Contribution of ammonia oxidizing microorganisms and effect of paranitrophenol on ammonia oxidation of nitrifying sludge

Miss Papitchaya Srithep

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การมีส่วนร่วมของจุลินทรีย์ที่ออกซิไดซ์แอมโมเนียและผลกระทบของสารพาราไนโตรฟีนอลต่อกระบวนการแอมโมเนียออกซิเดชันของตะกอนไนตริไฟอิง

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By	Miss Papitchaya Srithep
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Thesis Advisor	Associate Professor Tawan Limpiyakorn, Ph.D.

Accepted by the Graduate School, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

.....Dean of the Graduate School

(Associate ProfessorSunait Chutintaranond, Ph.D.)

THESIS COMMITTEE

.....Chairman

(Associate Professor Ekawan Luepromchai, Ph.D.)

.....Thesis Advisor

(Associate Professor Tawan Limpiyakorn, Ph.D.)

.....Examiner

(Professor Alissara Reungsang, Ph.D.)

.....Examiner

(Sorawit Powtongsook, Ph.D.)

.....Examiner

(Parinda Thayanukul, Ph.D.)

.....External Examiner

(Suwat Soonglerdsongpha, Ph.D.)

ปพิชญา ศรีเทพ : การมีส่วนร่วมของจุลินทรีย์ที่ออกซิไดซ์แอมโมเนียและผลกระทบของสารพาราไนโตรฟีนอลต่อกระบวนการแอมโมเนียออกซิเดชันของตะกอนไนตริไฟอิง (Contribution of ammonia oxidizing microorganisms and effect of paranitrophenol onammonia oxidation of nitrifying sludge) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. ตะวัน ลิมปิยากร, 220 หน้า.

การออกซิไดซ์แอมโมเนียเป็นไนตรต์เป็นขั้นตอนเริ่มแรกและเป็นขั้นตอนจำกัดอัตราของกระบวนการกำจัดไนโตรเจนในระบบบำบัดน้ำเสีย อย่างไรก็ตามยังไม่เป็นที่ทราบแน่ชัดว่าระหว่างแอมโมเนียออกซิไดซิงอาร์เคีย (AOA) และแอมโมเนียออกซิงไดซิงแบคทีเรีย (AOB) จุลินทรีย์กลุ่มใดที่มีส่วนร่วมในการออกซิไดซ์แอมโมเนียเป็นไนตรต์ในระบบบำบัดน้ำเสีย งานวิจัยนี้เริ่มจากการเดินระบบถังปฏิกรณ์ไนตริไฟอิง 2 ถัง (NRI และ NRII) ที่ใช้ตะกอนเริ่มต้นและการเดินระบบแตกต่างกัน ส่งผลให้ถังปฏิกรณ์ทั้ง 2 ถัง มีสัดส่วนประชากร AOA และ AOB ในถังแตกต่างกัน โดยในถัง NRI พบจำนวน AOA มากกว่า AOB ในขณะที่ถัง NRII พบเฉพาะ AOB เท่านั้น กลุ่มประชากร AOA ที่พบในถัง NRI จัดอยู่ในกลุ่มของ *Nitrososhaera sister* ภายในกลุ่ม 1.1b *Thaumacheota* จากนั้นได้ใช้สาร Allythiourea (ATU) และ 2-Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO) ซึ่งมีคุณสมบัติในการยับยั้ง AOB และ AOA ตามลำดับ ในรูปแบบสารเดี่ยวและสารผสม กับตะกอนจากถังปฏิกรณ์ทั้ง 2 ถัง เพื่อศึกษาการออกซิไดซ์แอมโมเนียของ AOA และ AOB ผลการทดลองชี้ให้เห็นว่า ในถัง NRI ทั้ง AOA และ AOB มีส่วนร่วมกันในการออกซิไดซ์แอมโมเนีย ในขณะที่ AOB เป็นจุลินทรีย์หลักที่ออกซิไดซ์แอมโมเนียในถัง NRII ผลการใช้ DNA-stable isotope probing (DNA-SIP) โดยใช้ ^{13}C-HCO_3^- กับตะกอนจากถัง NRI พบการดึง ^{13}C เข้าสู่ยีน *amoA* ของ AOA และ AOB ซึ่งแสดงให้เห็นว่าจุลินทรีย์ทั้งสองกลุ่มน่าจะสามารถดำรงชีวิตแบบออโตโทรประหว่างการออกซิไดซ์แอมโมเนียได้ นอกจากนี้การใช้ DNA-SIP พบว่า AOA สามารถดึง ^{13}C เข้าสู่ยีน *amoA* เมื่อมี ATU ความเข้มข้น 80 ไมโครโมลาร์ในระบบ ในขณะที่ AOB ไม่สามารถเจริญเติบโตได้ในสภาวะดังกล่าว ซึ่งเป็นการยืนยันว่าสามารถใช้ ATU ที่ความเข้มข้น 80 ไมโครโมลาร์ ในการศึกษาการออกซิไดซ์แอมโมเนียของ AOA ได้ จากนั้นได้ใช้ ATU กับตะกอนจากโรงบำบัดน้ำเสีย 5 โรง ซึ่งแต่ละโรงมีจำนวนประชากร AOA และ AOB แตกต่างกัน ผลการทดลองแสดงให้เห็นว่า AOB เป็นจุลินทรีย์หลักที่ออกซิไดซ์แอมโมเนียในตะกอนจากโรงบำบัดทั้ง 5 โรง สำหรับตัวอย่างตะกอนที่มีจำนวน AOA มากกว่า AOB นั้น AOA ก็มีส่วนร่วมในการออกซิไดซ์แอมโมเนียเช่นกัน โดยคิดเป็นประมาณ 20% ภายใต้สภาวะที่มี ATU ความเข้มข้น 80 ไมโครโมลาร์ในระบบ ผลการยับยั้งการออกซิไดซ์แอมโมเนียโดยสารพาราไนโตรฟีนอล (PNP) กับตะกอนจากถัง NRII พบว่า PNP ที่ความเข้มข้นสูงกว่า 50 มิลลิกรัมต่อลิตร ยับยั้งการออกซิไดซ์แอมโมเนียโดยสมบูรณ์ การศึกษาเซลล์แบคทีเรียมีชีวิต และเซลล์จุลินทรีย์กลุ่มไนตริไฟอิงด้วยเทคนิค fluorescence in situ hybridization (FISH) ชี้ให้เห็นว่า PNP ที่ความเข้มข้น 10 และ 200 มิลลิกรัมต่อลิตร มีแนวโน้มลดจำนวนเซลล์แบคทีเรียมีชีวิตและ เซลล์ AOB ลง ผลที่ได้จากงานวิจัยนี้สามารถนำไปประยุกต์ใช้ในการปรับปรุงการออกแบบและการเดินระบบบำบัดน้ำเสียในอนาคต

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ปีการศึกษา 2559 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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KEYWORDS: AMMONIA-OXIDIZING ARCHAEA; AMMONIA-OXIDIZING BACTERIA; ATU; DNA-SIP; NITRIFYING REACTOR; PTIO; WASTEWATER TREATMENT; PARANITROPHENOL

PAPITCHAYA SRITHEP: Contribution of ammonia oxidizing microorganisms and effect of paranitrophenol onammonia oxidation of nitrifying sludge. ADVISOR: ASSOC. PROF. TAWAN LIMPIYAKORN, Ph.D., 220 pp.

The oxidation of ammonia to nitrite is the initial and rate-limiting step for most biological nitrogen removal approaches in wastewater treatment. However, the contribution of ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) to ammonia oxidation in wastewater treatment plants (WWTPs) has yet been clearly clarified. In this study, two laboratory nitrifying reactors (NRI and NRII) were seeded and operated under different conditions; therefore, different proportions of AOA and AOB arose in both reactors. AOA *amoA* genes outnumbered AOB *amoA* genes in reactor NRI, while only AOB *amoA* genes were the only detectable ammonia oxidizer in reactor NRII. The AOA *amoA* gene sequences from reactor NRI belonged to the *Nitrososhaera sister* cluster within the Group 1.1b *Thaumacheota*. Allythiourea (ATU) and 2-Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO), which were shown in previous studies to specifically inhibit AOB and AOA, respectively, were applied individually and as a mixture to observe the ammonia-oxidizing activity of AOA and AOB in NRI and NRII sludge. The results demonstrated that AOA and AOB jointly oxidized ammonia in NRI sludge, while AOB played the main role in ammonia oxidation in NRII sludge. DNA stable isotope probing (DNA-SIP) with ^{13}C-HCO_3^- was performed on NRI sludge. The ^{13}C was incorporated into AOA and AOB *amoA* genes implying that both microorganisms may perform autotrophy during ammonia oxidation. DNA-SIP also showed that AOA can incorporate the ^{13}C into the *amoA* genes while AOB cannot grow when 80 μM ATU was added. The results confirmed that ATU of 80 μM can be applied to clarify the ammonia-oxidizing activity of AOA in NRI sludge. ATU was applied to sludge from 5 full-scale WWTPs where the numbers of AOA and AOB *amoA* genes in the sludge varied. The results demonstrated that AOB played the main role in ammonia oxidation in all sludge. In the sludge that AOA outnumbered AOB, AOA involved around 20% of ammonia oxidation under presence of ATU at 80 μM. Inhibitory effect of paranitrophenol (PNP) was studied with sludge from reactor NRII. PNP at concentrations of ≥ 50 mgL^{-1} showed complete inhibition of ammonia oxidation. Analyses of bacterial cell viability and active nitrifying microorganisms using fluorescence in situ hybridization (FISH) technique indicated that PNP at concentrations of 10 and 200 mgL^{-1} tended to reduce bacterial cells and active AOB. The findings of this study can further lead to an improvement of wastewater treatment design and operation.

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

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AOA	Ammonia oxidizing archaea
AOB	Ammonia oxidizing bacteria
ATU	Allylthiourea
DO	Dissolved oxygen
DNA-SIP	DNA stable isotope probing
HRT	Hydraulic retention time
mL	Milliliter
mgL^{-1}	Milligram per Liter
$mgNL^{-1}$	Milligram nitrogen per Liter
MLSS	Mixed liquor suspended solid
NR	nitrifying reactors
PNP	*p*-Nitrophenol
PTIO	2-phenyl-4, 4, 5, 5-tetramethylimidazoline-1-oxyl 3-oxide
WWTPs	wastewater treatment plant

CHAPTER I

Introduction

1.1 Background

The oxidation of ammonia to nitrite is considered to be the initial step for all biological nitrogen removal approaches. After ammonia is oxidized to nitrite, several subsequent processes are available for a choice of section. For example, nitrite can be further oxidized to nitrate, then nitrate is reduced to nitrogen gas by nitrate denitrification. Alternatively, nitrite itself can directly be reduced to nitrogen gas by nitrite denitrification or be reduced coupling ammonia by Annamox microorganisms. In comparison to other microorganisms in nitrogen removal processes, ammonia oxidizers is believed to be the rate-limiting step due to the slower growth rate and higher sensitivity to environmental stresses.

It had long been considered that ammonia oxidizing bacteria (AOB) is the key contributor for ammonia oxidation in global nitrogen cycle. During the past ten years, this believe has been changed drastically after the discovery of an *amoA*-like gene on an archaeal-associated scaffold in sea (Venter et al. 2004) and the isolation of the first ammonia oxidizing archaea (AOA) from an environmental sample (Konneke et al. 2005a). After that, a numbers of works have focused on these microorganisms in several natural environmental samples. After a numbers of sequences are available, researchers have proposed to place AOA in a newly found phylum, Thaumacheota, in the Archaea domain (Avrahami et al. 2011). Thus far, a few enriched cultures have been available for some branches of Thaumacheota, for example *Nitrosopumilus maritimus* from marine environments (Konneke et al. 2005a), *Candidatus* Nitrososphaera viennensis (Tourna et al. 2011) and *Candidatus* Nitrosocosmicus franlandus (Lehtovirta-Morley et al. 2016) from soil environments.

