# อิทธิพลของซิลเวอร์อนุภาคขนาดนาโนต่อจลนพลศาสตร์ของกระบวนการไนตริฟิเคชันและแอมโมเนียออกซิเดชันในตะกอนเร่ง

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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# INFLUENCE OF SILVER NANOPARTICLES ON NITRIFICATION KINETICS AND AMMONIA OXIDATION IN ACTIVATED SLUDGE

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A Thesis Submitted in Partial Fulfillment of the Requirements
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| Thesis Advisor | Sumana Siripattanakul, Ph.D. |
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เหงียน ทั่น ฆ่าว: อิทธิพลของซิลเวอร์อนุภาคขนาดนาโนต่อจลนพลศาสตร์ของกระบวนการไนตริฟิเคชันและแอมโมเนียออกซิเดชันในตะกอนเร่ง. (INFLUENCE OF SILVER NANOPARTICLES ON NITRIFICATION KINETICS AND AMMONIA OXIDATION IN ACTIVATED SLUDGE) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: อ.ดร. สุมนา สิริพัฒนากุล, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: อ.ดร. ตะวัน ลิมปิยากร, 95 หน้า.

ไนตริฟิเคชันเป็นกระบวนการกำจัดไนโตรเจนจากน้ำเสียทางชีวภาพซึ่งได้รับความนิยมสูง กระบวนการดังกล่าวประกอบด้วยการทำงานสองขั้นตอน อันได้แก่ แอมโมเนียออกซิเดชันและไนไตรท์ออกซิเดชัน โดยแอมโมเนียถูกเปลี่ยนเป็นไนไตรท์โดยจุลินทรีย์แอมโมเนียออกซิไดซิงแล้วจากนั้นไนไตรท์ถูกเปลี่ยนเป็นไนเตรทโดยจุลินทรีย์ไนไตรท์ออกซิไดซิง จากที่รู้กันทั่วไปว่ากระบวนการออกซิเดชันของแอมโมเนียเป็นขั้นตอนที่จำกัดอัตราการเกิดปฏิกิริยา (rate-limiting) และมีความอ่อนไหวส่งผลให้ขั้นตอนนี้มีความสำคัญต่อระบบบำบัดน้ำเสียทั่วไป สาเหตุที่กระบวนนี้มีข้อจำกัดเนื่องจากจุลินทรีย์แอมโมเนียออกซิไดซิงมีความอ่อนไหวต่อสารอาหารต่าง ๆ มากโดยเฉพาะสารพิษ สำหรับซิลเวอร์อนุภาคขนาดนาโน (silver nano-particles; AgNPs) เป็นหนึ่งในสารพิษที่มีการผลิตและการใช้ประโยชน์เพิ่มมากขึ้น การศึกษานี้เป็นการศึกษาถึงผลของซิลเวอร์อนุภาคขนาดนาโน ในการยับยั้งกระบวนการไนตริฟิเคชันและแอมโมเนียออกซิเดชันในตะกอนเร่งไนตริฟายโดยใช้วิธีการติดตามการหายใจ (respirometric method) การศึกษาทดลองที่ช่วงความเข้มข้นของซิลเวอร์อนุภาคขนาดนาโนและแอมโมเนียเริ่มต้นระหว่าง 0.25 to 10 mg/L และ 14 to 280 mg/L ตามลำดับ ภายใต้สภาวะที่มีซิลเวอร์อนุภาคขนาดนาโนกระบวนการไนตริฟิเคชันและแอมโมเนียออกซิเดชันมีค่าอัตราการใช้ออกซิเจนสูงสุด (maximum oxygen uptake rate; $OUR_{max}$) และค่าคงที่การอิ่มตัว (half saturation constant; $K_s$) ลดลง พฤติกรรมของซิลเวอร์อนุภาคขนาดนาโนเป็นไปตามแบบจำลองการยับยั้งแบบไม่มีการแข่งขันโดยตรง (uncompetitive inhibition) กระบวนการไนตริฟิเคชันและแอมโมเนียออกซิเดชันมี $K_s$ เท่ากับ 5.42 และ 15.88 mg$NH_4^+$-N/L และค่าคงที่การยับยั้ง (inhibition coefficients; $K_i$) เท่ากับ 8.3 และ 21.7mg/L ตามลำดับ โดยเมื่อซิลเวอร์อนุภาคขนาดนาโนเพิ่มขึ้นในช่วง 0.25 ถึง 10 mg/L การยับยั้งอัตราการหายใจของจุลินทรีย์ไนตริฟายและจุลินทรีย์แอมโมเนียออกซิไดซิง เท่ากับ ร้อยละ 8 ± 4.8 ถึง 38 ± 14.2 และ ร้อยละ 16 ± 8.2 ถึง 38 ± 11.3 ภายใน 0.5 hr ตามลำดับ จากผลการทดลองพบว่าซิลเวอร์อนุภาคขนาดนาโนส่งผลต่อกระบวนการไนตริฟิเคชันและแอมโมเนียออกซิเดชันเพียงบางส่วนเท่านั้น โดยเมื่อซิลเวอร์อนุภาคขนาดนาโนเพิ่มขึ้นการยับยั้งอัตราการหายใจเพิ่มขึ้นเช่นกัน ความเข้มข้นของแอมโมเนียและซิลเวอร์อนุภาคขนาดนาโนมีผลต่อกิจกรรมไนตริฟายอิงและแอมโมเนียออกซิไดซ์ในลักษณะเสริมฤทธิ์ ในการทดลองที่ความเข้มข้นของแอมโมเนียเริ่มต้นสูง (280mg/L) กิจกรรมไนตริฟายอิงและแอมโมเนียออกซิไดซ์ถูกยับยั้งด้วยซิลเวอร์อนุภาคขนาดนาโนสูงถึงร้อยละ 48 และ 41 ภายใน 0.5 hr ตามลำดับ ผลการทดลองนี้บ่งชี้ได้ว่าซิลเวอร์อนุภาคขนาดนาโนเป็นอันตรายต่อการกำจัดไนโตรเจนในโรงบำบัดน้ำเสีย ดังนั้นซิลเวอร์อนุภาคขนาดนาโนควรได้รับการพิจารณาเป็นสารอันตรายซึ่งในการจัดการปลดปล่อยสู่สิ่งแวดล้อมควรมีการควบคุมเป็นพิเศษด้วยการกำหนดมาตรการทางกฎหมายทั้งในการผลิตและใช้ประโยชน์ต่อไป

ลายมือชื่อนิสิต.....

สาขาวิชาการจัดการสิ่งแวดล้อม ลายมือชื่ออาจารย์ที่ปรึกษาวิทยานิพนธ์หลัก.....

ปีการศึกษา 2553 ลายมือชื่ออาจารย์ที่ปรึกษาวิทยานิพนธ์ร่วม .....

## #5287623020: MAJOR ENRONMENTAL MANAGEMENT
KEYWORDS : AMMONIA OXIDIZING MICROORGANISM, SILVER NANOPARTICLE/ INHIBITION COEFFICIENT/ NITRIFYING ACTIVATED SLUDGE

NGUYEN THANH GIAO: INFLUENCE OF SILVER NANOPARTICLES ON NITRIFICATION KINETICS AND AMMONIA OXIDATION IN ACTIVATED SLUDGE. THESIS ADVISOR: SUMANA SIRIPATTANAKUL, PhD; THESIS CO-ADVISOR: TAWAN LIMPIYAKORN, Ph.D, 95 PP.

Nitrification is widely applied process for biological removal of nitrogen from wastewaters. The process comprises of two-steps: ammonia oxidation and nitrite oxidation. Ammonia ($NH_3$) is oxidized to nitrite ($NO_2^-$) by ammonia-oxidizing microorganisms (AOM) and then nitrite is subsequently oxidized to nitrate ($NO_3^-$) by nitrite-oxidizing microorganisms (NOM). Of which, ammonia oxidation is known as a rate-limiting and more sensitive step because of the microorganism behaviors thus plays an essential important role in any wastewater treatment facilities. Ammonia oxidizing microorganisms are reported to be very sensitive to non-growth substrates including toxic substances. Silver nanoparticles (AgNPs) are one of the toxic substances that have significantly increased in production and use, recently. In this study, the inhibitory effect of AgNPs on nitrification and ammonia oxidation in enriched nitrifying activated sludge was investigated by using respirometric method. The initial concentrations of AgNPs and ammonia ranged from 0.25 to 10 mg/L and 14 to 280 mg/L, respectively, for both processes. Under the presence of AgNPs, the maximum oxygen uptake rate ($OUR_{max}$) and half saturation constant ($K_s$) of both nitrification and ammonia oxidation were declined. The effect behavior of AgNPs on the both processes was proved to follow uncompetitive inhibition model. The kinetic parameters were estimated for half saturation constants ($K_s$) without AgNPs for nitrification and ammonia oxidation and were 5.42 and 15.88 mg$NH_4^+$-N/L while the inhibition coefficients ($K_I$) were 8.3 and 21.7mg/L, respectively. Increasing AgNPs from 0.25-10 mg/L inhibited 8 ± 4.8% - 38 ± 14.2 and 16 ± 8.2% - 38 ± 11.3% respiration rate of nitrifying and ammonia oxidizing activity, respectively within 0.5h. Silver nanoparticles only partially influenced on nitrification and ammonium oxidation in the manner that higher AgNPs resulted in higher inhibition of respiration rate. Silver nanoparticle and ammonia concentration could synergize inhibitory effect on nitrifying and ammonia oxidizing activity. At high ammonium concentration (280mg/L), nitrifying and ammonia oxidizing activity were inhibited by AgNPs up to 48 and 41% within 0.5h, respectively. The findings indicated that AgNPs could harm nitrogen removal in wastewater treatment plants. Thus, AgNPs should be considered as hazardous substance and special attention should be made in the management of discharge of AgNPs into the environments through enhancing implementation of regulations of production and use.

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 ……..…..… | xi |
| LIST OF ABBREVIATIONS……. | xiii |
| CHAPTER I: INTRODUCTION …….. | 1 |
| 1.1 Background and motivation…….……..... | 1 |
| 1.2 Objectives…….……..... | 3 |
| 1.3 Hypothesis…….…… | 3 |
| 1.4 Scope of the study……..... | 3 |
| CHAPTER II: LITERATURE REVIEW. …..……..... | 4 |
| 2.1 Overview of products containing silver or/and AgNPs.……... | 4 |
| 2.1.1 Definition of nanoparticles……...….…….. | 4 |
| 2.1.2 Types of products using AgNPs…...……... | 5 |
| 2.1.2.1 AgNPs in children products…… | 5 |
| 2.1.2.2 AgNPs in dietary supplements and food related items… | 6 |
| 2.1.2.3 AgNPs in clothing and textiles…… | 7 |
| 2.1.2.4 AgNPs in comestics and personal care products……. | 7 |
| 2.1.2.5 AgNPs in hospital and medical applications……... | 7 |
| 2.1.3 Sources of AgNPs contamination……. | 8 |
| 2.2 Forms of AgNPs in the environment…… | 10 |

**Page**

2.3 Fate and transport of AgNPs in the environment ……..…….. 11

2.4 Characteristics of antimicrobial activity of AgNPs...…….. 12

2.4.1 In vitro effect……..……...….……. 15

2.4.2 In vivo effect……..……...….…….. 17

2.5 Occurrence of AgNPs in the environment……...…….. 19

2.5.1 In natural water……..……...….…….. 19

2.5.2 In soil……..……...….…….. 20

2.5.3 In wastewater treatment plant..……...….…….. 22

2.6 Nitrification process……..……...…….. 21

2.6.1 Factors affecting on nitrification process…...….…….. 22

2.6.2 Phylogeny of ammonia-oxidizing microorganisms…….. 24

2.6.2.1 Ammonia-oxidizing bacteria (AOB)……. 25

2.6.2.2 Ammonia-oxidizing archaea (AOA)……. 26

2.6.2.3 Differences between archaea and bacteria…….…… 29

2.7 Real-time PCR....……... 32

2.8 Methods for characterization of AgNPs…….....…….. 33

2.9 Respirometry…….. 35

CHAPTER III: MATERIAL AND METHODS…….……..... 38

3.1 Experimental framework …….. 38

3.2 Material and methods……..... 40

3.2.1 Enrichment of nitrifying activated sludge (NAS)…..…..…… 40

3.2.2 Quantification of *amo*A genes of AOB and AOB …..... 40

3.2.3 Investigation of inhibitory kinetics of AgNPs on nitrification and ammonia oxidation in activated sludge…….....……... 42

3.2.3.1 Chemical preparation……. 42

3.2.3.2 Reactor setup and operation…….. 42

3.2.3.3 Kinetic analysis…….. 43

**Page**

3.2.3.4 Determination of inhibitory kinetics of nitrification by AgNPs...…….. 45

3.2.3.5 Determination of inhibitory kinetics of ammonia oxidation by AgNPs.…….. 46

3.2.3.6 Analytical methods……. 47

CHAPTER IV: RESULTS AND DISCUSSION……..... 50

4.1 Enrichment of nitrifying activated sludge……. 50

4.2 Nitrifying and ammonia oxidizing activity without AgNPs…...….. 52

4.3 Inhibitory effect of AgNPs on nitrifying and ammonia oxidizing activity...……... 58

4.4 Inhibitory kinetics of AgNPs on nitrification and ammonia oxidation……..…… 61

4.5 Quantification of inhibition (%) caused by AgNPs on nitrification and ammonia oxidation…… 66

CHAPTER V: CONCLUSIONS AND RECOMMENDATIONS……..……. 69

5.1 Conclusions……... 69

5.2 Recommendations…….. 70

REFERENCES……... 71

APPENDICES……..…….. 81

APPENDIX A…….. 82

APPENDIX B…….. 84

APPENDIX C…….. 92

Biography……..……. 95

# LIST OF TABLES


| Table | | Page |
|---|---|---|
| 2.1 | Forms of silver……..... | 10 |
| 2.2 | Inhibitory concentrations of AgNPs on some selected species…… | 13 |
| 2.3 | Median L(E)C50 values for synthetic AgNPs towards different groups of organisms……..... | 13 |
| 2.4 | Summary of *in vitro* and *in vivo* effect of AgNPs in selected species……. | 14 |
| 2.5 | The differences between archaea and bacteria……... | 29 |
| 2.6 | Summary of the archaeal phospholipid compared with bacterial phospholipid……. | 30 |
| 2.7 | Biochemical adaptation mechanisms such as low permeability membrane and specific catabolic pathways enable archaea in their evolution……... | 31 |
| 3.1 | Specific primers for AOB and AOA used in the study…… | 41 |
| 4.1 | Summary of inhibition models……..……... | 61 |
| 4.2 | $OUR_{max}$ and Ks of nitrifying activity with AgNPs…….. | 62 |
| 4.3 | $OUR_{max}$ and Ks of ammonia oxidizing activity with AgNPs……... | 62 |
| 4.4 | Summarized $K_i$ values of some heavy metals……. | 64 |

# LIST OF FIGURES


| Figure | | Page |
|---|---|---|
| 2.1 | *amoA*-based phylogenetic tree of the betaproteobacterial AOB…… | 25 |
| 2.2 | Phylogenetic tree of AOA…….. | 26 |
| 2.3 | Neighbor joining tree of AOA amoA sequences from full-scale wastewater treatment plants…… | 28 |
| 3.1 | Framework of the study…….. | 38 |
| 3.2 | Schematic diagram of experiment tasks……... | 39 |
| 3.3 | Schematic diagram of preliminary study…… | 41 |
| 3.4 | Schematic diagram of the experiment 1……. | 45 |
| 3.5 | Schematic diagram of the experiment 2……. | 46 |
| 3.6 | The plasmon absorption band of 100–1000 mg/L the synthesized AgNPs……. | 48 |
| 3.7 | TEM images of the synthesized AgNPs at the TEM magnification of 150,000……... | 49 |
| 4.1 | Concentrations of $NH_4^+$-N and $NO_3^-$-N in the reactor during enrichment of NAS……. | 51 |
| 4.2 | Copy numbers of *amo*A genes of AOA and AOB in the reactor during enrichment of NAS……. | 51 |
| 4.3 | Dissolved oxygen consumption by heterotrophs…… | 52 |
| 4.4 | Test of DO sink for ammonia oxidation experiment…….. | 53 |
| 4.5 | Test of DO sink for nitrification experiment……. | 53 |
| 4.6 | DO consumption under different $NH_4^+$-N concentration in nitrification……. | 54 |
| 4.7 | DO consumption under different $NH_4^+$-N concentration in ammonium oxidation……. | 55 |
| 4.8 | Maximum OUR and Ks for nitrification…… | 57 |
| 4.9 | Maximum OUR and Ks for ammonia oxidation…… | 56 |
| 4.10 | OUR versus initial $NH_4^+$-N concentrations in nitrification tests..…….. | 60 |

**Page**


| | | |
|---|---|---|
| 4.11 | OUR versus initial $NH_4^+$-N concentrations in ammonia oxidation tests……..…….. | 60 |
| 4.12 | Lineweaver-Burk for nitrification tests…….. | 63 |
| 4.13 | Lineweaver-Burk for ammonia oxidation tests……... | 63 |
| 4.14 | Inhibition (%) caused by AgNPs in nitrification…… | 68 |
| 4.15 | Inhibition (%) caused by AgNPs in ammonium oxidation…… | 68 |

# LIST OF ABBREVIATIONS

| **Abbreviations** | **In full words** |
|---|---|
| AgNPs | Silver nanoparticles |
| FDA | Food and Drug Administration |
| $Ag^+$ | Silver ion |
| SSD | Silver sulfadiazine |
| $AgNO_3$ | Silver nitrate |
| AgCl | Silver chloride |
| DO | Dissolved oxygen |
| NPs | Nanoparticles |
| LC50 | Lethal concentration causing 50 death of experimental organisms |
| MIC | The minimum inhibitory concentration |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| AuNPs | Gold nanoparticles |
| DNA | Deoxyribonucleic acid |
| RNA | Ribonucleic acid |
| cDNA | Complementary Deoxyribonucleic acid |
| PSNPs | Polystyrene nanoparticles |
| mRNA | Messenger RNA |
| $Ag_2CO_3$ | Silver carbonate |
| PCRs | Polymerase chain reactions |
| SEM and TEM | The scanning and transmission electron miscrocopic |
| OECD | Organization for Economic Cooperation and Development |
| GLP | Good Laboratory Practice |
| AgENs | Engineered nanoparticles |
| WWTPs | Wastewater treatment plants |
| AOA | Ammonia oxidizing archaea |

| | |
|---|---|
| AOB | Ammonia oxidizing bacteria |
| HRT | Hydraulic retention time |
| SRT | Solid retention time |
| PCR | Polymerase Chain Reaction |
| RT-PCR | Real-time Polymerase Chain Reaction |
| SNP | Single nucleotide polymorphism |
| UV-vis | Ultraviolet-visible |
| DLS | Dynamic Light Scattering |
| XRD | X-ray diffraction |
| ESR | Electron Spin Resonance spectrometer |
| EDS | Energy-dispersed Spectroscopy |
| XPS | X-ray photoelectron Spectrophotometry |
| ISE | Ion Selective Elcetrode |
| ICP-OES | inductively coupled plasma-optical emission spectroscopy |
| FT-IR | Fourier Transform-Infrared Spectroscopy |
| USEPA | United States Environmental Protection Agency |
| RCRA | The resource recovery and conservation act |
| NAS | Enrichment of nitrifying activated sludge |
| mg/L | Milligram per litter |
| mM | Millimolar |
| Ki | Inhibitory coefficient or inhibitory constant |
| Ks | Half saturation coefficient/constant |
| OUR | Oxygen Uptake Rate |
| $OUR_{max}$ | Maximum Oxygen Uptake Rate |
| MLVSS | Mixed liquor volatile suspended solid |
| NOEC | No observable effect concentration |
| LOEC | Lowest observable effect concentration |

# CHAPTER I

# INTRODUCTION

## 1.1 Background and motivation

Nanotechnology has gained a great deal of public interest because of the needs and applications of nanomaterials in many areas of human activities including industry, agriculture, business, medicine, and public health (Ray et al., 2009). Silver nanoparticles (AgNPs), one of the most well-liked nanomaterials, have been used broadly for enhancing consumer products because of its strong antimicrobial property. Silver nanoparticles with their unique physical and chemical properties have been developed as alternative antibacterial agents (Sharma et al., 2009; Ray et al., 2009). It has used in diverse applications such as a component in wound dressings, medical device coatings, textile fabrics, cosmetic products, and detergents (Rai et al., 2009). As a result, nanotoxicity research is gaining attention.

In a prior study, it was indicated that up to 15% of total silver in the form of $Ag^{+}$ or AgNPs could be released from biocidal plastic and textile into water (Blaser, 2008). Another recent study confirmed that AgNPs were easily released from AgNPs-coated socks during the washing process (Benn and Westerhoff, 2008). Based on AgNPs discharge information from the earlier studies and a large amount of AgNPs utilization, AgNPs likely entered wastewater collection and treatment systems leading to failure in the wastewater treatment systems (Choi et al., 2009).

Most of studies on effect of AgNPs have been focused on testing its toxicity on pure microorganisms, such as *Escherichia coli* (Sondi and Salopek-Sondi, 2004; Yang et al., 2009), *Bacillus subtilis* (Ruparelia et al., 2008), *Staphylococcus aureus* (Kim et al., 2007; Ruparelia et al., 2008), *Candida sp.* (Panacek et al., 2009), *yeast* (Kim et al., 2007) and animal species, such as Oryzias latipes (Wise, 2010), Zebra fish (Lee et al., 2007) , and human cells for example reducing and inhibiting formation of extracellular HBV DNA and intracellular HBV RNA of HepAD38 (Lu et al., 2008), inhibiting oxidation

based biological process of cytochrome P450 (Sereemaspun et al., 2008), causing damage to chromosome in HepG2 cells (Kawata et al., 2009), causing death associated with aptosis in HeLa cells (Miura and Shinohara, 2009), causing oxidative stress (Kim et al., 2009), and penetrating and damaging skin cells (Laresea et al., 2009). Thus far, there have been only a few studies on influence of AgNPs on the wastewater treatment systems and microorganisms in the systems (Choi et al., 2008).

It is known that nitrification is one of the most sensitive processes in wastewater treatment systems. The process comprises of two-steps. Ammonia ($NH_3$) is oxidized to nitrite ($NO_2^-$) by ammonia-oxidizing microorganisms (AOM) and then nitrite is subsequently oxidized to nitrate ($NO_3^-$) by nitrite-oxidizing microorganisms (NOM).Both AOB and AOA share some similarities, such as doing the same task in the nitrification process and having slow growth rates and yields (Aoi et al., 2000; Hu et al., 2002b; You et al., 2009). Also, they are very sensitive to environmental conditions, such as ammonium loadings, dissolved oxygen concentration, pH, temperature, solid retention time, hydraulic retention time and highly affected by toxic compounds; for example, cyanide, thiourea, phenol, silver, mercury, nickel, chromium, copper, and zinc (Aoi et al., 2000; Hu et al., 2002b; Bitton, 2005; Malgorzata et al., 2006; Limpiyakorn et al., 2007; Lydmark et al., 2007; Sonthiphand and Limpiyakorn, 2009). However, AOA and AOB have differences in their components of lipid membranes (Ulrih et al., 2009) and biochemical adaptations (Valentine, 2002). What will be likely happen if toxic substances introduced to the wastewater treatment plant systems? Recent study found that AgNPs of only 1 mg/L inhibited microbial growth in nitrification process by approximately 80% (Choi et al., 2008) but not mentioned how the impact differed between ammonia oxidation and nitrification. The previous studies focused only the properties of AgNPs and environmental conditions to inhibition of microbial growth (Choi et al., 2008; Choi et al., 2009), there was no published nitrification inhibition kinetic information. Therefore, this study will investigate the ammonia oxidation and nitrification inhibitory kinetics by AgNPs. The work includes the inhibitory kinetics of complete nitrification and only ammonia oxidation processes.

## 1.2 Objectives

1.2.1 To determine inhibitory kinetics of nitrification by AgNPs,

1.2.2 To determine inhibitory kinetics of ammonia oxidation by AgNPs.

## 1.3 Hypotheses

AgNPs inhibit nitrification and ammonia oxidation activities of activated sludge.

## 1.4 Scope of the study

1.4.1 The activated sludge was collected from Siphraya Municipal Wastewater Treatment Plant (Bangkok, Thailand).

1.4.2 Nitrification and ammonia oxidation tests were run by using respirometric method.

1.4.3 Abundance of AOA and AOB was investigated based on *amoA* gene expression using real-time polymerase chain reaction (RT-PCR) technique.

1.4.4 Gene primers specific to groups of AOB and AOA will be used for PCR amplification of *amoA* genes.

# CHAPTER II

# LITERATURE REVIEW

## 2.1 Overview of products containing silver or/and silver nanoparticles

### 2.1.1 Definition of nanoparticles

The first definition of "Nano-technology" was termed by Professor Norio Taniguchi in 1974 from Tokyo Science University of Japan. "Nano-technology" was defined as the production technology to get the extra high accuracy and ultra fine dimensions, i.e. the preciseness and fineness of the order of 1 nm (nanometer), $10^{-9}$ m, in length. The name of nanotechnology originates from this nanometer (Taniguchi, 1974).

Nanotechnology is a new and developing rapidly around the world. It offers the advantages for many fields, such as medicine, consumer products, energy, materials and manufacturing. Manipulating matter at the atomic or molecular scales leads to form new materials, structures, and devices owing the unique physical and chemical properties associated with nanoscale structures. Nano object is defined as material with one, two, or three external dimensions in the size range from approximately 1-100 nm. Nanoparticle (NP) is a nano object with all three external dimensions at the nanoscale. Similarly, AgNPs is defined as silver particles measured by nanometer.

Unique properties in nanoscale are the most advantages in nanomaterial technology; however, this scale can also result in the introduction of new toxicological risks. Nanoparticles have a very large surface area which typically results in greater chemical reactivity, biological activity, and catalytic behavior compared to larger particles of the same chemical composition (Garnett and Kallinteri, 2006). Organisms may uptake more NPs into cells, tissues, and organs due to the fact that the greater bioavailability of NPs than that of larger particles. Materials which measure less than 300 nm can be taken up by individual cells (Garnett and Kallinteri, 2006) while less than 70nm-sized particles can even be taken up by nuclei of cell and cause major damage (Chen and Mikecz, 2005).

### 2.1.2 Types of products using AgNPs

The worldwide production of silver in 2007 was up to 28,000 tons, of which, approximately 500 tons are AgNPs (Mueller and Nowack, 2008). The majority of silver is used in industry (38.2%), as jewelry and silverware (32.5%) and in the photographic industry (23.8%). Germany alone used about 8,000 kg of silver in 2007, of which 6,600 kg are used for water treatment purposes. In the United States, more than $4 \times 10^6$ tons of silver were consumed in 2000 (Sharma et al., 2009). The European market for silver containing biocidal products is produced to reach 110-230 tons of silver by 2010 (Blaser, 2008).

