridized with NIT3 (*Nitrobacter*; red). The target cells complimentary to both Cy3-labeled probe (red) and Alexa Flour 488-labeled probe (green), are shown in yellow. NOTE: Non bright green and red signals were suggested to be non-active target cells, autofluorescence of cell lysate, exopolymeric substance or/and precipitated salt..... 126

6.2 Diagram indicate the position of heterotrophs and autotrophic AOB in FISH images from Fig. 6.1 (a, c, e)..... 127

6.3 Degradation rate of PNP during the first batch test with entrapped cells from C/N0, C/N0.4, and C/N0.8 reactors at initial TAN concentrations of 50 mg N l^{-1} and 100 mg N l^{-1}..... 129

6.4 Degradation rate of PNP during the 2st batch test with entrapped cells from C/N0, C/N0.4, and C/N0.8 reactors at initial TAN concentrations of 50 mgN l^{-1} and 100 mgN l^{-1}. Noted that 86 µM ATU was added into the test with initial PNP concentrations of 4 mg l^{-1} and 8 mg l^{-1}. 129

6.5 Degradation of PNP by plain bead (no cells; abiotic control test)..... 130

6.6 Rate of NH_3 oxidation during the 1st batch test with entrapped cells from C/N0, C/N0.4, and C/N0.8 reactors at initial TAN concentrations of 50 mgN l^{-1} and 100 mgN l^{-1}..... 133

Figure Page

6.7 Rate of NH_3 oxidation during the 2nd batch test with entrapped cells from C/N0, C/N0.4, and C/N0.8 reactors at initial TAN concentrations of 50 mgN l^{-1} and 100 mgN l^{-1}..... 133

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

LIST OF ABBREVIATIONS

AOA	ammonia oxidizing archaea
AOB	ammonia oxidizing bacteria
DO	dissolved oxygen
EN	entrapment of nitrifying sludge
ENN	entrapment of non-nitrifying sludge
EPNI	entrapment of partial nitrifying sludge I
EPNII	entrapment of partial nitrifying sludge II
FA	free ammonia
FISH	fluorescence in situ hybridization
FNA	free nitrous acid
mg l^{-1}	milligram per liter
µg l^{-1}	microgram per liter
MLSS	mixed-liquor suspended solid
mM	millimolar
N sludge	nitrifying sludge
NN sludge	non-nitrifying sludge
ng	nanogram
nm	nanometer
NOB	nitrite oxidizing bacteria
PCR	polymerase chain reaction
PNI sludge	partial nitrifying sludge I
PNII sludge	partial nitrifying sludge II
PNP	*p*-nitrophenol
SBNR	shortcut biological nitrogen removal
VSS	volatile suspended solid
vol	volume
WWTP	wastewater treatment plant
wt	weight

ศูนย์วิทยทรัพยากร
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CHAPTER I

INTRODUCTION

1.1 State of Problem

Treatment of ammonium rich wastewater, such as supernatant from municipal wastewater sludge digesters, landfill leachate, and high ammonia industrial wastewater has received attention due to stringent discharge regulations and economic aspects (Egli et al., 2003). The operational costs of biological nitrogen removal of ammonium rich wastewater are mainly on the aeration and organic carbon requirements for nitrification and denitrification, respectively. In 1995, shortcut biological nitrogen removal (SBNR) was developed in order to reduce these costs (STOWA, 1995). The main concept of SBNR is based on the fact that nitrite is an intermediate for both nitrification and denitrification steps. Thus, it will be convenient to partially nitrify up to nitrite in the nitrification step called partial nitrification and then to denitrify from nitrite in the denitrification step (Fig 1.1).

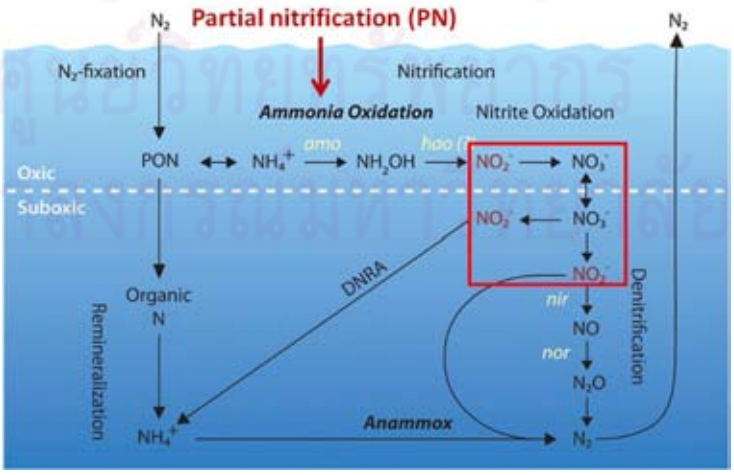

Fig. 1.1 Shortcut biological nitrogen removal (SBNR)

To completely nitrify each mol of nitrogen in the traditional nitrification process, ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) use 1.5 and 0.5 mol of oxygen, respectively. In the traditional denitrification process, denitrifying bacteria require sufficient organic carbon to denitrify nitrate to nitrite and finally to nitrogen gas. SBNR has become more attractive as a way to reduce oxygen demand and organic carbon requirement compared to conventional nitrification and denitrification. There have been a number of recently developed processes based on the concept of SBNR to remove nitrogen from ammonium rich wastewater and low carbon/nitrogen wastewater (C/N < 3.5 - 4) including the single reactor high activity ammonia removal over nitrite (SHARON®) process (Hellinga et al., 1998) and the combined SHARON and anaerobic ammonium oxidation (SHARON-ANAMMOX®) process (van Dongen et al., 2001).

The most critical condition needed to achieve partial nitrification or nitritation, the first step of SBNR process, is to inhibit the production of nitrate or nitratation without excessively retarding the nitritation rate. It has extensively been known that AOB are autotrophic bacteria with very low growth rates and yields. Thus, an unintentional washing out of AOB should be avoided when generating and maintaining nitritation reactors. The prolonged biomass retention may be obtained in a variety of cell immobilization techniques (Wijffels and Tramper, 1995). The immobilization by entrapping AOBs in polymeric substances such as polyvinyl alcohol (PVA) would ensure their retention within the treatment system. The application of nitrifying bacterial immobilization for nitrogen removal has been extensively studied (Cao et al., 2002; Vogelsang et al., 1997; Leenen et al., 1996; Wijffels and Tramper, 1995; Chen and Lin, 1994). Immobilized cells have many advantages over suspended cells including higher cell concentrations, higher conversion rates, and protections from toxic effects and temperature shocks. (Rostron et al., 2001; Vogelsang et al., 1997; Yang et al., 1997; Chen and Lin, 1994)

Although retaining AOB and washing out/inhibiting NOB simultaneously to generate and maintain partial nitrification are difficult (Blackburne et al., 2007), a number of strategies has been studied and suggested including controlling temperature

at 30 – 40^{o}C, controlling dilution rate between the growth rates of AOB and NOB (Hellinga et al., 1998), terminating aeration prior to complete nitrification in sequencing batch operation (Peng et al., 2004; Yoo et al., 1999), promoting free nitrous acid (Prakasam and Loehr, 1972), hydroxylamine addition (Hao and Chen, 1994; Yang and Allman, 1992), fulvic acids addition (Zhang et al., 2000). Among them, limiting dissolved oxygen (DO) concentration (Sliekers et al., 2005; Wyffels et al., 2004) is the most common strategy for partial nitrification. Low DO concentrations are effective because AOB have lower oxygen saturation coefficients than NOB (Wiesmann, 1994) and in turn have higher affinity for oxygen compared with NOB. Therefore, NOB could be significantly inhibited within the lower DO environment such as within sludge floc or within biofilm.

Biofilm can give resistance to oxygen transfer through the biofilm/bulk liquid interface (Picioreanu et al., 1997). Moreover, the spatial distribution of a member of AOB genus *Nitrosomonas* and of NOB genus *Nitrobacter* determined by a fluorescence in situ hybridization (FISH) technique and their oxygen utilization measured by a microelectrode revealed that genus *Nitrosomonas* formed dense layer clusters in the outer layer of the biofilm, while genus *Nitrobacter* are dispersed in close vicinity to the *Nitrosomonas* clusters and the adjacent layer (Schramm et al., 1996). The limitation of DO within the inner part of the biofilm, where NOB exist, mostly is responsible for the complete inhibition of NOB activity in the nitrifying biofilm (Kim et al., 2003).

To the best of our knowledge, there has recently only two peer reviewed article that addresses nitrite accumulation in an immobilized cell reactor (Yan and Hu, 2009; Isaka et al., 2007) Moreover, there have been only a few previous studies which were dedicated to investigating the spatial distribution of immobilized nitrifying bacteria using either an immunological technique such as fluorescent-antibody (FA) labeling (Uemoto et al., 2000; Hunik et al., 1993) or a molecular technique such as FISH (Isaka et al., 2007; Vogelsang et al., 2002). These studies showed that the spatial distribution of immobilized nitrifying bacteria is in a similar arrangement as that of nitrifying biofilm. The biofilm-like layer structure in the periphery of the

entrapped bead is developed based on a competition between species for space and access to substrate such as oxygen (Vogelsang et al., 2002).

Besides lowering DO, promoting free ammonia is also a typical alternative to achieve nitritation (Villaverde et al., 2000; 1997; Fdz-Planco et al., 1996). The inhibitory effect of free ammonia on NOB activities has been extensively reported (Philips et al., 2002; Balmelle et al., 1992) and is linked to nitrite accumulation (Villaverde et al., 2000). Although AOB activities can also be inhibited by free ammonia, NOB activities are much more sensitive to free ammonia than AOB (Abeling and Seyfried, 1992).

In engineering aspects, inoculums used is a critical factor to determine the duration of and the succession of system startup. Although inhibiting NOB and maintain AOB growth so as to partial nitrification achieved are needed to startup nitritation reactor, but NOB still can be persisted as which leading to a significant unwanted nitrate production in biofilm based-nitritation reactor (Wang et al., 2009). Although effect of inoculums on the system performance have been previously demonstrated in both suspended cell and biofilm nitritation reactors (Bartrolí et al., 2011; Chen et al., 2010; Terada et al., 2010) in immobilized cell reactor has never been studied.

Ammonia rich wastewaters containing toxic organic compounds was typical in effluent from several industries such as textile, organic chemical synthesis, petrochemical, resin producing, and pharmaceuticals industries (Olmos et al., 2004). Toxic nitroaromatic compound like *p*-nitrophenol (PNP) was found often in high ammonia industrial wastewater such as pharmaceuticals wastewater (ammonia in wastewater: 80 – 500 mgN l^{-1}; PNP in wastewater: < 10 – 2300 µg l^{-1}; Gupta et al., 2006). PNP is one of the U.S. EPA's priority pollutant which its toxicity value as EC50 is 64 mg/l (Tomei et al., 2004). EPA recommended restricting PNP level in natural waters at below 10 ng l^{-1} (Kuscu and Sponza, 2007). It is hardly biodegradable compound and can stable in both surface and subsurface water due to high solubility and low partitioning coefficient. PNP can create a significant health risks due to their

mutagenic and carcinogenic activity and may bioaccumulate in the food chain (Rezouga et al., 2009; Yi et al., 2006; Tomei et al., 2004). The high inhibitory effect of PNP on AOB have been reported (Zhang et al., 2010; Blum and Speece, 1991). The concentration that inhibited the AOB activity by 50% (IC50) of PNP is 2.6 mg l^{-1} (Blum and Speece, 1991). This indicated that PNP can have a lot of negative effects on the stability of partial nitrification. High fluctuation in quality of industrial effluent can lead to a shock loading problem in industrial wastewater treatment process. This operation problem can be highly adverse and may result in a complete process failure. An inability to recover the AOB activity after experience with shock load of toxic organic compound often lead to a serious drop or failure in nitrification (Amor et al., 2005; Texier and Gomez, 2002; Winther-Nielsen and la Cour Jansen, 1996; Benmoussa et al., 1986). Sensitivity with the instant loading of toxic compound could limit applications of partial nitrification for treating ammonia in real industrial effluent (Suárez-Ojeda, et al., 2010). Thus, the study about operational technique for dealing with this problem in partial nitrification reactor is worth being investigated.

Although the performances of both suspended cell and biofilm nitritation reactors have been reported in the past several years (Sinha and Annachhatre, 2007; Philips et al., 2002), nitritation by immobilized cells for treating ammonium rich wastewater has not been investigated. With the economical benefits of SBNR over the conventional nitrification - denitrification and several advantages of immobilized cells over suspended cells, the application of PVA immobilized nitrifying bacteria for treating ammonium rich wastewater based on two typical strategies, limiting DO and promoting free ammonia accumulation in bulk liquid, is worth being investigated. Specific issues that should be addressed include effect of inoculum history, instability conditions caused by the shock load of toxic compounds, the actual activity of working bacteria, and the bacterial community. More understanding in the actual mechanisms to achieve and maintain stable partial nitrification is a main outcome of this research. This outcome may be further applied to help setting up the design criteria for a novel ammonia removal process over nitrite by immobilized nitrifying biomass.

1.2 Objectives

Task 1 Effects of DO (dissolved oxygen) and FA (free ammonia) on the activities of ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) by respirometric assays.

a) To examine the activities of polyvinyl alcohol (PVA) entrapped AOB and NOB under various DO and FA concentrations.

Task 2 Strategies for achieving partial nitrification in continuous flow entrapped cell nitritation reactors.

a) To compare duration required for achieving stable partial nitrification by entrapped non-nitrifying sludge (ENN), entrapped nitrifying sludge (EN), and entrapped partial nitrifying sludge (EPN).

b) To investigate the abundance and spatial distribution of nitrifying-microorganisms in the gel beads

c) To examine the use of DO/FA ratio to control partial nitrification in a long term operation of entrapped cell nitritation reactors.

Task 3 Effect of heterotrophs on activity of AOB in entrapped cell under condition of *p*-nitrophenol (PNP) inhibition.

a) To examine PNP degradation rate and ammonia oxidation rate in an aerated batch reactor with entrapped cells from organic loaded-nitritation reactors at various initial PNP and ammonia concentrations.

b) To investigate the spatial distribution of nitrifying-microorganisms in the gel beads from organic loaded-nitritation reactors.

1.3 Hypotheses

Task 1 Effects of DO and FA on the activities of AOB and NOB by respirometric assays.

Under the conditions with lower DO or high FA ,

a) The activities of AOB and NOB in both suspended and immobilized forms will be deteriorates.

b) The activities of NOB will be more deteriorated resulting in the lower activity of NOB than AOB for both suspended and immobilized forms.

Task 2 Strategies for achieving partial nitrification in continuous flow entrapped cell nitritation reactors.

a) The duration required for achieving partial nitrification of high ammonia wastewater by entrapped cell system can be reduced by enriching ENN or/and EN as opposed to enriching partial nitrifying sludge and entrapping it.

b) The abundance and spatial distribution of nitrifying-microorganisms in ENN, EN, and EPN will be different.

c) DO/FA ratio can be used as a control parameter to maintain effective partial nitrification in high ammonia wastewater using entrapped cell system.

Task 3 Effect of heterotrophs on activity of AOB in entrapped cell under condition of *p*-nitrophenol (PNP) inhibition.

a) The PNP degradation by heterotrophs in entrapped cells from organic loaded-nitritation reactor can reduce an inhibitory effect of PNP on the AOB activity.

b) The most of AOB clusters in entrapped cells from organic loaded-nitritation reactor will be within the outer layers of the gel beads while the heterotrophs layers will be adjacent to the AOB layers at the outermost parts of the gel beads.

1.4 Scopes of study

a) Unless otherwise states, all reactor start up and operation along this study was conducted in laboratory scale reactor under room temperature.

b) Synthetic wastewater without organic used to be the influent in all experiment shown in chapter 4 and 5.

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CHAPTER II

BACKGROUND AND LITERATURE REVIEW

2.1 Biological nitrogen removal

2.1.1 Conventional biological nitrogen removal (CBNR)

Nitrogenous wastewater, generally in the form of ammonium or organic nitrogen, is usually treated using CBNR. For treating relatively low nitrogen-containing wastewater (total nitrogen concentration (TAN) < 100mg N l^{-1}), CBNR process is recommended as it provides high efficiency as well as good process stability and reliability.

2.1.1.1 Complete nitrification

CBNR is based on autotrophic nitrification followed by heterotrophic denitrification. In the first step of nitrification, ammonia oxidizing bacteria (AOB) oxidize ammonia (NH_4^+) to nitrite (NO_2^-) by ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO) enzyme, so called nitritation (equation 1). In the second step, nitrite oxidizing bacteria (NOB) oxidize NO_2^- to nitrate (NO_3^-) with the involvement of nitrite oxidoreductase (NOR) enzyme, so called nitratation (equation 3). Autotrophic nitrifying bacteria use carbon dioxide and NH_4^+ or NO_3^- as the carbon and nitrogen source for growth, respectively (equation 2 and 4).

Nitritation:

Energy:

$$NH_4^+ + 1.5O_2 \longrightarrow NO_2^- + 2H^+ + 2H_2O \quad ; \Delta G^o = -270 \text{ kJ/mol of } NH_4^+\text{-N} \qquad (1)$$

Cell synthesis (AOB):

$$15CO_2 + 13NH_4^+ \longrightarrow 10NO_2^- + 3C_5H_7NO_2 + 23H^+ + 4H_2O \quad (2)$$

Nitratation:

Energy:

$$NO_2^- + 0.5O_2 \longrightarrow NO_3^- ; \Delta G^o = -80 \text{ kJ/mol of } NO_2^-\text{-N} \quad (3)$$

Cell synthesis (NOB):

$$5CO_2 + NH_4^+ + 10NO_2^- + 2H_2O \longrightarrow 10NO_3^- + C_5H_7NO_2 + H^+ \quad (4)$$

By combining energy and cell synthesis reactions (equation 1 and 3 for nitritation, equation 2 and 4 for nitratation) and by using the carbonate equilibrium system:

Nitritation:

$$80.7NH_4^+ + 114.55O_2 + 160.4HCO_3^- \longrightarrow C_5H_7NO_2 + 79.7NO_2^- + 82.7H_2O + 155.4H_2CO_3 \quad (5)$$

Nitratation:

$$134.5NO_2^- + NH_4^+ + 62.25O_2 + HCO_3^- + 4H_2CO_3 \longrightarrow C_5H_7NO_2 + 134.5NO_3^- + 3H_2O \quad (6)$$

The overall reaction for complete nitrification is found by combining nitritation and nitratation (equation 5 and 6):

Nitrification:

$$NH_4^+ + 1.86O_2 + 1.98HCO_3^- \longrightarrow 0.02C_5H_7NO_2 + 0.98NO_3^- + 1.88H_2CO_3 + 1.04H_2O \quad (7)$$

The kinetic of nitrification process is dependent mainly on substrate and dissolved oxygen (DO) concentrations. It is normally described by a Double Monod expression (equation 8). Table 2.1 showed kinetic parameters for each process at 20^oC.

$$SOUR = SOUR_{MAX}\left(\frac{[S]}{K_S + [S]}\right)\left(\frac{[DO]}{K_O + [DO]}\right) \quad (8)$$

where SOUR is specific oxygen uptake rate for nitritation or nitratation (mgO_2 [mgVSS h]$^{-1}$), $SOUR_{MAX}$ is maximum SOUR for nitritation or nitratation ($SOUR_{MAX,AOB}$, $SOUR_{MAX,NOB}$) (mg O_2 [mgVSS h]$^{-1}$), [S] is substrate concentration (TAN or NO_2^-) (mgN l^{-1}), [DO] is DO concentration (mgO_2 l^{-1}), K_S is half saturation coefficient for substrate ($K_{S,AOB}$,$K_{S,NOB}$) (mgN l^{-1}), and K_O is half saturation coefficient for oxygen ($K_{O,AOB}$, $K_{O,NOB}$) (mgO_2 l^{-1}).

Table 2.1 Kinetic parameters for nitrification at 20°C (adjusted from Henze et al., 2002)

Kinetic parameter	Symbol	Unit	Nitritation	Nitratation	Nitrification
Maximum specific growth rate	μ_{MAX}	d^{-1}	0.6-0.8	0.6-1.0	0.6-0.8
Half saturation constant for substrate	K_S	mgN l^{-1}	0.3-0.7	0.8-1.2	0.3-0.7
Half saturation constant for oxygen	K_O	gO_2 l^{-1}	0.5-1.0	0.5-1.5	0.5-1.0
Yield	Y	gVSS gN^{-1}	0.10-0.12	0.05-0.07	0.15-0.20
Decay	b	d^{-1}	0.03-0.06	0.03-0.06	0.03-0.06

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2.1.1.2 Denitrification

In denitrification, NO_3^- is reduced to gaseous nitrogen (N_2) with a variety of electron donors, such as methanol, acetate, or organic substances in wastewater under anoxic condition by heterotrophs which are widespread among the groups of Proteobacteria (equation 9). Equation 10 showed reaction for cell synthesis of denitrifier by using NH_4^+ as a nitrogen source. Denitrification was driven in stepwise

manner in which NO_3^- is sequentially reduced to NO_2^-, nitric oxide (NO), nitrous oxide (N_2O), and N_2 as shown in equation 11 - 14:

NO_3^- denitrification:

$$C_{18}H_{19}O_9N + 14NO_3^- + 14H^+ \longrightarrow 7N_2 + 17CO_2 + HCO_3^- + NH_4^+ + H_2O$$

$$; \Delta G^o = -103 \text{ kJ/e-eqv} \quad (9)$$

Cell synthesis (denitrifier) (NH_4^+ assimilation):

$$0.52C_{18}H_{19}O_9N + 3.28NO_3^- + 0.48NH_4^+ + 2.80H^+ \longrightarrow C_5H_7NO_2 + 1.64N_2 + 4.36CO_2 + 3.8H_2O \quad (10)$$

NO_3^- reduction:

$$NO_3^- + 2e^- + 2H^+ \longrightarrow NO_2^- + H_2O \quad (11)$$

NO_2^- reduction:

$$NO_2^- + e^- + 2H^+ \longrightarrow NO + H_2O \quad (12)$$

NO reduction:

$$2NO + 2e^- + 2H^+ \longrightarrow N_2O + H_2O \quad (13)$$

N_2O reduction:

$$N_2O + 2e^- + 2H^+ \longrightarrow N_2 + H_2O \quad (14)$$

2.1.1.3 Limitations of CBNR

Because nitrification and denitrification are carried out by different microorganisms under different conditions, they must be designed and operated in separate time sequences or spaces (Lee et al., 2001). For treating high nitrogenous wastewater (TAN > 100 mgN l^{-1}), a large volume of reactor and a high level of oxygen (2 moles of O_2/mole of NH_4^+-N as shown in equation 1 and 2) are required to accomplish complete nitrification. And, because the organic carbon present naturally in high nitrogen-containing wastewater is limited, a high level of external carbon

sources (ie. methanol, acetate) must be supplied for denitrification of wastewater with low C/N content (Tam et al., 1992). In overall, these increase significantly operational cost. Furthermore, most existing wastewater treatment facilities which not designed for nitrogen removal, meeting the demands of the complete nitrification and denitrification in these facilities can be difficult. Thus, many wastewater treatment plants do not meet the effluent standard of 10 mg N l^{-1} (Jetten et al., 2001). The limitations of low removal efficiency, high oxygen requirement, long retention time, and high external carbon source requirement are the driving forces to use CBNR, thus shortcut biological nitrogen removal (SBNR) must be developed to avoid these drawbacks.

2.1.2 Shortcut biological nitrogen removal

2.1.2.1 Shortcut biological nitrogen removal via nitrite denitrification

SBNR is the process in which intermediate NO_2^- occurred in nitrification be reduced to N_2 in denitrification step. With this principle, partial nitrification in which NH_4^+ is oxidized to intermediate NO_2^- is promoted to achieve an accumulation of NO_2^- instead of NO_3^- in aerobic condition (promote reaction in equation 5 instead of that in equation 5 and 6). In anoxic environment, the intermediate NO_2^- is reduced to N_2 by denitrifier (promote reaction in equation 11 instead of that in equation 10).

Compared with CBNR, SBNR has the following advantages (Beccari et al., 1983; Turk and Mavinic, 1989; Peng and Zhu, 2006):

1. 25% lower oxygen consumption in the aerobic phase implies 60% energy saving in the entire process (see Table 2.3).

2. The requirement for electron donors is as much as 40% lower in the anoxic phase (see Table 2.3).

3. NO_2^- denitrification rate is 1.5 to 2 times higher than NO_3^- denitrification rate.

SBNR is more appropriated to apply with high nitrogen wastewater or low C/N content such as sludge digester supernatant, piggery wastewater, landfill leachate, and some industrial wastewater (tannery, pharmaceutical, dye wastewater) (Turk and Mavinic, 1989; Villaverde et al., 1997).

Table 2.2 Concentrations of Chemical Oxygen Demand (COD), Biological Oxygen Demand (BOD), and total nitrogen in high nitrogen-containing wastewater (modified from Van Hulle et al., 2010).

Wastewater	COD ($mg\ l^{-1}$)	BOD ($mg\ l^{-1}$)	Total nitrogen ($mg\ l^{-1}$)	References
Tannery	300 - 1400	N.A.	50 - 200	Carucci et al., 1999
Landfill leachate	1300 - 1600	N.A.	160 - 270	Jokela et al., 2002
Slaughter house (after anaerobic pretreatment)	1400 - 2400	N.A.	170 - 200	Keller et al., 1997
Reject water	232 - 12587	81-750	260 - 958	Gil and Choi, 2004
Starch production (after anaerobic pretreatment)	3000	990	1060	Abeling and Seyfried, 1992
Piggery manure	3969	1730	1700	Obaja et al., 2003

N.A., not available

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

Table 2.3 Comparison of stoichiometrical requirement for oxygen and organic compound (as COD) in various BNR scheme (modified from Van Hulle et al., 2010)

Process scheme		Oxygen requirement ($gO_2\ gN^{-1}$)	COD requirement without cell assimilation ($gCOD\ gN^{-1}$)	COD requirement with cell assimilation ($gCOD\ gN^{-1}$)
CBNR	Complete nitrification + nitrate denitrification	4.57	2.86	4.0
SBNR	Partial nitrification + nitrite denitrification	3.43	1.72	2.4
SBNR	Partial nitritation + Anammox	1.72	-	-

2.1.2.2 Shortcut biological nitrogen removal via anaerobic ammonium oxidation (Anammox)

Anaerobic ammonium oxidation process is a new way to remove nitrogen from wastewater (Jetten et al., 1997). Under oxygen-free condition, anaerobic ammonium oxidation (anammox) bacteria remove nitrogen autotrophically by using NH4+ as an electron donor, NO_2^- as an electron acceptor and CO_2 as a main carbon source for their growth without using addition external organic carbon as shown in equation 15 (Jetten et al., 1999). The anammox process is carried out by two anammox bacteria that have been tentatively named as ‘‘Brocadia anammoxidans’’ and ‘‘Kuenenia stuttgartiensis’’ (Schmid et al., 2000). Anammox bacteria has a very low growth rate and yield compared with aerobic AOB, consequently long sludge age reactor was needed to retain their growth.

$$NH_4^+ + 1.32NO_2^- + 0.66HCO_3^- \longrightarrow 1.02N_2 + 2.03H_2O + 0.66CH_2O_{1.5}N_{0.15} + 0.26NO_3^- \quad (15)$$

2.2 Partial nitrification

To remove nitrogen using SBNR scheme, partial nitrification must be achieved first. Partial nitrification is the oxidation of NH_4^+ to NO_2^-, as the end-product. This requires the reduction of activity of NOB, without negatively affecting the AOB activity (promotion of reaction in equation 5 and suppression of reaction in equation 6).

2.2.1 Environmental factors governing partial nitrification

Many parameters have been suggested to maintain the accumulation of nitrite over nitrate, either individually or in combination with other factors. Environmental factors such as oxygen, temperature, pH and organic carbon have a strong influence on growth and activity of microorganisms.

2.2.1.1 Oxygen

From equation 1 and 2, stoichiometric requirement for oxygen is 3.43 gO_2 per 1 g of NH_4^+-N for nitritation, and 1.14 mgO_2 per 1 g of NO_2^--N for nitratation. However, AOB are stronger than NOB against low DO level as can be explained by the difference in half saturation constant for oxygen of both microorganisms ($Ko,_{AOB} < Ko,_{NOB}$, Table 2.4). Thus, nitrite accumulation can be achieved by maintaining the low DO concentration. Previous evidence achieving nitrite accumulation using low-DO-level maintaining strategy is summarized in Table 2.5.

Table 2.4 Half saturation constants for oxygen of AOB and NOB

$K_{O,AOB}$ ($mgO_2\ l^{-1}$)	$K_{O,NOB}$ ($mgO_2\ l^{-1}$)	Sludge culturing condition	References
0.03 – 0.48	0.704 – 5.312	Suspended mixed culture, measured at different oxygen tension, 25°C, pH 7.5	Laanbroek and Gerards,1993
0.224– 0.554	0.16 – 4.32	Suspended mixed culture, measured at different dilution rates and oxygen concentrations, 28°C	Laanbroek et al.,1994,
0.1616	0.544	Mixed culture immobilized in gel beads, 30°C, pH 7.4	Hunik et al.,1994
0.033	0.41	Activated sludge, pH 7.8	Blackburne et al.,2008
0.99	1.4	Activated sludge, 20°C, pH 8.0	Ciudad et al.,2006
0.3	1.1	-	Wiesmann,1994

Table 2.5 Previous evidence achieving nitrite accumulation using low DO level maintaining strategy (modified from Park, et al., 2010)

DO ($mgO_2\ l^{-1}$)	Efficiency	System	References
0.5	Inhibition of NOB activity	Suspended growth	Hanaki et al., 1990
1.5	100% as NO_2^-/NO_x^-, 50% of NH_4^+ removed	Biofilm airlift reactor	Garrido et al., 1997
2.0 – 5.0	100% as NO_2^-/NO_x^-, 60% of NH_4^+ removed	Biological aerated filter	Joo et al., 2000
0.5	90% as NO_2^-/NO_x^-, 100% of NH_4^+ removed	Completely stirred biofilm reactor	Bernet et al., 2001
0.7	93% as NO_2^-/NO_x^-, 67% of NH_4^+ removed	Activated sludge	Ruiz et al., 2003
1.0	100% as NO_2^-/NO_x^-	Biofilm airlift reactor	Kim et al., 2003
1.4	75% as NO_2^-/NO_x^-, 95% of NH_4^+ removed	Biofilm airlift reactor	Ciudad et al., 2005
2.0 – 4.0	100% as NO_2^-/NO_x^-, 50% of NH_4^+ removed	Moving bed biofilm reactor	Fux et al., 2004
< 2.0	93% as NO_2^-/NO_x^-, 88% of NH_4^+ removed	Activated sludge with biofilm carriers	Chung et al., 2007
< 3.0	100% as NO_2^-/NO_x^-, 50% of NH_4^+ removed	Sequencing batch reactor	Gali et al., 2007

DO ($mgO_2\ l^{-1}$)	Efficiency	System	References
0.4	15% - 95% as NO_2^-/NO_x^-	Biofilm airlift reactor	Blackburne et al., 2008
5.0	93% as NO_2^-/NO_x^-, 43% of NH_4^+ removed	Up-flow reactor with biomass carrier	Yamamoto et al., 2008

Hanaki et al. (1990) suggested that NOB activity in a suspended growth system at 25ºC was strongly inhibited by low DO (< 0.5 $mgO_2\ l^{-1}$). Their findings indicated that in the full nitrification system, low DO levels did not affect the overall ammonia oxidation. This is because the growth yield of AOB became double compare to at high DO and this compensated the reduction of specific activity of AOB at low DO levels. In contrast, nitrite oxidation was strongly inhibited by low DO levels. The growth yield of NOB was unchanged. Thus, the low DO levels result in highering growth yields of AOB, while the growth yield of NOB was unchanged under this condition. This can possibly give rise to nitrite accumulate without affecting ammonia oxidation (accumulation of nitrite up to 60 $mgN\ l^{-1}$ at HRT of 2.0-3.8 d^{-1}).

Stenstrom and Poduska (1980) suggested no clear defined optimum DO concentration for full nitrification. The optimum DO concentration is dependent on other operational parameters such as sludge age. At higher sludge age, nitrification can be achieved at DO concentrations in the range of 0.5–1.0 $mgO_2\ l^{-1}$, while at lower sludge age, higher DO concentrations are required. However, they suggested that the lowest DO concentration at which full nitrification can occur appears to be approximately 0.3 $mgO_2\ l^{-1}$.

Leu et al. (1998) suggested that in the inner layer of biofilms, oxygen deficiency that is a result of NH_4^+ oxidation can cause nitrite accumulation. This means that NOB activity is strongly influenced by the oxygen-limiting level in the deeper parts of the biofilms. However, Harada et al. (1987) found that nitrite accumulation in bulk liquid phase increased with a decrease in biofilm thickness. Possibly, this may be explained by the hypothesis that in thick biofilms, O_2 is not only transported by diffusion, but also by advection through channels. When the O_2 supply

was limited, nitrite was accumulated in a mixed culture of AOB and NOB (Laanbroek and Gerards, 1993). By controlling the DO to low values, the oxidation of nitrite to nitrate can be controlled. This is possiblly owing to the higher affinity to oxygen of AOB than NOB (Garrido et al., 1997).

Cecen and Gonenc (1995) found that bulk O_2/NH_4^+ ratio rather than the NH_4^+ concentration alone is a more meaningful parameter to control nitrite accumulation. In nitrification, they found a considerable degree of nitrite accumulation at bulk O_2/ NH_4^+ ratios lower than 5. This finding corresponded to those reported in the study of Bougard et al. (2006). Bougard et al. (2006) concluded that the combined O_2 and NH_4^+ control strategy is more appropriate than either one since the shift in temperature set point strongly affected the composition of the microbial ecosystem present in the reactor while active control of oxygen and ammonia does not. Bernet et al. (2005) found that at bulk O_2/NH_4^+ ratio between 0.05 and 0.1, 80% of nitrite accumulation was achieved.

2.2.1.2 Temperature

The temperatures between 35 and 45^oC are reported for optimal partial nitritation (Van Hulle et al. 2007). Hellinga et al. (1998) found that temperatures above 25^oC lead to an increase in the specific growth rate of AOB, which become over NOB. A number of studies reported activation energy of AOB between 72 and 60 kJ mol^{-1} and for NOB, it is between 43 and 47 kJ mol^{-1} at between 7 and 30^oC (Jetten et al., 1999, Helder et al., 1983, Knowles et al., 1965, Stratton, et al.,1967). These indicated that the AOB activity will increase faster than the NOB activity. However, Yamamoto et al. (2008) found that nitrite accumulation can also achieved and maintained at lower temperature levels of between 15 and 30^oC and below 15^oC, the activity decreased.

2.2.1.3 Free Ammonia (FA) and free nitrous acid (FNA)

Free ammonia and free nitrous acid are the unionized forms of ammonium ion (NH_4^+) and nitrite ion (NO_2^-). They are the real substrate and inhibitor for AOB and NOB (Susuki et al., 1974, Anthonisen, et al., 1976). FA or FNA concentrations can be calculated from total ammonia nitrogen or total nitrite nitrogen, which is the sum of the ionized and unionized form, by incorporating an actual pH and temperature (^oC) (Anthonisen et al., 1976):

$$FA = \frac{(\text{total ammonia concentration}) * 10^{pH}}{(e^{(6344/(273+Temp))} + 10^{pH})} \quad (16)$$

$$FNA = \frac{(\text{nitrite ion concentration})}{(e^{(-2300/(273+Temp))} * 10^{pH})} \quad (17)$$

From equation 16 and 17, they indicated that the ratio between the ionized and un-ionized forms of both ammonia and nitrite is determined by acid-base equilibrium. FA fraction increases with increasing pH and/or temperature, while the fraction of FNA increases with decreasing pH and/or temperature.

Anthonisen et al. (1967) concluded that AOB are inhibited at FA concentrations between 8 and 120 mgN l^{-1} while inhibition on NOB activity was found at FA concentrations between 0.08 and 0.82 mgN l^{-1}(Anthonisen et al., 1976). Although some previous study confirmed that nitrite accumulation could be achieved by regulating pH to control FA concentration (Peng et al., 2006). Chung et al.(2006) found that a FA concentration of between 5 and 10 mgN l^{-1} was most efficient in inhibiting the NOB activity without slowing down the AOB activity (Chung et al., 2006). Han et al. (2003) suggested that FA has only an inhibition effect on NOB, but does not kill them. And NOB can recover activity after a period of cultivation (Han et al., 2003). Ford et al. (1980) reported that both ammonia and nitrite oxidation activities would be inhibited under the condition of FA concentration higher than 24 mgN l^{-1}, but they would recover as soon as FA level was below the threshold

concentration, and the system could operate in spite of FA concentration of 56 mgNl^{-1} (Ford et al.,1980). For FNA inhibition, Anthonisen et al. (1976) suggested that AOB are inhibited at FNA concentrations between 0.2 and 2.8 mgN l^{-1} while inhibition on NOB activity was found at FNA concentrations between 0.06 and 0.83 mgN l^{-1} (Anthonisen et al., 1976). Vadivelu et al. (2007) studied FA and FNA inhibition on catabolism and anabolism of *Nitrosomonas* and *Nitrobacter*, some important finding was summarized on Table 2.6 (Vadivelu et al., 2007).

Table 2.6 FA and FNA inhibition on *Nitrosomonas* and *Nitrobacter* (adjusted from Vadivelu et al., 2007).

Substrate inhibitor	**AOB (*Nitrosomonas*)**		**NOB (*Nitrobacter*)**	
	Catabolism	**Anabolism**	**Catabolism**	**Anabolism**
FA	No inhibition at up to 16.0 mgN l^{-1}	No inhibition at up to 16.0 mgN l^{-1}	Inhibited by 12% at 6.0 – 9.0 mgN l^{-1}	Likely inhibited completely at above 6.0 mgN l^{-1}
FNA	50% inhibition at 0.40 – 0.63 mgN l^{-1}	Likely inhibited completely at 0.40 mgN l^{-1}	No inhibition at up to 0.04 mgN l^{-1}	Likely stopped completely at 0.02 mgN l^{-1}

2.2.1.4 Sludge age

Partial nitrification in suspended-growth system can be achieved easily by appropriate regulation sludge age because of the difference in minimum sludge age requirement between AOB and NOB. van Kempen et al. (2001) suggested that, at temperature above 30^{o}C, operating at sludge age between 1 day and 2.5 days can washout the NOB population while simultaneously still maintain AOB growth (van Kempen et al., 2001). However, successful partial nitrification under long sludge age were also reported in some literatures. Pollice et al. (2002) indicated that sludge age was a critical parameter for partial nitrification when the oxygen supply was not limiting. Under limited oxygen supply, complete and stable nitritation was obtained, independent of the sludge age. And the sludge age only showed some influence on the kinetics of NH_4^+ oxidation under oxygen limitation. Zeng et al. (2004) found that

stable nitrite accumulations were also maintained in treating domestic wastewater under normal or even low temperature (less than 13^{o}C) with 30 days long sludge age (Zeng et al., 2004).

2.2.1.5 Organic carbon

Organic carbon has been reported to affect nitrification performance. When organic matter is present, heterotrophs compete with nitrifiers for oxygen (Zhang et al., 1995), and generally it is heterotrophs who rule this competition due to their higher affinity to oxygen over the nitrifiers. The activity of nitrifiers in a fluidized bed reactor was inhibited by an increase in C/N ratio (Fdz-Polanco et al., 2000, Okabe et al., 1996b). The proportion of nitrifiers decreased with an increasing C/N ratio (Satoh et al., 2000). A higher influent C/N ratio retarded accumulation of nitrifying bacteria and resulted in a considerably longer start up period for nitrification (Okabe et al., 1996b). An exponential decrease of the nitrification rate with an increased influent COD/N ratio was observed in a study on nitrogen removal from high-strength ammonia industrial wastewater (Carrera et al., 2004). For the same sludge age, the ammonia oxidation efficiency decreased at higher COD concentrations but at a constant COD concentration efficiency restored again by increasing sludge age (Hanaki et al., 1990). A moderate increase of sludge age to between two and three days can help reducing influence of heterotrophs on ammonia oxidation.

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

2.3 Microorganisms involved in nitrification

2.3.1 Ammonia oxidizing bacteria (AOB)

Comparative 16S rRNA gene sequence analyses of cultured AOB found that members of physiological group are limited to two monophyletic lineages within the *Proteobacteria: Gammaproteobacteria* and *Betaproteobacteria. Nitrosococcus oceani* is member in the *Gammaproteobacteria*, despite members of the genera *Nitrosomonas* (including *Nitrosococcus mobilis*), *Nitrosospira, Nitrosolobus* and

Nitrosovibrio from a closely related grouping within the *Betaproteobacteria* (Purkhold et al., 2000). Table 2.7 summarized saturation constants for ammonia (as TAN and FA) from literature. Wastewater treatment plants (WWTPs) harbor a diversity of AOB of the *Betaproteobacteria*. From fluorescence in situ hybridization (FISH) results, it indicated that some nitrifying WWTPs were dominated by a single AOB species (Juretschko et al., 1998) while other plants harbored at least five different co-existing AOB populations which are present in significant numbers (Daims et al., 2001).

Table 2.7 Half saturation constants for ammonia of AOB pure cultures

AOB	$K_{S,AOB}$ (µM as NH_3)	References
Nitrosomonas europaea	553 877 - 1960 30 - 61	Marten-Habbena et al., 2009 Laanbroek et al., 1993 Koops et al., 2006
Nitrosomonas oligotropha *Nitrosomonas ureae*	1.9 - 4.2	Koops et al., 2006
Nitrosomonas oceani	101.4	Marten-Habbena et al., 2009
Nitrosomonas marina *Nitrosomonas aestuari*	50 - 52	Koops et al., 2006
Nitrosomonas communis	14-43	Koops et al., 2006
Nitrosomonas briensis	2.4 ± 0.8	Bollmann et al., 2005
Nitrosomonas eutropha	30 - 61	Koops et al., 2006
Nitrosospira	6 - 11	Jiang Q.Q., 1999
Nitrosomonas cryotolerans	42 - 59	Koops et al., 2006
Nitrosococcus mobilis	49 - 59	Koops et al., 2006

From a review of Wagner and Loy (2002), it was shown that almost all recognized lineages of betaproteobacterial AOB can be found in WWTPs. However, the *Nitrosomonas europaea/Nitrosomonas eutropha*-lineage, the *Nitrosococcus mobilis*-lineage, and the *Nitrosomonas marina* cluster are most frequently detected. *Nitrosomonas* (including *Nitrosococcus mobilis*) and not *Nitrosospiras* (including the genera *Nitrosospira*, *Nitrosolobus* and *Nitrosovibrio*) are important for NH_4^+ oxidation in WWTPs. The 193 *amoA* clones from several nitrifying WWTPs are

affiliated with the genera *Nitrosomonas* while only 6 *amoA* clones cluster with the genera *Nitrosospira* (Loy et al., 2002).

Juretschko et al. (1998) found that in an industrial WWTP the dominant AOB was *Nitrosococcus mobilis*, a bacterium which was previously considered to occur in brackish water only (Juretschko et al., 1998). Subsequently, *Nitrosococcus mobilis* was also detected in significant numbers in a nitrifying sequencing batch biofilm reactor (Daims et al., 2001). In contrast, *Nitrosospira* related AOB were found to be dominant *in situ* in a laboratory scale fluidized bed reactor (Schramm et al., 1998). Although *Nitrosospira* was also reported in a polymerase chain reaction (PCR)-based study as important AOB genus in WWTPs (Hiorns et al., 1995), this finding could not be confirmed by FISH analyses of various WWTPs and by a large *amoA*-based AOB diversity survey in WWTPs (Purkhold et al., 2000).

2.3.2 Ammonia oxidizing archaea (AOA)

Cultivation-independent molecular surveys showed that members of the kingdom Crenarchaeota within the domain Archaea represent a substantial component of microbial communities in aquatic and terrestrial environments. This is the first link between *amo*-like genes and mesophilic Crenarchaeota. This evidence also led to the hypothesis that non-thermophilic Crenarchaeota of soil could be ammonia oxidizers (Schleper et al., 2005). Candidatus “Nitrosopumilus maritimus” is phylogenetically placed within the ‘marine’ group 1.1a lineage and can grows chemolithotrophically, using ammonia as a sole energy source, and seems to grow at similar rates and densities as cultured AOB with near-stoichiometric conversion of ammonia to nitrite (Könneke et al., 2005).

AOA have recently been detected in nitrifying wastewater treatment bioreactors used to remove ammonia from wastewater by using PCR primers targeting archaeal *amo*A gene (Park et al., 2006). Park et al. found that all of the archaeal amoA sequences are most closely related to those obtained from sediments and soils. They suggested that the presence of AOA appears to be dependent upon

some WWTP's operating condition such as DO concentration and sludge age. However, AOA *amoA* gene have not been obtained from all WWTPs with low DO concentrations or long sludge age in the study of Park et al. Urakawa et al. (2008). found that phylogenetic diversity and species richness of AOA are greater than those of AOB in a marine filtration system and low temperatures significantly reduce the diversity of AOA and of AOB (Urakawa et al., 2008).

2.3.3 Nitrite oxidizing bacteria (NOB)

NOB are chemolithotrophic, belonging to one of the four different genera *Nitrobacter* (alpha subclass of *Proteobacteria*), *Nitrococcus* (gamma subclass of *Proteobacteria*), *Nitrospina* (delta subclass of *Proteobacteria*), and *Nitrospira* (phylum *Nitrospira*). In the past time, it had been believed that *Nitrobacter* is the main microorganisms that are responsible for oxidizing nitrite to nitrate in wastewater treatment plants (Henze et al., 1997). However, *Nitrobacter* could not be detected by FISH with specific 16S rRNAtargeted probes in various nitrifying WWTPs (Wagner et al., 1996). Using the full cycle rRNA approach the occurrence of yet uncultured *Nitrospira*-like NOB in nitrifying WWTPs could be demonstrated (Juretschko et al., 1998). Juretschko et al. (1998) found *Nitrospira* is dominant NOB in activated sludge. Combination of FISH and microautoradiography (FISH-MAR) showed that the *Nitrospira*-like NOB in activated sludge can fix carbon dioxide and also grow mixotrophically using organic compound such as pyruvate (Daims et al., 2001).

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

Table 2.8 Kinetic parameters for *Nitrospira* and *Nitrobacter* (modified from Blackburne et al., 2007b).

	OUR_{MAX} (mg $gVSS^{-1}$ h^{-1})	$K_{O,NOB}$ (mgO_2 l^{-1})	$K_{S,NOB}$ (mgN l^{-1})	Yield (gVSS gN^{-1})
Nitrospira	32±2 [h)]	0.13±0.06 [a)]	0.12±0.02 [a)]	N.A. [a),b),h)]
			0.22±0.04 [a)]	
			0.15±0.02 [a)]	
			0.14 [b)]	
Nitrobacter	289±15 [h)]	0.17 – 5.3 [c),d)]	0.3 – 1.9 [e),f)]	0.02 – 0.084 [e),f),d)]

Note : Reference [a)] Manser, 2005; [b)] Schramm et al., 1999; [c)] Laanbroek and Gerards, 1993; [d)] Laanbroek et al., 1994; [e)] Beccari et al., 1979; [f)] Alleman, 1984; [g)] Blackburne et al., 2007a; [h)] Blackburne et al., 2007b

Nitrobacter was abundant in high nitrite concentration environment (Nogueira and Melo, 2006). Kim and Kim (2006) showed that genus *Nitrobacter* is R-strategists that can grow in high nitrite and oxygen concentration. *Nitrospira* were K-strategists adapted to low nitrite and oxygen concentration (see Table 2.8). Wagner et al. (2002) suggested that *Nitrospiras* will outcompete *Nitrobacter* in most full scale nitrifying WWTPs in which nitrite concentrations are low. While nitrifying WWTPs with temporally or spatially elevated nitrite concentrations, ie. nitrifying sequencing batch reactors, both NOB should be able to coexist. Daims et al. (2001) found that coexistence of *Nitrobacter* and *Nitrospira*-like bacteria was observed by FISH in a nitrifying sequencing batch biofilm reactor (Daims et al., 2001).

2.4 Current practical implementation of partial nitrification in SBNR system

In SBNR process, partial nitrification step is needed up front followed by of nitrite denitritation or Anammox. Partial nitrification can be implemented in separate reactors (partial nitrification + Anammox) or in single reactor together with the following process.

2.4.1 Separate reactor system

For separate reactor system, partial nitrification and Anammox are activated in separated spaces. An equal molar of influent NH_4^+ is converted to NO_2^- by AOB in the first reactor, while in the second reactor, an equal molar of the remaining NH_4^+ and NO_2^- becomes substrate for active Anammox. The advantage of this separate configuration over single reactor are: 1) higher flexibility and stability to maintain process performance and 2) higher resistance to toxic or organic biodegradable compounds since the compounds can be also degraded in the partial nitrification reactor (Lackner et al., 2008). Main application of separate reactor system is preparing a stable, Anammox-suited influent, which is an equal molar of NH_4^+ to NO_2^- ratio of 1:1.32 according to the stoichiometry (Strous et al., 1998). Both flow-through reactor, such as chemostat, and high sludge age reactor, for example, membrane bioreactor (MBR) or SBR reactor are the common configuration for partial nitrification with separate reactor system (see Table 2.7). For biofilm system, such as in MBR, it is difficult to control exact sludge age; subsequently, stable nitrite accumulation is more difficult to be generate even under O_2 limited concentrations (Xue et al., 2009). Thus, suspended growth systems are more desirable for full scale application.

Van Dongen et al. (2001) used SHARON process to prepare for Anammox-suited influent (Van Dongen et al., 2001). SHARON process can achieve partial nitrification in ammonia-rich wastewater such as side stream waste by using high FA loading under 1 – 2 days sludge age at temperature of 35^oC in chemostat reactor. Van Dongen et al.(2001) found that 53% of NH_4^+ oxidized to NO_2^- without pH control resulting in a NO_2^- to NH_4^+ ratio of 1.13:1. At the present time, although SHARON process can be applied successfully at full scale, some disadvantages of SHARON such as 1) inability of the system to deal with fluctuated waste stream which came from the effluent of sludge digester operating with short HRT or effluent from industrial effluent and 2) the maximum volumetric loading rate of SHARON reactor be limited due to limited HRT at 1 - 1.2 days . Uncoupling between sludge age and

HRT to lower HRT than one day is impossible in SHARON process which resulting in limiting volumetric nitrogen loading compared with the attached growth reactor at similar effluent quality. Other disadvantages of SHARON for real application are Protozoa invasion-related problem. Moreover, maintaining high operating temperature is needed to maintain the AOB overgrown NOB.

Van Hulle et al. (2010) suggested four operational strategies to prepare Anammox influent (or in other words, to achieve stable partial nitrification) which are: 1) low O_2 concentration (< $0.5 mgO_2\ l^{-1}$), (2) high pH (7.5–8.5), 3) high temperature (> 25 ^{o}C), and 4) limiting nitrification time to terminate NH_4^+ oxidation before its depletion and to prevent inorganic carbon limitation (Van Hulle et al., 2010).

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

Table 2.9 Operating condition and performance of partial nitrification in separate reactor system. (modified from Van Hulle et al., 2010)

Reactor	Wastewater	pH	Temperature (^oC)	DO ($mgO_2\ l^{-1}$)	SRT (d)	HRT (d)	N load ($kgN\ m^{-3}d^{-1}$)	NO_2^-/NH_4^+ removed	NO_3^- in the effluent (%)	References
SHARON	Sludge digester supernatant	6.6 – 7.2	29	2.7	1.05 – 1.18	1.05 – 1.181	0.56	1.4	Negligible	Fux et al., 2002
SHARON	Synthetic	7.1	35	-	1 – 1.5	1.54	1.5	1	-	Van Hulle et al., 2005
SHARON	Sludge digester supernatant	6.7	35	-	1	1	1.2	0.74	113	van Dongen et al., 2001
SHARON	Urine	9.2	30	2.5 - 4	4.8	4.8	1.580	1	Negligible	Udert et al., 2003
SHARON	Digested effluent of fish canning	7.5	35	>2	1	1	0.1	1	No NO_3^-	Mosquera-Corral et al., 2005
SBR	Urine	6 – 8.8	24.5	2 – 4.5	>30	4	0.560	1	Negligible	Udert et al., 2003
SBR	Landfill leachate	6.8 – 7.1	36	2	3 - 7	1.5	1.5	0.6 – 1.5	<5	Ganigue et al., 2007
Biofilm	Pre-filtered digested liquor of swine wastewater	-	25	5	13	1	1.0	1.38	<5	Yamamoto et al., 2008
Biofilm	Digested liquid manure	7.5 - 8	30	2.5 – 6.5	-	-	3.8	1.22	2.3	Qiao et al., 2010
MBR	synthetic	8	35	<0.6	35	0.23	-	1.30	Trace	Feng et al., 2007
MBR	synthetic	8	35	0.3 – 0.5	-	0.67	0.450	1	-	Xue et al., 2009
MBR	Pre-filtered sludge digester supernant	7.9	30	<0.2	Varying	0.58 - 1	0.73 – 1.45	1.13	-	Liang and Liu, 2008

2.4.2 Single reactor system

In most single reactor system, AOB and Anammox are co-cultured under microaerobic conditions to obtain partial nitrification and avoid oxygen inhibition to Anammox activity (Strous et al., 1997). Different kind of systems such as sequencing batch, granular sludge or biofilm systems were used to obtain this microaerobic conditions.