For wastewater treatment plant (WWTP) field of study, AOA was found for the first time in activated sludge bioreactors in the USA by Park et al. (2006). During 2006-2017, AOA was found in WWTPs in many worldwide countries; for example, the USA (Wells et al. 2009) Hong Kong (Jin et al. 2010; Ye and Zhang 2011; Zhang et al. 2009) Thailand (Limpiyakorn et al. 2011; Sonthiphand and Limpiyakorn 2011),Turkey (Yapsakli et al. 2011), China (Bai et al. 2012; Gao et al. 2013; Guo et al. 2014; Zhang et al. 2009; Zhang et al. 2015), Singapore (Zhang et al. 2009), countries in Europe (Mussmann et al. 2011), and Canada (Sauder et al. 2012). Very recently, AOA was able to isolated from municipal WWTPs in China and Canada, respectively (Li et al. 2016; Sauder et al. 2017).

The contribution of AOA to ammonia oxidation in WWTPs has still been in argument. In 2011, Mussmann et al. (2011) et al demonstrated that AOA in one refinery WWTP in England may not perform ammonia oxidation and may rely on other route to maintain life in the WWTP. However, *Candidatus* Nitrosotenuis cloacae and *Candidatus* Nitrosocosmicus exaquare have recently been obtained from WWTPs in China and Canada under autotrophic growth condition (Li et al. 2016; Sauder et al. 2017). Both cultures demonstrated the ability to oxidize ammonia to nitrite.

Regarding WWTPs that AOA and AOB coexist; it is hard to differentiate the activity of AOA and AOB directly because it needs sophisticated technique that allow observing target microorganisms in mixed microbial community structure. Selective inhibitors or specific inhibitors for AOA and AOB may be applicable to differentiate the activity of AOA and AOB by allowing one microorganism to perform activity, while inhibit the activity of the other. This is because AOA and AOB perform ammonia oxidation with different pathways and are different in cell structure and properties (Shen et al. 2013; Walker et al. 2010). During ammonia oxidation process, AOB used hydroxylamine as an intermediate but nitric oxide was an intermediate for ammonia oxidation of AOA (He et al. 2012; Shen et al. 2013; Stahl and de la Torre 2012).

Regarding cell structure and properties, membrane lipid of archaea has ether bonds instead of ester bonds as found in bacteria. Moreover, cell wall of bacteria contain peptidoglycan compared to archaea. Some recent studies used selective inhibitors to clarify the contributions of AOA and AOB to ammonia oxidation in environmental samples; including soil, sediment and manure (Lehtovirta-Morley et al. 2013; Shen et al. 2013; Taylor et al. 2010; Tourna et al. 2008) To date, no study has reported the contributions of AOB and AOA to ammonia oxidation in WWTPs.

Most ammonia oxidation inhibitors target AMO enzyme, but may rely on different mechanisms; for examples, act as copper chelating inhibitor in the active AMO (such as allylthiourea, ATU; amidinothiourea, ASU) (Shen et al. 2013), act as bacteriostatic agent (such as dicyanamide, DCD) (Shen et al. 2013), and bind to the complex of membrane-bound proteins and inactivating the AMO (such as 3,4-dimethylpyrazole phosphate, DMPP) (Kleineidam et al. 2011). Antibiotic agents react with folic acid and biosynthesis in bacteria; such as, sulfonamide, kanamycin, streptomycin, ampicillin and carbenicillin (Sauder et al. 2017; Shen et al. 2013; Tourna et al. 2011). Some compounds act as a scavenger of nitric oxide (NO) such as 2-phenyl-4, 4, 5, 5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO) (Shen et al. 2013).

Allylthiourea (ATU) was commonly used as an inhibitor for ammonia oxidation. It is a copper chelating inhibitor of the active AMO. Differences in inhibition threshold was observed between AOA and AOB because of the differences of amino acid sequences in the active center of AMO or content of copper-containing protein in the genomes of both microorganisms (Li et al. 2016; Shen et al. 2013). At low concentration, ATU has strong effect on AOB; for example, in pure culture (Martens-Habbena et al. 2015), marine (Santoro and Casciotti 2011) and soil (Taylor et al. 2010) studies. However, AOA was affected to ATU at higher concentration as compared to AOB (Martens-Habbena et al. 2015). 2-phenyl-4, 4, 5, 5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO) inhibits ammonia oxidation by acting as a NO scavenger. PTIO was a

specific inhibitor for AOA because NO is an intermediate of ammonia oxidation by AOA (Walker et al. 2010). PTIO had strong effect on activity of AOA culture; including, *Nitrososphaera viennensis, Nitrosopumilus maritimus* strain HCA1, *Nitrosopumilus maritimus* stain SCM1 and *Candidatus* Nitrosocosmicus exaquare, whereas PTIO unaffected on β-AOB, γ-AOB strains (Martens-Habbena et al. 2015).

However, the application of only specific inhibitors for AOA or AOB is sometimes unable to differentiate the activity of both microorganisms clearly. We probably need to apply specific inhibitors in a mixture form or use in-situ activity investigation technique in combination. Up to date, the techniques, that allow direct activity investigation, reply on applying labeled compounds into complex community and perform phylogenetic identification of microorganisms that uptake the labelled compounds; for example, MAR-FISH, Raman-FISH, SIMS-FISH and stable isotope probing (SIP). However, direct identification of ammonia-oxidizing activity by using the above techniques is not simple since signal may come from other microorganism which is able to incorporate ammonia as the nitrogen source for biomass synthesis. In this case, DNA-stable isotope probing (DNA-SIP) is probably an appropriate technique with labeled inorganic carbon to observe the incorporation of the compound for biomass synthesis during ammonia oxidation. This technique has been applied for several environmental samples; for example, soil (Zhang et al. 2012), drinking water treatment (Niu et al. 2013), freshwater sediment (Wu et al. 2013) and AOA isolation from WWTPs (Li et al. 2016). Few studies used specific inhibitors for AOA and AOB in combination to DNA-SIP; for example, applying DCD to acid soil sample (Zhang et al. 2012) and applying DMPP to agricultural soils (Shi et al. 2016). The combination of specific inhibitors and DNA-SIP can provide clear result.

For all above reasons, this study investigated the relative contributions of AOA and AOB to ammonia oxidation in laboratory nitrifying reactors and full-scale WWTPs

using specific inhibitors and DNA-SIP. In addition, the study aimed to observe the effect of paranitrophenol (PNP) on ammonia oxidation of the nitrifying reactors. Since, PNP has been reported as priority pollutant by United States Environmental Protection Agency (USEPA). PNP is able to cause teratogenic, carcinogenic, and mutagenic to organisms (Guo et al. 2014). Therefore, U.S.EPA listed PNP as a considering pollutant and restricted the concentration of PNP in natural water to be less than 10 ngL^{-1}. In addition, industrial effluent should discharge monthly average concentration of PNP of lower than 162 μgL^{-1} (1.9 $mgL^{-1}Y^{-1}$). Some studies reported that some of polycyclic aromatic hydrocarbons can reduce soil nitrification by acting as the ammonium monooxygenase's suicide substrate such as nitrobenzene (Sverdrup et al. 2002). In this way, the presence of cyclic N compounds, such as PNP, in soil is likely to affect the nitrification of ammonium oxidizers. To date, few studies provided knowledge on inhibitory effect of PNP on ammonia oxidation. Therefore, the threshold of PNP concentration that inhibits ammonia oxidizing is yet clearly clarified.

1.2 Research Objectives

The final goal of this study is to understand the roles of ammonia oxidizing archaea (AOA) and ammonia oxidizing bacteria (AOB) in ammonia oxidation in nitrifying reactors and full-scale WWTPs. Therefore, the objectives of this study are

1. To optimize concentrations of selective inhibitors to differentiate ammonia-oxidizing activity of AOA and AOB in nitrifying reactors
2. To identify the ammonia-oxidizing activity and growth of AOA and AOB in nitrifying reactors and full-scale WWTPs
3. To study inhibitory effect of paranitrophenol (PNP) on ammonia oxidation of nitrifying reactors

1.3 Research Hypothesis

1. AOA and AOB in nitrifying reactors oxidize ammonia as an energy source and utilize inorganic carbon as a carbon source.

2. Ammonia oxidizers are able to perform activity at low concentrations of PNP.

CHAPTER II

Literature Review

2.1 Ammonia oxidation and microorganisms involved in ammonia oxidation

Nitrification plays an important role in nitrogen removal in wastewater treatment plants (WWTPs). Nitrification is a two-step process; ammonia is changed to nitrite via ammonia oxidation process by ammonia oxidizing bacteria (AOB) and ammonia oxidizing archaea (AOA) and nitrite is changed to nitrate via nitrite oxidation process by nitrite oxidizing bacteria (NOB) (Figure 1). Ammonia oxidation process is the rate-limiting step of nitrogen removal in WWTPs since the growth of AOA and AOB is slow and more sensitive to environment factors than other microorganisms.

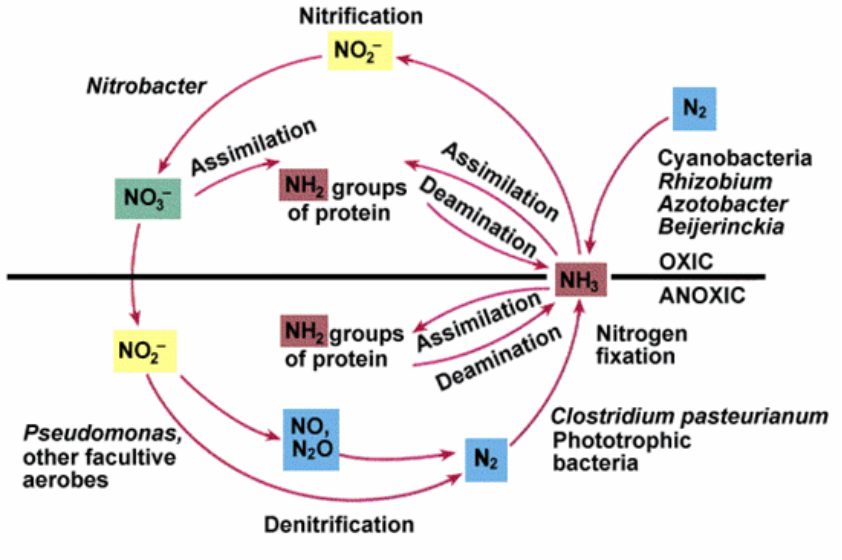
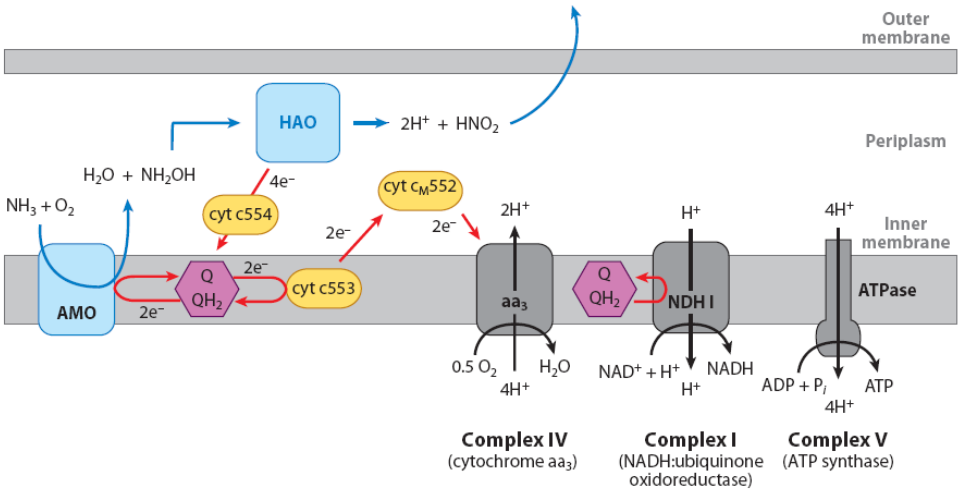


Figure 1 Nitrogen cycle
(http://www.siamchemi.com)

It was long time believed that AOB is the key contributor for ammonia oxidation in ecosystem and the *amoA* gene for ammonia monooxygenase of AOB is the key enzyme for ammonia oxidation. Recently, this concept has been changed since the discovery of *amoA*-like gene in sea water (Venter et al. 2004) and the isolation of AOA from a marine aquarium tank (Konneke et al. 2005b) and soil samples (Tourna et al. 2011).