#### 2.1.2.1 AgNPs in children products

There are several AgNPs found in children products such as strollers, toys, wet wipes, pencils, baby bottles, nipples and teeth brushers as an antimicrobial agent. The idea of using strong antimicrobials in children's products is of concern, especially for health-related problems. Children can be particularly susceptible to being contacted with AgNPs because of certain reasons. First, they are undergoing development in terms of metabolism and excretion of toxicants; second, they have greater dermal absorption due to greater surface area to volume ratio; next, children are likely to be frequently enhanced hand-to-mouth activities; and finally, they can be closely contact with toys. Thus, increasing risk from non-dietary ingestion is as a consequence (Mark, 2010). Therefore, more special attention needs be given to children's interactions with AgNPs.

#### 2.1.2.2 AgNPs in dietary supplements and food related items

Nanotechnology has been widely used in the production of food products, such as packaging and food containing items. These items are often coated with antimicrobial nanomaterials. However, these NPs containing products do not have properly labeling to show the properties of material being used. This explained why consumers have very little information on food products which they are purchasing.

*Food supplements***:** there is an emerging health and environmental concerns that products on the market contain ingredients without knowing their properties. The products may contain nanomaterials due to its antimicrobial function. In the United States, the governments are facing with barriers to develop effective regulations to control nanotechnology-based dietary supplements. For instance, the Food and Drug Administration (FDA) is doing the best to regulate the safety of dietary supplements using nanomaterials but the lack of information, financing and unclear responsibility in some critical areas. These issues are the severely limited. The failure of governments to require comprehensive safety testing of toxicity risks in nano additives is likely to cause health risk for the exposure of the public in the next few years.

*Food storage and food contact materials***:** One of the favorable commercial applications of nanotechnology within the food sector is in packaging. An approximately 500 product packaging using nanomaterials are estimated to be in the market now while nanotechnology is predicted to be used in the manufacturing of 25% of all food packaging within the next decades (Stones, 2009). Nanomaterials with antibacterial properties applying in packaging and nano-sensor technologies have been promoted as greater food safety delivery by detecting or eliminating bacterial and toxin contamination of food. There is a risk to consumers if AgNPs could move from food contact materials into food or drink once ingested. Few studies have been done on this concern, and indicated that AgNPs could migrate into food, but at an insignificant level (Chaudhry et al., 2008).

*Household appliances*: Silver nanoparticles have also been incorporated in various inner surfaces of domestic refrigerators in an apparent attempt to prevent

microbial growth and maintain a clean and hygienic environment. Other household appliances include air and water purifiers, washing machines, and computer parts and hardware. Similarly, antibacterial coatings containing AgNPs have been applied to kitchenwares, cutting boards, and tablewares.

#### 2.1.2.3 AgNPs in clothing and textiles

Other forms of AgNPs containing products are textiles and clothing. Textile and clothing products containing AgNPs, such as socks, pants, shorts, swimming suits, shoes, various business wears, sportswear, jackets, slippers, hats, gloves, and bath towels (Benn and Westerhoff, 2008). Silver nanoparticles are also embedded into textiles and fabrics for furniture, beddings and mattresses and for industrial material use. Some companies are claiming that the AgNPs used in their products remains long term in the product so it can protect users from bacterial infections. A study showed that AgNPs contained in socks, gloves and jackets can easily be washed out (Benn and Westerhoff, 2008) and could go into the natural environments.

#### 2.1.2.4 AgNPs in cosmetics and personal care products

Cosmetics and personal care products containing AgNPs comprise of soap, toothpaste, shampoo, facial masks, creams, skin whiteners, menstrual pads, hair dryers, hair straighteners, curling irons, hair brushes, and electric razors. At present, little information of releasing of AgNPs from using these products has been studied (Benn and Westerhoff, 2008).

#### 2.1.2.5 AgNPs in hospital and medical applications

The discovery and use of silver for hospital and medical applications has been known for long time ago. Silver has been used in wound treatment as early as the 18th century, during which silver nitrate ($AgNO_3$) was used in the treatment of ulcers. Silver has also been used to induce abortions, cauterize wounds and remove calluses and warts. Soluble silver was the most common form in medical use. The compounds could

be used to treat mental illness, epilepsy, nicotine addiction, gastroenteritis, and infectious diseases, including syphilis and gonorrhea (Bouwmeester et al., 2009).

During the early 19th century silver ions ($Ag^+$) were used for their antimicrobial properties and were approved for wound management by the FDA in the 1920s. After the introduction of penicillin in the 1940s, antibiotics were used to replace silver for infectious treatments. The 1960s was the time for silver to be selected again for the management of burns in the form of 0.5% $AgNO_3$ solution. In 1968 $AgNO_3$ was combined with a sulphonamide antibiotic to produce silver sulfadiazine (SSD) cream, which acts as a broad spectrum silver-based antibacterial. This continues to be prescribed to-date chiefly for the management of burns (Chopra, 2007).

### 2.1.3 Sources of AgNPs contamination

A study estimated that probably 300,000 kg of discharged silver entered ecosystems each year worldwide (Bouwmeester et al., 2009). Recently, AgNPs have begun to increase dramatically, for example, by using a computer model, Blaser et al. (2008) analyzed the risk caused by AgNPs due to textile and plastic uses to freshwater ecosystems and predicted that in the future 15% of the total silver released into water in the European Union.

One of the sources that release AgNPs is from the washing machine. Nowadays, the behaviors of consumers have been positively changed in terms of energy and water saving, especially, when they are washing their clothes by using machines. For instance, instead of using hot water, cold water is used. The problem associated with this replacement is the fungi and bacteria cannot be cleaned. In order to meet demand of consumers and solve the problem, the washing machines have been improved by adding silver to their products. Silver ions will be released during wash through electrolysis process. The addition of $Ag^+$ to be thought of not only solves the fungi and bacteria problems but also creates a good feeling to the clothes. As consequently, the silver released during washing is increased to about 0.05 mg/L. The discharged silver is believed to migrate to wastewater treatment facilities and end up in sewage sludge. It may also be spread on agricultural lands (Blaser, 2008).

Textile is also the main source of AgNPs. For examples, the amount of Ag leached from the socks ranged from 0.3 to 377 µg/g (Geranio et al., 2009) that is somewhat higher than that was reported by Benn and Westerhoff (2008) (1 to 68 µg/g). This difference may come from extraction procedure and the speed of agitation used in their experiments. The study showed that Ag in the particulate fraction of greater than 450 nm in diameter is probably the most predominant form of Ag released into the washing liquor and subsequently to wastewater. During washing the synergistic effects of chemical agents and the mechanical stresses may enhance the Ag release from a textile. A comparison of the fabric samples indicates that the manufacturing processes play a determining role in the amount and form of Ag leached into washing water.

## 2.2 Forms of AgNPs in the environments

The forms of silver in the environment and their attributes are summarized as shown in Table 2.1.

Table 2.1 Forms of silver *(Kahru and Dubourguier, 2009)*

| Type of silver | Approximate size | Attributes |
|---|---|---|
| 1. Elemental/metallic (a single atom) | 0.288 nm | Not found as single atom in nature, normally found as an aggregate. Elemental silver has no oxidation state. |
| 2. Silver ion (ionic) | 0.258 nm | Toxic, may dissolve in water, may have positive or negative charge. |
| 3. AgNPs | 1-100 nm | May release ions and/or be toxic on its own |
| 4. Colloidal | 1-1000 nm | A mixture of different sized particles, suspended in fluid, may contain nanoparticulate silver or silver ions or both |
| 5. Inorganic silver compounds/silver salts e.g. silver chloride, silver oxide | Depends | Not easily dissolved, can be nanosized |
| 6. Organic silver compounds e.g. silver proteins | Depends | Covalent, almost impossible to dissolve |

## 2.3 Fate and transport of AgNPs in the environment

Manufactured nanomaterials will enter the environment through intentional and unintentional releases such as atmospheric emissions and solid or liquid waste streams from production facilities. In addition, nanomaterials in paints, fabrics, and personal and health care products including sunscreens and cosmetics, enter the environment in proportional to their use. Emitted nanomaterials will ultimately deposit on land and water surface. Nanomaterials that reach in the land have the potential to contaminate soil and migrate into surface and ground waters. Particles in solid wastes, wastewater effluents, direct discharges, or accidental spillages can be transported to aquatic systems by wind or rainwater runoff. The biggest release in the environment can come from spillages associated with the transportation of manufactured nanomaterials from production facilities to other manufacturing sites, intentional releases for environmental applications, and diffuse releases associated with wear and erosion from general use (Blaser, 2008).

Silver does not often occur in the environment. It may be released through weathering process and raining. Silver may be present in form of monovalent to trivalent oxidation states. The trivalent state is unstable in aquatic environments. The monovalent is normally found to combine with sulfide, bicarbonate or sulfate or chlorides and sulfates complexes. Silver salts, such as $AgNO_3$ and AgCl are well dissolved in water (Bouwmeester et al., 2009). Once after-use-silver has been discharged into the environment, it is governed by environmental conditions, such as organic matter content, concentration of sulfide and pH (Choi et al., 2008). The presence of natural organic matter, chloride, sulfide, sulphate, and hardness may lower silver toxicity. The mobility of silver increases with dissolved oxygen (DO) and pH increase. The transport and disperse of silver enhanced when it exists in free form or form of colloids.

Reactivity of AgNPs may be different depending on their surface structure and shape. The surface properties of AgNPs are also one of the most important factors that influence their stability and mobility. As in aquatic systems, discharged AgNPs tend to combine with sediment particles to form the larger ones. Therefore, sediment is considered as the important source of sink for AgNPs when released to the environment. Logically, benthic organisms are key receptors for AgNPs. There is only a small

proportion of the total mass of AgNPs for direct uptake throughout the water phase. In this phase aggregation may play significant role in causing toxicity especially to algae (Navarro et al., 2008). The ability of silver and AgNPs to bind to other toxic substances, toxicity effect on groundwater, air pollution, accumulation along the food chain, differential effects in saltwater as well as freshwater has not been fully addressed.

## 2.4 Characteristics of antimicrobial activity of AgNPs

The AgNPs were classified as “extremely toxic” because its lethal concentration ($LC_{50}$) is less than 0.1 mg/L (Kahru and Dubourguier, 2009). Their antibacterial effects have been shown to depend not only on size (Morones et al., 2005) but also on shape (Pal et al., 2007). The size of the NPs implies that it has a large surface area to come in contact with the bacterial cells and hence, it will have a higher percentage of interaction than the bigger particles (Pal et al., 2007; Morones et al., 2005). The AgNPs of smaller than 10 nm interact with bacteria and produce electronic effects which enhance the reactivity of NPs. Thus, it is concluded that the bactericidal effect of AgNPs is size dependent (Morones et al., 2005).

The antimicrobial efficacy of the NPs also depends on their shapes. This property can be confirmed by studying the inhibition of bacterial growth by differentially shaped NPs (Pal et al., 2007), for instance, truncated triangular shape showed bacterial inhibition with silver content of 1 µg whereas spherical was 12.5 µg and much more needed for the rod shaped particles (50 to 100 µg). In other words, the AgNPs with different shapes have different effect levels on bacterial cells.

Due to its toxicity, AgNPs can be widely used as antifungal (Panacek et al., 2009), bactericidal agents (Lee et al., 2009). Currently, several studies have been continuously carried out to elucidate the *in vitro*, *in vivo* effects of AgNPs on organisms. Table below listed out some previous studies about the antimicrobial activity of AgNPs.

Table 2.2 Inhibitory concentrations of AgNPs on some selected species

| Type of organisms | MIC (mg/L) | References |
|---|---|---|
| *Candida albicans I* | 0.42 | (Panacek et al., 2009) |
| *Candida albicans II* | 0.21 | |
| *Candida parapsilosis* | 1.69 | |
| *Candida tropicalis* | 0.84 | |
| *Escherichia coli* | 120 | (Ruparelia et al., 2008) |
| *Bacillus subtilis* | 40 | |
| *Staphylococus aureus* | 120 | |
| *Nitrifying bacteria* | 1.0 | (Choi et al., 2008) |
| *Yeast* | 0.70 | (Kim et al., 2007) |
| *Escherichia. coli* | 0.35 | |
| *Staphylococus. aureus* | 3.50 | |

*Note: The minimum inhibitory concentration (MIC) is defined as the lowest concentration of material that inhibits the growth of an organism.*

Table 2.3 Median L(E)C50 values for synthetic AgNPs towards different groups of organisms*(Kahru and Dubourguier, 2009).*

| Group of organisms | mgAg/L (nano Ag) | LOEC | NOEC |
|---|---|---|---|
| Crustaceans | 0.04 | 0.004 | 0.0004 |
| Bacteria | 7.60 | 0.076 | 0.0076 |
| Algae | 0.23 | 0.023 | 0.0023 |
| Fish | 7.10 | 0.071 | 0.0071 |
| Ciliates | 39 | 0.39 | 0.039 |

*Note:*

- *NOEC: No observable effect concentration, corresponding to the highest administered dose that produces no detectable response in test animals, NOEC = LOEC/10*
- *LOEC: Lowest observable effect concentration, corresponding to the lowest administered dose capable of producing a measurable increase in the frequency of pathological changes,* $LOEC = EC_{50}/10$

- *The classification is based on median L(E)C50 value of the most sensitive organism used: <0.1 mg/L= extremely toxic to aquatic organisms; 0.1-1.0 mg/L= very toxic to aquatic organisms; 1-10 mg/L= toxic to aquatic organisms; 10-100 mg/L= harmful to aquatic organisms; >100 mg/L= non-toxic to aquatic organisms.*

Table 2.4 Summary of *in vitro* and *in vivo* effect of AgNPs in selected organisms

| **In vitro/vivo** | **Type of cell** | **Target organ** | **Effect** | **Reference** |
|---|---|---|---|---|
| In vitro | Human cells | HepAD38 | - Reduce formation of DNA and inhibit the formation of RNA | (Lu et al., 2008) |
| | Human enzymes | Cytochrome P450 | - Inhibit oxidation based biological process | (Sereemaspun et al., 2008) |
| | Human cells | HepG2 | - Demage to chromosome<br>- Induce abnormal morphology | (Kawata et al., 2009) |
| | Human cells | HeLa cells | - Cause cell death associated with apotosis | (Miura and Shinohara, 2009) |
| | Human cells | HepAD38, mRNA | - Cause oxidative stress | (Kim et al., 2009) |
| | Human cells | Skin cells | - Penaltrate and cause demage skin | (Laresea et al., 2009) |
| | Escherichia Coli | E. coli cell | - Cause cell damage by pits formation | (Sondi and Salopek-Sondi, 2004) |
| | Aquatic species | Oryzias latipes cell | - Induce chromosomal aberrations and aneuploidy | (Wise, 2010) |
| In vivo | Zebra Fish | Embryos | - Increase deformation rate of embryos thus causing mortality and hatching delay | (Lee et al., 2009) |
| | Sprague-Dawley Rat | Liver and kidney | - Accumulate in liver and kidney and cause demages | (Kim et al., 2008) |
| | Drosophila melanogaster | DNA | - Produce malondialdehyde as the end product of lipid peroxidation | (Ahamed et al., 2010) |

### 2.4.1 *In vitro* effect

Several studies confirmed that AgNPs potentially affect human cells. It was proved that AgNPs could inhibit the *in vitro* production of hepatilis B virus (HBV) RNA and extracellular virions. Lu et al. (2008) used monodisperse AgNPs with the mean diameters of approximate 10 nm and 50 nm that were prepared from reduction of $AgNO_3$ in HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer. HepAD38 cell line was used as infection model to determine the *in vitro* anti-HBV activities of the AgNPs. The findings indicated that both AgNP sizes were able to reduce the extracellular HBV DNA formation of HepAD38 cells by greater than 50% as compared to the control. The results showed that AgNPs had little effect on the amount of HBV covalently closed circular DNA, but could inhibit the formation of intracellular HBV RNA. The authors believed that the viral and silver systems were in nano size range, and thus they could directly interact with each other, with DNA and with RNA of viruses. A cell level study was conducted by Sereemaspun et al. (2008) by using gold nanoparticles (AuNPs) and AgNPs because their popular use in hospital treatments. Such metallic nanomaterials are used for therapeutic and diagnostic on the microsomes containing wild-type cDNA expressed human CYP450 enzymes CY1A2, 2C9, 2C19 and 3A4. The experimental results indicated that metallic NPs could penaltrate across the microsomal membrane and inhibit oxidation based biological process thus exhibit inhibition of human cytochrome P450 enzymes. Kawata et al. (2009) evaluated *in vitro* toxicity of AgNPs at noncytotoxic doses in human hepatoma cell line, HepG2, based on cell viability assay, micronucleus test, and DNA microarray analysis. Polystyrene nanoparticles (PSNPs) and silver carbonate ($Ag_2CO_3$) were used to compare the toxicity with that of AgNPs. The findings suggested that AgNPs cause much stronger damages to chromosome than that of PSNPs, $Ag_2CO_3$, and $Ag^+$. It was demonstrated that AgNPs accelerated cell proliferation at low doses (less than 0.5 mg/L). However, only AgNPs exposure exhibited a significant cytotoxicity at higher doses (greater than 1 mg/L) and induced abnormal cellular morphology, displaying cellular shrinkage and acquisition of an irregular shape. In addition, only AgNPs exposure increased the frequency of micronucleus formation up to $47.9 \pm 3.2\%$ of binucleated cells. It was concluded that both AgNPs and $Ag^+$ contributed to the toxic effect of AgNPs.

Silver nanoparticles also caused problem for DNA replication fidelity and cell damage. A study was carried by Yang and his colleauges (2009) by using cell cultures of *Escherichia coli* transformed with the wild-type rpsL gene that then treated with three types of AgNPs (silver nanopowder, silver-copper nanopowder, and colloidal silver). Molecular technique, polymerase chain reactions (PCRs), was used to quantify the replication fidelity of the rpsL gene in the presence of AgNPs. Yang et al found some similar results of that of Lu et al. (2008). The replication fidelity of the rpsL gene was differentially compromised by all three kinds of AgNPs compared with the control. This was because AgNPs bound with genomic DNA thus compromised DNA replication fidelity. In 2004, Sondi and Salopek-Sondi studied antimicrobial activity of AgNPs against *Escherichia coli* as a model for gram-negative bacteria. From the scanning and transmission electron miscrocopic (SEM and TEM) micrographs, formation of aggregates composed of AgNPs and dead bacterial cells were observed. Silver nanoparticles interacted with the building elements of the bacterial membrane and caused damage to the cells (forming of pits).

Yen et al. (2009) proved that AgNPs could enter the cells and cause stresses. They were both trapped in vesicles in the cytoplasma, in particlular, due to its negatively charged AuNPs could adsorb serum protein and resulted in higher cytotoxicity and immunological response of gold as compared to AgNPs. Mirura and Shinohara (2009) observed the cytotoxic effect of AgNPs in HeLa cells. They found that the AgNPs-induced toxicity was lower than that of $AgNO_3$. The apoptosis was associated with the cell death. Furthermore, the well-known oxidative stress-related genes, ho-1 and mt-2A, were up-regulated by AgNPs treatment. Oxidative stress-dependent toxicity of AgNPs in human hepatoma cells was also studied by Kim et al. (2009). Toxicity caused by $Ag^+$ was recorded to be in parallel to AgNPs. Chemical analysis confirmed that AgNPs contain a negligible amount of $Ag^+$. Metal-responsive metallothionein 1b (MT1b) mRNA expression was not induced in AgNP-treated cells, while it was induced in $AgNO_3$-treated cells. Silver nanoparticles agglomerated in the cytoplasm and nuclei of treated cells, and induced intracellular oxidative stress. It exhibited cytotoxicity with potency comparable to that of $Ag^+$ in *in vitro* cytotoxicity assays. However, the toxicity of AgNPs

was prevented by use of the antioxidant N-acetylcysteine, and AgNP-induced DNA damage was also prevented by N-acetylcysteine.

Silver nanoparticles can cause cytotoxicity and genotoxicity for aquatic species. Wise et al. (2009) conducted the experiment on the effect of AgNPs (30 nm in diameters) in Oryzias latipes cell line. The findings showed that AgNPs at concentration of 5 $\mu g/cm^2$ induced 0.1% survival of the fish cells in a colony forming assay. It was also found that AgNPs induced chromosomal aberrations and aneuploidy, for instance, with the concentration of 0.3 $\mu g/cm^2$ resulted in damage in 15.8% of metaphases and 24 total aberrations in 100 metaphases. Thus it was concluded that AgNPs are cytotoxicity and genotoxic to fish cells. Recently, Larese and colleagues (2009) performed a study to evaluate *in vitro* skin penetration of AgNPs by using Franz diffusion cell method with intact and damaged human skin. Physiological solution was used as receiving phase and 70 $\mu g/cm^2$ of AgNPs coated with polyvinylpirrolidone dispersed in synthetic sweat were applied as donor phase to the outer surface of the skin for 24 h. By using TEM, it was concluded that AgNPs absorption through intact and damaged skin was in trace concentration but detectable. It was expected that there was possible an increasing permeation of silver applied as NPs.

### *2.4.2 In vivo* effect

Nanoparticles may have different effects on human health in relation to conjugate bulk materials. Increase in biological activity of NPs can be beneficial, detrimental or both. Many NPs are small enough to have an access to skin, lungs, and brain (Panyala et al., 2008). Exposure of metal containing NPs to human lung cells generated reactive oxygen species which can lead to oxidative stress and thus cellular damage (Panyala et al., 2008). However, no sufficient information is available on the adverse effects of NPs on human health, but studies are ongoing to address this matter.

Silver nanoparticles could affect on developmental phase of zebra fish. Silver nanoparticles administered *in vivo* to zebra fish embryos increased deformation rates, and led to death. The developmental stage inside embryos can be damaged by AgNPs (Lee et al., 2007) as a result of inducing altered physiology including the degeneration of body parts and an increase in mortality and hatching delay. The research thus concluded that

AgNPs induced a dose-dependent toxicity in zebra fish embryos, which impacts normal development.

Silver nanoparticles could also affect on liver and kidney of rats. The oral toxicity of AgNPs is of particular concern to ensure public and consumer health. Kim et al. (2008) conducted study tested the oral toxicity of AgNPs (60 nm) over a period of 28 days in Sprague-Dawley rats following Organization for Economic Cooperation and Development (OECD) test guideline 407 with Good Laboratory Practice (GLP) application. Eight-week-old rats (283g for the males and 192 g for the females) were divided into four 4 groups including vehicle control, low-dose group (30 mg/kg), middle-dose group (300 mg/kg), and high-dose group (1000 mg/kg). After 28 days of exposure, the blood biochemistry and hematology were investigated, along with a histopathological examination and silver distribution study. The results indicated that exposure to over 300 mg of AgNPs may result in slight liver damage. Silver nanoparticles did not induce genetic toxicity in male and female rat bone marrow *in vivo*. Nonetheless, there was a dose-dependent accumulation of silver content in all the tissues examined for example. The AgNPs were accumulated in the kidneys of the tested rats with a twofold increase in the female kidneys when compared with the male kidneys.

Most recently, a study was undertaken by Ahamed et al. (2010) to examine the toxicological effects of well-characterized polysaccharide coated 10 nm AgNPs on heat shock stress, oxidative stress, DNA damage and apoptosis in *Drosophila melanogaster*. AgNPs at the concentrations of 50 and 100 mg/L were used in the experiments by well mixing with the meal of larvae of *Drosophila melanogaster* for 24 and 48 h. Heat shock stress, oxidative stress, DNA damage and apoptosis were observed during the experiment period. It was found that the rate of lipid peroxidation was accelerated through the production of malondialdehyde as the end product of lipid peroxidation. This information was confirmed because of a dramatic decline of antioxidant glutathione content in AgNPs exposed organisms. In addition, activities of antioxidant enzyme superoxide dismutase and catalase were also significantly higher in the organisms exposed to AgNPs. Furthermore, AgNPs up-regulated the cell cycle checkpoint p53 and cell signaling protein p38 that are involved in the DNA damage repair pathway. Moreover, activities of caspase-3 and caspase-9, markers of apoptosis were significantly higher in AgNPs

exposed organisms. Further studies should be carried out to provide sufficient information for the assessment of *in vivo* effect of AgNPs.

## *2.5* Occurrence of AgNPs in the environment

### 2.5.1 In water

The sensitivity of aquatic organisms is individually different. Study on the toxicity of AgNPs to aquatic organisms is still limited and tends to focus on some key species such as zebra fish, invertebrates, and some algae. Toxicity of AgNPs is a result of its characteristic and $Ag^+$ and depends on their concentration. Silver ions react with thiol, a molecular group that includes a bonded sulfur and hydrogen atom (-SH) in biomolecules. For instance in fish, $Ag^+$ block the active absorption of sodium and chlorine as well as causing sublethal effects. In water fleas $Ag^+$ disturbed ion regulation via a competitive inhibition of $Na^+$ (sodium) uptake (Hogstrand et al., 2002).

In natural environment, $Ag^+$ tends to form stable complexes to both inorganic, such as chloride, thiosulphate and sulphide, and organic, for instance, monomeric thiols, and natural organic matters. Silver thiosulphate was thought to be relatively inactive. However, silver thiosulphate complexes could be transported across cell membranes in *Chlamydomonas reinhardtii* and *Pseudokirchneriella subcapitata*, two freshwater algae species, and that this led to increased toxicity (Hiriart-Baer et al., 2006).

Navarro et al. (2008) investigated the toxicity of AgNPs versus $Ag^+$ to *Chlamydomonas reinhardtii* and found that $Ag^+$ appeared to be 18 times more toxic than the AgNPs. However, several studies revealed that AgNPs appeared more toxic than the $Ag^+$ alone because it contributed to the overall toxicity of silver to the algae by providing a continuous source of $Ag^+$.

Toxicity of silver engineered nanoparticles (AgENs) was tested on marine algae diatom *Thalassiosira weissflogii* (Miao et al., 2009). The researchers found that AgENs formed non-toxic aggregates in seawater. Free $Ag^+$ was reduced by diafiltration or thiol complexation thus no effect was observed. Although the magnitude of AgENs was found to be greater than background level in natural aquatic environment but it was detoxified by secretion of polysaccharide-rich algal expolymeric substances. A recent research reported that AgNPs did not have any or very little impact on estuarine sediment bacterial

diversity (Bradford et al., 2009). The possible reasons for these could be included environmental factors, in particular the negatively cloride ions in estuary water affecting the chemistry and behavior of AgNPs (Bradford et al., 2009).

### 2.5.2 In soil

Toxicity of AgNPs in soil appears to be dependent on physiochemical soil properties and sediment properties. It is slowly being investigated. There was a variation in the content of silver in soil. The variation heavily depends on whether it is impacted by industry contamination (2.2 mg/kg to 44 mg/kg) or not (<1 mg kg) (Jacobson et al., 2005). However, land use patterns influenced on the mobility of metals, any changes in use, for example fertilizers could result in a reduction in pH to below 4. In addition, organic matter was believed a dominant factor in silver sorption (Jacobson et al., 2005).