In biofilm system, under very low oxygen level condition, NOB activity is prevented due to their lower oxygen affinity compared to AOB and lower nitrite affinity compared to Anammox. An active AOB in the outer layers of the biofilm can produce suitable amounts of nitrite for the Anammox that are active in the inner parts (Wyffels et al., 2004). In biofilm system, substrate transfer resistance is always the rate limiting step of the process. Sliekers et al. suggested that as long as NH_4^+ level outside the biofilm is much higher than the O_2 or NO_2^- level, NH_4^+ diffusion into the biofilm will not limit the process rate. If the NO_2^- produced in the outer layer is mainly consumed in the inner layer, O_2 is the main limiting factor controlling the overall rate (Sliekers et al., 2003). The OLAND process (Kuai and Verstraete, 1998) and the CANON process (Third et al., 2001) are the single reactor system based SBNR. The OLAND process were assumed that NH_4^+ oxidation mainly performed by AOB under microaerobic conditions while in the CANON process, Anammox were assumed to be responsible. Normally, single reactor system have higher volumetric nitrogen removal rate than the separated system. Consequently, lower capital costs are required since no additional partial nitrification reactor volume is needed. However, some previous study reported an inability and incomplete removal for treating high loaded wastewaters due to difficulties to regulate DO (Hao et al., 2001).

2.5. Immobilized cell

2.5.1 An overview

In wastewater treatment application, immobilized cell has several advantages over suspended cells such as higher cell retention, higher conversion rate, higher resistance to an adverse environmental conditions, and easier for solid/liquid separation (Wijffels and Tramper, 1995). Currently, there are three main techniques to form an immobilized cell which include cell attachment, gel encapsulation and gel entrapment (Cassidy et al., 1996). For gel entrapment technique, cell is mixed completely and retained in a spherical or cubic form of polymeric gel matrix. Both natural and synthetic polymers are normally used as gel matrix for entrapment. The natural polymers are, for example polysaccharides made from algae or seaweed, such as calcium alginate, carrageenan, agarose, and gelatin. Polyvinyl alcohol, cellulose triacetate, and polyacrylamide are the mostly used synthetic polymers (Siripattanakul and Khan, 2010). Polyvinyl alcohol (PVA) is a non-toxic synthetic polymer. For gel entrapment, many techniques are available for PVA gelation, such as boric acid-PVA, freeze and thaw of PVA, and PVA-phosphorylation (Hashimoto and Furukawa, 1987; Lozinsky and Plieva, 1998). PVA-phosphorylation is less time consuming and cell disruption than others. Spherical PVA gel bead is formed by cross linkage between PVA and boron and after that, a sodium phosphate is used to improve the durability of the gel bead by increasing the strength of the surface of gel bead by PVA-phosphorylation mechanism.

2.5.2 Application of immobilized cell for nitrogen removal in wastewater

Isaka et al. (2007) studied nitrification of landfill leachate using Polyethylene glycol-immobilized sewage sludge at low temperature ($10^{o}C$) and high DO concentrations (> 7 mgO_2 l^{-1}). The communities of nitrifying bacteria were investigated by using a FISH technique in 3 mm cubical entrapped gel carrier in a 0.71 kg N m^{-3} day^{-1} loading airlift reactor for nitrification on the 400^{th} – 500^{th} days after starting up. They found that the dominant AOB was *Nitrosomonas* sp. which

was 60% of all bacteria while *Nitrosospira* sp. was much lesser detected, represented only 2.1% of all bacteria. All experiments gave quite stable nitrification rate after 3 months. However, complete NH_4^+ removal could not be achieved; almost the same NH_4^+ concentrations were detected in the effluent with different NH_4^+ loadings. They suggested that this was because *Nitrosomonas* sp., which usually has low affinity for ammonium, is the dominant of AOB in the reactor. The organism is known to be inefficient at a low ammonia condition. A significant level of stable nitrite accumulation, which resulted from an inhibitory effect of the high amount of free ammonia in landfill leachate, was also observed. This work indicated that highly active AOB can be maintained at high concentrations in entrapped gel carrier even at low temperature operation.

Vogelsang et al. (2002) investigated changes of communities and spatial distribution of nitrifying bacteria within nitrifying sludge immobilized with gel bead of sodium alginate and PVA with stilbazolium groups after 0, 30 and 80 days of enrichment in a nitrification medium. The nitrifying communities analysis through a FISH technique showed that *Nitrosospira* sp. were the dominant AOB while no members of the genus *Nitrosomonas* were detected, although it has been reported as the generally most abundant AOB in wastewater treatment plants. For NOB, *Nitrobacter* sp. dominated while a few cells of *Nitrospira* sp. were also detected. Most nitrifying bacteria formed typically dense clusters of hundreds and thousands of cells. The AOB generally appeared at larger sizes of cluster than NOB. For the spatial distribution of nitrifying bacteria, a biofilm-like structure appeared within the gel bead, with the nitrifying biomass concentration in the outer 100 µm layer of the gel bead higher than in the core region of the bead. AOB and NOB appeared in separate, but often closely associated colonies. For the dynamics of the amount of nitrifying biomass during the enrichment, they found that after the first 30 days of enrichment, nitrifying biomass was grown around 10 times more than the non-enriched gel bead. However, the growth of nitrifying biomass appeared differently after the next 50 days; 30% less increase of the nitrifying biomass in the outer layer was observed while within the core of the beads, it decreased by approximately one third. Moreover, the numbers of AOB and NOB were in the same quantity in all experiments. The

fractional ratio of nitrifying bacteria relative to all bacteria changed from 20% for the non-enriched culture to 64% after 30 days of cultivation. After 80 days of cultivation, it was reduced to 35% even there was 30% increase in the nitrifying biomass from day 30 to 80. Moreover, some growth of heterotrophs biomass was also observed mainly within the outer 300 μm layer of the gel beads.

Uemoto et al. (2000) co-immobilized *Nitrosomonas europaea* and *Paracoccus denitrificans* in the exterior and interior layers of tubular PVA, respectively. The research focused on the effect of DO on the spatial distributions of two different pure culture bacteria within the tubular gel and process performance. Results showed that when free *N. europaea* cells were exposed to either 50% purity O_2 or pure O_2, ammonia oxidation was inhibited and their activities could not be recovered. In contrast, the ammonia oxidation rate of immobilized *N. europaea* cells accelerated. It was suggested that polymeric gel protected *N. europaea* from the toxic effects of pure oxygen. Fluorescence-Antibody labeling technique (FA labeling) revealed that small colonies of both microorganisms were distributed throughout the tubular gel before acclimating to oxygen. However, when the tubular gel were acclimated to 20%, 50%, and pure O_2, *N. europaea* were bundled within a region extending from the external surface of the gel to a depth of 200 micrometers, a depth of 50 to 200 micrometres and a depth of 120 to 300 micrometres, respectively while *P. denitrificans* concentrated in a region extending from the internal surface of the gel to a depth of 80 micrometers in all experiments.

Hunik et al. (1993) was successful in modifying an FA labeling technique to investigate the spatial distribution of *Nitrosomonas europaea* and *Nitrobacter agilis* cells immobilized in carrageenan gel beads. They found that after 49 days of in a continuously operated airlift loop reactor with a nitrification medium at 30^{o}C, the biomass concentration in the outer 140 μm layer of the bead is relatively high while a low biomass concentration region is in the center of the bead. Moreover, the result from the estimation of the amount of biomass showed that both pure AOB and NOB cultures were present in the same quantity which is around 40% of total biomass.

2.6. *p*-nitrophenol

p-nitrophenol (PNP), also called 4-Nitrophenol or 4-hydroxynitrobenzene, is a phenolic compound that has a nitro group at the opposite position of hydroxy group on the benzene ring. It is manufactured chemical that does not occur naturally in the environment. It is usually used mainly to produce drugs, fungicides, dyes, and to darken leather (ATSDR, 2001).

2.6.1 Properties

PNP shows two polymorphs in the crystalline state. The alpha-form is colorless pillars, unstable at room temperature, and stable toward sunlight. The beta-form is yellow pillars, stable at room temperature, and gradually turns red upon irradiation of sunlight. PNP normally exists as a mixture of these two forms. In aqueous solution, PNP has a dissociation constant (p*K*a) of 7.08 at 22°C. Solution of PNP alone appears colorless or pale yellow, whereas its phenolic salts tend to develop a bright yellow color. The physico-chemical properties of PNP are presented in Table 2.10.

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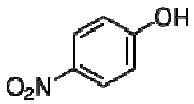
Table 2.10 Physical and chemical properties of *p*-nitrophenols

Property	**Characteristic**
Structure	
Chemical Formula	$C_6H_5NO_3$
CAS number	100-02-7
Molar Weight (MW)	139.11 g mol^{-1}
Color	colorless or yellow pillars
Melting point	113–114 °C
Boiling point	279 °C, 552 K, 534 °F
Density at 20° C	1.270
Solubility in water (g l^{-1}) at 15°C at 20°C at 25 °C	10 11.6 16
Partition Coefficient: Log Kow	1.91
Vapour pressure at 20°C (mm Hg):	<1

Source: Agency for Toxic Substances and Disease Registry (ATSDR), 2003.

2.6.2 Fate in environments

When released into the soil, PNP is expected to have a half-life of less than 1 day while a half-life between 1 and 10 days was expected when released into the water. PNP is not expected to significantly bioaccumulation. Most of PNP enter the environment during manufacturing and processing. It can be formed in the air as a result of the breakdown of many other manufactured chemicals. Moreover, it readily break down in surface water but take a long time to break down in deep soil and in groundwater (Environmental Health & Safety, 2009).

2.6.3 Effect on human health and ecosystems

p-nitrophenol is corrosive to the eyes, is a potential skin irritant, and its inhalation toxicity is not known. It is of relatively high acute toxicity by the oral route and moderately toxic by the dermal route. No food uses are registered so dietary risk is not anticipated. However, based on reviews of the generic data, EPA has some concerns about potential handler dermal and inhalation exposure during treatment of leather and cork products for use by the military. For environmental and ecosystem assessment, *p*-nitrophenol is slightly to moderately toxic to birds and aquatic animals. Wild animals or plants are not likely to be exposed to paranitrophenol, however, since it is not applied outside of a factory. *p*-nitrophenol thus is not expected to pose a risk to nontarget organisms (US.EPA. ,1998).

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CHAPTER III

EXPERIMENTAL FRAMEWORK

3.1 Experimental framework

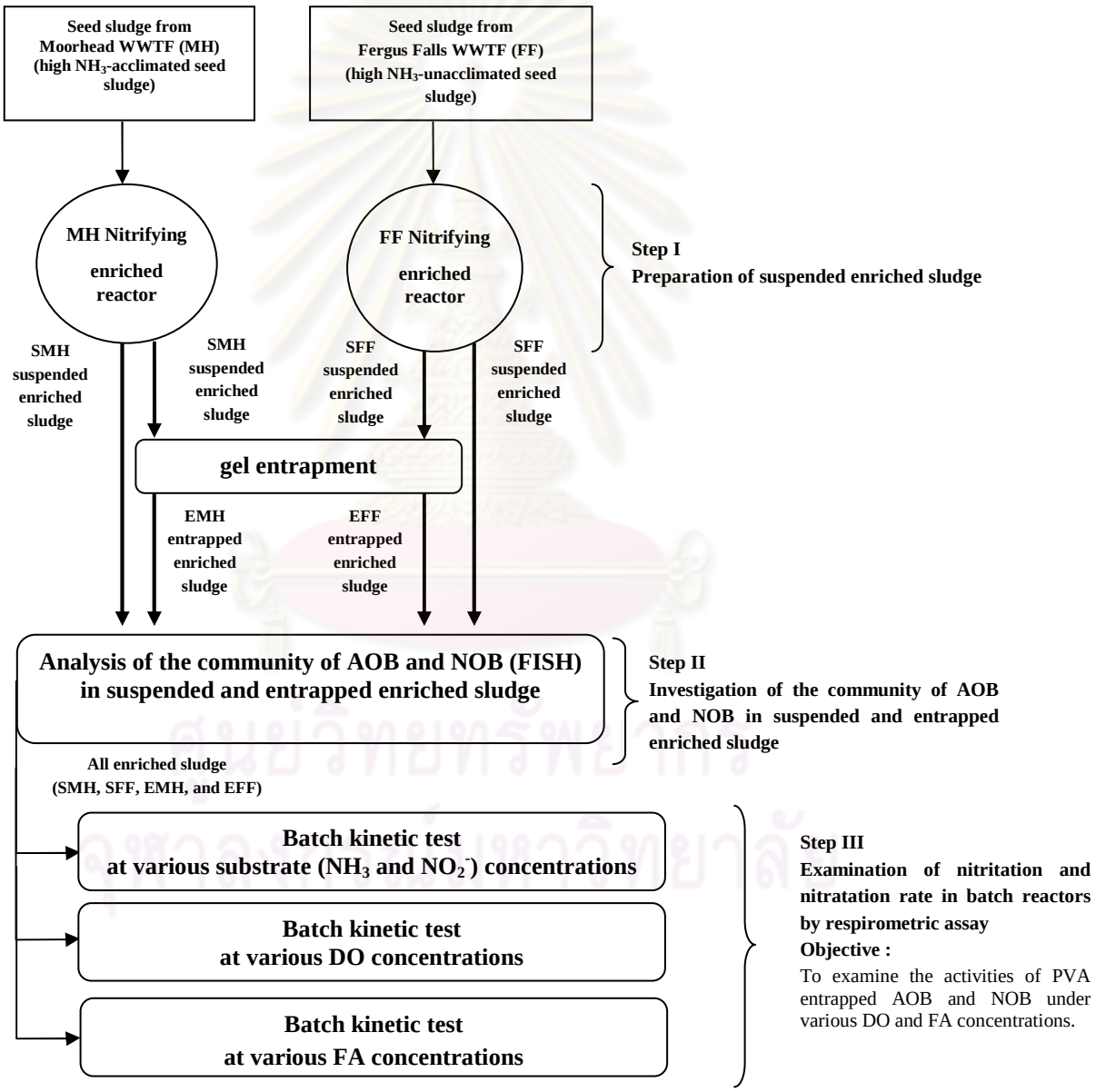

Fig. 3.1 Experimental framework of Task 1 (Chapter IV)

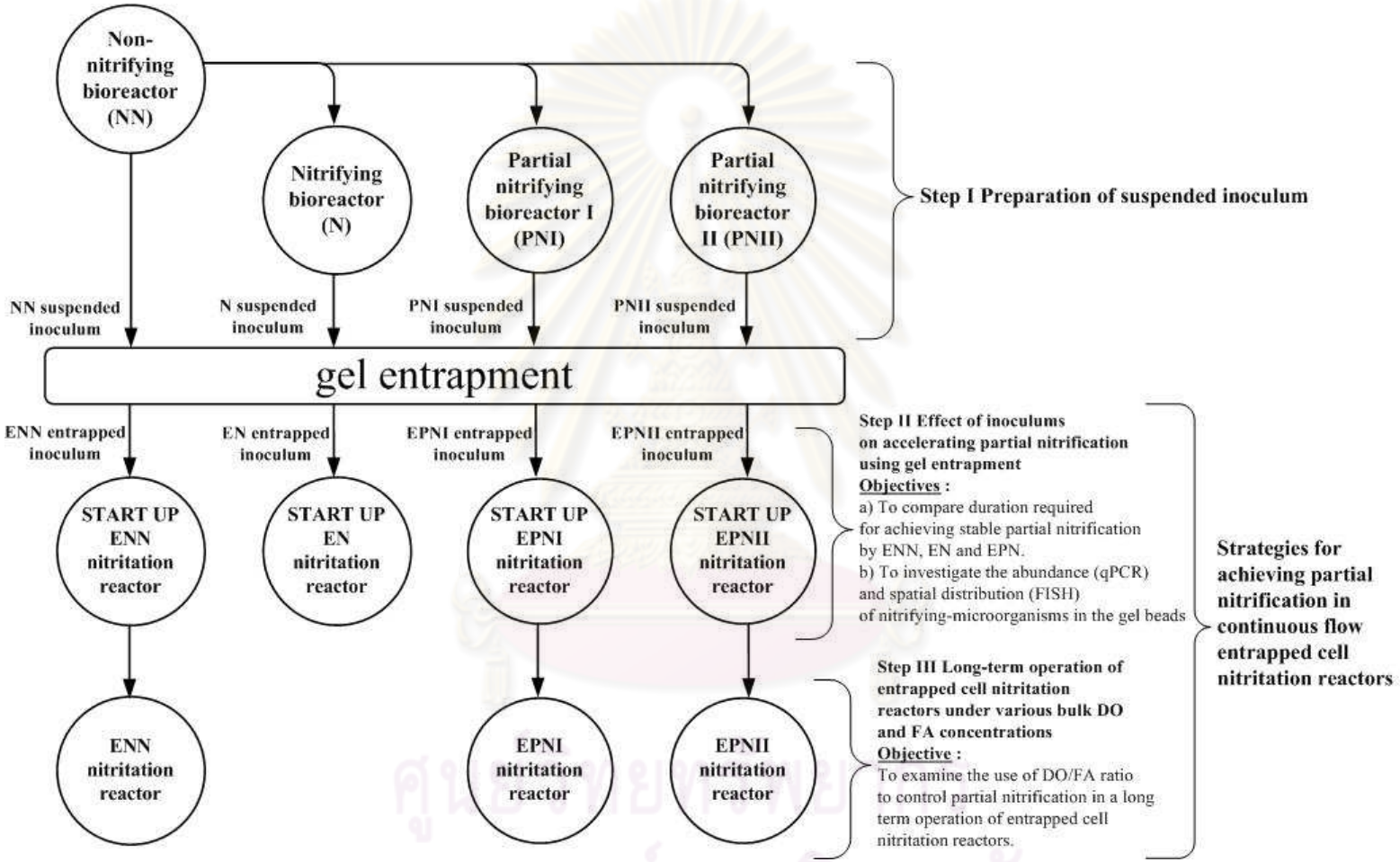

Fig 3.2 Experimental framework of Task 2 (Chapter V)

38

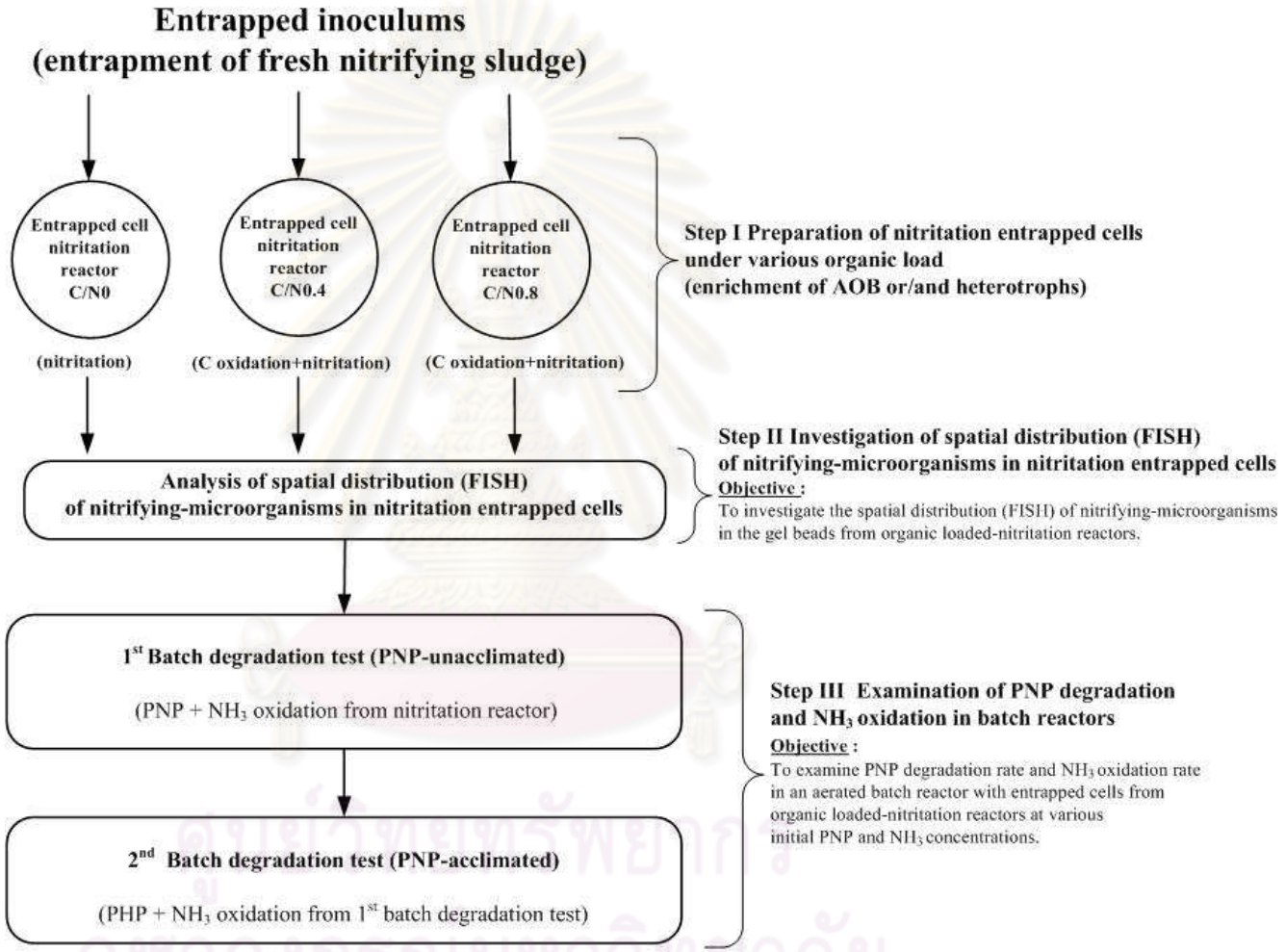

Fig. 3.3 Experimental framework of Task 3 (Chapter VI)

CHAPTER IV

RELATIONSHIP BETWEEN RESPIROMETRIC ACTIVITY AND COMMUNITY OF ENTRAPPED NITRIFYING BACTERIA: IMPLICATIONS FOR PARTIAL NITRIFICATION

4.1 Introduction

Partial nitrification of ammonium rich wastewater is now a common practice. To generate and maintain partial nitrification, the ammonia oxidizing bacteria (AOB) activity has to be maintained while simultaneously the activity of the nitrite oxidizing bacteria (NOB) needs to be inhibited. For suspended growth systems, at high ammonium or limited dissolved oxygen (DO) concentrations, the activities of NOB or the nitratation rate are suppressed due to ammonia inhibition or lower affinity for oxygen than AOB and thus, nitrite accumulates in the systems. The effectiveness of these two common strategies depends on what specific species of nitrifying bacteria are in the reactor and how they interact with environmental conditions such as their affinity to substrate and ability to tolerate inhibiting conditions.

For attached growth systems, which have less substrate transfer and space for growth compared to suspended growth systems, nitrite accumulation depends on other factors such as cell localization (Okabe et al., 2004). Recently, there have been studies suggesting that microbial localization within biofilm has significant effects on its substrate diffusion resistance or substrate utilization (Kim et al., 2003; Schramm et al., 1996; Tanaka et al., 1992). For example, Okabe et al. (1996) reported that heterotrophs out competed nitrifiers for oxygen and space. As a result, hetetorophs dominated the outer most biofilm while nitrifying bacteria were found only in the inner layer causing reduced nitrification (Okabe et al., 1996).

Cell entrapment within polymeric gels such as polyvinyl alcohol (PVA) and carrageenan has been extensively applied for nitrification of wastewater (Cao et al., 2002; Wijffels et al., 1995; Hunik et al., 1993). Nitrification in full scale wastewater treatment plants by using polyethylene glycol (PEG) entrapped cells has been practiced in Japan and the United States (Emori et al., 1996). Moreover, advantages of entrapped nitrifying bacteria over corresponding suspended cells including effective protection against cell washout, higher conversion rates, higher cell concentrations, ease of cell separation, and protection from unfavorable conditions have been reported (Chen and Lin, 2004; Rostron et al., 2001) .

Although there have been a number of studies and applications of entrapped cells for nitrification, there have been limited peer reviewed articles that address partial nitrification by entrapped cell (Yan and Hu , 2009 ; Hill and Khan, 2008; Isaka et al., 2007; Rostron et al., 2001). Isaka et al. (2007) reported nitrite accumulation in their study which used PEG-entrapped cells to treat landfill leachate (150–200 mg l^{-1} of NH_3-N) at low temperatures. Hill and Khan (2008) found that by using calcium alginate-entrapped cells, more than 70% of ammonia in anaerobic sludge digester supernatant was converted to nitrite even under an unlimited dissolved oxygen condition in bulk liquid. Although these studies showed the possibility for applying entrapped cells for partial nitrification, they did not provide any understandings on the kinetics of nitritation and nitratation by entrapped cells and in situ nitrifying bacterial community within the entrapment matrix to elucidate on how to control partial nitrification by entrapped cells, which could be different from suspended cells.

Nitritation and nitratation can become the rate limiting step at different environmental conditions. An understanding of the kinetics of these two steps is needed for setting up design criteria and control strategy for partial nitrification. Although there have been a number of kinetic studies of nitritation and nitratation processes with suspended cells (Ahn et al., 2008; Chandran and Smets, 2000), such work has never been conducted for entrapped cells. The knowledge on the kinetics of

these two steps for entrapped cells, particularly associated with the control strategy, could be used to achieve effective partial nitrification.

The main objectives of this study were to examine nitritation and nitratation kinetics based on oxygen uptake rate of enriched nitrifying sludge entrapped in PVA and to observe how they are related to bacterial community and population. A batch respirometric assay coupled with nitritation and nitratation chemical inhibitors was used to determine the bacterial activities. A fluorescence in situ hybridization (FISH) technique was chosen for probing nitrifying bacteria within the entrapment matrix since the nitritation and nitratation kinetics are related to the make-up of nitrifying bacterial community. This study was also conducted with suspended cells in parallel.

4.2 Materials and methods

4.2.1 Enrichment medium and synthetic wastewater

An enrichment medium, which was synthetic wastewater with a total ammonia nitrogen (TAN) level of 150 mg N l^{-1} (0.72 g of $(NH_4)_2$ SO_4 per liter), was used for enriching nitrifying bacteria. The synthetic wastewater contained $NaHCO_3$ (1.5 g), Na_2HPO_4 (4.05 g), K_2HPO_4 (2.1 g), $MgSO_4 \cdot 7H_2O$ (0.05 g), $CaCl_2 \cdot 2H_2O$ (0.01 g), and $FeSO_4 \cdot 7H_2O$ (0.09 g) in one liter of distilled water. The synthetic wastewater plus ammonia ($(NH_4)_2SO_4$) and/or nitrite ($NaNO_2$) at various concentrations was used for respirometric experiments. All chemicals were purchased from VWR international, Inc. (West Chester, PA, USA).

4.2.2 Enrichment and entrapment of nitrifying sludge

Activated sludge obtained from the Moorhead (MH) Wastewater Treatment Facility (WWTF) in Moorhead, Minnesota, USA (High purity oxygen activated sludge system, solid retention times of 3 days) and Fergus Falls (FF) WWTF in Fergus Falls, Minnesota, USA (Complete-mix activated sludge system, solid retention times of 5 days) were enriched separately for nitrifying bacteria. The Moorhead

WWTF had a return flow of sludge digester supernatant, which was very high in ammonia (average NH_3-N concentration of 1813 mg N l^{-1} and average NH_3-N pulse loading of 222 kg N d^{-1}) while the Fergus Falls WWTF did not practice the return of digester supernatant. Two 17 l laboratory-scale enrichment reactors, one for each sludge, were aerated and operated in a two day cycle fill and draw mode at room temperature (22–23°C). The reactors were fed with the enrichment medium with a pH adjustment between 7.5 and 8.2. After 10 weeks, more than 95% of TAN was removed and no nitrite accumulation was observed in both reactors, then the enriched sludge was harvested.

The harvested biomass was entrapped in phosphorylated PVA gel beads at a cell-to-matrix ratio of 4% (w/v) (g of centrifuged mixed liquor volatile suspended solids/ml of media) according to the method by Chen and Lin (1994). In brief, a PVA aqueous solution (20%, w/v) was mixed thoroughly with an equal volume of concentrated enriched sludge with volatile suspended solid (VSS) concentration of 70 g l^{-1}. The mixture was dropped into a saturated boric acid solution at a rate of 0.83 ml min^{-1} resulting in a droplet diameter of 3–4 mm. About one hour after the dropping ended, the formed beads were then transferred to a 1 M sodium orthophosphate solution (pH 7) for 2 h for hardening. After that, each batch of the entrapped cells was returned to the enrichment reactor for maintenance.

4.2.3 Respirometric assay

A 300 ml completely sealed glass vessel with a port at the top for the insertion of a DO probe was employed for respirometry. The DO probe was connected through a RS-232 port to a personnel computer, which was used for storing and monitoring all data transmitted by the probe. For each test, the suspended cells or entrapped cell beads previously washed with the synthetic wastewater without nitrogen supplement were added to the vessel, which was later filled up with the synthetic wastewater previously aerated for 1–2 min. The pH of the synthetic wastewater was adjusted between 7.9 and 8.2 and was maintained by the phosphate buffer (part of the synthetic wastewater). In the vessel, the concentration of suspended cells was 0.47 ± 0.03 g

VSS l^{-1} while around 6.0–9.0 g wet entrapped cell beads (30 beads) were used. The vessel was magnetically stirred and operated at room temperature (22–23°C). DO depletion in the vessel due to substrate utilization was monitored over time and the specific oxygen uptake rate (SOUR) was determined. The SOUR was determined twice for every substrate concentration tested.

The kinetic of nitrification process is dependent mainly on substrate and DO concentrations. It is normally described by a Double Monod expression (Eq. (1)). For each experiment, the data were fitted to Eq. (1) using a nonlinear regression module in SIGMAPLOT® 10.0 to determine maximum SOUR and half saturation coefficients for nitritation and nitratation.

$$SOUR = SOUR_{MAX}\left(\frac{[S]}{K_S + [S]}\right)\left(\frac{[DO]}{K_O + [DO]}\right) \quad (1)$$

where SOUR is specific oxygen uptake rate for nitritation or nitratation (mg O_2 [mg VSS h]$^{-1}$), $SOUR_{MAX}$ is maximum SOUR for nitritation or nitratation ($SOUR_{MAX,AOB}$, $SOUR_{MAX,NOB}$) (mgO_2 [mg VSS h]$^{-1}$), [S] is substrate concentration (TAN or NO_2^-) (mgN l^{-1}), [DO] is DO concentration (mgO_2 l^{-1}), K_S is half saturation coefficient for substrate ($K_{S,AOB}$, $K_{S,NOB}$) (mgN l^{-1}), and K_O is half saturation coefficient for oxygen ($K_{O,AOB}$, $K_{O,NOB}$) (mgO_2 l^{-1}).

4.2.3.1. Effect of substrate (ammonia and nitrite) concentrations

To investigate the effect of substrate concentrations, two tests, nitritation and nitratation, were conducted. For the nitritation test, the synthetic wastewater which had initial ammonia concentrations of 0, 3, 5, 10, 20, 40, 80, 150, 300 mg N l^{-1} was used to study the influence of ammonia on nitritation rate by both suspended and entrapped cells. To inhibit nitrite oxidation in the nitritation test, sodium azide was added at a concentration of 50 µM. For the nitratation test, the initial nitrite concentrations of synthetic wastewater were 0, 5, 10, 20, 40, 80, 120, 150 mg N l^{-1} to study the influence of nitrite on nitratation rate by both types of cells. Allylthiourea

(ATU) was added simultaneously at a concentration of 86 µM to inhibit ammonia oxidation (Ginestet,1998) .To avoid the occurrence of DO limitation in the vessel, the experiment was terminated at 4.0 $mgO_2\ l^{-1}$ for suspended cells and 6.0 $mgO_2\ l^{-1.}$ for entrapped cells. The initial DO was at 7.5 $mgO_2\ l^{-1.}$

4.2.3.2 Effect of bulk DO concentration

Experiments to examine the effect of bulk DO concentration were conducted in the same manner as described in Subsection 4.2.3.1. An initial ammonia concentration of 150 mgN l^{-1} and an initial nitrite concentration of 120 mgN l^{-1} were chosen for the nitritation and nitratation tests, respectively.

4.2.3.3 Inhibitory effect of free ammonia

A free ammonia (FA) inhibition on ammonia oxidation was tested using synthetic wastewater at the initial FA concentrations of 0, 0.6, 1.2, 2.4, 4.9, 9.8, 18.3, 36.6, 61.1, 91.7, 122.2 mgN l^{-1} with 50 µM sodium azide. For the nitratation test, the initial FA concentrations of synthetic wastewater were 0, 0.6, 1.2, 2.4, 4.9, 9.8, 18.3, 36.6, 61.1 mgN l^{-1} while the nitrite concentration was 120 mgN l^{-1}. Ammonia oxidation was inhibited by adding ATU in the same manner as described in Subsection 4.2.3.1.

4.2.4 Chemical analyses

DO (Thermo Orion 850A meter, Thermo Orion DO 083005D probe), pH (Thermo Orion 250A + pH Electrode), and VSS were determined according to Standard Methods (American Public Health Association, 1998). Ammonia and nitrate nitrogen were analyzed using ion selective electrodes (Thermo Orion 250A+, VWR SympHony Ammonia Combination Electrode, VWR SympHony Nitrate Ion Selective Electrode, VWR SympHony Double Junction Reference Half-Cell) in accordance with Standard Methods for the Examination of Water and Wastewater (APHA et al.,

1998). Nitrite was analyzed colorimetrically using standard HACH NitriVer® 2 reagents according to the method specified by the manufacturer (HACH).

4.2.5 Community analysis of ammonia oxidizing and nitrite oxidizing bacteria

4.2.5.1 Preparations of suspended and entrapped cell samples

Both suspended and entrapped cells were collected during their maintenance in the enrichment reactors, one or two days before they were used in the respirometric experiments. After the collection, they were suspended in a phosphate buffered saline (PBS) solution and then fixed in a solution of 4% paraformaldehyde in PBS (pH 7.2) at 4 °C for 9 h and 18 h, respectively. Fixed entrapped cells were embedded in a Tissue-Tek OCT compound (Sakura Finetek USA Inc.) and cut into 10 µm sections at −18 °C using a cryomicrotome (Leica CM 1950) and then immobilized onto a Poly-L Lysine coating slide. The slides were air-dried and dehydrated by 50%, 80% and 98% (v/v) ethanol (3 min each) successively.

4.2.5.2 Fluorescent 16S rRNA targeted oligonucleotide probes and in situ hybridization

Oligonucleotide probes used in this study were commercially synthesized and fluorescently 5' labeled with Oregon Green 488 or Rhodamine Red (ThermoHybaid, Ulm, Germany). The FISH assay was performed according to the protocol described by Amann et al. (1995) at 46 °C for 2.5 h in hybridization buffer (0.9 M NaCl, 20 mM tris–hydrochloride, 0.01% sodium dodecyl sulfate (SDS), formamide) containing 5 ng of probe μl^{-1} in a humidity chamber. A negative control (no probe) was included for every sample to observe autofluorescence. After hybridization, the slides were rinsed and immersed in pre-warmed washing buffer (20 mM tris–hydrochloride, 0.01% SDS, NaCl) at 48 °C for 10 min. After washing, the slides were rinsed briefly with DI water, air-dried, and mounted with a cover slip using an anti-fading solution (SlowFade, Antifade kit, Molecular Probes, Eugene,

OR). The oligonucleotide probes used in this study and hybridization conditions including the formamide concentration in the hybridization buffer and NaCl concentration in the washing buffer are shown in Table 4.1. In addition, for simultaneous hybridization with the probes which require different hybridization stringencies, two hybridizations were performed successively with the probe requiring higher stringency first (Wagner et. al, 1996)

Table 4.1 Oligonucleotide probes used.

Probe	Sequence (5' to 3')	Label	Target organisms	Formamide (%)	NaCl (M)	References
EUB338	GCTGCCTCCCGTAGGAGT	Oregon Green 488	Most bacteria	15	0.318	Amann et al.,1990
Nso190	CGATCCCCTGCTTTTCTCC	Rhodamine Red	Many but not all ammonia oxidizing *β-Proteobacteria*	40	0.056	Mobarry et al.,1996
Nse1472	ACCCCAGTCATGACCCCC	Oregon Green 488	*Nitrosomonas europaea, Nitrosomonas eutropha, Nitrosomonas halophila*	50	0.028	Juretschko et al.,1998
Ntspa 662	GGAATTCCGCGCTCCTCT	Oregon Green 488	*Nitrospira* genus	35	0.08	Daims et al., 2001
Nit3	CCTGTGCTCCATGCTCCG	Rhodamine Red	*Nitrobacter spp.*	40	0.056	Wagner et al.,1996

4.2.5.3 Confocal scanning laser microscopy and image analysis

The FISH samples were observed using an Olympus inverted microscope (Olympus IX81) mounted with an Olympus FluoView FV300 (Olympus, Germany) confocal laser scanning microscopy (CSLM) module, equipped with argon (488 nm) and two helium-neon (543 nm) laser units. Barrier filters, BA 505–525 and BA 565IF, were used to collect the excited fluorescence of the Oregon Green 488-labeled probes and the Rhodamine Red-labeled probes, respectively. The DAIME software (Daims et al., 2005) was used as an image analysis tool to help in determining the relative area taken up by target cells complimentary to the specific probe compared to the area of cells complimentary to the EUB338 probe. The average area fraction was determined by evaluating at least nine representative microscopic fields.

4.3 Results and discussion

4.3.1 Effect of substrate concentration

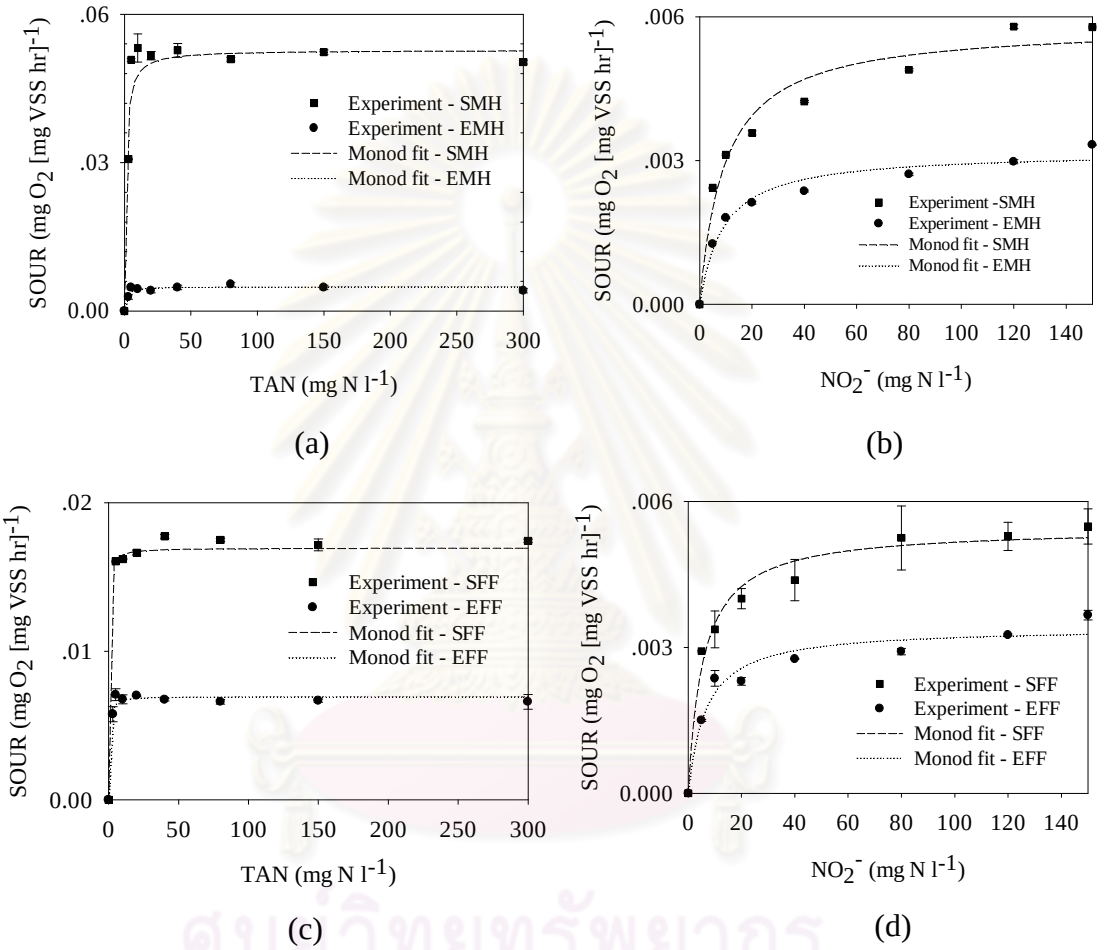

Fig. 4.1 SOUR of entrapped and suspended nitrifiers as a function of total ammonia concentration [(a) for SMH and EMH sludge, and (c) for SFF and EFF sludge] and nitrite concentration [(b) for SMH and EMH sludge, and (d) for SFF and EFF sludge]. The prefixes S and E prior to MH or FF represent suspended and entrapped cells, respectively.

Fig. 4.1 shows the average SOUR for nitritation and nitratation of MH and FF suspended cells (SMH and SFF) and entrapped cells (EMH and EFF) as a function of substrate concentration. The error bars represent the minimum and maximum values.

Note that some error bars are not visible because the difference in results from the duplicate experiments was very small. The SOUR of both the entrapped and suspended cells followed a Monod type equation with respect to substrate concentration. At substrate concentrations of 0–10 mgN l^{-1} TAN for nitritation and 0 – 40 mgN l^{-1} NO_2^- for nitratation, the AOB and NOB growth was limited by substrate and SOUR depended on the substrate concentration. At higher substrate concentrations (>10 mgN l^{-1} TAN for nitritation and >40 mgN l^{-1} NO_2^- for nitratation), their growth and SOUR reached the maximum and were independent from substrate concentration. The Monod fitting parameters of the data are summarized in Table 4.2. The $SOUR_{MAX}$ of suspended cells were clearly higher than those of corresponding entrapped cells while the half saturation coefficients for substrate of entrapped cells were higher probably because of the internal substrate transfer limitation within the entrapment gel (Carrera et al., 2004). There was only one case that did not follow the trend; the half saturation coefficient for nitrite of SMH was higher than that of EMH which could be due to uncontrollable variability of the respirometric assay.

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Table 4.2 Summary of Monod kinetic parameters.

Process	Symbol	Definition	Unit	Sludge	Type of cell	
					Suspended (R^2)	Entrapped (R^2)
Nitritation	$SOUR_{MAX,S,AOB}$	Maximum specific oxygen uptake rate of AOB for ammonia	mgO_2 $[mgVSS\ hr]^{-1}$	Moorhead WWTF	$5.28\times10^{-2}\pm1.06\times10^{-3}$ (0.94)	$4.90\times10^{-3}\pm1.58\times10^{-4}$ (0.85)
				Fergus Falls WWTF	$1.69\times10^{-2}\pm1.48\times10^{-4}$ (0.99)	$6.96\times10^{-3}\pm1.05\times10^{-4}$ (0.96)
	$K_{S,AOB}$	Half saturation coefficient of AOB for ammonia	$mgN\ l^{-1}$	Moorhead WWTF	$1.06\pm2.54\times10^{-1}$ (0.94)	$1.29\pm4.41\times10^{-1}$ (0.85)
				Fergus Falls WWTF	$2.79\times10^{-1}\pm1.30\times10^{-1}$ (0.99)	$3.77\times10^{-1}\pm1.47\times10^{-1}$ (0.96)
	$SOUR_{MAX,DO,AOB}$	Maximum specific oxygen uptake rate of AOB for oxygen	mgO_2 $[mgVSS\ hr]^{-1}$	Moorhead WWTF	$1.65\times10^{-2}\pm1.54\times10^{-4}$ (0.99)	a
				Fergus Falls WWTF	$1.26\times10^{-2}\pm2.29\times10^{-4}$ (0.87)	a
	$K_{O,AOB}$	Half saturation coefficient of AOB for oxygen	$mgO_2\ l^{-1}$	Moorhead WWTF	$3.34\times10^{-1}\pm1.89\times10^{-2}$ (0.99)	a
				Fergus Falls WWTF	$3.25\times10^{-1}\pm3.76\times10^{-2}$ (0.87)	a
Nitratation	$SOUR_{MAX,S,NOB}$	Maximum specific oxygen uptake rate of NOB for nitrite	mgO_2 $[mgVSS\ hr]^{-1}$	Moorhead WWTF	$5.83\times10^{-3}\pm1.94\times10^{-4}$ (0.97)	$3.19\times10^{-3}\pm9.26\times10^{-5}$ (0.97)
				Fergus Falls WWTF	$5.46\times10^{-3}\pm1.72\times10^{-4}$ (0.96)	$3.41\times10^{-3}\pm1.18\times10^{-4}$ (0.95)
	$K_{S,NOB}$	Half saturation coefficient of NOB for nitrite	$mgN\ l^{-1}$	Moorhead WWTF	9.59±1.42 (0.97)	8.82±1.17 (0.97)
				Fergus Falls WWTF	$5.66\pm9.56\times10^{-1}$ (0.96)	6.45±1.14 (0.95)
	$SOUR_{MAX,DO,NOB}$	Maximum specific oxygen uptake rate of NOB for oxygen	mgO_2 $[mgVSS\ hr]^{-1}$	Moorhead WWTF	$6.00\times10^{-3}\pm6.71\times10^{-5}$ (0.97)	$4.23\times10^{-3}\pm2.40\times10^{-4}$ (0.92)
				Fergus Falls WWTF	$5.50\times10^{-3}\pm3.76\times10^{-4}$ (0.69)	$4.53\times10^{-3}\pm1.40\times10^{-4}$ (0.98)
	$K_{O,NOB}$	Half saturation coefficient of NOB for oxygen	$mgO_2\ l^{-1}$	Moorhead WWTF	$3.57\times10^{-1}\pm2.47\times10^{-2}$ (0.97)	$3.53\pm4.48\times10^{-1}$ (0.92)
				Fergus Falls WWTF	$9.67\times10^{-1}\pm2.38\times10^{-1}$(0.69)	$3.38\pm2.18\times10^{-1}$ (0.98)

[a] The SOUR of entrapped cells for nitritation did not follow a Monod type equation with respect to substrate concentrations.

ศูนย์วิทยทรัพยากร
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For suspended cells, the values of half saturation coefficients for total ammonia nitrogen ($K_{S,AOB}$) between 2.79×10^{-1} and 1.06 mgN l^{-1} and nitrite ($K_{S,NOB}$) between 5.66 and 9.59 mgN l^{-1} were agreeable with those reported in previous studies (Carrera et al., 2004; Sanchez et al., 2001; Schramm et al., 1999; Laanbroek et al.,1994). When comparing between nitritation and nitratation, the $SOUR_{MAX}$ for nitritation (5.28×10^{-2} mgO_2 $mgVSS^{-1}$ h^{-1} for SMH and 1.69×10^{-2} mgO_2 $mgVSS^{-1}$ h^{-1} for SFF) were higher than those of corresponding nitratation (5.83×10^{-3} mgO_2 $mgVSS^{-1}$ h^{-1} for SMH and 5.46×10^{-3} mgO_2 $mgVSS^{-1}$ h^{-1} for SFF). This is because the amount of AOB was higher than that of NOB as discussed later in Subsection 4.3.4. The magnitudes of $SOUR_{MAX}$ for nitritation and nitratation of SMH were higher than those of SFF. This observation is agreeable with the FISH results (Subsection 4.3.4) which show higher fractions of AOB and NOB in SMH than those in SFF. The seed sludge from the Moorhead WWTF was acclimated to high ammonia and should be abundant in high growth rate nitrifier species (r-strategist). As a result, after the enrichment under the same condition, there were more AOB and NOB in SMH than those in SFF.

For entrapped cells, the $SOUR_{MAX}$ for nitritation (4.90×10^{-3} mgO_2 $mgVSS^{-1}$ h^{-1} for EMH and 6.96×10^{-3} mgO_2 $mgVSS^{-1}$ h^{-1} for EFF) were higher than those of corresponding nitratation (3.19×10^{-3} mgO_2 $mgVSS^{-1}$ h^{-1} for EMH and 3.41×10^{-3} mgO_2 $mgVSS^{-1}$ h^{-1} for EFF) agreeing with an observation that the amounts of AOB in both EMH and EFF were higher than that of NOB (see Subsection 4.3.4). Although the source of sludge affected the activity of suspended cells (SMH and SFF), this behavior was less evident for entrapped cells (EMH and EFF). The magnitudes of $SOUR_{MAX}$ of EMH and EFF were closer. However, EMH had lower $SOUR_{MAX}$ than EFF while higher fractions of AOB and NOB in EMH than those in EFF were observed (see Subsection 4.3.4). This suggests that there was no relationship between the change in the activity of entrapped nitrifiers and the change in the amount of nitrifiers in the entrapment. It is possible that cell protection against adverse effects provided by entrapment matrix (Wijffels and Tramper, 1995) controlled the nitrifier activity through substrate and/or oxygen diffusion.

4.3.2 Effect of bulk DO concentration

Effects of DO concentration on SOUR for nitritation and nitratation are presented in Fig. 4.2 and summarized in Table 4.2. Note that there are no error bars in Fig. 4.2 because the measurement of DO concentration could not be controlled to be at the same values between the duplicate experiments. As shown in Fig. 4.2, the nitrifier activity was affected differently by DO between suspended and entrapped cells.

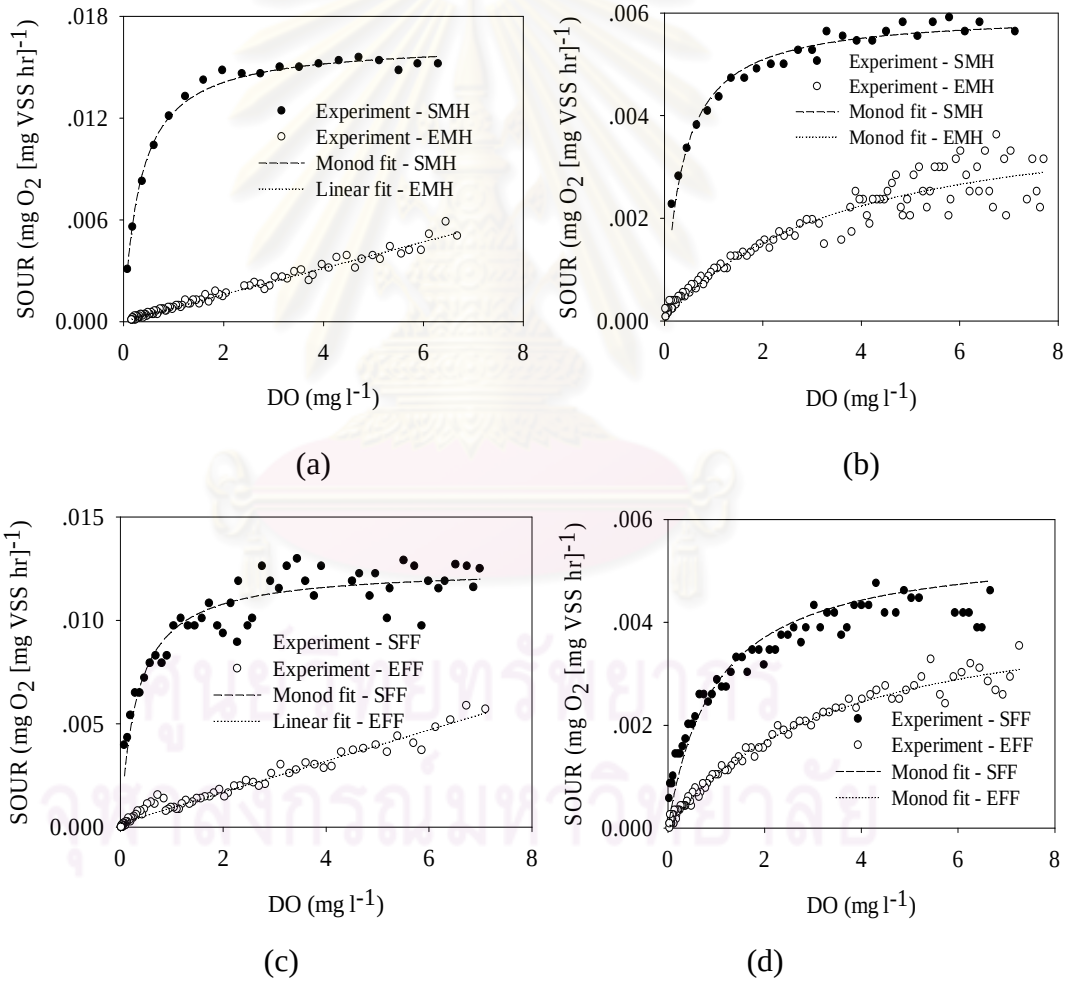


Fig. 4.2 SOUR of entrapped and suspended nitrifiers as a function of DO concentration in nitritation [(a) for SMH and EMH sludge, and (c) for SFF and EFF sludge] and in nitratation [(b) for SMH and EMH sludge, and (d) for SFF and EFF sludge]. The prefixes S and E prior to MH or FF represent suspended and entrapped cells, respectively.