AOA and AOB are different in cell structure and properties and oxidize ammonia with different pathways (Shen et al. 2013; Walker et al. 2010). For example, membrane lipid of archaea has ether bonds rather than ester bonds of bacteria. Moreover, only cell wall of bacteria contain peptidoglycan as compared to archaea. AOB have hydroxylamine as an intermediate but AOA have nitrite oxide as an intermediate for ammonia oxidation (Figure 2) (He et al. 2012; Shen et al. 2013; Stahl and de la Torre 2012).

(a) AOB



(b) AOA

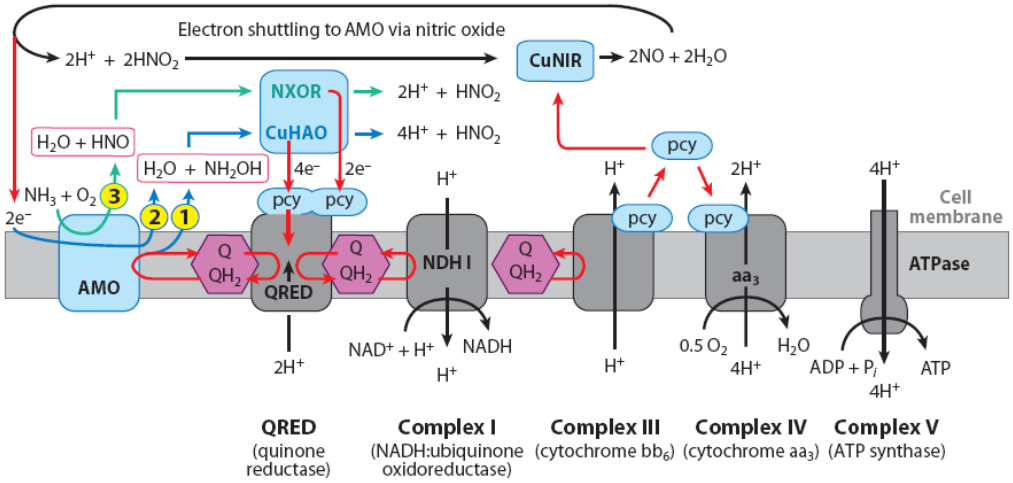


Figure 2 Proposed respiratory pathway of AOB (a) and AOA (b) (Stahl and de la Torre 2012)

2.2 Abundance of AOA in wastewater treatment plants

AOA was first reported in USA WWTSs (Park et al. 2006). Later studies then observed the relative abundance of AOA and AOB gene markers in WWTSs and laboratory nitrifying reactors (Gao et al. 2013; Kleineidam et al. 2011; Limpiyakorn et al. 2011; Mussmann et al. 2011; Zhang et al. 2015). In some systems, AOA *amoA* genes were found to outcompete AOB *amoA* genes (Limpiyakorn et al. 2011; Mussmann et al. 2011; Zhang et al. 2015).

2.3 Methods to differentiate activity of AOA and AOB

2.3.1 Ammonia concentration measurement using specific inhibitors

The simplest ammonia- oxidizing activity measurement is to measure the decrease in ammonia concentration or increase in concentrations of nitrite and nitrate. Because AOA and AOB perform ammonia oxidation with different pathways and they

are different in cell structure and properties (Shen et al. 2013; Walker et al. 2010), a few compounds can be used as selective inhibitors allowing only one group of microorganisms to perform ammonia oxidation at a time. However, specific inhibitor for AOA and AOB is still unidentified clearly in term of what chemicals and what concentrations should be applied. Previous studies used various types of chemicals; including allylthiourea, acetylene, dicyanamide, antibiotics and PTIO to differentiate the ammonia-oxidizing activity of AOA and AOB in enriched AOA and AOB cultures and environmental samples (Kleineidam et al. 2011; Lehtovirta-Morley et al. 2013; Li et al. 2016; Martens-Habbena et al. 2015; Nicol and Prosser 2011; O'Callaghan et al. 2010; Offre et al. 2009; Sauder et al. 2017; Shen et al. 2013; Sonthiphand and Neufeld 2014; Taylor et al. 2010; Tourna et al. 2008; Zhang et al. 2012). Some recent studies used ammonia oxidation inhibitors in combination to molecular technique to provide more accurately results (Wang and Gu 2014; Zhang et al. 2012).

Most inhibitors target AMO enzyme, the enzyme for ammonia oxidation. However, each inhibitor has different mechanism to inhibit the AMO enzyme. This includes acting as copper chelating inhibitor in the active AMO, such as allylthiourea, (ATU) and amidinothiourea (ASU)(Shen et al. 2013), acting as bacteriostatic agent, such as dicyanamide (DCD) (Shen et al. 2013), and binding to the complex of membrane-bound proteins and inactivating the AMO enzyme, such as 3,4- dimethylpyrazole phosphate (DMPP) (Kleineidam et al. 2011). Antibiotic agent reacts with folic acid biosynthesis in Bacteria, such as sulfonamide, kanamycin, streptomycin ampicillin and carbenicillin (Sauder et al. 2017; Shen et al. 2013; Tourna et al. 2011). Some compounds act as a scavenger of nitric oxide (NO) such as 2- phenyl- 4, 4, 5, 5- tetramethylimidazoline- 1- oxyl 3- oxide (PTIO) (Shen et al. 2013). The ammonia oxidation inhibitors used in several previous studies was show in Table 1-5.

Table 1 Inhibitory effect of inhibitors

Inhibitor	Type of sample	Concentration	Nitrite concentration
Dicyandiamide (DCD)	*Nitrosotalea devanaterra*	5, 10 mM [(1)]	not change
	Ca. Nitrososphaera viennensis	1.5 mM DCD [(2)]	not change
	N. multiformis ATCC 25196T	0.1 mM DCD [(2)]	not change
Nitrapirin	Ca. Nitrososphaera viennensis	50-173 uM [(2)]	not change
	N. multiformis ATCC 25196T	70-173 uM [(2)]	not change
Acetylene	Soil	10, 100, 10000 Pa [(3)]	Nitrite + Nitrate not change
Sulfathiozole (ST)	Ca. Nitrososphaera viennensis	300-1500 µM [(2)]	Nitrite ↑
Sulfathiozole (ST)	Ca. Nitrososphaera viennensis	300-1500 µM [(2)]	Nitrite ↑
	N. multiformis ATCC 25196T	4-300 µM [(2)]	not change

Note Ref: [(1)] Lehtovirta-orley L et al (2013) ; [(2)] Shen et al (2013) ; [(3)] Offre et al (2009)

Table 2 Inhibition effect of ATU on AOB

Inhibitor	Type of sample	Concentration	Nitrite concentration /ammonia concentration
Allythiourea (ATU)	*N. multiformis* ATCC 25196T	4-300 µM [2]	Nitrite not change (Completely inhibition)
		50, 200, 300 uM [2]	Nitrite not change (Completely inhibition)
	Nitrosotalea devanaterra	50, 100 uM [1]	Nitrite not change (Completely inhibition)
	N. multiformis ATCC 25196T	0, 0.1, 0.2, 0.3, 0.4 uM [2]	Nitrite ↑ (No inhibition)
	Nitrosomonas europaea, Nitrosomonas oligotropha, Nitrosomonas ureae, Nitrosomonas cryotolerans, and *Nitrosospira multiformis*	>3.3 ([3])	Ammonia not change (Completely inhibition)

Note Ref: [1] Lehtovirta-orley L et al (2013) ; [2] Shen et al (2013) ; [3] Martens-Habbena et al. 2015

Table 3 Inhibition effect of ATU on AOA

Inhibitor	Type of sample	Concentration	Nitrite concentration /ammonia concentration
Allythiourea (ATU)	*Candidatus* Nitrososphaera viennensis	0, 80, 300, 500 μM [(2)]	Nitrite ↑ (No inhibition)
	Candidatus Nitrosotenuis cloacae	<100 μM[(1)] 500 μM[(1)] 700 μM	Ammonia change (No inhibition) Ammonia change (Partial inhibition) Ammonia not change (Completely inhibition)
	Candidatus Nitrosocosmicus exaquare	<30 μM[(3)] 100-300 μM [(3)] 1000 μM [(3)]	Ammonia change (No inhibition) Ammonia change (Partial inhibition) Ammonia not change (Completely inhibition)

Note Ref: [(1)] Lehtovirta-orley L et al (2013) ; [(2)] Shen et al (2013) ; [(3)] Martens-Habbena et al. 2015

Table 4 Inhibition effect of PTIO on AOB

Inhibitor	Type of sample	Concentration	Nitrite concentration /ammonia concentration
PTIO	*N. multiformis* ATCC 25196T	0, 0.1, 0.2, 0.3, 0.4 uM [(2)]	Nitrite ↑ (No inhibition)
	Nitrosomonas europaea, Nitrosomonas oligotropha, Nitrosomonas ureae, Nitrosomonas cryotolerans, and *Nitrosospira multiformis*	100 μM [(1)]	Nitrite ↑ (No inhibition)

Note Ref: [(1)] Martens-Habbena et al. 2015; [(2)] Shen et al (2013)

Table 5 Inhibition effect of PTIO on AOA

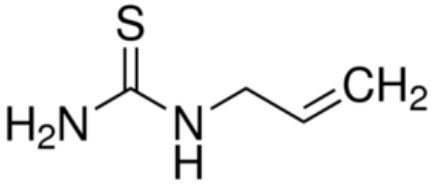
Inhibitor	Type of sample	Concentration	Nitrite concentration /ammonia concentration
PTIO PTIO	*Nitrososphaera viennensis*	50 µM [(2)]	Nitrite not change (No inhibition)
	Candidatus Nitrosocosmicus exaquare	100 µM [(3)]	Ammonia not change (Completely inhibition)
	Nitrosopumilus maritimus strain HCA1 and strain SCM1	100 µM [(1)]	Ammonia not change (Completely inhibition)
	Nitrososphaera viennensis	50 µM [(2)]	Nitrite not change (No inhibition)

Note Ref: [(1)] Lehtovirta-orley L et al (2013) ; [(2)] Shen et al (2013) ; [(3)] Martens-Habbena et al. 2015

2.3.2.1 Allylthiourea (ATU)

Allylthiourea (ATU) was commonly used as an inhibitor for ammonia oxidation. Chemical and physical properties are shown in Table 6. ATU is a copper chelating inhibitor in the active AMO (Shen et al. 2013). Different inhibition threshold between AOA and AOB was observed because of the differences in amino acid sequences in the active center of AMO or content of copper-containing protein in their genome of both microorganisms (Li et al. 2016; Shen et al. 2013). At low concentrations of ATU, it has strong effect to AOB; for example, AOB pure cultures (Martens-Habbena et al. 2015) and marine (Santoro and Casciotti 2011) and soil (Taylor et al. 2010) samples (See Table 2). AOA was affected by ATU at much higher concentrations as compared to AOB; for example AOA isolates and environmental samples (Martens-Habbena et al. 2015) (See Table 3).