Currently, very little information has been considered on the effect of AgNPs on soil microbial communities in real soils or *in situ*. Murata et al. (2005) reported that silver disturbed the growth of soil microbes even in the much smaller concentration in comparison to other heavy metals. Ammonifying and nitrogen bacteria play a crucial role in assimilating nitrogen in soil, particularly susceptible to toxic substances as silver. Silver ions have been proved to inhibit enzymes needed for nitrifying bacteria (Ratte, 1999).

Silver could slow down and prohibit denitrifying bacteria activity. Denitrifying bacteria play an important role in removing nitrate from water contaminated by excessive fertilizer use, in some soils, wetlands and other wet environments through converting nitrate to nitrogen gas to the atmosphere. However, silver disrupted activity of the bacteria (Throbäck et al., 2007) implying that the ecosystems may be at risk. As a result, plant productivity could be reduced; eutrophication in rivers, lakes and marine ecosystems may become more likely to happen and damage the ecosystems.

Nematodes suffered negatively impact by AgNPs. Nematodes are widely found in soils and play a critical role in the soil food web. Their functions include primary production, decomposition, energy flow, and nutrient cycling. Nematode abundance also

serves as a useful indicator in natural ecosystems to the presence of soil pollutants and ecological disturbances. Several ecological toxicity tests have been developed for this purpose. The study of Wang and his colleagues (2009) was the first to investigate the effect of metal oxide NPs on nematodes *Caenorhabditis elegans (C.elegans).* The findings showed that both AgNPs and bulk silver were toxic to nematode and resulted in impaired growth and reproductive ability.

### 2.5.3 In wastewater treatment plant

The disposal of silver products into wastewater raises a number of concerns as the resulting sewage sludge may be used on agricultural soils or disposed as solid waste in landfills. The final destination of discharged silver may be subsoil and groundwater and then would probably cause health problem. Silver toxicity in water is determined by the concentrations of $Ag^{+}$. This is typically low in wastewater treatment systems and in the natural environment, partly due to silver's tendency to form strong bonds with various ligands such as chloride, sulfide, thiosulfate, and dissolved organic carbon. Silver nanoparticles may have a variety of fates in wastewater, including being converted into ionic form, forming a complex with other ions, molecules, or molecular groups, agglomerating or remaining in NPs form (Zhang et al., 2007; Blaser, 2008).

Wastewater treatment relies on heterotrophic microorganisms for organic and nutrient removal, while autotrophic microorganisms play an important role in nitrification. However, they can be easily harmed by AgNPs. Choi and coworkers (2008) evaluated the effect of AgNPs, $Ag^{+}$ and colloid AgCl on heterotrophic and autotrophic growth and found that nitrifying bacteria were especially susceptible to inhibition by AgNPs. Silver nanoparticles showed inhibition of autotrophic bacterial growth was almost twice that of $Ag^{+}$ and colloids (Choi et al., 2008). Heterotrophic bacteria in contrast were more susceptible to $Ag^{+}$ versus AgNPs and colloid AgCl. It was suggested that the accumulation of AgNPs may have detrimental effects on the activities of microorganisms in wastewater treatment. Thus, regulation of AgNPs should be strictly considered.

## 2.6 Nitrification process

Nitrification is a key process in wastewater treatment systems. Biological removal of nitrogen from wastewater involves nitrification of ammonia ($NH_3$) to nitrite ($NO_2^-$) and nitrate ($NO_3^-$) followed by denitrification of nitrite and nitrate to nitrogen gas ($N_2$) which is released into the atmosphere (Aoi et al., 2000). Ammonia oxidation is critical to global nitrogen cycling and is often thought to be driven by only ammonia-oxidzing bacteria. It also is considered the rate-limiting step of nitrification in a variety of environments. The recent finding of new ammonia-oxidizing organisms belonging to the archeal domain challenges the traditional perception. Two major microbial groups are now believed to be involved in aerobic autotrophic ammonia oxidation: chemolithotropic ammonia oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA). The presence of AOA in activated sludge opens new opportunities for enhancing and elucidating its role of ammonia removal in wastewater treatment plants and wetlands (You et al., 2009).

### 2.6.1 Factors affecting on nitrification process

Nitrification bacteria must have dissolved oxygen (DO) to perform their work. Nitrification occurs only under aerobic conditions at DO levels of 1.0 mg/L or more. At DO concentration less than 0.5 mg/L, the growth rate is minimal. When DO is greater than 2 mg/L the specific oxygen uptake rate will be constant and become maximum. Nitrification requires a long retention time, a low food to microorganism ratio (F:M), a high mean cell residence time (measured sludge age), and adequate alkalinity buffering (Aoi et al., 2000).

Nitrifying bacteria were found to exhibit various organizational forms under different conditions of substrate composition and concentration (Aoi et al., 2000; Lydmark et al., 2007). Ammonia-oxidizing bacteria were dominant in ammonia-rich inorganic wastewater, while heterotrophic bacteria and ammonia-oxidizing bacteria were localized at different positions in the biofilm in organic wastewater (Aoi et al., 2000). Laboratory scale study with continuous-flow reactors indicated that the concentration of ammonium and nitrite has affected on the communities and populations of ammonia-oxidizing bacteria (Limpiyakorn et al., 2007). The findings showed that *Nitrosomonas*

*oligotropha* cluster was predominated in the reactors with lower ammonium loads (2, 5 and 10 mM $NH_4^+$-N) and without nitrite accumulation (0 and 2 mM $NO_2^-$ -N). In contrast, *Nitrosomonas europaea* was dominant in the reactor with the highest ammonium load (30 mM $NH_4^+$-N) and with the accumulation of nitrite (12-22 mM $NO_2^-$ -N).

The nitrification process produces acid. This acid formation lowers the pH of the biological population in the aeration tank and causes a reduction of the growth rate of nitrifying bacteria. The optimum pH for Nitrosomonas and Nitrobacter species is between 7.5 and 8.5; most treatment plants are able to effectively nitrify with a pH of 6.5 to 7.0. Nitrification stops at a pH below 6.0. However, a series of studies revealed different niches for different groups of AOB (and also AOA) in soils with pH between 4.5 and 7.5 (Junier et al., 2010). The nitrification reaction (the conversion of ammonia to nitrate) consumes 7.1 mg/L of alkalinity as $CaCO_3$ for each mg/L of ammonia nitrogen oxidized. An alkalinity of no less than 50-100 mg/L is required to insure adequate buffering (Aoi et al., 2000). It was reported that pH can cause small change in the community structure of mircroorganisms (Junier et al., 2010).

Water temperature also affects the rate of nitrification (Malgorzata et al., 2006). Nitrification reaches a maximum rate at temperatures between 30 and 35$^o$C. At temperatures of 40$^o$C and higher, nitrification rates fall to near zero. At temperatures below 20$^o$C, nitrification proceeds at a slower rate, but will continue at temperatures of 10$^o$C and less. However, if nitrification is lost, it will not resume until the temperature increases to well over 10$^o$C. Some of the most toxic compounds to nitrifiers include cyanide, thiourea, phenol and heavy metals such as silver, mercury, nickel, chromium, copper and zinc (Hu et al., 2002a; Murata et al., 2007; Ruyters et al., 2010).

The following equations describe the nitrification process

Catabolic reaction

$$NH_4^+ + 1.5O_2 \longrightarrow 2H^+ + 2H_2O + NO_2^-$$

$$NO_2^- + 0.5O_2 \longrightarrow NO_3^-$$

Cell synthesis reaction

$$5CH_2O + HCO_3^- + NH_4^+ \longrightarrow C_5H_7NO_2 + 4H_2O + CO_2$$

### 2.6.2 Phylogeny of ammonia-oxidizing microorganisms

Heterotrophic ammonia oxidizers and chemolithotrophic AOB were traditionally thought to be responsible for ammonia oxidization (Aoi et al., 2000). Recent researches have showed that there are two major microbial groups involved in ammonia oxidation. They are ammonia oxidizing bacteria and ammonia oxidizing archaea. The discovery of AOA in natural and engineered systems demonstrates the role of AOA in nutrient removal and global nitrogen cycling.

### 2.6.2.1 Ammonia Oxidizing Bacteria (AOB)

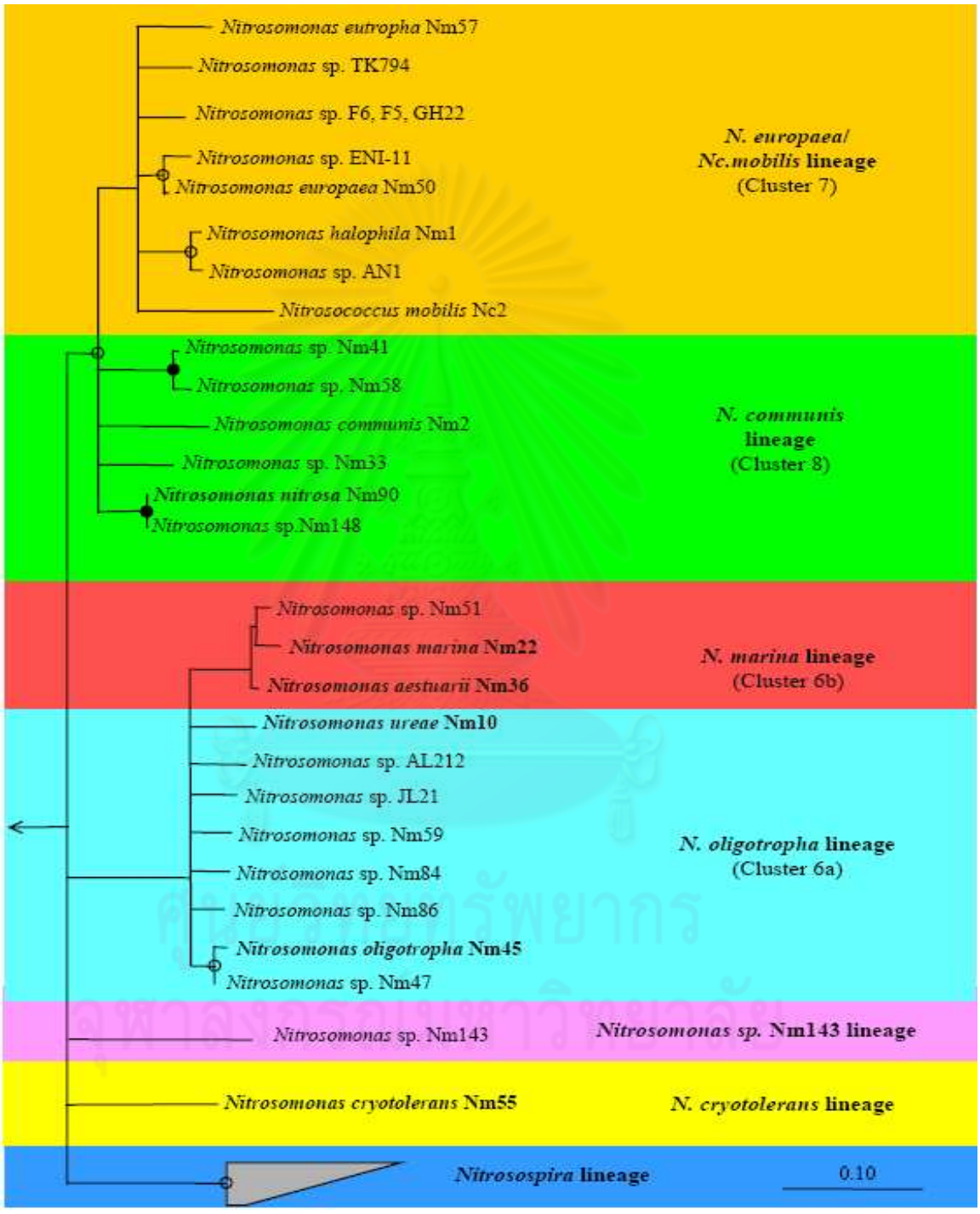


Figure 2.1 *amoA*-based phylogenetic tree of the betaproteobacterial AOB (Koops *et al.*, 2003).

### 2.6.2.2 Ammonia Oxidizing Archaea (AOA)

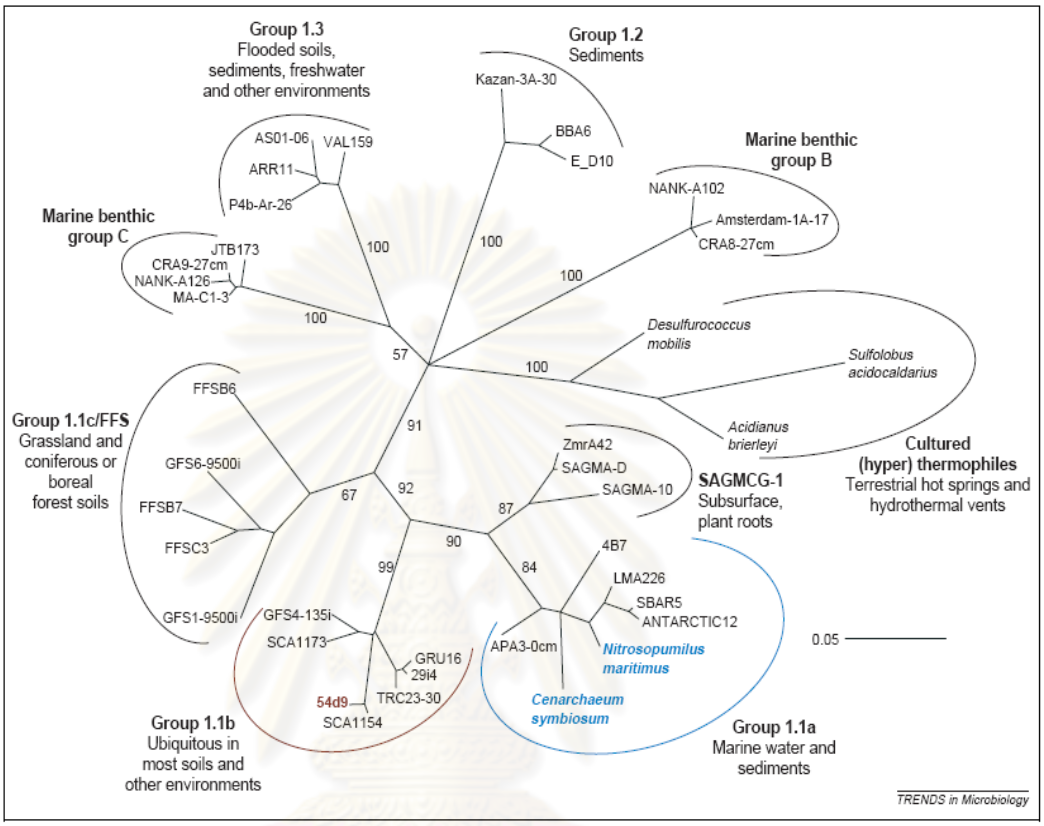


Figure 2.2 Phylogenetic tree of AOA (Nicol and Schleper, 2006).

The three domains of life on Earth include the two prokaryotic groups, Archaea and Bacteria. The Archaea are distinguished from bacteria based on phylogenetic and biochemical differences, but currently there is no unifying ecological principle to differentiate these groups (Valentine, 2002). Cultivated archaea can be divided into five broad physiological (halophiles, thermophiles and acidophiles) or metabolic (nitrifiers and methanogens) groups (Valentine, 2002). Various molecule tools have been applied to study AOB and AOA and determine their abundance and community structure changes from natural and engineered systems. AOA are to be found to thrive in various habitats including hot/thermal springs, marine and fresh waters, soils and wastewater treatments systems, where they may outnumber their counterpart, AOB (You et al., 2009). There was presence of AOA in nitrifying wastewater treatment

bioreactors by using PCR primers targeting archaea *amoA* genes to retrieve and compare 75 sequences from wastewater treatment plants across the U.S (Park et al., 2006). The study also indicated that all of the archaeal *amoA* sequences are distributed primarily in four major phylogenetic clusters and are most closely related to the characteristics of soils and sediments. The presence of AOA appears to be dependent upon oxygen concentration and solids retention time (SRT) in wastewater treatment plants. There may be many other factors controlling the presence of AOA, but the temperature is likely an important factor controlling the growth and diversity of AOA in engineered systems (Urakawa et al., 2008). Recently, the community of AOA was observed intensively both in municipal and industrial wastewater treatment plants (Sonthiphand and Limpiyakorn, 2009). Beside 4 clusters (A, B, C, and D) were found by Park et al. (2006) from the activated sludge, there 8 other clusters were also found (Figure 2.3). Of eight other clusters found, the cluster K was the most dominant. It was reported that the communities of AOA in the industrial WWTPs were less diverse than those in the municipal WWTPs. The study found various clusters of AOA in municipal wastewater treatment plants (6 plants) with the influent ammonium concentration ranged from 5 to 13 mg-N/l while only two out of four industrial plants that could find the AOA with moderate ammonium loads (40-70 mg-N/l). Other than ammonium loads, the operational conditions such as hydraulic retention time (HRT) and solid retention time (SRT) also play a decisive role in the presence of AOA community as reported that SRT greater than 15 days and HRT greater than 24 hrs contained AOA communities in their system whereas no AOA was observed if SRT and HRT less than 15 days and 24 hrs, respectively.

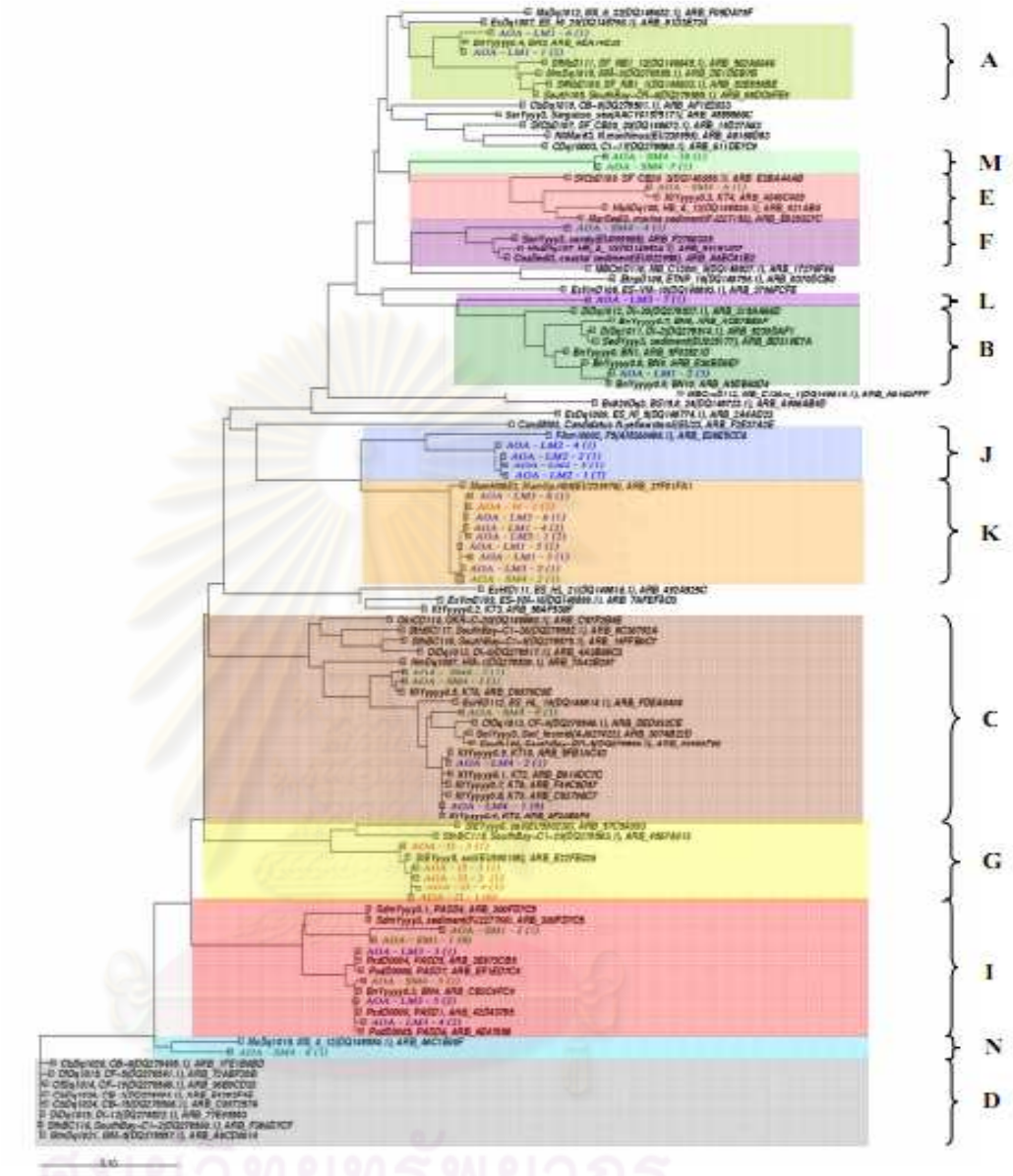


Figure 2.3 Neighbor joining tree of AOA *amoA* sequences from full-scale WWTPs (Sonthiphand and Limpiyakorn, 2009).

### 2.6.2.3 Differences between archaea and bacteria

Table 2.5 The differences between Archaea and bacteria (Valentine, 2002)

| Categories | Archaea | Bacteria |
|---|---|---|
| **Genomes** | - Smaller | - Larger |
| **Diversity** | - Less | - More |
| **Lipid membranes** | - Lipids consist of isoprenoidal alcohols (branched carbon chains) that are ether-linked to glycerol.<br>- Tetraether-based membranes<br>- Monolayer-saturated membranes<br>- Less permeable to ions | - Lipids consist of fatty acids esterified (straight carbon chains) to a glycerol moiety.<br>- Diether- based membranes<br>- Bilayers-saturated membranes<br>- More permeable to ions |
| **Pathogenic** | - Rare or nonexistent | - Better suited to pathogenesis |
| **Living conditions** | - Physically or geochemically extreme and stable conditions | - Variables |
| **Cell wall** | - Varies in composition; contains no peptidoglycan. | - Contains peptidoglycan |
| **Antibiotic sensitivity:** *penicillins, tetrecylins, and aminoglycosides kanamycin, chloramphenicol, rifampicin, and anisomycin.* | - No<br>- Ribosomes behave more like eukaryotic ribosomes | - Yes<br>- Ribosomes behave less like eukaryotic ribosomes |

The Archaea as the third domain of living organisms. This domain is subdivided into three kingdoms such as Crenarchaeotes, Euryarchaeotes, and Korarchaeotes. Although the archaea are considered to be prokaryotic cells, they possess certain characteristics that are different from those of bacteria or eukaryotes. Their membranes are made of branched hydrocarbon chains attached to glycerol by ether linkages. Their cell walls do not contain peptidoglycan. Their rRNA is different from eukaryotic and prokaryotic rRNA. It appears that archaea are more closely related to eukaryotes than to bacteria. As regards their metabolism, archaea may range from organotrophs (use of

organic compounds as a source of carbon and energy) to chemoautotrophs (use of $CO_2$ as a carbon source).

Most of the archaea live in extreme environments and are called extremophiles. They include the thermophiles, hyperthermophiles, psychrophiles, acidophiles, alkaliphiles, and halophiles. Thus, their unique products are of great interest to biotechnologists. Archaeal enzymes display attractive properties such as tolerance to high and low temperatures, high salt concentrations, high hydrostatic pressures, and organic solvents.

Table 2.6 Summary of the archaeal phospholipid compared with bacterial phospholipid *(Ulrih et al., 2009)*

| | **Components of lipid** | **Archaea** | **Bacteria** |
|---|---|---|---|
| - | Phospholipids | sn-glycerol-1-phosphate (G-1-P) | sn-glycerol-3-phosphate (G-3-P) |
| - | Hydrocarbon chains bound to glycerol moiety at position | sn-2,3-radyl | sn-1,2-radyl |
| - | Hydrocarbon chains are bonded to glycerol moiety by | Ether linkages | Ester linkages |
| - | Hydrocarbon chains of polar lipids types | Highly methyl-branched isoprenoids and isoprenoids | Straight chain fatty acids |
| - | Biopolar lipids with a tetraether core that span through membranes | Found | Rarely found |

Table 2.7 Biochemical adaptation mechanisms such as low-permeability membrane and specific catabolic pathways enable archaea in their evolution (Valentine, 2002)

| Biochemical adaptations | Archaea | Bacteria |
|---|---|---|
| **1. Lipid membranes**<br>*Primary adaptation*<br>Use specific membrane structures to reduce energy loss from maintaing chemiosmotic potential | - Lipids consist of isoprenoidal alcohols that are ether-linked to glycerol.<br>- Tetraether- based membranes<br>- Monolayer-saturated membranes<br>- Less permeable to ions and less energy lost | - Lipids consist of fatty acids esterified to a glycerol moiety.<br>- Diether- based membranes<br>- Bilayers-unsaturated membranes<br>- More permeable to ions and more energy lost |
| **2. Catabolic specificity**<br>*Secondary adaptation*<br>Their capacity to cope with chronic energy stress | - ***Environmental exclusivity***: halophiles, hyperthermophiles and thermoacidophiles (coping with temperature, salinity and acidity).<br>- ***Metabolic exclusivity***: methanogens and methane oxidizers.<br>- ***Singularity of catabolism*** | - Environmental exclusivity gives way to competition with bacteria as conditions become moderate and archaeal success becomes more dependent on catabolic adaptations. |
| **3. Energy conservation**<br>*Secondary adaptation*<br>The capacity to conserve small amounts of metabolically useful energy during catabolism is a key to survival in anaerobic, energy stressed environment. | - Common mechanisms are methanogenesis, anaerobic methane oxidation, proton reduction coupled directly to proton translocation, $H_2$-dependent sulfur reduction and phototrophy.<br>- $H_2/CO_2$ methanogenesis is among the best understood of the archaeal catabolic pathways. | |

## 2.7 Real-time PCR

The scientific, medical and diagnostic communities have been presented the most powerful tool for quantitative nucleic acids analysis: real-time PCR. This new technique is a refinement of the original Polymerase Chain Reaction (PCR). By PCR essentially any nucleic acid sequence present in a complex sample can be amplified in a cyclic process to generate a large number of identical copies that can readily be analyzed. In real-time PCR the amount of product formed is monitored during the course of the reaction by monitoring the fluorescence of dyes or probes introduced into the reaction that is proportional to the amount of product formed, and the number of amplification cycles required to obtain a particular amount of DNA molecules is registered. Typical uses of real-time PCR include pathogen detection, gene expression analysis, single nucleotide polymorphism (SNP) analysis, analysis of chromosome aberrations, and most recently also protein detection by real-time immuno PCR.

In aerobic AOB and AOA, ammonium is first oxidized by the membrane-bound enzyme ammonia monooxygenase (Arp et al., 2002; Könneke et al., 2005). In the case of AOB, the second and energy-producing step is the oxidation of hydroxylamine to nitrite by hydroxylamine oxidoreductase (Arp et al., 2002). In AOA, an alternative mechanism for channeling electrons has been proposed, since available genome information does not support the existence of genes homologous to *hao* and other essential proteins found in AOB (Hallam et al., 2006a).