For suspended cells, at <2 $mgO_2\ l^{-1}$ DO, the SOUR for nitritation and nitratation were limited by the DO concentration. When DO >2 $mgO_2\ l^{-1}$, the SOUR were constant and became maximum. The $SOUR_{MAX}$ values of SMH were higher than those of SFF. The explanations for these observations were the same as those given in Section 4.3.1. The half saturation coefficient for oxygen in nitritation ($K_{O,AOB}$ = 3.34×10^{-1} and 3.25×10^{-1} $mgO_2\ l^{-1}$) and nitratation ($K_{O,NOB}$ = 3.57×10^{-1} and 9.67×10^{-1} $mgO_2\ l^{-1}$) for SMH and SFF sludge were comparable to the published values ($K_{O,AOB}$ = 0.22 – 0.56 $mgO_2\ l^{-1}$ and $K_{O,NOB}$ = 0.17– 4.32 $mgO_2\ l^{-1}$) (Laanbroek et al.,1994). The half saturation coefficients for oxygen in nitritation were lower than those of nitratation suggesting that under oxygen limited environment (as in partial nitrifying condition), AOB in SMH and SFF sludge would outcompete NOB for oxygen and NOB would eventually be washed out of the reactor.

For entrapped cells, the SOUR for nitratation followed the Monod kinetics with respect to DO concentration. NOB in both EMH and EFF needed DO around 4 $mgO_2\ l^{-1}$ to achieve their maximum utilization while the SOUR of entrapped AOB for both EMH and EFF were related to DO in the first order manner and they needed more oxygen to reach saturation compared to NOB. These observations indicate that bulk DO concentration is more vital for entrapped cells than corresponding suspended cells and DO transfer from bulk liquid to the bead is critical for entrapped nitrifiers. Higher bulk DO concentrations and higher DO transfer would lead to more growth for nitrifiers. Moreover, under low DO which intensified oxygen transfer limitation, the activity of entrapped AOB would be more suppressed than the activity of entrapped NOB. These results suggest that it might not be possible to achieve partial nitrification in entrapped cells system by using DO limitation as a sole control strategy.

4.3.3 Inhibitory effect of free ammonia

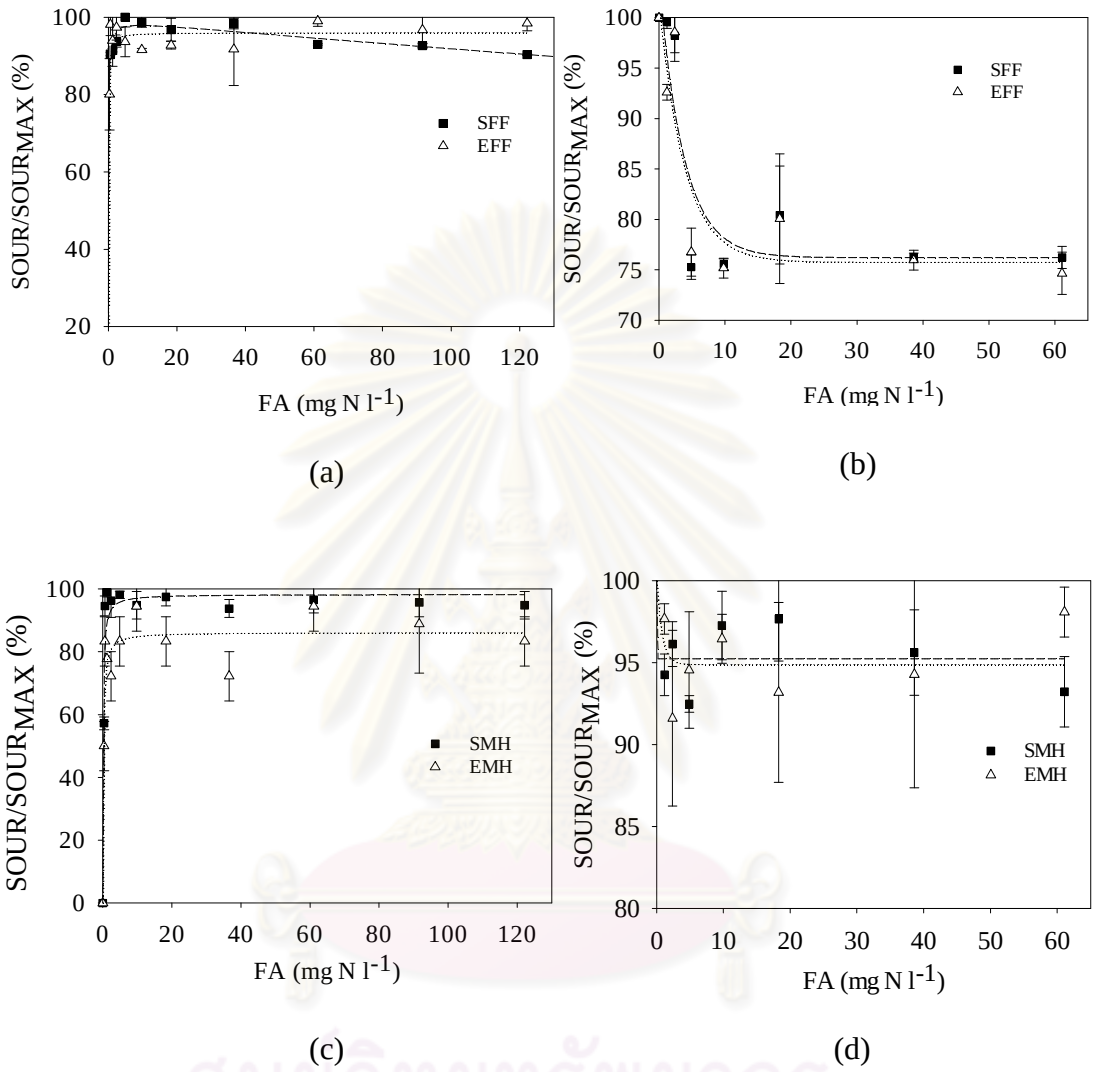


Fig. 4.3 SOUR/$SOUR_{MAX}$ of entrapped and suspended nitrifiers as a function of free ammonia concentration in nitritation [(a) for SFF and EFF sludge, and (c) for SMH and EMH sludge] and in nitratation [(b) for SFF and EFF sludge, and (d) for SMH and EMH sludge]. The prefixes S and E prior to FF or MH represent suspended and entrapped cells, respectively.

As shown in Fig. 4.3, the inhibitory effect of FA was observed in both sludge used in this study but the level of FA inhibition was different depending on the source of sludge. For both MH and FF sludge, the results for suspended cells and entrapped cells were similar. Although the cell protection by entrapment matrix was frequently

reported (Isaka et al., 2004; Wijffels and Tramper, 1995), this study shows that the entrapment did not help with FA inhibition on both nitritation and nitratation.

For FF sludge (Fig. 4.3a and b), the FA concentration range of 60–120 mgN l^{-1} had an inhibitory effect on AOB activity while the nitrite oxidation was inhibited at 0.6–60 mgN l^{-1} FA. These ranges of concentration were agreeable with a previous investigation (Anthonisen et al., 1976) which reported a possible FA inhibition at higher than 10 mgN l^{-1} FA for AOB, and higher than 0.1–1.0 mgN l^{-1} FA for NOB. Fig. 4.3 clearly shows that nitratation was inhibited more than nitritaton (SOUR/ $SOUR_{MAX}$ reduction of 10% for nitritation and 23% for nitratation). According to several previous studies, both AOB and NOB are sensitive to FA (Ahn et al., 2008; Sinha and Annachhatre, 2007; Anthonisen et al., 1976).

For MH suspended cells and entrapped cells (Fig. 4.3c and d), nitritation was not adversely affected by FA concentration up to 120 mgN l^{-1} (highest level used in this study for nitritation) and nitratation experienced only 5% inhibition. Based on these results, both AOB and NOB in MH sludge adapted to FA better than FF sludge. Although there is no supporting information, it is likely that the higher FA tolerance in MH sludge (SMH and EMH) could be a result of a nitrifier community that was more acclimated to high ammonia. An investigation on the bacterial communities of SMH and SFF sludge at the species level might confirm this explanation.

In this study, NOB were more sensitive to FA compared to AOB and a lower range of FA concentration was sufficient for inhibiting nitratation. It was found that an inhibitory effect started to affect NOB activity at 0.6 mgN l^{-1} FA which was also reported by Villaverde et al. (2000). Moreover, the inhibition on nitratation stabilized at FA > 10 mgN l^{-1} for FF sludge and at FA > 4 mgN l^{-1} for MH sludge. This limited inhibitory effect on NOB activity was reported in previous studies (Vadivelu et al., 2007; Turk and Mavinic, 1986). Vadivelu et al. (2007) found that the activity of enriched *Nitrobacter* culture was reduced by only 12% at 4 mgN l^{-1} FA and remained at the same level even after FA concentration was raised to 9 mgN l^{-1}. This suggests the ability of NOB to adapt to FA inhibition. The FA inhibition by itself might not be

an effective method to maintain nitrite accumulation in both suspended and entrapped cell systems.

4.3.4 Community analysis of ammonia oxidizing and nitrite oxidizing bacteria

The FISH assay was performed to observe the communities of AOB and NOB in both MH and FF sludge. The fractions of AOB and NOB were determined from the relative areas of the NSO190 probe and the combination of Ntspa662 and Nit3 probes to the EUB338 probe (see Table 4.1). The fraction of heterotrophs was estimated from the remaining relative area other than those of AOB and NOB.

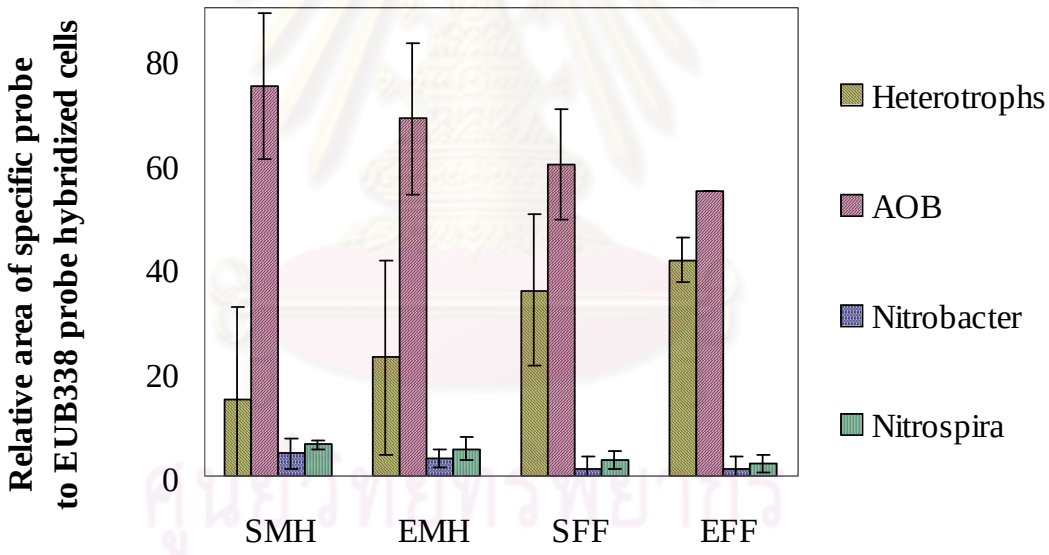


Fig. 4.4 Bacterial community structures of suspended and entrapped MH and FF sludge determined by FISH. The percentages of AOB, *Nitrospira* genus, and *Nitrobacter* spp., in sludge were determined from the relative areas of NSO190, Ntspa662 and Nit3 probes to the EUB338 probe (all bacteria), respectively. The fraction of heterotrophs was estimated from the remaining relative area other than those of AOB and NOB. The average relative area and standard deviation were determined by evaluating at least nine representative microscopic fields.

As shown in Fig. 4.4, the fractions of AOB in both suspended cells (74.0±14.0% for SMH and 59.9±10.7% for SFF) and entrapped cells (68.7±14.6% for EMH and 54.8±0.1% for EFF) were quite similar. The fractions of NOB in suspended cells (10.3±3.7% for SMH and 4.4±4% for SFF) and entrapped cells (8.5±3.9% for EMH and 3.7±4.1% for EFF) were also close. The cell entrapment did not change the fractions of AOB and NOB in the sludge and consequently the difference in nitrification kinetics between suspended cells and entrapped cells could be the results of internal substrate and oxygen transfer limitations within the entrapment.

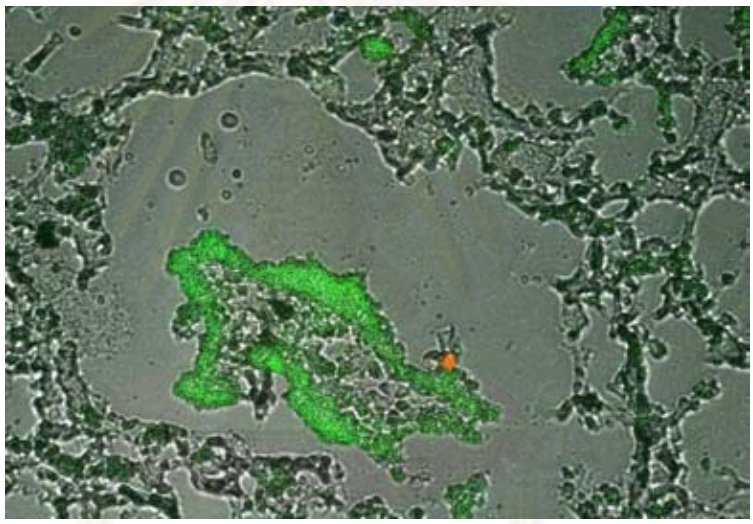
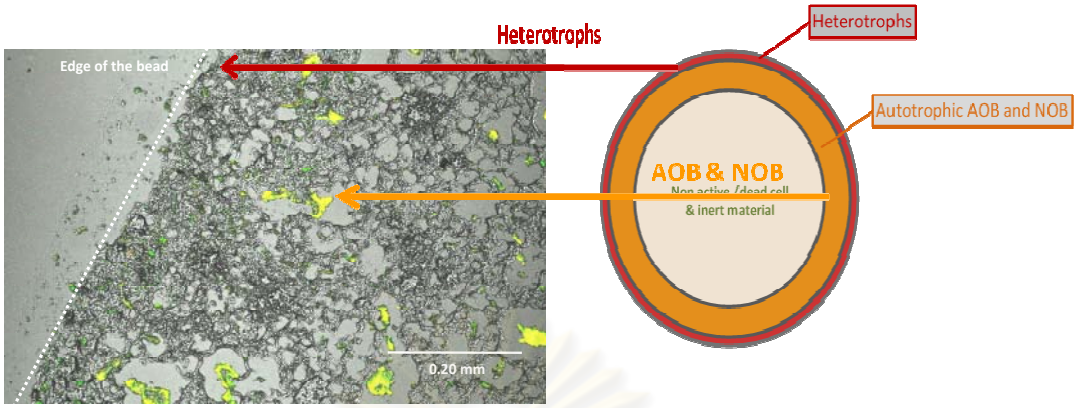
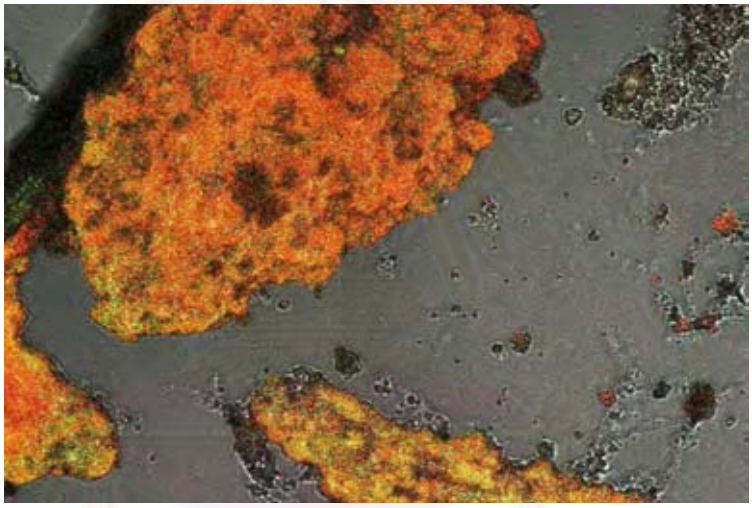


Fig. 4.5 Example of FISH-CLSM images of entrapped nitrifiers in Moorhead sludge, hybridized with Nse1472, specific for the detection of *Nitrosomonas europaea* (in green) and Nit3, specific for the detection of *nitrobacter* spp. (in red).



(a)



(b)

Fig. 4.6 Example of FISH-CLSM images of nitrifiers in Moorhead sludge: (a) entrapped nitrifiers, hybridized with NSO190 (in red) and EUB338, specific for the detection of all bacteria (in green). The target cells complimentary to both Rhodamine Red-labeled probe (in red) and Oregon Green 488-labeled probe (in green), are in yellow. The observed bacteria in green, which stayed around the peripheral layer of the bead, were heterotrophs and (b) suspended nitrifiers, hybridized with NSO190, specific for the detection of beta-proteobacteria AOBs (in red) and Nse1472, specific for the detection of *Nitrosomonas europaea* (in green).

The segregation between AOB and NOB within the entrapment was observed (Fig. 4.5). Large and dense clusters of AOB stayed around huge voids. While smaller clusters of NOB were adjacent to the AOB clusters to receive the substrate (nitrite). These arrangements agree with the results of SOUR of EMH and EFF with respect to DO concentration in Subsection 4.3.2 (Fig. 4.2). AOB within the bead had more oxygen transfer limitation effect than NOB resulting in the difference in sizes of AOB and NOB clusters within the bead. The AOB cluster was larger and denser, and oxygen needed more time and faced more resistance to diffuse into it. These differences in the density and size of AOB and NOB clusters were frequently found in nitrifying biofilm (Okabe et al., 2004; Schramm et al., 1996).

An unknown group of bacteria, likely heterotrophs, was also observed. The fractions of heterotrophs in entrapped cells were 22.8±18.6% and 41.5±4.3% for EMH and EFF, respectively (Fig. 4.4). Under high sludge age and substrate limiting condition as within the bead, soluble microbial products (SMP) are produced substantially and can become the source of organic carbon for heterotrophic growth. This explanation is agreeable with the observed spatial arrangement of heterotrophic bacteria in this study, which was around the carbon source (SMP excreted from nitrifiers) and oxygen source (the peripheral layer of the bead) (Fig. 4.6a). Oxygen competition and co-existence between nitrifiers and heterotrophs observed in this study have been found in nitrifying biofilm with no external carbon (Kindaichi et al., 2005; Okabe et al.2005).

Fig. 4.6b is the FISH-CLSM result which clearly indicates Nitrosomonas europaea as a dominant AOB in the SMH sludge. The result for the FF sludge was similar (data not shown). Due to high ammonia oxidation activity (r-strategist) and high nitrite tolerance, members of the *N. europaea*–Nitrosococcus *mobilis* cluster are usually found in wastewater treatment systems with high ammonia loads and/or high nitrite accumulation (Ahn et al., 2008; Dytczak et al., 2008; Limpiyakorn et al., 2007; Wagner et al., 1996). For NOB community, *Nitrobacter spp.* was the dominant NOB (6.0±2.9% for SMH and 3.0±2.3% for SFF) while Nitrospira was found in smaller proportions (4.3±0.8% for SMH and 1.4±1.7% for SFF) in both SMH and SFF sludge

(Fig. 4.4). *Nitrobacter spp.* is known to have high nitrite oxidation activity (r-strategist) while Nitrospira has lower activity (k-strategist) (Schramm et al., 1996). Co-existence between *Nitrobacter* and *Nitrospira* in high nitrite fluctuation environment as in nitrifying sequencing batch reactors has been reported (Dytczak et al., 2009). This study, which involved the sequencing batch operation for enrichment, also observed both genus and additionally discovered the dominance of *Nitrobacter* over *Nitrospira.*

4.4 Conclusion

Effects of substrate, DO and FA concentrations on nitritation and nitratation in both entrapped and suspended cells systems were determined by a respirometric assay. Two nitrifying sludge enriched from high ammonia acclimated and unacclimated seed sludge, were used in this study. For both nitritation and nitratation, the $SOUR_{MAX}$ and the affinity for substrate and oxygen of suspended cells were higher than those of corresponding entrapped cells. Under DO limiting conditions, the SOUR for nitratation was reduced more than that for nitritation for suspended cells because of higher oxygen affinity of AOB than NOB, while for entrapped cells, the results were the opposite. The FA inhibitory effect was observed in the un-acclimated sludge at significant levels but the acclimated sludge was not inhibited by high FA. These results lead to a conclusion that FA inhibition or DO limitation can be used to maintain nitrite accumulation in entrapped cell systems but might not be effective strategies, which need a detailed investigation to identify one. The fractions of AOB and NOB in both entrapped cells and suspended cells were comparable. N. *europaea* was the dominant AOB while *Nitrobacter* spp. was the dominant NOB for both suspended and entrapped cells. The competition for space and oxygen led to segregations among AOB, NOB and heterotrophs within the entrapment. Heterotrophic bacteria stayed within the peripheral layer of the bead to take oxygen from bulk liquid and soluble microbial products excreted from nitrifiers. Large clusters of AOB stayed in huge voids and smaller clusters of NOB were adjacent to the AOB clusters to receive nitrite. This study suggests that internal substrate and

oxygen transfers would be two of the most important factors in controlling nitritation kinetics of entrapped nitrifying sludge.

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER V

EFFECT OF INOCULUM AND DO/FA RATIO ON ACHIEVING PARTIAL NITRIFICATION USING GEL ENTRAPMENT

5.1 Introduction

Shortcut biological nitrogen removal (SBNR) is a cost effective innovative process to treat low carbon or/and high nitrogen wastewater (C/N < 3.5 - 4). For SBNR, partial nitrification (or nitritation) is believed to be the rate-limiting step of the overall SBNR. Partial nitrification can be achieved by the oxidation of ammonia (NH_3) to nitrite (NO_2^-) without further oxidation of NO_2^- to nitrate (NO_3^-) (Hellinga et al., 1998). Therefore, the activity of ammonia oxidizing bacteria (AOB) needs to be promoted over the activity of nitrite-oxidizing bacteria (NOB). The most two common strategies to achieve partial nitrification under normal temperature are to maintain oxygen (O_2)-limiting and/or free ammonia (FA)-accumulating conditions (Sinha and Annachhatre, 2007). Under low O_2 condition, growth of AOB can be higher than NOB due to higher O_2 affinity of AOB than that of NOB. At certain levels of FA, the activity of NOB is inhibited, while the activity of AOB is not.

Although partial nitrification can be achieved by controlling either O_2 or FA concentrations in the systems (van Hulle et al., 2010), a number of studies showed that partial nitrification efficiency (as measured by the ratio between the concentration of NO_2^- in bulk liquid and the amount of NH_3 removed in the systems) varied considerably, when only one of the parameters was selected to maintain partial nitrification. For O_2, the partial nitrification efficiencies varied between 50% and 95% in biofilm systems even at the concentrations of dissolved oxygen (DO) in bulk liquid were controlled at a similar level (95% at 1.4 mg O_2 l^{-1} DO, 50% at 1.5 mg O_2 l^{-1} DO: Ciudad et al., 2005; Garrido et al., 1997). A wide range of FA concentrations of 0.05

– 20 mg N l^{-1} have been recommended to inhibit the activity of NOB (Vadivelu et al., 2007; Villaverde et al., 2000; Abeling and Seyfried, 1992; Turk and Mavinic, 1989; Alleman and Irvine, 1980; Verstraete et al., 1977; Anthonisen et al. 1976; Prakasam and Loehr, 1972; Murray and Watson, 1965). Vadivelu et al. (2007) reported that although FA concentration was controlled between 4 and 9 mg N l^{-1}, the activity of NOB was not fully inhibited (only 88%). Based on the above mentioned information, it is unavoidable to incorporate both controlling parameters to maintain highly efficient partial nitrification.

Recently, cell entrapment in gel has been proposed to be a potential means to achieve partial nitrification under ammonia-rich environment (Rongsayamanont et al., 2010). Although the mechanisms behind partial nitrification by entrapped cells are unclear, an O_2-limiting condition occurring inside the gel beads which is more favorable for AOB than NOB is likely the main contributing factor (Rongsayamanont et al., 2010). Advantages of entrapped cell systems over suspended cell systems include better cell maintenance from washing out, higher cell protection from toxic substances, higher conversion rate of substrate, and easier liquid-cell separation (Chen and Lin, 1994). The applications of entrapped cells for nitrification has been demonstrated at several full-scale wastewater treatment plants (WWTP) in Japan (Isaka et al., 2007).

For entrapped cell and biofilm systems where most cells being retained in system, the profile of inoculum (in terms of original bacterial community and composition) can be a curtail factor to determine the duration needed for starting up partial nitrification system. A previous study in a biofilm system found that although the activity of NOB is inhibited, their cells can still be persistent and later lead to unexpected NO_3^- production (Wang et al., 2009). Rongsayamanont et al. (2010) showed the influence of culture history of inoculum on the resultant nitrifying microorganism composition and the nitrification kinetics of an enriched nitrifying culture (Rongsayamanont et al., 2010).

The first objective of this study is to investigate the effect of inoculum on accelerating partial nitrification in entrapped cell reactor. Moreover, the effect of inoculum on the abundance and spatial distribution of nitrifying microorganisms was also carried out. Suspended cells include 1) nitrifying (N) sludge, 2) partial nitrifying (PNI) sludge and 3) partial nitrifying (PNII) sludge were prepared, then all three types of suspended cells and another one as non-nitrifying (NN) sludge were used as 'suspended inocula'. The four types of suspended inoculum were later entrapped into the polyvinyl alcohol (PVA) gel called 'entrapped inocula'. Four entrapped inocula were used to start up entrapped cells nitritation reactors under identical conditions at DO concentrations of 2 and 3 mg O_2 l^{-1} to promote partial nitrification. Along the start-up period, NO_2^- accumulation was observed to determine effect of inoculum on accelerating partial nitrification. Quantitative polymerase chain reaction (qPCR) technique was used for quantifying the numbers of AOB, ammonia oxidizing archaea (AOA), and NOB to investigate the effect of inoculum on the community and population of nitrifying microorganism in nitritation entrapped cell. A fluorescence in situ hybridization (FISH) technique was used for probing nitrifying microorganisms (AOB and NOB) within the entrapment matrix to observe the effect of inoculum on the spatial distribution of nitrifying microorganism in nitritation entrapped cell.

The second objective of this study is to observe the effect of bulk DO and/or FA concentrations on partial nitrification efficiency in an entrapped cell system. After finishing the start-up of entrapped cell nitritation reactors, three entrapped cell reactors, which were inoculated with entrapped cells originated from NN, PNI and PNII sludge, were further operated for long periods under various bulk DO and FA concentrations. During the operational period, NO_2^- accumulation at steady state operation was monitored.

5.2 Materials and Methods

5.2.1 Synthetic wastewater

Two types of synthetic wastewater were used, one for suspended cell (inoculums) reactors and the other for entrapped cell reactors. The synthetic wastewater for suspended cell reactors, which is an inorganic medium solution with a total ammonia nitrogen (TAN) level of 70 mgN l^{-1} (0.34 g of $(NH_4)_2SO_4$ per liter), 250 mgN l^{-1} (1.20 g of $(NH_4)_2SO_4$ per liter), and 950 mgN l^{-1} (4.56 g of $(NH_4)_2SO_4$ per liter) were used as the influent for preparing nitrifying (N), partial nitrifying I (PNI) and partial nitrifying II (PNII) sludge, respectively. Synthetic wastewater for entrapped cell reactors, which was inorganic medium solution with a TAN level of 625 mgN l^{-1} (3.0 g of $(NH_4)_2SO_4$ per liter), was used as influent for its start up and long-term operation. Inorganic medium solution contained $NaHCO_3$ (9.8 g per gTAN), Na_2HPO_4 (4.05 g), K_2HPO_4 (2.1 g), $MgSO_4 \cdot 7H_2O$ (0.05 g), $CaCl_2 \cdot 2H_2O$ (0.01 g), and $FeSO_4 \cdot 7H_2O$ (0.09 g) in one liter of deionized (DI) water. All chemicals were purchased from Carlo Erba Reagenti (Milan, Italy).

5.2.2 Preparation of suspended and entrapped inocula

Three types of suspended inocula were experimented: non-nitrifying (NN), nitrifying (N), partial nitrifying (PNI and PNII). Fresh NN inoculum was taken from an aeration basin of a full-scale municipal activated sludge WWTP (a contact stabilization process) in Bangkok. The N, and PNI and PNII inocula were prepared from the NN inoculum. For the WWTP, the average influent and effluent concentrations of biochemical oxygen demand (BOD) were 57.4 and 5.5 mg l^{-1}. The average ammonium concentrations in the influent and effluent were less than 1 mg N l^{-1} (August 2008). Mixed liquor suspended solid (MLSS) and mixed liquor volatile suspended solid (MLVSS) were 5,006 and 2,653 mg l^{-1}, respectively. The descriptions and operation of the laboratory-scale reactors used to prepare the suspended inocula are shown in Table 5.1. Note that the difference between PNI and PNII was preparing PNI with no sludge wasting while PNII prepared with the selective sludge wasting to

limit the numbers of NOB. After steady state conditions, with respect to the concentrations of both inorganic nitrogen and biomass, the suspended inocula were harvested.

All three prepared suspended inocula (N, PNI, and PNII sludge) together with the fresh NN sludge taken directly from the WWTP were entrapped in phosphorylated PVA (PPVA) gel beads at a cell-to-matrix ratio of 4% w/v (g of centrifuged MLVSS/ml of media) according to the procedure described by Chen and Lin (Chen and Lin, 1994). Briefly, a mixture at an equal volume between a PVA aqueous solution (20% w/v) and a sludge suspension of 70 g volatile suspended solid (VSS) per liter was prepared. Then, the mixture was dropped into a saturated boric acid solution at a rate of 0.83 ml/min resulting in a droplet diameter of 3 – 4 mm. About one hour after the dropping ended, the formed beads were transferred to a 1 M sodium orthophosphate solution (pH 7) and kept for 2 hr to allow hardening. With this protocol, the gel beads had about the same sizes (approximately 6 mm in diameter). Entrapped cells originated from NN, N, PNI and PNII sludge were called ENN, EN, EPNI and EPNII and were used as the entrapped inocula for four corresponding entrapped cell nitritation reactors (ENN, EN, EPNI and EPNII reactors).

Table 5.1 Setup and operation of laboratory-scale reactors for preparation of suspended inoculums

Reactor	Influent	Operating conditions								
	NH_3 ($mgN\ l^{-1}$)	Type of reactor	NH_3 Load ($gN\ m^{-3}d^{-1}$)	HRT (d)	SRT (d)	MLVSS ($mg\ l^{-1}$)	DO ($mg\ l^{-1}$)	pH	temp (^oC)	Operating period (d)
N	71.4±1.2	SBR	30	2.25	no sludge wasting	850	> 4	8.1±0.1	25±1	76
PNI	252.0±1.1	SBR	500	0.5	no sludge wasting	1225	< 1	7.9±0.2	36±1	70
PNII	949.9±1.8	CSTR	540	1.5 - 2.0		430	230	8.0±0.1	35±1	73

5.2.3 Setup of entrapped cell nitritation reactors

Entrapped cell nitritation reactors have an effective volume of 3 liters. The reactors were stirred completely using mechanical stirrers at 250 rpm (IKA RW20D, IKA-Werke GmbH & Co., Germany). Air was supplied to each reactor through an ON/OFF air blower which was connected to an oxygen transmitter (O_2 4100e, Mettler Toledo, USA) equipped with a DO probe (O_2 Sensor InPro 6820, Mettler Toledo, USA) to control the DO concentration in the reactor. pH was controlled by a pH controller (Liquitron DP 5000, LMI Milton Roy, USA) equipped with a pH probe (Orion 9156DJWP, Thermo scientific, UK) as well as HCl and NaOH solutions.

5.2.4 Start up of entrapped cell nitritation reactors

The influent used to start-up the entrapped cell nitritation reactors is the organic-free synthetic wastewater containing TAN of 625.7 mg N l^{-1}. All reactors were operated under the same conditions. Hydraulic retention time was 1.2 days resulting in an influent NH_3 loading rate of 520 g N m^{-3} d^{-1}. The reactors were operated at room temperature (24-26^{o}C), while the pH level was strictly controlled at 8.0±0.1. . The start-up period was divided into 2 phases: phase I with the DO concentration in bulk solution of 3 mg O_2 l^{-1} and phase II with DO concentration in bulk solution of 2 mg O_2 l^{-1}. In phase II, bulk DO concentration was reduced into 2 mg O_2 l^{-1} to see the effect of O_2 level in achieving partial nitrification. During the start-up period, the supernatant was collected, filtered through a glass fiber filter (GF/C, Whatman) and analyzed for TAN, NO_2^- and NO_3^- concentrations daily. To observe spatial distribution of AOB and NOB in the gel beads, the beads were collected after the end of phases I and II of start up and analyzed using FISH technique. Also, after the end of phase II, gel beads were taken to quantify the numbers of nitrifying microorganisms as AOB, AOA, *Nitrospira*, and *Nitrobacter* in the cell entrapment matrix.

5.2.5 Long-term operation of entrapped cell nitritation reactors under various DO and FA concentrations

After starting up partial nitrification, the ENN, EPNI and EPNII reactors were further operated to investigate the effect of DO and/or FA concentration on the partial nitrification. All three reactors were operated under identical conditions. The influent was the synthetic wastewater containing 624.9 mg N l^{-1}TAN. HRT was 3.0 days resulting in an influent NH_3 loading rate of 208 g N m^{-3} d^{-1}. The reactors were operated at room temperature (24-26^{o}C). The DO concentration in the reactor was varied: 0.5±0.1, 1.0±0.1, 2.0±0.1 mgO_2 l^{-1}. To vary FA concentration, pH was adjusted to 6.5±0.1, 7.2±0.1, 7.8±0.1, 8.2±0.1. During the operational period, the supernatant was collected, filtered through a glass fiber filter (GF/C, Whatman) and analyzed for TAN, NO_2^- and NO_3^- concentrations daily.

5.2.6 Analyses

DO (WTW Oxi 340i meter, WTW Cellox 325 probe, Germany), pH (HACH Sension1 pH Electrode, USA), and VSS were determined following the Standard Methods for the Examination of Water and Wastewater (APHA et al. 1998). TAN was analyzed using ion selective electrodes (WTW GmbH, NH_4 500/2, Germany) and NO_2^- and NO_3^- were analyzed using a UV visible spectrophotometer (Thermo Electron Corporation, Hexious α, Cambridge, UK) in accordance with the Standard Methods for the Examination of Water and Wastewater (APHA et al. 1998).

5.2.7 Abundance of AOB, AOA and NOB in cell entrapment matrix determination using quantitative PCR

For each entrapped cell sample, two gel beads were suspended in an ice-cold phosphate buffered saline (PBS) solution. Later, the beads were transferred and fixed in a paraformaldehyde solution (4% in PBS at pH 7.2) at 4^{o}C for 12 h. After washing three times and resuspending in a ice-cold PBS solution, fixed gel beads were allow to be dissolved completely in a PBS solution at 70^{o}C for 5 min. Immediately, DNA of

the released cells was extracted using Fast-DNA SPIN kits for soil (QBiogene, USA). Agarose gel (1.5%) electrophoresis (Bio-Rad, Spain) was used to verify the products from DNA extraction.

For each sample of extracted DNA, DNA was prepared into three dilutions at 10-fold dilution factor. For AOB, *amoA* genes were quantified using the primers amoA 1F and amoA 2R and the 16S rRNA genes were analyzed using the primers CTO 189A/Bf , CTO189Cf , and CTO 654r. The range of 7.2×10^1 to 7.2×10^7 copies of the pGEM-T Easy Vector (Promega, USA) inserted with the *amoA* gene fragment of the clone AOB-NAS10-360-4 (an accession number of GU980134) was used as the standard DNA for the AOB *amoA* genes. The pGEM-T Easy Vector possessing the 16S rRNA gene fragment of *Nitrosospira multiformis* (an accession number of X90822) in the range of 4.4×10^1 to 4.4×10^7 copies was used as the standard DNA for AOB 16S rRNA gene. For AOA, *amoA* genes were quantified using the primers Arch-amoAF and Arch-amoAR. The range of 5.0×10^1 to 5.0×10^7 copies of the pGEM-T Easy Vector possessing the *amoA* gene fragment of the archaea *amoA* clone AOA-S-4 (an accession number of GQ390338) was used as the standard DNA for the AOA *amoA* gene quantification. For NOB, the 16S rRNA genes were quantified with the primers P338F and Ntspa0685 for *Nitrospira* and with the primers P338F and NIT3 for *Nitrobacter*. The standard DNA, which was the pGEM-T Easy Vector possessing the 16S rRNA gene fragment of *Nitrospira* and *Nitrobacter*, were prepared in the ranges of 7.7×10^1 to 7.7×10^7 copies and 2.5×10^1 to 2.5×10^7 copies, respectively. The sequences of all PCR primers and their target genes used in this study are shown in Table 5.2.

An Mx3005P instrument (Stratagene, USA) with a Brilliant II SYBR Green QPCR Master Mix (Stratagene, USA) was used to perform qPCR. For AOB and AOA, the PCR condition, 95°C for 10 min, followed by 40 cycles of 30 s at 95°C, 60 s at 56°C and 30 s at 72°C, was used to amplify the AOB *amoA* genes, AOB 16S rRNA, and AOA *amoA* genes. For NOB, the PCR condition, 95°C for 5 min, followed by 40 cycles of 90 s at 95°C, 30 s at 65°C and 60 s at 72°C, was used to amplify the 16S rRNA gene of *Nitrobacter* and *Nitrospira*. For every cycle of PCR

amplifications, fluorescence signal was captured at 78 °C for 15 s. At the end of every qPCR reaction, dissociation curves were plotted to confirm the single target fragment of the PCR amplified products.

To calculate the cell numbers of AOA and AOB from the observed number of archaeal and bacterial amoA genes, it was assumed, based on the numbers of archaeal and bacterial amoA gene copies found in enriched AOA and isolated AOB culture, that the AOA and AOB cell possessed one copy and 2.5 copies of the amoA gene per genome, respectively (Hallam et al., 2006; Norton et al. 2002). To calculate the cell numbers of *Nitrospira* and *Nitrobacter* from the observed number of 16S rRNA genes, it was assumed that the *Nitrospira* and *Nitrobacter* cell possessed one copy of the 16S rRNA gene per genome based on the numbers of 16S rRNA gene copies found in enriched *Nitrospira* and isolated *Nitrobacter* culture (Lücker et al., 2010; Starkenburg et al., 2006).

5.2.8 Spatial distribution of AOB and NOB in cell entrapment matrix determination using fluorescence *in situ* hybridization (FISH)

Spatial distribution of microorganisms in gel beads was analyzed using FISH. Protocols for cell fixing, sectioning, hybridizing and washing are described in Chapter 4. Briefly, for each sample, one gel bead was suspended in an ice-cold PBS solution. Later, the bead was transfer and fixed in a paraformaldehyde solution (4% in PBS at pH 7.2) at 4^{o}C for 12 h. After washing three times and resuspending in a ice-cold PBS solution, the fixed gel bead was embedded in a rapid freezing compound (Tissue-Tek OCT, Sakura Finetek USA Inc., USA) and cut into five micron sections at -20^{o}C using a cryomicrotome (Leica CM 1950, Germany), then immobilized onto a polymer coating slide.

Oligonucleotide probes used in this study were labeled at 5’ ends with Alexaflour 488 or Cy3 (ThermoHybaid, Ulm, Germany). Hybridization was performed at 46^{o}C for 1.5 h in buffer (0.9 M NaCl, 20 mM Tris-HCl, 0.01% sodium dodecyl sulfate (SDS), formamide) containing 5 ng of probe μl^{-1}. The cut section of

the gel bead was immersed in pre-warmed washing buffer (20 mM Tris-HCl, 0.01% SDS, NaCl) at 48^{o}C for 10 min and subsequently rinsed shortly with DI water, air dried and mounted with an anti-fading solution (SlowFade, Antifade kit, Molecular Probes, Eugene, OR, USA). The oligonucleotide probes and their corresponding hybridization conditions are shown in Table 5.3. For simultaneous hybridization, two hybridizations were performed successively with the probe requiring higher stringency performed first (Wagner et al., 1996). The hybridized sample was observed using a fluorescence inverted microscope (Olympus IX81, Japan) with DP2-BSW software for image processing. Alexaflour 488–labelled probe was visualized by excitation between 460 and 495 nm and collection of fluorescence emission at 510 nm. Cy3-labelled probe was excited at between 530 and 550 nm and its fluorescence emission was collected at 575 nm.

Table 5.2 PCR primers used in this study

PCR Primer	Sequences (5'- 3')	Target genes	References
P338F	ACTCCTACGGGAGGCAGCAG	Bacterial 16S rRNA	Ovreås et al (1997)
amoA 1F amoA 2R	GGGGTTTCTACTGGTGGT CCCCTCKGSAAAGCCTTCTTC	AOB *amoA* gene	Rotthauwe (1997)
CTO 189A/Bf CTO 189Cf CTO 654r	GGAGRAAAGCAGGGGATCG GGAGGAAAGTAGGGGATCG CTAGCYTTGTAGTTTCAAACGC	AOB 16S rRNA gene	Kowalchuck et al (1997)
Arch-amoAF Arch-amoAR	STAATGGTCTGGCTTAGACG GCGGCCATCCATCTGTATGT	AOA *amoA* gene	Francis et al (2005)
Ntspa0685	CGGGAATTCCGCGCTC	*Nitrospira* 16S rRNA gene	Regan et al (2002)
NIT3	CCTGTGCTCCATGCTCCG	*Nitrobacter* 16S rRNA gene	Wagner et al (1996)

Table 5.3 Labeled 16SrRNA oligonucleotide probes used in this study

Probe	Sequences (5' to 3')	Label	Target organisms	Formamide (%)	References
EUB338	GCTGCCTCCCGTAGGAGT	Cy3/AF	Most bacteria	15	Amann et al. (1990)
Nso190	CGATCCCCTGCTTTTCTCC	Cy3/AF	*β-AOB* (Many but not all)	40	Mobarry et al. (1996)
Nse1472	ACCCCAGTCATGACCCCC	Cy3	*Nitrosomonas europaea, Nitrosomonas eutropha, Nitrosomonas halophila*	50	Juretschko et al. (1998)
Ntspa662	GGAATTCCGCGCTCCTCT	Cy3	*Nitrospira* genus	35	Daims et al. (2001)
Nit3	CCTGTGCTCCATGCTCCG	Cy3	*Nitrobacter spp.*	40	Wagner et al. (1996)

5.3 Results and discussion

5.3.1 Preparation of suspended inocula

Suspended inocula used for the preparation of entrapped cell inocula included the fresh NN sludge, which was taken directly from the WWTP and N, PNI, and PNII sludge, which were prepared by enriching the fresh NN sludge in the laboratory-scale reactors. Operating conditions and steady-state performance of the reactors used for the preparation of N, PNI, and PNII sludge are shown in Tables 5.1 and 5.4. Abundance and spatial distribution of the target microorganisms are shown in Fig. 5.1 and 5.2, respectively.

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Table 5.4 Operating conditions and steady-state performance of laboratory-scale reactors during preparation of suspended inocula (N, PNI, and PNII sludge) (see monitoring results in Fig. A-1 to A-3 in Appendix A)

Reactor	Influent	Operating conditions									Effluent			Removal efficiency			
	NH_3 ($mgNl^{-1}$)	Type of reactor	NH_3 Load ($gN\ m^{-3}d^{-1}$)	HRT (d)	SRT (d)	MLVSS (mgl^{-1})	Actual DO (mgl^{-1})	Actual pH	Actual temp (^oC)	Operating period (d)	NH_3 ($mgNl^{-1}$)	NO_2^- ($mgNl^{-1}$)	NO_3^- ($mgNl^{-1}$)	N loss (%)	NH_3 removal (%)	NH_3 removal load ($gN\ m^{-3}d^{-1}$)	Eff NO_2^- /NH_3 removed (%)
N	71.4±1.2	SBR	30	2.25	no sludge wasting	850	> 4	8.1±0.1	25±1	76	0.6±0.7 (1)	1.3±0.3 (2)	67.5±0.6 (95)	2	99	30	2
PNI	252.0±1.1	SBR	500	0.5	no sludge wasting	1225	< 1	7.9±0.2	36±1	70	28.9±3.6 (11)	184.7±4.6 (73)	19.1±2.8 (8)	8	89	400	83
PNII	949.9±1.8	CSTR	540	1.5 - 2.0		430	> 5	8.0±0.1	35±1	73	34.6±11.7 (4)	832.5±20.1 (88)	60.6±11.6 (6)	2	96	510	91

Note : Values in parenthesis are the percentage of the species to total nitrogen.

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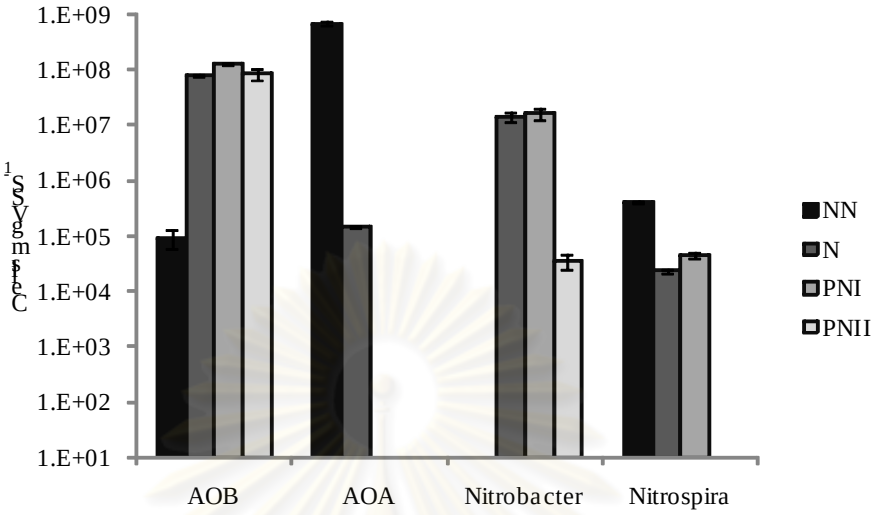

Fig. 5.1 Abundance of ammonia oxidizing bacteria (AOB), ammonia oxidizing archaea (AOA), *Nitrobacter* (NOB) and *Nitrospira* (NOB) in suspended inocula at the end of the preparation periods. Limit of detection (LOD) for the bacterial and archaeal *amoA* genes was 6.0×10^{1} and 6.9×10^{3} cells $mgVSS^{-1}$, respectively. The LOD for *Nitrobacter* and *Nitrospira* 16S rRNA genes was 1.7×10^{3} and 5.1×10^{3} cells $mgVSS^{-1}$, respectively.

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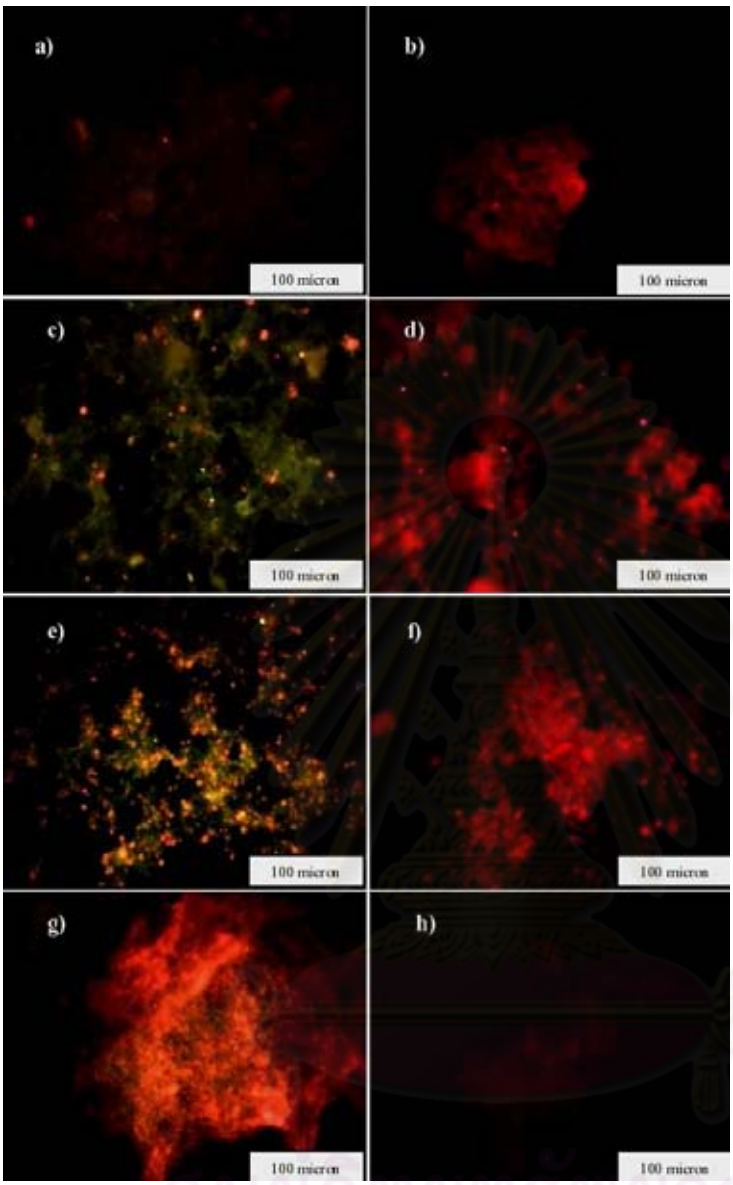

Fig. 5.2 FISH image showing nitrifying microorganisms in suspended inocula: NN sludge (a, b), N sludge (c, d), PNI sludge (e, f), and PNII sludge (g, h). Left-side (a, c, e, g) are suspended inocula, hybridized with NSO190 (beta-proteobacterial AOB; green) and EUB338 (all bacteria*;* red). Right-side is suspended inocula, hybridized with Ntspa662 (*Nitrospira*; red) (b) and NIT3 (*Nitrobacter*; red) (d, f, h). The target cells complimentary to both Cy3-labeled probe (red) and Alexa Flour 488-labeled probe (green), are shown in orange-yellow. Non bright green and red signals arenon-active target cells, autofluorescence of cell lysate, exopolymeric substance and/or and precipitated salts.

NN sludge was pre-defined as the sludge lack of nitrifying activity. Fig. 5.1 showed that NN sludge contained low numbers of AOB ($9.5x10^4 \pm 7.1x10^4$ cells mg VSS^{-1}) corresponding to the FISH image (Fig. 5.2a) in which no signal of active AOB was observed. There are two possible explanations for this. Firstly, very limited levels of NH_3 in the influent of the WWTP (less than 1 mg N l^{-1}) during the sampling period led to insufficient substrate available for AOB growth. These levels of NH_3 were much less than the typical level for low-strength municipal wastewater (around 20 mg N l^{-1}) (Henze et al., 2008). The reduction of NH_3 concentration in the influent of the WWTP may cause by the degradation of NH_3 during long transportation of wastewater from household to the WWTP or the dilution of wastewater with stormwater during wet season. Secondly, NN sludge was harvested from a contact stabilized activated sludge process which is developed mainly for the removal of BOD. This process may limit complete nitrification ability due to the short retention time maintained for the system (Metcalf and Eddy, 2003).