Table 6 Chemical and physical Properties

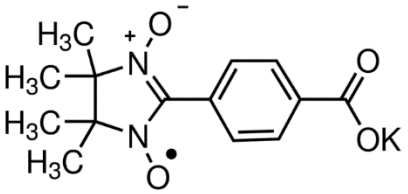
Chemical and physical Properties	
Molecular Formula	$C_4H_8N_2S$
Chemical structure	
Molecular Weight:	116.182 g/mol
Melting Point	70-77.7 ^{O}C
Solubility	10 to 50 mg mL^{-1} at $20^{O}C$

Ref https://pubchem.ncbi.nlm.nih.gov/compound/allylthiourea

2.3.2.2 2-phenyl-4, 4, 5, 5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO)

2-phenyl-4, 4, 5, 5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO) (Table 7) inhibits ammonia oxidation by acting as a nitric oxide (NO) scavenger. PTIO was specific inhibitor for AOA because NO is an intermediate of ammonia oxidation by AOA (Walker et al. 2010). PTIO had strong effect to AOA activity in pure culture; including, *Nitrososphaera viennensis, Nitrosopumilus maritimus* Strain HCA1, *Nitrosopumilus maritimus* Stain SCM1 and *Candidatus* Nitrosocosmicus exaquare, whereas unaffected on β-AOB, γ-AOB strains (Martens-Habbena et al. 2015) (Table4-5).

Table 7 Chemical and physical Properties

Chemical and physical Properties	
Molecular Formula	$C_{14}H_{16}KN_2O_4$
Chemical structure	
Molecular Weight:	315.39 g/mol
Solubility	Highly soluble in water.

Ref https://pubchem.ncbi.nlm.nih.gov/compound/2733502

According Table 1, the inhibitory effect on AOA and AOB depends on type of inhibitors (mechanism of inhibitor), concentration of inhibitors and type of environmental samples. Up to date, ATU and PTIO are shown to be good specific inhibitor for AOB and AOA, respectively (Tables 2-4) (Lehtovirta-Morley et al. 2013; Li et al. 2016; Martens-Habbena et al. 2015; Sauder et al. 2017; Shen et al. 2013; Sonthiphand and Neufeld 2014).

2.3.2 Quantifying transcriptional activity

RNA (rRNA and mRNA) is a better indicator of activity than DNA because ribosome has more stable structure and ribosome content in metabolically inactive cells can be maintained at high level. Change in the abundance of gene transcripts may not relate directly to actual measured process activity. However, ratio of gene transcript to gene abundance is able to show the better correlation with process measurement. Quantitative PCR analysis of mRNA transcription and gene abundance can be useful for determining changes in potential activity (Jia and Conrad 2009). There is some studies on ammonia oxidizing activity in soil samples by quantifying transcriptional(Nicol and Prosser 2011).

2.3.3 Stable isotope probing (SIP)

SIP identify which active microorganism assimilate a stable isotope substrate during incubation. For an identification of autotrophic activity of ammonia oxidation, incubation of 13C- labeled compound is performed in form of ^{13}C- labeled carbon dioxide or bicarbonate. SIP measures assimilation of inorganic carbon by ammonia oxidizers which is autotroph but it is not a direct measurement of ammonia- oxidizing activity. After incubation, ^{12}C- and ^{13}C- labeled DNA or RNA extract is fractionated and identified for microorganisms who incorporate inorganic carbon by quantification of specific gene numbers (qPCR) or observation of fingerprinting (PCR-DGGE, PCR-cloning) of gene markers.

RNA- SIP may provide more sensitivity than DNA- SIP because 13C can be incorporated into RNA transcripted without cell division. Sequence analysis of functional genes in ^{13}C- labeled RNA provides information on which microorganism is actively specific gene, rather than DNA.

SIP analysis has been used to determine ammonia oxidizers in microcosms containing soil, sediment and granular activated carbon incubated with inorganic

carbon inform of $^{13}CO2$ or 13C-bicarbonate solution (Jia and Conrad 2009; Lehtovirta-Morley et al. 2013; Niu et al. 2013; Wu et al. 2013; Zhang et al. 2010).

2.4 Environmental factors affecting the activity and growth of ammonia oxidizers

2.4.1 Ammonia concentration

Ammonia half saturation constant (Ks) of AOA is lower than the Ks of AOB (Figure 3). Figure 4 showed that maximum specific growth rate (μ_{max}) of AOA was in the similar range of the μ_{max} of *Nitrosospira* and *Nitrosomonas oligotropha* which are AOB with high affinity to ammonia. However, μ_{max} of these AOB is lower than the μ_{max} of *Nitrosomonas europaea-Nitrosococcus mobilis* cluster which are AOB with low affinity to ammonia. This indicated that with limiting ammonia concentration in a system, only AOA can be grown. Both of AOA and AOB with high affinity to ammonia are able to grow together when the ammonium level is close to the Ks for AOB (Limpiyakorn et al. 2013).

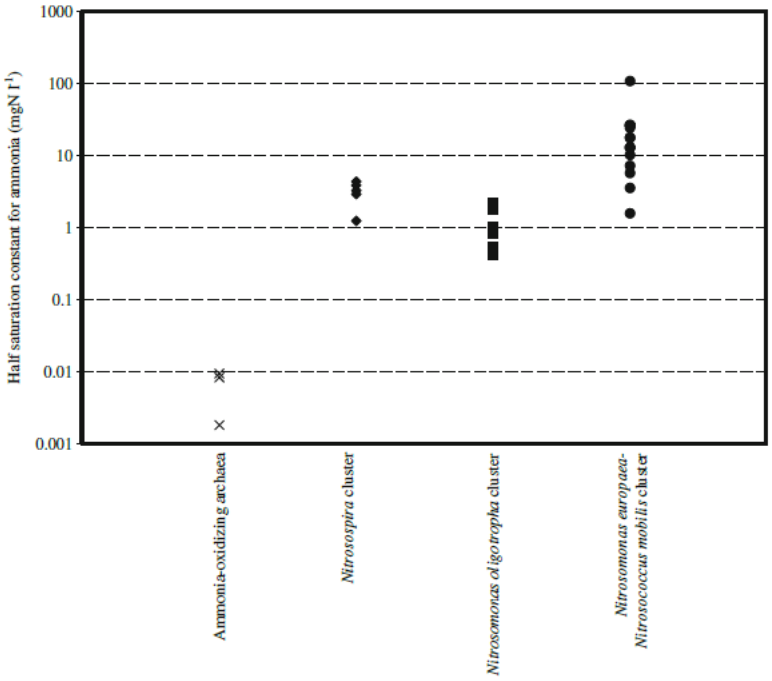


Figure 3 Half saturation constant of ammonia oxidizers culture (Limpiyakorn et al. 2013)

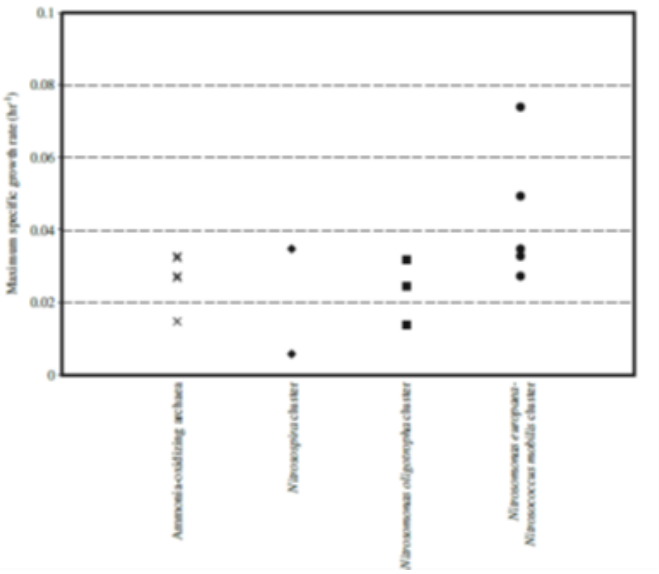


Figure 4 Maximum specific growth rate of ammonia oxidizers culture (Limpiyakorn et al. 2013)

2.4.2 Temperature

AOB can grow over a wide temperature range, but the optimal temperature for pure culture is at 30^{O}C. Moreover, temperature can be differentiating AOB diversity (Wu et al. 2013). Similarly, the activity of AOA is controlled by temperature. For example, the optimal temperature for the growth of *Nitrososphaera viennens* is 37^{O}C (Tourna et al., 2011), but for some species of AOA, *Nitrosocaldus yellostonii*, can grow at temperature up to 74^{O}C (de la Torre et al. 2008). Wu et al (2013) studied on the active bacteria and archaeal ammonia oxidizers in sediment microcosms from eutrophic freshwater Lake Taihu (China) by using SIP and molecular community analysis. The sediments were incubated at different temperatures (4, 15, 25, and 37^{O}C) for up to 8 weeks. The result showed that incubation at different temperatures was affected to nitrite and nitrate accumulation, although ammonium reduction was the same. After 8 weeks incubation, the number of AOB increased in all microcosms indicated that AOB still active in all different temperature incubation. The ^{13}C labeling of AOB was found at low and high temperature. In contrast, ^{13}C labeling of *Nitrososphaera*-like archaea and change in the composition and abundance of *amoA* AOA gene was observed at 37^{O}C, indicating autotrophic growth of AOA under warmer condition. From these researches, it was found that temperature affected to growth and activity of ammonia oxidizers.

2.4.3 Carbon source

During 2007- 2010, it had been believed that AOA is able to grow autotrophically and the growth of AOA depend on the ammonia concentration. *Nitrosopumilus maritimus*, the first AOA isolated culture, obtained from a marine environment grew on ammonia as an energy source and organic compounds were found to inhibit their growth (Konneke et al. 2005b). *Nitrososhaera viennesis*, the

second isolated AOA, were isolated from a soil sample and were found to grow on ammonia or urea as an energy source. Furthermore, the growth rate of this strain can be accelerated with an addition of low amounts of pyruvate or grown in co-culture with bacteria (Tourna et al. 2011).

In WWTPs, Mußmann et al (2011) studied the presence of *amoA*-encoding archaea (AEA) and AOB in 52 municipal and industrial wastewater treatment plants. AEA is the term originally used for *amoA*-carrying thaumarchaeotes and it is used instead of AOA in this study because archaea is able to oxidize ammonia. They found high abundance of AEA in 4 industrial WWTPs and in one plant, AEA closely related to soil group 1.1b outnumbered AOB up to 10,000-fold. Afterwards, they combined FISH and microautoradiography to detect activity of $^{14}CO_2$ fixation. The result showed $^{14}CO_2$ fixation in AOB. Furthermore, *in situ* transcription of archaeal *amoA*, and very weak *in situ* labeling of crenarchaeol after addition of $^{13}CO_2$, was independent of the addition of ammonium. The result showed weak labeling signal for AOA. It means that AEA is unable to fix CO_2 and may be unable to uptake ammonia. So, the results of this study demonstrated that *amoA*-carrying groupl. 1b Thaumarchaeota in this study was not obligate chemolithoautotrophs. However, *Candidatus* Nitrosotenuis cloacae and *Candidatus* Nitrosocosmicus exaquare have recently been obtained from WWTPs in China and Canada under autotrophic growth condition (Li et al. 2016) (Sauder et al. 2017).

In case of other environmental studies, SIP microcosm present the direct evidence for autotrophic growth of AOA in agricultural soil (Jia and Conrad 2009; Zhang et al. 2010; Xia et al. 2011), acidic soils (Lehtovirta et al. 2011; He et al. 2012), freshwater sediment (Wu et al. 2013) and granular activated carbon for drinking water (Niu et al. 2013). It is still unclear whether AOA in WWTPs also performed autotrophic or mixotrophic lifestyle.

2.3.4 Dissolve Oxygen

The occurrence of AOA is demonstrated in activated sludge bioreactor with low DO concentrations (<6.3 μM) under oxic-anoxic conditions (Park et al. 2006). Ye and Zhang (2011) studied on the diversity and abundance of AOA and AOB under different DO level and ammonia loading in a nitrification reactor treating ammonia-rich saline wastewater during operated almost 1 year. All reactors operated under low DO levels (0.15-0.5 mg/L) and high nitrogen loading (0.26-0.52 kgN m^3 day-1). The number of AOB is higher than AOA. AOA number still quantified even though high ammonia concentration in all reactor.

2.4 Paranitrophenol (PNP)

PNP is one of nitro-aromatic compounds that have been widely used and highly toxic. It widely used as a main raw material in many types of industries such as petrochemical and polymer, dye, pharmaceutical industry, insecticides, pesticides and dying (Bhatti et al. 2002; Goh et al. 2009; Suja et al. 2012). Due to its widespread and frequent use, PNP was contaminated in industrial wastewater and groundwater.