In all known AOB, the genes encoding the enzyme AMO belong to an operon with the structure amoCAB (Chain et al., 2003; Stein et al., 2007). Multiple copies of the operon are present in the genomes of beta-AOB (Chain et al., 2003; Stein et al., 2007), whereas a single copy has been reported for gamma-AOB (Klotz et al., 2006). Despite the potential of using the whole amoCAB operon for molecular studies, only a portion of the gene *amoA* has been generally used as a molecular marker to study the diversity of AOB (Rotthauwe et al., 1997). It has been argued that this region, which is relatively short (around 450 bp) and highly conserved, provides less resolution than the 16S rRNA gene (Koops et al., 2003; Purkhold et al., 2003). For environmental studies, the

advantages of the higher specificity of the *amo*A approach may outweigh this disadvantage.

As mentioned above, homologs to the *amo*A gene were initially detected in genomic community studies and cultured AOA (Könneke et al., 2005; Treusch et al., 2005). Homologs to *amo*C and *amo*B also have been found, although the structure of an *amo*CAB operon has not been observed in AOA (Nicol and Schleper, 2006). Several primer sets have been developed to amplify archaeal *amo*A (Könneke et al., 2005; Treusch et al., 2005), allowing the identification and quantification of AOA. Currently, archaeal *amo*A sequences are submitted to the GenBank at a faster pace than bacterial *amo*A sequences.

## 2.8 Methods for characterization of AgNPs

The important characteristics of AgNPs are size, shape, charge, surface charge or zeta potential and its distribution in the media.

For characterization of AgNPs, several advanced techniques have been used. Ultraviolet-visible (UV-vis) spectrophotometer is one of the most useful tools. This tool can help users to scan the wavelength peak to choose the best absorption spectra of the AgNPs. Choi et al. (2008) scanned the aliquot of the prepared AgNPs in the range of wavelength from 250 to 700 nm to obtain absorption spectra at 400 nm and at 425 nm by using UV-vis spectroscopy (Cary 50, Varian, and CA). Different sizes and shapes can also exhibit different wavelengths. For example, in the study of inhibition of human cytochrome P450 enzymes by metallic NPs (gold and silver), UV-vis spectroscopy (Shimadzu, Japan) was performed to confirm AgNPs with the spherical shape and with the average size of 15 nm that was peaked at the wavelength of 440 nm (Sereemaspun et al., 2008), and 25 nm-sized AgNPs peaked at 420 nm (Panacek et al., 2009) that was measured by using UV–Vis Spectroscopy with the Specord S600 spectrophotometer (Analytik Jena AG). In other study, time-dependent formation of AgNPs was measured by using UV-vis Spectrophotometry (Cary 300 UV-vis spectrophotometer, Varian, Inc., Palp Alto, California) (Fayaz, 2009).

Nanodimensions of AgNPs including distribution, size and shape are characterized by Transmission Electron Microscopy (TEM). Various brands of TEM have been used and most commonly are H-7650, Hitachi, Japan (Sereemaspun et al., 2008); JEOL 2000 FX MARK II, Tokyo, Japan (Fayaz, 2009); and JEM-2010, Jeol (Panacek et al., 2009). Other advanced equipments have some similar functions with TEM such as Dynamic Light Scattering (DLS), X-ray diffraction (XRD), Electron Spin Resonance (ESR) spectrometer. The presence of AgNPs can be confirmed by Energy-dispersed Spectroscopy (EDS) and the reduction of $Ag^{+}$ to metallic silver characterized by X-ray photoelectron Spectrophotometry (XPS). These can be found in the study of Fayaz (2009).

Surface charge or zeta potential is another important characteristic contributing to the toxicity of AgNPs. The zeta potential of the suspended particle solution was often measured by using Electrophoretic Light Scattering Spectrophotometer (Chae et al., 2009) or Zetasizer NanoZS (Sereemaspun et al., 2008).

Silver ions are contributed to overall toxicity of AgNPs. There are two common methods to measure $Ag^{+}$. Gu et al. (2009) has successfully used Ion Selective Elcetrode (ISE) because it is cheap and easy to use. The ISEs are membrane electrodes that selectively respond to particular ions in the presence of other ions. These include probes that measure specific ions and gasses in solution. The basic ISE setup includes a meter (capable of detecting millivolts), a probe (selective for each analyte of interest), and various consumables used for pH or ionic strength adjustments. Another option is using inductively coupled plasma-optical emission spectroscopy (ICP-OES). This equipment is extremely expensive and quiet complicate to use. However, most of heavy metals in liquid phase can be determined with high precision and accuracy by using ICP-OES (Chae et al., 2009).

The interaction between nanoparticles and microbes needs to be visualized as well. Choi et al. (2008) observed this interaction through FEI Quanta 600F ESEM (with the resolution of 3 nm at 30 kV, FEI Company, OR) equipped with a scanning transmission electron microscopy (STEM) detector in the study of the inhibitory effects of AgNPs, $Ag^{+}$, and AgCl colloids on microbial growth. The interaction between protein-

AgNPs was also analyzed by Fourier Transform-Infrared Spectroscopy (FT-IR) (Fayaz, 2009).

## 2.9 Respirometry

In the biological treatment, we are aware of the capability that microorganisms have to metabolize organic substances. However, many substances that are present in wastewater exert inhibitory or toxic effects on the microorganisms in a wastewater treatment plants. In the context of biological wastewater treatment, inhibition is defined as impairment of the enzymatic system of the living cell or direct manages to the cell structure, resulting in slowing down of the cell activity. When the inhibited biochemical reactions are vital to the cell then the agent is identified as toxic. The effect of toxicity is manifested in microorganisms' increase difficulty to take up nutrients and decrease in growth rate or the ultimate death of the cell. If the toxicity is present, it does not necessarily mean that the microorganism dies but that its activity diminishes. The level of inhibition represents almost continuum leading all the way to death. Since, in a treatment system there are many species, a certain level of toxicity will affect the different microorganisms to various degrees; some would not be able to adapt and eventually will disappear, while others remain in the system. As a result, the composition of the microbial population would change to a new state of affair.

Respirometry is one of the methods used to study impacts of toxic substances (Cokgor et al., 2007). In the presence of inhibitory substances the biomass activity decreases. One clear manifestation of the activity is the oxygen consumption rate in aerobic processes which is recognized as the respiration rate. The respirometric test for inhibitory environments or for a specific substance is based on the measurement of a standard decrement in the respiration rate: the difference in respiration relative to a non-inhibitory situation.

Relation to respirometric test, Monod equation was successfully applied (Downing et al., 1964) to calculate nitrification kinetic values such as half saturation coeffiient (Ks, also known as equilibrium or affinity coefficient), the yield coefficient (k), and the maximum specific growth rate ($\mu_{max}$). Monod established that (1) the mass of

organisms generated is a fixed fraction of the mass of substrate utilized and (2) the specific rate of growth, i.e the rate of growth per unit mass of organisms per unit time, is related to the concentration of substrate surrounding the organisms. The relation of substrate utilization and growth rate (without toxic substance) can be described as following:

$$\mu = \mu_{max} \frac{[S]}{Ks + [S]} - b \qquad \text{Eq. (2.1)}$$

where μ is specific biomass growth rate (gVSS/gVSS.d), $\mu_{max}$ is maximum growth rate of biomass (gVSS/gVSS.d), b is the specific biomass decay rate (gVSS/gVSS.d), [S] is substrate concentration and Ks is the half saturation constant.

***Effect of inhibitors on enzyme activity***

The effect of inhibitors on enzyme activity is described by competitive, uncompetitive and non competitive.

- Competitive Inhibition: In competitive inhibition, the inhibitor (I) and the substrate (S) compete for the same reactive site on the enzyme (E). In the presence of a competitive inhibitor I, the reaction rate r is given by Eq. (2.2):

$$r_s = r_{max} \frac{[S]}{[S] + K_s(1 + \frac{[I]}{K_i})} \qquad \text{Eq. (2.2)}$$

$$\frac{1}{r_s} = \frac{K_s}{r_{max}}(1 + \frac{[I]}{K_i})\frac{1}{[S]} + \frac{1}{r_{max}} \qquad \text{Eq. (2.3)}$$

- Uncompetitive Inhibition: In uncompetitive inhibition the inhibitor binds to the enzyme–substrate complex (ES) but not to the free enzyme. The equation for this is given below:

$$r_s = r_{max} \frac{[S]}{(Ks + [S](1 + \frac{[I]}{K_i})} \qquad \text{Eq. (2.4)}$$

$$\frac{1}{r_s} = \frac{K_s}{r_{\max}} \frac{1}{[S]} + \frac{1}{r_{\max}} (1 + \frac{[I]}{K_i}) \qquad \text{Eq. (2.5)}$$

Many of the known toxic compounds regulated by the United States Environmental Protection Agency (USEPA, RCRA) fall under the category of non-competitive inhibitor (Bitton, 2005).

- Non-competitive inhibitor that the inhibitor (I) does not compete for active site; once attached somewhere else on the enzyme it changes its composition and prevents the product from being formed. The kinetic expression can be described by the following equation:

$$r_s = r_{\max} \frac{[S]}{(Ks + [S])(1 + \frac{[I]}{K_i})} \qquad \text{Eq. (2.6)}$$

$$\frac{1}{r_s} = \frac{K_s}{r_{\max}} (1 + \frac{[I]}{K_i}) \frac{1}{[S]} + \frac{1}{r_{\max}} (1 + \frac{[I]}{K_i}) \qquad \text{Eq. (2.7)}$$

where $r_s$ is the specific substrate utilization rate (gS/L.h), $r_{max}$ is maximum substrate utilization rate (gS/L.h), S is substrate (g/L), I is the concentration of inhibitor (g/g), $K_s$ is half saturation concentration coefficient, known as equilibrium or affinity coefficient, $K_i$ (g/L) represents the inhibitory coefficient.

# CHAPTER III

# MATERIAL AND METHODS

## 3.1 Experimental Framework

The framework of this study was illustrated as shown in Figure 3.1. This study aimed to investigate effect of AgNPs on ammonia oxidation and nitrification processes. Biochemical (respirometry) technique was used in this study. Based on the framework, experimental tasks were summarized as shown in Figure 3.2.

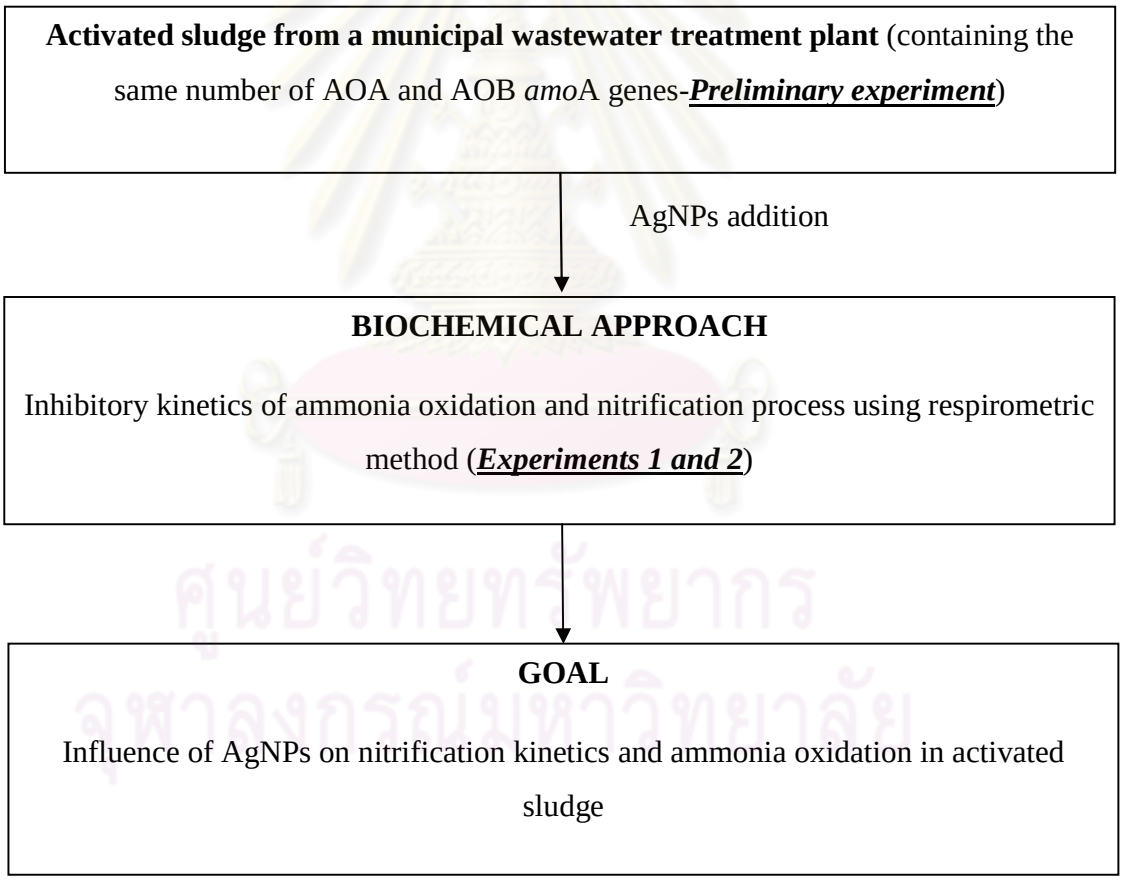


Figure 3.1 Framework for the study

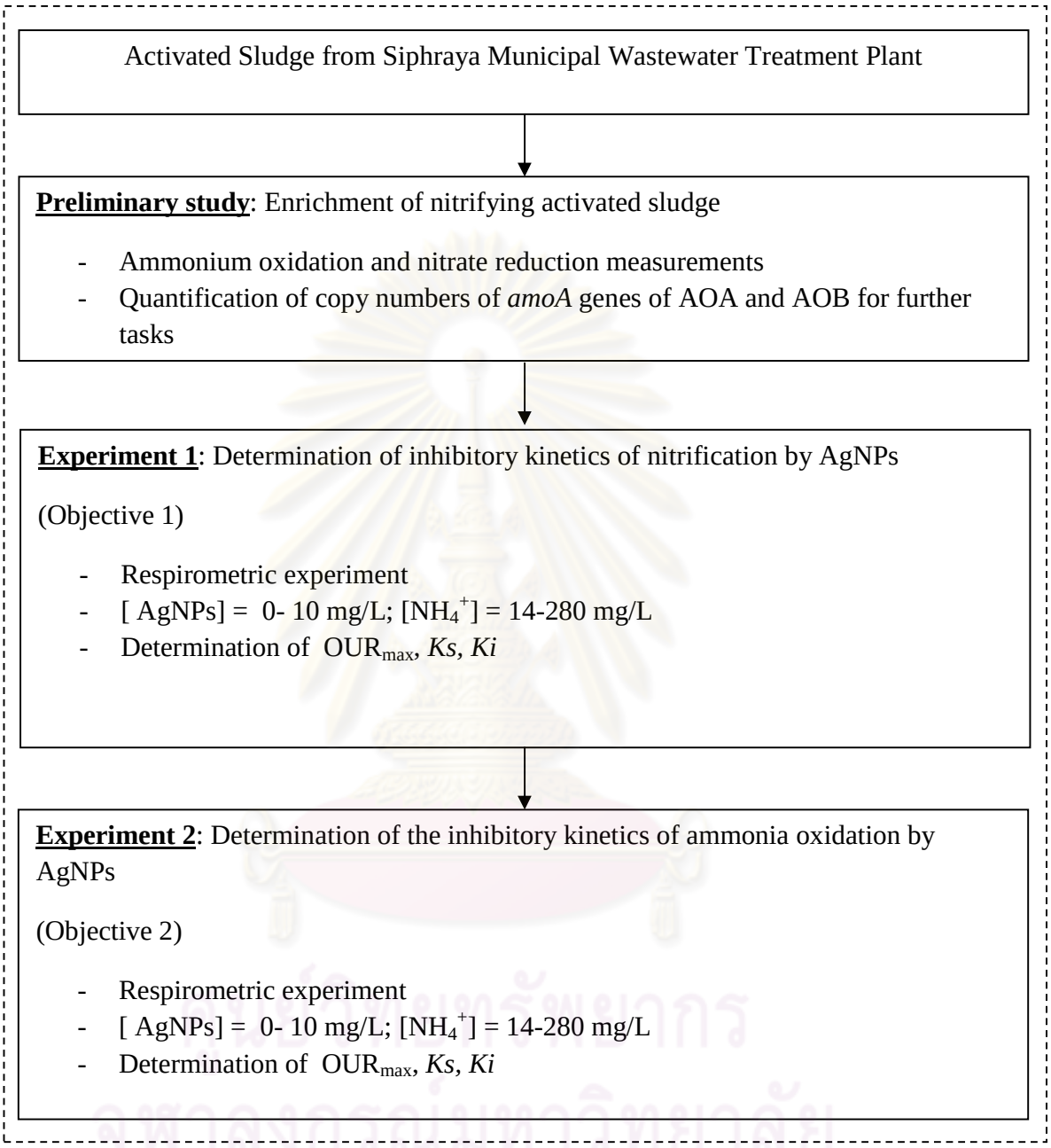


Figure 3.2 Schematic diagram of experimental tasks

## 3.2 Material and methods

### 3.2.1 Enrichment of nitrifying activated sludge (NAS)

The activated sludge was collected from Siphraya Municipal Wastewater Treatment Plant (Bangkok, Thailand) and enriched in 12-L reactors in sequencing batch mode for 6 months before use. The hydraulic retention time and solid retention time of the reactors were maintained at 2 days and 24 days, respectively. The reactors were operated under aerobic condition in which oxygen was supplied from air pump. The parameters in the reactors such as temperature, pH, dissolved oxygen (DO) were maintained at 25-30$^0$C, 7-8, and above 2 mg/L, respectively. The activity of NAS was monitored by measuring the decrease of ammonium ($NH_4^+$-N) and increase of nitrate ($NO_3^-$-N) in the reactors. The stock sludge was used for the respirometric experiments when the ammonia utilization rate reached steady state.

### 3.2.2 Quantification of *amo*A genes of AOB and AOA (Preliminary study)

**Sample preparation and DNA extraction:** Sludge of approximately 2 mg of MLSS was transferred into a 2 ml Eppendorf tubes and centrifuged at 14,000 rpm for 10 min. The supernatant was removed, and the pellet was kept at -20 $^0$C until analysis. DNA was extracted from samples using Fast-DNA SPIN kits for soil (QBiogene, Solon, Ohio, USA) according to the manufacturer's instructions. The product from DNA extraction was verified by electrophoresis in 2% agarose (Bio-Rad, Spain). The steps for AOB and AOA analysis are briefly introduced in the Figure 3.3.

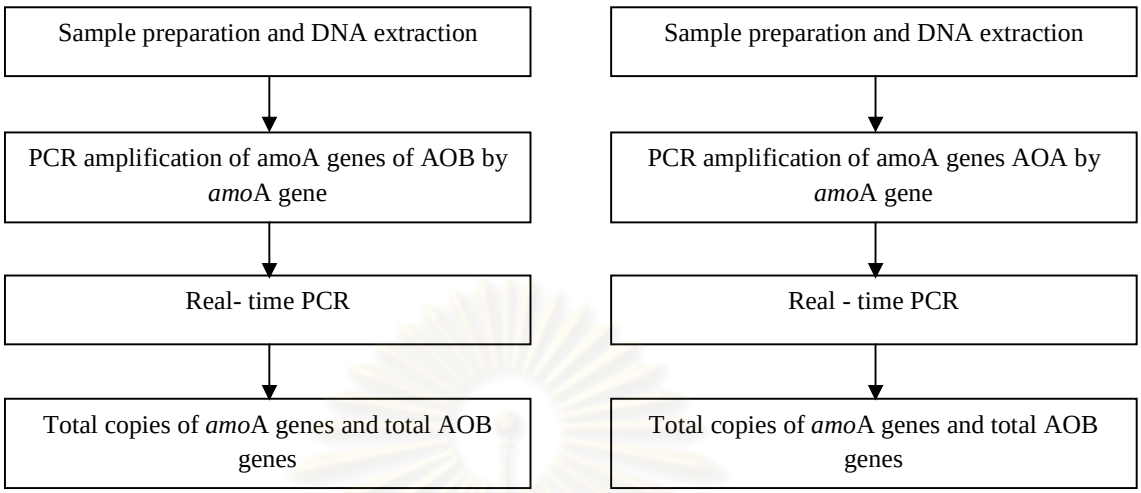


Figure 3.3 Schematic diagram of preliminary study

Table 3.1 Specific primers for AOB and AOA used in this study

| Targeting gene | Primer | Nucleotide sequence (5/–3/) | Reference |
|---|---|---|---|
| AOB *amo*A gene | amoA 1F | GGGGTTTCTACTGGTGGT | (Rotthauwe et al., 1997) |
| | amoA 2R | CCCCTCTGCAAAGCCTTCTTC | (Rotthauwe et al., 1997) |
| AOA *amo*A gene | Arch-amoAF | STAATGGTCTGGCTTAGACG | (Francis et al., 2005) |
| | Arch-amoAR | GCGGCCATCCATCTGTATGT | (Francis et al., 2005) |

**Real-time PCR:** The extracted DNA was prepared for four different 10-fold dilutions; each dilution was quantified in duplicates using an Mx3005P instrument (Stratagene, USA) with a Brilliant II SYBR Green QPCR Master Mix (Stratagene, USA). Quantification of archaeal *amoA* genes was performed using the primers Arch-amoAF (5'-STAATGGTCTGGCTTAGACG-3') and Arch-amoAR (5'-GCGGCCATCCATCTGTATGT-3') ((Francis et al., 2005). The PCR mixture contained 12.5 µl of the QPCR master mix, 1 µl of each primer (0.4 µM), and 1 µl of each sample, which composed a total volume of 25 µl. The PCR conditions were 95 °C for 10 min,

followed by 40 cycles of 60s at 95 °C, 60s at 56 °C, 30s at 72 °C, and data capture for 15 s at 78 °C. Standard DNA was the pGEM-T Easy Vector (Promega, WI, USA) possessing the *amoA* gene fragment of archaea *amoA* clone AOA-S-4 (an accession number of GQ390338) prepared in a range of $2.7 \times 10^1$ to $2.7 \times 10^7$ copies. Bacterial *amoA* genes were quantified using the primers *amoA* 1F (5'-GGGGTTTCTACTGGTGGT-3') and *amoA* 2R (5'- CCCCTCTGCAAAGCCTTCTTC-3') (Rotthauwe et al., 1997). The PCR mixtures and conditions were the same as those used for the archaeal *amoA* genes. Standard DNA was pGEM-T Easy Vector (Promega, USA) with an inserted *amoA* gene fragment of the clone AOB-NAS10-360-4 (an accession number of GU980134) prepared in a range of $4 \times 10^1$ to $4 \times 10^7$ copies.

### 3.2.3 Investigation of inhibitory kinetics of AgNPs on nitrification and ammonia oxidation in activated sludge

#### 3.2.3.1 Chemical preparation

Enrichment medium was described elsewhere (Limpiyakorn et al., 2007). A synthetic stock feed medium contained 0.33 g/L $(NH_4)_2SO_4$, 4.04 g/L $Na_2HPO_4$, 2.1 g/L $K_2HPO_4$, 0.75 g/L $NaHCO_3$, and trace inorganic salt solution 1mL. The solution comprised 40 g/L $MgSO_4.7H_2O$, 40 g/L $CaCl_2.2H_2O$, 200 g/L $KH_2PO_4$, 1 g/L $FeSO_4.7H_2O$, 0.1 g/L $Na_2MoO_4$, 0.2 g/L $MnCl_2.4H_2O$, 0.02 g/L $CuSO_4.5H_2O$, 0.1 g/L $ZnSO_4.7H_2O$, and 0.002 g/L $CoCl_2.6H_2O$. All chemicals were purchased from Merck Company (Darmstadt, Germany) via local distributor.

#### 3.2.3.2 Reactor setup and operation

Activated sludge was determined mixed liquor volatile suspended solids also known as cells (MLVSS, $mg.L^{-1}$). The activated sludge was then centrifuged and washed with distilled water and inorganic medium 5 times in order to eliminate organic matters. The centrifuged activated sludge was then used in the respirometric experiments.

A 250-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 centrifuged activated sludge with MLVSS of 110 ± 14 mg/L was added to the vessel, which was later filled up with synthetic wastewater. The synthetic wastewater formulation was the same with the stock feed, prepared by phosphate buffer with pH range from 7.8 to 8.2. Sodium azide ($NaN_3$) was added to the final concentration of 24 µM to inhibit nitrite oxidizers (Ginestet et al., 1998). The vessel was magnetically stirred and operated at room temperature (23-25$^oC$). The DO depletion in the vessel due to ammonium utilization was monitored and recorded every minute. The DO concentration was used for determination of Oxygen Uptake Rate (OUR). In addition, $NH_4^+$, and $NO_2^-$, $NO_3^-$ concentration were measured before and after the respirometric experiments. This was to confirm the activity of microorganisms in the experiments.

For the tests with AgNPs, six duplicate experiments were performed. Each of experiment was carried out with the initial concentrations of N-$NH_4^+$ ranged from 14-280 mg/L at fixed AgNPs. The initial AgNP concentrations were 0.25, 0.5, 1, 3, 5, and 10 mg/L. The vessel was aerated until it reached saturation at dissolved oxygen concentration of 7-8 mg/L. The blank experiments were performed as the same manner but no AgNPs were supplied. The oxygen consumption by heterotrophs also was tested by using the treatment without ammonium. The DO depletion was used for OUR determination through linear regression analysis. The OURs were used for nitrification kinetic modeling as described in following subsection.

**3.2.3.3 Kinetic analysis**

Oxygen Uptake Rate (OUR) was determined based on the change of dissolved oxygen over the time.

The OUR was then used for determination of $OUR_{max}$ and $K_s$ values by fitting the OUR and initial N-$NH_4^+$ concentrations into Monod equation (equation 3.1) by using nonlinear regression module in SigmaPlot version 11.0 (Sigmaplot, Dick Mitchell, SYSTAT, Inc.).

$$OUR = OUR_{max} \frac{[S]}{Ks+[S]} \quad (3.1)$$

where OUR is the oxygen uptake rate (mg-$O_2$/L.$min^{-1}$); $OUR_{max}$ is the maximum oxygen uptake rate (mg-$O_2$/L.$min^{-1}$), S is the growth-substrate concentration (mg N-$NH_4^+$/L), Ks is the half saturation coefficient for growth-substrate (mg N-$NH_4^+$/L).