In contrast to AOB, AOA in NN sludge were found in high number ($7.1x10^8 \pm 1.3x10^8$ cells mg VSS^{-1}), 10^3 times higher than the AOB numbers. Several studies reported high abundance of AOA in full-scale WWTPs with low NH_3 levels (Limpiyakorn et al., 2011; Sonthiphand and Limpiyakorn, 2011; Park et al., 2006). Up to present, only two studies reported K_S for NH_3 of AOA; one is for the only available AOA isolate, *Candidatus Nitrosopumilus maritimus*, 1.86 µg N l^{-1} (Martens-Habbena et al., 2009) and the other for an enriched AOA culture, 8.54 µg N l^{-1} (Park et al., 2010). According to Limpiyakorn et al. (2011), AOB with high affinity to NH_3 like members of *Nitrosomonas oligotropha* cluster dominated in full-scale municipal WWTPs and the K_s values for this AOB cluster are between 0.42 and 1.4 mg N l^{-1}(Bollmann et al., 2001; Stech et al., 1995), which are much higher than those of AOA. As a result, under limiting NH_3 environments AOA can outcompete AOB for NH_3.

For NOB in NN sludge, only members of genus *Nitrospira* were found ($4.3x10^5 \pm 2.7x10^4$ cells mg VSS^{-1}), while no *Nitrobacter* was detected. From the FISH image, active *Nitrospira* was found a very limited level (Fig. 5.2b) while no signal of

active *Nitrobacter* was observed (data not shown). Previous research showed the predomination of *Nitrospira* over *Nitrobacter* in the environments low in NO_2^- levels (Kim and Kim, 2006; Schramm et al., 1999). This was a result of the higher NO_2^- affinity of the *Nitrospira* than that of the *Nitrobacter* (K_s for NO_2^- between 0.12 and 0.22 mg N l^{-1} for *Nitrospira*; Manser et al., 2005; Schramm et al., 1999, and between 0.3 and 1.9 mg N l^{-1} for *Nitrobacter*; Beccari et al., 1979; Alleman, 1984). Thus, in the WWTPs, where the level of NO_2^- was found minimally (< 0.1 mg N l^{-1}) *Nitrospira* outcompetes *Nitrobacter* (Wagner et al., 2002).

The N sludge was prepared by promoting complete nitrification in the NN sludge. This can be achieved by providing sufficient DO (> 4 mg O_2 l^{-1}) and sludge age (no sludge wasting) to support activity of both AOB and NOB (Tables 5.1 and 5.4). N sludge fully oxidized NH_3 to NO_3^- (99% NH_3 removal and effluent NO_2^-/NH_3 removed = 2%) (Table 5.4). The high nitrification efficiency led to the increase in numbers AOB in N sludge from the NN sludge (10^3 times higher, $8.2x10^7 \pm 3.4x10^6$ cells mg VSS^{-1} in N sludge and $9.5x10^4 \pm 7.1x10^4$ cells mg VSS^{-1} in NN sludge). The FISH image also showed that active AOB in N sludge was higher than that in the NN sludge (Fig. 5.2a and c). In contrast to AOB, the number of AOA in N sludge was 10^3 times lower than that of the NN sludge ($1.5x10^5 \pm 7.0x10^3$ cells mg VSS^{-1} in N sludge and $7.1x10^8 \pm 1.3x10^8$ cells mg VSS^{-1} in NN sludge). The washing out of AOA from the reactor caused by substrate inhibition likely occurred. Due to the variation in NH_3 levels in the reactor during a batch operating cycle (data not shown), the NH_3 peak after the filling period (71.4 mgN l^{-1}) could temporally inhibit the growth of AOA, subsequently the consortium of ammonia oxidizing microorganisms shifted from AOA-dominated NN sludge to AOB-dominated N sludge. A growth inhibition level for isolated AOA, *Candidatus Nitrosopumilus maritimus*, is at 28 mg N/l (Martens-Habbena et al., 2009). AOA likely played a main role in NH_3 oxidation in the N sludge.

For NOB abundance in N sludge, Fig. 5.1 shows that the number of *Nitrobacter* was 10^3 times higher than of the *Nitrospira* number ($1.5x10^7 \pm 5.0x10^6$ cells mg VSS^{-1} for *Nitrobacter* and $2.4x10^4 \pm 4.3x10^3$ cells mg VSS^{-1} for *Nitrospira*).

The results from FISH analysis were also in agreement. Active *Nitrobacter* was present (Fig. 5.2c), while no signal of active *Nitrospira* was observed (data not shown). Change in dominant NOB from *Nitrospira* in the NN sludge to *Nitrobacter* in the N sludge was likely to be caused by the NO_2^- concentration in the reactor (data not shown). The NO_2^- peaks which took place during the feeding time of batch operation along the study period (13 mg l^{-1}) could acclimate NOB which are tolerant to high NO_2^- levels. Consequently, lower NO_2^- affinity or r-strategist NOB, as *Nitrobacter*, instead of the K-strategist NOB (*Nitrospira*), became the dominant NOB in the N sludge (as discussed above). Although the numbers of AOB and NOB in the N sludge rose in the similar orders ($8.2x10^7 \pm 3.4x10^6$ cells mg VSS^{-1} for AOB and $1.5x10^7 \pm 5.0x10^6$ cells mg VSS^{-1} for *Nitrobactor*), FISH analysis revealed that active *Nitrobacter* in the N sludge showed weaker signal intensity than the active AOB (Fig. 5.2c and d). This was probably due to low sensitivity of FISH techniques to detect microorganisms in environmental samples. Daims and Wagner (2010) showed that the sensitivity of FISH is approximately 10^3 -10^4 active cells ml $sludge^{-1}$. Lower signal of active NOB as compared to AOB, even under a favorable growth condition for both microorganisms (full nitrification), is likely because of the lower maximum specific growth rate (μ_{MAX}) of NOB at temperature above 14^oC (Hunik et al., 1994).

PNI sludge was prepared by enriching the NN sludge under DO-limiting condition (< 1 mg O_2 l^{-1}) with sufficiently high NH_3 load to promote partial nitrification (activity of AOB over that of NOB) (Tables 5.1 and 5.4). In PNI reactor, a high amount of NH_3 was nitrified to NO_2^- (89% NH_3 removal and effluent NO_2^-/NH_3 removed = 83%) (Table 5.4). For PNI sludge, the oxidation of NH_3 to NO_2^- was governed by only the limited DO, not NH_3. This condition remained until NH_3 became exhausted. The Ks values for O_2 of AOB were found to be less than those of NOB (0.03-0.99 mg N l^{-1} for AOB and 0.41-5.3 mg N l^{-1} for NOB; Blackburne et al., 2008; Ciudad et al., 2005; Wiesmann, 1994; Laanbroek and Gerards, 1993). Due to the lower O_2 affinity of NOB, NOB is more sensitive with O_2 availability than AOB especially under an O_2-limiting condition. As a result, the activity of AOB was promoted, while at the same time NOB activity was suppressed. A high amount of NH_3 oxidized in the reactor led to the increase in AOB number in the PNI sludge to

10^4 times higher than in the NN sludge ($1.3x10^8 \pm 9.0x10^6$ cells mg VSS^{-1} in PNI sludge and $9.5x10^4 \pm 7.1x10^4$ cells mg VSS^{-1} in NN sludge). The FISH image showed higher signal of active AOB in the PNI sludge than in the NN sludge (Fig. 5.2a and e).

The abundance of AOA in the PNI sludge decreased from the NN sludge (Not detected for PNI sludge and $7.1x10^8 \pm 1.2x10^8$ cells mg VSS^{-1} in NN sludge). Complete washing out of AOA from the PNI sludge could be due to two possible reasons. One is substrate inhibition. The NH_3 peaks (252.0-28.9 mgN l^{-1}) appeared at the beginning of each cycle could repeatedly inhibited growth of AOA (as discussed above). The other possible reason is due to free nitrous acid (FNA) inhibition. Anthonisen et al. (1976) suggested that AOB are inhibited at the threshold FNA concentrations between 0.2 and 2.8 mg N l^{-1}, while the inhibition on NOB activity was found at the concentrations between 0.06 and 0.83 mg N l^{-1} (Anthonisen et al., 1976). However, much higher sensitivity of AOA to FNA was reported (complete inhibition of AOA, *Candidatus Nitrosopumilus maritimus* at FNA of 0.0026 mg N l^{-1}; Konneke et al., 2005). As shown in Table 5.4, an average NO_2^- concentration of 184.7 mg N l^{-1} in the reactor corresponded to 0.004 mg N l^{-1} of FNA. This level of FNA could strongly inhibit activity of AOA, but not AOB.

As a result of suppressing the growth of NOB during the preparation of PNI sludge, *Nitrobacter*, as the dominant NOB in the reactor, was 10 times lower that the number of AOB ($1.7x10^7 \pm 6.9x10^6$ cells mg VSS^{-1} for *Nitrobacter* and $1.3x10^8 \pm 9.0x10^6$ cells mg VSS^{-1} for AOB). This corresponds to the FISH results that active *Nitrobacter* was observed at lower signals than active AOB (Fig. 5.2e and f). Although a high level of partial nitrification (effluent NO_2^-/NH_3 removed = 83%) indicated nearly complete inhibition of nitratation, under long sludge age operation (no sludge wasting) as in this study NOB were not washed out from the reactor as confirmed by the existence of high numbers of *Nitrobacter* and *Nitrospira* in the PNI sludge ($1.7x10^7 \pm 6.9x10^6$ cells mg VSS^{-1} for *Nitrobacter* and $4.5x10^4 \pm 1.0x10^4$ cells mg VSS^{-1} for *Nitrospira*). In addition, the shift in the dominant NOB from *Nitrospira* found in the NN sludge to *Nitrobacter* in the PNI sludge agrees with the high accumulation of NO_2^- in the reactor. Correspondingly, active *Nitrobacter* was

observed (Fig. 5.2f) without a signal of active *Nitrospira* (data not shown) in FISH images. High NO_2^- environment in the reactor could acclimate and change the community of NOB .

PNII sludge was prepared in a reactor without sludge recycling. Hunik et al. (1994) suggested that at temperature above 14^oC, μ_{MAX} of AOB is higher than that of NOB. Moreover, the growth of AOB can largely be increased compared to NOB at higher temperature levels (but not above 40^oC) (Hunik et al., 1994). Thus, when maintaining high temperature, NOB can be washed out while AOB cells can be maintained if in the reactor without sludge recycling, dilution rate was controlled to be higher than the growth rate of NOB but lower than that the growth rate of AOB. From the practical point of view, a reactor with sludge age between 1 and 2.5 days can achieve high levels of partial nitrification when the temperature is above 30^oC (van Kempen et al., 2001). As a result of using low sludge age (1.5 - 2 days), PNII sludge nitrified NH_3 to NO_2^- at high level (96% NH_3 removal and effluent NO_2^-/NH_3 removed = 91%) (Table 5.4). This resulted in 10^3 times higher abundance of AOB in the PNII sludge than that in the NN sludge ($8.9x10^7 \pm 4.0x10^7$ cells mg VSS^{-1} in PNII sludge and $9.5x10^4 \pm 7.1x10^4$ cells mg VSS^{-1} in NN sludge). In the FISH images, active AOB in the PNII sludge was also higher than in the NN sludge (Fig. 5.2a and g). The number of AOA was not detected in the PNII sludge and while it was $7.1x10^8 \pm 1.2x10^8$ cells mg VSS^{-1} in the NN sludge. Similar to the PNI reactor, complete washing out of AOA from the PNII reactor is probably caused by continuously inhibiting AOA by high concentrations of NH_3 and FNA in the reactor (34.6 mg N l^{-1} of NH_3 and 0.015 mg N $l^{-1}$for FNA) (Table 5.4).

For the number of the dominant NOB in PNII sludge, *Nitrobacter* was 10^3 times lower than that of AOB ($8.9x10^7 \pm 4.0x10^7$ cells mg VSS^{-1} for AOB and $3.7x10^4 \pm 3.0x10^4$ cells mg VSS^{-1} for *Nitrobacter*). FISH results also agree with this. No active cell of either *Nitrobacter* or *Nitrospira* was observed while active AOB cells were found at high levels (Fig. 5.2g and h). Similar to the PNI sludge, *Nitrobacter*, not *Nitrospira* was the dominant NOB in the PNII sludge which corresponded to the high accumulated NO_2^- levels in the reactor ($3.7x10^4 \pm 3.0x10^4$

cells mg VSS^{-1} of *Nitrobacter* and not detected for *Nitrospira*). Although NOB (*Nitrobacter*) activity was completely inhibited, their cells were not washed out from the reactor. Comparing to NN sludge, the dominant NOB found in the PNII sludge was 10 times lower those in the NN sludge ($3.7x10^4 \pm 3.0x10^4$ cells mg VSS^{-1} of *Nitrobacter* in the PNII sludge and $4.3x10^5 \pm 2.7x10^4$ cells mg VSS^{-1} of *Nitrospira* in the NN sludge).

When comparing among different suspended inocula, sludge with higher NH_3 removal loads (PNII > PNI > N > NN sludge, 510, 200, 30, and 0 g N m^{-3} d^{-1}, respectively) contained more active AOB (PNII > PNI > N > NN sludge) as suggested by the using FISH results. However, qPCR results disagreed (PNI > N ≈ PNII > NN sludge, $1.6x10^{11} \pm 1.1x10^{10}$, $6.9x10^{10} \pm 7.1x10^9$, $3.8.0x10^{10} \pm 1.7x10^{10}$, and $2.5x10^8 \pm 1.9x10^8$ cells l^{-1}, respectively). It should be noted that qPCR included genes belonging to active and inactive, viable and nonviables cells in the quantification, while FISH detected only 16S rRNA within ribosomes of active cells. Although the analysis of AOB at species levels was not performed in this study, evidence from previous studies implied that *Nitrosomonas oligotropha* is likely to be the dominant AOB in NN, and N sludge which received low NH_3 loading rate (30 g N m^{-3} d^{-1}) and *Nitrosomonas europaea* may be the dominant AOB in PNI (NH_3 loading rate of 500 g N m^{-3} d^{-1}) and PNII sludge (NH_3 loading rate of 540 g N m^{-3} d^{-1}). Limpiyakorn et al. (2007) suggested that AOB with high affinity to NH_3 such as *Nitrosomonas oligotropha* comprised majority of AOB in nitrifying activated sludge acclimated with low NH_3 influent load (< 33 g N m^{-3} d^{-1}) while *Nitrosomonas europaea* cluster which has lower NH_3 affinity is the dominated AOB in sludge acclimated with high NH_3 loading rate (> 100 gN m^{-3} d^{-1}).

5.3.2 Abundance and spatial distribution of nitrifying microorganisms in entrapped inoculums

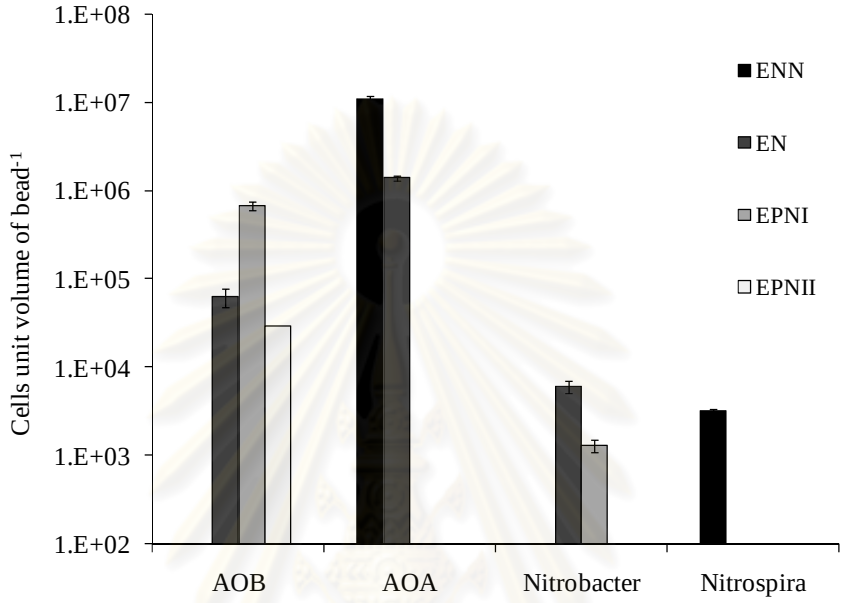

Fig. 5.3 Abundance of AOB, AOA, *Nitrobacter* (NOB) and *Nitrospira* (NOB) in entrapped inocula (before starting up nitritation reactors). The LOD for the AOB and AOA *amoA* genes were 1.45×10^2 and 1.01×10^2 cells/unit volume of bead, respectively. The LOD for *Nitrobacter* and *Nitrospira* 16S rRNA genes were 5.16×10^2 and 1.54×10^3 cells/unit volume of bead, respectively.

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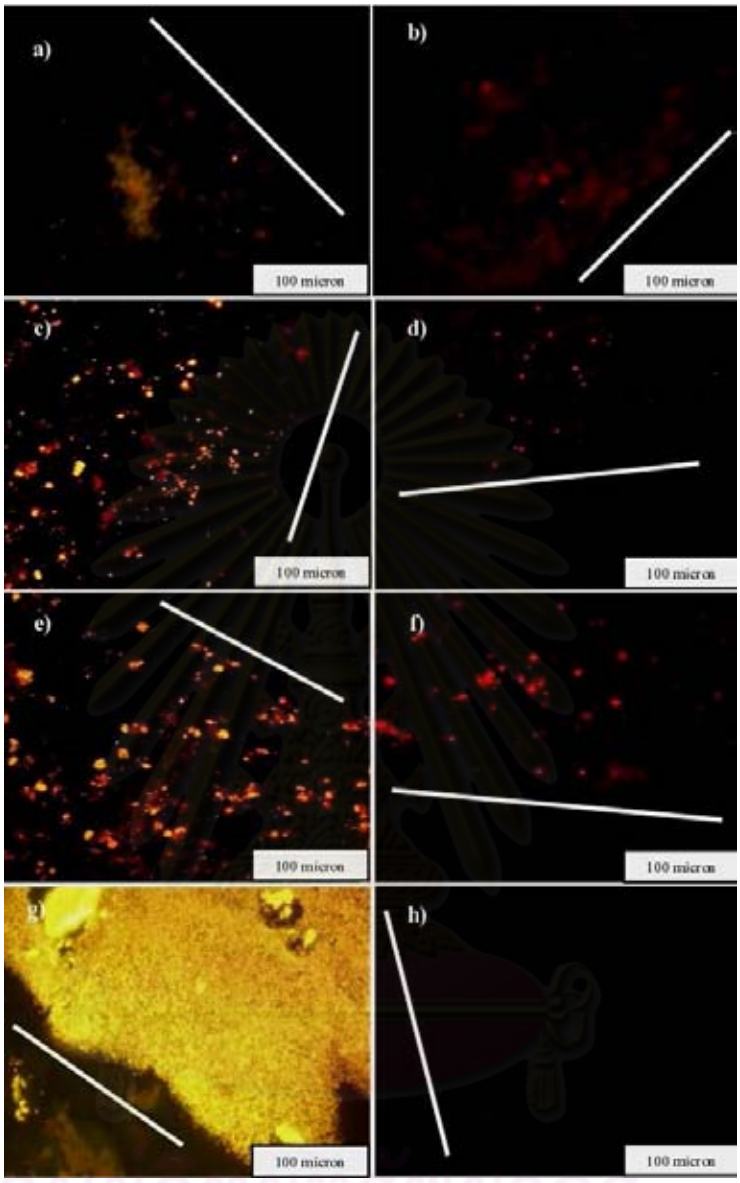

Fig. 5.4 FISH image showing nitrifying microorganisms in entrapped inocula: ENN entrapped cell (a, b), EN entrapped cell (c, d), EPNI entrapped cell (e, f), and EPNII entrapped cell (g, h). Left-side images are entrapped cells, hybridized with NSO190 (beta-proteobacterial AOB; green) and EUB338 (all bacteria*;* red). Right-side images are entrapped cells, hybridized with Ntspa662 (*Nitrospira*; red) (b) and hybridized with NIT3 (*Nitrobacter*; red) (d, f, h). The target cells complimentary to both Cy3-labeled probe (red) and Alexa Flour 488-labeled probe (green), are shown in orange-yellow. NOTE: Non bright green and red signals are non-active target cells, autofluorescence of cell lysate, exopolymeric substance or/and and precipitated salts. And white line indicates the edge of entrapped cells.

Fig. 5.1 and 5.3 showed that the proportions of nitrifying microorganisms in their consortium were similar between in the suspended and entrapped inoculums. For AOB, their abundance in suspended inoculums was $1.3x10^{8}\pm9.0x10^{6}$ in PNI sludge ≈ $8.9x10^{7}\pm4.0x10^{7}$ in PNII sludge ≈ $8.2x10^{7}\pm8.4x10^{6}$ in N sludge > $9.5x10^{4}\pm7.1x10^{4}$ cells mg VSS^{-1} in NN sludge which was similar to those found in entrapped inocula $6.8x10^{5}\pm1.5x10^{5}$ in EPNI > $2.9x10^{4}\pm4.7x10^{2}$ in EPNII ≈ $6.2x10^{4}\pm2.9x10^{4}$ in EN > LOD cells/unit volume of bead in ENN. For AOA, the abundance was $7.1x10^{8}\pm1.3x10^{8}$ in NN sludge > $1.5x10^{5}\pm7.0x10^{3}$ in N sludge > LOD cells $mgVSS^{-1}$ in PNII and PNI sludge and for entrapped inocula $1.1x10^{7}\pm2.1x10^{6}$ in ENN > $1.4x10^{6}\pm1.9x10^{5}$ for EN > LOD cells/unit volume of bead for EPNII and EPNI. For NOB, the numbers of *Nitrobacter* were $1.5x10^{7}\pm5.0x10^{6}$ in N sludge ≈ $1.7x10^{7}\pm6.9x10^{6}$ in PNI sludge > $3.7x10^{4}\pm2.0x10^{4}$ in PNII sludge > LOD cells $mgVSS^{-1}$ in NN sludge, which were in the same order as found for entrapped inoculums ($6.0x10^{3}\pm1.9x10^{3}$ in EN ≈ $1.3x10^{3}\pm4.1x10^{2}$ in EPNI > LOD cells/unit volume of bead in EPNII and ENN. Abundance of *Nitrospira* was $4.3x10^{5}\pm2.7x10^{4}$ in NN sludge > $4.5x10^{4}\pm1.0x10^{4}$ in PNI sludge ≈ $2.4x10^{4}\pm4.3x10^{3}$ in N sludge > LOD cells $mgVSS^{-1}$ in PNII sludge and $3.2x10^{3}\pm4.4x10^{2}$ in ENN > LOD cells/unit volume of bead in EPNI, EN and EPNII). This indicates that effect of gel entrapment on the consortium of nitrifying microorganisms was minimal. However, there was one exception for AOB in EPNI which was found at a high level ($6.8x10^{5}\pm1.5x10^{5}$ cells /unit volume of bead; Fig. 5.3). This was possibly due to non-homogeneity taken place during gel entrapment.

FISH results suggested that the numbers of active nitrifying microorganisms found in entrapped inoculums were similar to those in suspended inocula. For AOB, the abundance of AOB in entrapped inoculums was EPNII > EPNI > EN > ENN (Fig. 5.4) corresponding to those found in suspended inocula (PNII > PNI > N > NN sludge, Fig. 5.2) which agreed with the NH_3 removal load in the suspended inoculums reactors (see Subsection 5.3.1). The same results were found for NOB. The abundance of active dominant NOB in entrapped inocula (EPNI > EN > ENN > EPNII) was not different from those occurred in suspended inocula (PNI > N > NN > PNII sludge).

This confirms that the effect of gel entrapment on active nitrifying microorganism was negligible.

5.3.3 Effect of inoculums on accelerating partial nitrification using gel entrapment

Effect of inoculum on accelerating partial nitrification was examined during the start up period of entrapped cell nitritation reactors. For each reactor, the start-up period was divided into 2 phases: phase I with the DO concentration in bulk solution of 3 mg O_2 l^{-1} (18 – 27 days) and phase II with DO concentration in bulk solution of 2 mg O_2 l^{-1} (17 – 31 days). In phase II, O_2 concentration was reduced to see the effect of O_2 level in achieving partial nitrification. Overall, the results showed that NH_3 removal among the reactors at the end of phase I varied between 46 and 71%. NO_2^- accumulation among the reactors also differed especially for the EPNII reactor, which exhibited much higher nitrite accumulation than the others (14 – 28% for ENN, EN, EPNI, but 91% for EPNII). However, when DO concentration was reduced from 3 mg O_2 l^{-1} in phase I to 2 mg O_2 l^{-1} in phase II, NH_3 removal in all reactors improved and was in a similar range of NH_3 removal efficiencies (between 50 and 64%). NO_2^- accumulation was also higher at the end of phase II for all reactors excepting EPNII (65 – 66% in ENN, EN, EPNI but 87% in EPNII).

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Table 5.5 Operating conditions and steady-state performance of entrapped cell nitritation reactors (ENN, EN, EPNI, and EPNII reactors) during start up periods.

Reactor	Influent		Operating condition			Phase I (DO = 3 mgO_2 l^{-1})						
						Operating period (d)	Effluent			Removal efficiency		
	NH_3 (mgN l^{-1})	NH_3 load (gN $m^{-3}d^{-1}$)	HRT (d)	pH	Temp (^oC)		TAN (mgN l^{-1})	NO_2^- (mgN l^{-1})	NO_3^- (mgN l^{-1})	Total nitrogen loss (%)	NH_3 removal (%)	Eff NO_2^-/NH_3 removed (%)
ENN	625.7±3.2	520	1.2	8.1±0.1	25±1	23	234.1±11.2 (37)	59.0±4.3 (9)	267.8±16.0 (43)	11	63	15
EN						23	351.0±20.6 (56)	77.2±6.6 (12)	140.1±14.9 (22)	10	46	28
EPNI						27	182.8±8.4 (29)	61.7±6.3 (10)	345.8±29.9 (55)	6	71	14
EPNII						18	262.7±14.7 (42)	329.4±14.6 (53)	25.7±5.2 (4)	1	58	91
Reactor	Influent		Operating condition			Phase II (DO = 2 mgO_2 l^{-1})						
						Operation period (d)	Effluent			Removal efficiency		
	NH_3 (mgN l^{-1})	NH_3 load (gN $m^{-3}d^{-1}$)	HRT (d)	pH	Temp (^oC)		TAN (mgN l^{-1})	NO_2^- (mgN l^{-1})	NO_3^- (mgN l^{-1})	Total nitrogen loss (%)	NH_3 removal (%)	Eff NO_2^-/NH_3 removed (%)
ENN	625.7±3.2	520	1.2	8.1±0.1	25±1	23	225.5±16.7 (36)	260.0±18.4 (42)	82.9±13.8 (13)	9	64	65
EN						31	244.1±25.4 (39)	245.9±24.0 (39)	87.0±28.0 (14)	8	61	65
EPNI						31	311.3±8.7 (50)	207.6±17.2 (33)	39.5±15.9 (6)	11	50	66
EPNII						17	268.0±4.9 (43)	309.4±2.4 (49)	39.2±2.4 (6)	2	57	87

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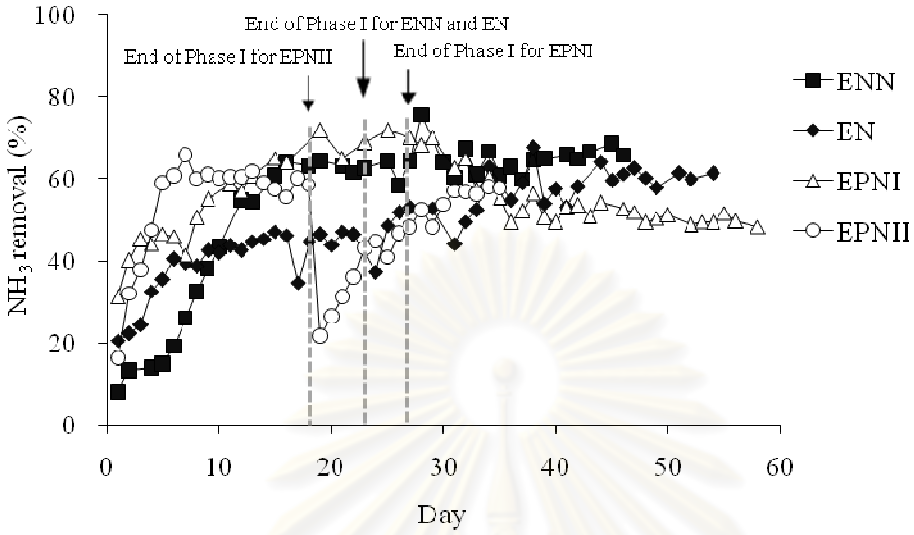

Fig. 5.5 Percent NH_3 removal during start up of entrapped cell nitritation reactors using ENN, EN, EPNI and EPNII entrapped inocula.

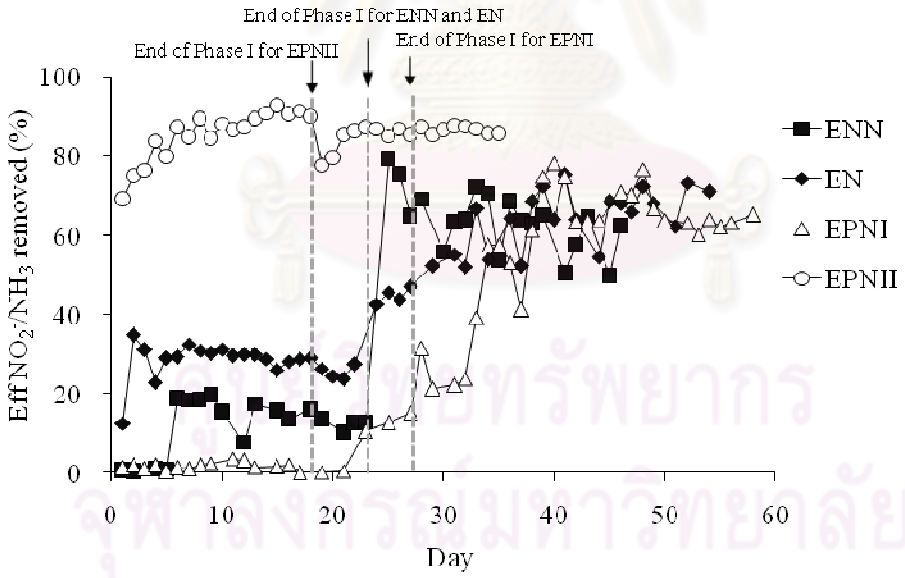

Fig. 5.6 Nitritation level (ratio of NO_2^- produced per NH_3 removed) during start up of entrapped cell nitritation reactors using ENN, EN, EPNI and EPNII entrapped inocula.

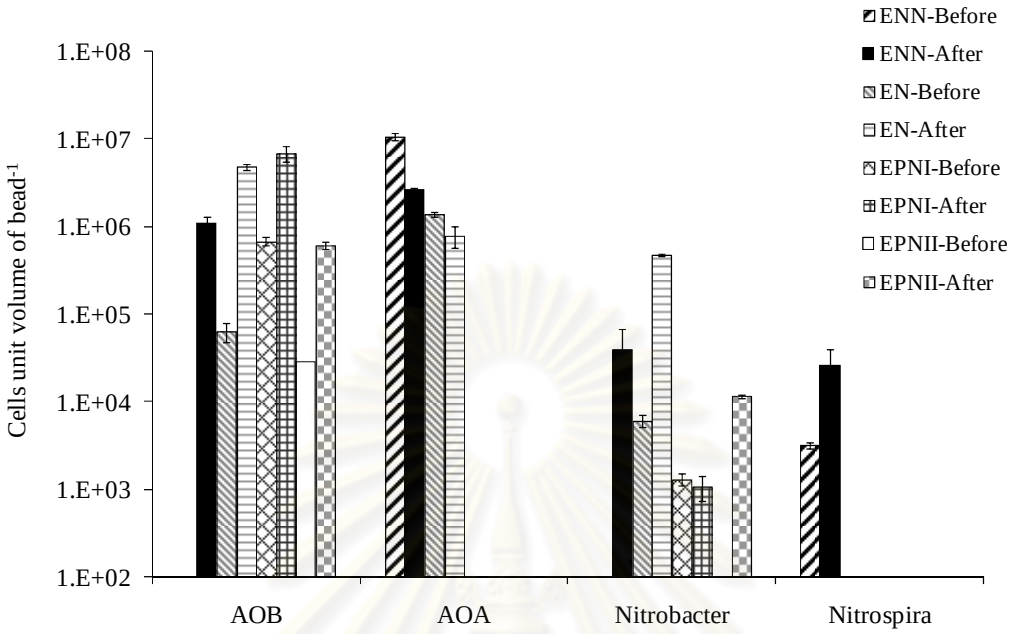

Fig. 5.7 Abundance of AOB, AOA, *Nitrobacter* (NOB) and *Nitrospira* (NOB) of entrapped cells before and after (at the end of phase II) start up of nitritation reactor. The LOD for the bacterial and archaeal *amoA* genes were 1.45×10^2 and 1.01×10^2 cells /unit volume of bead, respectively. The LOD for *Nitrobacter* and *Nitrospira* 16S rRNA genes were 5.16×10^2 and 1.54×10^3 cells/unit volume of bead, respectively.

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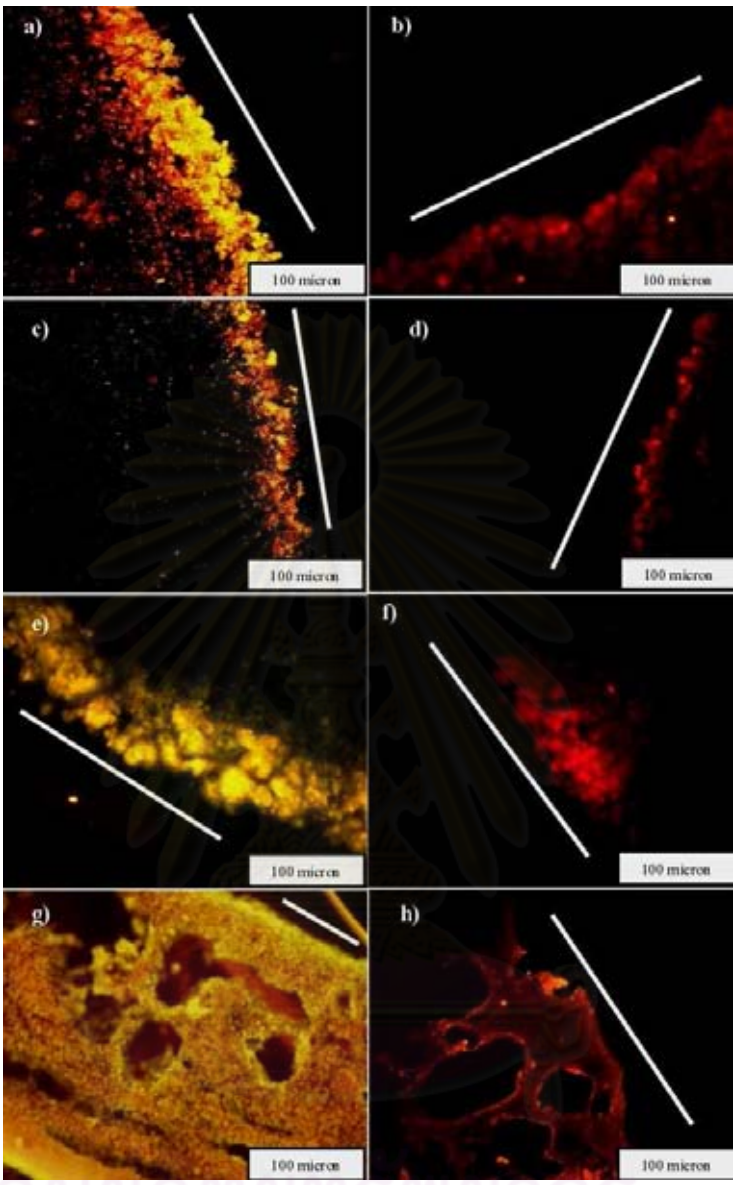

Fig. 5.8 FISH images showing nitrifying microorganisms in entrapped harvested after phase I: ENN entrapped cell (a, b), EN entrapped cell (c, d), EPNI entrapped cell (e, f), and EPNII entrapped cell (g, h). Left-side images are entrapped cells, hybridized with NSO190 (beta-proteobacterial AOB; green) and EUB338 (all bacteria*;* red), Right-side images (b, d, f, h) are entrapped cells, hybridized with NIT3 (*Nitrobacter*; red). The target cells complimentary to both Cy3-labeled probe (red) and Alexa Flour 488-labeled probe (green), are shown in orange-yellow. NOTE: Non bright green and red signals are non-active target cells, autofluorescence of cell lysate, exopolymeric substance or/and and precipitated salts. And white line indicates the edge of entrapped cells.

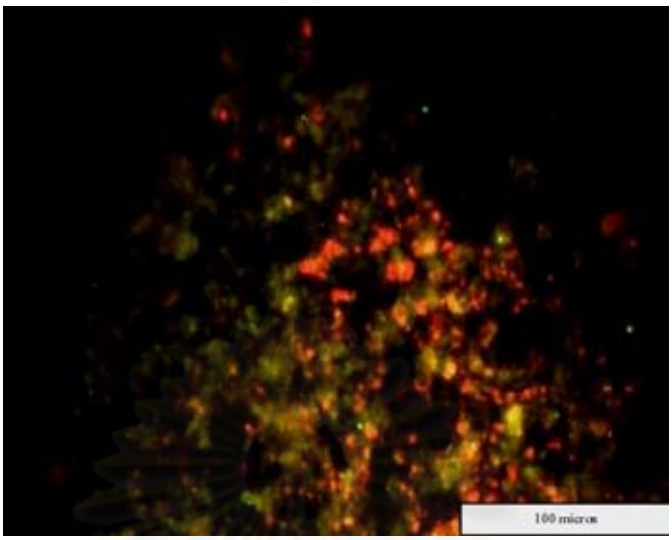

Fig. 5.9 FISH image showing nitrifying microorganisms in an inner part of ENN entrapped cells harvested after phase I. Entrapped cell was hybridized with NSO190 (beta-proteobacterial AOB; green) and EUB338 (all bacteria*;* red). The target cells complimentary to both Cy3-labeled probe (red) and Alexa Flour 488-labeled probe (green), are shown in orange-yellow. NOTE: Non bright green and red signals are non-active target cells, autofluorescence of cell lysate, exopolymeric substance or/and and precipitated salts.

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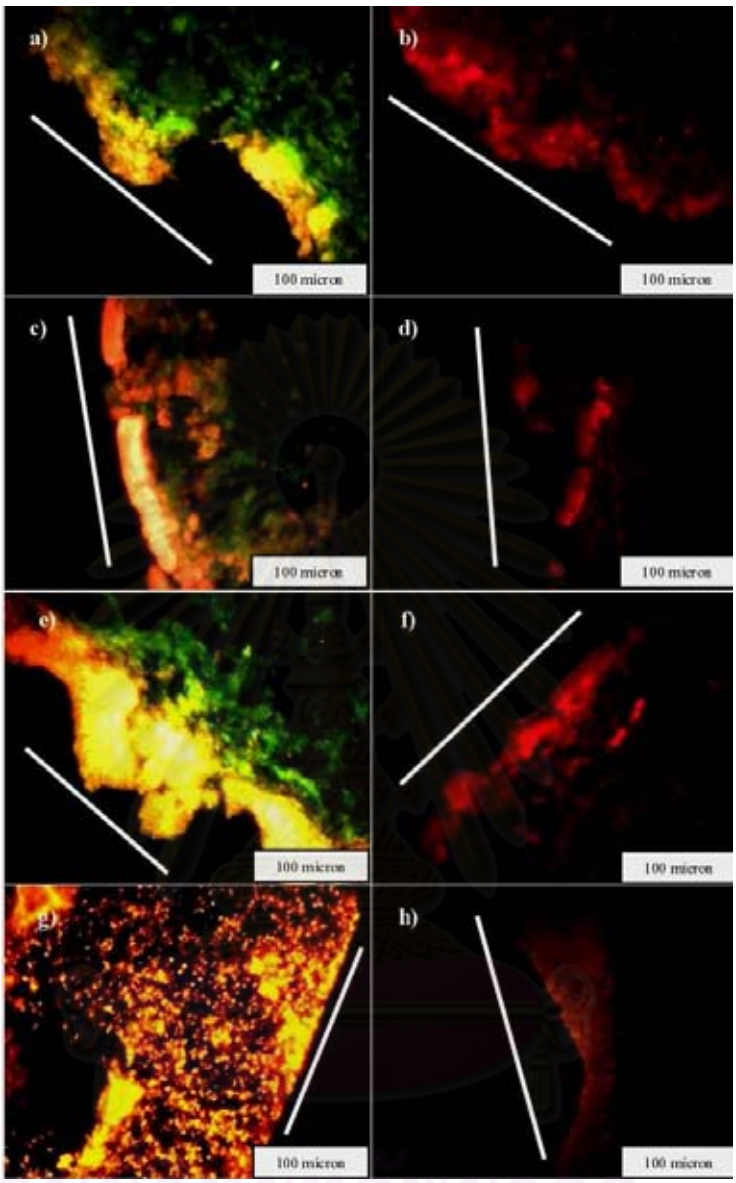

Fig. 5.10 FISH images showing nitrifying microorganisms in entrapped cells harvested after phase II: ENN entrapped cell (a, b), EN entrapped cell (c, d), EPNI entrapped cell (e, f), and EPNII entrapped cell (g, h). Left-side images are entrapped cells, hybridized with NSO190 (beta-proteobacterial AOB; green) and EUB338 (all bacteria*;* red), Right-side images (b, d, f, h) are entrapped cells, hybridized with NIT3 (*Nitrobacter*; red). The target cells complimentary to both Cy3-labeled probe (red) and Alexa Flour 488-labeled probe (green), are shown in orange-yellow. NOTE: Non bright green and red signals are non-active target cells, autofluorescence of cell lysate, exopolymeric substance or/and and precipitated salts. And white line indicates the edge of entrapped cells.

5.3.3.1 Ammonia removal

A. Phase I (DO = 3 mg O_2 l^{-1})

Fig. 5.4, 5.5 and 5.7 showed that during the initial period (1st week) of operation, the removal of NH_3 (26%, 39%, 42%, and 66% for ENN, EN, EPNI, and EPNII, respectively) depended on the abundance of active AOB cells as observed by FISH results rather than the number of AOB examined using qPCR in the gel beads (NH_3 removal: EPNII > EPNI > EN > ENN; abundance of active AOB: EPNII > EPNI > EN > ENN; numbers of AOB: $6.8x10^5 \pm 1.5x10^5$ [EPNI] > $2.9x10^4 \pm 4.7x10^2$ [EPNII] ≈ $6.2x10^4 \pm 2.9x10^4$ [EN] > LOD [ENN]) cells/unit volume of bead). The discrepancy found between both methods is because qPCR quantifies all genes belonging to both viable and nonviable cells, while FISH selects only 16S rRNA of active cells.

After the 1st week of operation toward the end of phase I (3rd – 4th week) (Table 5.5 and Fig. 5.5), the percentage of NH_3 removal of all reactors increased and ranged 46 to 71% (63%, 46%, 71%, and 58% for ENN, EN, EPNI, and EPNII, respectively). In this study, residual concentrations of NH_3 in bulk liquid of all reactors remained at high levels (> 150 mgN l^{-1}) throughout the operation period. Under this high NH_3 environment, O_2 can be limited in the gel beads due to much lower concentration of DO compared to NH_3 which resulted in probably shorter penetration depth of O_2 than NH_3 into the gel bead and consequently promotion of partial nitrification. The O_2 limiting condition in the gel beads is evidenced by FISH results at the beginning and the end of phase I (Fig. 5.4 and 5.8). At the beginning of phase I, active AOB clusters distributed throughout the entire beads which resulted from thoroughly mixing between PVA and suspended inoculum during the cell entrapment (Fig. 5.4). In contrast, at the end of phase I, active AOB clusters appeared only around the peripheral layers of the gel beads where sufficient O_2 was available (Fig. 5.8). This indicates that AOB relocated from the O_2-limiting zones in the inner parts of the gel beads to the O_2 available

zones at the outer parts of the beads. The relocation of AOB in the gel beads could occur two ways. First, AOB in the O_2-limiting zones died off due to the lack of an electron acceptor; in contrast AOB in the O_2 available zones become flourishing leading to the change in AOB density along the depth of the gel beads. Second, AOB may move themselves from the O_2-limiting zones to the O_2 available zones in the gel beads. Some previous studies (Vlaeminck et al., 2007 and Philips et al., 2002) found that nitrifying microorganisms can move themselves towards more favorable zones where the O_2 level reactivates their activity (Vlaeminck et al., 2007; Philips et al., 2002). The relocation of AOB in the gel beads occurred in all types of the gel beads except for EPNII.

For EPNII, no difference in spatial distribution of AOB in the gel beads between at the beginning and end of phase I was observed (Fig. 5.4g and 5.8g). At the end of phase I, active AOB clusters distributed throughout the whole bead as found at the beginning of phase I. This suggests that O_2 may not be limited in the EPNII beads. It should be noted that the PNII sludge was cultured in a reactor with extremely low SRT (1.5 - 2 days) leading to the formation of looser aggregates due to the lack of exopolymeric substance produced. This character could be carried over to EPNII. Fig. 5.4g, 5.8g, and 5.10g show a lack of autofluorescence indicating that exopolymeric substance and cell lysate did not occur in the gel beads of EPNII.

With an exception of EPNII, when comparing among the EN, ENN, and EPNI reactors, the NH_3 removal efficiency at the end of phase I was found to depend on the abundance of active AOB in the spherical parts of the gel beads as observed by FISH (NH_3 removal: 71%[EPNI] > 63%[ENN] > 46%[EN], Table 5.5 and Fig. 5.5 ; abundance of active AOB: EPNI > ENN > EN, Fig. 5.8a, c, and e). At the end of phase I, EPNI was found to have higher abundance of active AOB in the peripheral part of the gel beads. This may be caused by the fact that the microorganisms in EPNI originated from the PNI suspended inoculum which was incubated under partial nitrification condition

for a certain period of time. Therefore, they required less time to accelerate their activity during phase I.

For ENN, it is surprising to have high NH_3 removal efficiency in this reactor where non-nitrifying inoculum was incorporated. However, although small numbers of AOB were observed at the beginning of phase I (Fig. 5.4a), high density of active AOB appeared in the peripheral parts of the gel beads at the end of phase I. The interaction between heterotrophs and AOB in the ENN beads during phase I operation, may lead to the proliferation of active AOB cells at the peripheral parts of the gel beads at the end of phase I. ENN beads originated from NN sludge taken directly from the municipal WWTP where several carbonaceous organics were present. Although an organic compound was not provided in the entrapped cell nitritration reactor, heterotrophs can still proliferate in the gel beads by utilizing organic-containing byproducts from the exogenous substrate utilization and the endogenous decay of their cells and nitrifying microorganisms. Thus, under the O_2 limiting condition in the gel beads, heterotrophs which have higher affinity to O_2 than nitrifying microorganisms, can outcompete the nitrifying microorganisms for O_2 and subsequently, enforced the nitrifying microorganisms to move toward the outer zones of the gel beads. This explanation was supported by FISH images (Fig. 5.8a and 5.9). At the end of phase I, high abundance of active AOB in the outer part of ENN beads was clearly observed (Fig. 5.8a), while no active AOB cells were found in the inner parts of the gel beads, in spite heterotrophs were found in this area (Fig. 5.9).

For EN, at the end of phase I, NH_3 removal was found to be the lowest in this reactor comparing to others (Table 5.5 and Fig. 5.5). The low abundance of active AOB cells was found in the peripheral parts of the gel beads at the end of phase I (Fig. 5.8c). The reason for the low abundance of active AOB cells is unclear. However, it should be noted that the N suspended inoculum was incubated with lower ammonium influent (71.4 ± 1.2 mgN l^{-1}) resulting in low ammonium level in the reactor (0.6 ± 0.7 mgN l^{-1}) (Table 5.4).

Unlike other prepared inocula, AOB with high affinity to NH_3 (for example *Nitrosomonas oligotropha*) were expected to dominate in this inoculum. However, during the start up of entrapped nitritation reactor, high influent NH_3 loading rate (520 gN m^{-3} d^{-1}) was provided leading to high NH_3 levels in the reactor (351.0±20.6 mgN l^{-1})(Table 5.5). Under this condition, the growth of AOB with high affinity to NH_3 can be deteriorated leading to the shift of the dominant AOB species from AOB with high affinity to NH_3 to AOB with low affinity to NH_3. During the transition, both types of AOB competed with one another for substrate (NH_3 and O_2) and space leading to the lower NH_3 removal in this reactor comparing to others (Table 5.5 and Fig. 5.5).

B. Phase II (DO = 2 mgO_2 l^{-1})

At the end of phase II, NH_3 removal for all reactors was 50 to 64% (64%, 61%, 50%, and 57% for ENN, EN, EPNI, and EPNII, respectively). Similar to phase I, NH_3 removal efficiency were expected to be controlled by the availability of O_2 in the gel beads around AOB aggregates. It is impossible to relate NH_3 removal efficiency to the number of active AOB in each type of gel beads based on FISH images (Fig. 5.10) because the difference in signal of active cells in each image was unclear. However, the number of AOB estimated by qPCR technique, showed similar numbers of AOB in all entrapped cells at the end of phase II ($4.8x10^6$±$7.2x10^5$[EN] ≈ $1.1x10^6$±$4.4x10^5$[ENN] ≈ $6.8x10^6$±$2.7x10^6$[EPNI] ≈ $6.1x10^5$±$1.1x10^5$[EPNI] cells/unit volume of bead Fig. 5.7). After operating all reactors under identical conditions for a long period of time, AOB community structure in all reactors was expected to be the same. This was driven by similar O_2 availability in the gel beads around AOB aggregates in all reactors; consequently, NH_3 removal efficiencies were not different.

Comparing the NH_3 removal by each reactor between at the end of phase I and II (Table 5.5) suggested no difference for the ENN and EPNII reactors (from 63% to 64% for ENN and from 58% to 57% for EPNII).

However for the EN reactor, NH_3 removal efficiency increased at the end of phase II compared to at the end of phase I (from 46% to 61%). This is in contrast to the EPNI reactor, where NH_3 removal decreased at the end of phase II (from 71% to 50%). For reactors ENN and EPNII, no difference in NH_3 removal between both phases implied that although DO level was reduced in phase II (2 mg O_2 l^{-1}), this level of O_2 was still sufficient to serve AOB to grow with the maximum NH_3 utilization rate. FISH results (Fig. 5.8[a, g] and Fig. 5.10[a, g]) also showed no difference in spatial distribution of active AOB cells in both types of the gel beads between at the end of phases I and II.

For the EN reactor, NH_3 removal efficiency increased in phase II compared to phase I. During phase I, the lower NH_3 removal efficiency is expected to be due to unstable AOB community in the gel beads. During the transition period in phase I, AOB with low affinity to NH_3 competed with AOB with high affinity to NH_3 (originated during preparation of the suspended inoculum) for substrate (NH_3 and O_2). However, after long operation toward the end of phase II, the AOB community structure became stable leading to higher NH_3 removal at the end of phase II compared to at the end of phase I.

For EPNI, the DO level maintained in phase II might not sufficient to serve AOB to grow under a maximum NH_3 utilization rate resulting in decreasing NH_3 removal at the end of phase II. Higher abundance of AOB in the EPNI entrapped inoculum required more DO than the other entrapped inocula (Fig. 5.7). Obviously, much higher density of active AOB layers in the EPNI beads at the end of phase II than other entrapped cells was observed in FISH images (Fig. 5.10a, c, e and g).

Only in the N and NN suspended inocula, AOA were found and continued to appear in the EN and ENN entrapped inocula toward the end of phase II operation. However, their numbers decreased 10 times during the start up period (Fig. 5.7) ($1.4x10^6 \pm 1.9x10^5$ to $7.8x10^5 \pm 4.2x10^5$ cells/unit volume of bead for EN and from $1.1x10^7 \pm 2.1x10^6$ to $2.6x10^6 \pm 2.4x10^5$ cells/unit volume

of bead for ENN). High NH_3 levels in the reactors during the start up period could inhibit AOA growth as described earlier.

5.3.3.2 Nitrite accumulation

A. Phase I (DO = 3 mgO_2 l^{-1})

Table 5.5 and Fig. 5.6 show that at the DO concentration of 3 mg O_2 l^{-1}, very high NO_2^- accumulation took place only in the EPNII reactor (91%), while in the other reactors only 14-28% of NH_3 removed was oxidized to NO_2^- (15, 28, and 14% for ENN, EN, and EPNI, respectively). For EPNII reactor, the high NO_2^- accumulation might be because the inoculum of this reactor was grown by wasting NOB cells for a certain period of time resulting in very low abundance of active NOB as indicated by FISH (Fig. 5.4h and 5.8h). This led to no active NOB (*Nitrobacter*) in EPNII at the end of phase I. For ENN, EN, and EPNI reactors, the accumulation of NO_2^- can be controlled by inhibiting NOB activity with some environmental conditions; however, in this study only the O_2 availability and FA level are the determinative factors to promote partial nitrification.