2.4.1 Property and toxicity of PNP

Table 8 Physical and chemical properties

Physical and chemical properties	
Molar mass	139.109 g/mol
Density	1.479 g/cm3 (20 °C)
Melting point	113°C
Solubility	16 g/L (25 °C)
Vapor pressure	0.0054 mm Hg (20 °C)
Sediment-water sorption partition coefficient	1.15
Soil sorption partition coefficient	1.43

Ref Agency for Toxic Substances and Disease Registry U.S. Public Health Service (1992)

Table 9 Potential Health Effects

Acute health effects	
Eye	Irritates and damaging to eye for direct contact
Skin	Occurrence of skin inflammation for direct contact
Inhalation	Irritates to respiratory system for exposure. Irritates and damaging to lung for further irritation to body.
Ingestion	Harmful effect may observe for ingestion. Fatal or serious damage to body likely to occurs if ingested for 150 g.
Chronic health effects	
	For body's organ and biochemical system, repeat exposure can cause adverse effect. For respiratory system, repeat exposure can cause irritation, difficulty breathing and disease to airways.

Source: Material safety data sheet, Santa Cruz Biotechnology, Chemwatch, 2010

2.4.2 Effect of PNP on human health

According to Bhatti et al. (2002), PNP was detected as a metabolite in urine of parathion; therefore, it was detected as a biomarker for pesticide exposure. the concentration and exposure time is the key factor for adverse health effect of PNP to human health. Blood disorder causes by exposure of PNP at high concentration and longer time (Health and Environmental Effects, 1980).

2.4.3 Effect of PNP on microorganisms

Several works reported influence of various concentrations of PNP on microorganisms (<1990 HEITKAMP et al. pdf> ; Sverdrup et al. 2002). High concentrations of PNP can inhibit growth of PNP-degrading microrganisms. According

to a study of Bhatti et al. (2002), by feeding influent with PNP at a concentration of 700 mgL^{-1}, the PNP concentration in the effluent increased and no observe on degradation of PNP residual. The growth of *pseudomonas putida* PNP1 also completely inhibit by 600 mgL^{-1} PNP.

In contrast, a study of treatment of PNP by using immobilized cell with *Pseudomonas* sp, showing that 91-93% removal efficiency at concentration PNP in a range of 630-1800 mgL^{-1}. However, increasing PNP in range of 2100 -2500 mgL^{-1} lead to acute toxic effect to immobilized microorganisms Heitkamp et al. (1990)

Not only PNP-degrading microorganism, but also high concentration of PNP can inhibit ammonia oxidation of ammonia-oxidizing microorganisms. Some polycyclic aromatic hydrocarbons were reported to decrease soil nitrification by acting as the ammonium monooxygenase's suicide substrate such as nitrobenzene (<1990 HEITKAMP et al.pdf> ; Sverdrup et al. 2002). In 2010, Zhang et al. (2010) investigated the inhibitory effect of aromatic compounds, including PNP, on nitrification in soil samples. They found that this chemical could inhibit nitrification up to approximately 60% and 70% for the addition of 13.9 and 69.5 mg PNP kg^{-1}soil. Guo et al. (2014) studied short-term effect of nitrophenol on WWTPs sludge enriched for more than 200 days. The impact of exposure was tested by adding PNP only one time. They found that biomass concentration decreased, sludge deflocculated, COD also increased, and ammonia concentration accumulated. The concentration of PNP profile was 6-34 mgL^{-1} along the test. These reports supported that PNP had high possibility of inhibiting ammonia oxidation in soil and WWTPs.

CHAPTER III

METHODOLOGY

3.1 Overview of experiments

This study was separated into three experiments: 1) contribution of ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) to ammonia oxidation in nitrifying reactors, 2) ammonia-oxidizing activity of sludge from full-scale wastewater treatment plants (WWTPs) and 3) effect of paranitrophenol (PNP) on ammonia oxidation of sludge from nitrifying reactors (Figure 5).

Experiment 1: Contribution of ammonia-oxidizing archaea and ammonia-oxidizing bacteria to ammonia oxidation in nitrifying reactors

Contribution of AOA and AOB to ammonia oxidation in WWTPs has yet been known clearly. Experiment 1 aimed to investigate the roles of these two microorganisms in ammonia oxidation in two-laboratory nitrifying reactors. In addition, Experiment 1 optimized concentrations of ATU (allylthiourea) and PTIO (2-phenyl-4, 4, 5, 5-tetramethylimidazoline-1-oxyl 3-oxide) to be used as selective inhibitors for AOB and AOA, respectively in the nitrifying reactors. The nitrifying reactors were initiated and operated under different conditions, leading to distinct proportions of AOA and AOB in the reactors. The reactors were started up in 2012 and had been running for around 3 years before the start of this experiment. Therefore, heterotrophic microorganisms existing in the reactors were expected to be much lower than what generally occurred in full-scale WWTPs. This provided supportive condition to study the selective inhibitors since the effect of heterotrophic microorganisms on the

selective inhibitors was reduced. The study period of Experiment 1 took around 18 months.

The first part of Experiment 1 is long-term monitoring of abundance and diversity of AOA and AOB in the nitrifying reactors. Sludge samples were collected from both reactors around once per month for analysis of numbers of AOA and AOB *amoA* genes by qPCR, twice per study period (month1 and month6) for diversity of AOA and AOB *amoA* genes by PCR-cloning-sequencing, and at the end of experiment 1 for cell morphology observation by scanning electron microscopy (SEM).

The second part is to clarify the contribution of AOA and AOB to ammonia oxidation in the nitrifying reactors using ATU and PTIO. ATU was used in a range of 10-2000 μM and PTIO was applied in a rage of 50-300 μM. The addition part was adding ATU (20, 80, 150 and 2000 μM) and PTIO (100 μM) together.

The last part is to examine autotrophic ammonia-oxidizing activity and growth of AOA and AOB using DNA-SIP technique. Sludge from NRI was collected and incubated with label (^{13}C-HCO_3^-) and unlabeled (^{12}C-HCO_3^-) inorganic carbon. Together with the two incubations, an additional DNA-SIP incubation was carried out with ^{13}C-HCO_3^- and ATU at the concentration of 80 μM. This third incubation was performed to investigate ATU specificity at this concentration to selectively inhibit AOB, not AOA.

Experiment 2: Ammonia-oxidizing activity of sludge from full-scale wastewater treatment plants

ATU at the same concentrations range as in Experiment 1 (10-2000 μM) was applied to sludge from 5 full-scale WWTPs to observe ammonia-oxidizing activity of AOA in the sludge. The criteria to select the WWTPs was the numbers of AOA and AOB *amoA* genes in the sludge.

Experiment 3: Effect of paranitrophenol (PNP) on ammonia oxidation of sludge from nitrifying reactors

In experiment 3, effect of PNP concentration on ammonia oxidation was studied with sludge from NRI and NRII. The sludge from NRI and NRII was first quantified for the numbers of AOA and AOB *amoA* genes by qPCR, characterized for microbial communities using Miseq (next generation sequencing), and analyzed active nitrifying microorganisms using fluorescence in situ hybridization (FISH) technique. Inhibitory effect on ammonia oxidation was test with PNP in a range of 1-400 mgL^{-1} under initial ammonia concentrations of 7, 14, and 70 $mgNL^{-1}$. Then, some selected PNP concentrations was used to observe cell viability and active nitrifying microorganisms after PNP exposure.

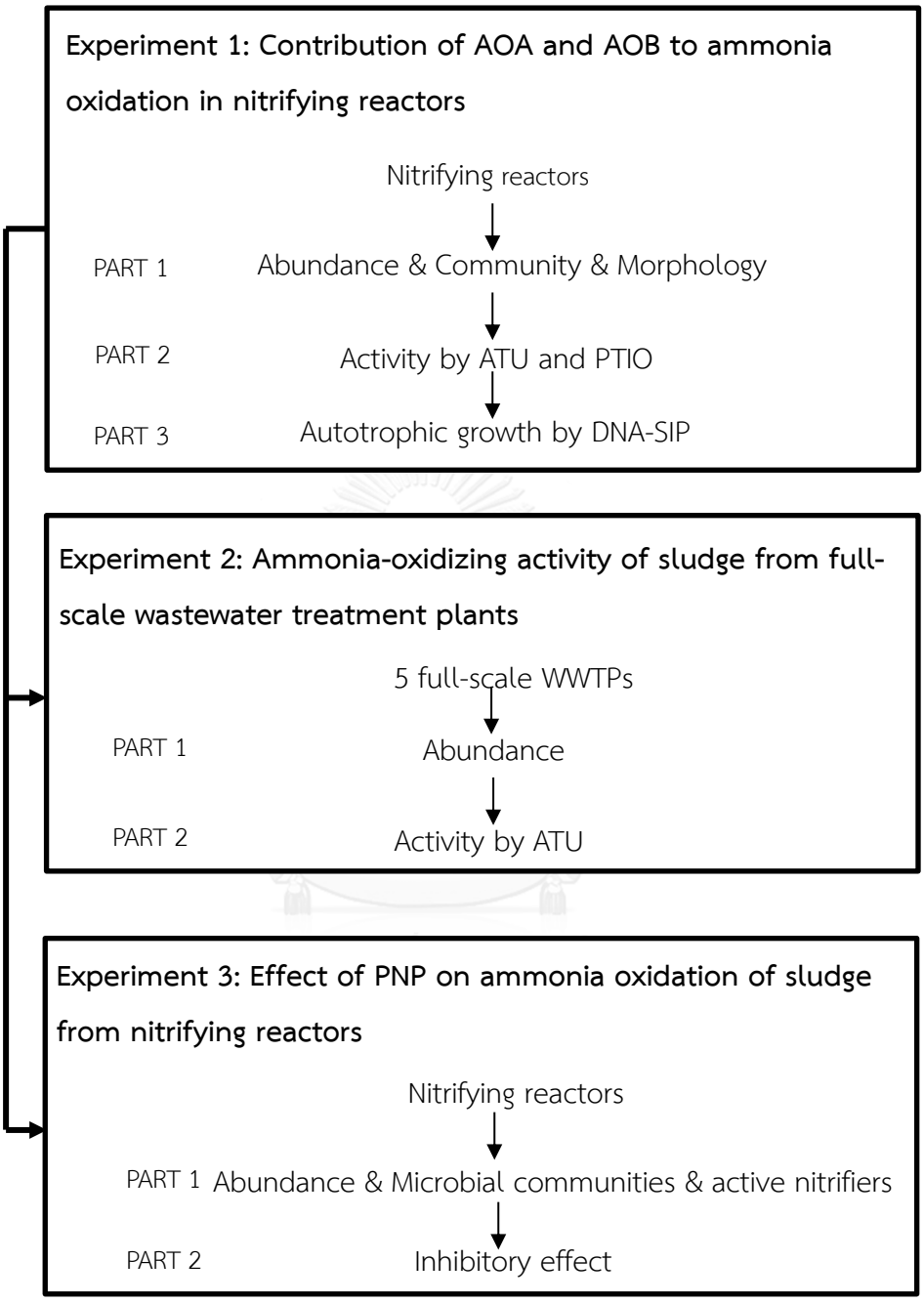


Figure 5 Experimental framework

3.2 Experiment 1: Contribution of ammonia-oxidizing archaea and ammonia-oxidizing bacteria to ammonia oxidation in nitrifying reactors

3.2.1 Part 1: Long-term monitoring of diversity and abundance of AOA and AOB

3.2.1.1 Nitrifying reactor

Two nitrifying reactors, NRI and NRII (Figure 6), were operated in continuous-feed mode without sludge recycle. The reactors had an effective volume of 5 L. NRI was seeded with sludge collected from a municipal WWTS (Sonthiphand and Limpiyakorn 2011). Agarose gel check of the PCR products amplified by specific primers targeting AOA and AOB *amoA* genes indicated that AOA and AOB coexisted in the seed sludge. NRI was fed with an inorganic medium containing 28 $mgNL^{-1}$ of ammonia. Details of the inorganic medium were described in Table 10-15. The medium composition was modified from (Konneke et al. 2005b; Tourna et al. 2011). The seed sludge for NRII was obtained from an industrial WWTP. PCR screening indicated that only AOB, and no AOA, could be detected in the seed sludge. The reactor was supplied with an inorganic medium modified by (Rongsayamanont et al. 2010) (see Table 16). The ammonia concentration of the medium was 420 $mgNL^{-1}$. Table 17 summarizes characteristics and operation parameters of NRI and NRII.