Under the presence of AgNPs the equation (3.1) is rewritten as the following:

$$OUR = OUR_{\max} \frac{[S]}{Ks + [S](1 + \frac{[I]}{K_i})} \quad (3.2)$$

where [I] is the concentration of AgNPs (mg/L), $K_i$ is inhibitory coefficient (mg/L). The equation 3.2 can be taken the reciprocal of both sides to give the Lineweaver-Burk model as below

$$\frac{1}{OUR} = \frac{K_s}{OUR_{\max}} \frac{1}{[S]} + \frac{1}{OUR_{\max}} \left(1 + \frac{[I]}{K_i}\right) \quad (3.3)$$

Enzyme Kinetic Modules incorporated in the SigmaPlot software (Sigmaplot, Dick Mitchell, SYSTAT, Inc.) was applied solve for $K_i$ based on equation (3.3). The data entry was given in the Appendix C.

Additionally, the quantification of percentage of inhibition caused by AgNPs was calculated by comparing OUR of AgNPs experiments with the control as the following

$$Inhibition(\%) = \frac{(OUR_{control} - OUR_{AgNPs}) * 100}{OUR_{control}} \quad (3.4)$$

### 3.2.3.4 Determination of inhibitory kinetics of nitrification by AgNPs (Experiment 1)

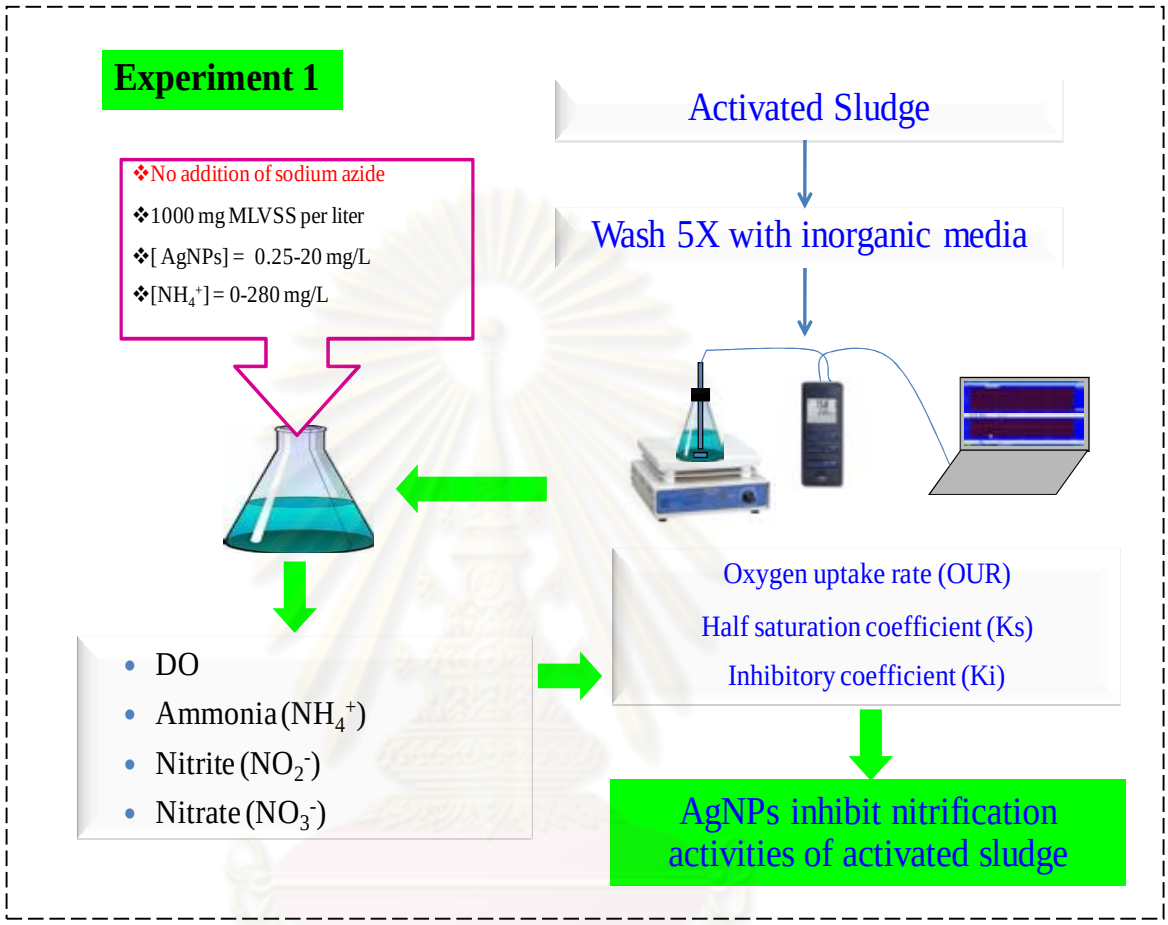


Figure 3.4 Schematic diagram of experiment 1

By setting up the respirometric experiments, inhibitory kinetics of nitrification by AgNPs were investigated at initial AgNPs concentrations ranged from 0-10 mg/L with varying initial N-$NH_4^+$ concentrations of 0-280 mg N/L. Nitrification inhibitory kinetic parameters included maximum oxygen uptake rate ($OUR_{max}$), half saturation coefficient (Ks), and inhibitory coefficient (*Ki*) were estimated by using equations in the subsection 3.2.3.2.. In addition, the concentration of $NH_4^+$-N, $NO_2^-$-N and $NO_3^-$-N were measured before and after each experiment in order to confirm the utilization of $NH_4^+$-N done by microbes.

### 3.2.3.5 Determination of inhibitory kinetics of ammonia oxidation by AgNPs (Experiment 2)

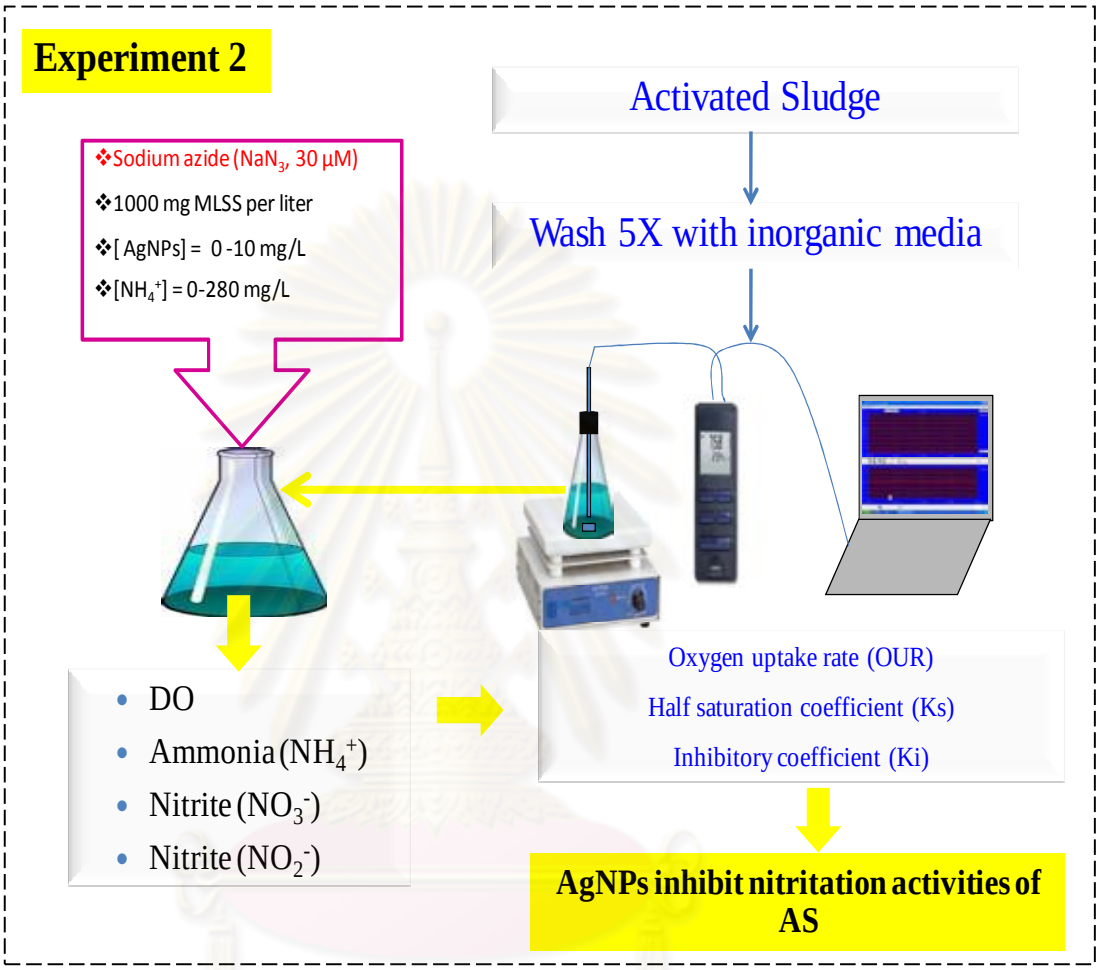


Figure 3.5 Schematic diagram of experiment 2

In this experiment, the concentration of AgNPs and $NH_4^+$ ranged from 0-10 mg/L and 0-280 mg/L, respectively. The tests were run in the same manner with the experiment 1. However, sodium azide at the concentration of 24µM was added to selectively study ammonia oxidation activity. Maximum oxygen uptake rate ($OUR_{max}$), half saturation coefficient (Ks), and inhibitory coefficient (Ki) were also calculated. Similarly, the concentration of $NH_4^+$-N, $NO_2^-$-N and $NO_3^-$-N was measured before and after each experiment in order to confirm the utilization of $NH_4^+$ done by microbes.

### 3.2.3.6 Analytical methods

**Measurement of ammonium:** The parameter measurement is followed Phenate method (APHA, 1998). The sample was diluted with distilled water. Five ml of diluted sample and 0.2 mL of phenol solution (mix 11.1 mL liquefied phenol (≥89%) with 95% (v/v) ethyl alcohol to a final volume of 100 mL) were added and mixed. Sodium nitroprusside solution of 0.2 mL (0.5% w/v: dissolve 0.5 g of sodium nitropusside in 100 mL of deionized water), and 0.5 mL of oxidizing solution (Mix 100 mL alkaline citrate solution: dissolve 200 g of trisodium citrate and 10 g of sodium hydroxide in 1000 mL of deionized water with 25 mL of sodium hypochloride) were added into the tube. Sample will be covered with plastic wrap or paraffin wrapper film and kept at room temperature in subdued light for at least 1 hr to develop color. Sample was measured for absorbance at 640 nm with UV visible spectrophotometers (Thermo Electron Corporation, Hexious α, Cambridge, UK).

**Measurement of nitrite:** The parameter measurement is followed Colorimetric method (APHA, 1998). Sample was filtered and diluted with deionized water when need. Take 5ml of diluted sample and 0.1mL of Sulphanilamide solution (dissolve 5 g of Sulphanilamide and 50 mL of hydrochloric in 500 mL) were added, and allowed to react 5 min, then 0.1 mL of NNED solution (dissolve 1 g of (N-(1-Naphthyl)-Ethylenediamine Dihydrochloride in 1000mL of de-ionized water) was added and allowed at room temperature in subdued light for at least 1 hr for color development. Developed color was measured for absorbance at 543 nm with UV visible spectrophotometers (Thermo Electron Corporation, Hexious α, Cambridge, UK) (Colorimetric method, Standard Method for the Examination of Water and Will betewater $20^{th}$ edition).

**Measurement of nitrate:** The parameter measurement was followed Ultraviolet Spectrophotometric Screening method (APHA, 1998). Sample was filtrated and diluted with deionized water when need. 5 mL of filtered and diluted sample was measured for absorbance at 220 nm to obtain $NO_3^-$ reading and absorbance at 275 nm to determine interference due to dissolved organic matter with UV visible spectrophotometers (Thermo Electron Corporation, Hexious α, Cambridge, UK)

(Ultraviolet Spectrophotometric Screening Method, Standard Method for the Examination of Water and Will betewater 20th edition).

**Characteristics of AgNPs:** Silver nanoparticles were provided by Sereemaspun et al. (2008) which the synthesis procedure previously descibed by Enustun and Turkevich (1963) and Van Hyning and Zukoski (1998). Briefly, AgNPs were made by reducing $AgNO_3$ with $NaBH_4$. The target final concentration produced is 1000 ppm. The solution then contained in dark bottle and stored at 4°C in the fridge for further use. According to Sereemaspun et al. (2008), the AgNPs were characterized by using Transmission Electron Microscopy (TEM) H-7650 (Hitachi, Japan) and UV-visible spectroscopy (Shimadzu, Japan). The zeta potential or surface charge of AgNPs will be determined by Zetasizer NanoZS (Malvern, UK). As a result, AgNPs were peaked at the wavelength of 403 nm (Figure 3.6) with its characteristics are spherical shape, diameter of 15 nm (Figure 3.7) and zeta potential is -14.4 ±1.1 mV. It must be noted that the AgNPs used in this study have the size and shape similar to commercial AgNPs.

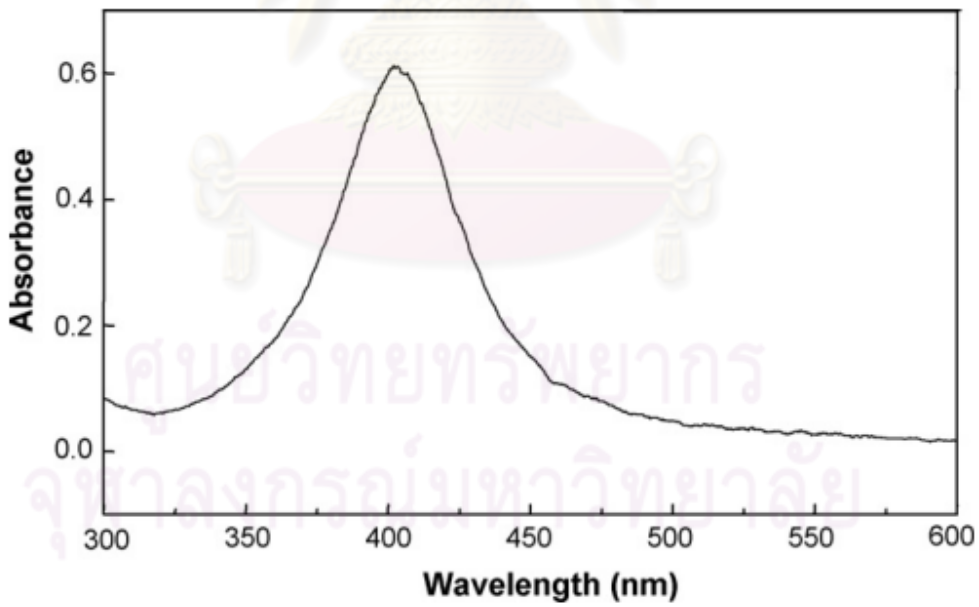


Figure 3.6 The plasmon absorption band of 100–1000 mg/L the synthesized AgNPs ($\lambda_{max}$ = 403 nm) (Wittaya Ngeontaea et al., 2009).

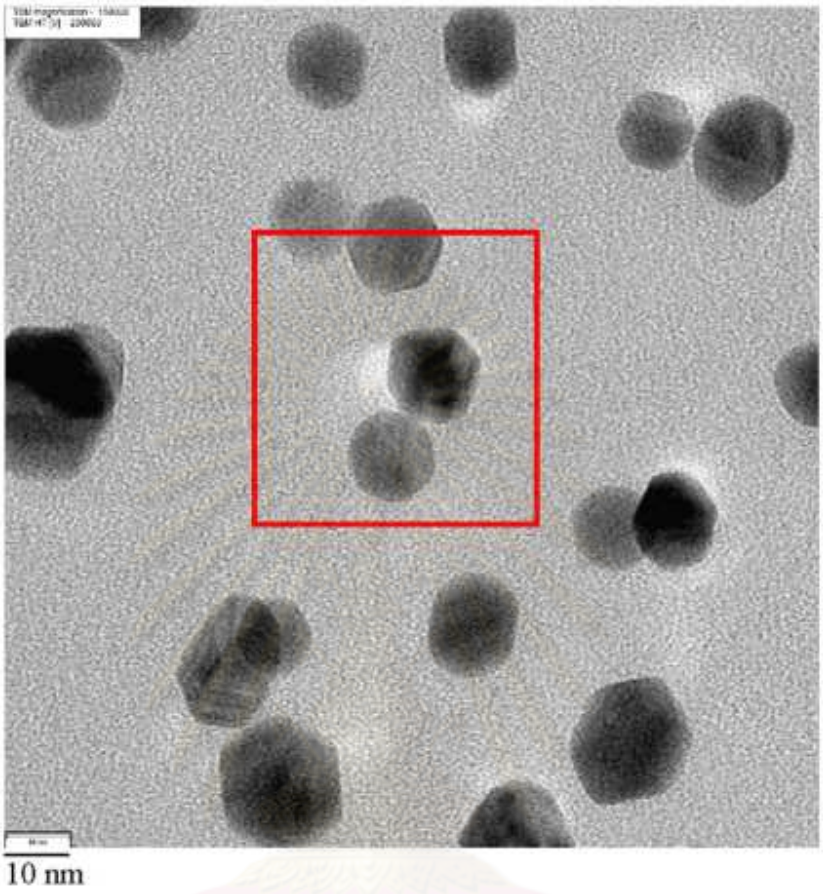


Figure 3.7 TEM images of the synthesized AgNPs at the TEM magnification of 150,000 (Wittaya Ngeontaea et al., 2009).

# CHAPTER IV

# RESULTS AND DISCUSSION

## 4.1 Enrichment of nitrifying activated sludge (NAS)

Enrichment of NAS is useful for laboratory-scale study of wastewater treatment plant. A previous study showed that three enriched NAS feeding with inorganic medium containing different $NH_4^+$-N concentrations (28, 180, and 420 mg $NH_4^+$-N/L) reached steady state after 22 days, 37 days, and 15 days of operation, respectively. In all cases, $NH_4^+$-N concentration was totally consumed (Sonthiphand and Limpiyakorn, 2009). Previous studies also indicated that measurement of ammonium and nitrate concentrations at the end of each cycle could assess the nitrification efficiency (Ploychankul et al., 2010). In this study, NAS was enriched at the initial ammonium concentration of approximately 70 mg$NH_4^+$-N/L. The ammonium and nitrate concentrations in the enrichment reactor were measured (Figure 4.1). As can be seen, the activity of the nitrifying microorganisms was stable because all the ammonium was completely oxidized and converted to nitrate (average nitrate concentration of 69 ± 2.8 mg $NO_3^-$-N/L). The average $NH_4^+$-N concentration was 72.8 mg $NH_4^+$-N /L. The measured data indicated that the NAS performed full nitrification (95%) because most of $NH_4^+$-N concentration was converted to nitrate.

During NAS enrichment, numbers of *amo*A genes of AOA and AOB were investigated to confirm the presence of those communities in NAS. The numbers of *amo*A genes of AOA and AOB were quantified monthly using real-time PCR method. The result was given in Figure 4.2. The copy numbers of *amo*A genes of AOA fluctuated within the range of $2.25 \times 10^5 \pm 5.63 \times 10^4$ to $5.13 \times 10^8 \pm 7.86 \times 10^7$ (average = $1.29 \times 10^8 \pm 2.22 \times 10^8$, copies/mg MLSS) while the copies of *amo*A genes of AOB ranged from $1.63 \times 10^7 \pm 3.55 \times 10^4$ to $9.32 \times 10^8 \pm 1.50 \times 10^8$ (average = $2.74 \times 10^7 \pm 1.06 \times 10^7$, copies/mg MLSS) (see Appendix A). The magnitude of difference between AOB and

AOA was in the range of 0.8 – 1.7 (mean value was 1.3) during the whole operating periods indicating that the cells increased with time but at the end it was quite stable.

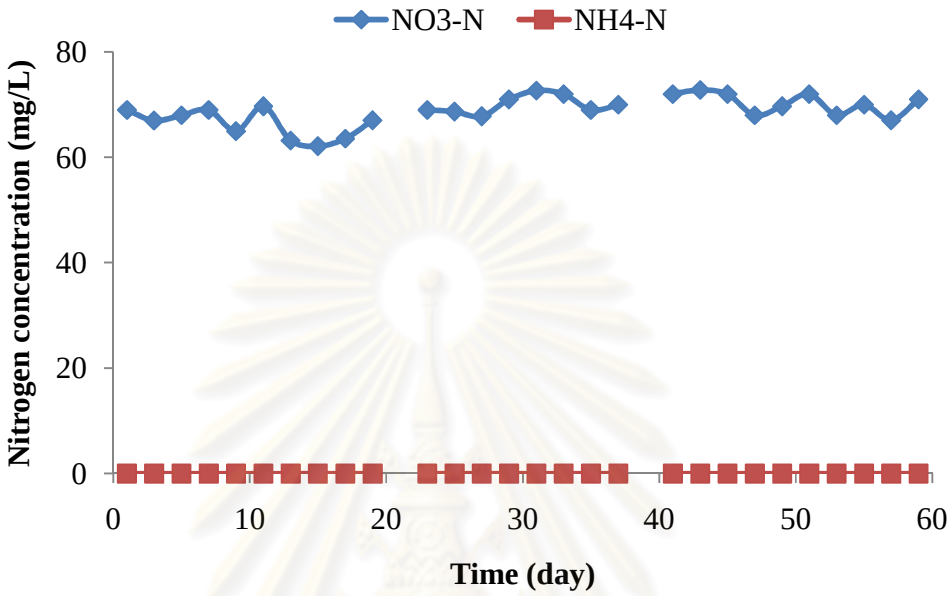


Figure 4.1 Concentrations of $NH_4^+$-N and $NO_3^-$-N in the reactor during enrichment of nitrifying activated sludge

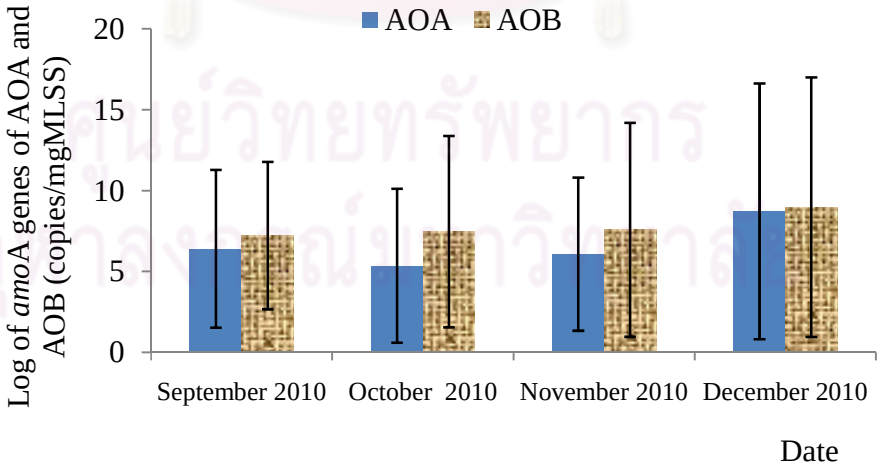


Figure 4.2 Copy numbers of *amo*A genes of AOA and AOB in the reactor during enrichment of NAS

## 4.2 Nitrifying and ammonia oxidizing activity without AgNPs

It is known that nitrifying microorganisms need oxygen to survive and function; consequently, measurement of oxygen uptake rate has been applied for studying such microbial activities (Cokgor et al., 2007; Lewandowski et al., 1985). However, not only nitrifying microbes used dissolved oxygen but also heterotrophs. In addition, there is a possibility that dissolved oxygen also reduced by an abiotic factor. Therefore, a series of control experiments were set up. In order to check heterotrophic activity, the control experiment in synthetic wastewater without ammonium and AgNPs addition was used. The result in Figure 4.3 showed that dissolved oxygen was constant at around 7.5 mg/L (the oxygen uptake rate of approximately 0 mg-$O_2$/L.min). This indicated that heterotrophs insignificantly competed for dissolved oxygen with nitrifying microbes in this study. The control experiment in synthetic wastewater with ammonium and AgNPs addition but without nitrifying cells was also performed to check the dissolved oxygen sink by abiotic factors in the vessels of ammonia oxidation and nitrification. The results in Figure 4.4 and 4.5 indicated that abiotic factors did not reduce dissolved oxygen in both ammonia oxidation and nitrification, respectively (the oxygen uptake rate of approximately 0 mg-$O_2$/L.min).