The FA levels accumulated in ENN, EN and EPNI reactors (12.5 – 24.2 mg N l^{-1}) were found to be around the upper range reported to inhibit the activity of NOB (0.05 to 20 mg N l^{-1}, Vadivelu et al., 2007; Abeling and Seyfried, 1992; Turk and Mavinic, 1989; Alleman and Irvine, 1980; Verstraete et al., 1977; Anthonisen et al. 1976; Prakasam and Loehr, 1972; Murray and Watson, 1965). Although effective levels of FA were maintained, NO_2^- accumulation did not occur fully in the reactors. This indicates that using this range of FA levels alone was not enough to promote partial nitrification. It must be noted that one advantage of using entrapped cell system is that the gel matrix can help protecting the cells from toxic substances. In this case, the gel matrix can reduce the levels of FA that came into contact with the cells. However, in the reactor with higher FA level (EN reactor), slightly higher

NO_2^- accumulation was found (NO_2^- accumulation of 28%, 15%, and 14% and FA levels of 24.2, 16.1, and 12.5 mg N l^{-1} for EN, ENN, and EPNI, respectively).

O_2 availability in the gel beads was governed by the penetration of O_2 from bulk solution into the gel matrix. This suggestion was supported by FISH results (Fig. 5.4 and 5.8). Active NOB in ENN (*Nitrospira*, Fig. 5.4b), EN (*Nitrobacter*, Fig. 5.4d), and EPNI (*Nitrobacter*, Fig. 5.4f) entrapped inocula distributed throughout the entire bead; however, at the end of phase I active NOB in ENN, EN, and EPNI (*Nitrobacter*, Fig. 5.8b, d and f) were observed only within the peripheral of the gel beads. Similar to AOB, NOB relocated themselves from the O_2-limiting zones in the inner part of the gel beads to the O_2 available zones at the outer part of the beads. Comparing to AOB (Fig. 5.8a – f), NOB aggregates were found underneath AOB aggregates in smaller and looser forms. This suggests that O_2 was limited in the gel beads and NOB were outcompeted by AOB for O_2 and space resulting in losing partially the nitrite-oxidizing activity.

In addition, at the end of phase I, r-strategist and high NO_2^- tolerable NOB, *Nitrobacter*, took over the majority of NOB from the k-strategist and low NO_2^- tolerable, *Nitrospira*. Therefore, *Nitrobacter* became the main player for NO_2^- oxidation in all reactors (high NO_2^- environments, see discussion in Subsection 5.3.1). Nonetheless, NO_2^- accumulation at the end of phase I was not dependent on the abundance of active NOB cells in the gel beads at the beginning of phase I as observed by FISH (NO_2^- accumulation: 28% [EN] > 15% [ENN] ≈ 14% [EPNI]; abundance of active NOB: EPNI > EN > ENN; see Table 5.5, Fig. 5.4b, d and f). Therefore, it can be concluded that under the DO concentration of 3 mg O_2 l^{-1}, partial nitrification was promoted mainly by O_2-limiting conditions in the gel beads, while the FA level is slightly involved. In addition, EPNII showed the effect of inoculum type on accelerating partial nitrification.

B. Phase II (DO = 2 mg O_2 l^{-1})

When the DO concentration decreased to 2 mg O_2 l^{-1}, the accumulation of NO_2^- improved in all reactors (Table 5.5 and Fig. 5.6). Very high NO_2^- accumulation was still observed in EPNII reactor (87%), while for ENN, EN, and EPNI, NO_2^- accumulation was 65%, 65%, and 66% which increased 15%, 28%, and 14% compared to at the end of phase I. The reason for very high NO_2^- accumulation in EPNII is because the entrapped cells were enriched under partial nitrification leading to the absence of active NOB in the entrapped cells as exhibited by FISH results (Fig. 5.10h). For ENN, EN, and EPNI, the results indicated that the reduction of O_2 in the gel beads improved partial nitrification. FISH analysis (Fig. 5.10) showed the supportive results. Similar to at the end of phase I, active NOB in ENN, EN, and EPNI (Fig. 5.10b, d and f) were observed only within the peripheral layer of the gel beads at the end of phase II. The FA level accumulated in ENN, EN and EPNI reactors (15.5 – 21.4 mg N l^{-1}) fell within the upper range found to inhibit NOB activity (0.05 to 20 mg N l^{-1}). Smaller and looser forms of NOB cluster were obviously found at below the AOB cell layer (Fig. 5.10a - f). This implied that NOB were outcompeted for O_2 and space by AOB under O_2-limited in the gel beads and resulting in losing partially the nitrite-oxidizing activity. Among the reactors, the degree of NO_2^- accumulation is not related to the levels of FA in the reactors (FA: 21.4[EPNI] > 16.8[EN] ≈ 15.5[ENN] mg N l^{-1}). This may be because the gel matrix reduced the levels of FA in bulk liquid that came into contact with the cells and/or NOB adapted themselves to tolerate to the FA level (during 6 - 8 weeks of operation).

Similar to phase I, the dominant NOB in ENN, EN, and EPNI at the end of phase II was *Nitrobacter* cluster (Fig. 5.10b, d and f), while no *Nitrospira* was observed. *Nitrobacter* rather than *Nitrospira* was the dominant active NOB and played a main role in NO_2^- oxidation in the reactors.

5.3.3.3 Comparison among the entrapped inoculums

When considering the duration required to achieve stable partial nitrification (Table 5.5 and Fig. 5.6), the order for NO_2^- accumulation efficiency is EPNII < ENN < EN = EPNI (15 days for NO_2^- accumulation at 91% by EPNII, 30 days for NO_2^- accumulation at 64% by ENN, 40 days for NO_2^- accumulation at 66% by EPNI and 42 days for NO_2^- accumulation at 65% by EN). It was found that type of inoculum accelerated partial nitrification. EPNII entrapped inoculum, as the positive control inoculum, is readily for partial nitrification as it contained high numbers of AOB and small numbers of NOB (Fig. 5.3). For other entrapped cells where NOB existed in high numbers (ENN, EN, and ENPI), no different result was observed, however, partial nitrification could be achieved within comparable period (between 30 and 42 days). For entrapped cells containing high NOB numbers (ENN, EN, and ENPI), the level of O_2 strongly influence their performance, while FA levels in bulk liquid were not important. Results showed that, cell entrapment can be an effective way to accelerate partial nitrification for high NH_3 wastewater even under ambient temperature and typical bulk DO operating condition (2 mg N l^{-1} DO).

5.3.3.4 Application of gel entrapment for partial nitrification

Because of sufficiently high loading rates of NH_3 used in the experiments, the average molar ratio between NO_2^- and NH_3 in the effluent from all reactors was close to 1.0 (average values of 1.1 for ENN, 1.0 for EN, 0.7 for EPNI, and 1.1 for EPNII reactors). The effluent of the partial nitrification reactors is proper for further treatment by the Anammox process (a molar NH_3 to NO_2^- ratio of 1:1.32 according to the stoichiometry, equation 15 in Chapter 2). However, van Kempen et al. (2001) recommended the ratio close to 1:1 in order to prevent NO_2^- inhibition on the activity of Anammox.

5.3.4 Long-term operation of entrapped cell nitritation reactors under various bulk DO and FA concentrations

5.3.4.1 Ammonia removal

Tables 5.6-5.8 show operating conditions and performance of entrapped cell nitritation reactors and the percentage of NH_3 removal during steady-state conditions of various concentrations of bulk DO and pH is summarized in Figure 5.11.

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Table 5.6 Summary of operating conditions and performance of ENN entrapped cell nitritation reactor (see monitoring results in Fig. A-4)

Operating condition				Effluent					Removal efficiency			
Phase	Day	DO (mg l^{-1})	pH	TAN (mgN l^{-1})	NO_2^- (mgN l^{-1})	NO_3^- (mgN l^{-1})	FA (mgN l^{-1})	FNA (mgN l^{-1})	Total nitrogen (%)	NH_3 removal (%)	Eff NO_2^-/NH_3 removed (%)	DO/FA (%)
1/8.5	1-11	1.0	8.5	10.1±5.0 (2)	532.6±8.7 (85)	37.2±13.4 (6)	1.6	0.004	93	98	86	0.6
1/7.8	12-21		7.8	66.0±8.9 (11)	360.1±19.2 (58)	165.6±19.4 (26)	2.4	0.013	95	89	64	0.4
1/7.2	22-32		7.2	94.4±1.1 (15)	225.1±13.4 (36)	277.9±26.3 (44)	0.9	0.032	95	85	42	1.1
1/6.5	33-50		6.5	267.0±15.0 (43)	15.3±10.9 (2)	305.4±19.0 (49)	0.5	0.011	94	57	4	2.0
2/6.5	51-65	2.0	6.5	32.3±19.6 (5)	82.6±22.2 (13)	471.1±23.6 (75)	0.1	0.059	93	95	14	33.4
2/7.2	66-81		7.2	18.1±8.1 (3)	83.1±9.5 (13)	500.4±15.7 (80)	0.2	0.012	96	97	14	12.0
2/7.8	82-95		7.8	7.6±1.7 (1)	86.1±3.4 (14)	494.7±33.4 (79)	0.3	0.003	94	99	14	7.4
2/8.5	96-118		8.5	7.8±2.6 (1)	42.9±6.0 (7)	535.1±24.2 (86)	1.2	0.0003	94	99	7	1.6
0.5/8.5	119-139	0.5	8.5	262.8±23.3 (42)	251.0±20.6 (40)	101.2±30.4 (16)	41.2	0.002	98	58	69	0.01
0.5/7.8	140-159		7.8	258.8±22.0 (41)	316.5±14.5 (51)	30.9±12.1 (5)	9.3	0.011	97	59	87	0.1
0.5/7.2	160-175		7.2	284.3±11.7 (45)	275.6±15.3 (44)	42.2±11.0 (7)	2.6	0.039	96	55	81	0.2
0.5/6.5	176-190		6.5	507.6±10.1 (81)	68.7±16.3 (11)	19.8±12.6 (3)	0.9	0.049	95	19	58	0.5

Note : Values in parenthesis are the percentage of the species to total nitrogen.

Table 5.7 Summary of operating conditions and performance of EPNI entrapped cell nitritation reactor (see monitoring results in Fig. A-5)

Operating condition				Effluent					Removal efficiency			
Phase	Day	DO (mg l^{-1})	pH	TAN (mgN l^{-1})	NO_2^- (mgN l^{-1})	NO_3^- (mgN l^{-1})	FA (mgN l^{-1})	FNA (mgN l^{-1})	Total nitrogen (%)	NH_3 removal (%)	Eff NO_2^-/NH_3 removed (%)	DO/FA (%)
1/8.5	1-18	1.0	8.5	25.0±1.6 (4)	520.5±17.7 (83)	38.3±4.4 (6)	3.9	0.004	93	96	87	0.3
1/7.8	19-33		7.8	32.1±6.3 (5)	363.1±19.3 (58)	180.3±18.9 (29)	1.1	0.013	92	95	61	0.9
1/7.2	34-49		7.2	95.9±10.9 (15)	204.3±7.6 (33)	296.0±25.5 (47)	0.9	0.029	95	85	39	1.1
1/6.5	50-63		6.5	351.8±19.2 (56)	55.1±13.9 (9)	163.5±5.4 (26)	0.7	0.039	91	44	20	1.5
2/6.5	64-81	2.0	6.5	336.9±26.4 (54)	18.6±7.4 (3)	240.5±40.2 (38)	0.6	0.013	95	46	7	3.2
2/7.2	82-107		7.2	18.8±4.1 (3)	28.0±9.9 (4)	567.4±12.7 (91)	0.2	0.004	98	97	5	11.5
2/7.8	108-127		7.8	20.3±5.1 (3)	23.3±7.2 (4)	571.4±12.6 (91)	0.7	0.001	98	97	4	2.8
2/8.5	128-143		8.5	7.1±1.5 (1)	21.3±4.0 (3)	570.4±12.4 (91)	1.1	0.0002	95	99	3	1.8
0.5/8.5	144-158	0.5	8.5	248.2±7.8 (40)	268.8±5.5 (43)	87.9±21.1 (14)	38.9	0.002	97	60	71	0.01
0.5/7.8	159-177		7.8	455.4±7.8 (73)	128.4±18.2 (21)	27.7±2.1 (4)	16.3	0.005	98	27	75	0.03
0.5/7.2	178-193		7.2	560.2±5.0 (90)	37.8±6.3 (6)	4.9±0.7 (1)	5.2	0.005	97	10	58	0.1
0.5/6.5	194-210		6.5	577.8±7.7 (92)	20.8±4.4 (3)	4.6±1.1 (1)	1.1	0.015	96	8	44	0.5

Note : Values in parenthesis are the percentage of the species to total nitrogen.

Table 5.8 Summary of operating conditions and performance of EPNII entrapped cell nitritation reactor (see monitoring results in Fig. A-6)

Operating condition				Effluent					Removal efficiency			
Phase	Day	DO (mg l^{-1})	pH	TAN (mgN l^{-1})	NO_2^- (mgN l^{-1})	NO_3^- (mgN l^{-1})	FA (mgN l^{-1})	FNA (mgN l^{-1})	Total nitrogen (%)	NH_3 removal (%)	Eff NO_2^-/NH_3 removed (%)	DO/FA (%)
1/8.5	1-14	1.0	8.5	20.9±3.0 (3)	20.9±4.8 (3)	569.8±3.4 (91)	3.3	0.0001	97	97	4	0.3
1/7.8	15-33		7.8	15.6±3.2 (2)	4.6±3.3 (1)	599.1±8.7 (96)	0.6	0.0002	99	98	1	1.8
1/7.2	34-47		7.2	19.3±5.9 (3)	0.9±0.3 (0)	5887±11.5 (94)	0.2	0.0001	97	97	0	5.6
1/6.5	48-61		6.5	602.3 ±8.0 (96)	0.3±0.2 (0)	15.8±5.9 (3)	1.1	0.0002	99	4	2	0.9
2/6.5	62-74	2.0	6.5	109.5±6.2 (18)	0.3±0.2 (0)	489.2±14.6 (78)	0.2	0.0002	96	83	0	9.8
2/7.2	75-83		7.2	11.8±0.9 (2)	0.5±0.1 (0)	602.0±7.8 (96)	0.1	0.0001	98	98	0	18.4
2/7.8	84-92		7.8	4.4±0.2 (1)	0.3±0.1 (0)	603.8±9.3 (97)	0.2	0.00001	98	99	0	12.6
2/8.5	93-101		8.5	1.8±0.1 (0)	0.1±0.0 (0)	621.6±2.0 (99)	0.3	0.000001	99	100	0	7.1
0.5/8.5	102-115	0.5	8.5	252.1±2.7 (40)	201.2 ±6.3 (32)	142.7±3.6 (23)	39.5	0.001	95	60	54	0.01
0.5/7.8	116-128		7.8	445.9±5.3 (71)	96.6±3.3 (15)	67.3±0.5 (11)	15.9	0.003	97	29	54	0.03
0.5/7.2	129-139		7.2	560.3±1.3 (90)	31.1±0.7 (5)	24.0±3.7 (4)	5.2	0.004	99	11	48	0.1
0.5/6.5	140-150		6.5	587.4±3.3 (94)	13.9±0.5 (2)	8.2±1.0 (1)	1.1	0.010	97	6	36	0.5

Note : Values in parenthesis are the percentage of the species to total nitrogen.

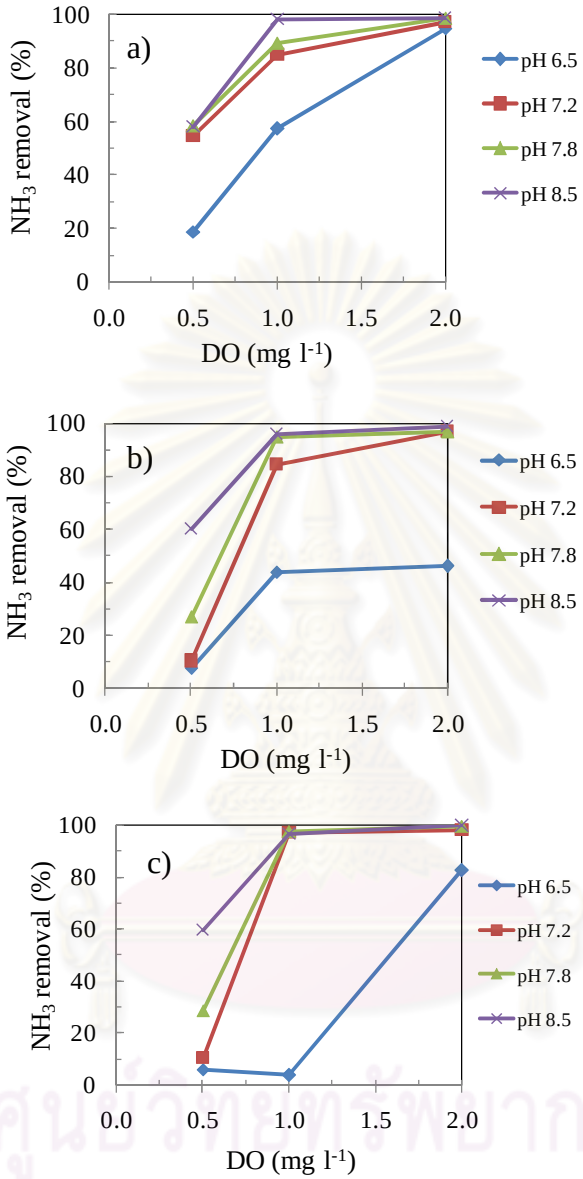

Fig. 5.11 Percentage of NH_3 removal during steady-state conditions at different concentrations of bulk DO and pH of a) ENN, b) EPNI, and c) EPNII entrapped cell nitritation reactors.

Figure 5.11 suggests that although pH levels varied from 7.2 to 8.5, all reactors exhibited high NH_3 removal whent high DO concentrations were provided. With an exception at pH 6.5, more than 91% of average NH_3 removal was achieved at the DO concentrations of 1.0 and 2.0 mg O_2 l^{-1} in all reactors. With an exception at pH level of 6.5, NH_3 removal diminished in all reactors at the lower DO concentration (0.5 mg O_2 l^{-1} DO) under all pH (average values < 57%) (ENN: 55 – 59% [average 57±2%]; EPNI: 10 – 60% [average 32±25%]; EPNII: 11 – 60% [average 33±25%]). This indicates that NH_3 removal efficiency depended largely on the bulk DO concentration. At lower than the threshold level (1.0 mgO_2 l^{-1}), NH_3 removal was limited by the diffusion of O_2 from bulk solution into the entrapped cell while no difference in the NH_3 removal efficiency was obviously observed at higher level of bulk DO (between 1.0 and 2.0 mgO_2 l^{-1}).

Under the same DO levels, NH_3 removal tended to increase with increasing pH. At the DO concentrations of 0.5 and 1.0 mg O_2 l^{-1}, the NH_3 removal efficiencies of EPNI and EPNII depended fairly on the pH while for ENN, the effect of pH on the NH_3 removal efficiency was less than that of EPNI and EPNII. NH_3 removal was sensitive to acidic pH. This was probably because the suspended inocula of EPNI and EPNII were familiar with the alkaline condition than acidic condition because previously these inocula were cultured under alkaline condition to promote AOB activity (pH 7.0±0.1 for NN, pH 7.9±0.2 for PNI and 8.0±0.1 for PNII sludge). At the lower DO level of 0.5 mg O_2 l^{-1}, NH_3 removal was more sensitive to acidic pH than at the higher DO concentrations (1.0 and 2.0 mg O_2 l^{-1}). Based on Double Monod expression for NH_3 oxidation (see equation 8, chapter 2), NH_3 utilization rate depends on both the maximum NH_3 utilization rate and the concentration of limiting substrate (either NH_3 or O_2). Inappropriate pH level in the reactor can reduce the maximum NH_3 utilization rate of ammonia oxidation (Park et al., 2007). Resulted from both non-optimal pH levels and limiting O_2 level (0.5 mg O_2 l^{-1}) in the reactors, NH_3 utilization rate was reduced. At the pH level of 6.5, the NH_3 removal efficiency dropped in all reactors as compared to other pH levels. Too low pH (≤ pH 6.5) strongly deteriorated NH_3 removal. An optimum pH range for nitrification is between

7.0 and 8.5 with sharp reduction outside this range (Henze et al., 2008). Between pH 7.0 and 8.5, higher nitrification rate was achieved at higher pH (Henze et al., 2002).

For inhibitory effects of FA and FNA on NH_3 removal, Table 5.6 to 5.8 show that the higher FA accumulated in the reactors did not reduce the NH_3 removal in all reactors. In addition, the concentration of FNA was lower than the threshold level found to inhibit NH_3 oxidation (between 0.2 and 2.8 mg N l^{-1}) and NO_2^- oxidation (between 0.06 and 0.83 mg N l^{-1}; Anthonisen et al. 1976). Based on the results, it can be concluded that NH_3 removal efficiency of entrapped cell nitritation reactors (ENN, EPNI and EPNII) depended on O_2 limitation and pH level.

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5.3.4.2 Nitrite accumulation

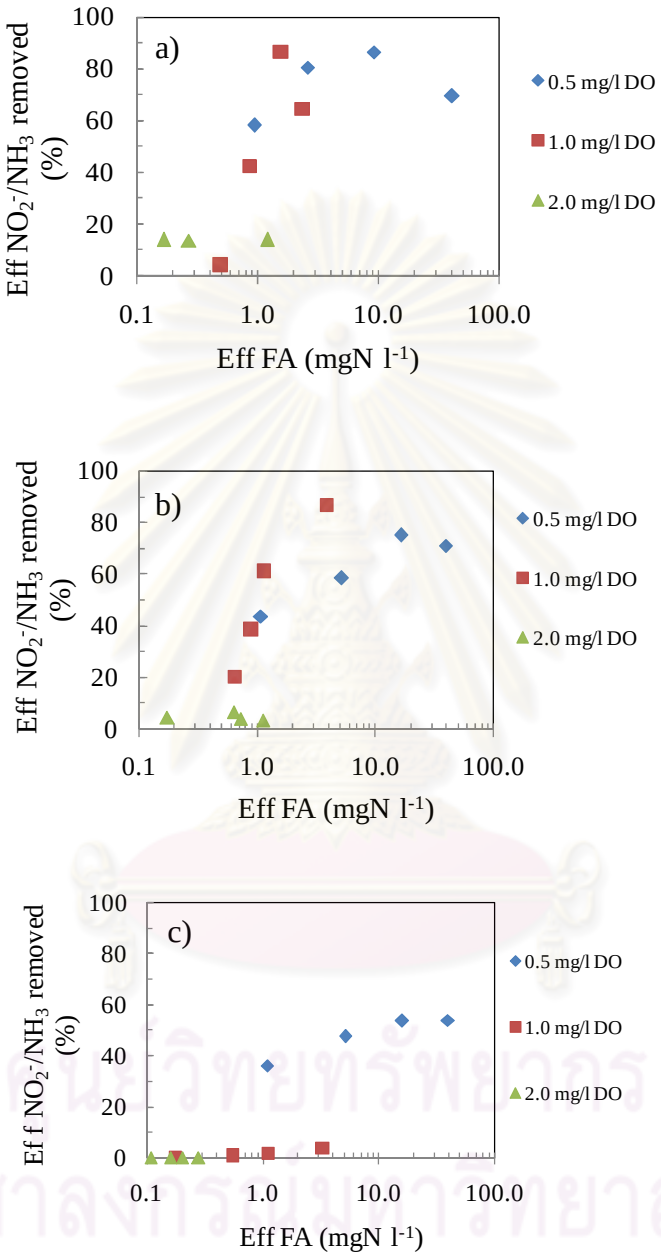

Fig. 5.12 Nitrite accumulation indicated by percentage of effluent NO_2^- concentration per NH_3 removed during steady-state conditions at various concentrations of bulk DO and effluent FA concentration of a) ENN, b) EPNI, and c) EPNII entrapped cell-nitritation reactors.

For all reactors, higher NO_2^- accumulation can be achieved at lower concentration of bulk DO (NO_2^- accumulation at 0.5 mg O_2 l^{-1} DO: 58 – 87% [average 74±13%] for ENN, 44 – 75% [average 62±14%] for EPNI, and 36 – 54% [average 48±8%] for EPNII; at 1.0 mgO_2 l^{-1} DO: 4 – 86% [average 49±35%] for ENN, 20 – 87% [average 52±29%] for EPNI, and 2 - 4% [average 2±2%] for EPNII; at 2.0 mgO_2 l^{-1} DO: 7 – 14% [average 12±4%] for ENN, 3 – 7% [average 5±2%] for EPNI, and 0% for EPNII) (Table 5.6 to 5.8 and Fig. 5.12). Based on these results, it is clear that the activity of NOB was suffered by O_2-limiting condition.

For an inhibitory effect of FA, higher NO_2^- accumulation was found at the higher concentrations of effluent FA (or accumulated FA). As shown in Fig. 5.12, FA inhibition started to affect NO_2^- oxidation at FA levels of above 0.6 - 0.9 mg N l^{-1} in all reactors which was a range reported in previous studies (0.05 – 20 mgN l^{-1}: Vadivelu et al., 2007; Villaverde et al., 2000; Abeling and Seyfried, 1992; Turk and Mavinic, 1989; Alleman and Irvine, 1980; Verstraete et al., 1977; Anthonisen et al. 1976; Prakasam and Loehr, 1972; Murray and Watson, 1965). FA is an unionized form of NH_3 and its concentration is regulated by both the concentrations of NH_3 and pH. Thus, higher FA levels were found in the reactors operated at higher pH (Percent FA per NH_3 under 25^oC: 15.8%, 3.6%, 0.9% and 0.2% at pH 8.5, 7.8, 7.2, and 6.5, respectively). Therefore, for certain values of pH, higher FA accumulation was found in the reactors at higher NH_3 accumulation as reactors operated at DO limiting condition for NH_3 oxidation (0.5 mg O_2 l^{-1}, see discussion in Subsection 5.3.4.1).

As shown in Fig. 5.12, although higher NO_2^- accumulation was found at the higher level of FA but a level of inhibitory effect of FA on NO_2^- oxidation was different among the bulk DO concentrations in reactor. The levels of FA (0.6 - 0.9 mg N l^{-1}) appeared to inhibit NOB activity when the reactors were operated at DO of 1.0 mg O_2 l^{-1}. Under DO of 1.0 mg O_2 l^{-1}, NO_2^- accumulation was substantially increased with increasing FA level (at 1.0 mg O_2 l^{-1} DO: FA increased from 0.5 to 1.6 mg N l^{-1}, NO_2^- accumulation increased from 4 to 86% for ENN; FA increased from 0.7 to 3.9 mg N l^{-1}, NO_2^- accumulation increased from 20 to 87% for EPNI; FA increased from 0.2 to 3.3 mg N l^{-1}, NO_2^- accumulation increased from 0 to 4% for EPNII). NO_2^-

accumulation under DO of 1.0 mgO_2 l^{-1} was regulated by FA inhibition on NO_2^- oxidation.

At the DO concentration of 2.0 mg O_2 l^{-1}, NO_2^- accumulation was low and did not depend on the level of FA that found mostly at lower than the range of FA started to inhibit NO_2^- oxidation (lower than 0.6 - 0.9 mgN l^{-1}) (at 2.0 mg O_2 l^{-1} DO: FA increased from 0.1 to 1.2 mg N l^{-1} but NO_2^- accumulation stayed between 7 – 14% [average 12±4%] for ENN; FA increased from 0.2 to 1.1 mg N l^{-1} but NO_2^- accumulation stayed between 3 and 7% [average 5±2%] for EPNI; FA increased from 0.1 to 0.3 mg N l^{-1} but NO_2^- accumulation stayed at 0% for EPNII). Under 2.0 mg O_2 l^{-1} DO, low NO_2^- accumulation could be caused by non-O_2 limiting condition due to high level of bulk DO or by non-FA inhibiting condition due to low level of FA.

At the DO concentration of 0.5 mg O_2 l^{-1}, the level of FA was high and found mostly at much higher than the range of FA that inhibits NO_2^- oxidation (higher than 0.6 - 0.9 mg N l^{-1}). However, NO_2^- accumulation was found to be high and obviously, less depended on the level of FA compared to that under 1.0 mg O_2 l^{-1} DO (at 0.5 mg O_2 l^{-1} DO: FA increased from 0.9 to 41.2 mg N l^{-1}, NO_2^- accumulation increased from 58 to 87% for ENN; FA increased from 1.1 to 38.9 mg N l^{-1}, NO_2^- accumulation increased from 44 to 77% for EPNI; FA increased from 1.1 to 39.5 mg N l^{-1}, NO_2^- accumulation increased from 36 to 54% for EPNII). NO_2^- accumulation under DO 0.5 mg O_2 l^{-1}, which was high and less sensitive to FA inhibition, could be caused by O_2 limitation due to low level of bulk DO rather than by FA inhibition. Thus, under DO of 0.5 mg O_2 l^{-1}, O_2 limitation had more effect on NO_2^- accumulation than the FA inhibition.

When comparing among the reactors, similar degrees of NO_2^- accumulation were achieved in ENN and EPNI, while the lower accumulation of NO_2^- was observed in EPNII reactor (average values for NO_2^- accumulation at 0.5 mgO_2 l^{-1} DO: 74±13% for ENN, 62±14% for EPNI, and 48±8% for EPNII; at 1.0 mgO_2 l^{-1} DO: 49±35% for ENN, 52±29% for EPNI, and 2±2% for EPNII; at 2.0 mgO_2 l^{-1} DO: 12±4% for ENN, 5±2% for EPNI, and 0% for EPNII). The reason for this is unclear as all reactors were

operated under the same levels of DO in bulk solution and the FA levels in the reactors under certain DO fell into similar ranges. The control parameter for partial nitrification in the entrapped cell system is the O_2 levels in the gel beads around NOB aggregates, not the DO levels in bulk solution. As mentioned in Section 5.3.3, FISH analysis (Fig. 5.10g) suggests that at the end of the start-up period (phase II), the EPNII gel beads experienced with less O_2 limitation than the other gel beads (ENN and EPNI) due to the formation of looser aggregates of PNII sludge (see discussion in Subsection 5.3.3.1). In this case, O_2 levels in the gel beads of EPNII may not low enough to inhibit NOB activity as in the other reactors. Nevertheless, FISH analysis at the end of this part of experiment is needed to confirm. Unfortunately, it was not performed here.

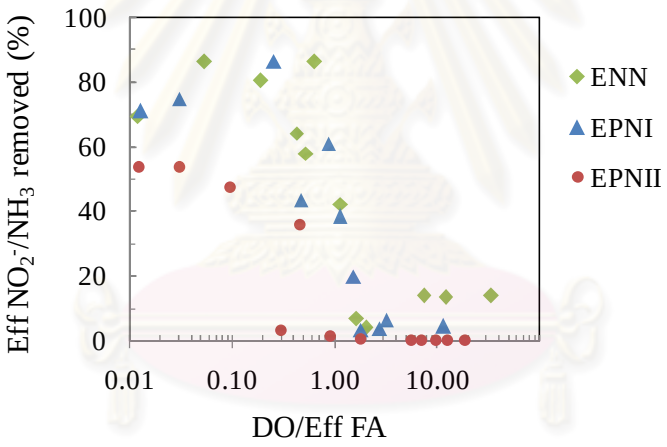

Fig. 5.13 Nitrite accumulation indicated by percentage of effluent NO_2^- concentration per NH_3 removed during steady-state conditions at various ratios of bulk DO and effluent FA concentration.

Based on the results mentioned above, it can be concluded that NO_2^- accumulation in the entrapped cell nitritation reactors depended on both concentrations of bulk DO and FA. consequently, it is not possible to control a desired level of nitritation in the reactors using either the concentration of bulk DO or FA alone. Thus, a relative ratio of both parameters (ratio of DO/effluent FA) is proposed to be the control parameter for partial nitrification. As shown in Fig. 5.13, NO_2^- accumulation became higher at the lower ratio of DO/effluent FA in spite of the

concentration of DO and FA. The link between NO_2^- accumulation and the ratio of DO/effluent FA can obviously be separated into three distinct zones. With high ratios of DO/effluent FA (DO/effluent FA: ≥ 1.6 [33.4 – 1.6], ≥ 1.8 [11.5 – 1.8], ≥ 0.9 [18.4 – 0.9] for ENN, EPNI, EPNII, respectively), low NO_2^- accumulation were achieved (NO_2^- accumulation: 4 – 14% [average 11±5%], 3 – 7% [average 5±1%], 0 – 2% [average 0%] for ENN, EPNI, EPNII, respectively). At medium ratios of DO/effluent FA (1.6 ≥ DO/effluent FA ≥ 0.6, 1.8 ≥ DO/effluent FA ≥ 0.3, 0.9 ≥ DO/effluent FA ≥ 0.1 for ENN, EPNI, EPNII, respectively), NO_2^- accumulation was very sensitive to DO/effluent FA ratio (NO_2^- accumulation: 14 – 87% [average 45±40%], 7 – 87% [average 42±29%], 2 – 47% [average 22±23%] for ENN, EPNI, EPNII). At low DO/effluent FA ratios (DO/effluent FA: ≤ 0.6 [0.6 – 0.01], ≤ 0.3 [0.3 – 0.01], ≤ 0.1 [0.1 – 0.01] for ENN, EPNI, EPNII, respectively), NO_2^- accumulation was high and less sensitive to the DO/effluent FA ratios as compared to the medium zone (effluent NO_2^-/NH_3 removed: 69 – 87% [average 74±12%], 71 – 87% [average 78±8%], 47 – 53% [average 52±4%] for ENN, EPNI, EPNII, respectively). Therefore, it is possible to use the DO/effluent FA ratio to control a desired level of nitritation in entrapped cell nitritation reactors by adjusting the bulk DO concentrations in the reactors under certain values of pH and effluent concentrations of NH_3 to achieve the selected DO/effluent FA ratios.

For directly applying the results from this study to prepare Anammox-suited effluent, the effluent of the entrapped-cell nitritation reactors needs to contain almost equal molar of NH_3 to NO_2^-. An Anammox process which is the next logical treatment step after the entrapped-cell nitritation reactors requires influent with a molar ratio of NH_3 and NO_2^- close to 1:1 – 1.32. The optimum value of DO/effluent FA ratio can obviously be selected from Fig. 5.13 and the criteria to select are the highest NO_2^- accumulation under the NH_3 removal efficiency of close to 50% (Tables 5.6 to 5.8). The optimum value for DO/effluent FA ratio was 0.01 that provided the NH_3 removal efficiency of 58% and the NO_2^- accumulation of 69% resulting in the molar NH_3:NO_2^- ratio of 1:1.0 for ENN. The value is 0.01 for EPNI (NH_3 removal efficiency of 60%; NO_2^- accumulation of 71%; molar NH_3:NO_2^- ratio of 1:1.1), and

0.01 for EPNII (NH_3 removal of 60%; NO_2^- accumulation of 54%; molar NH_3:NO_2^- ratio of 1:0.9). The ratio of 0.01 can be used with all inoculums types.

5.4 Conclusions

In this chapter, the effect of different entrapped inocula, include entrapment of non-nitrifying (ENN), nitrifying (EN), partial nitrifying (EPNI) sludge and partial nitrifying (EPNII), on accelerating partial nitrification were investigated during start-up periods in continuous-flow reactors fed with high NH_3 wastewater. EPNII served as the positive control inoculums as it contained high numbers of AOB but tiny numbers of NOB. Results show that entrapped cells containing very low numbers of NOB (positive control, EPNII) can achieve NO_2^- accumulation at higher level and shorter timeframe (91% of NO_2^- accumulation after 15 days of operation) than entrapped cells containing high NOB abundance (ENN, EN, and ENPI). This finding showed the effect of inoculum type on accelerating partial nitrification. However, entrapped cells where NOB existed in high numbers could achieve stable NO_2^- accumulation under ambient temperature and normal bulk DO operating condition (2 mg N l^{-1} DO) at comparable level and timeframe (after 30 - 42 days, 65 – 66% of NO_2^- accumulation was stably reached). This success was because the levels of the available O_2 strongly controlled the performances at the presence of NH_3 at high concentration (O_2 is a limiting substrate). A step for preparing sludge which is readily for nitrification or partial nitrification did not benefit partial nitrification by entrapped cells. Limited O_2 penetration depth into gel bead caused AOB and NOB to relocate themselves from the O_2-limiting zones in the inner parts of the gel beads to the O_2 available zones at the outer parts of the gel beads after start-up. Due to the competition for space and limiting substrate (O_2), NOB aggregates were found underneath AOB aggregates in the smaller and looser forms and resulting in some loss in partial nitrification.

Effects of bulk DO or/and FA concentrations on NO_2^- accumulation in entrapped cell system were observed during both the start-up and operational periods. Results showed that NH_3 removal efficiency depended largely on the bulk DO

concentration. Under the same DO level, NH_3 removal efficiency tended to increase with increasing pH. The NH_3 removal efficiencies of EPNI and EPNII were more sensitive to acidic pH than that of ENN due to their previous adaptation to the alkaline condition. NH_3 removal of nitritation entrapped cell governed by the DO availability and pH level and was not suppressed by FA inhibition.

Higher NO_2^- accumulation was found at a lower concentration of bulk DO due to NO_2^- oxidation suppression by low O_2 and also, at a higher level of FA due to NO_2^- oxidation inhibition by FA. However, an inhibitory effect of FA on NO_2^- oxidation was dependent on the bulk DO concentration in the reactor. NO_2^- oxidation was suppressed mainly by low O_2 when the reactor was operated under DO of 0.5 mg O_2 l^{-1}, but mainly by FA when the reactor was operated under 1.0 mgO_2 l^{-1} DO. While under high DO (2.0 mg O_2 l^{-1}), NO_2^- accumulation was very low due to non-O_2 limiting or non-FA inhibiting condition. Similar degrees of NO_2^- accumulation were achieved in ENN and EPNI, while lower accumulation of NO_2^- was observed in EPNII reactor. As a result of less O_2 limitation during the start-up than the other gel beads (ENN and EPNI), O_2 levels in the gel beads of EPNII was speculated not to be low enough to inhibit NOB activity as in the other reactors.

The accumulation of NO_2^- by entrapped cells depended on both concentrations of bulk DO and FA. A relative ratio of both parameters (ratio of DO/effluent FA) is proposed to be a control parameter for partial nitrification. NO_2^- accumulation becomes higher at lower ratios of DO/effluent FA regardless of the concentrations of DO and FA. Therefore, it is possible to use the DO/effluent FA ratio to control a desired level of nitritation in entrapped cell nitritation reactors by easily adjusting the bulk DO concentration in the reactors.

CHAPTER VI

EFFECT OF HETEROTROPHS ON ACTIVITY OF AOB IN GEL BEADS UNDER CONDITION OF *p*-NITROPHENOL INHIBITION

6.1 Introduction

Nitritation or oxidation of ammonia (NH_3) to nitrite (NO_2^-) is driven by autotrophic ammonia oxidizing bacteria (AOB). For shortcut biological nitrogen removal (SBNR), partial nitrification is believed to be the rate-limiting step of the overall process which can be achieved by promoting the oxidation of NH_3 to NO_2 without further oxidation of NO_2^- to NO_3^- (Hellinga et al., 1998). Comparing to conventional biological nitrogen removal (CBNR), SBNR is a cost effective innovative process scheme suitable for low carbon and/or high ammonia nitrogen wastewater (C/N ratio < 3.5 - 4). Recently, an entrapment of cells into gel beads is proposed to be an effective alternative method to attain stable partial nitrification at ambient temperature (Rongsayamanont et al., 2010; Yan and Hu, 2009). Entrapped cells provide several advantages over the suspended cells, including easier to promote nitrite accumulation due to low oxygen in the cell entrapment matrix (Yan and Hu, 2009) and higher protection of low growth and yield cells, such as AOB, from washing out (Rostron et al., 2001; Vogelsang et al., 1997; Yang et al., 1997; Chen and Lin, 1994).

Toxic nitroaromatic compound like *p*-nitrophenol (PNP) was found often in high ammonia industrial wastewater such as pharmaceuticals wastewater (ammonia in wastewater: 80 – 500 mgN l^{-1}; PNP in wastewater: < 10 – 2300 µg l^{-1}; Gupta et al., 2006). PNP is one of the U.S. EPA's priority pollutant which its toxicity value as EC50 is 64 mg/l (Tomei et al., 2004). EPA recommended restricting PNP level in natural waters at below 10 ng l^{-1} (Kuscu and Sponza, 2007). It is hardly biodegradable

compound and can stable in both surface and subsurface water due to high solubility and low partitioning coefficient. PNP can create a significant health risks due to their mutagenic and carcinogenic activity and may bioaccumulate in the food chain (Rezouga et al., 2009; Yi et al., 2006; Tomei et al., 2004). The high inhibitory effect of PNP on AOB have been reported (Zhang et al., 2010; Blum and Speece, 1991). The concentration that inhibited the AOB activity by 50% (IC50) of PNP is 2.6 mg l^{-1} (Blum and Speece, 1991). This indicated that PNP can have strong negative effects on the stability of partial nitrification during the treating of NH_3 in industrial wastewater.

High fluctuation in quality of industrial effluent can lead to a shock loading problem in industrial wastewater treatment process. This operation problem can be highly adverse and may result in a complete process failure. An inability to recover the AOB activity after experience with shock load of toxic organic compound often lead to a serious drop or failure in nitrification (Amor et al., 2005; Texier and Gomez, 2002; Winther-Nielsen and la Cour Jansen, 1996; Benmoussa et al., 1986). Sensitivity with the instant loading of toxic compound could limit applications of partial nitrification for treating ammonia in real industrial effluent (Suárez-Ojeda, et al., 2010).

Biofilm can give more tolerance to toxicant than suspended cell (de Beer et al., 1994). The reduced toxic sensitivity of bacteria in the deeper part of biofilm was suspected that as a result from reduction of toxicant penetration by the bacteria and extracellular polymeric substances at the outer part of biofilm (de Beer et al., 1994; Xu et al., 1996). Several studies reported good resistance to shock load of toxicant in biofilm reactor (Rajbhandari and Annachhatre, 2004; Borghei and Hosseini, 2004; Wobus and Röske, 2000). For the case of entrapped cell in which biofilm-like layer structure is developed in its outer part due to competition between species for space and substrate, bacterial layer structure in entrapped cell may also reduce toxic sensitivity of bacteria in the deeper part in the same manner with biofilm and consequently, give more resistance to shock load of toxicant. Chapter 4 showed that the relative localization of heterotrophs and AOB in nitrifying sludge for entrapped cell system rather than for suspended cell system was arranged in biofilm-

like layer structure by which following their physiology and the substrate availability. Heterotrophs can overgrow and take up more oxygen at the peripheral of entrapped cell compared with AOB in which at inner part and adjacent with heterotrophs cluster (Rongsayamanont et al., 2010). Thus, in entrapped mixed nitrifying sludge, it was speculated that heterotrophs at the outer part may possibly reduce the penetration of toxicant and subsequently reduce toxic sensitivity of AOB in the deeper part under toxic condition.

This study aims to investigate the effect of heterotrophs on the activity of AOB in gel beads under the inhibiting condition from a model toxic chemical, PNP. Three identically operated nitritation reactors receiving influent with different organic loads were setted up and start-up by gel entrapment of activated sludge to get three types of 'nitritation entrapped cells' which have a different amounts of heterotrophs in the gel beads. AOB and heterotrophs in nitritation entrapped cells was probed using fluorescence *in situ* hybridization (FISH) to see effect of different organic loads on their relative localization. Two sequentially batch tests (first and second batch test) for PNP degradation and NH_3 oxidation were setted up and performed to investigate effect of heterotrophs on the activity of AOB under condition simulated the PNP shock load with different types of nitritation entrapped cells.

6.2 Materials and Methods

6.2.1 Synthetic wastewater for entrapped cell nitritation reactors

Organic-free synthetic wastewater contained $NaHCO_3$ (4.9 g), Na_2HPO_4 (4.05 g), K_2HPO_4 (2.1 g), $MgSO_4 \cdot 7H_2O$ (0.05 g), $CaCl_2 \cdot 2H_2O$ (0.01 g), and $FeSO_4 \cdot 7H_2O$ (0.09 g) in one liter of deionized (DI) water. Before using as the influent for entrapped cell nitritation reactors, $(NH_4)_2SO_4$ was added to achieve a final concentration of 500 mg N l^{-1} TAN. In addition, sodium acetate was provided to obtain the desired concentration of dissolved organic carbon (DOC) resulting in different value of the organic loading rates for each reactor. All chemicals were purchased from Carlo Erba Reagenti (Milan, Italy).

6.2.2 Cell entrapment

Fresh activated sludge was taken from the aeration basin of a full scale municipal wastewater treatment plant (WWTP) in Bangkok and entrapped in phosphorylated PVA (PPVA) gel beads at a cell-to-matrix ratio of 4% w/v (g of centrifuged mixed liquor volatile suspended solids/ml of media) following the protocol recommended by Chen and Lin (1994). Briefly, a mixture at an equal volume between a PVA aqueous solution (20% w/v) and a sludge suspension of 70 g volatile suspended solid (VSS) per liter was prepared. Then, the mixture was dropped into a saturated boric acid solution at a rate of 0.83 ml min^{-1} resulting in a droplet diameter of 3 – 4 mm. About one hour after the dropping ended, the formed beads were transferred to a 1 M sodium orthophosphate solution (pH 7) and kept for 2 hr to allow hardening. The entrapped cells were then used as entrapped inocula for nitritation reactors.

6.2.3 Setup and start-up of entrapped cell nitritation reactor

Three laboratory-scale continuous flow reactors, each of which has an effective volume of 3 l, were supplied with an equal amount of gel beads (approximately 900 beads). The reactors were stirred completely using mechanical stirrers (IKA RW20D, IKA-Werke GmbH & Co., Germany). Air was supplied to each reactor through the ON/OFF air blower which was connected to an oxygen transmitter (O_2 4100e, Mettler Toledo, USA) equipped with a DO probe (O_2 Sensor InPro 6820, Mettler Toledo, USA) to control for a desired dissolved oxygen (DO) concentration in the reactor. pH was controlled by a pH controller system (Liquitron DP 5000, LMI Milton Roy, USA) consisting of a pH probe (Orion 9156DJWP, Thermo scientific, UK) and HCl and NaOH solution dosing mechanism (for pH adjustment).

All three reactors were operated under the same conditions except that they received the influent with different C/N mass ratios (0, 0.4 and 0.8). These reactors will be referred to as C/N0, C/N0.4, and C/N0.8. The influent for the three reactors contained the same TAN concentration of 505 mg N l^{-1} but different concentrations of

sodium acetate (0, 221, and 430 mg C l^{-1}) resulting in the organic loading rates of 0, 0.29 and 0.57 kg C m^{-3} d^{-1}. Hydraulic retention time (HRT) was 1.2 days for all reactors leading to an influent NH_3 loading rate of 0.67 kgNH_3-N m^{-3} d^{-1}. All reactors were operated at room temperature (24^oC-26^oC) while pH level was strictly controlled at 8.5±0.1. The DO concentration was controlled at 2±0.1 mg l^{-1}.

Every two days, the effluent was sampled to analyze for DOC, TAN, NO_2^- and nitrate (NO_3^-) concentrations. To observe spatial distribution of AOB and NOB in the gel beads, the gel beads were collected after the reactors reached the steady state condition and analyzed using FISH technique.

6.2.4 Effect of heterotrophs on activity of AOB in gel beads under condition of PNP inhibition

To investigate effect of heterotrophs on the activity of AOB in entrapped cells under condition of PNP inhibition, two sequential tests were setted up and performed in 500 ml aerated glass reactors with effective volumes of 450 ml. First batch test was setted up to investigate effect of heterotrophs on the activity of AOB under condition simulated the first time of PNP shock load in reactor by using entrapped cells which have not been previously acclimatized with PNP. To do this, an equal weight of PNP un-acclimated gel beads (around 80 beads) harvested from C/N0, C/N0.4 and C/N0.8 reactors, which have never been received PNP before, were put into the batch reactors. The media was prepared from the organic-free synthetic wastewater by adding NH_3 and PNP to obtain the final concentrations of NH_3 of 50 or 100 mg N l^{-1} TAN and PNP of 0, 2, 4, 6, 8 or 10 mg l^{-1}. A PNP abiotic control test was prepared with the beads carrying no cell to investigate the PNP lost from volatilization and adsorption. Aeration and mixing were provided by small diffusers connected to air blower so that average DO concentrations in all reactors were above 6.0 mg O_2 l^{-1} at room temperature to ensure sufficient oxygen. pH of the media was adjusted initially to be between 7.8 and 8.0 and maintained using phosphate buffering.

After finishing the first batch test, which the ability of the gel beads for PNP degradation have been demonstrated, PNP-acclimated gel beads from each reactor were harvested and washed with the synthetic wastewater for three times to simulate nonappearance of PNP in wastewater. Washed gel beads were again put into the batch reactors containing the corresponding NH_3 and PNP concentrations as conducted in the first batch test to investigate effect of heterotrophs on the activity of AOB under condition simulated the second time of PNP shock load in reactor by using entrapped cells which have been previously acclimatized with PNP. In second batch test, Ammonia monooxygenase enzyme (AMO) inactivator, allylthiourea (ATU), was added at a concentration of 86 µM in the reactors containing 4 and 8 mg l^{-1} PNP to observe the PNP degradation with non-AMO enzyme. Other setup and operation in the second batch tests were the same as in the first batch tests.

At an appropriate interval (every 1 - 2 days), supernatant was sampled, filtered and analyzed for PNP, TAN, NO_2^- and NO_3^- concentrations to observe the degradation of PNP and the oxidation of NH_3. Due to an incomplete degradation of PNP, the degradation rate of PNP was determined when the PNP was removed 90% of its initial concentration. The rate of NH_3 oxidation was the zero order rate constant which determined from a slope of linear relationship between the concentrations of NO_2^-- N and NO_3^--N combined and time.

6.2.5 Analyses

For TAN, NO_2^- and NO_3^- concentrations, supernatant was filtered through a GF/C Whatman filter paper. TAN was analyzed using an ion selective electrode (WTW GmbH, NH_4 500/2, and Germany). NO_2^- and NO_3^- were analyzed using UV visible spectrophotometer (Thermo Electron Corporation, Hexious α, Cambridge, UK) in accordance with Standard Methods for the Examination of Water and Wastewater (APHA et al. 1998).

For DOC and PNP concentrations, a 0.45 µm pore-size PTFE filter was used to filter the supernatant. DOC was analyzed using a total organic carbon analyzer

(TOC-V CPH 220V, Shimadzu, Japan). PNP was analyzed using an UV visible spectrophotometer at a wavelength of 400 nm (Thermo Electron Corporation, Hexious α, Cambridge, UK) after adjusting the pH of filtrate to higher than 9.5 with 1 M NaOH according to a method by Spain and Gibson (1991). DO (WTW Oxi 340i meter, WTW Cellox 325 probe, Germany), pH (HACH Sension1 pH Electrode, USA), and VSS were determined according to Standard Methods for the Examination of Water and Wastewater (APHA et al. 1998).

6.2.6 Analysis of spatial distribution of AOB and NOB in nitritation entrapped cell using FISH

For each sample, one gel beads was suspended in an ice-cold PBS solution. Later, bead was transfer and fixed at 4^{o}C in a paraformaldehyde solution (4% in PBS at pH 7.2) for 12 h. After washing three times and resuspending in a ice-cold PBS solution, fixed gel beads were embedded in a rapid freezing compound (Tissue-Tek OCT, Sakura Finetek USA Inc., USA) and cut into five micron sections at -20^{o}C using a cryomicrotome (Leica CM 1950, Germany) and then immobilized onto a polymer coating slide.

Oligonucleotide probes used in this study were 5' labeled with Alexaflour 488 or Cy3 (ThermoHybaid, Ulm, Germany). The hybridization was performed at 46^{o}C for 1.5 h in buffer (0.9 M NaCl, 20 mM Tris-HCl, 0.01% sodium dodecyl sulfate (SDS), formamide) containing 5 ng of probe μl^{-1}. Then, the section was immersed at 48^{o}C in pre-warmed washing buffer (20 mM Tris-HCl, 0.01% SDS, NaCl) for 10 min and subsequently rinsed shortly with DI water, air dried and mounted with an anti-fading solution (SlowFade, Antifade kit, Molecular Probes, Eugene, OR, USA). The oligonucleotide probes and their corresponding hybridization conditions are shown in Table 6.1. For simultaneous hybridization, two hybridizations were performed successively with the probe requiring higher stringency performed first (Wagner et al., 1996). The FISH samples were observed using a fluorescence inverted microscope (Olympus IX81, Japan) with DP2-BSW software for image processing. Alexaflour 488–labelled probe was visualized by excitation between 460 and 495 nm and

collection of fluorescence emission at 510 nm. Cy3-labelled probe was excited at between 530 and 550 nm and its fluorescence emission was collected at 575 nm.