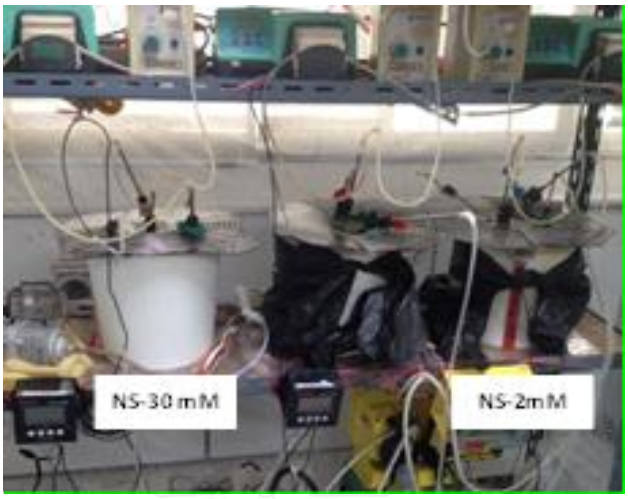


Figure 6 Nitrifying enrichment reactors

Table 10 Composition of 1L of media for NRI reactor

Chemical	weight (g) or volume (mL)
NaCl	1 g
$MgCl_2 \cdot 6H_2O$	0.4 g
$CaCl_2 \cdot 2H_2O$	0.1 g
KCl	0.5 g
KH_2PO_4	0.2 g
$NaHCO_3$ 1M	2mL
Nonchelated trace element mixture (see Table 11)	1 mL
Vitamin mixture solution (see Table12)	1mL
Thiamin solution (see Table13)	1mL
Vitamin B12 solution (see Table14)	1mL
selenite-tungstate solution (see Table 15)	1 mL

Table 11 Composition of nonchelated trace element mixture

Chemical	weight (mg) or volume (mL)
HCl (25% =7.7 M)	12.5 mL
$FeSO_4 \cdot 7H20$	2100 mg
H_3BO	30 mg
$MnCl_2 \cdot 4H_20$	100 mg
$CoCl_2 \cdot 6H_20$	190 mg
$NiCl_2 \cdot 6H_20$	24 mg
$CuCl_2 \cdot 2H_20$	2 mg
$ZnS0_4 \cdot 7H_20$	144 mg
$Na_2MoO_4 \cdot 2H_20$	36 mg
Distilled water	987 mL

Table 12 Composition of vitamin mixture solution

Chemical	weight (mg) or volume (mL)
Sodium phosphate buffer (10 mM pH7.1)	100 mL
4-Aminobenzoic acid	4 mg
D (+)-Biotin	8 mg
Nicotinic acid	10 mg
Calcium D (+)-pantothenate	5 mg
Pyridoxine dihydrochloride	15 mg

Table 13 Composition of thiamine solution

Chemical	weight (mg) or volume (mL)
thiamine chloride dihydrochloride	10 mg
sodium phosphate buffer pH 3.4	100 mL

Table 14 Composition of vitamin B12 solution

Chemical	weight (mg) or volume (mL)
Cyanocobalamine	5 mg
Distilled water	100 mL

Table 15 Composition of selenite-tungstate solution

Chemical	weight (mg) or volume (mL)
NaOH	0.4 g
$Na_2SeO_3 \cdot 5H_20$	6 mg
$Na_2WO_4 \cdot 2H_20$	8 mg
Distilled water	1000 mL

Table 16 Composition of 1L of media for NRII reactor

Chemical	weight (g)
$NaHCO_3$	1.5
Na_2HPO_4	4.05
K_2HPO_4	2.1
$MgSO_4 \cdot 7H_2O$	0.05
$CaCl_2 \cdot 2H_2O$	0.01
$FeSO_4 \cdot 7H_2O$	0.09

Table 17 Summary of characteristics and operational parameters of NRI and NRII

	NRI	NRII
Characteristics of seed sludge		
1. Source	Municipal WWTP	Industrial WWTP
2. PCR detection ▪ AOA *amoA* gene ▪ AOB *amoA* gene	 Detected Detected	 Not detected Detected
Operational parameters		
1.Effective volume (L)	5	5
2. Initial NH_4^+-N ($mgNL^{-1}$)	28	480
3. Inorganic media	See Table 3.1-3.6	See Table 3.7
4. Hydraulic retention time (d)	5	4
5. Temperature (^OC)	Room temperature	Room temperature
6. pH	7.3-7.8	7.3-7.8
7. DO (mgL^{-1})	>2	>2

Concentrations of ammonia, nitrite and nitrate along 3 years before this study		
1. Average NH_4^+-N ($mgNL^{-1}$)	0.09 ± 0.05	15.27 ± 9.03
2. Average NO_2^--N ($mgNL^{-1}$)	< LOD (0.1)	12.26 ± 9.03
3. Average NO_3^--N ($mgNL^{-1}$)	28.18 ± 4.39	370.98 ± 25.35

3.2.1.2 Sample collection and DNA extraction

Sludge samples were collected from NRI and NRII around once per month. Approximately 2 mg dry weight of sludge was transferred to a 1.7-mL tube and centrifuged at 14,000 g for 10-15 min. The supernatant was discarded, and the pellet was used for DNA extraction. See section 3.5.1 for DNA extraction method.

3.2.1.3 Quantification of AOA and AOB amoA genes

See method in section 3.5.2

3.2.1.4 Analysis of AOA and AOB amoA gene sequences

See method in section 3.5.3

3.2.1.5 Measurement of the ammonium, nitrite, and nitrate concentrations

See method in section 3.5.5

3.2.1.6 Scanning electron microscopy

See method in section 3.5.4

3.2.2 Part 2: Ammonia-oxidizing activity under the presence of ATU and PTIO

3.2.2.1 Ammonia-oxidizing activity under the presence of ATU and PTIO

Tests of ammonia-oxidizing activity were created in 250-mL Erlenmeyer flasks containing 70 $mgMLSSL^{-1}$ of NRI or NRII sludge, 200 mL of the inorganic medium, and 2 mL of HEPES buffer solution (1M HEPES and 0.6M NaOH). The inorganic medium had the initial ammonia concentration of 7 $mgNL^{-1}$ and the compositions were as described for NRI and NRII (Table 10 and Table 16, respectively).

Different concentrations of ATU and PTIO were added to separate treatments. ATU concentrations were 10, 30, 50, 80, 100, 150, 200, 500, 1000, and 2000 µM (Figure 7a) PTIO concentrations were 50, 100, and 300 µM (Figure 7b). In addition, some treatments prepared with sludge from either reactor were dosed with a mixture of ATU (100 µM) and PTIO (30, 80, 150, and 2000 µM).

All treatments including the controls with ATU and the mixture of ATU and PTIO were performed in triplicate sets and the treatments with PTIO were performed in duplicate sets. Flasks were incubated in the dark at room temperature (~28°C). A liquid sample of each flask was collected for triplicate measurements of ammonia concentration.

(a) ATU (b) PTIO

Figure 7Ammonia-oxidizing activity under the presence of (a) ATU and (b) PTIO

3.2.2.2 Measurement of the ammonium, nitrite, and nitrate concentrations

See method in section 3.5.5

3.2.2.3 Percent inhibition of ammonia oxidation

Percent inhibition of ammonia oxidation was calculated by comparing remaining ammonia concentrations in the tests to the control (without adding ATU) at a time an ammonia concentration of the control reached around 0 $mgNL^{-1}$. Percent inhibition of ammonia oxidation was calculated follows this equation

$$\%\, Inhibtion\ of\ ammonia\ oxidation\ = 100 - \left[\frac{\left(\frac{C-D}{C}\right)\times 100}{\left(\frac{A-B}{A}\right)\times 100}\right]$$

Where A is initial ammonia concentration in the control (without adding ATU)

B is final ammonia concentration in the control (without adding ATU)

C is initial ammonia concentration in the test (with ATU)

D is final ammonia concentration in the test (with ATU)

3.2.3 Part 3: Incorporation of ^{13}C-HCO_3^- by AOA and AOB during ammonia oxidation of NRI sludge under the absence and presence of ATU

3.2.3.1 DNA-SIP incubation

DNA-SIP incubation was performed for NRI sludge in continuous-flow reactors with an effective volume of 1 L without sludge recycle (Figure 8). At the start of incubation, sludge was added to the reactors to achieve a MLSS concentration of 70 mgL^{-1}. The reactors were supplied with the inorganic medium as described above for NRI reactor, but ammonia concentration in the medium was modified to 14 $mgNL^{-1}$. Incubation was performed in separated sets with ^{12}C-HCO_3^- and ^{13}C-HCO_3^- as carbon

sources in the medium. Flow rate through the reactors was adjusted to achieve a HRT of 5 days. Mixing was provided around 12 hr a day. Other aspects of reactor set-up followed (Niu et al. 2013). The presence of ^{12}C in the inorganic medium was avoided by using water aerated by CO_2-free air at pH ≤ 3 during medium preparation, then adjusting the medium pH to 7.4-7.8. Aerobic conditions were maintained by aerating the reactors with CO_2-free air twice a day. The incubation was carried out in the dark and at room temperature. One additional incubation was prepared in the same manner as described above with ^{13}C- HCO_3^- as the carbon source, but ATU at the concentration of 80 µM was added to this incubation in order to observe the effect of ATU at this concentration on AOA and AOB.

For all incubations, liquid samples were collected for measurements of ammonia, nitrite, and nitrate concentrations. Sludge samples were taken on day 21 of incubation for downstream analysis.

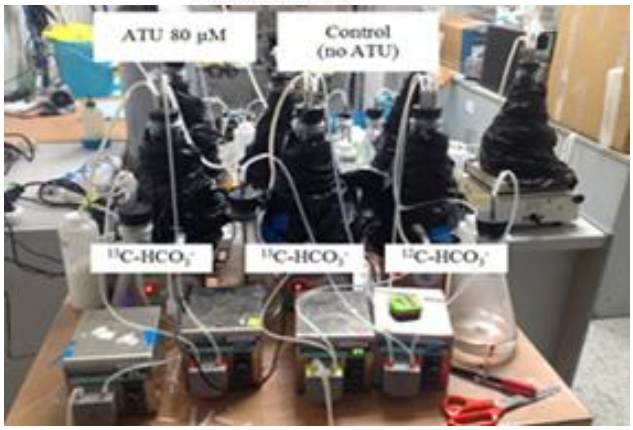


Figure 8 DNA-SIP reactor setup

3.2.3.2 Sample collection and DNA extraction

For DNA-SIP incubation, 400 mL water samples were filtrated through a 0.2 µm filter paper which was then cut into small pieces. DNA was extracted from the samples using Fast-DNA SPIN kits for soil (QBiogene, USA). See details for DNA extraction method in section 3.5.1

3.2.3.3 Measurement of the ammonium, nitrite, and nitrate concentrations

See method in 3.5.5

3.2.3.4 Quantification of AOA and AOB amoA genes

See method in 3.5.2

3.2.3.5 Separation of ^{12}C- and ^{13}C-DNA SIP

See method in 3.5.6

3.3 Experiment 2: Ammonia-oxidizing activity of sludge from full-scale wastewater treatment plants

3.3.1 Full-scale wastewater treatment plants

Sludge was taken from 5 full-scale WWTPs. The plants treated municipal wastewater and located in Bangkok area (Table 18).