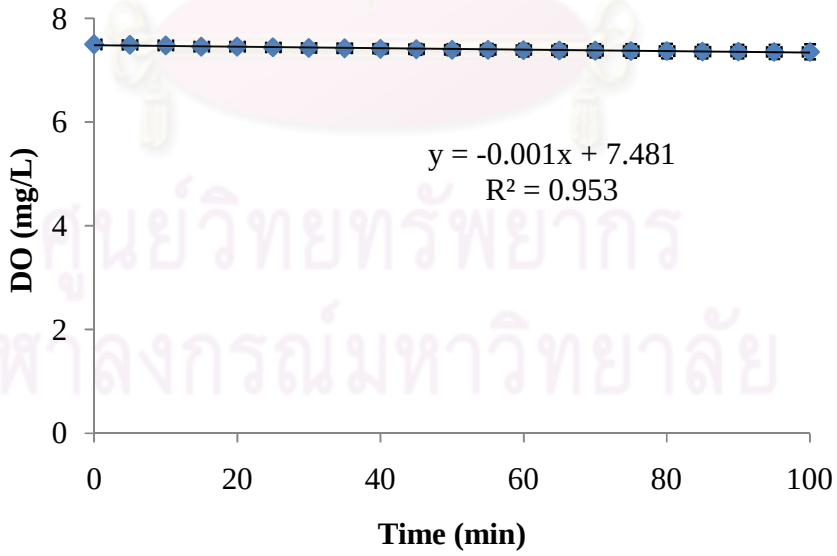


Figure 4.3 Dissolved oxygen consumption by heterotrophs

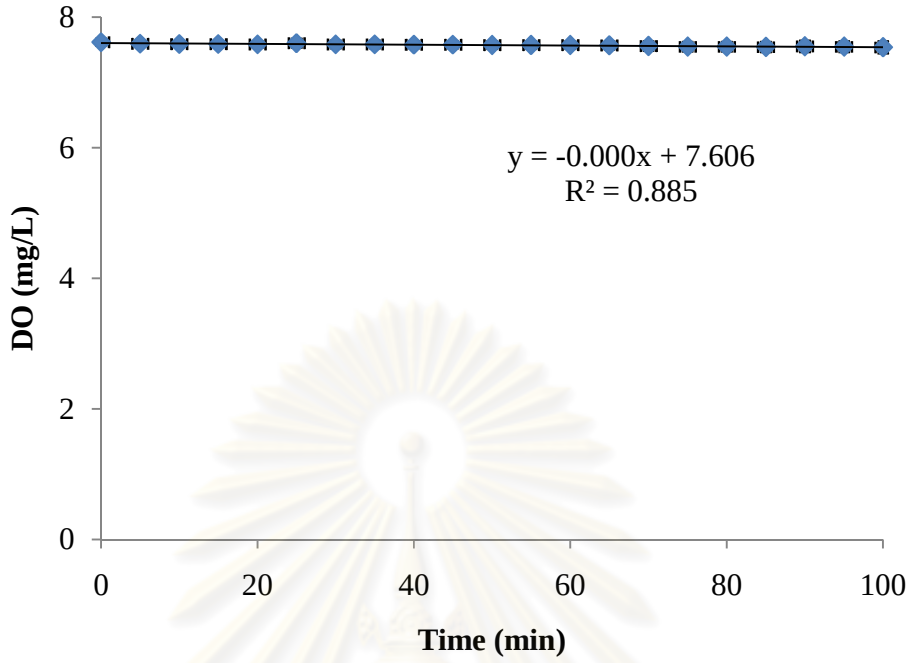


Figure 4.4 Test of dissolved oxygen sink for ammonia oxidation experiment

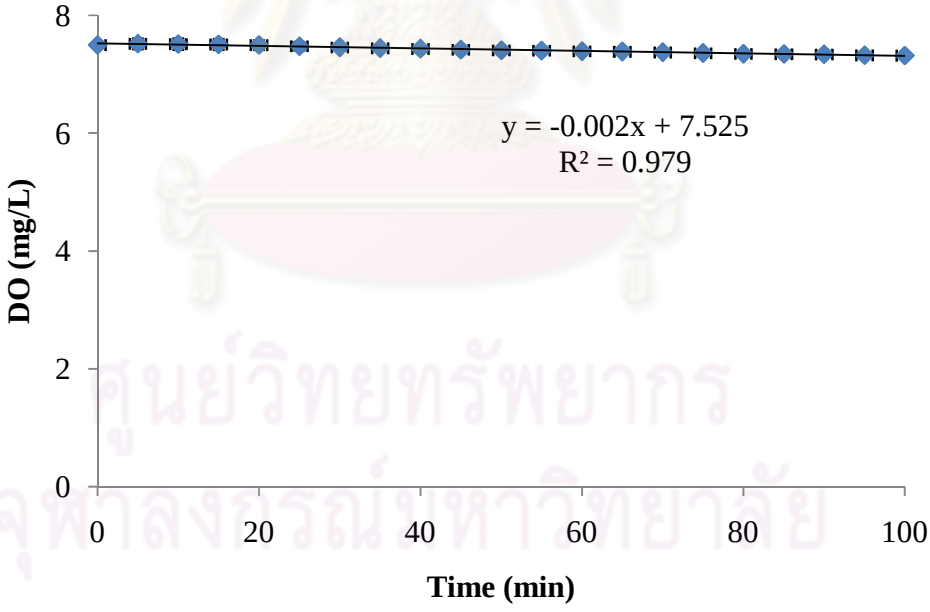


Figure 4.5 Test of oxygen sink for nitrification experiment

The ammonia oxidation and nitrification tests in synthetic wastewater with different ammonium concentrations were performed. The rate of oxygen consumption was expressed by slope of the linear line that represents for depletion of dissolved oxygen with respect to time. In this study, an increasing trend of the slopes was observed in nitrification tests which were demonstrated in Figure 4.6. The slopes for the tests with initial ammonium concentrations of 0, 5, 10, 50, 70, and 280 mg/L were 0.001, 0.093, 0.106, 0.165, 0.207, and 0.227 mg-$O_2$/L.min, respectively. This is similar to a prior study. Ploychankul et al. (2010) reported that dissolved oxygen decreased quickly within the first 40-100 min for the nitrification test without AgNPs.

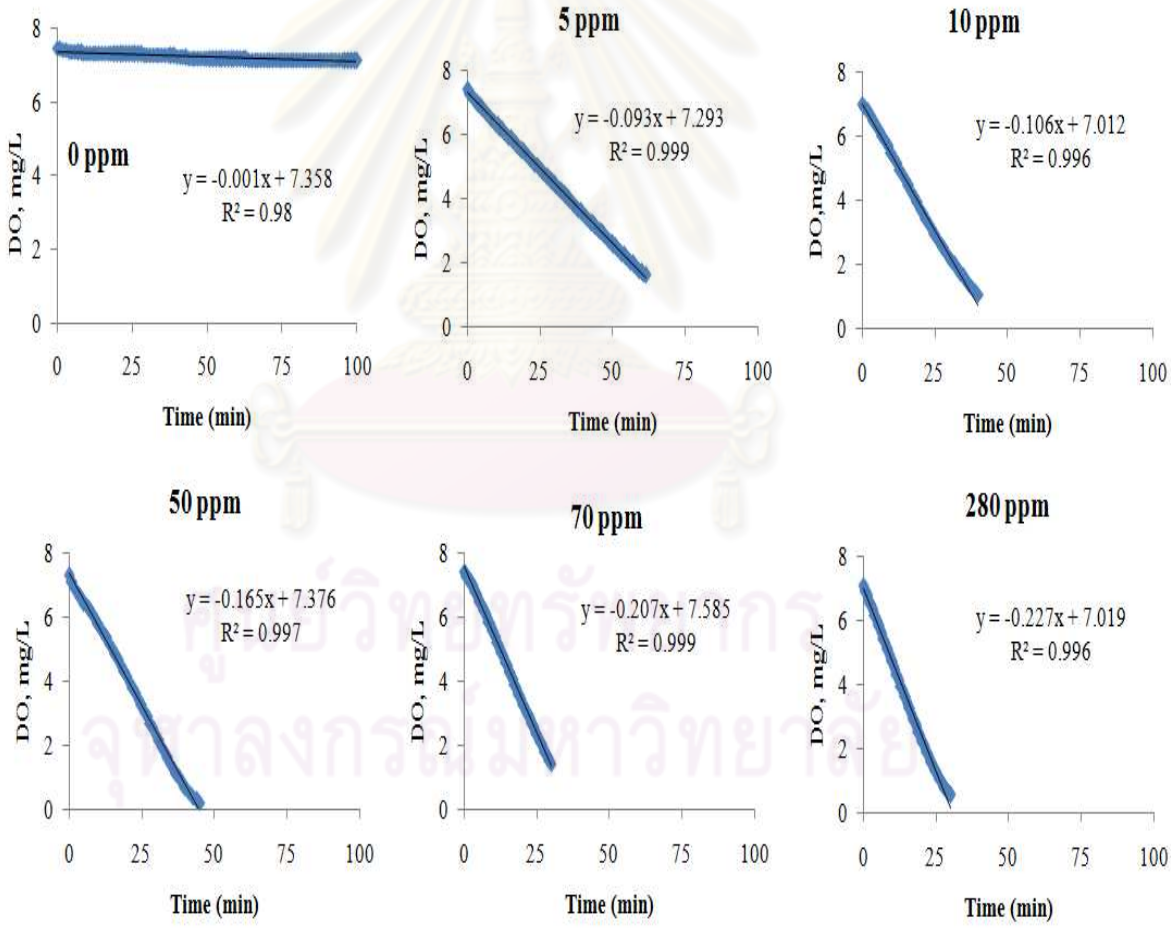


Figure 4.6 DO consumption under different $NH_4^+$-N concentrations in nitrification

Oxygen consumption in the ammonia oxidation tests was showed in Figure 4.7. The slopes were 0.002, 0.064, 0.123, 0.125, 0.193 and 0.198 mg-$O_2$/L.min corresponded to $NH_4^+$-N concentrations of 0, 5, 10, 50, 70, and 280 mg/L. The results indicated that the initial $NH_4^+$-N concentrations influenced ammonia oxidation activity. The higher $NH_4^+$-N concentration resulted in the higher ammonia oxidation rate. However, oxygen consumption rate slowly increase and tend to be constant when $NH_4^+$-N concentration higher than 70 mg/L. The rate increased only 0.005 mg-$O_2$/L.min (from 0.193 to 0.198 mg-$O_2$/L.min) while the $NH_4^+$-N concentration increased 4 times (70 mg/L to 280 mg/L).

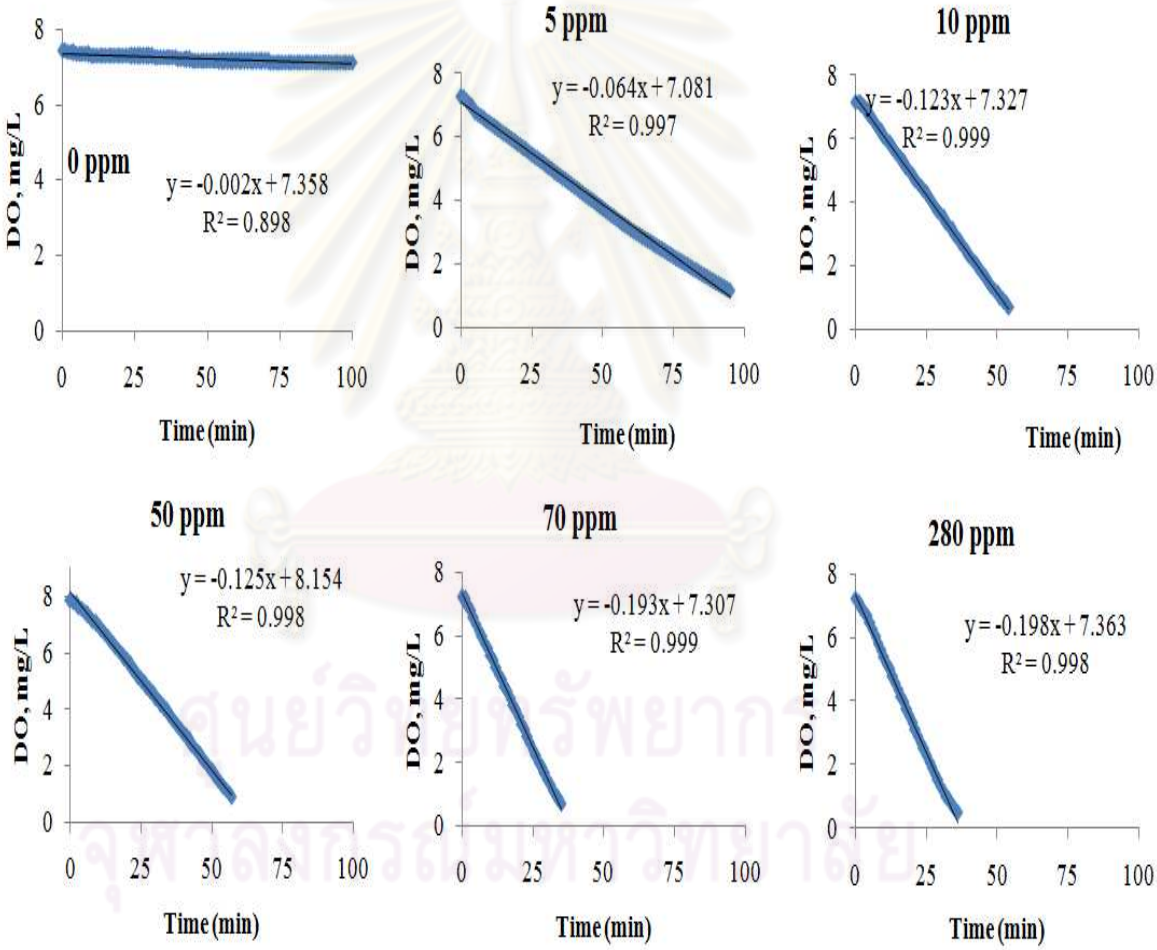


Figure 4.7 DO consumption under different $NH_4^+$-N concentration in ammonia oxidation

Monod model was applied to estimate the kinetic parameters of maximum oxygen uptake rate ($OUR_{max}$) and half saturation constant (*Ks*) for nitrification (Figure 4.8) ammonia oxidation (Figure 4.9). The estimated $OUR_{max}$ and *Ks* were 0.212 mg- $O_2$/L.min and 5.42 mg $NH_4^+$-N/L for nitrification and 0.227 mg-$O_2$/L.min and 15.9 mg $NH_4^+$-N/L for ammonia oxidation process. The maximum oxygen uptake rate in nitrification was slightly smaller (6.6%) than that of ammonia oxidation. This could be because of interference of sodium azide which was added to selectively study respiration of ammonia oxidation activity in the ammonia oxidation process. Similarly, half saturation constant in nitrification was much lower when compared with that of ammonia oxidation. This is not only found in this study but also was reported elsewhere (Carrera et al., 2004). The half saturation constants for nitrification and ammonia oxidation were 1.6 mgN/L and 11 mgN/L, respectively. In most of studies of half saturation constant in pure or mixed culture in nitrification process, the Ks values of AOB are greater than that of NOB. In addition, AOB play main role in ammonia oxidation while NOB for nitrification. As consequently, the Ks value of ammonia oxidation was found to be greater than nitrification in this study.

Most of the studies focused on estimation of Ks values of nitrification process. The values of Ks were found to be 17.8 mgN/L, and 26.5 mgN/L for two different reactors (Racz et al., 2010) while to be in the range of 66-70 mgN/L (Bilge and Cecen, 2007) for the nitrification process. The difference of Ks values in the nitrification process could be because in the prior study, different activated sludge which containing different microbial communities was used leading to differ in Ks values.

Half saturation constant, Ks, expressed the niche separation among the microorganisms and has been widely used to predict that which types of microorganisms play a main role in the ammonia oxidation or nitrite oxidation in such environments. For example, in ammonia oxidation process, microorganisms with high Ks value meaning that low affinity to $NH_3$ explaining their dominance in environments where $NH_3$ concentration are high. The process of nitrification in wastewater treatment is widely accepted as a two-step process. In the first step ammonia is oxidized to nitrite, a process considered to be carried out mainly by the *Nitrosomonas* sp., while in the second step the

*Nitrobacter* sp. oxidizes the nitrite to nitrate. In all cases, ammonia oxidation process consumes more dissolved oxygen than nitrite oxidation. For example, study kinetics of pure culture of AOB and NOB of those species showed that values of Ks were 0.62 mg-N/L, and 0.22 mgN/L (Kantartzi et al., 2006) while in the mixed culture were found to be 9.1 mgN/L and 4.85 mgN/L (Fang et al., 2009), respectively. In normal condition, it is likely that NOB detemine the nitrification processs while AOB determine the ammonia oxidation process. In the liteature Ks of ammonia oxidation is always greater than that of nitrite oxidation therefore the Ks of ammonia oxidation was greater than Ks of nitrification. This finding was similar to that of Carrera and his coworkers (2008). There are many species involved in ammonia oxidation, each species has different affinity to substrate leading to different Ks values were found in some literature. For instance, Ks values for ammonium oxidizing microorganisms ranged from high to low with pure culture study, high Ks values fell in the range of 0.42-0.85 mgN/L and low Ks values were 0.03-0.06 mgN/L (Koops and Roser, 2001). For nitrite oxidation, Ks values ranged from 0.49-1.10 mgN/L (Koops and Roser, 2001).

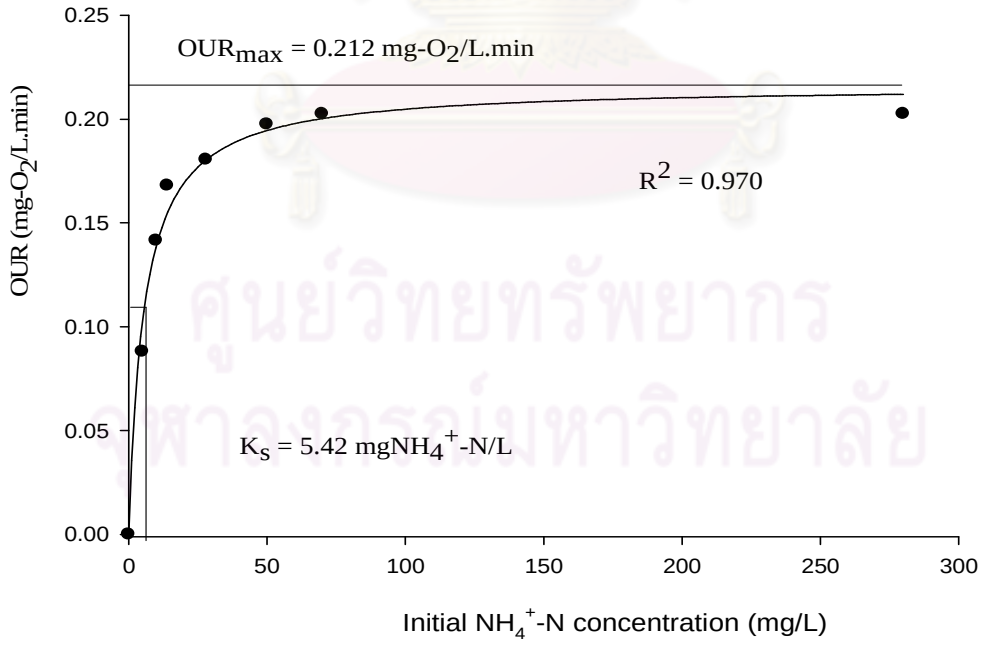


Figure 4.8 Maximum OUR and Ks for nitrification

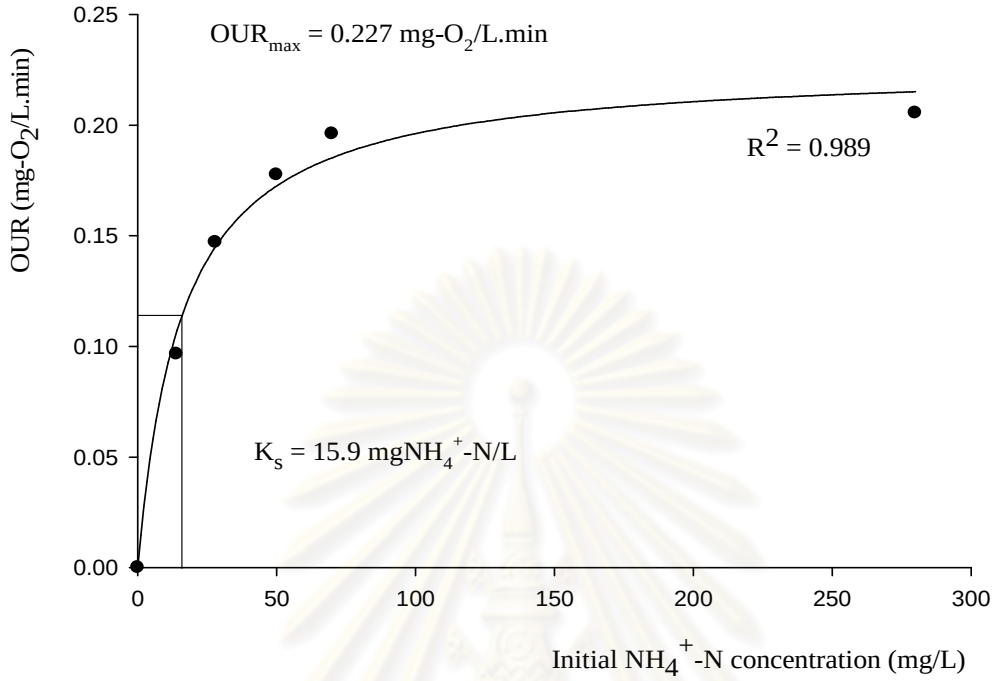


Figure 4.9 Maximum OUR and Ks for ammonia oxidation

## 4.3 Inhibitory effect of AgNPs on nitrifying and ammonia oxidizing activity

Influence of AgNPs (0 to 10 ppm) and $NH_4^+$-N (0 to 280 mg/L) concentrations on oxygen uptake rates of nitrification and ammonia oxidation was performed (see data in appendix B). The oxygen uptake rates influenced by AgNPs and $NH_4^+$-N on nitrification and ammonia oxidation were showed in Figures 4.10 and 4.11, respectively. Silver nanoparticles reduced dissolved oxygen consumption ability of nitrifying microbes in both nitrification and ammonia oxidation processes. At lower $NH_4^+$-N concentrations (14-70 mg/L), the effect of AgNPs on nitrifying and ammonia oxidizing activity was dependent on its doses. In most of the cases, the oxygen uptake rates were decreased when concentration of AgNPs increased within a fixed $NH_4^+$-N concentration. For example, at the $NH_4^+$-N concentration of 14 mg/L, a decreasing oxygen uptake rate trend was recorded at 0.168, 0.147, 0.137, 0.133 and 0.134 mg$O_2$/L.min respected to the AgNPs concentrations of 0, 0.25, 0.5, 1 and 10 mg/L, respectively, for nitrification while 0.097, 0.093, 0.088, 0.085, and 0.085 mg$O_2$/L.min corresponding to the AgNPs

concentrations of 0, 0.25, 0.5, 1 and 5 mg/L in ammonia oxidation. Considering $NH_4^+$-N concentrations of 28 mg/L and 70 mg/L which were selected to represent for the $NH_4^+$-N concentration in most of the municipal and industrial wastewater treatment plants, the similar trend also was found. However, the more intensive effect of AgNPs (1-10 mg/L) on nitrification and ammonia oxidation was found when the $NH_4^+$-N concentration was increased to 280 mg/L. For instance, at the $NH_4^+$-N concentration of 280 mg/L, the oxygen uptake rates were 0.203, 0.198, 0.188, 0.163, 0.122, 0.081, and 0.080 mg-$O_2$/L.min corresponding to AgNPs concentrations of 0, 0.25, 0.35, 0.5, 1, 3, 10 mg/L for the nitrification tests. Regarding to ammonia oxidation tests, the oxygen uptake rates were 0.206, 0.186, 0.140, 0.094, 0.095, 0.115, and 0.103 mg-$O_2$/L.min respected to 0, 0.25, 0.5, 1, 3, 5, and 10 mg/L of AgNPs concentrations. This may be because of the integration effect of AgNPs and $NH_4^+$-N which caused additional stresses on respiration rate in the nitrification and ammonia oxidation processes. Related to toxicity of AgNPs, Ploychankul et al. (2010) reported that suspended cells were very sensitive to AgNPs and when its concentration was raised, ammonia oxidizing activity was reduced apparently thus inhibited significantly nitrification process. Similar conclusion was made for heavy metals that increasing dose of heavy metals resulted in increasing of oxygen uptake rate inhibition (Volskay and Grady, 1988; Cecen et al., 2010). An additional experiment was performed in which the concentration of AgNPs were increased up to 200 mg/L (data not shown), the result indicated that AgNPs only partially inhibited the oxygen uptake rate of nitrifying and ammonia oxidizing activity in NAS. Silver nanoparticles caused higher inhibition level when the exposure time increased. For example, respiration rate was inhibited by 41.4% and 46.5% at the beginning and after one month of exposure to AgNPs, respectively (Hu et al., 2010). There was no information about the recovery of nitrifying and ammonia oxidizing activity in the AgNPs contaminated activated sludge system, however, it is predicted that the activity of microbes may return to normal condition in the longer time because AgNPs may be washed out.

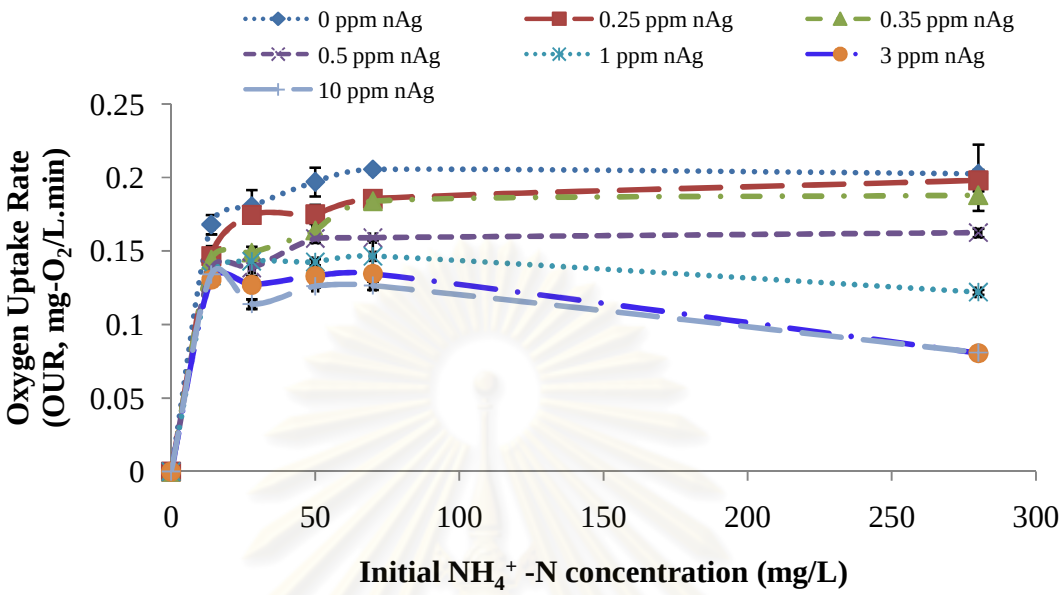


Figure 4.10 OUR versus initial $NH_4^+$-N concentrations in nitrification tests

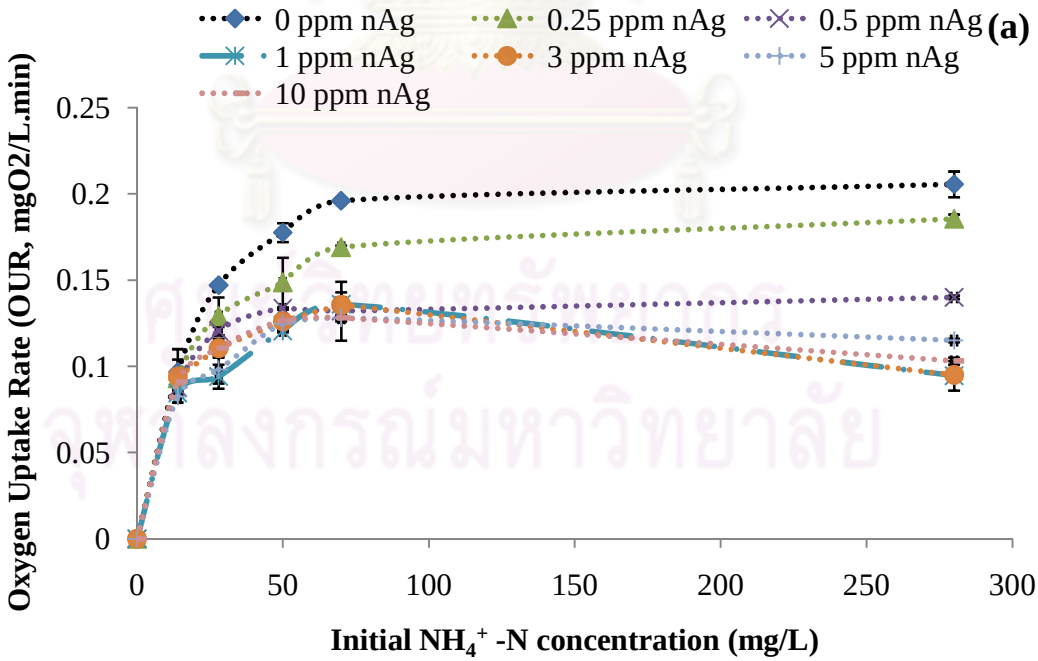


Figure 4.11 OUR versus initial $NH_4^+$-N concentrations in ammonia oxidation tests

## 4.4 Inhibitory kinetics of AgNPs on nitrification and ammonia oxidation

Varying concentrations of AgNPs and $NH_4^+$-N resulted in apparent OURs changed followed the Michaelis-Menten type as showed in Figure 4.10 and Figure 4.11 for nitrification and ammonia oxidation, respectively. The Mechaelis-Menten equation is the fundamental equation of Enzymes kinetics and describes a rectangular hyperbolic dependence of velocity on substrate. It has the dual nature because it is a combination of zero-order and first-order kinetics. When substrate is low, the equation for rate is first-order in substrate and when substrate is high, the equation for rate is zero-order. In this study, an increasing AgNPs led to a decreasing of oxygen uptake rates indicating that the inhibitory effect of AgNPs on nitrification and ammonia oxidation processes.