Table 6.1 Labeled 16SrRNA oligonucleotide probes used in this study

Probe	Sequences (5' to 3')	Label	Target organisms	Formamide (%)	References
EUB338	GCTGCCTCCCGTAGGAGT	Cy3/AF	Most bacteria	15	Amann et al. (1990)
Nso190	CGATCCCCTGCTTTTCTCC	Cy3/AF	*β-AOB* (many but not all)	40	Mobarry et al. (1996)
Ntspa662	GGAATTCCGCGCTCCTCT	Cy3	*Nitrospira* genus	35	Daims et al. (2001)
Nit3	CCTGTGCTCCATGCTCCG	Cy3	*Nitrobacter spp.*	40	Wagner et al. (1996)

6.3 Results and discussion

6.3.1 Preparation of nitritation entrapped cells

Nitritation entrapped cells were prepared in laboratory-scale continuous-flow reactors receiving different organic loading rates. Operating conditions and steady-state performance of the reactors are shown in Fig. B-1 to B-3 in Appendix B and summarized in Table 6.2.

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Table 6.2 Operating conditions and steady-state performance of entrapped cell nitritation reactors

Reactor	Influent					Operating conditions				
	DOC ($mgC\ l^{-1}$)	TAN ($mgN\ l^{-1}$)	Organic C load ($kgC\ m^{-3}d^{-1}$)	NH_3 load ($kgN\ m^{-3}d^{-1}$)	C/N	HRT (d)	DO ($mg\ l^{-1}$)	pH	Temp (^oC)	Operating Period (d)
C/N0	0	505.2 ± 2.2	0	0.67	0	0.75	2.0±0.1	8.5±0.1	25±1	38
C/N0.4	221.1 ± 3.5		0.29		0.44					
C/N0.8	430.1 ± 5.8		0.57		0.85					

Reactor	Effluent				Removal efficiency				
	DOC ($mgC\ l^{-1}$)	TAN ($mgN\ l^{-1}$)	NO_2^- ($mgN\ l^{-1}$)	NO_3^- ($mgN\ l^{-1}$)	Total nitrogen (%)	Total nitrogen loss (%)	DOC removal (%)	TAN removal (%)	Eff NO_2^-/TAN removed (%)
C/N0	-	39.4 ± 15.1 (8)	342.4 ± 18.1 (68)	76.7 ± 35.2 (15)	91	9	-	92	74
C/N0.4	6.1 ± 3.2	51.5 ± 12.6 (10)	261.4 ± 15.0 (52)	86.2 ± 21.2 (17)	79	21	97	90	58
C/N0.8	9.5 ± 4.2	50.7 ± 16.1 (10)	257.2 ± 10.7 (51)	82.1 ± 20.8 (16)	77	23	98	90	57

Note : Values in parenthesis are the percentage of the species to total nitrogen.

The results showed that, in all reactors, NH_3 was removed significantly (92, 90, and 90% TAN removal for C/N0, C/N0.4, and C/N0.8 reactors, respectively) and in the reactors receiving organic (C/N0.4 and C/N0.8 reactors), DOC were well removed. Although NH_3 was present, DOC was removed at very high levels in the organic loaded nitritation reactors (between 97% and 98% for C/N0.4 and C/N0.8 reactors). This indicates that organic carbon can be removed simultaneously with NH_3 under the operating conditions in this study (the NH_3 loading rate of 0.67 kg NH_3-N m^{-3} d^{-1} and influent C/N ratios from 0.4 to 0.8). In addition, success NH_3 removal in all reactors implied that the range of influent C/N ratios used did not influence NH_3 oxidation. This suggests that sufficient O_2 was provided to maintain AOB activity under all influent C/N ratios; in spite of the known fact that heterotrophs can easily outcompete AOB for O_2. Zhu and Chen (2001) found that nitrification rate in mixed nitrifying biofilm largely depended on the influent C/N ratio. The rate of nitrification can be strongly reduced if the influent C/N ratio is higher than 1.0 (Zhu and Chen, 2001). Okabe et al. (1996) found that in mixed nitrifying biofilm, although heterotrophs outcompeted nitrifier for O_2 and space when increasing the C/N ratio, NH_3 utilization rate was not inhibited if the C/N ratio was 1.5 or less.

Partial nitrification was achieved in all reactors (effluent NO_2^-/TAN reduction of 78, 58, and 57% for C/N0, C/N0.4, and C/N0.8 reactors, respectively). This is possibly due to the O_2-limiting conditions in the gel beads (see Chapter 5), However, a reduced NO_2^- accumulation was observed in the reactors supplied with organic carbon. NO_2^- accumulated less in the reactors with higher OLR (68, 52, and 51% of TAN for C/N0, C/N0.4, and C/N0.8 reactors, respectively); in contrast, NO_3^- accumulation was found at similar levels for all reactors (between 15% and 17% of TAN). The difference in the amounts of NO_2^- accumulation between the reactors supplied with organic carbon and the organic carbon free reactor was 81.0 and 85.2 mg N l^{-1} for C/N0.4, and C/N0.8 reactors, respectively. These values were similar to the difference in the amounts of nitrogen loss between both types of reactors (60.6 and 70.7 mg N l^{-1} for C/N0.4, and C/N0.8 reactors, respectively). The main NO_2^- loss pathway could be denitritation of NO_2^- to nitrogen gas by heterotrophic denitrifier under low O_2 condition which occurred in the inner parts of the gel beads. Previous

studies showed that denitrification of nitrite can occur at the inner parts of the aerated nitrifying biofilm or aggregates where the oxygen levels were limited due to the activity of heterotrophs at the outer parts (Virdis et al., 2011; Meyer et al., 2005; Satoh et al., 2005; Tseng and Chang, 2002). NOB can have mixotrophic growth pattern by using both organic compound and NO_2^- as their energy sources (Jie et al., 2008; Bock, et al., 1990; Steinmuller and Bock, 1977; 1976). Thus, if NOB in the entrapped cells grew in the similar pattern of mixotrophical condition, it is possible that an increase in active NOB (see Fig. 6.1[d, f]) under mixotrophic growth could contribute to the minor NO_2^- loss (other than the main NO_2^- loss by denitritation of NO_2^- to nitrogen gas) or the difference in the amounts of NO_2^- accumulation between the reactors supplied with organic carbon and the organic carbon free reactor (20.4 and 14.5 mg N l^{-1} for C/N0.4, and C/N0.8 reactors, respectively) other than by denitritation.

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6.3.2 Spatial distribution of AOB, NOB, and other bacteria in nitritation entrapped cells

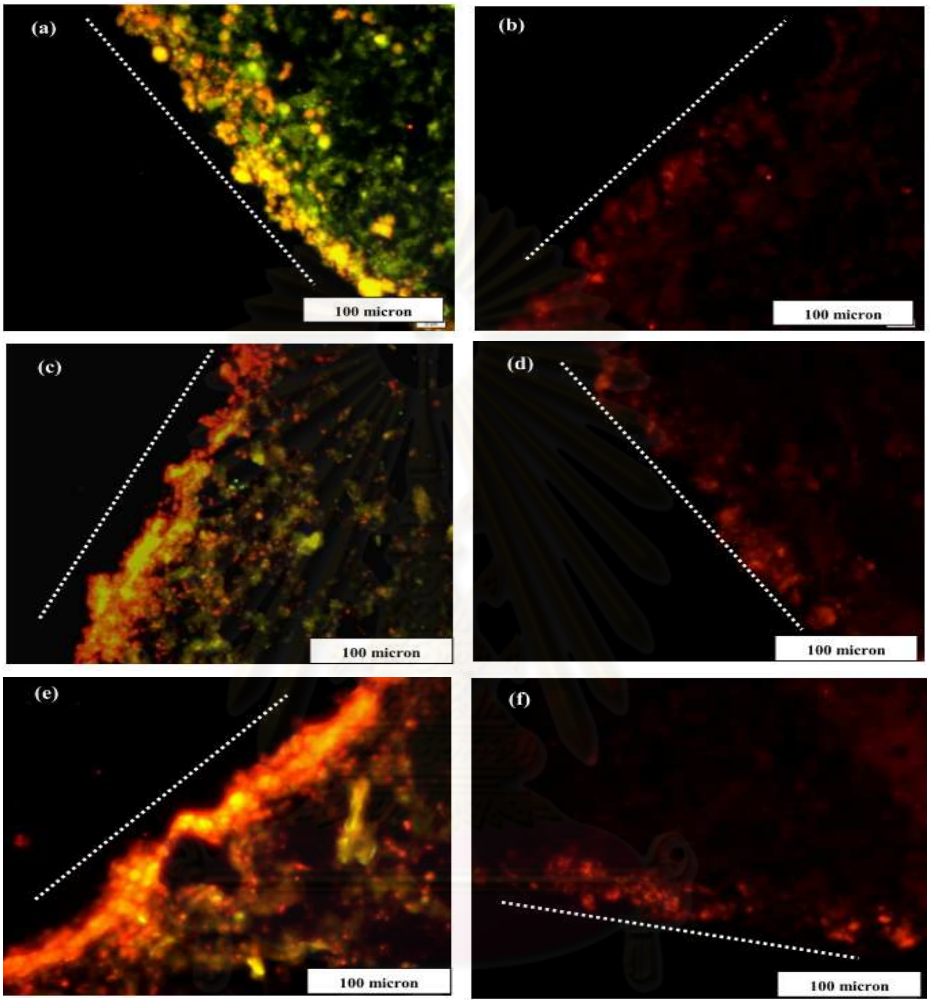

Fig. 6.1 FISH images showing spatial distributions of AOB, NOB and other bacteria in nitritation entrapped cells from C/N0 (a, b), C/N0.4 (c, d) and C/N0.8 (e, f) reactors. Images in the left column (a, c, e) are entrapped cells, hybridized with NSO190 (beta-proteobacterial AOB; green) and EUB338 (all bacteria; red). Images in the right column (b, d, f) are entrapped cells, hybridized with NIT3 (*Nitrobacter*; red). The target cells complimentary to both Cy3-labeled probe (red) and Alexa Flour 488-labeled probe (green), are shown in yellow. NOTE: Non bright green and red signals were suggested to be non-active target cells, autofluorescence of cell lysate, exopolymeric substance or/and precipitated salts. And dotted line indicates the edge of the bead.

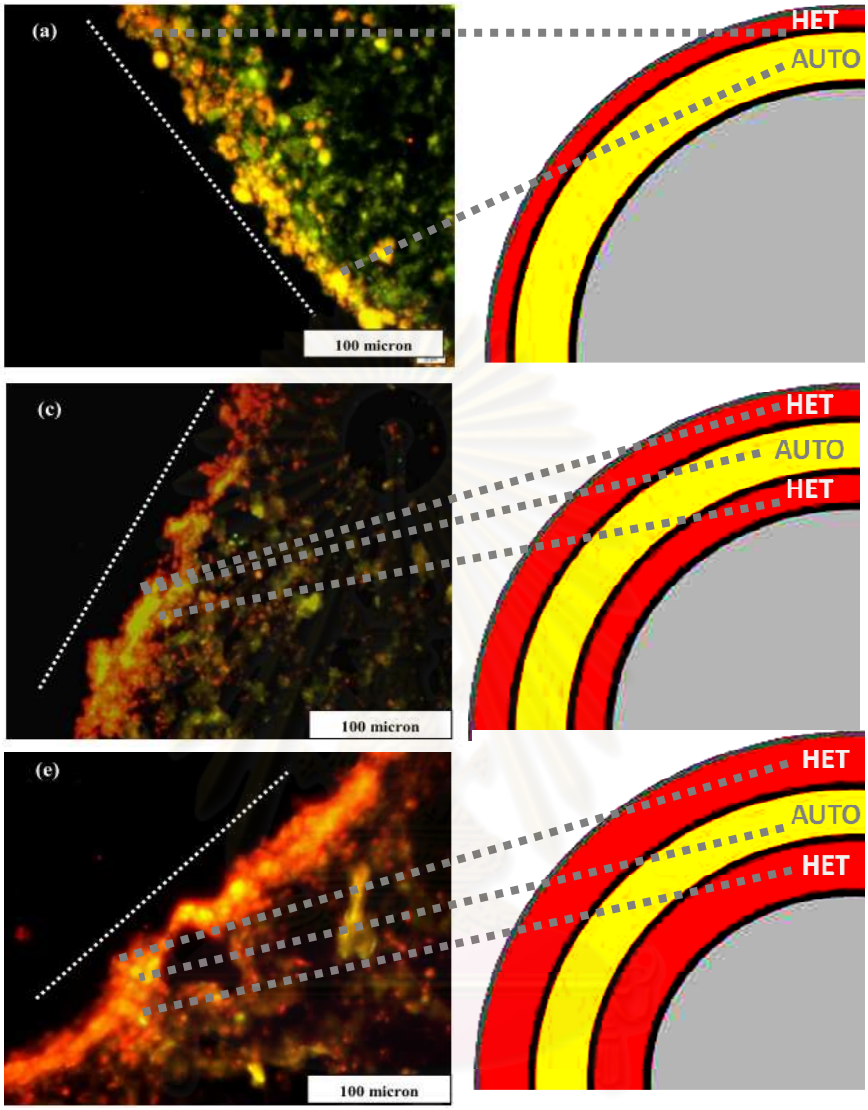

Fig. 6.2 Diagram indicate the position of heterotrophs and autotrophic AOB in FISH images from Fig. 6.1 (a, c, e).

Five weeks after steady state conditions, spatial distribution of AOB, NOB, and other bacteria in nitritation entrapped cells of C/N0, C/N0.4 and C/N0.8 reactors were investigated using FISH. As shown in Fig. 6.1 and 6.2, in all reactors, the spatial distribution of AOB and NOB in the nitritation entrapped cells was governed by the physiology of microorganisms and substrate availability in the gel beads. Because of limited depth of the O_2 penetration in the gel beads, active AOB formed dense layers at the higher O_2 available zones which are around the peripheral of the gel beads (Fig. 6.1[a, c, d] and Fig. 6.2). Active NOB cells formed the smaller and looser clusters

because they have lower O_2 affinity than AOB (Fig. 6.1[b, d, f]). Moreover, with relative distribution of AOB in Fig. 6.1(a, c, e) and NOB in Fig. 6.1(b, d, f), active NOB clusters were found underneath the AOB clusters because NOB utilized NO_2^- which is produced from AOB during ammonia oxidation. The arrangement of AOB and NOB clusters in the gel beads strongly supported NO_2^- accumulation in the entrapped cell system, especially with high ammonium load environments as found in this study. When a high ammonium load was supplied, the growth of AOB can be limited by oxygen. As a result, denser layers of AOB formed in the outer parts of the gel beads to facilitate oxygen uptake. This leads to limited oxygen in the inner parts and thus NOB cannot function resulting in higher nitrite accumulation in the system (see Chapter 5). As shown in Fig. 6.1 (a, c, e) and Fig. 6.2, AOB in the gel beads taken from all reactors showed very bright signal. This result corresponded to the ammonia oxidizing activity which was high in all reactors during the steady conditions (see Table 6.2). As shown in Fig. 6.1 [b, d, f], Fig. 6.2 and Table 6.2, when the OLR or influent C/N ratio increased, higher amounts of active NOB were observed because its mixotrophic growth as discussed above in Subsection 6.3.1.

As shown in Fig. 6.1 (a, c, e) and Fig. 6.2, the majority of bacteria other than AOB was suggested to be heterotrophs. Higher amounts of active heterotrophs were observed in the organic loaded-reactors (C/N0.4 and C/N0.8 reactors) compared to the C/N0 reactor. Active heterotrophic layers of the C/N0.8 reactor were slightly denser than those of the C/N0.4 reactor suggesting that the amount of active heterotrophs positively is related to the OLR or the influent C/N ratio. Most heterotroph clusters were found in the most outer layers of the gel beads and covered over the AOB clusters. However, small clusters of heterotrophs also distributed at the inner zones of the gel beads in the organic loaded reactors (C/N0.4 and C/N0.8 reactors). These observations can be explained by the fact that heterotrophs can outcompete AOB for O_2 and space due to their higher growth rate and lower O_2 affinity. Thus, when organic carbon is available, heterotrophs can outgrow AOB in high O_2 areas which is around the most outer parts of the gel beads. Ecophysiological-dependent arrangement found in our gel beads was similar to that usually found in high substrate

gradient multispecies aggregate, such as in mixed nitrifying biofilm (Satoh et al., 2000; Okabe et al., 1996; Zhang and Bishop, 1996; Zhang et al., 1995).

6.3.3 Degradation of PNP and oxidation of NH_3 in batch tests

6.3.3.1 Degradation of PNP in batch tests

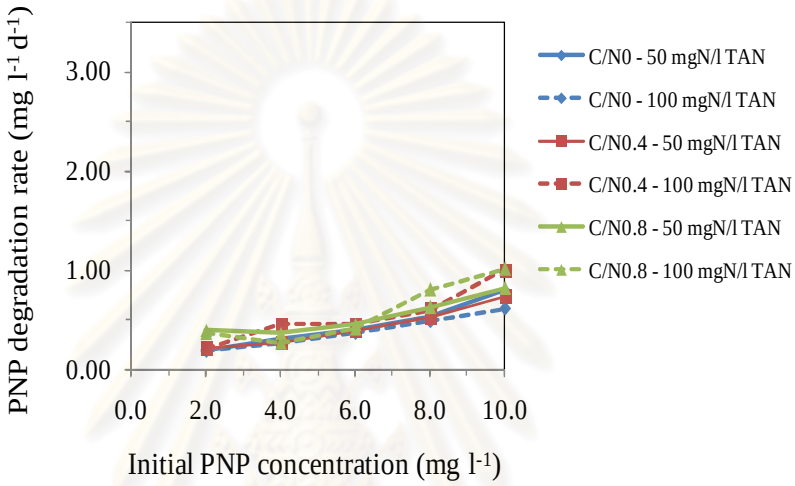

Fig. 6.3 Degradation rate of PNP during the first batch test with entrapped cells from C/N0, C/N0.4, and C/N0.8 reactors at initial TAN concentrations of 50 mg N l^{-1} and 100 mg N l^{-1}.

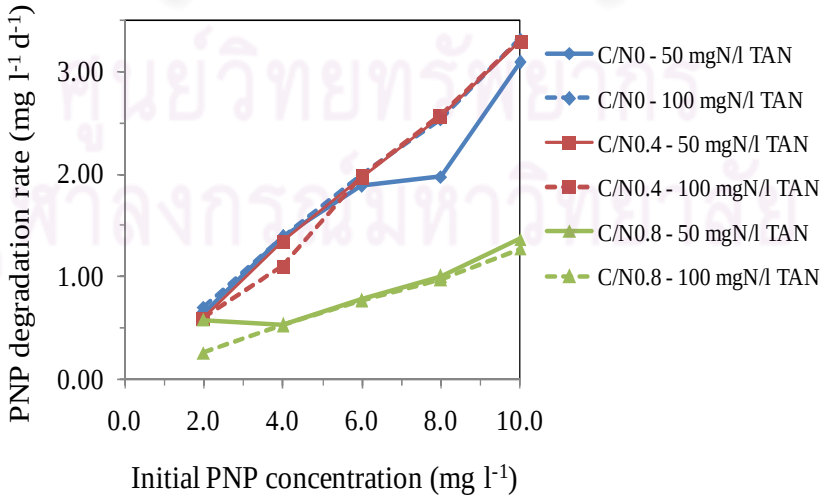

Fig. 6.4 Degradation rate of PNP during the 2st batch test with entrapped cells from C/N0, C/N0.4, and C/N0.8 reactors at initial TAN concentrations of 50

mgN l^{-1} and 100 mgN l^{-1}. Noted that 86 µM ATU was added into the test with initial PNP concentrations of 4 mg l^{-1} and 8 mg l^{-1}.

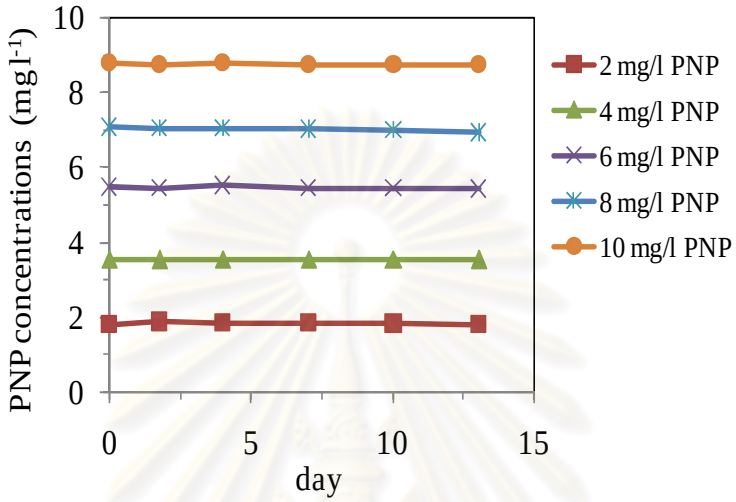

Fig. 6.5 Degradation of PNP by plain bead (no cells; abiotic control test).

Fig. 6.3 and 6.4 (as well as Table B-1 to B-3 in Appendix B) show the degradation rate of PNP during the first and second batch tests. The results showed clearly that the un-acclimated (the first batch test) and acclimated (the second batch test) entrapped cells can degrade PNP and the PNP was degraded mainly by biotic degradation. This was confirmed by limited loss of PNP in the abiotic control test using plain beads (Fig. 6.5). Some phenolic compounds such as phenol, *o*-cresol, 2,5-dimethylphenol are known to be co-substrates for AMO enzyme (Hooper et al., 1997). To confirm the inability of AOB to cometabolise PNP during NH_3 oxidation, 86 µM of ATU was added into the 2nd batch test with the initial PNP concentrations of 4 and 8 mg l^{-1} to inactivate the AMO enzyme. The results showed that no substantial NH_3 oxidation was observed (Fig. B-7 to B-12 in Appendix B). However, PNP degradation still occurred at significant levels (Fig.6.4) indicating that PNP was not degraded through cometabolism of AMO enzyme, but was degraded by PNP-degrading microorganisms that utilize PNP as main energy and carbon sources. Previously, activated sludge from municipal WWTPs has been used as inocula for acclimatizing PNP-degrading microorganisms (Tomei and Annesini, 2005; Tomei et al., 2004; 2003). Although biodegradation of PNP by heterotrophs was achieved, its

degradation pathway was not elucidated in these studies. To the best of our knowledge, two different pathways for PNP degradation have been reported, but are for pure cultures. Spain and Gibson (1991) reported the following PNP degradation pathway for *Moraxella* sp.: PNP → *p*-benzoquinone → hydroquinone → γ-hydroxymuconic semialdehyde → maleylacetic acid → β-ketoadipic acid. While, another pathway was proposed by Jain et al. (1994) for *Arthrobacter* sp.: PNP → *p*-nitrocatechol/*p*-nitroresorcinol → 1,2,4-benzenetriol → maleylacetic acid → β-ketoadipic acid.

As shown in Fig. 6.3, the degradation rates of PNP in the first batch test improved slightly at the higher initial PNP concentrations. PNP-degrading microorganisms may grow little faster at the higher PNP levels. There was no large difference in PNP degradation rates among the entrapped cells from different reactors implying that the abundance of PNP-degrading microorganisms in all un-acclimated entrapped cells were not different. Initial NH_3 concentrations were found not to affect the PNP degradation rates. For the first batch test, very slight improvement of PNP degradation rates was observed in the entrapped cells harvested from the organic loaded-reactors (C/N0.4 and C/N0.8 reactors) compared to the non organic loaded-reactor (C/N0 reactor). This phenomenon was more obvious at the higher initial PNP concentration (10 mg l^{-1}). According to the FISH results, the numbers of heterotrophs at the outer parts of the gel beads of C/N0.4 and C/N0.8 reactors were more than that of the C/N0 reactor. The higher numbers of cells created resistance for PNP to penetrate into the gel bead as a result of higher diffusion limitation. This led to a higher concentration gradient of PNP in the gel beads of the organic loaded-reactors than the non organic loaded-reactor. Subsequently, this helped reducing the concentrations of PNP inside the gel beads and allowed PNP-degrading microorganisms in the gel beads of the organic loaded-reactors to survive more and degrade PNP.

When comparing between the first and second batch tests, the degradation rates of PNP in the second batch test were higher than in the first batch test (Fig. 6.4). The numbers of PNP-degrading microorganisms might increase during the first batch

test. Wiggins et al. (1987) reported that acclimation of PNP-degrading microorganisms to mineralize PNP in sewage required time to attain sufficient cells to do the task. Similar to the first batch test, the degradation rates of PNP in the second batch test were also higher at the higher PNP concentrations than those at the lower PNP concentrations. In addition, no large differences were found in the degradation rates with different initial NH_3 concentrations. However, there was similar level in the degradation rates among the entrapped cells from the different reactors. The degradation rates of the C/N0 and C/N0.4 reactors were much higher than those of the C/N0.8 reactor. Although the higher concentration gradient of PNP in the gel beads of the C/N0.8 reactor than the C/N0 reactor can help reducing the concentrations of PNP inside the gel beads and allow PNP-degrading microorganisms in the gel beads of the C/N0.8 reactor to degrade PNP more during the acclimation period (the first batch test). However, after sufficient PNP-degrading microorganisms had already developed to mostly complete PNP degradation in the first batch test, the higher numbers of heterotrophs which appeared initially in the C/N0.8 gel beads might limit space and O_2 (and/or other nutrients) for PNP-degrading microorganisms compared to what occurred in the C/N0, and C/N0.4 gel beads. Consequently, PNP-degrading microorganisms in the C/N0.8 gel beads had lower PNP degradation in the second batch test than the microorganisms in the C/N0 and C/N0.4 gel beads. Zaidi et al. (1996) found that increasing the cell inoculum size reduced the degradation rate, and extended the PNP mineralization ability.

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6.3.3.2 Oxidation of NH_3 in batch tests

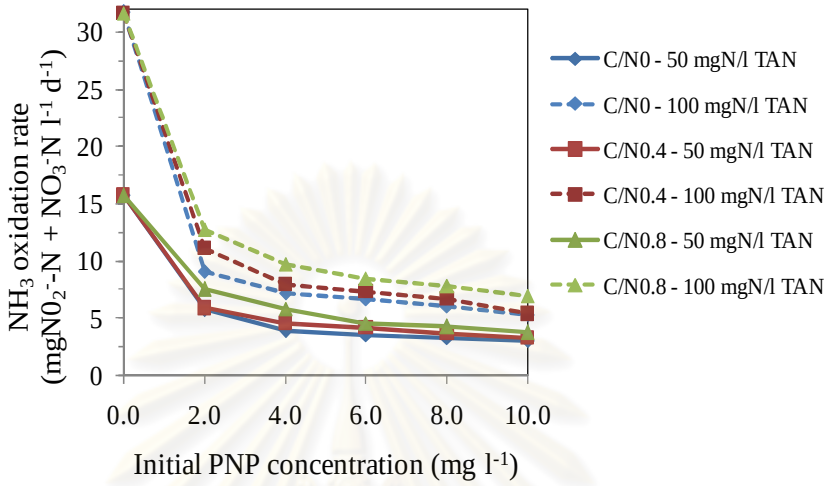

Fig. 6.6 Rate of NH_3 oxidation during the 1st batch test with entrapped cells from C/N0, C/N0.4, and C/N0.8 reactors at initial TAN concentrations of 50 mgN l^{-1} and 100 mgN l^{-1}

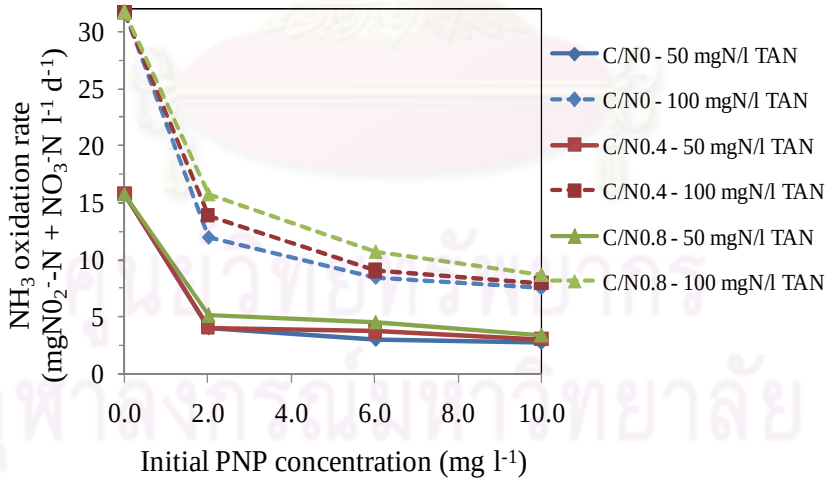

Fig. 6.7 Rate of NH_3 oxidation during the 2nd batch test with entrapped cells from C/N0, C/N0.4, and C/N0.8 reactors at initial TAN concentrations of 50 mgN l^{-1} and 100 mgN l^{-1}.

Fig. 6.6 and 6.7 (as well as Table B-4 in Appendix B) showed the rate of NH_3 oxidation during the first and second batch tests. The results showed that, for all

tests, the NH_3 oxidation rate decreased when initial PNP concentration increased. This indicates that an inhibitory effect of PNP on NH_3 oxidation was higher at the higher initial concentrations of PNP. Strong inhibitions on AOB activity by phenolic compound, such as phenol and chlorophenol have been reported (Amor et al., 2005; Texier and Gomez, 2002; Winther-Nielsen and la Cour Jansen, 1996; Benmoussa et al., 1986). However, the results in this study showed that an inhibitory effect of PNP was slightly sensitive to the initial PNP concentration especially between 6 and 10 mg l^{-1}. This observation may be explained by the fact that the entrapped gel helps protecting AOB cells by reducing the PNP levels around the contact areas inside the gel beads. Protection of cells from toxic substances by gel entrapment has been demonstrated in several studies (Buchtmann et al., 1997; Pai et al., 1995; Ferschl et al., 1991; Bettmann and Rehm, 1985; Westmeier and Rehm, 1985; Bettmann and Rehm, 1984).

NH_3 oxidation rate by the gel bead of the higher OLR reactors was higher than of lower OLR reactor both in the first and second batch tests. However, this trend of NH_3 oxidation rate did not corresponded well with the PNP degradation rates (C/N0.8 = C/N0.4 = C/N0 in the first batch test and C/N0.8 < C/N0.4 = C/N0 in the second batch test). This suggests that the NH_3 oxidation is not related to the PNP degradation. Reducing the concentration of PNP due to the higher concentration gradient of PNP rather than the degradation by PNP-degrading microorganisms inside the gel beads of the higher OLR reactors was the main reason behind the higher NH_3 oxidation rate by the gel bead of the higher OLR reactors (Fig. 6.1 [a, c, e] and Fig. 6.2).

The rate of NH_3 oxidation for all tests increased with increasing initial NH_3 concentration. This indicates that NH_3 was still the rate-limiting substrates for NH_3 oxidation of all entrapped cells. At an initial NH_3 concentration of 50 mg N l^{-1}, the rates of NH_3 oxidation were similar between in the first and second batch tests. However, there was a substantial difference in NH_3 oxidation rate between the first and second batch tests, when an initial NH_3 concentration was higher (100 mg N l^{-1}). At an initial NH_3 concentration of 100 mg N l^{-1}, the rates of NH_3 oxidation of the

second batch test were higher than that of the first batch test. The reason for this is that the higher initial concentration of NH_3 may help in recovering NH_3 oxidation after the cells were exposed to PNP shock load during the first batch test. Although the mode of action of PNP on AMO enzyme has not yet known, it was found that several compounds with similar structure to phenolic compounds, such as o-cresol, 2,5-dimethylphenol can compete for active site on AMO enzyme (Hooper et al., 1997). Thus, when more numbers of NH_3 molecules were present, they can easily compete PNP molecules for active site on AMO enzyme. Another explanation is that NH_3 can have the regulatory effect on synthesis of a subunit of AMO enzyme even after inactivation of the AMO enzyme (Hyman and Arp, 1995; 1992; Hyman and Wood, 1985). Under the condition that AOB were unable to use NH_3 as an energy source, the AMO active site containing 27 kDa polypeptide was synthesized through a de novo synthesis pathway under the presence of NH_3. Thus, in our case, under higher initial concentrations of NH_3, the additional polypeptide of AMO enzyme may provide additional active sites available for NH_3 oxidizing by other active cell of AOB in entrapped cells.

6.4 Conclusion

Effect of heterotrophs on the activity of AOB in gel beads under the inhibiting condition from a model toxic chemical, PNP was investigated. Two sequentially tests, first and second batch test, for PNP degradation and NH_3 oxidation were setted up and performed to investigate effect of heterotrophs on the activity of AOB under condition simulated the first and second time of PNP shock load in batch reactor with three different types of nitritation entrapped cells which have a different amounts of heterotrophs in the gel beads. Results showed that high initial concentrations and the second shock pulse of PNP can improve the degradation rates of PNP by nitritation entrapped cells while no significant effect of initial NH_3 concentrations was observed. The degradation rate of PNP by PNP-unacclimated entrapped cells of different nitritation reactors (during the first batch test) was similar while for acclimated cells (during the second batch test), the PNP degradation rate of the C/N0.8 reactor was lower than that of C/N0, and 0.4 as a result of its higher competition for oxygen,

space and/or nutrient between PNP-degrading and non PNP-degrading microorganisms. NH_3 oxidation (or AOB activity) by entrapped cells of the organic loaded-reactors (C/N0.4 and 0.8) had more tolerance for a PNP-inhibiting condition than that from the non organic loaded-reactor. Reducing the concentration of PNP due to the higher concentration gradient of PNP inside the entrapped cells could be the main reason behind that higher tolerance for PNP-inhibiting condition. An inhibitory effect of PNP on NH_3 oxidation became higher at higher initial concentrations of PNP. However, higher initial concentrations of NH_3 could help in improving a recovery of the NH_3 oxidation of entrapped cells after a first time of PNP shock load .

Results from this part of research implied that a serious drop or failure of partial nitrification for treating ammonia in low organic carbon (but not organic carbon-free) and high ammonia (low C/N) industrial wastewater, which may be caused by an inability to recover the AOB activity after experience with shock load of strongly toxic organic compound in industrial effluent stream, can be partly prevented in the entrapped cell based-rather than the suspended cell based-reactor as a results from reducing the penetration of toxicant by the outer layer of heterotrophs and subsequently reduce toxic sensitivity of AOB in the deeper part of the biofilm-like layer structure. Moreover, maintaining high sufficient level of ammonia in entrapped cell reactor (ie. 100 mgN l^{-1} in this study) could be one way to improve a recovery of the NH_3 oxidation of entrapped cells after experience with shock load of toxicant.

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CHAPTER VII

CONCLUSIONS AND RECOMMENDATIONS

7.1 Conclusions

Shortcut biological nitrogen removal (SBNR) is a cost effective innovative process to treat low carbon or/and high nitrogen wastewater. Partial nitrification is believed to be the rate-limiting step of the overall SBNR. Partial nitrification can be achieved by the oxidation of ammonia (NH_3) to nitrite (NO_2^-) without further oxidation of NO_2^- to nitrate. Therefore, activity of ammonia oxidizing bacteria (AOB) needs to be promoted over the activity of nitrite-oxidizing bacteria (NOB). The most two common strategies to achieve partial nitrification under normal temperature condition are to maintain oxygen (O_2)-limiting or/and free ammonia (FA)-accumulating conditions in the systems. Under low O_2 condition, growth of AOB can be higher than NOB due to the distinct in the O_2 affinity between both microorganisms. In addition, at certain levels of FA, activity of NOB can be inhibited, while the activity of AOB does not. Gel entrapment has been proposed to be a potential means to achieve partial nitrification under ammonia-rich environment. However, the determining mechanisms behind its contribution on partial nitrification have still been unclear. With the economical benefits of SBNR over the conventional nitrification - denitrification and several advantages of immobilized cells over suspended cells, the application of polyvinyl alcohol (PVA) entrapped nitrifying bacteria for treating high NH_3 wastewater based on two typical strategies, limiting dissolved oxygen (DO) and promoting FA accumulation in bulk liquid, was investigated in this study. And specific issues include the actual activity and community of working bacteria (**Chapter 4**), inoculum history and process control strategy (**Chapter 5**) and an instability conditions caused by the shock load of toxic compounds (**Chapter 6**) were mainly addressed.

To examine whether and how the two most common strategies (DO limitation and FA inhibition) for achieving partial nitrification in suspended cell system can be applied for entrapped cell system, effects of substrates (DO and FA concentrations) on NH_3 and NO_2^- oxidation (nitritation and nitratation) in both suspended and entrapped cell systems of enriched nitrifying sludge were determined using batch respirometric assay in **Chapter 4**. A fluorescence in situ hybridization (FISH) technique was also used for probing nitrifying microorganisms within the entrapment matrix since the nitritation and nitratation kinetics are related to the make-up of nitrifying community. Results showed that for both nitritation and nitratation, the maximum specific oxygen uptake rate (as a result of substrate utilization) and the apparent affinity for substrate and oxygen of suspended cells were higher than those of the corresponding entrapped cells. Under DO-limiting conditions, the specific oxygen uptake rate for nitratation reduced more than that for nitritation for suspended cells because of the higher oxygen affinity of AOB than NOB. For entrapped cells, the results were the opposite. The FA inhibitory effect was observed in the NH_3-unacclimated sludge at significant levels but the acclimated sludge was not inhibited by high FA. Moreover, the competition for space and O_2 led to segregations among AOB, NOB and heterotrophs within the gel matrix. The results from **Chapter 4** implied that FA inhibition or DO limitation can be used to maintain NO_2^- accumulation in entrapped cell systems but might not be effective strategies, which need a detailed investigation to identify one. Moreover, internal substrate and oxygen transfers would be two of the most important factors in controlling nitritation kinetics of entrapped nitrifying sludge.

After finishing the batch experiment for nitritation and nitratation kinetic in **Chapter 4**, the experiment in **Chapter 5** was designed to find out the strategies to achieve partial nitrification in continuous-flow entrapped cell nitritation reactors. In the first part of **Chapter 5**, the effect of different entrapped inoculums, including entrapment of non-nitrifying (ENN), nitrifying (EN), partial nitrifying (EPNI) and partial nitrifying (EPNII) sludge, on accelerating partial nitrification were investigated during start-up periods of continuous-flow reactors fed with high NH_3 wastewater. Results show that entrapped cells containing very low numbers of NOB (EPNII) can achieve NO_2^- accumulation at higher level and shorter timeframe (91% of NO_2^-

accumulation after 15 days of operation) than entrapped cells containing high NOB abundance (ENN, EN, and ENPI). This finding showed the effect of inoculum type on accelerating partial nitrification. However, entrapped cells containing high numbers of NOB can achieve stable NO_2^- accumulation under ambient temperature and normal bulk DO (2 mgN l^{-1} DO) condition at comparable level and timeframe (65 – 66% of NO_2^- accumulation after 30 - 42 days of operation). The control factor is expected to be the levels of available O_2 under the presence of high NH_3 concentration. This indicated that a step for preparing sludge which is readily for nitrifying or partial nitrifying, was not needed for entrapped cells. Moreover, as a result of the O_2 gradient in the gel beads, both AOB and NOB relocated themselves from the O_2-limiting zones in the inner parts of the gel beads to the O_2 available zones at the outer parts of the gel beads after the start-up period. And as a result of the competition for space and O_2, NOB aggregates were found underneath AOB aggregates in the smaller and looser forms resulting in losing partially their nitrite-oxidizing activity.

In the second part of **Chapter 5**, the most two common strategies, DO limitation and FA inhibition, were applied for partial nitrification in continuous-flow entrapped cells reactors. Effect of bulk DO or/and FA concentrations on NO_2^- accumulation in entrapped cell system was observed during long term operational period with various bulk DO and FA concentrations after succession of start-up partial nitrification. The results from the first part of **Chapter 5** recommended using the ENN entrapped cells as they require no acclimation step. In the second part, EPNI and EPNII entrapped cells were studied in parallel with the ENN entrapped cells. Results showed that NH_3 removal efficiency depended largely on the bulk DO concentration. At lower than the threshold level (1.0 mgO_2 l^{-1}), NH_3 removal was limited by the diffusion of O_2 from bulk solution into the entrapped cell while no difference in the NH_3 removal efficiency was obviously observed at higher level of bulk DO (between 1.0 and 2.0 mgO_2 l^{-1}). Under the same DO levels, NH_3 removal efficiencies tended to increase with increasing the pH levels. However, the NH_3 removal efficiencies of EPNI and EPNII were more sensitive to the acidic pH than that of ENN because ENI and ENII were previously adapted under alkaline condition rather than acidic condition. Results also showed that NH_3 removal of the entrapped cells was not

suppressed by FA inhibition. Higher NO_2^- accumulation was found at the lower concentration of bulk DO and the higher concentration of FA. When the reactors were operated under DO 0.5 mgO_2 l^-1, $NO2^-$ oxidation was suppressed mainly by low O_2 level in the reactors. However, with higher DO level (<1.0 mgO_2 l^{-1}) $NO2^-$ oxidation was mainly inhibited by FA. With the DO level of 2.0 mgO_2 l^{-1}, NO_2^- accumulation was very low due high O_2 and low FA conditions. Because the accumulation of NO_2^- depended on both concentrations of bulk DO and FA, a relative ratio of both parameters (ratio of DO/effluent FA) rather than either one is recommended to use as a control parameter for partial nitrification. It is possible to use the DO/effluent FA ratio to control a desired level of nitritation in entrapped cell nitritation reactors by adjusting the bulk DO concentrations in the reactors under certain values of pH and effluent concentrations of NH_3 to achieve the selected DO/eff FA ratios.

Moreover, because instant loading of toxic compound could limit applications of partial nitrification, the deterioration of partial nitrification in industrial wastewater treatment system needs to be prevented. In **Chapter 6**, effect of heterotrophs on the activity of AOB in entrapped cell under the inhibiting condition from a model toxic chemical, PNP, was investigated. Two sequentially tests, 1^{st} and 2^{nd} batch test, were used to investigate effect of heterotrophs on the AOB activity under the 1^{st} and 2^{nd} time of PNP shock load with nitritation entrapped cells which contained a different amounts of heterotrophs. Results showed that high initial concentrations and the 2^{nd} shock pulse of PNP can improve the degradation rates of PNP by nitritation entrapped cells while no significant effect of initial NH_3 concentrations was observed. The degradation rate of PNP by PNP-unacclimated entrapped cells of different nitritation reactors (during the 1^{st} batch test) was similar while for acclimated cells (during the 2^{nd} batch test), the PNP degradation rate of the C/N0.8 reactor was lower than that of C/N0, and 0.4 as a result of its higher competition for oxygen, space and/or nutrient between PNP-degrading and non PNP-degrading microorganisms. NH_3 oxidation (or AOB activity) by entrapped cells of the organic loaded-reactors (C/N0.4 and 0.8) had more tolerance for a PNP-inhibiting condition than that from the non organic loaded-reactor. Reducing the concentration of PNP due to the higher concentration gradient of PNP inside the entrapped cells could be the main reason behind that higher

tolerance for PNP-inhibiting condition. An inhibitory effect of PNP on NH_3 oxidation became higher at higher initial concentrations of PNP. However, higher initial concentrations of NH_3 could help in improving a recovery of the NH_3 oxidation of entrapped cells after a first time of PNP shock load. Results from **Chapter 6** (and the results of spatial distribution of microorganisms in suspended cell from **Chapter 4**) implied that a serious drop or failure of partial nitrification for treating ammonia in low organic carbon (but not organic carbon-free) and high ammonia (low C/N) industrial wastewater, which may be caused by an inability to recover the AOB activity after experience with toxic shock, can be partly prevented in the entrapped cell based-rather than the suspended cell based-reactor as a results from reducing the penetration of toxicant by the outer layer of heterotrophs and subsequently reduce toxic sensitivity of AOB in the deeper part of the biofilm-like layer structure. Moreover, maintaining high sufficient level of NH_3 in entrapped cell reactor (ie. at NH_3 of 100 mgN l^{-1} in this study) could be one way to improve a recovery of the NH_3 oxidation of entrapped cells after experience with shock load of toxicant.

From this research, it was found that high partial nitrification (up to) can be achieved by entrapped cells. Oxygen-limiting condition within an entrapment matrix is suggested to be a main control factor for achieving while an inhibitory effect of free ammonia is also proven to be another one control factor, thus, a relative ratio between concentration of dissolved oxygen and free ammonia in bulk liquid can be used to be an effective parameter to control partial nitrification. For purposing on preparing Anammox process suited-effluent which has an equal molar ratio of ammonia and nitrite nitrogen, the optimum bulk dissolved oxygen to free ammonia ratio is 0.01. And with in situ microbial localization in gel bead, an adverse effect of shock load by toxic substance on partial nitrification also can be reduced by entrapped cell as a results from reducing the penetration of toxicant by the outer layer of heterotrophs and subsequently reduce toxic sensitivity of ammonia oxidizing bacteria in the deeper part of the biofilm-like layer structure.

7.2 Recommendations

a. Although PVA gel based-entrapped cell was used throughout this study, other kinds of gel entrapped cells, which have different physical and chemical characteristics such as calcium alginate and polyethylene glycol based-entrapped cells, may be also applied for achieving partial nitrification in a similar way.
b. A study in microbial community dynamic in species level of nitrifying microorganism during achieving partial nitrification, by using some molecular fingerprinting technique such as denaturing gradient gel electrophoresis (DGGE) or PCR-cloning-sequencing, could help to confirm the role of the predominated AOB and NOB in inoculums which can largely affect the acceleration of partial nitrification by cell entrapment.
c. Because the penetration of O_2 from bulk solution into the gel beads could cause high O_2 gradient which later help to accelerate partial nitrification, study on the population dynamic of nitrifying microorganisms in the peripheral of the gel beads is likely to provide some useful information to improve an understanding for achieving partial nitrification by entrapped cell.
d. In situ kinetic study of nitrifying microorganisms (which may be done by determining the change of an actual concentrations of substrate within the gel beads by microsensor) could provide invaluable information to confirm maintaining the AOB activity and suppressing the NOB activity within the gel bead during achieving partial nitrification.
e. The proposed nitritation control parameter, ratio of DO/effluent FA, should be validated at another loading rate of ammonia and bulk DO level in operating entrapped cell nitritation reactor to see the extent of its application.
f. Although nitritation entrapped cell developed in this study was mainly applied in the nitritation step, simultaneous nitritation and denitritation (or heterotrophic denitrification via nitrite pathway) was also evidence under the presence of organic carbon (see **Chapter 6**). Thus, study to find out the operational technique to control entrapped cell for simultaneous nitritation and denitritation in single reactor rather than nitritation is recommended for further study.

REFERENCES

Abeling, U. and Seyfried, C.F. (1992). Anaerobic-aerobic treatment of high-strength ammonium wastewater nitrogen removal via nitrite. Water. Sci. Technol. 26:1007–1015.

ATSDR (Agency for Toxic Substances and Disease Registry). 1990. Toxicological Profile for Nitrophenols (Draft) [Online]: Available from: http://msds.chem.ox.ac.uk/NI/4-nitrophenol.html. [2010, February 25].

Ahn, J.H., Yu, R., and Chandran, K. (2008). Distinctive microbial ecology and biokinetics of autotrophic ammonia and nitrite oxidation in a partial nitrification bioreactor. Biotechnol. Bio. Eng.100:1078–1087.

Alleman, J.E. (1984). Elevated nitrite occurrence in biolog-ical wastewater treatment systems. Water. Sci. Technol. 17:409–419.

Amann, R., Ludwig, W., and Schleifer, K.H. (1995). Phylogenetic identification and in-situ detection of individual microbial cells without cultivation. Microbiol. Rev. 59:143–169.

Amann, R.I, Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R., and Stahl D.A. (1990). A combination of 16S rRNA targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. 56:1919–1925.

American Public Health Association. (1998). Standard methods for the examination of water and wastewater. 20thed. Washington, DC: American Public Health Association.

Amor, L., Eiroa, M., Kennes, C. and Veiga, M.C. (2005). Phenol biodegradation and its effect on the nitrification process. Water Res. 39: 2915-20.

Anthonisen, A.C., Loehr, R.C., Prakasam, T.B.S., and Srinath, E.G. (1976). Inhibition of nitrification by ammonia and nitrous acid. J Wat Pollut Control Fed. 48, 5:835–852.

Balmelle, B., Nguyen, K.M., Capdeville, B., Cornier, J.C., and Deguin, A. (1992). Study of factors controlling nitrite buildup in biological processes for water nitrification. Water Sci Technol. 26: 1017–1025.

Bartrolí, A., Carrera, J., and Prez, J. (2011). Bioaugmentation as a tool for improving the start-up and stability of a pilot-scale partial nitrification biofilm airlift reactor. Bioresource Technol .102,6: 4370-4375.

Beccari, M., Marani, D., and Ramadori, R. (1979). A critical analysis of nitrification alternatives. Water Res. 13:185–192.

Beccari, M., Passino, R., Ramadori, R., Tandoi, V. (1983). Kinetics of dissimilatory nitrate and nitrite reduction in suspended growth culture .Journal of the Water Pollution Control Federation .55,1:58-64.

Benmoussa, H., Martin, G., Tonnard, F., Richard, Y., and Leprince, A. (1986). Inhibition study of the nitrification by organic compounds. Water Res. 20: 1465-1470.

Bernet, N., Dangcong, P., Delgenes, J. P., and Moletta, R. (2001). Nitrification at low oxygen concentration in biofilm reactor. J Environ Eng. 127, 3: 266–271.

Bernet, N., Sanchez, O., Cesbron, D., Steyer, J.P., and Delgenes, J.P. (2005). Modeling and control of nitrite accumulation in a nitrifying biofilm reactor. Biochem Eng J. 24:173–183.

Bettmann, H., and Rehm, H.J. (1984). Degradation of phenol by polymer entrapped microorganisms. Appl Microbiol Biotechnol. 20: 285-290.

Bettmann, H., and Rehm, H.J. (1985). Continuous degradation of phenol(s) by entrapped in polyacrylamidehydrazide. Appl Microbiol Biotechnol. 22: 389-393.

Bhushan, B. , Chauhan, A. , Samanta, S. K. and Jain, R. K. (2000) Kinetics of biodegradation of *p*-nitrophenol by different bacteria. Biochem. Biophys. Res. Commun. 274:626-630.

Blackburne, R., Yuan, Z., and Keller, J. (2007). Partial nitrification to nitrite using low dissolved oxygen concentration as the main selection factor. Biodegradation, doi: 10.1007/s10532-007-9136-4.

Blackburne, R., Vadivelu, V.M., Yuan, Z., and Keller, J. (2007a). Determination of growth rate and yield of nitrifying bacteria by measuring carbon dioxide uptake rate .Water Environ. Res. 79,12: 2437-2445.

Blackburne, R., Vadivelu, V.M., Yuan, Z., and Keller, J. (2007b). Kinetic characterisation of an enriched *Nitrospira* culture with comparison to Nitrobacter . Water Res. 41, 14: 3033-3042.

Blackburne, R., Yuan, Z., and Keller, J. (2008). Demonstration of nitrogen removal via nitrite in a sequencing batch reactor treating domestic wastewater. Water. Res. 42: 2166–2176.

Blum, D.J.W., and Speece, R.E. (1991). A database of chemical toxicity to environmental bacteria and its use in interspecies comparisons and correlations. Res J Water Pollut Control Fed. 63: 198-207.

Bock, E., Koops, H.P., Möller, U.C., and Rudert, M. (1990). A new facultatively nitrite oxidizing bacterium, *Nitrobacter vulgaris* sp. nov. Arch Microbiol. 153: 105-110.

Bollmann, A., and Laanbroek, H.J. (2001). Continuous culture enrichments of ammonia oxidizing bacteria at low ammonium concentrations. FEMS Microbiol Ecol. 37,3:211–221.

Bollmann, A., Schmidt, I., Aaron, M., Saunders, M.A. and Nicolaisen, H.M. (2005). Influence of Starvation on Potential Ammonia-Oxidizing Activity and *amoA* mRNA Levels of *Nitrosospira briensis*. Appl Environ Microbiol. 1276–1282.

Borghei, S.M., and Hosseini, S.H. (2004). The treatment of phenolic wastewater using a moving bed biofilm reactor. Process Biochemistry. 39,10:1177-1181.

Bougard, D., Bernot, N., Cheneby, D., and Delgenes, J.P. (2006). Nitrification of a high-strength wastewater in an inverse turbulent bed reactor:effect of temperature on nitrite accumulation. Process Biochem. 41:106–113.

Buchtmann, C., Kies, U., Deckwer, W.D., and Hecht, V. (1997). Performance of three phase fluidized bed reactor for quinoline degradation on various supports at steady state and dynamic conditions. Biotechnol Bioeng. 56: 295-303.