Table 18 *Characteristics of influent and effluent of full-scale WWTPs*

Parameter	Plant A	Plant B	Plant C	Plant D	Plant E
BOD in influent (mgL^{-1})	155	36.16	24.49	39	35.6-37.3
BOD in effluent (mgL^{-1})	9	6.8	9.01	10.92	10.6-11.8
Ammonia concentrations in influent ($mgNL^{-1}$)	-	4.15	7.99	4	4.0-5.1
Ammonia concentrations in effluent ($mgNL^{-1}$)	-	1.24	1.25	3.92	0.1-0.2
Treatment System	Suspended-growth system				

3.3.1.1 Sample collection and DNA extraction

Approximately 2 mg dry weight of sludge was transferred to a 1.7-mL tube and centrifuged at 14,000 g for 10-15 min. The supernatant was discarded, and the pellet was used for DNA extraction. DNA extraction method was shown in section 3.5.1.

3.3.1.2 Quantification of AOA and AOB amoA genes

See method in section 3.5.2

3.3.2 Ammonia-oxidizing activity under the presence of ATU

3.3.2.1 Ammonia-oxidizing activity

Tests of ammonia-oxidizing activity were created in 250-mL Erlenmeyer flasks containing 200 $mgMLSSL^{-1}$ of each sludge, 200 mL of the inorganic medium, and 2 mL of HEPES buffer solution (1M HEPES and 0.6M NaOH). The inorganic medium had the

initial ammonia concentration of 7 mgNL^{-1} and the compositions were as described for NRI (Table 10). ATU concentrations were 10, 30, 50, 80, 100, 150, 200, 500, 1000, and 2000 µM (Figure 9). Flasks were incubated in the dark at room temperature (~28°C). A liquid sample of each flask was collected for triplicate measurements of ammonia concentration.

Figure 9 Tests of ammonia-oxidizing activity of WWTP sludge under the presence of ATU

3.3.2.2 Measurement of the ammonium, nitrite, and nitrate concentrations

See method in section 3.5.5

3.3.2.3 Percent inhibition of ammonia oxidation

See calculation in section 3.2.2.3

3.4 Experiment 3: Effect of paranitrophenol (PNP) on ammonia oxidation of sludge from nitrifying reactors

3.4.1 Nitrifying reactor

3.4.1.1 Sample collection and DNA extraction

Sludge samples were collected from NRI an NRII at the beginning of PNP test. Approximately 2 mg dry weight of sludge was transferred to a 1.7-mL tube and centrifuged at 14,000 g for 10-15 min. The supernatant was discarded, and the pellet was used for DNA extraction. DNA extraction method was shown in section 3.5.1.

3.4.1.2 Quantification of AOA and AOB amoA genes

See method in section 3.5.2

3.4.1.3 Characterization of microbial communities in nitrifying reactors

See method in section 3.5.7

3.4.1.4 Fluorescence in situ hybridization (FISH) with 16S rRNA-targeted oligonucleotide probes

See method in section 3.5.8

3.4.2 Effect of PNP concentration on ammonia-oxidizing activity

3.4.2.1 Ammonia oxidizing activity under the presence of PNP

Tests of ammonia-oxidizing activity were created in 250-mL Erlenmeyer flasks containing 70 mgMLSSL^{-1} of each sludge, 200 mL of the inorganic medium, and 2 mL of HEPES buffer solution (1M HEPES and 0.6M NaOH). For NRI, the inorganic medium had the initial ammonia concentration of 7 mgNL^{-1} and the compositions were as described for NRI (Table 8). For NRII, the inorganic medium had the initial ammonia

concentration of 7, 14 and 70 mgNL^{-1} and the compositions were as described for NRII (Table 14). Different concentrations of PNP was added to separate treatments. PNP concentrations were1, 2., 5, 7.5, 10, 25, 50, 100, 200 and 400 mgL^{-1} (Figure 10). Flasks were incubated in the dark at room temperature (~28°C). A liquid sample of each flask was collected for triplicate measurements of ammonia concentration.

Figure 10 Tests of ammonia-oxidizing activity of nitrifying sludge under the presence of PNP

3.4.2.2 Measurement of the ammonium, nitrite, and nitrate concentrations

See method in section 3.5.5

3.4.2.3 Percent inhibition of ammonia oxidation

See calculation in section 3.2.2.3

3.4.2.4 Observation of bacteria cell viability and active nitrifying microorganisms

Bacterial cell viability and active nitrifying microorganisms were observed after exposing NRII sludge to PNP at concentrations of 10 and 200 mgL^{-1}. An initial ammonia concentration selected for the tests was 70 $mgNL^{-1}$. See method in sections 3.5.8 and 3.5.9.

3.4.2.5 Microbial community analysis

See method in section 3.5.8

3.5 Analytical Methods

3.5.1 DNA extraction

DNA was extracted from samples by using Fast-DNA SPIN kits for soil (QBiogene, USA). DNA extracts were checked by electrophoresis in 2% agarose (Bio-Rad, Spain) and DNA concentrations were determined using a NanoDrop 1000 Spectrophotometer (Thermo, USA).

3.5.2 Qualification of AOA and AOB *amoA* genes

The primer set for AOA *amoA* genes was Arch- amoAF (5′- STAATGGTCTGGCTTAGACG- 3′) and Arch- amoAR (5′- GCGGCCATCCATCTGTTGT- 3’) (Francis et al. 2005). The primer set for AOB *amoA* genes was amoA1F (5′- GGGGTTTCTACTGGTGGT- 3′) and amoA2R (5′- CCCCTCKGSAAAGCCTTCTTC- 3′) (Rotthauwe et al. 1997). qPCR was performed using Maxima SYBR Green qPCR Master Mix (Thermo, USA) in a 25 µL volume containing 12.5 µL of the Master Mix (2X), 0.4 µM of each primer and 1 µL of DNA template in Mx3005P instrument (Stratagene, USA). The qPCR conditions for both primer sets were as follows: 10 min at 95°C, 40 cycles of 30 sec at 95°C, 1 min at 53°C, 30 sec at 72°C, finally followed by data capture at 78°C for 15 sec. DNA standards used were the PCR-amplified products of the pGEM-T Easy Vector (Promega, USA) containing the *amoA* genes of AOA and AOB from NRI. A standard curve for each gene was prepared from tenfold serial dilutions in the range between 10^2 and 10^7 copies. The standard curves showed efficiency in ranges of 94-104 % (R^2=0.99) and 94-99% (R^2=0.99) for AOA and AOB *amoA* genes, respectively. For each sample, qPCR was performed in at least triplicate. The specificity of qPCR amplification was checked by melting curve analysis and agarose gel electrophoresis.

3.5.3 Analysis of AOA and AOB *amoA* gene sequences

The AOA *amoA* gene fragment was amplified using the primers CamoA-19f (5'-ATGGTCTGGYTWAGACG-3') and CamoA-616r (5'- GCC ATCCABCKRTANGTC CA -3') (Pester et al. 2012). The AOB *amoA* gene fragment was obtained using the primer set amoA1F and amoA2R (Rotthauwe et al. 1997). The PCR mixture was prepared using a Thermo polymerase (Thermo., USA) and amplified using a thermal cycler (Biorad Laboratories, USA). The PCR condition was 2 min at 94°C, 30–35 cycles of 30 sec at 94°C, 45 sec at 53°C, and 45 sec at 72°C, followed by 10 min of final extension at 72°C. The product was purified using a NucleoSpin Extract II Kit (Clontech Laboratories Inc., USA) and cloned using the pGEM-T Easy vector system (Promega, USA). For each sample, 13-16 clones were randomly selected for sequencing at Macrogen Inc., Korea. The analyzed sequences were calculated for an arrangement of operational taxonomic units (OTUs) based on 99% OTU identity using CD-HIT (Huang et al. 2010). Representative sequences from each OTU and selected reference sequences were aligned and analyzed with neighbor joining calculation using MAGA7 (Kumar et al. 2016).

3.5.4 Scanning electron microscopy

Sludge samples were prepared for SEM examination by being fixed in 0.1 M PBS with 4% glutaraldehyde overnight at 4°C, separated in half in liquid nitrogen, dehydrated using a series of ethanol solutions, and covered with gold under vacuum condition. The prepared samples were examined using SEM (JEOL, JSM-5410LV, Tokyo, Japan).

3.5.5 Measurement of the ammonium, nitrite, and nitrate concentrations

Ammonium concentrations were measured by using salicylate method (Verdouw et al. 1977). Nitrite concentrations were analyzed by colorimetric method

(Miranda et al. 2001). Nitrate was reduced to nitrite by vanadium (III) chloride and measured by using the method indicated for nitrite. The measurement was performed by VICTOR X Plate Reader (PerkinElmer, USA).

3.5.6 Separation of 12C- and 13C-DNA

12C- and 13C-DNA were separated by isopycnic centrifugation in a CsCl gradient solution (Neufeld et al. 2007; Niu et al. 2013). Briefly, extracted DNA samples (2.0 -3.0 mg), CsCl solution (7.163 M) and gradient buffer (0.1M Tris HCl, pH 8.0; 1mM EDTA; 0.1M KCl) were mixed to achieve the initial buoyant density of 1.700 gmL^{-1}. Ultracentrifugation was carried out in 8 ml polyallomer centrifuge tubes using a MLN-80 rotor (Beckman, USA) in Optima MAX-XP (Beckman, USA) at 178,000× g for 72 h at 20°C. Approximately 23 fractions of 250 µL solution were collected from the tube by feeding mineral oil to the top of the tube using a NE-1000 Single Syringe Pump (New Era, USA). Buoyant density of each fraction was measured using an AR200 digital refractometer (Reichert, USA). DNA was recovered by PEG, precipitated by ethanol, and dissolved in 25-30 mL TE buffer. Numbers of AOA and AOB *amoA* genes in each fraction were analyzed by qPCR.

3.5.7 Illumina MiSeq

16S rRNA gene fragments for bacteria and archaea were amplified using the primers 515F (5' GTGYCAGCMGCCGCGGTAA- 3') and 806R (5'-GGACTACHVGGGTWTCTAAT -3') (Ding et al. 2015). The PCR mixture was prepared using a Thermo polymerase (Thermo., USA) and amplified using a thermal cycler (Biorad Laboratories, USA). The PCR condition was 3 min at 95°C, 30–35 cycles of 30 sec at 95°C, 30 sec at 53°C, and 30 sec at 72°C, followed by 5 min of final extension at 72°C. The product was purified using a NucleoSpin Extract II Kit (Clontech Laboratories Inc., USA). 16S amplicons were submitted to the Omics Sciences an Bioinformation Center for analysis (BKK, Thailand).

3.5.8 Fluorescence *in situ* hybridization (FISH) with 16S rRNA-targeted oligonucleotide probes

4mL of sample was fixed in 8% paraformaldehyde solution for 18 h. Briefly, 10 µL of 16S rRNA-targeted oligonucleotide probe (Table 19) in hybridization buffer was added onto each spot of the samples on microscopic slide. The slides were hybridized at 46 °C for 2 h and wash the rest of buffer, after that stained with 0.9 µ M DAPI for 15 min. The hybridized samples were observed under CLSM (FluoView FV10i, Olympus, Japan). Analysis of image by using ImageJ (Broken Symmetry Software), the approximately 15 images/1 oligonucleotide probe were used to determine percent abundance of target microbes in total microbes via estimating an area.

Table 19 Oligonucleotide probes

Probe (Sequence 5′ to 3′)	Label	Target organisms	Formamide (%)
Nso190 (CGATCCCCTGCTTTTCTCC)	AF	β-Proteobacteria	55
Nit3 (CCTGTGCTCCATGCTCCG)	CY3	Nitrobacter	40
Ntspa662 (GGAATTCCGCGCTCCTCT)	CY3	Nitrospira	35

3.5.9 Live/dead cell observation

4mL of sample was stained with an appropriate mixture of the SYTO 9 and propidium iodide (PI) stains according to the manufacturer's protocol. (LIVE/DEAD® BacLight™ Bacterial Viability, Molecular Probes, Invitrogen) then observed under CLSM (FluoView FV10i, Olympus, Japan) (Choi et al. 2008). The excitation/emission maxima

for SYTO 9 and PI stains were 480/500 and 490/635 nm, respectively. Briefly, all bacterial cells were stained with SYTO 9 and show in green color but damaged cell or dead cell were stained with PI and show in red color. Approximately 20 images from CLSM of each sample were used for calculation of the areas of live and dead cells using the software ImageJ (Gu et al. 2014).