There are various kinds of inhibition including competitive, uncompetitive, non competitive and mixed inhibition. Each type will affect differently on plotting of Michaelis-Menten equation. The summary of inhibition model with the change of main model parameters was showed in Table 4.1. In the present study, the characteristic of inhibitory effect of AgNPs was predicted based on the changing of apparent $OUR_{max}$ and *Ks* values in the nitrification and ammonia oxidation processes as indicated in Table 4.2 and 4.3, respectively. It seemed that AgNPs have the characteristics of an uncompetitive inhibition because the apparent $OUR_{max}$ and *Ks* were both slightly decreased (Figure 4.10 and Figure 4.11).

Table 4.1 Summary of inhibition models (Bitton, 2005)

| **Inhibition type** | **$V_{max}$** | **$K_s$** |
|---|---|---|
| Competitive | No effect | Increases |
| Non-competitive | Decreases | No effect |
| Un-competitive | Decreases | Decreases |

Table 4. 2 $OUR_{max}$ and Ks values of nitrifying activity with AgNPs

| **AgNPs (mg/L)** | **$OUR_{max}$ (mg-$O_2$/L.min)** | **Ks (mg$NH_4^+$-N/L)** | **$R^2$** |
|---|---|---|---|
| 0 | 0.213 | 5.48 | 0.997 |
| 0.25 | 0.199 | 5.33 | 0.997 |
| 0.35 | 0.189 | 4.91 | 0.994 |
| 0.5 | 0.165 | 3.19 | 0.994 |
| 1 | 0.149 | 1.68 | 0.999 |
| 3 | 0.134 | 0.50 | 0.998 |

Table 4. 3 $OUR_{max}$ and Ks values of ammonia oxidizing activity with AgNPs

| **AgNPs (mg/L)** | **$OUR_{max}$ (mg-$O_2$/L.min)** | **Ks (mg$NH_4^+$-N/L)** | **$R^2$** |
|---|---|---|---|
| 0 | 0.227 | 16.00 | 0.988 |
| 0.25 | 0.198 | 16.15 | 0.988 |
| 0.5 | 0.164 | 15.87 | 0.994 |
| 1 | 0.156 | 9.99 | 0.980 |
| 3 | 0.152 | 8.82 | 0.998 |
| 5 | 0.150 | 12.79 | 0.994 |
| 10 | 0.145 | 8.33 | 0.972 |



Theoretically, in uncompetitive inhibition, the inhibitor is only able to bind to the enzyme-substrate complex (ES), not to the free enzyme (E). Based on that theory for this case, the $OUR_{max}$ decreased in the presence of AgNPs because some of the enzyme molecules will always be inactivated. However, the *Ks* also decreased because some of the substrate is always bound up in enzyme-substrate-inhibitor (ESI) complexes where it cannot be converted to product. The $OUR_{max}$ and the *Ks* are decreased by the same factor

(1+ I/*Ki*), so the ratio of *Ks*/$OUR_{max}$ does not change. This resulted in a Lineweaver-Burk plot with two parallel lines corresponding to the uninhibited and inhibited experiments as demonstrated in Figure 4.12 and Figure 4.13 for nitrification and ammonia oxidation, respectively. Regarding to these figures, higher AgNPs resulted in higher respiration rate in the nitrification and ammonia oxidation processes.

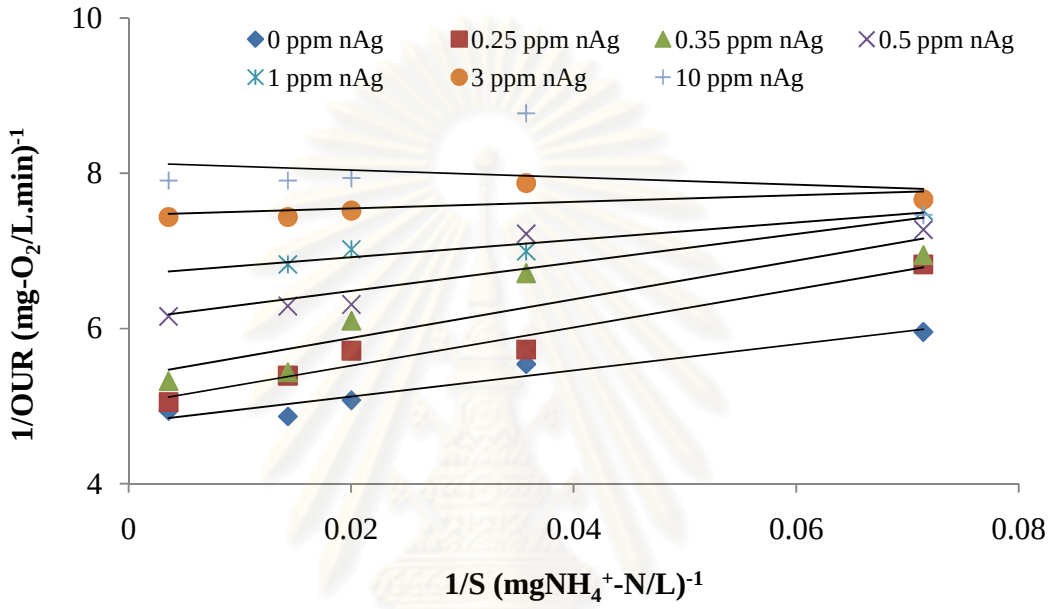


Figure 4.12 Lineweaver - Burk Plot for nitrification tests

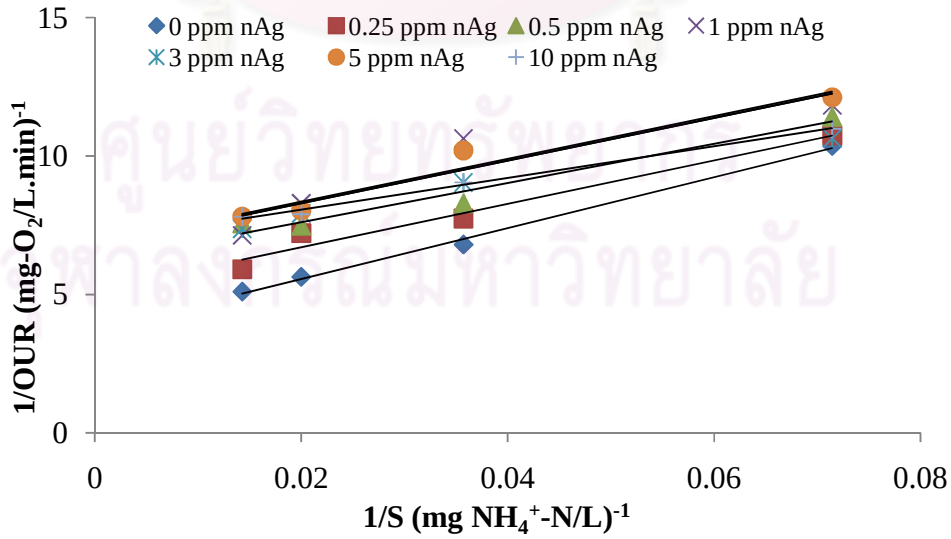


Figure 4.13 Lineweaver - Burk Plot for ammonia oxidation tests

For calculation of inhibitory coefficient, $K_i$, the experimental data of oxygen uptake rates and initial AgNPs concentration of both nitrification and ammonia oxidation were fitted into the SigmaPlot worksheet in the Enzyme Kinetic Modules (Appendix C). The calculated *Ki* values were 8.3 and 21.7 mg/L for nitrification and ammonia oxidation, respectively.

Table 4.4 Summarized $K_i$ values of some heavy metals

| **Heavy metals** | ***K*$_i$ (mg/L)** | **Test condition** | **References** |
|---|---|---|---|
| $Cr^{3+}$ | 161.9 | Biodegradation of MTBE using pure culture of *Pseudomonas aeruginosa* | (Lin et al., 2006) |
| $Zn^{2+}$ | 164.6 | | |
| $Mn^{2+}$ | 163.8 | | |
| $Cu^{2+}$ | 32.4 | | |
| $Cd^{2+}$ | 12 | Denitrification | (Gumaelius et al., 1996) |
| $Cr^{6+}$ | 1.2 | Nitrification | (Lewandowski et al., 1985) |
| AgNPs | 8.3 | Nitrification | This study |
| AgNPs | 21.7 | Ammonia oxidation | This study |

So far, there is no published report of *Ki* value for AgNPs in nitrification and ammonia oxidation processes in the activated sludge systems. Many studies have been focused on calculation of *Ki* values, but for heavy metal ions (Lewandowski et al., 1985; Gumaelius et al., 1996; Lin et al., 2006; Bitton, 2005) which was summarized in table 4.4. Effects of metal ions on biodegradation of methyl tert-butyl ether (MTBE) by using pure culture of *Pseudomonas aeruginosa* were studied by Lin et al. (2006). Accordingly, the inhibition coefficient *Ki* for trivalent chromium ($Cr^{3+}$), zinc ($Zn^{2+}$), and manganese ($Mn^{2+}$) were found not much different and fell in the range of 161-165 mg/L while the

inhibition coefficient for copper ($Cu^{2+}$) was recorded at the concentration of 32.4 mg/L. This indicated that $Cu^{2+}$ was much more toxic than $Cr^{3+}$, $Zn^{2+}$, and $Mn^{2+}$. *Ki* value of 12 mg/L also was estimated for cadmium ($Cd^{2+}$) in the denitrification process in activated sludge (Gumaelius et al., 1996) and $K_i$ for hexavalent chromium ($Cr^{6+}$) was found to be 1.2 mg/L in the biological reactors (Lewandowski et al., 1985). This information can be related to this study that different metal species affected microorganisms and microbial activities differently. It is very difficult to compare Ki values of AgNPs and the other heavy metals because its characteristics totally different. However, the calculated Ki values for nitrification and ammonia oxidation suggested that AgNPs are very toxic to nitrogen removal in biological reactors.

Silver nanoparticles exhibited inhibitory effect differently from heavy metals in some ways. First is the inhibitory characteristic. Most of heavy metals fell into noncompetitive inhibition (Bitton, 2005) while AgNPs was found to be uncompetitive behavior in the present study. Second is the stimulation effect. It was reported that some heavy metals are necessary to microorganisms thus they can stimulate the growth of the microbes, for instances, $Ni^{2+}$, $Co^{2+}$, $Cr^{6+}$ at the concentration of 10, 5, and 25 mg/L were found significantly stimulated the maximum growth rate in the activated sludge (Gikas, 2007; Gikas and Romanos, 2005; Gumaelius et al., 1996).

Silver nanoparticles exhibited more toxic than other nanoparticles including copper nanoparticles (CuNPs), gold nanoparticles (AuNPs), and zero valent iron (ZVI) nanoparticles. It was reported that CuNPs at the concentration of 10 mg/L caused no significantly decrease in respiration rate of activated sludge microorganisms (Ganesh et al., 2010). In another study, *Escherichia coli* was inactivated significantly by AgNPs while AuNPs did not show any inactivation ability (Ehre et al., 2009). For ZVI nanoparticles, the addition of its concentration up to 100 mg/L only caused slightly decrease in the richness of microbial communities but it returned to the initial state after 3 days and then the stimulation effect was observed (Thompson et al., 2010).

## 4.5 Quantification of inhibition (%) caused by AgNPs on nitrification and ammonia oxidation

Quantification of the inhibition of AgNPs for nitrification was also made in the present study as showed in Figure 4.14. For the both processes, increasing AgNPs concentration resulted in higher inhibition level. The average percentage of inhibition of oxygen uptake rate of nitrifying microbes corresponding to $NH_4^+$-N concentrations of 14, 28, 50, 70, and 280 mg/L was 19 ± 3.9%, 27 ± 7.7%, 26 ± 10%, 26 ± 11.8%, and 48 ± 18%, respectively. Silver nanoparticles at the concentrations of 0.25, 0.35, 0.5, 1, 3, and 10 mg/L caused average inhibition of 8 ± 4.8%, 13 ± 4.4%, 21 ± 2.2%, 28 ± 7.8%, 36 ± 14.4, and 38 ± 14.2, respectively. For ammonia oxidation, the data in Figure 4.15 indicated that AgNPs concentrations of 0.25, 0.5, 1, 3, 5, 10 mg/L accounted for an average inhibition of oxygen uptake rate of 16 ± 8.2%, 26 ± 7.3% and 38 ± 11.3%, 28 ± 13.6%, 30 ± 7.8%, and 28 ± 7.1%, respectively. Furthermore, the effect of $NH_4^+$-N concentration on oxygen uptake rate under the presence of AgNPs also was observed. The results showed that higher $NH_4^+$-N concentration resulted in higher inhibition rate of respiration of ammonia oxidizing microorganisms. The average percentage of inhibition was 8 ± 4.9%, 25 ± 8.9%, 26 ± 2.8%, 30 ± 7.9%, and 41 ± 17.2% for the concentrations of 14, 28, 50, 70 and 280 mg $NH_4^+$-N/L, respectively.

The impact of common heavy metals such as nikel divalent ($Ni^{2+}$) and chromium hexavalent ($Cr^{6+}$) in activated sludge has been studied. The results showed that $Cr^{6+}$ and $Ni^{2+}$ inhibited microbial oxygen uptake rate up to 15% and 40%, respectively, within 30 min (Cokgor et al., 2007). Respiration rate of nitrifying bacteria was found to be inhibited by up to 41% (Hu et al., 2010) and 86% (Choi et al., 2008) at the AgNPs concentration of 1 mg/L. In this study, the average inhibition caused by AgNPs on nitrifying and ammonia oxidizing was only 28% and 38% at the same concentration for nitrification and ammonia oxidation process, respectively. Hu and his colleauges (2010) also found that at a concentration of 0.4 and 0.75 mg/L total Ag inhibited the growth of nitrifying bacteria by 11.5% and 50%, respectively.

The discrepancies of AgNPs effects among the literature may be come from differences of cell concentration, characteristics of AgNPs, composition of communities

as well as exposure time in the activated sludge. In addition, dissimilarities among other studies and this study may be because of the presence of both Ammonium Oxidizing Bacteria (AOB) and Ammonia Oxidizing Archaea (AOA) in the cultivated culture. In previous study, it was also found that the original activated sludge used in this study comprised both AOA and AOB as mentioned in earlier sub-section. Also, it is known that AOA have been reported to be well tolerated in the extreme environments thus they may tolerate with the presence of AgNPs.

Several mechanisms have been proposed for effect of AgNPs on bacteria but not archaea. The growth inhibition of microbes may be related to the formation of free radicals from the surface of AgNPs (Panacek et al., 2009). Uncontrolled generation of free radicals can attach membrane lipids and then lead to a breakdown of membrane function (Choi et al., 2008). This may not be the reason for this study, because by nature, free radicals will consume oxygen in the experimental condition; however, the control experiment was conducted and proved that there was no dissolved oxygen consumption other than microbial activity. This means that AgNPs did not generate free radicals. The same conclusion was made that AgNPs only generated in the presence of light (Hu and Choi, 2008). Another suggested mechanism is the formation of “pits” in the cell wall of bacteria under the presence of AgNPs (Sondi and Salopek-Sondi, 2004; Choi et al., 2008). Furthermore, it was recommended that the nanoparticles preferably attack the respiratory chain, cell division finally leading to cell death (Sondi and Salopek-Sondi, 2004). Unfortunately, little information of AgNPs effect mechanisms has been proposed for the case of AOA.

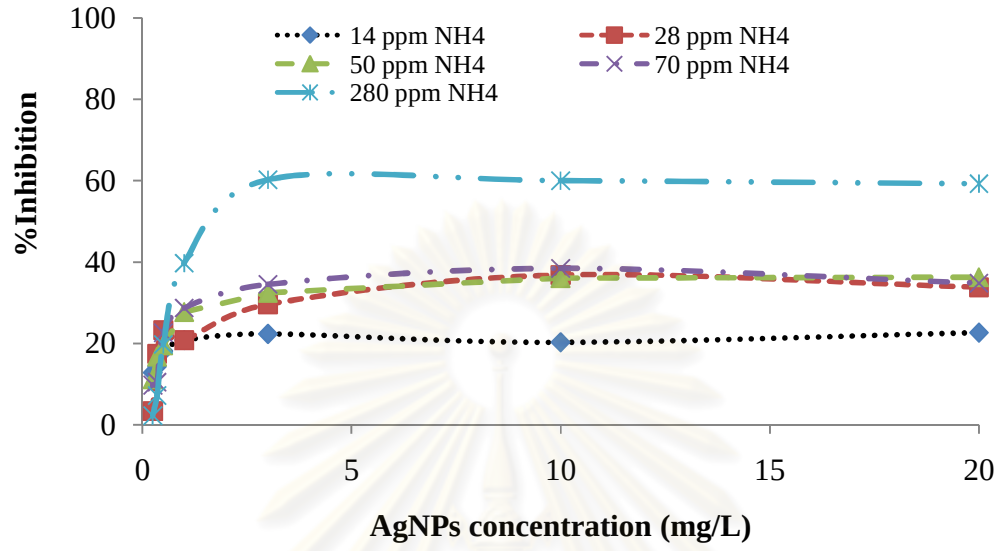


Figure 4.14 Inhibition (%) caused by AgNPs in nitrification

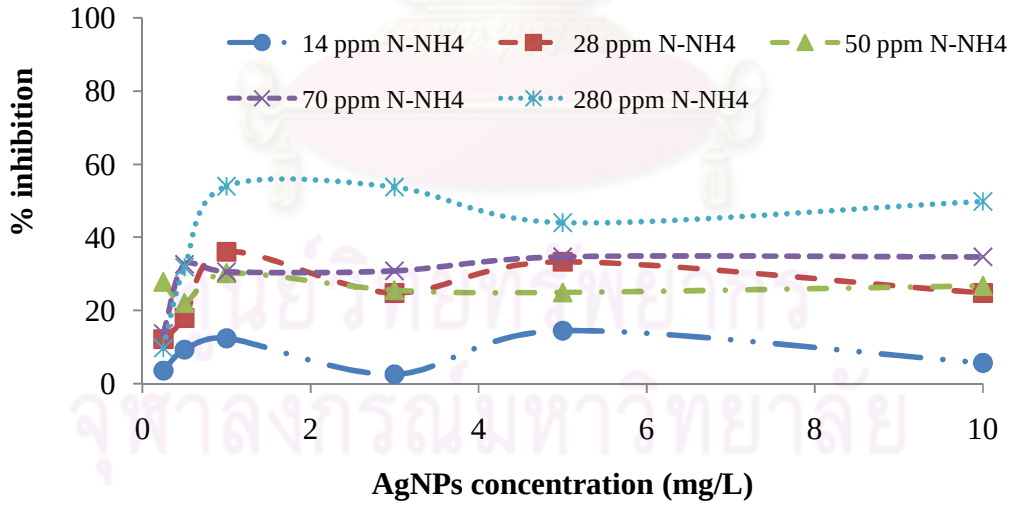


Figure 4.15 Inhibition (%) caused by AgNPs on ammonia oxidation

# CHAPTER V

# CONCLUSIONS AND RECOMMNEDATIONS

## 5.1 Conclusions

Nitrifying activated sludge was enriched by using inorganic media with the nitrification efficiency of 95%. The NAS is containing the average copy number of *amo*A genes of $1.29 \times 10^8 \pm 2.22 \times 10^8$ and $2.74 \times 10^7 \pm 1.06 \times 10^7$ copies/mg MLSS of AOA and AOB, respectively.

Influence of AgNPs on nitrification and ammonia oxidation was investigated by using respirometric method. Under the presence of AgNPs, the maximum oxygen uptake rate and half saturation constant of both nitrification and ammonia oxidation were slightly declined. The effect of AgNPs on the both followed uncompetitive inhibition model. This model was tested and confirmed by using Lineweaver-Burk plot (1/OUR vs 1/S) and the indicator was the parallel lines of with and without AgNPs.

Half saturation constants (Ks) without AgNPs for nitrification and ammonia oxidation were 5.42 and 15.88 mg $NH_4^+$-N/L, respectively. The inhibition coefficients (*Ki*) were 8.3 and 21.7 mg/L, respectively.

Silver nanoparticles partially influenced on nitrification and ammonia oxidation in the manner that higher AgNPs resulted in higher inhibition of respiration rate. There was the interacting effect of AgNPs and $NH_4^+$ -N concentration on nitrifying and ammonia oxidizing activity.

Increasing AgNPs concentrations resulted in higher inhibition level of nitrification and ammonia oxidation processes. For nitrification tests, AgNPs at the concentrations of 0.25, 0.35, 0.5, 1, 3, and 10 mg/L caused average inhibition of 8 ± 4.8%, 13 ± 4.4%, 21 ± 2.2%, 28 ± 7.8%, 36 ± 14.4, and 38 ± 14.2, respectively. For ammonia oxidation tests, AgNPs concentrations of 0.25, 0.5, 1, 3, 5, 10 mg/L inhibited an

average inhibition of oxygen uptake rate of 16 ± 8.2%, 26 ± 7.3% and 38 ± 11.3%, 28 ± 13.6%, 30 ± 7.8%, 28 ± 7.1%, respectively.

It is anticipated that the findings of this study will be useful in many practical applications that can result in improved biological reactor design and regulations of production and use of AgNPs products.

## 5.2 Recommendations

Silver nanoparticles should be considered as hazardous waste and thus special attention should be made in the management of discharge of AgNPs into the environments by enhancing regulations production and use.

Clarify between AOA and AOB who probably play the main role in ammonia oxidation under the presence of AgNPs by using specific functional mRNA *amoA* genes of AOA and AOB.

It is necessary to study the speciation of AgNPs in combination with the respirometric method to elucidate the mechanisms of the effect.

Future study should also focus on the direct detection and quantitative determination of AgNPs inside cells.

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# APPENDICES

# APPENDIX A

**Table A.1** Copy numbers of *amo*A genes of AOA and AOB in the reactor during enrichment of NAS

| Date | AOA (Mean ± SD) | AOB (Mean ± SD) |
|---|---|---|
| 02/09/2010 | $2.49 \times 10^6 \pm 7.59 \times 10^4$ | $1.63 \times 10^7 \pm 3.55 \times 10^4$ |
| 15/10/2010 | $2.25 \times 10^5 \pm 5.63 \times 10^4$ | $2.86 \times 10^7 \pm 8.20 \times 10^5$ |
| 15/11/2010 | $1.17 \times 10^6 \pm 5.39 \times 10^4$ | $3.74 \times 10^7 \pm 4.10 \times 10^6$ |
| 15/12/2010 | $5.13 \times 10^8 \pm 7.86 \times 10^7$ | $9.32 \times 10^8 \pm 1.50 \times 10^8$ |

**Table A.2** Nitrogen species (mg/L) in the nitrification tests

| Treatment | $NO_3^-$-N | $NO_2^-$-N | $NH_4^+$-N |
|---|---|---|---|
| **0I** | 0.00 | 0.00 | 110 |
| **0E** | 2.49 | 1.07 | 107 |
| **0.5I** | 0.00 | 0.00 | 100 |
| **0.5E** | 1.24 | 0.07 | 97 |
| **1I** | 0.00 | 0.00 | 99 |
| **1E** | 1.98 | 0.14 | 98 |
| **3I** | 0.01 | 0.00 | 97 |
| **3E** | 2.52 | 0.08 | 96 |
| **5I** | 0.00 | 0.00 | 106 |
| **5E** | 2.75 | 0.14 | 102 |

# APPENDIX B

Table B.1 Oxygen Uptake Rate (mg-$O_2$/L.min) in nitrification tests

| $NH_4^+$-N (mg/L) | AgNPs (mg/L) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **Replication** | **0** | **0.25** | **0.35** | **0.5** | **1** | **3** | **10** | **20** |
| **14** | Rep 1 | 0.160 | 0.138 | 0.141 | 0.126 | 0.128 | 0.126 | 0.126 | 0.137 |
| | Rep 2 | 0.176 | 0.155 | 0.147 | 0.149 | 0.138 | 0.135 | 0.142 | 0.123 |
| | Rep 3 | 0.168 | 0.147 | 0.144 | 0.138 | 0.133 | 0.131 | 0.134 | 0.130 |
| **28** | Rep 1 | 0.167 | 0.171 | 0.144 | 0.132 | 0.143 | 0.125 | 0.110 | 0.121 |
| | Rep 2 | 0.194 | 0.178 | 0.154 | 0.145 | 0.143 | 0.129 | 0.118 | 0.118 |
| | Rep 3 | 0.181 | 0.175 | 0.149 | 0.139 | 0.143 | 0.127 | 0.114 | 0.120 |
| **50** | Rep 1 | 0.175 | 0.167 | 0.157 | 0.155 | 0.140 | 0.130 | 0.125 | 0.119 |
| | Rep 2 | 0.199 | 0.183 | 0.171 | 0.162 | 0.145 | 0.136 | 0.127 | 0.132 |
| | Rep 3 | 0.187 | 0.175 | 0.164 | 0.159 | 0.143 | 0.133 | 0.126 | 0.126 |
| **70** | Rep 1 | 0.204 | 0.181 | 0.177 | 0.158 | 0.133 | 0.136 | 0.123 | 0.124 |
| | Rep 2 | 0.207 | 0.190 | 0.191 | 0.160 | 0.160 | 0.133 | 0.130 | 0.144 |
| | Rep 3 | 0.206 | 0.186 | 0.184 | 0.159 | 0.147 | 0.135 | 0.127 | 0.134 |
| **280** | Rep 1 | 0.178 | 0.189 | 0.175 | 0.159 | 0.120 | 0.085 | 0.078 | 0.084 |
| | Rep 2 | 0.227 | 0.207 | 0.201 | 0.166 | 0.124 | 0.076 | 0.084 | 0.081 |
| | Rep 3 | 0.203 | 0.198 | 0.188 | 0.163 | 0.122 | 0.081 | 0.081 | 0.083 |