Cao., G., Zhao, Q., Sun, X., and Zhang,T. (2002). Characterization of nitrifying and denitrifying bacteria coimmobilized in PVA and kinetics model of biological nitrogen removal by coimmobilized cells. Enzyme Microb Technol. 30:49–55.

Carrera, J., Jubany I, Carvallo, L., Chamy, R., and Lafuente, J. (2004). Kinetic models for nitrification inhibition by ammonium and nitrite in a suspended and an immobilised biomass systems. Process Biochem. 39:1159–1165.

Carucci, A.,Chiavola, A.,Majone,M., and Rolle E. (1999). Treatment of tannery wastewater in a sequencing batch reactor. Water Sci Technol. 40, 1: 253–259.

Cassidy, M.B., Lee, H., and Trevors, J.T. (1996). Environmental applications of immobilized microbial cells: A review. J Ind Microbiol. 16, 2: 79-101.

Cecen, F., and Gonenc¸ I.E. (1995). Criteria for nitrification and denitrification of high-strength wastes in 2 up-flow submerged filters. Water Environ Res. 67, 2:132–142.

Chandran, K., and Smets, B.F. (2000). Single-step nitrification models erroneously describe batch ammonia oxidation profiles when nitrite oxidation becomes rate limiting. Biotechnol. Bioeng. 68:396–406.

Chen, K.C, and Lin, Y.F. (1994). Immobilization of microorganisms with phosphorylated polyvinyl alcohol (PVA) gel. Enzyme Microb Technol. 16: 79–83.

Chen, J., Zheng, P., Yu, Y., Mahmood, Q., and Tang, C. (2010). Enrichment of high activity nitrifers to enhance partial nitrification process. Bioresource Technol.101, 19: 7293-7298 .

Chung, J., and Bae, W. (2002). Nitrite reduction by a mixed culture under conditions relevant to shortcut biological nitrogen removal. Biodegradation. 13, 3: 163–170.

Chung, J. Bae, W. Lee, Y. W. and Rittmann, B. E. (2007). Shortcut biological nitrogen removal in hybrid biofilm/suspended growth reactors. Process Biochem. 42,3: 320–328.

Ciudad, G., et al. (2005). Partial nitrification of high ammonia concentration wastewater as a part of a shortcut biological nitrogen removal process. Process Biochem. 40: 1715–1719.

Daims, H., Nielsen, J.L., Nielsen, P.H., Schleifer. K.H., and Wagner, M. (2001). In *situ* characterization of *Nitrospira*-like nitrite-oxidizing bacteria active in wastewater treatment plants. Appl Environ Microbiol. 67:5273–5284.

Daims H, Lücker S, and Wagner M. (2005). DAIME. A novel image analysis program for microbial ecology and biofilm research. Environ Microbiol .8:200–213.

de Beer, D., Srinivasan, R., and Stewart, P.S. (1994). Direct measurement of chlorine penetration into biofilms during disinfection. Appl Environ Microbiol 60:4339–4344.

Dytczak, M.A., Londry, K.L., and Oleszkiewicz, J.A. (2008). Activated sludge operational regime has significant impact on the type of nitrifying community and its nitrification rates. Water Res. 42:2320–2328.

Egli, K., Langer, C., Siegrist, H.R., Zehnder, A.J.B., Wagner, M., and van der Meer J.R. (2003). Community analysis of ammonia and nitrite oxidizers during start-up of nitritation reactors. Appl Environ Microbiol. 69: 3213–3222.

Emori, H., Mikawa, K., Hamaya, M, Yamaguchi, T., Tanaka, K., and Takeshima, T. (1996) .PEGASUS Innovative biological nitrogen removal process using entrapped nitrifiers. In: Wijffels RH, Buitelaar RM, Bucke C, Tramper J, editors. Immobilized Cells: Basics and Applications, Elsevier, Amsterdam; 546–555.

Erguder, T.H, Boon, B.N., Wittebolle, L., Marzorati, M., Verstraete, W. (2009). Environmental factors shaping the ecological niches of ammoniaoxidizing archaea. FEMS Microbiol Rev. 33:855–869.

Fdz-Polanco, F., Villaverde, S., and Garcia, P.A. (1994). Temperature effect on nitrifyin.g bacteria activity in biofilters:activation and free ammonia inhibition. Water Sci Technol. 30, 11:121–130.

Fdz-Polanco, F., Méndez, E., Urueña, M.A., Villaverde, S., and García, P.A. (2000). Spatial distribution of heterotrophs and nitrifiers in a submerged biofilter for nitrification. Water Res. 34, 16: 4081-4089.

Feng, Y.J., Tseng, S.K., Hsia, T.H., Ho, C.M., and Chou, W.P. (2007). Partial nitrification of ammonium-rich wastewater as pretreatment for anaerobic ammonium oxidation (Anammox) using membrane aeration bioreactor. J Biosci. Biioeng.104:182–187.

Ferschl, A., Loidl, M., Ditzelmiiller, G., Hinteregger, C., and Streichsbier, F. (1991). Continuous degradation of 3-chloroaniline by calcium-alginate-entrapped cells of CA28: Influence of additional substrates. Appl Microbiol Biotechnol. 35: 544-550.

Fux, C., Marchesi,V., Brunner, I, and Siegrist, H. (2004). Anaerobic ammonium oxidation of ammonium-rich waste streams in fixed-bed reactors. Water Sci. Technol. 49, 11–12:77–82.

Gali, A., Dosta,J., van Loosdrecht,M.C.M., and Mata-Alvarez, J.(2007). Two ways to achieve an anammox influent from real reject water treatment at lab-scale: Partial SBR nitrification and SHARON process. Process Biochem. 42:715–720.

Ganigue, R., Lopez, H., Balaguer, M.D., and Colprim, J. (2007). Partial ammonium oxidation to nitrite of high ammonium content urban land fill leachates. Water Res. 41: 3317–3326.

Garrido, J.M., van Benthum, W.A.J., van Loosdrecht M.C.M., and Heijnen, J.J. (1997). Influence of dissolved oxygen concentration on nitrite accumulation in a biofilm airlift suspension reactor. Biotechnol. Bioeng. 53:168–178.

Gil, K.I., and Choi, E., (2004). Nitrogen removal by recycle water nitritation as an attractive alternative for retrofit technologies in municipal wastewater treatment plants. Water Sci Technol. 49, 5– 6:39–46.

Ginestet, P., Audic, J.M., Urbain, V., and Block, J.C. (1998). Estimation of nitrifying bacterial activities by measuring oxygen uptake in the presence of the metabolic inhibitors allylthiourea and azide. Appl Environ Microbiol. 64:2266–2268.

Gupta, S.K., Gupta, S.K. and Hung, Y.-T. (2006). Treatment of pharmaceutical wastes. In: Wang, L.K., Hung, Y.-T., Lo, H.H., and Yapijakis, C., editors. Waste treatment in the process industries, CRC Press; p. 167 – 233.

Hallam, S.J., et al. (2006). Genomic analysis of the uncultivated marine crenarchaeote Cenarchaeum symbiosum. Proceedings of the National Academy of Sciences .103,48: 18296–18301.

Hanaki, K., Wantawin, C., and Ohgaki, S. (1990). Nitrification at low-levels of dissolved oxygen with and without organic loading in a suspended-growth reactor. Water Res. 24:297–302.

Hao, X., Heijnen, J.J., and van Loosdrecht, M.C.M. (2001). Sensitivity analysis of a biofilm model describing a one-stage completely autotrophic nitrogen removal (CANON) process. Biotechnol Bioeng. 77, 3:266–277.

Harada, H., Ando, H., and Momonoi, K. (1987). Process analysis of fluidized-bed biofilms reactor for denitrification. Water Sci Technol. 19, 1–2:151–162.

Hashimoto, S., and Furukawa, K. (1987). Immobilization of activated sludge by PVA-boric acid method. Biotechnol Bioeng. 30: 52-59

Helder, W., and De Vries, R.T.P. (1983). Estuarine nitrite maxima and nitrifying bacteria (Ems-Dollard estuary) .Netherlands Journal of Sea Research. 17, 1:1-18.

Hellinga, C., Schellen, A.A.J.C., van Loosdrecht, M.C.M., and Heijnen, J.J. (1998). The SHARON process: an innovative method for nitrogen removal from ammonium-rich wastewater. Water Sci. Technol. 37:135–142.

Hellinga, C., van Loosdrecht, M.C.M., and Heijnen, J.J. (1999). Model based design of a novel process for nitrogen removal from concentrated flows. Math. Comput. Modell. 5, 4:351–371.

Henze, M. (1997). Trends in advanced wastewater treatment. Water Sci. Technol. 35, 10:1-4.

Henze, M., Harremoës, P., Jansen, J. la C. and Arvin, E. (2002). Wastewater treatment –Biological and chemicalprocesses. Third Edition. Springer Verlag. Berlin Heidelberg. ISBN: 3-540-42228-5. p. 89 – 109.

Hill, B.C., and Khan, E. (2008). A comparative study of immobilized nitrifying and co-immobilized nitrifying and denitrifying bacteria for ammonia removal from sludge digester supernatant. Water Air Soil Pollut. 195:23–33.

Hiorns, W.D., et al. (1995). Amplification of 16S ribosomal RNA genes of autotrophic ammonia-oxidizing bacteria demonstrates the ubiquity of nitrosospiras in the environment. Microbiology. 141: 2793–2800.

Ho, C.M., Tseng, S.K., and Chang, Y.J. (2002). Simultaneous nitrification and denitrification using an autotrophic membrane-immobilized biofilm reactor. Lett Appl Microbiol. 35: 481-485.

Hooper, A.B., Vanelli, T., Bermann, D.J., and Arciero, D.M. (1997). Enzymology of the oxidation of ammonia to nitrite by bacteria. A Van Leeuw J Microb. 71: 59-67.

Hunik, J.H., Tramper, J., and Wijffels, R.H. (1994). A strategy to scale-up nitrification processes with immobilized cells of Nitrosomonas europaea and Nitrobacter agilis. Bioprocess Eng. 11:73–82.

Hunik, J.H., van den Hoogen, M.P., de Boer W., Smit, M., and Tramper, J. (1993). Quantitative determination of the spatial distribution of *Nitrosomonas europaea* and *Nitrobacter agilis* cells immobilized in K-carrageenan gel beads by a specific fluorescent antibody labelling technique. Appl Environ Microbiol. 59:1951–1954.

Hyman, M.R., and Arp, D.J. (1992). $^{14}C_2H_2$ and $^{14}CO_2$ -labeling studies of the de novo synthesis of polypeptides by *Nitrosomonas europaea* during recovery from acetylene and light inactivation of ammonia monooxygenase. J Biol Chem .267: 1534-1545.

Hyman, M.R., and Arp, D.J. (1995). Effects of ammonia on the de novo synthesis of polypeptides in cells of *Nitrosomonas europaea* denied ammonia as an energy source. J Bacteriol .177: 4974-4979.

Hyman, M.R., and Wood, P.M. (1985). Suicidal inactivation and labelling of ammonia monooxygenase by acetylene. Biochem J. 227: 719-725.

Isaka, K., Yoshie. S., Sumino, T., Inamori, Y.,and Tsuneda, S. (2007). Nitrification of landfill leachate using immobilized nitrifying bacteria at low temperatures. Biochem Eng J. 37:49–55.

Jain, R.K., Dreisbach, J.H., and Spain, J.C. (1994). Biodegradation of p-nitrophenol via 1,2,4-benzenetriol by an *Arthrobacter sp.*. Appl Environ Microbiol. 60: 3030-3032.

Jetten, M.S.M., Horn, S.J., and van Loosdrecht, M.C.M. (1997). Towards a more sustainable wastewater treatment system. Water Sci Technol. 3, 9: 171–180.

Jetten, M.S.M., et al. (1999). The anaerobic oxidation of ammonium. FEMS Microbiol. Rev. 22:421–437.

Jetten, M.S.M., Wagner, M., Fuerst, J., Van Loosdrecht, M., Kuenen, G., and Strous, M. (2001). Microbiology and application of the anaerobic ammonium oxidation ('anammox') process. Curr Opin Biotechnol. 12,3:283-288.

Jiang, Q.Q. and Bakken, L.R. (1999). Comparison of *Nitrosospira* strains isolated from terrestrial environments. FEMS Microbiol. Ecol. 30: 171-186.

Jokela, J., Kettunen, R., Sormunen, K., and Rintal, J. (2002). Biological nitrogen removal from municipal landfill leachate: low-cost nitrification in biofilters and laboratory scale in-situ denitrification. Water Res. 36:4079–4087.

Jones SH, and Alexander M. (1988). Effects of inorganic nutrients on the acclimation period preceding mineralization of organic chemicals in lake water. Appl Environ Microbiol 54: 3177-3179.

Joo, S.H, Kim, D.J., Yoo, I.K, Park, K., and Cha, G.C. (2000). Partial nitrification in an up-flow biological aerated filter by O_2 limitation. Biotechnol. Lett. 22:937–940.

Juretschko, S., et al. (1998). Combined molecular and conventional analysis of nitrifying bacterium diversity in activated sludge: *Nitrosococcus mobilis* and *Nitrospira*-like bacteria as dominant populations. Appl Environ Microbiol. 64:3042–3051.

Keener, W.K., and Arp, D.J. (1994). Transformations of aromatic compounds by *Nitrosomonas europaea*. Appl Environ Microbiol. 60:1914–1920.

Keller, J., Subramaniam, K., Gösswein, J., and Greenfield, P. (1997). Nutrient removal from industrial wastewater using single tank sequencing batch reactors. Water Sci Technol. 35, 6:137–144.

Kim, D.J, Chang, J.S., Lee, D.I., Han, D.W., Yoo. I.K., and Cha, G.C. (2003). Nitrification of high strength ammonia wastewater and nitrite accumulation characteristics. Water Sci Technol. 47:45–51.

Kim, D.J, and Kim, S.H. (2006). Effect of nitrite concentration on the distribution and competition of nitrite-oxidizing bacteria in nitratation reactor systems and their kinetic characteristics. Water Res. 40:887–894.

Kindaichi, T., Ito, T., and Okabe, S. (2004). Ecophysiological interaction between nitrifying bacteria and heterotrophic bacteria in autotrophic nitrifying biofilms as determined by microautoradiography-fluorescence in situ hybridization. Appl. Environ. Microbiol. 70:1641–1650.

Knowles, G., Downing, A.L., and Barrett, M.J. (1965). Determination of kinetic constants for nitrifying bacteria in mixed culture, with the aid of electronic computer, J Gen Microbiol. 38:263–278.

Könneke, M., Bernhard, A.E., De La Torre, J.R., Walker, C.B., Waterbury, J.B., and Stahl, D.A. (2005). Isolation of an autotrophic ammonia-oxidizing marine archaeon. Nature. 437, 7058:543-546.

Koops, H. P., Purkhold, U., Pommeren, R. A., Timmermann, G., and Wagner, M. (2006). The Lithoautotrophic Ammonia-Oxidizing Bacteria. Dworkin et al. (eds.), The Prokaryotes: An Evolving Electronic Resource For the Microbiological Community (third edition). 5: 778-811. New York.

Kuai, L., and Verstraete, W. (1998). Ammonium removal by the oxygen-limited autotrophic nitrification–denitrification system. Appl Environ. Microbiol. 64:4500 – 4506.

Kuscu, O.S., and Sponza, D.T. (2007). Performance of p-nitrophenol (p-NP) fed sequential anaerobic migrating blanket reactor (AMBR)/aerobic completely stirred tank reactor (CSTR) system under increasing organic loading conditions. Enzyme Microb Tech. 40: 1026-1034.

Laanbroek, H.J. and Gerards, S. (1993). Competition for limiting amounts of oxygen between *Nitrosomonas europaea* and *Nitrobacter winogradsky* grown in mixed continuous cultures. Arch Microbiol. 159:453–459.

Laanbroek, H.J., Bodelier, P.L.E., and Gerards, S. (1994). Oxygen consumption kinetics of *Nitrosomonas europaea* and *Nitrobacter hamburgensis* grown in mixed continuous cultures at different oxygen concentrations. Arch Microbiol. 161:156–162.

Lackner, S.,Terada, A., and Smets, B.F (2008). Heterotrophic activity compromises autotrophic nitrogen removal in membrane-aerated biofilms: results of a modelling study. Water Res. 42:1102–1112.

Lee, D.U., Lee, I.S., Choi, Y.D., and Bae, J.H. (2001). Effects of external carbon source and empty bed contact time on simultaneous heterotrophic and sulfur-utilizing autotrophic denitrification. Process Biochem. 36, 12:1215-1224.

Leenen, E.J.T.M., Dos Santos, V.A.P.M., Grolle, K.C.F., Tramper, J., and Wijffels R.H. (1996). Characteristics of and selection criteria for cell immobilization in wastewater treatment. Water Res. 30: 2985–2996.

Leu, H.G., Lee, C.D., Ouyang, C.F., and Tseng, H.T.(1998). Effects of organic matter on the conversion rates of nitrogenous compounds in a channel reactor under various flow conditions. Water Res. 32, 3:891–899.

Lewis, D.L., Kollig, H., and Hodson, R.E. (1986). Nutrient limitation and adaptation of microbial populations to chemical transformation. Appl Environ Microbiol. 51: 598-603.

Liang, Z. and Liu, J. (2008). Landfill leachate treatment with a novel process: Anaerobic ammonium oxidation (Anammox) combined with soil infiltration system. J Hazard Mater. 151:202–212.

Limpiyakorn, T., Sakamoto, Y., Kurisu, F., and Yagi, O. (2007). Effect of ammonia and nitrite concentrations on communities and populations of ammonia-oxidizing bacteria in laboratory-scale continuous-flow reactors. FEMS Microbiol Ecol. 60:501–512.

Lozinsky, V.I., and Plieva, F.M. (1998). Poly(vinyl alcohol) cryogels employed as matrices for cell immobilization. 3. Overview of recent research and developments. Enzyme Microb Te. 23:227-242.

Lucker, S., et al. (2010). A Nitrospira metagenome illuminates the physiology and evolution of globally important nitrite-oxidizing bacteria. Proceedings of the National Academy of Sciences of the United States of America. 107,30: 13479-13484.

Manser, R., Gujer, W., and Siegrist, H. (2005). Consequences of mass transfer effects on the kinetics of nitrifiers. Water Res. 39: 4633–4642.

Martens, H.W., Berube, P.M., Urakawa, H., Torre, J.R., and Stahl D.A. (2009). Ammonia oxidation kinetics determine niche separation of nitrifying archaea and bacteria. Nature. 461:976-981.

Metcalf and Eddy Inc. (2002). Wastewater engineering: treatment and reuse, 4th ed. New York, NY, USA: McGraw Hill. Revised by Tchobanoglous G, Burton FL, Stensel HD.

Meyer, R.L., Zeng, R.J., Giugliano, V., and Blackall, L.L. (2005). Challenges for simultaneous nitrification, denitrification, and phosphorus removal in microbial aggregates: mass transfer limitation and nitrous oxide production. FEMS Microbiol Ecol. 52: 329-338.

Mobarry, B.K., Wagner, M., Urbain, V., Rittmann, B.E., and Stahl, D.A. (1996). Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. Appl Environ. Microbiol. 62:2156–2162.

Mosquera-Corral, A., González, F., Campos, J.L., and Méndez, R. (2005). Partial nitrification in a HARON reactor in the presence of salts and organic carbon compounds. Process Biochem. 40:3109–3118.

Moussa, M.S., Hooijmans, C.M., Lubberding, H.J., Gijzen, H.J., and van Loosdrecht, M.C.M. (2005). Modelling nitrification, heterotrophic growth and predation in activated sludge. Water Res. 39: 5080-5098.

Nogueira, R., and Melo, L.F. (2006). Competition between *Nitrospira spp.* and *Nitrobacter spp.* in nitrite oxidising bioreactors. Biotechnol Bioeng. 95:169–175.

Norton, J.M., Alzerreca, J.J., Suwa, Y., and Klotz, M.G. (2002). Diversity of ammonia monooxygenase operon in autotrophic ammonia oxidizing bacteria. Arch Microbiol. 177:139–149.

Obaja, D., Macé, S., Costa, J., Sans, C., and Mata-Alvarez, J. (2003). Nitrification, denitrification and biological phosphorus removal in piggery wastewater using a sequencing batch reactor. Bioresour Technol. 87:103–111.

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

Okabe, S., Hiratia, K., Ozawa, Y., and Watanabe, Y. (1996a). Spatial microbial distributions of nitrifiers and heterotrophs in mixed-population biofilms. Biotechnol Bioeng. 50:24–35.

Okabe, S., Oozawa, Y., Hirata, K., and Watanabe, Y. (1996b). Relationship between population dynamics of nitrifiers in biofilms and reactor performance at various C:N ratios. Water Res. 30: 1563-1572.

Okabe, S., Kindaichi, T., and Ito, T. (2005). Fate of 14C-labeled microbial products derived from nitrifying bacteria in autotrophic nitrifying biofilms. Appl. Environ. Microbiol. 71:3987–3994.

Okabe, S., Satoh, H., Ito, T., and Watanabe, Y. (2004). Analysis of microbial community structure and in situ activity of nitrifying biofilms. J Water and Environt Technol. 2:65–74.

Olmos, A., Olguin, P., Fajardo, C., Razo, E., Monroy, O. (2004).Physicochemical characterization of spent caustic from the OXIMER process and sour waters from mexican oil refineries. Energy and Fuels. 18, 2:302-304.

Pai, S.L., Hsu, Y.L., Chong, N.M., Sheu, C.S, and Chen, C.H. (1995). Continuous degradation of phenol by immobilized on granular activated carbon and in calcium alginate. Biores Technol. 51: 37-42.

Park, H.D., Wells, G.F., Bae, H., Criddle, C.S. and Francis, C.A. (2006). Occurrence of ammonia-oxidizing archaea in wastewater treatment plant bioreactors. Appl. Environ. Microbiol. 72, 5643–5647.

Park, S., Bae, W., Chung, J., and Baek S.C. (2007). Empirical model of the pH dependence of the maximum specific nitrification rate. Process Biochemistry. 42,12:1671-1676.

Park, S., Bae , W., and Rittmann E.B. (2010). Operational Boundaries for Nitrite Accumulation in Nitrification Based on Minimum/Maximum Substrate Concentrations That Include Effects of Oxygen Limitation, pH, and Free Ammonia and Free Nitrous Acid Inhibition. Environ Sci Technol. 44:335–342.

Peng, Y.Z., et al. (2004). Nitrite accumulation by aeration controlled in sequencing batch reactors treating domestic wastewater. Water Sci Technol. 50: 35–43.

Peng, Y.and Zhu, G. (2006). Biological nitrogen removal with nitrification and denitrification via nitrite pathway. Appl Microbiol Biotechnol. 73:15–26.

Picioreanu, C., van Loosdrecht, M.C.M., and Heijnen, J.J. (1997). Modelling the effect of oxygen concentration on nitrite accumulation in a biofilm airlift suspension reactor. Water Sci Technol. 36: 147–156.

Philips, S., Laanbroek, H.J., and Verstraete, W. (2002). Origin, causes and effects of increased nitrite concentrations in aquatic environments. Rev Environ Sci Biotechnol. 1: 115–141.

Pollice, A., Tandoi, V., and Lestingi, C. (2002). Influence of aeration and sludge retention time on ammonium oxidation to nitrite and nitrate. Water Res. 36:2541–2546.

Prakasam, T.B.S. and Loehr, R.C. (1972). Microbial nitrification and denitrification in concentrated wastes. Water Res. 6: 859–869.

Purkhold, U., Pommerening-Roser, A., Juretschko, S., Schmidt, M.C., Koops, H.P., and Wagner, M. (2000). Phylogeny of all recognized species of ammonia-oxidisers based on comparative 16SrRNA and AmoA sequence analysis: implications for molecular diversity survey. Appl Environ Microbiol. 66: 5368–5382.

Qiao, S., et al. (2010). High-rate nitrogen removal from livestock manure digester liquor by combined partial nitritation–anammox process. Biodegradation. 21:11–20.

Rajbhandari, B.K., and Annachhatre, A.P. (2004). Modeling response of nitrifying biofilm to inhibitory shock loads. Water Science and Technology. 50:6, 53-60.

Rezouga, F., Hamdi, M., and Sperandio, M. (2009). Variability of kinetic parameters due to biomass acclimation: Case of para-nitrophenol biodegradation. Bioresource Technol. 100: 5021-5029.

Rongsayamanont, C., Limpiyakorn, T., Law, B., and Khan, E. (2010). Relationship between respirometric activity and community of entrapped nitrifying bacteria: implications for partial nitrification. Enzyme Microb and Technol. 46: 229-236.

Rostron, W.M., Stuckey, D.C., and Young, A.A. (2001). Nitrification of high strength ammonia wastewater: comparative study of immobilisation media. Water Res. 35: 1169-1178.

Ruiz, G., Jeison, D., and Chamy, R. (2003). Nitrification with high nitrite accumulation for the treatment of wastewater with high ammonia concentration. Water Res. 37:1371–1377.

Sanchez, O., Marti, M.C., Aspe, E., and Roeckel, M. (2001). Nitrification rates in a saline medium at different dissolved oxygen concentrations. Biotechnol. Lett. 23:1597–1602.

Satoh, H., Okabe, S., Norimatsu, N., and Watanabe, Y. (2000). Significance of substrate C/N ratio on structure and activity of nitrifying biofilms determined by in situ hybridization and the use of microelectrodes. Water Sci Technol. 41: 317-321.

Satoh, H., Ono, H., Rulin, B., Kamo, J., Okabe, S., and Fukushi, K. (2004). Macroscale and microscale analyses of nitrification and denitrification in biofilms attached on membrane aerated biofilm reactors. Water Res. 38: 1633-1641.

Satoh, H., Sasaki, Y., Nakamura, Y.,Okabe, S., and Suzuki, T. (2005). Use of microelectrodes to investigate the effects of 2-chlorophenol on microbial activities in biofilms, Biotechnol Bioeng. 91: 133-138.

Schleper, C., Jurgens, G., and Jonuscheit, M. (2005). Genomic studies of uncultivated archaea. Nat Rev Microbiol. 3: 479–488.

Schmid, M., Twachtmann, M., Klein, Strous, M., Juretschko S. and Jetten M., (2002). Molecular evidence for genus level diversity of bacteria capable of catalyzing anaerobic ammonium oxidation, System Appl Microbiol 23: 93–96.

Schramm, A., Larsen. L.H., Revsbech, N.P., Ramsing, N.B., Amann, R.I., and Schleifer, K.H. (1996). Structure and function of a nitrifying biofilm as determined by in situ hybridization and the use of microelectrodes. Appl Environ Microbiol. 62: 4641-4647.

Schramm, A., de Beer, D., Wagner, M.,and Amann, R. (1998). Identification and activities in situ of *Nitrosospir*a and *Nitrospira spp*. as dominant populations in a nitrifying fluidized bed reactor. Appl Environ Microbiol. 64:3480–3485.

Schramm, A., de Beer, D., van den Heuvel, J.C., Ottengraf, S., and Amann, R., (1999). Microscale distribution of populations and activities of Nitrosospira and Nitrospira spp. along a macroscale gradient in a nitrifying bioreactor: quantification by in situ hybridization and the use of microsensors. Appl Environ Microbiol. 65:3690–3696.

Sinha, B., and Annachhatre, A.P. (2007). Partial nitrification-operational parameters and microorganisms involved. Rev Environ Sci Biotechnol. 6:285–313.

Siripattanakul, S. and Khan, E. (2010). Fundamentals and Applications of Entrapped Cell Bioaugmentation for Contaminant Removal. Shah V. (eds.). Emerging Environmental Technologies. 2:147-169. Nestherlands Springer,

Sliekers, A., Third, K., Abma., W., Kuenen, J.G., and Jetten, M.S.M. (2003). CANON and Anammox in a gaslift reactor. FEMS Lett. 218:339–344.

Spain, J.C., and Gibson, D.T. (1991). Pathway for biodegradation of p-nitrophenol in a Moraxella sp. Appl Environ Microbiol. 57: 812-819.

Starkenburg, S.R., et al. (2006). Genome sequence of the chemolithoautotrophic nitrite-oxidizing bacterium *Nitrobacter winogradskyi* Nb-255. Appl Environ. Microbiol. 72, 3: 2050–2063.

Steinmuller, W., and Bock, E. (1976). Growth of Nitrobacter in the presence of organic matter. I, Mixotrophic growth. Arch Microbiol .108: 299-304.

Steinmuller, W., and Bock, E. (1977). Enzymatic studies on autotrophically, mixotrophically and heterotrophically grown Nitrobacter agilis with special reference to nitrite oxidase. Arch Microbiol.115: 51-54.

Stenstrom, M.K., and Poduska, R.A. (1980). The Effect of Dissolved Oxygen concentration on Nitrification. Water Res. 14:643.

Stephenson, T., Lester, J.N., and Perry, R. (1984). Acclimatisation to nitrilotriacetic acid in the activated sludge process. Chemosphere .13: 1033-1040.

STOWA.1995. Treatment of nitrogen rich return flows of sewage treatment plants. Evaluation of Dutch pilot plant research projects (in Dutch). STOWA report: 95–108.

Stratton, F.E., and Mc Carty, P.L. (1967). Microbiological aspects of ammonia oxidation of swine waste. Can J Microbiol. 37: 918–923.

Strous, M., Van Gerven, E., Kuenen, J.G., and Jetten, M.S.M. (1997). Effects of aerobic and microaerobic conditions on anaerobic ammonium-oxidizing (Anammox) sludge. Appl Environ Microbiol. 63:2446–2448.

Strous, M., Heijnen, J.J., Kuenen, J.G., and Jetten, M.S.M. (1998). The sequencing batch reactor as a powerful tool for the study of slowly growing anaerobic ammonium-oxidizing icroorganisms, Appl Microbiol Biotechnol. 50:589–596.

Suzuki, I., Dular, U., and Kwok, S.C. (1974). Ammonia or ammonium ion as substrate for oxidation by *N.* europaea cells and extracts. J Bacteriol. 120: 556–558.

Suárez-Ojeda, M.E., Guisasola, A., and Carrera, J. (2010). Inhibitory impact of quinone-like compounds over partial nitrification. Chemosphere. 80, 4: 474-480 .

Tam, N.F.Y., Wong, Y.S., and Leung, G. (1992) .Effect of exogenous carbon sources on removal of inorganic nutrients by the nitrification denitrification process. Water Res .26:1229-1236.

Tanaka, H., and Dunn, I.J. (1992). Kinetics of biofilm nitrification. Biotechnol Bioeng. 24:669–689.

Terada, A., Lackner, S., Kristensen, K., and Smets, B.F. (2010). Inoculum effects on community composition and nitritation performance of autotrophic nitrifying biofilm reactors with counter-diffusion geometry. Environ Microbiol. 12, 10: 2858-2872.

Texier, A.C., and Gomez, J. (2002). Tolerance of nitrifying sludge to *p*-cresol. Biotechnol Lett. 24: 321-324.

Third, K.A., Sliekers, O., Kuenen, J.G., and Jetten, M.S.M. (2001). The CANON system Completely Autotrophic Nitrogen-removal Over Nitrite) under ammonium limitation: interaction and competition between three groups of bacteria. Syst Appl Microbiol. 24:588–596.

Tokutomi, T. (2004). Operation of a nitrite-type airlift reactor at low DO concentration. Water Sci Technol. 49,5–6: 81–88.

Tomei, M.C., Annesini, M.C., Luberti, R., Cento, G., and Senia, A. (2003). Kinetics of 4-nitrophenol biodegradation in a sequencing batch reactor. Water Res. 37: 3803-3814.

Tomei, M.C., Annesinib, M.C., and Bussolett, S. (2004). 4-Nitrophenol biodegradation in a sequencing batch reactor: kinetic study and effect of filling time. Water Res. 38: 375–384.

Tomei, M.C., and Annesini, M.C. (2005). 4-Nitrophenol biodegradation in a sequencing batch reactor operating with aerobic-anoxic cycles. Environ Sci Technol. 39: 5059-5065.

Turk, O., and Mavinic, D.S. (1989). Maintaining nitrite build-up in a system acclimated to free ammonia. Water Res. 23:1383–1388.

Udert, K., Fux, C., Munster, M., Larsen, T., Siegrist, H., and Gujer, W. (2003). Nitrification and autotrophic denitrification of source-separated urine. Water Sci. Technol. 48, 1: 119–130.

Uemoto, H., Ando, A., and Saiki H. (2000). Effect of oxygen concentration on nitrogen removal by *Nitrosomonas europaea* and *Paracoccus denitrificans* immobilized within tubular polymeric gel. J of Biosci and Bioeng . 90: 654–660.

U.S. Environmental Protection Agency. (2000). Technology Transfer Network Air Toxics Web Site: 4-nitrophenol. [Online]: Available from http://www.epa.gov/ttnatw01/hlthef/nitrophe.html . [2010, February 25].

Urakawa, H., Tajima, Y., Numata, Y., Tsuneda, S. (2008). Low Temperature Decreases the Phylogenetic Diversity of Ammonia-Oxidizing Archaea and Bacteria in Aquarium Biofiltration Systems. Appl. Environ. Microbiol. 74: 894-900.

Vadivelu, V.M., Keller, J., and Yuan, Z. (2007). Effect of free ammonia on the respiration and growth processes of an enriched *Nitrobacter* culture. Water Res. 41:826–834.

van Dongen U., Jetten, M.S.M., and van Loosdrecht, M.C.M. (2001). The SHARON ® -Anammox® process for treatment of ammonium rich wastewater. Water Sci Technol. 44, 1: 153–160.

van Hulle, S.W.H., Volcke, E.I.P., López Teruel, J., Donckels, B., van Loosdrecht, M.C.M., and Vanrolleghem, P.A. (2007). Influence of temperature and pH on the kinetics of the SHARON nitritation process. J Chem Technol Biotechnol. 82: 471–480.

van Hulle, S.W.H., et al. (2005) Construction, start-up and operation of a continuously aerated laboratory-scale SHARON reactor in view of coupling with an Anammox reactor. Water SA . 31:327–334.

van Hulle, S.W.H., Vandeweyer, H.J.P., Meesschaert, B.D., Vanrolleghem, P.A., Dejans, P., and Dumoulin, A. (2010). Engineering aspects and practical application of autotrophic nitrogen removal from nitrogen rich streams . Chem Eng J .162,1: 1-20.

van Kempen, R., Mulder, J.W., and van Loosdrecht, M.C.M. (2001). Overview: full scale experience of the SHARON process for treatment of rejection water of digested sludge dewatering. Water Sci Technol. 44:145–152.

Vadivelu, V.M., Keller, J., and Z. Yuan. (2007). Free ammonia and free nitrous acid inhibition on the anabolic and catabolic processes of Nitrosomonas and Nitrobacter. Water Sci Technol. 56, 7:89–97.

Villaverde, S., and Garcia-encina, P.A., and Fdz-Polanco, F. (1997). Influence of pH over nitrifying biofilms activity in submerged biofilters. Water Res 31,5:1180–1186.

Villaverde, S., Fdz-Polanco, F., and Garcia-encina, P.A. (2000). Nitrifying biofilms acclimation to free ammonia in submerged biofilters Start-up influence. Water Res. 34:602–610.

Vogelsang, C., Husby, A., and Østgaard K. (1997). Functional stability of temperature-compensated nitrification in domestic wastewater treatment obtained with PVA-BQ/alginate gel entrapment. Water Res. 31: 1659–1664.

Vogelsang, C., Schramm, A., Picioreanu, C., van Loosdrecht, M.C.M., and Østgaard K. (2002). Microbial community analysis by FISH for mathematical modeling of selective enrichment of gel-entrapped nitrifiers obtained from domestic wastewater. Hydrobiologia. 469: 165-178.

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

Wagner, M., and Loy, A. (2002). Bacterial community composition and function in sewage treatment system. Environ Biotechnol. 13:218–227.

Wang, R., et al. (2009). Nitritation performance and biofilm development of co-and counter-diffusion biofilm reactors: modeling and experimental comparison. Wat Res. 43,10: 2699-709.

Westmeier, F., and Rehm, H.J. (1985). Biodegradation of 4-chlorophenol by entrapped sp. A 7-2. Appl Microbiol Biotechnol. 22: 301-305.

Wiesmann, U. (1994). Biological nitrogen removal from wastewater. Advances in Biochemical Engineering/Biotechnology. 51: 113–154.

Wiggins, B.A., Jones, S.H., and Alexander, M. (1987). Explanations for the acclimation period preceding the mineralization of organic chemicals in aquatic environments. Appl Environ Microbiol. 53: 791-796.

Wijffels, R.H., and Tramper, J. (1995). Nitrification by immobilized cells. Enzyme Microb Technol. 17:482–492.

Winther-Nielsen, M., and la Cour Jansen, J. (1996). The role of the sludge in nitrification inhibition tests. Water Sci Technol. 33: 93-100.

Wyffels, S., et al. (2004). Modelling and simulation of oxygen-limited partial nitritation in a membrane-assisted bioreactor (MBR), Biotechnol. Bioeng. 86:531–542.

Xu, X, Stewart, PS, and Chen, X. (1996). Transport limitation of chlorine disinfection of Pseudomonas aeruginosa entrapped in alginate beads. Biotechnol Bioeng 49:93–100.

Xue, Y., Yang, F., Liu, S., and Fu, Z. (2009). The influence of controlling factors on the start-up and operation for partial nitrification in membrane bioreactor. Bioresour Technol. 100:1055–1060.

Yamamoto, T., Takaki, K., Koyama, T., and Furukawa, K. (2008). Long-term stability of partial nitritation of swine wastewater digester liquor and its subsequent treatment by Anammox. Bioresour Technol. 99, 14:6419–6425.

Yan, J., and Hu, Y.Y. (2009). Partial nitrification to nitrite for treating ammonium-rich organic wastewater by immobilized biomass system. Bioresource Technol.100: 2341-2347.

Yang, L. and Alleman, J.E. (1992). Investigation of batchwise nitrite build-up by an enriched nitrification culture. Water Sci Technol. 26: 997–1005.

Yang, P.Y., Zhang, Z,Q,, and Jeong, B.G. (1997). Simultaneous removal of carbon and nitrogen using an entrapped-mixed-microbial-cell process. Water Res. 31: 2617-2625.

Yi, S., Zhuang, Q.W., Wu, B., Tay. S.T.L., and Tay, J.H. (2006). Biodegradation of *p*-nitrophenol by aerobic granules in a sequencing batch reactor. Environ Sci Technol. 40: 2396-2401.

Yoo, H., Ahn, K., Lee H., Lee, K., Kwak, Y., and Song, K. (1999). Nitrogen removal from synthetic wastewater by simultaneous nitrification and denitrification (SND) via nitrite in an intermittently-aerated reactor. Water Res. 33: 145–154.

Zaidi, B.R.,Imam, S.H., and Greene, R.V. (1996). Accelerated biodegradation of high and low concentrations of p- nitrophenol (PNP) by bacterial inoculation in industrial wastewater: The role of inoculum size on acclimation period. Curr Microbiol. 33: 292-296.

Zeng, R.J., Lemaire, R., Yuan, Z., and J. Keller. (2004) . A novel wastewater treatment process: simultaneous nitrification, denitrification and phosphorus removal, Water Sci. Technol. 50 (10), pp. 163–170

Zhang, T.C., Fu, Y.C., and Bishop PL. (1995). Competition for substrate and space in biofilms. Water Environ Res .67: 992-1003.

Zhang, T.C., and Bishop, P.L. (1996). Evaluation of substrate and pH effects in a nitrifying biofilm. Water Environ Res .68: 1107-1115.

Zhang, S.Y., Wang, J.S., Jiang, Z.C., and Chen, M.X. (2000). Nitrite accumulation in an attapulgas clay biofilm reactor by fulvic acids. Bioresource Technol. 73: 91–93.

Zhang, L.L., Wu, Z.J., Shi, Y.F., Chen, L.J., Song, Y.C., and Juan, Y.H. (2010) .Inhibitory Effects of Aromatic Compounds on Soil Nitrification . Pedosphere 20: 326-333.

Zhu, S., and Chen, S. (2001) . Effects of organic carbon on nitrification rate in fixed film biofilters. Aquacult Eng. 25: 1-11.

Appendices

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

Appendix A

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

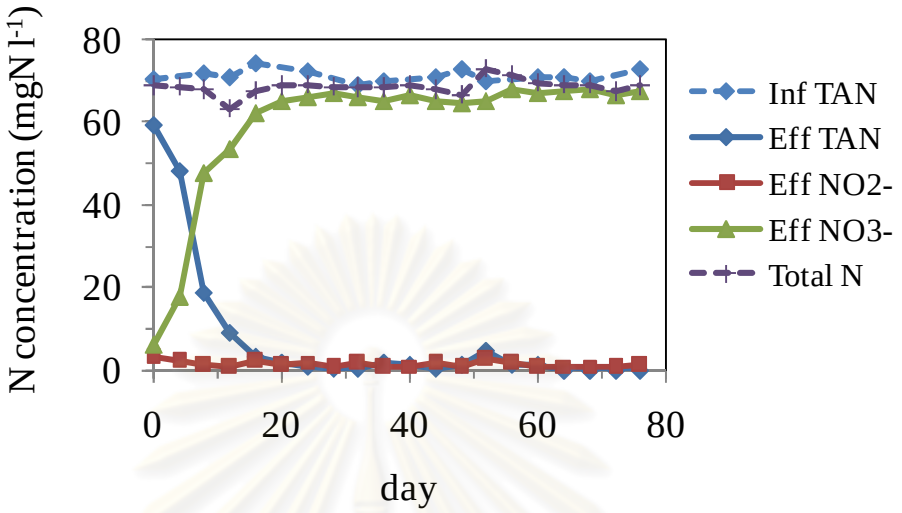

Fig. A-1 Monitoring results of laboratory-scale reactors during preparation of N sludge.

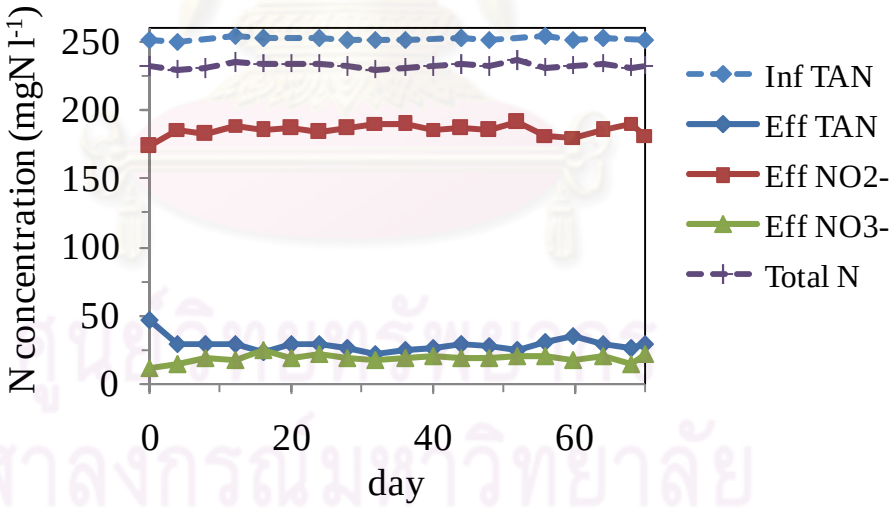

Fig. A-2 Monitoring results of laboratory-scale reactors during preparation of PNI sludge.

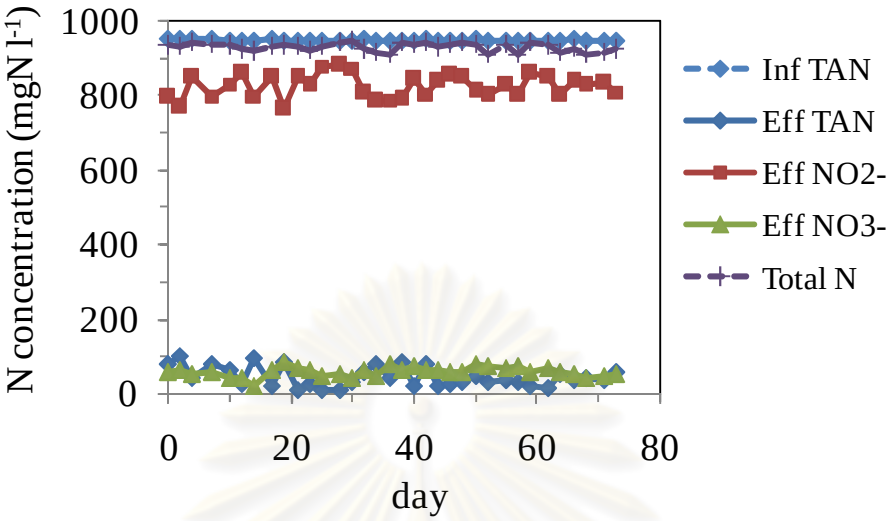

Fig. A-3 Monitoring results of laboratory-scale reactors during preparation of PNII sludge.

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

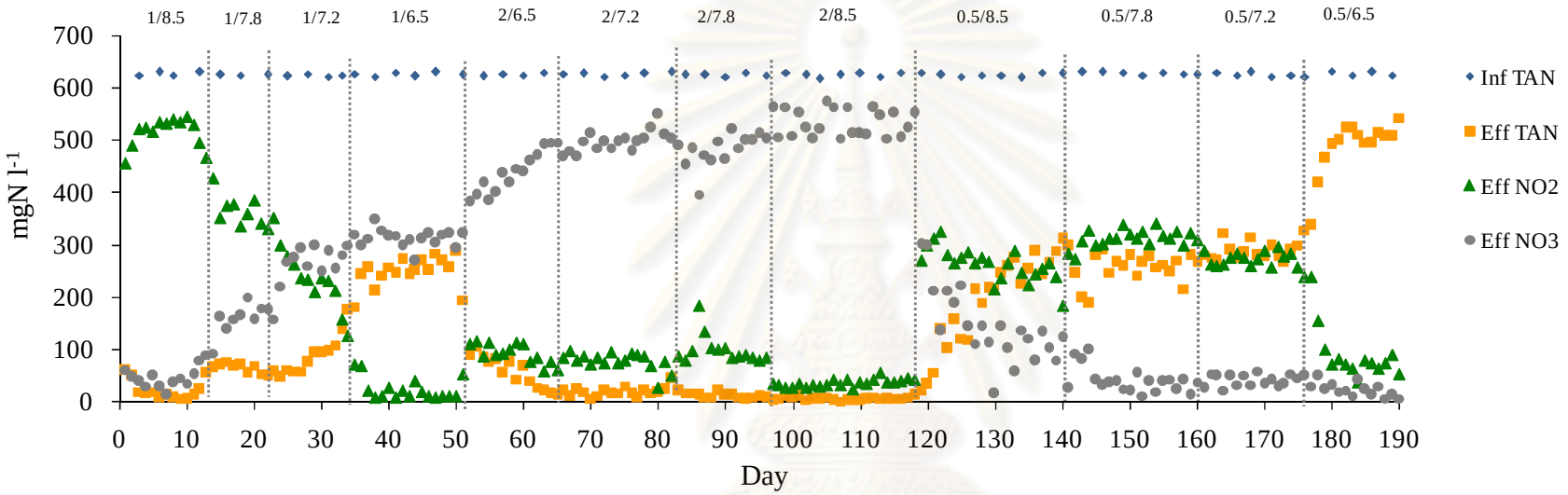

Fig.A-4 Monitoring results of long term operating ENN nitritation reactor at various bulk DO concentrations and pH

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

172

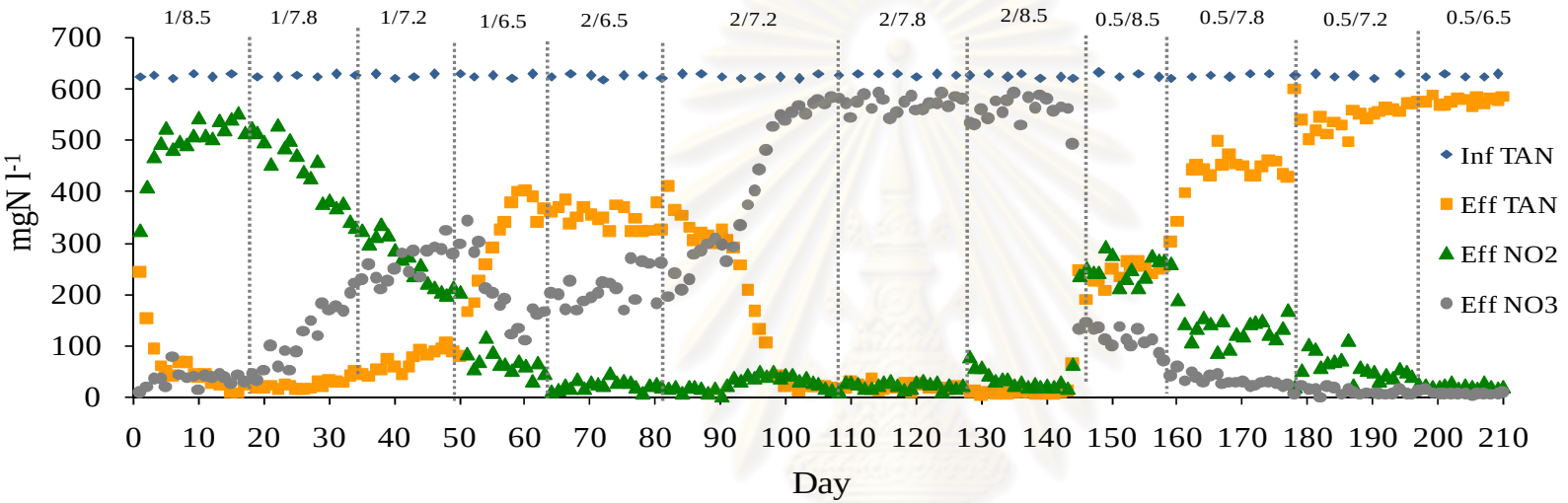

Fig.A-5 Monitoring results of long term operating EPNI nitritation reactor at various bulk DO concentrations and pH

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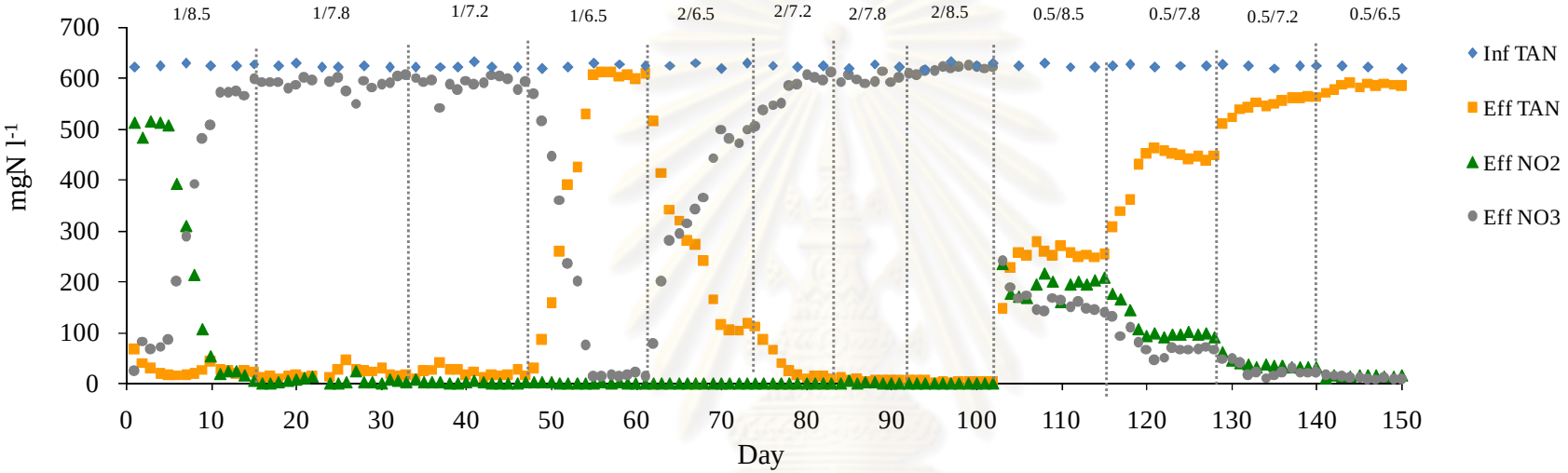

Fig.A-6 Monitoring results of long term operating EPNII nitritation reactor at various bulk DO concentrations and pH