Table B.2 Oxygen Uptake Rate (mg-$O_2$/L.min) in ammonia oxidation tests

| $NH_4^+$-N (mg/L) | AgNPs (mg/L) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **Replication** | **0** | **0.25** | **0.5** | **1** | **3** | **5** | **10** | **No $NaN_3$** |
| **14** | Rep 1 | 0.110 | 0.082 | 0.093 | 0.085 | 0.095 | 0.086 | 0.087 | 0.123 |
| | Rep 2 | 0.083 | 0.104 | 0.082 | 0.084 | 0.093 | 0.079 | 0.095 | 0.132 |
| | Rep 3 | 0.097 | 0.093 | 0.088 | 0.085 | 0.094 | 0.083 | 0.091 | 0.128 |
| **28** | Rep 1 | 0.146 | 0.140 | 0.118 | 0.101 | 0.109 | 0.106 | 0.116 | 0.177 |
| | Rep 2 | 0.148 | 0.118 | 0.123 | 0.087 | 0.112 | 0.090 | 0.105 | 0.156 |
| | Rep 3 | 0.147 | 0.129 | 0.121 | 0.094 | 0.111 | 0.098 | 0.111 | 0.167 |
| **50** | Rep 1 | 0.172 | 0.124 | 0.134 | 0.120 | 0.128 | 0.129 | 0.126 | 0.184 |
| | Rep 2 | 0.183 | 0.153 | 0.133 | 0.121 | 0.124 | 0.120 | 0.127 | 0.189 |
| | Rep 3 | 0.178 | 0.139 | 0.134 | 0.121 | 0.126 | 0.125 | 0.127 | 0.187 |
| **70** | Rep 1 | 0.195 | 0.168 | 0.115 | 0.132 | 0.128 | 0.126 | 0.127 | 0.215 |
| | Rep 2 | 0.197 | 0.170 | 0.149 | 0.140 | 0.143 | 0.130 | 0.129 | 0.198 |
| | Rep 3 | 0.196 | 0.169 | 0.132 | 0.136 | 0.136 | 0.128 | 0.128 | 0.207 |
| **280** | Rep 1 | 0.198 | 0.183 | 0.139 | 0.103 | 0.095 | 0.114 | 0.101 | 0.208 |
| | Rep 2 | 0.213 | 0.188 | 0.141 | 0.086 | 0.095 | 0.116 | 0.105 | 0.207 |
| | Rep 3 | 0.206 | 0.186 | 0.140 | 0.095 | 0.095 | 0.115 | 0.103 | 0.208 |

Table B.3 MLVSS (mg/L) in nitrification tests

| AgNPs (mg/L) | $NH_4^+$-N (mg/L) | | | | | |
|---|---|---|---|---|---|---|
| | **0** | **14** | **28** | **50** | **70** | **280** |
| **0** | 105 | 124 | 110 | 115 | 116 | 163 |
| **0.25** | 105 | 143 | 103 | 123 | 112 | 130 |
| **0.35** | 105 | 138 | 68 | 128 | 108 | 101 |
| **0.5** | 105 | 106 | 120 | 110 | 115 | 102 |
| **1** | 105 | 151 | 125 | 134 | 108 | 150 |
| **3** | 105 | 136 | 120 | 118 | 102 | 123 |
| **10** | 105 | 107 | 111 | 125 | 93 | 126 |
| **20** | 105 | 101 | 119 | 109 | 149 | 105 |

Table B.4 MLVSS (mg/L) in ammonia oxidation tests

| AgNPs (mg/L) | $NH_4^+$-N (mg/L) | | | | | |
|---|---|---|---|---|---|---|
| | **0** | **14** | **28** | **50** | **70** | **280** |
| **0** | 100 | 119 | 97 | 106 | 97 | 97 |
| **0.25** | 100 | 136 | 87 | 123 | 95 | 102 |
| **0.5** | 100 | 126 | 93 | 96 | 136 | 106 |
| **1** | 100 | 142 | 96 | 94 | 122 | 120 |
| **3** | 100 | 109 | 108 | 88 | 107 | 119 |
| **5** | 100 | 116 | 106 | 130 | 107 | 121 |
| **10** | 100 | 108 | 101 | 112 | 89 | 108 |
| **No NaN3** | 100 | 104 | 105 | 90 | 111 | 105 |

Table B.5 pH in nitrification tests

| $NH_4^+$-N (mg/L) | AgNPs (mg/L) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **When** | **0** | **0.25** | **0.35** | **0.5** | **1** | **3** | **10** | **20** |
| **14** | Initial | 7.72 | 7.76 | 7.74 | 7.76 | 7.77 | 7.74 | 7.75 | 7.75 |
| | End | 7.62 | 7.71 | 7.75 | 7.76 | 7.76 | 7.74 | 7.73 | 7.73 |
| **28** | Initial | 7.71 | 7.85 | 7.8 | 7.83 | 7.84 | 7.87 | 7.84 | 7.88 |
| | End | 7.89 | 7.91 | 7.86 | 7.87 | 7.86 | 7.83 | 7.83 | 7.83 |
| **50** | Initial | 7.76 | 7.77 | 7.99 | 7.97 | 7.75 | 7.76 | 7.8 | 7.79 |
| | End | 7.76 | 7.76 | 7.91 | 7.93 | 7.73 | 7.58 | 7.78 | 7.79 |
| **70** | Initial | 7.77 | 7.72 | 8.01 | 8 | 7.98 | 7.99 | 7.97 | 8.07 |
| | End | 7.74 | 7.8 | 7.89 | 7.93 | 7.97 | 7.91 | 7.93 | 7.96 |
| **280** | Initial | 7.82 | 7.96 | 7.79 | 7.92 | 7.75 | 7.7 | 7.78 | 7.77 |
| | End | 7.67 | 8 | 7.7 | 7.88 | 7.69 | 7.83 | 7.83 | 7.73 |

Table B.6 pH in ammonia oxidation tests

| $NH_4^+$-N (mg/L) | AgNPs (mg/L) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **When** | **0** | **0.25** | **0.5** | **1** | **3** | **5** | **10** | **No $NaN_3$** |
| **14** | Initial | 7.66 | 7.7 | 7.69 | 7.67 | 7.67 | 7.67 | 7.66 | 7.65 |
| | End | 7.63 | 7.64 | 7.65 | 7.67 | 7.66 | 7.67 | 7.59 | 7.63 |
| **28** | Initial | 7.64 | 7.65 | 7.67 | 7.66 | 7.68 | 7.67 | 7.67 | 7.63 |
| | End | 7.65 | 7.65 | 7.67 | 7.67 | 7.69 | 7.7 | 7.65 | 7.63 |
| **50** | Initial | 7.67 | 7.67 | 7.69 | 7.69 | 7.69 | 7.69 | 7.68 | 7.67 |
| | End | 7.68 | 7.69 | 7.70 | 7.70 | 7.68 | 7.67 | 7.69 | 7.64 |
| **70** | Initial | 7.84 | 7.84 | 7.81 | 7.79 | 7.82 | 7.82 | 7.81 | 7.82 |
| | End | 7.8 | 7.79 | 7.8 | 7.82 | 7.84 | 7.84 | 7.81 | 7.79 |
| **280** | Initial | 7.79 | 7.81 | 7.87 | 8.01 | 7.86 | 7.9 | 7.98 | 7.85 |
| | End | 7.78 | 7.75 | 7.81 | 7.92 | 7.78 | 7.8 | 7.83 | 7.77 |

Table B.7 Nitrogen species in nitrification tests

| $NH_4^+$-N (mg/L) | AgNPs (mg/L) | $NO_3^-$-N | | $NO_2^-$-N | | $NH_4^+$-N | |
|---|---|---|---|---|---|---|---|
| | | **Initial** | **End** | **Initial** | **End** | **Initial** | **End** |
| | 0 | 1.39 | 1.49 | 0.063 | 0.067 | 12.15 | 11.16 |
| | 0.25 | 1.06 | 1.16 | 0.307 | 0.243 | 11.47 | 13.01 |
| | 0.35 | 0.69 | 1.10 | 0.113 | 0.136 | 11.51 | 13.37 |
| **14** | 0.5 | 0.73 | 0.93 | 0.074 | 0.078 | 13.42 | 12.86 |
| | 1 | 0.63 | 0.80 | 0.071 | 0.066 | 13.47 | 13.01 |
| | 3 | 0.97 | 0.94 | 0.069 | 0.071 | 14.43 | 13.78 |
| | 10 | 0.79 | 1.05 | 0.069 | 0.081 | 14.77 | 13.78 |
| | 0 | 1.45 | 1.52 | 0.365 | 0.242 | 27.15 | 26.33 |
| | 0.25 | 1.28 | 1.28 | 0.303 | 0.414 | 31.17 | 27.26 |
| | 0.35 | 0.87 | 0.89 | 0.090 | 0.138 | 30.81 | 29.20 |
| **28** | 0.5 | 0.92 | 0.80 | 0.066 | 0.077 | 30.58 | 30.00 |
| | 1 | 0.98 | 0.84 | 0.068 | 0.080 | 30.58 | 30.23 |
| | 3 | 1.04 | 0.76 | 0.071 | 0.072 | 30.81 | 30.11 |
| | 10 | 1.28 | 1.01 | 0.106 | 0.093 | 30.81 | 30.11 |
| | 0 | 1.36 | 1.52 | 0.070 | 0.140 | 53.71 | 51.69 |
| | 0.25 | 1.47 | 1.13 | 0.260 | 0.349 | 54.95 | 54.33 |
| | 0.35 | 1.69 | 1.07 | 0.070 | 0.200 | 55.17 | 53.71 |
| **50** | 0.5 | 1.08 | 0.91 | 0.153 | 0.141 | 56.02 | 55.80 |
| | 1 | 0.85 | 0.82 | 0.073 | 0.072 | 54.33 | 51.89 |
| | 3 | 0.92 | 1.09 | 0.076 | 0.077 | 53.50 | 51.69 |
| | 10 | 0.84 | 1.11 | 0.109 | 0.098 | 43.50 | 42.35 |
| | 0 | 1.36 | 1.45 | 0.380 | 0.460 | 76.40 | 74.95 |
| | 0.25 | 0.58 | 1.23 | 0.513 | 0.411 | 74.67 | 73.81 |
| | 0.35 | 1.03 | 0.97 | 0.299 | 0.342 | 74.95 | 72.41 |
| **70** | 0.5 | 1.40 | 0.81 | 0.215 | 0.299 | 67.59 | 70.77 |
| | 1 | 1.20 | 0.13 | 0.146 | 0.261 | 72.97 | 67.59 |
| | 3 | 0.87 | 0.75 | 0.241 | 0.319 | 69.16 | 70.77 |
| | 10 | 0.67 | 0.88 | 0.241 | 0.341 | 68.11 | 68.11 |
| | 0 | 0.92 | 1.10 | 0.366 | 0.455 | 280.02 | 277.88 |
| | 0.25 | 0.90 | 0.65 | 0.604 | 0.320 | 277.88 | 278.95 |
| | 0.35 | 0.81 | 0.59 | 0.484 | 0.358 | 267.44 | 265.40 |
| **280** | 0.5 | 0.64 | 0.43 | 0.383 | 0.324 | 270.53 | 267.44 |
| | 1 | 1.24 | 0.21 | 0.369 | 0.346 | 261.36 | 258.37 |
| | 3 | 0.56 | 0.61 | 0.361 | 0.369 | 278.95 | 276.82 |
| | 10 | 1.07 | 1.08 | 0.526 | 0.326 | 278.95 | 280.02 |

Table B.8 Nitrogen species in ammonia oxidation tests

| $NH_4^+$-N (mg/L) | AgNPs (mg/L) | $NO_3^-$-N | | $NO_2^-$-N | | $NH_4^+$-N | |
|---|---|---|---|---|---|---|---|
| | | Initial | End | Initial | End | Initial | End |
| 14 | No NaN3 | 9.99 | 10.63 | 0.014 | 0.088 | 12.77 | 11.60 |
| | 0 | 2.24 | 6.25 | 0.066 | 0.103 | 12.57 | 12.33 |
| | 0.25 | 4.49 | 5.88 | 0.015 | 0.135 | 12.52 | 12.43 |
| | 0.5 | 1.95 | 3.86 | 0.017 | 0.022 | 12.77 | 12.15 |
| | 1 | 2.11 | 3.43 | 0.015 | 0.027 | 12.77 | 12.19 |
| | 3 | 3.65 | 3.66 | 0.024 | 0.047 | 12.77 | 12.43 |
| | 5 | 3.16 | 3.88 | 0.054 | 0.078 | 11.16 | 11.69 |
| | 10 | 5.03 | 5.20 | 0.068 | 0.094 | 12.01 | 10.66 |
| 28 | No NaN3 | 11.10 | 11.21 | 0.002 | 0.023 | 28.32 | 26.95 |
| | 0 | 4.31 | 4.87 | 0.532 | 0.554 | 27.26 | 26.74 |
| | 0.25 | 3.51 | 4.17 | 0.423 | 0.490 | 26.84 | 25.64 |
| | 0.5 | 3.77 | 4.07 | 0.016 | 0.027 | 26.74 | 25.15 |
| | 1 | 2.67 | 0.08 | 0.024 | 0.030 | 26.44 | 25.83 |
| | 3 | 3.13 | 3.23 | 0.024 | 0.061 | 26.95 | 28.00 |
| | 5 | 2.89 | 3.66 | 0.070 | 0.188 | 26.23 | 26.84 |
| | 10 | 4.60 | 4.01 | 0.137 | 0.147 | 26.23 | 25.93 |
| 50 | No NaN3 | 11.21 | 11.18 | 5.492 | 5.952 | 46.79 | 44.34 |
| | 0 | 6.08 | 5.80 | 5.587 | 5.932 | 48.24 | 45.90 |
| | 0.25 | 3.22 | 3.48 | 1.461 | 2.103 | 46.79 | 45.55 |
| | 0.5 | 3.15 | 3.37 | 1.272 | 2.374 | 47.51 | 47.15 |
| | 1 | 1.90 | 1.97 | 0.379 | 2.286 | 42.51 | 43.34 |
| | 3 | 2.04 | 2.19 | 0.386 | 1.380 | 42.84 | 44.51 |
| | 5 | 1.78 | 3.85 | 0.298 | 1.515 | 43.84 | 42.03 |
| | 10 | 2.50 | 4.15 | 0.413 | 1.874 | 43.84 | 40.91 |
| 70 | No NaN3 | 11.45 | 11.28 | 5.925 | 6.094 | 69.96 | 67.07 |
| | 0 | 11.23 | 10.81 | 4.964 | 5.451 | 66.30 | 65.05 |
| | 0.25 | 4.41 | 10.77 | 1.799 | 2.807 | 66.30 | 64.30 |
| | 0.5 | 2.15 | 2.21 | 0.521 | 1.089 | 70.23 | 67.33 |
| | 1 | 2.57 | 4.62 | 1.245 | 1.846 | 67.33 | 67.59 |
| | 3 | 2.80 | 3.83 | 1.657 | 2.496 | 66.30 | 67.59 |
| | 5 | 2.65 | 4.39 | 2.699 | 3.172 | 74.38 | 72.41 |
| | 10 | 3.79 | 4.89 | 2.374 | 3.436 | 65.55 | 66.56 |
| 280 | No NaN3 | 7.48 | 7.58 | 6.006 | 7.034 | 243.94 | 240.23 |
| | 0 | 6.70 | 6.92 | 5.316 | 6.290 | 238.40 | 235.67 |
| | 0.25 | 2.95 | 1.83 | 0.778 | 1.339 | 242.08 | 239.31 |
| | 0.5 | 4.47 | 4.68 | 0.284 | 0.656 | 243.01 | 239.31 |
| | 1 | 2.31 | 0.95 | 1.211 | 1.671 | 241.16 | 238.40 |
| | 3 | 2.11 | 1.35 | 1.603 | 2.212 | 242.08 | 240.23 |
| | 5 | 2.64 | 1.98 | 2.415 | 3.253 | 248.66 | 247.71 |
| | 10 | 2.71 | 3.74 | 1.819 | 2.374 | 240.23 | 239.31 |

# APPENDIX C

Table C.1 SigmaPlot worksheet for Ki estimation for nitrification tests

| $NH_4^+$-N(mg/L) | AgNPs | OUR11 | OUR12 | OUR13 | OUR21 | OUR22 | OUR23 | OUR31 | OUR32 | OUR33 |
|---|---|---|---|---|---|---|---|---|---|---|
| 14 | 0.25 | 0.138 | 0.155 | 0.147 | 0.141 | 0.147 | 0.144 | 0.126 | 0.149 | 0.138 |
| 28 | 0.35 | 0.171 | 0.178 | 0.175 | 0.144 | 0.154 | 0.149 | 0.132 | 0.145 | 0.139 |
| 50 | 0.5 | 0.167 | 0.183 | 0.175 | 0.157 | 0.171 | 0.164 | 0.155 | 0.162 | 0.159 |
| 70 | 1 | 0.181 | 0.190 | 0.186 | 0.177 | 0.191 | 0.184 | 0.158 | 0.160 | 0.159 |
| 280 | 3 | 0.189 | 0.207 | 0.198 | 0.175 | 0.201 | 0.188 | 0.159 | 0.166 | 0.1625 |
| $NH_4^+$-N(mg/L) | AgNPs | OUR41 | OUR42 | OUR43 | OUR51 | OUR52 | OUR53 | OUR61 | OUR62 | OUR63 |
| 14 | 0.25 | 0.128 | 0.138 | 0.133 | 0.126 | 0.135 | 0.131 | 0.126 | 0.142 | 0.134 |
| 28 | 0.35 | 0.143 | 0.143 | 0.143 | 0.125 | 0.129 | 0.127 | 0.110 | 0.118 | 0.114 |
| 50 | 0.5 | 0.140 | 0.145 | 0.143 | 0.130 | 0.136 | 0.133 | 0.125 | 0.127 | 0.126 |
| 70 | 1 | 0.133 | 0.160 | 0.147 | 0.136 | 0.133 | 0.135 | 0.123 | 0.130 | 0.127 |
| 280 | 3 | 0.120 | 0.124 | 0.122 | 0.085 | 0.076 | 0.081 | 0.078 | 0.084 | 0.081 |

Table C.2 SigmaPlot worksheet for Ki estimation for ammonia oxidation tests

| $NH_4^+$-N(mg/L) | AgNPs | OUR11 | OUR12 | OUR13 | OUR21 | OUR22 | OUR23 | OUR31 | OUR32 | OUR33 |
|---|---|---|---|---|---|---|---|---|---|---|
| 14 | 0.25 | 0.082 | 0.104 | 0.093 | 0.093 | 0.082 | 0.088 | 0.085 | 0.084 | 0.085 |
| 28 | 0.5 | 0.140 | 0.118 | 0.129 | 0.118 | 0.123 | 0.121 | 0.101 | 0.087 | 0.094 |
| 50 | 1 | 0.124 | 0.153 | 0.139 | 0.134 | 0.133 | 0.134 | 0.120 | 0.121 | 0.121 |
| 70 | 3 | 0.168 | 0.170 | 0.169 | 0.115 | 0.149 | 0.132 | 0.132 | 0.140 | 0.136 |
| 280 | 5 | 0.183 | 0.188 | 0.186 | 0.139 | 0.141 | 0.140 | 0.103 | 0.086 | 0.095 |
| $NH_4^+$-N(mg/L) | AgNPs | OUR41 | OUR42 | OUR43 | OUR51 | OUR52 | OUR53 | OUR61 | OUR62 | OUR63 |
| 14 | 0.25 | 0.095 | 0.093 | 0.094 | 0.086 | 0.079 | 0.083 | 0.087 | 0.095 | 0.091 |
| 28 | 0.5 | 0.109 | 0.112 | 0.111 | 0.106 | 0.090 | 0.098 | 0.116 | 0.105 | 0.111 |
| 50 | 1 | 0.128 | 0.124 | 0.126 | 0.129 | 0.120 | 0.125 | 0.126 | 0.127 | 0.127 |
| 70 | 3 | 0.128 | 0.143 | 0.136 | 0.126 | 0.130 | 0.128 | 0.127 | 0.129 | 0.128 |
| 280 | 5 | 0.095 | 0.095 | 0.095 | 0.114 | 0.116 | 0.115 | 0.101 | 0.105 | 0.103 |

*Notes: Data is entered in S, I, multiple-replicate-OUR format where S is a column of substrate values, I is a column of inhibitor values and multiple-replicate-OURs are groups of columns containing replicate OUR values. Each group contains the same number of columns for replicates. Replicates are entered row-wise. For example, the worksheet in Table C.2 shows substrate and inhibitor values in columns 1 and 2, respectively. There are 5 groups of replicate OUR values corresponding to the 5 inhibitor values. Each group contains 3 columns of replicates.*

Table C.3 Effect of AgNPs and $NH_4^+$-N on nitrification tests

| AgNPs (mg/L) | $NH_4^+$-N(mg/L) (Mean ± SD) | | | | |
|---|---|---|---|---|---|
| | 14 | 28 | 50 | 70 | 280 |
| Control | 0.168 ± 0.008$^{b(a)}$ | 0.1805 ± 0.014$^{ab(a)}$ | 0.187 ± 0.012$^{ab(a)}$ | 0.2055 ± 0.002$^{a(a)}$ | 0.2025 ± 0.025$^{a(a)}$ |
| 0.25 | 0.1465 ± 0.008$^{c(b)}$ | 0.1745 ± 0.003$^{ab(a)}$ | 0.175 ± 0.008$^{ab(b)}$ | 0.1855 ± 0.005$^{a(b)}$ | 0.198 ± 0.009$^{a(a)}$ |
| 0.35 | 0.144 ± 0.003$^{c(bc)}$ | 0.149 ± 0.005$^{c(b)}$ | 0.164 ± 0.007$^{b(bc)}$ | 0.184 ± 0.007$^{a(b)}$ | 0.188 ± 0.013$^{a(a)}$ |
| 0.5 | 0.1375 ± 0.012$^{b(bc)}$ | 0.1385 ± 0.006$^{b(b)}$ | 0.1585 ± 0.004$^{a(c)}$ | 0.159 ± 0.001$^{a(c)}$ | 0.1625 ± 0.004$^{a(b)}$ |
| 1 | 0.133 ± 0.005$^{bc(bc)}$ | 0.143 ± 0.000$^{ab(b)}$ | 0.1425 ± 0.002$^{ab(d)}$ | 0.1465 ± 0.014$^{a(d)}$ | 0.122 ± 0.002$^{c(c)}$ |
| 3 | 0.1305 ± 0.005$^{ab(bc)}$ | 0.127 ± 0.002$^{bc(c)}$ | 0.133 ± 0.003$^{ab(de)}$ | 0.1345 ± 0.002$^{a(e)}$ | 0.0805 ± 0.005$^{c(d)}$ |
| 10 | 0.134 ± 0.008$^{a(bc)}$ | 0.114 ± 0.004$^{b(d)}$ | 0.126 ± 0.001$^{a(e)}$ | 0.1265 ± 0.004$^{a(e)}$ | 0.081 ± 0.003$^{c(d)}$ |
| 20 | 0.130 ± 0.007$^{ab(c)}$ | 0.1195 ± 0.002$^{b(cd)}$ | 0.1255 ± 0.007$^{ab(e)}$ | 0.134 ± 0.010$^{a(e)}$ | 0.0825 ± 0.020$^{c(d)}$ |

Table C.4 Effect of AgNPs and $NH_4^+$-N on ammonia oxidation tests

| AgNPs (mg/L) | $NH_4^+$-N(mg/L) (Mean ± SD) | | | | |
|---|---|---|---|---|---|
| | 14 | 28 | 50 | 70 | 280 |
| Control | 0.0965±0.014 $^{a(d)}$ | 0.1470 ± 0.001 $^{a(c)}$ | 0.1775 ± 0.006 $^{a(b)}$ | 0.1960 ± 0.001 $^{a(a)}$ | 0.2055 ± 0.008 $^{a(a)}$ |
| 0.25 | 0.0930 ± 0.011 $^{a(c)}$ | 0.129 ± 0.011 $^{b(b)}$ | 0.1385 ± 0.015 $^{b(b)}$ | 0.1690 ± 0.001 $^{b(a)}$ | 0.1855 ± 0.003 $^{b(a)}$ |
| 0.5 | 0.0875 ± 0.055 $^{a(c)}$ | 0.1210 ± 0.003 $^{bc(ab)}$ | 0.1335 ± 0.001 $^{b(a)}$ | 0.1320 ± 0.017 $^{c(a)}$ | 0.1400 ± 0.001 $^{c(a)}$ |
| 1 | 0.0845 ± 0.005 $^{a(d)}$ | 0.0940 ± 0.007 $^{d(bc)}$ | 0.1205 ± 0.001 $^{cd(b)}$ | 0.1360 ± 0.004 $^{c(a)}$ | 0.0945 ± 0.009 $^{e(d)}$ |
| 3 | 0.0940 ± 0.001 $^{a(d)}$ | 0.1105 ± 0.002 $^{c(c)}$ | 0.1260 ± 0.002 $^{c(b)}$ | 0.1355 ± 0.008 $^{c(a)}$ | 0.0950 ± 0.000 $^{e(d)}$ |
| 5 | 0.0825 ± 0.004 $^{a(d)}$ | 0.098 ± 0.008 $^{d(c)}$ | 0.1245 ± 0.005 $^{c(a)}$ | 0.1280 ± 0.002 $^{c(a)}$ | 0.1150 ± 0.001 $^{d(b)}$ |
| 10 | 0.0910 ± 0.004 $^{a(d)}$ | 0.1105 ± 0.005 $^{c(b)}$ | 0.1265 ± 0.001 $^{bc(a)}$ | 0.1280 ± 0.001 $^{c(a)}$ | 0.1030 ± 0.002 $^{e(c)}$ |

*Note: Letters indicate differences ($p<0.05$). Letters in the parentheses show the differences for Oxygen Uptake Rate (OUR) respect to initial $NH_4^+$-N concentration. Letters without parentheses indicate the differences for Oxygen Uptake Rate (OUR) respect to initial AgNPs.*

# BIOGRAPHY

Mr. Nguyen Thanh Giao was born on 20 December 1982 in Co Do district, Can Tho city, Vietnam. He attended university in 2000 and earned his Bachelor degree in Environmental Science that was awarded on 15 August 2005 by the Rector of Can Tho University, Can Tho city, Vietnam. After graduation, Mr Giao has been recruiting to work at the Department of Environmental and Natural Resources Management, College of Environment and Natural Resources, Can Tho University. He has been working there as a research and teaching assistant for Can Tho University - University of Aarhus Link in Environmental Sciences (CAULES) Project funded by DANIDA, Denmark. He joined the international program of Exchange Cultural in 2005 at Sydney University, Australia for one month. He was then selected to go to Plant Biology Department, Aarhus University, Denmark to attend a short training course in three months about Water and Soil and Sediment Analysis in the laboratory. In 2009, he applied and was awarded a master scholarship from Chulalongkorn University, Thailand in the program of “Scholarship Programs for Neighboring Countries”. He studied in the field of Environmental Management – the International Program at National Center of Excellence for Environmental and Hazardous Waste Management, Chulalongkorn University, Bangkok, Thailand. In order to fulfill requirements for his master degree he carried out the thesis entitled “INFLUENCE OF SILVER NANOPARTICLES ON NITRIFICATION KINETICS AND AMMONIA OXIDATION IN ACTIVATED SLUDGE” as a partial need.