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APPENDIX B

ศูนย์วิทยทรัพยากร
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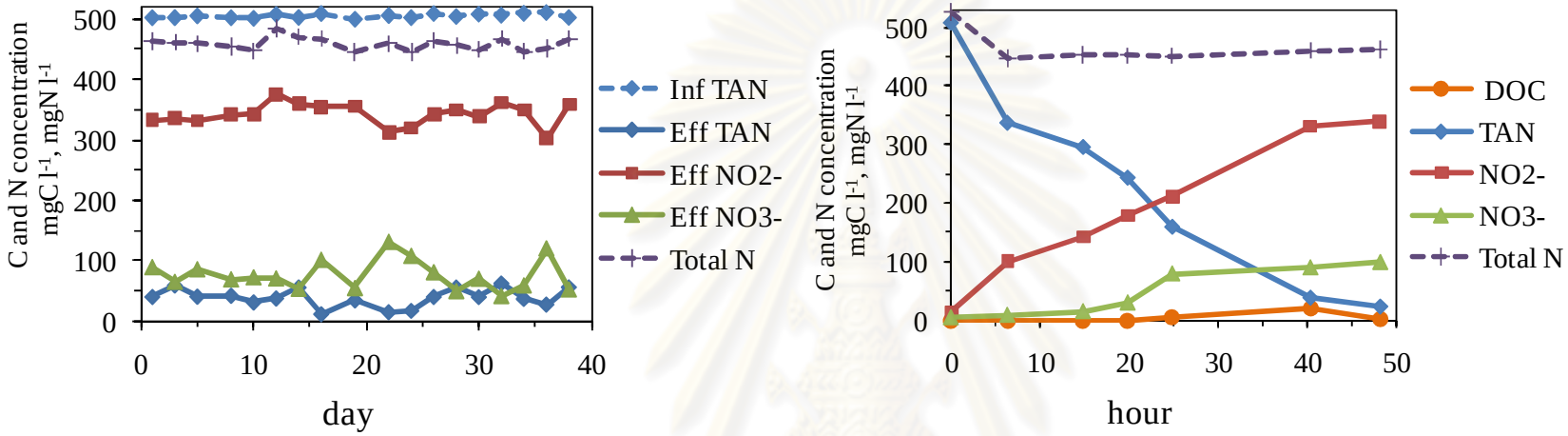

Fig. B-1 C and N concentrations in CN0 entrapped cell nitritation reactor during steady state (Right) and each cycle (Left)

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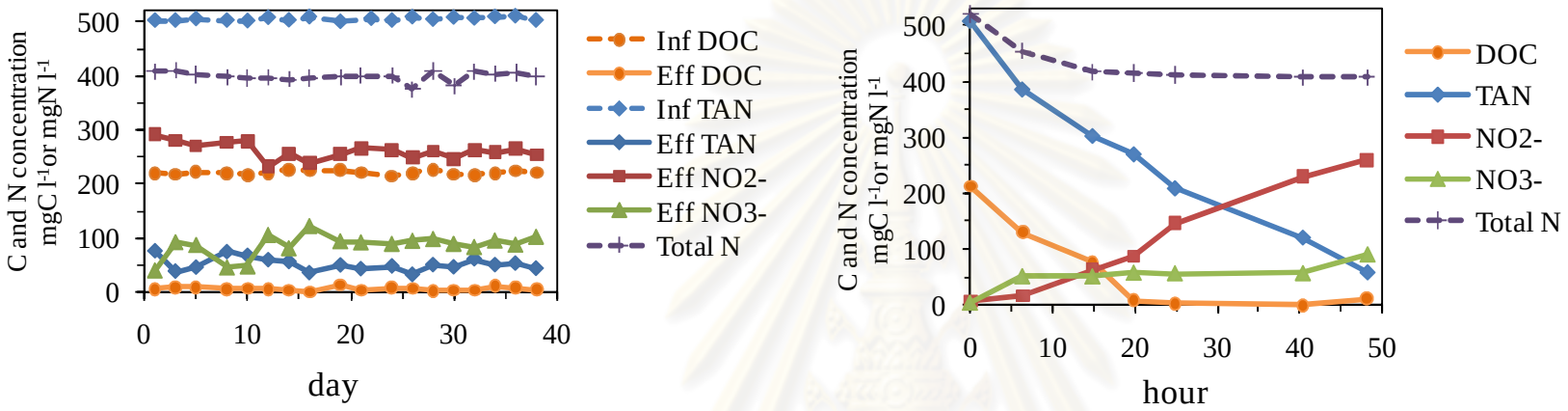

Fig. B-2 C and N concentrations in C/N0.4 entrapped cell nitritation reactor during steady state (Right) and each cycle (Left)

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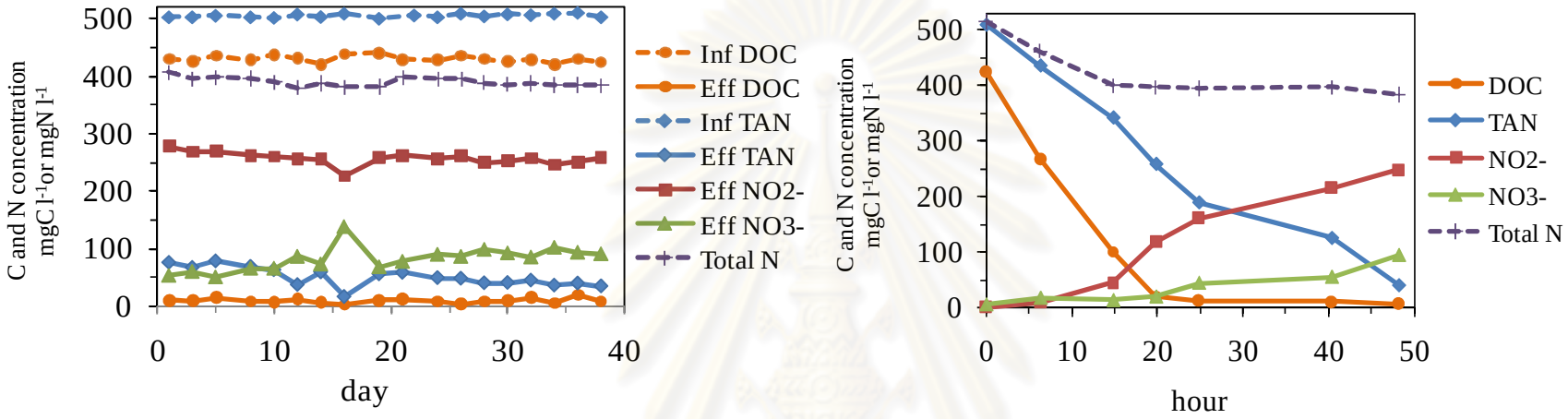

Fig. B-3 C and N concentrations in C/N0.8 entrapped cell nitritation reactor during steady state (Right) and each cycle (Left)

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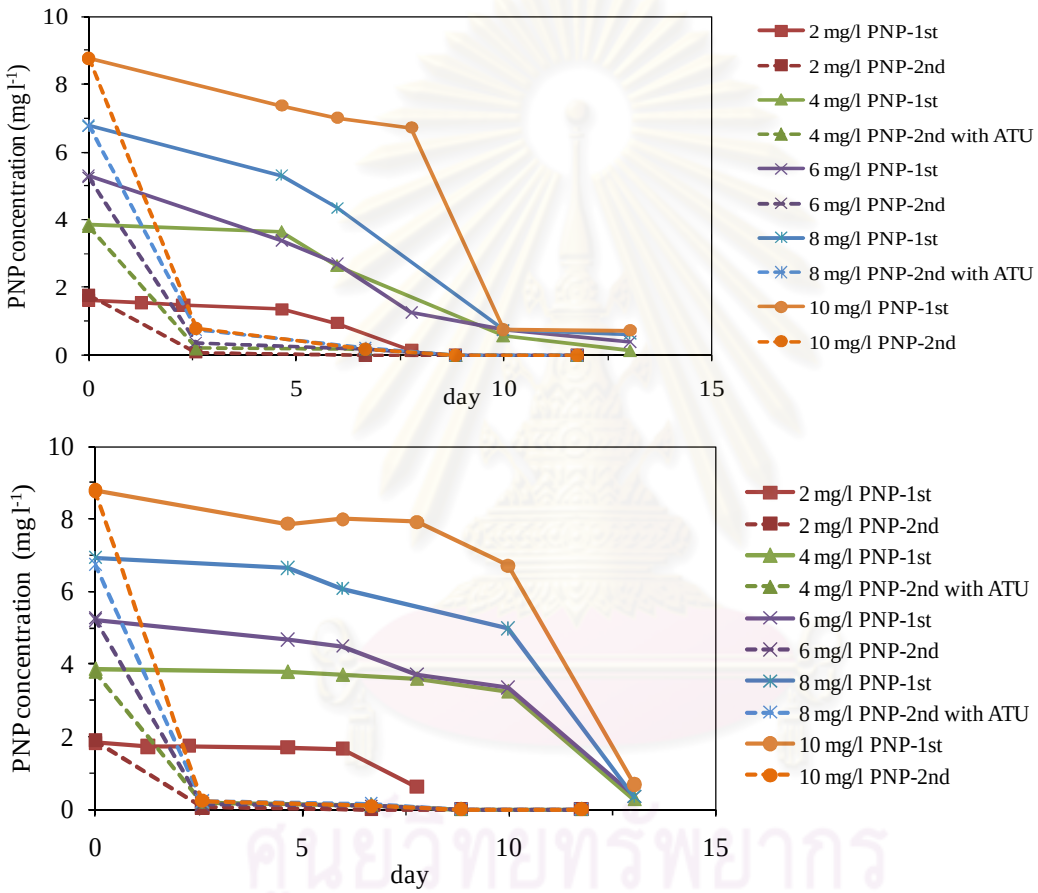

Fig. B-4 Degradation of PNP (1st and 2nd batch test) by C/N0 entrapped cells at initial TAN concentrations of 50 mgN l^{-1} (Above) and 100 mgN l^{-1} (Below)

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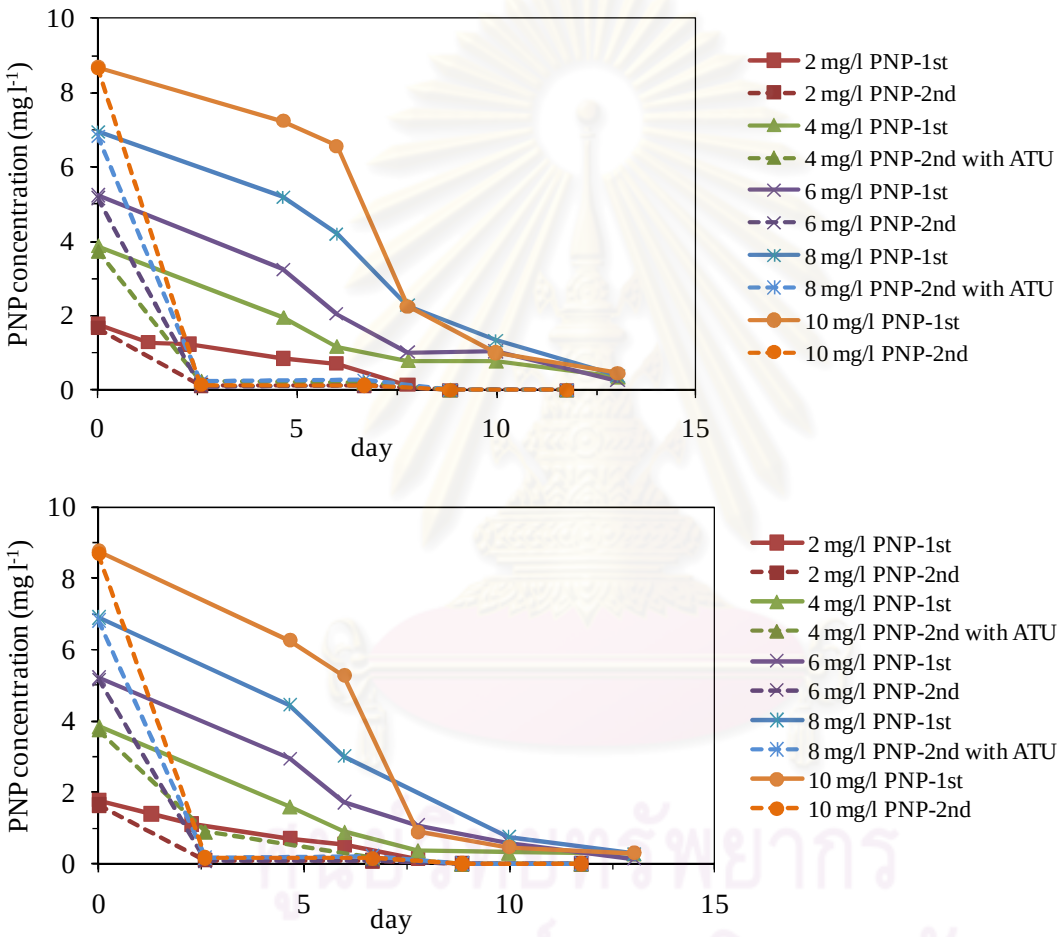

Fig. B-5 Degradation of PNP (1st and 2nd batch test) by C/N0.4 entrapped cells at initial TAN concentrations of 50 mgN l^{-1} (Above) and 100 mgN l^{-1} (Below)

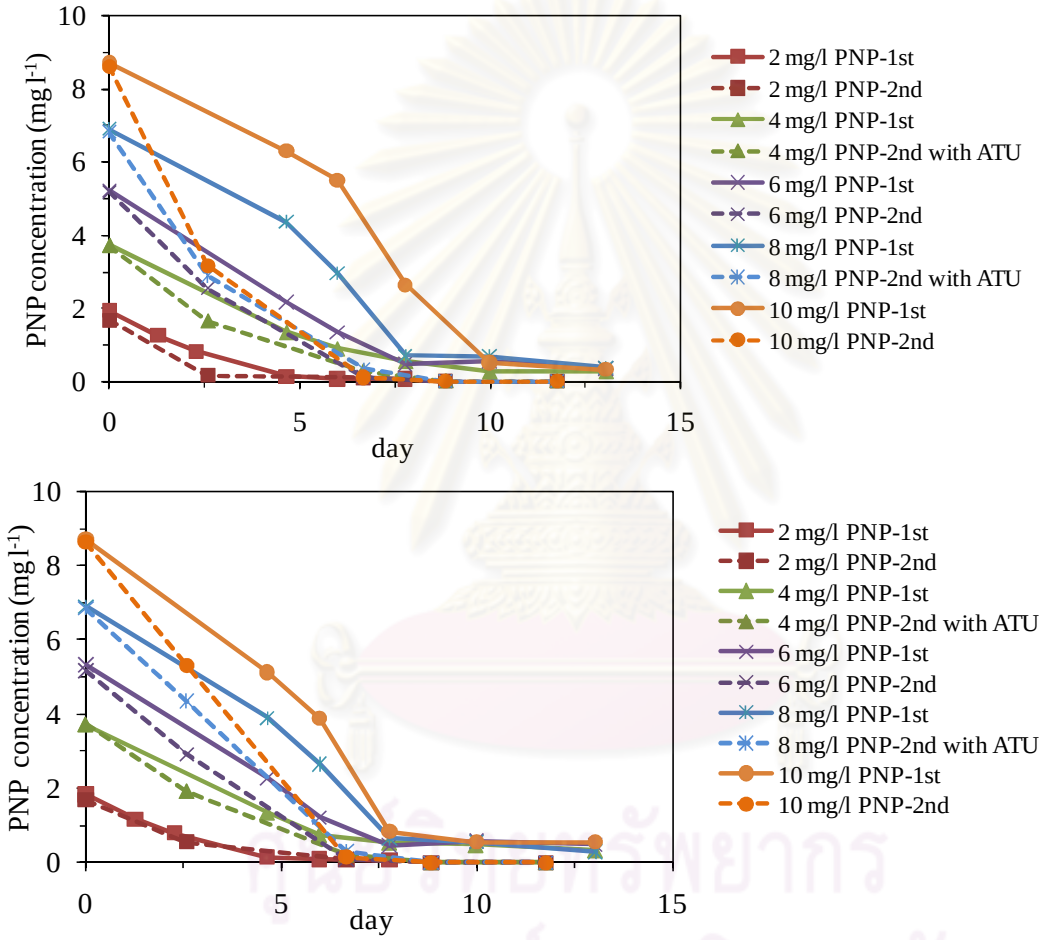

Fig. B-6 Degradation of PNP (1st and 2nd batch test) by C/N0.8 entrapped cells at initial TAN concentrations of 50 mgN l^{-1} (Above) and 100 mgN l^{-1} (Below)

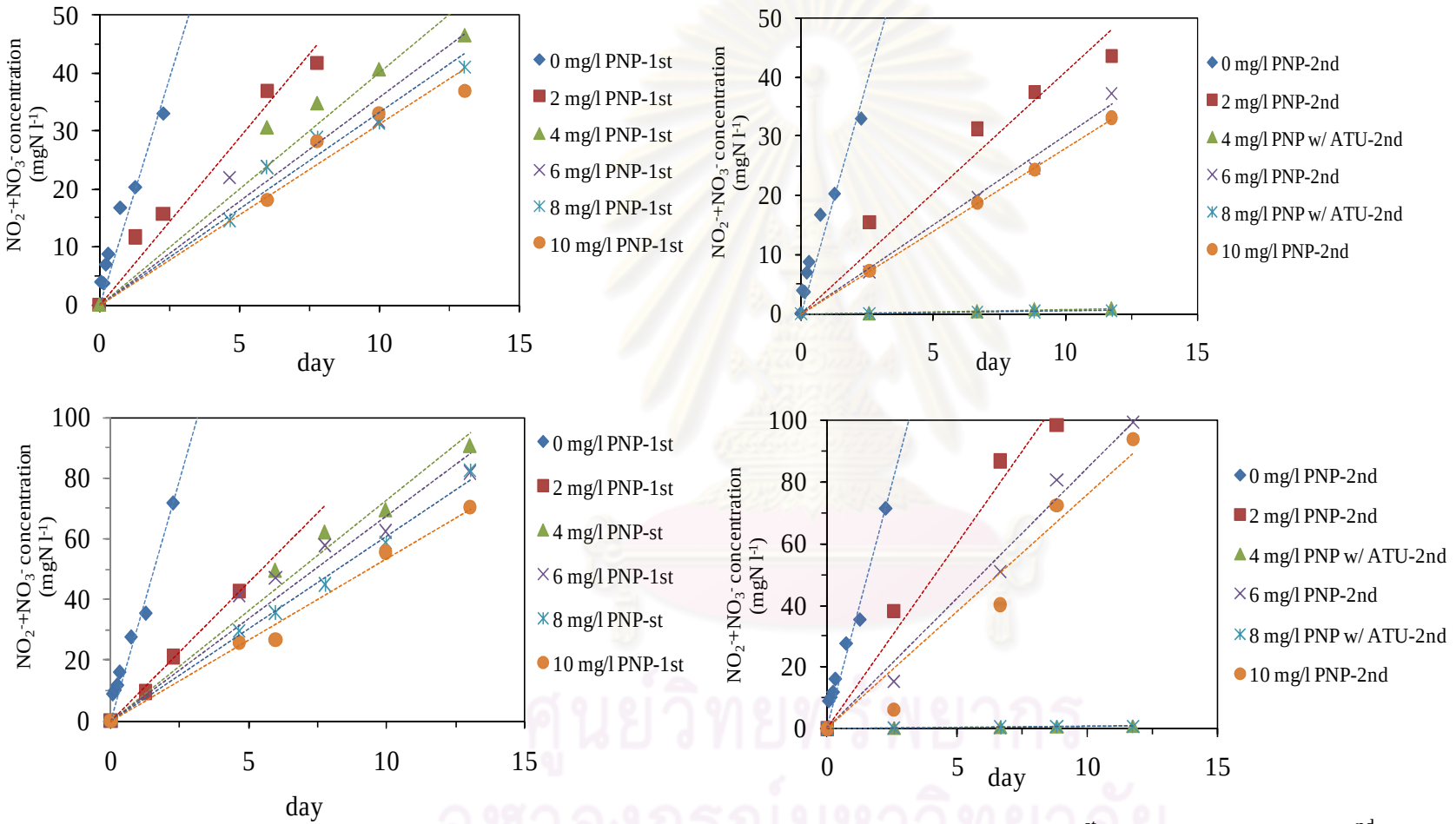

Fig. B-7 Concentrations of nitrite plus nitrate during PNP degradation by C/N0 entrapped cells; 1st batch test (Left) and 2nd batch test (Right); initial TAN concentrations of 50 mgN l^{-1} (Top) and 100 mgN l^{-1} (Bottom)

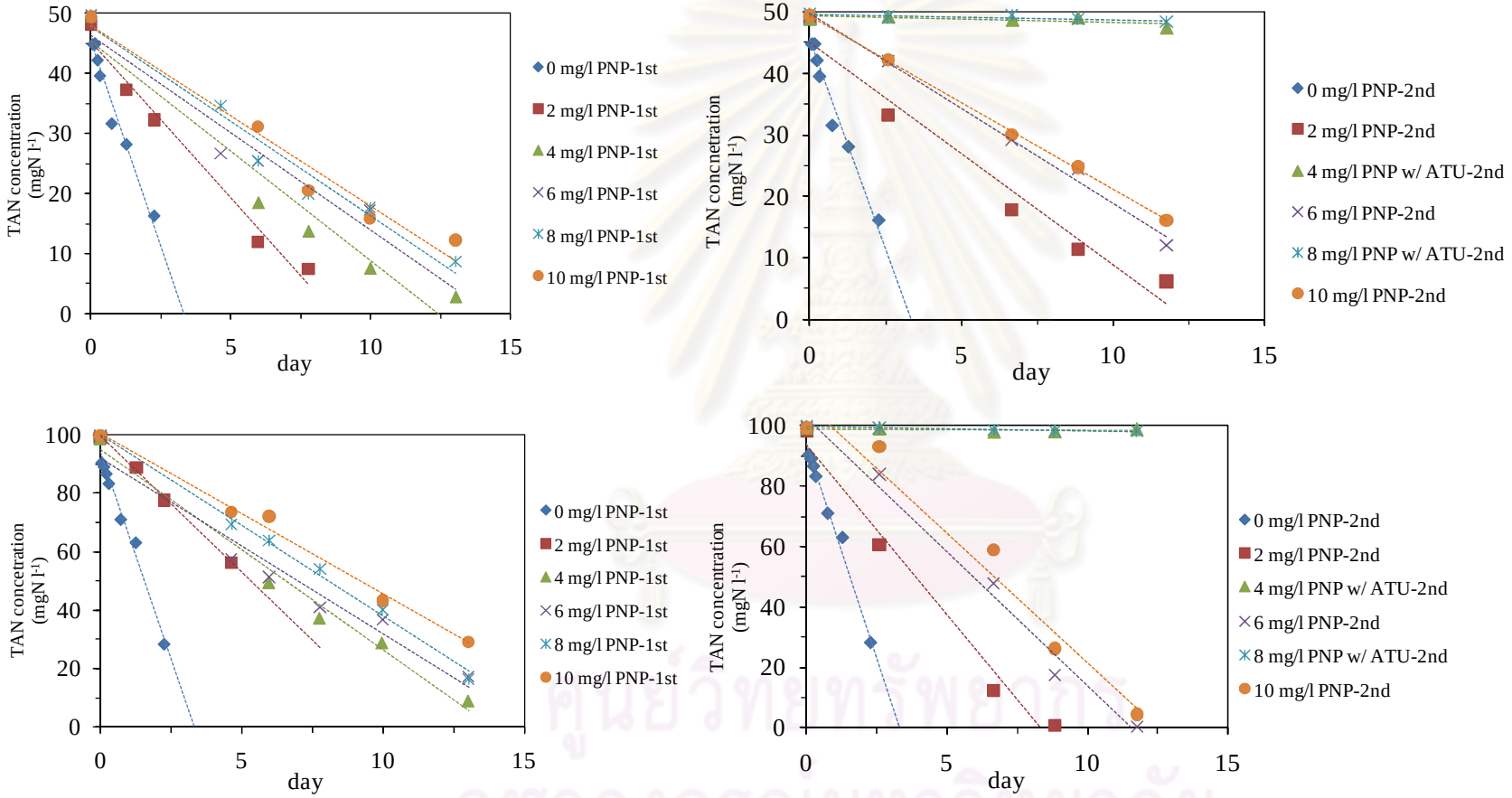

Fig. B-8 Concentrations of TAN during PNP degradation by C/N0 entrapped cells; 1st batch test (Left) and 2nd batch test (Right); initial TAN concentrations of 50 mgN l^{-1} (Top) and 100 mgN l^{-1} (Bottom)

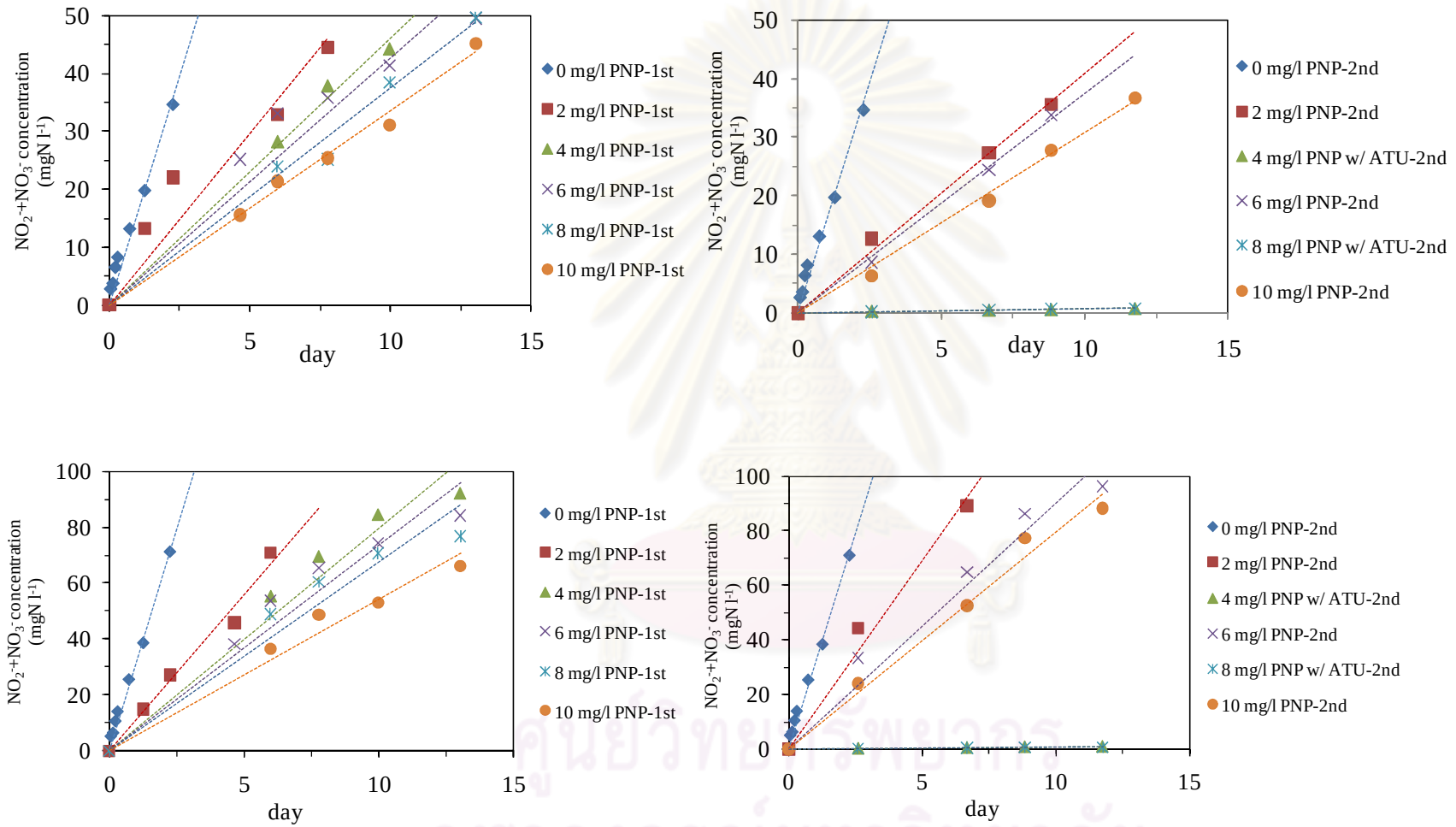

Fig. B-9 Concentrations of nitrite plus nitrate during PNP degradation by C/N0.4 entrapped cells; 1st batch test (Left) and 2nd batch test (Right); initial TAN concentrations of 50 mgN l^{-1} (Top) and 100 mgN l^{-1} (Bottom)

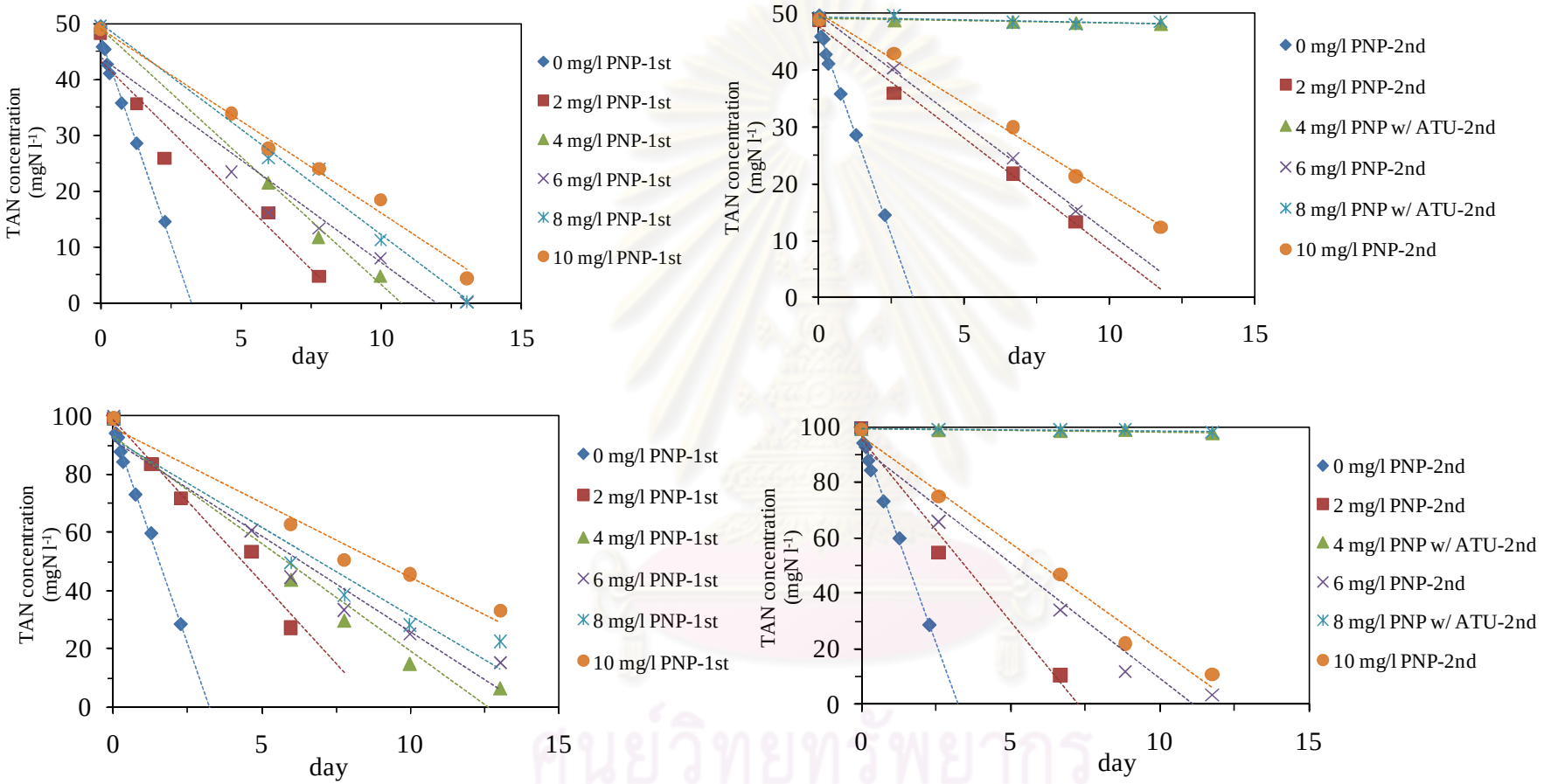

Fig. B-10 Concentrations of TAN during PNP degradation by C/N0.4 entrapped cells; 1st batch test (Left) and 2nd batch test (Right); initial TAN concentrations of 50 mgN l^{-1} (Top) and 100 mgN l^{-1} (Bottom)

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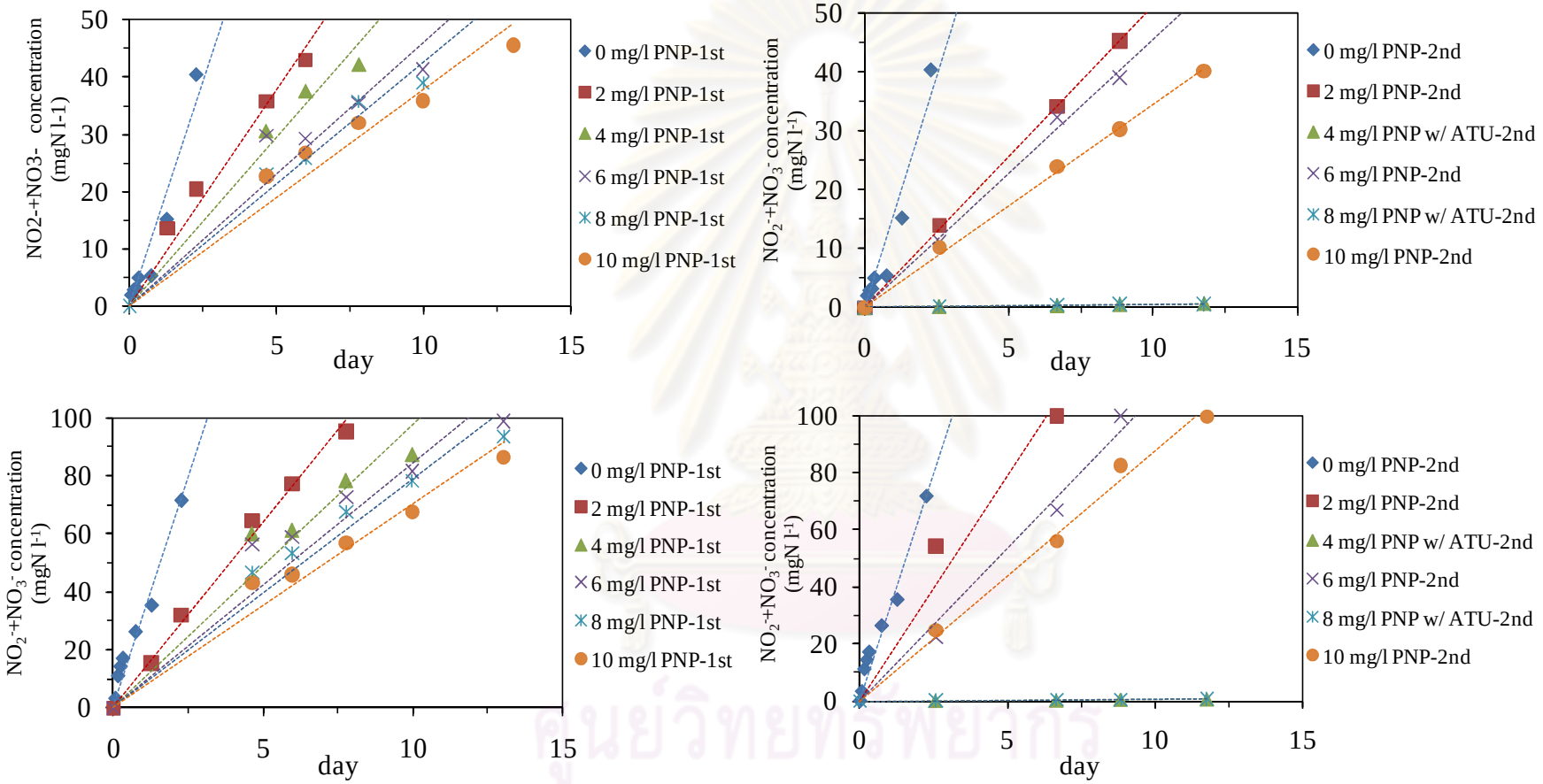

Fig. B-11 Concentrations of nitrite plus nitrate during PNP degradation by C/N0.8 entrapped cells; 1st batch test (Left) and 2nd batch test (Right); initial TAN concentrations of 50 mgN l^{-1} (Top) and 100 mgN l^{-1} (Bottom)

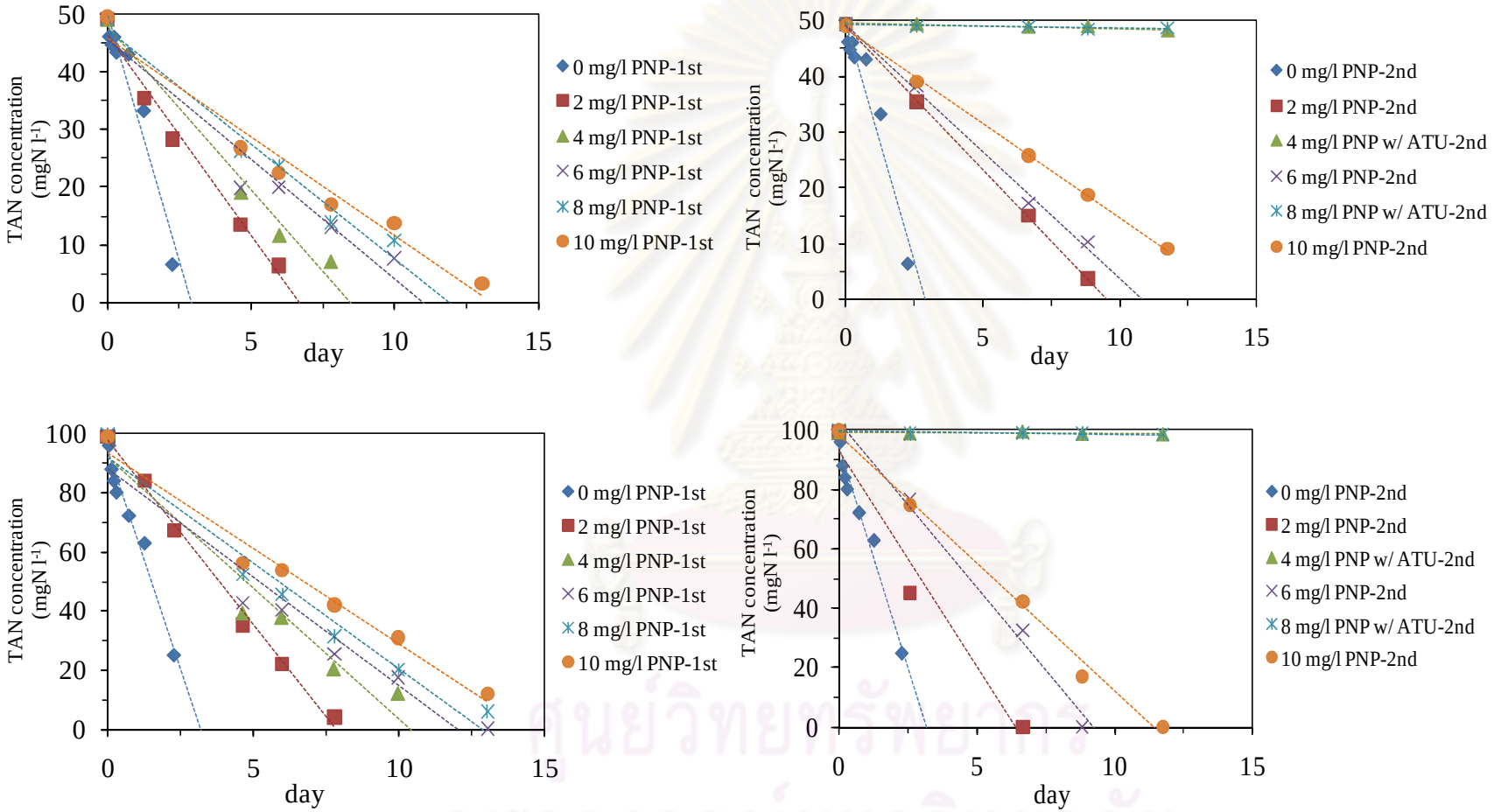

Fig. B-12 Concentrations of TAN during PNP degradation by C/N0.8 entrapped cells; 1st batch test (Left) and 2nd batch test (Right); initial TAN concentrations of 50 mgN l^{-1} (Top) and 100 mgN l^{-1} (Bottom)

Table B-1 PNP removed, the degradation time and degradation rate of PNP by C/N0 entrapped cells

C/N0 - 1st batch test										
Initial PNP ($mg\ l^{-1}$)	Initial TAN 50 $mgN\ l^{-1}$					Initial TAN 100 $mgN\ l^{-1}$				
	PNP at 0 hr ($mg\ l^{-1}$)	10% of PNP at 0 hr ($mg\ l^{-1}$)	PNP removed ($mg\ l^{-1}$)	Degradation time (d)	Degradation rate ($mg\ l^{-1}\ d^{-1}$)	PNP at 0 hr ($mg\ l^{-1}$)	10% of PNP at 0 hr ($mg\ l^{-1}$)	PNP removed ($mg\ l^{-1}$)	Degradation time (d)	Degradation rate ($mg\ l^{-1}\ d^{-1}$)
2.0	1.61	0.16	1.45	7.7	0.19	1.83	0.18	1.65	8.5	0.19
4.0	3.86	0.39	3.47	11.3	0.31	3.86	0.39	3.48	12.9	0.27
6.0	5.30	0.53	4.77	11.8	0.40	5.22	0.52	4.70	12.9	0.36
8.0	6.77	0.68	6.10	11.6	0.53	6.94	0.69	6.24	12.8	0.49
10.0	8.77	0.88	7.89	9.9	0.79	8.78	0.88	7.90	12.9	0.61

C/N0 - 2nd batch test										
Initial PNP ($mg\ l^{-1}$)	Initial TAN 50 $mgN\ l^{-1}$					Initial TAN 100 $mgN\ l^{-1}$				
	PNP at 0 hr ($mg\ l^{-1}$)	10% of PNP at 0 hr ($mg\ l^{-1}$)	PNP removed ($mg\ l^{-1}$)	Degradation time (d)	Degradation rate ($mg\ l^{-1}\ d^{-1}$)	PNP at 0 hr ($mg\ l^{-1}$)	10% of PNP at 0 hr ($mg\ l^{-1}$)	PNP removed ($mg\ l^{-1}$)	Degradation time (d)	Degradation rate ($mg\ l^{-1}\ d^{-1}$)
2.0	1.77	0.18	1.59	2.4	0.65	1.90	0.19	1.71	2.4	0.71
4.0	3.78	0.38	3.40	2.5	1.39	3.79	0.38	3.41	2.5	1.39
6.0	5.23	0.52	4.71	2.5	1.88	5.27	0.53	4.74	2.4	1.99
8.0	6.78	0.68	6.10	3.1	1.97	6.75	0.68	6.08	2.4	2.53
10.0	8.76	0.88	7.88	2.6	3.08	8.78	0.88	7.90	2.4	3.31

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Table B-2 PNP removed, the degradation time and degradation rate of PNP by C/N0.4 entrapped cells

C/N0.4 - 1st batch test										
Initial PNP ($mg\ l^{-1}$)	Initial TAN 50 $mgN\ l^{-1}$					Initial TAN 100 $mgN\ l^{-1}$				
	PNP at 0 hr ($mg\ l^{-1}$)	10% of PNP at 0 hr ($mg\ l^{-1}$)	PNP removed ($mg\ l^{-1}$)	Degradation time (d)	Degradation rate ($mg\ l^{-1}\ d^{-1}$)	PNP at 0 hr ($mg\ l^{-1}$)	10% of PNP at 0 hr ($mg\ l^{-1}$)	PNP removed ($mg\ l^{-1}$)	Degradation time (d)	Degradation rate ($mg\ l^{-1}\ d^{-1}$)
2.0	1.79	0.18	1.61	7.7	0.21	1.79	0.18	1.61	7.6	0.21
4.0	3.87	0.39	3.48	12.7	0.27	3.85	0.38	3.46	7.6	0.46
6.0	5.24	0.52	4.72	12.0	0.39	5.22	0.52	4.70	10.4	0.45
8.0	6.94	0.69	6.25	12.0	0.52	6.90	0.69	6.21	10.4	0.60
10.0	8.69	0.87	7.82	10.7	0.73	8.76	0.88	7.88	7.8	1.01

C/N0.4 - 2nd batch test										
Initial PNP ($mg\ l^{-1}$)	Initial TAN 50 $mgN\ l^{-1}$					Initial TAN 100 $mgN\ l^{-1}$				
	PNP at 0 hr ($mg\ l^{-1}$)	10% of PNP at 0 hr ($mg\ l^{-1}$)	PNP removed ($mg\ l^{-1}$)	Degradation time (d)	Degradation rate ($mg\ l^{-1}\ d^{-1}$)	PNP at 0 hr ($mg\ l^{-1}$)	10% of PNP at 0 hr ($mg\ l^{-1}$)	PNP removed ($mg\ l^{-1}$)	Degradation time (d)	Degradation rate ($mg\ l^{-1}\ d^{-1}$)
2.0	1.67	0.17	1.50	2.5	0.60	1.66	0.17	1.49	2.5	0.60
4.0	3.74	0.37	3.37	2.5	1.36	3.74	0.37	3.37	3.0	1.11
6.0	5.17	0.52	4.66	2.4	1.96	5.17	0.52	4.66	2.4	1.97
8.0	6.83	0.68	6.15	2.4	2.56	6.83	0.68	6.14	2.4	2.58
10.0	8.66	0.87	7.80	2.4	3.30	8.69	0.87	7.82	2.4	3.30

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Table B-3 PNP removed, the degradation time and degradation rate of PNP by C/N0.8 entrapped cells

C/N0.8 - 1st batch test										
Initial PNP ($mg\ l^{-1}$)	Initial TAN 50 $mgN\ l^{-1}$					Initial TAN 100 $mgN\ l^{-1}$				
	PNP at 0 hr ($mg\ l^{-1}$)	10% of PNP at 0 hr ($mg\ l^{-1}$)	PNP removed ($mg\ l^{-1}$)	Degradation time (d)	Degradation rate ($mg\ l^{-1}\ d^{-1}$)	PNP at 0 hr ($mg\ l^{-1}$)	10% of PNP at 0 hr ($mg\ l^{-1}$)	PNP removed ($mg\ l^{-1}$)	Degradation time (d)	Degradation rate ($mg\ l^{-1}\ d^{-1}$)
2.0	1.94	0.19	1.75	4.5	0.39	1.84	0.18	1.66	4.5	0.37
4.0	3.73	0.37	3.36	9.2	0.36	3.72	0.37	3.35	12.4	0.27
6.0	5.23	0.52	4.71	10.3	0.46	5.32	0.53	4.79	11.6	0.41
8.0	6.92	0.69	6.23	10.0	0.63	6.90	0.69	6.21	7.7	0.80
10.0	8.72	0.87	7.85	9.6	0.82	8.71	0.87	7.84	7.8	1.01

C/N0.8 - 2nd batch test										
Initial PNP ($mg\ l^{-1}$)	Initial TAN 50 $mgN\ l^{-1}$					Initial TAN 100 $mgN\ l^{-1}$				
	PNP at 0 hr ($mg\ l^{-1}$)	10% of PNP at 0 hr ($mg\ l^{-1}$)	PNP removed ($mg\ l^{-1}$)	Degradation time (d)	Degradation rate ($mg\ l^{-1}\ d^{-1}$)	PNP at 0 hr ($mg\ l^{-1}$)	10% of PNP at 0 hr ($mg\ l^{-1}$)	PNP removed ($mg\ l^{-1}$)	Degradation time (d)	Degradation rate ($mg\ l^{-1}\ d^{-1}$)
2.0	1.67	0.17	1.50	2.6	0.58	1.69	0.17	1.52	5.9	0.26
4.0	3.71	0.37	3.34	6.4	0.52	3.73	0.37	3.36	6.3	0.53
6.0	5.19	0.52	4.67	6.0	0.78	5.17	0.52	4.66	6.1	0.77
8.0	6.83	0.68	6.14	6.1	1.00	6.84	0.68	6.16	6.3	0.98
10.0	8.62	0.86	7.76	5.7	1.37	8.63	0.86	7.76	6.1	1.27

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Table B-4 Ammonia oxidation rate of C/N0, C/N0.4 and C/N0.8 entrapped cells

	C/N	Initial TAN (mgN l^{-1})	Initial PNP (mg l^{-1})					
			0	2	4	6	8	10
			Ammonia oxidation rate (mg [NO_2^- + NO_3^-] l^{-1} day^{-1})					
1st batch test	0	50.00	15.78	5.79	4.00	3.57	3.32	3.11
	0.4		15.75	5.919	4.613	7.584	9.14	12.79
	0.8		15.75	4.613	5.886	7.27	7.966	9.78
	0	100.00	31.76	4.364	4.611	6.75	7.346	8.47
	0.4		31.7	3.742	4.272	6.10	6.752	7.90
	0.8		31.73	3.348	3.787	5.37	5.433	7.02

C/N	Initial TAN (mgN l^{-1})	Initial PNP (mg l^{-1})					
		0	2	4	6	8	10
		R Square for ammonia oxidation rate					
0	50	0.917	0.966	0.930	0.908	0.967	0.961
0.4		0.969	0.901	0.993	0.910	0.985	0.991
0.8		0.931	0.973	0.974	0.908	0.967	0.943
0	100	0.949	0.995	0.978	0.937	0.994	0.988
0.4		0.986	0.981	0.937	0.930	0.926	0.968
0.8		0.951	0.992	0.994	0.904	0.948	0.962

	C/N	Initial TAN (mgN l^{-1})	Initial PNP (mg l^{-1})					
			0	2	4 with ATU	6	8 with ATU	10
			Ammonia oxidation rate (mg [NO_2^- + NO_3^-] l^{-1} day^{-1})					
2nd batch test	0	50	15.78	4.09	0.08	3.01	0.05	2.80
	0.4		15.75	4.091	0.064	3.746	0.07	3.071
	0.8		15.75	5.145	0.056	4.557	0.052	3.46
	0	100	31.76	11.98	0.07	8.45	0.06	7.59
	0.4		31.7	13.85	0.087	9.026	0.062	7.98
	0.8		31.73	15.75	0.06	10.72	0.08	8.74

C/N	Initial TAN (mgN l^{-1})	Initial PNP (mg l^{-1})					
		0	2	4 with ATU	6	8 with ATU	10
		R Square for ammonia oxidation rate					
0	50	0.917	0.951	.0.977	0.990	0.983	0.999
0.4		0.969	0.993	0.956	0.997	0.972	0.994
0.8		0.931	0.999	0.978	0.994	0.966	0.997
0	100	0.943	0.975	0.985	0.984	0.996	0.949
0.4		0.986	0.979	0.985	0.958	0.940	0.970
0.8		0.951	0.957	0.968	0.987	0.983	0.993

BIOGRAPHY

Mr. Chaiwat Rongsayamanont was born on March 6, 1977 in Bangkok, Thailand. He received his bachelor degree in civil engineering from Mahidol University and later, pursued the master degree study in environmental engineering at Chiang Mai University. Then, he started his Ph.D in international environmental management program of NCE-EHWM, Chulalongkorn University, since 2005-2010.

Journal publications

Rongsayamanont C., Limpiyakorn T., Law B., and Khan E. (2010) "Relationship Between Respirometric Activity and Community of Entrapped Nitrifying Bacteria: Implications for Partial Nitrification", Enzyme and Microbial Technology Vol 46, pp 229-236.

Journal publications (not directly related to dissertation)

Kayee P., Sonthiphand P., **Rongsayamanont C.**, and Limpiyakorn T. (2011) "Archaeal amoA genes outnumber bacterial amoA genes in municipal wastewater treatment plants in Bangkok ", Microbial Ecology (Accepted)

Limpiyakorn, T., Sonthiphand, P., **Rongsayamanont C.**, and Polprasert C. (2011) "Abundance of amoA genes of ammonia-oxidizing microorganisms archaea and bacteria in activated sludge of full-scale wastewater treatment plants". Bioresource Technol 102: 3694–3701.

Conferences and proceeding publications

Rongsayamanont C., Khan E., "Kinetic of Nitrification in Gel-Entrapped Biomass", The 2nd Annual Joint Student Environmental Conference, American Water Work Association/Water Environment Federation (AWWA/WEF) Student Chapter, Fargo, North Dakota, USA, August 2008 (Oral).

Rongsayamanont C., Limpiyakorn T., and Khan K. (2011) Cell entrapment: an efficient way to achieve partial nitrification for ammonium-rich wastewater. Proceeding on Korea-Thai Environmental Engineering Conference, January 2011, Bangkok, Thailand (Oral).

Rongsayamanont C., Limpiyakorn T., and Khan K. (2011) Effect of inoculums on achieving partial nitrification using gel entrapment. Proceeding on IWA's specialist conference on 'Microbes in Wastewater and Waste Treatment, Bioremediation and Energy Production', January 2011, Goa, India (Oral).