CHAPTER IV

RESULTS AND DISCUSSIONS

4.1 Experiment 1: Contribution of ammonia-oxidizing archaea and ammonia-oxidizing bacteria to ammonia oxidation in nitrifying reactors

Contribution of AOA and AOB to ammonia oxidation in WWTSs has yet been known clearly. Experiment 1 aimed to investigate the roles of these two microorganisms in ammonia oxidation in two-laboratory nitrifying reactors. In addition, Experiment 1 optimized concentrations of ATU (allylthiourea) and PTIO (2-phenyl-4, 4, 5, 5-tetramethylimidazoline-1-oxyl 3-oxide) to be used as selective inhibitors for AOB and AOA, respectively, in the nitrifying reactors. The nitrifying reactors were initiated and operated under different conditions, leading to distinct proportions of AOA and AOB in the reactors. The reactors were started up in 2012 and had been running for around 3 years before the start of this experiment. Therefore, heterotrophic microorganisms existing in the reactors were expected to be much lower than what generally occurred in full-scale WWTPs. This provided supportive condition to study the selective inhibitors since the effect of heterotrophic microorganisms on the selective inhibitors was reduced. The study period of Experiment 1 took around 18 months.

The first part of Experiment 1 is long-term monitoring of abundance and diversity of AOA and AOB in the nitrifying reactors. Sludge samples were collected from both reactors around once per month for analysis of numbers of AOA and AOB *amoA* genes by qPCR, twice per study period (month1 and month6) for diversity of AOA and AOB *amoA* genes by PCR-cloning-sequencing, and at the end of Experiment 1 for cell morphology observation by scanning electron microscopy (SEM).

The second part is to clarify the contribution of AOA and AOB to ammonia oxidation in the nitrifying reactors using ATU and PTIO. ATU was used in a range of 10-2000 μM and PTIO was applied in a rage of 50-300 μM.

The last part is to examine autotrophic ammonia-oxidizing activity and growth of AOA and AOB using DNA-SIP technique. Sludge from NRI was collected and incubated with label (^{13}C-HCO_3^-) and unlabeled (^{12}C-HCO_3^-) inorganic carbon. Together with the two incubations, an additional DNA-SIP incubation was carried out with ^{13}C-HCO_3^- and ATU at the concentration of 80 μM. This third incubation was performed to investigate ATU specificity at this concentration to selectively inhibit AOB, not AOA.

4.1.1 Part 1: Long-term monitoring of abundance and diversity of AOA and AOB in nitrifying reactors

4.1.1.1 Nitrifying reactors

1. NRI

During the 14-month study period, ammonia concentration in the effluent of NRI was 0.07 ± 0.03 mgNL^{-1}. Nitrite concentration in the effluent remained below < LOD of 0.1 mgNL^{-1}. Nitrate concentration was 26.17 ± 4.44 mgNL^{-1}, which was in balance with the range of ammonia concentration in the influent.

2. NRII

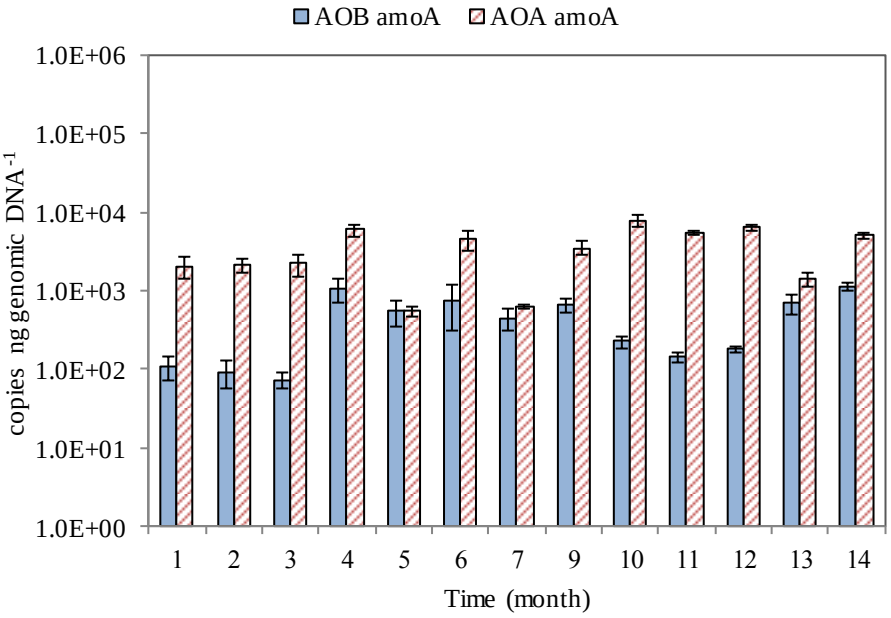
For NRII, during the 7-month study period, the reactor effluent ammonia concentration was 14.19 ± 9.54 mgNL^{-1}. The nitrite concentration was 6.24 ± 6.79 mgNL^{-1} and the nitrate concentration was 370.12 ± 26.19 mgNL^{-1}. The summation of ammonia, nitrite, and nitrate concentrations in the effluent water was close to the influent ammonia concentration.

4.1.1.2 Abundance and diversity of AOA and AOB amoA genes in nitrifying reactors

1. NRI

The numbers of AOA *amoA* genes ranged between $5.53 \times 10^2 \pm 9.34 \times 10^1$ and $7.88 \times 10^3 \pm 1.17 \times 10^3$ copies ng genomic DNA^{-1}, while AOB *amoA* genes were between $7.38 \times 10^1 \pm 1.79 \times 10^1$ and $1.16 \times 10^3 \pm 1.39 \times 10^2$ copies ng genomic DNA^{-1} (Figure11). The AOA *amoA* genes were found, on average, to outnumber AOB *amoA* genes16 times during the study period (Figure 12).

(a)



(b)

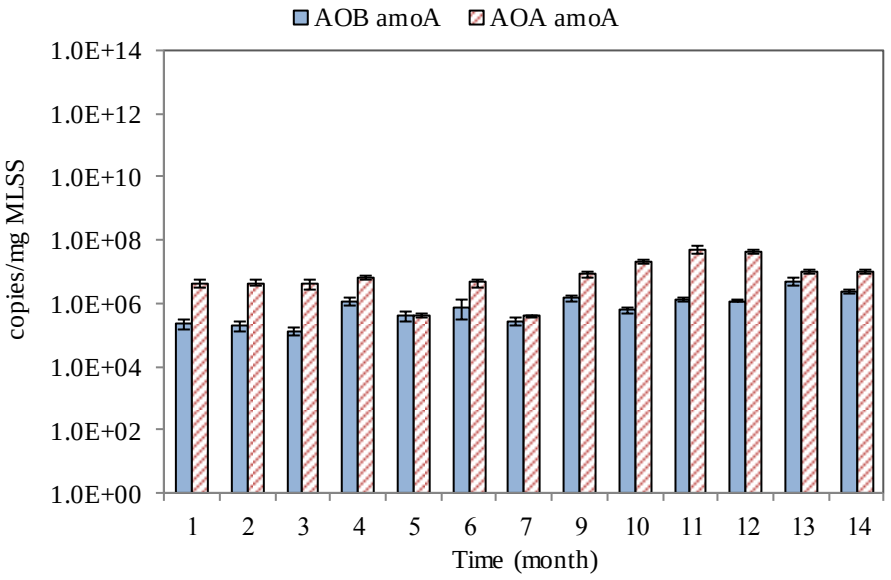


Figure 11 Abundace of AOB and AOA *amoA* genes in NRI

a) copies ng genomic DNA^{-1} and b) copies $mgMLSS^{-1}$

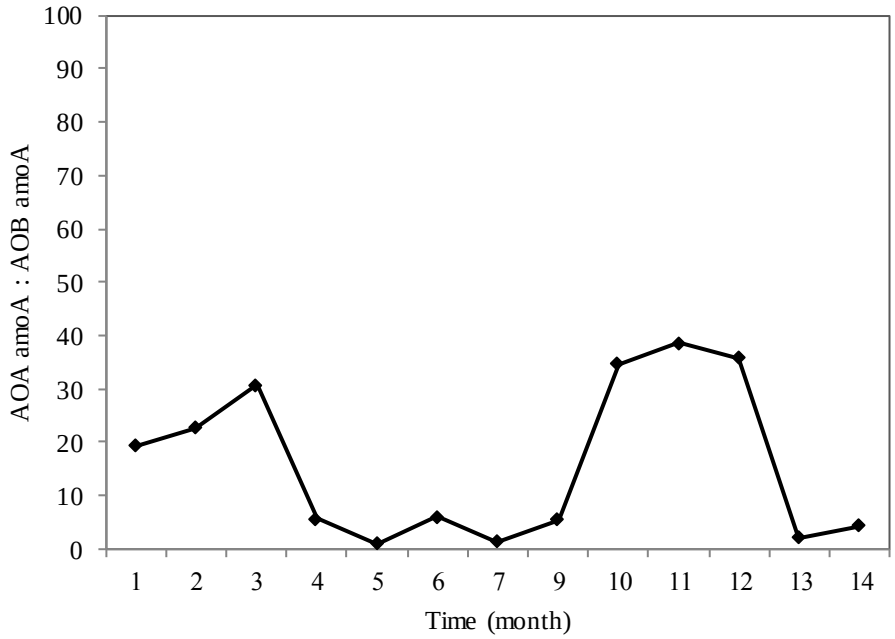
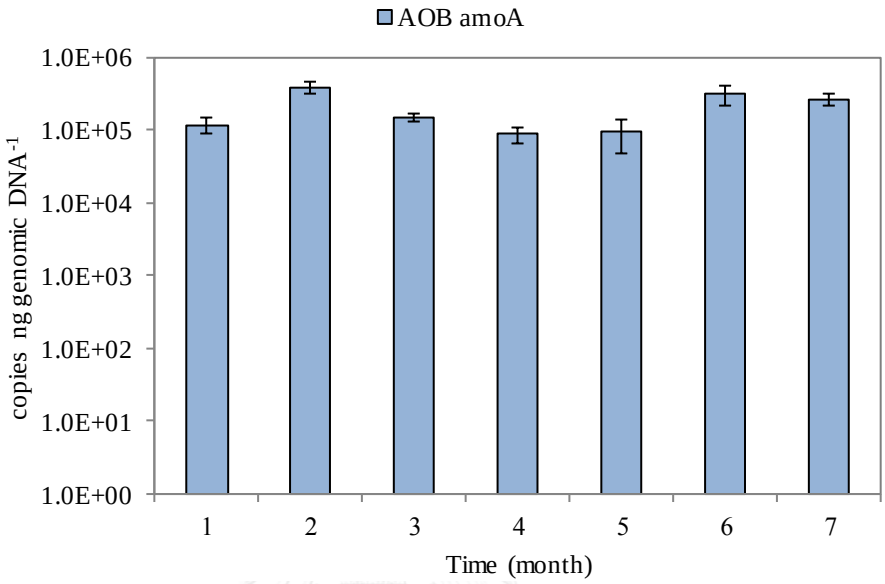


Figure 12 Ratios of AOA *amoA* : AOB *amoA* in NRI

2. NRII

AOA *amoA* gene numbers were lower than the LOD (approximately 4.29 copies ng genomic DNA^{-1}) at all sampling intervals (Figure 11). Also, no AOA *amoA* gene sequence could be retrieved from this reactor. The number of AOB *amoA* genes ranged between $8.76 \times 10^4 \pm 2.08 \times 10^4$ and $3.85 \times 10^5 \pm 6.56 \times 10^4$ copies ng genomic DNA^{-1} (Figure 13)..

(a)



(b)

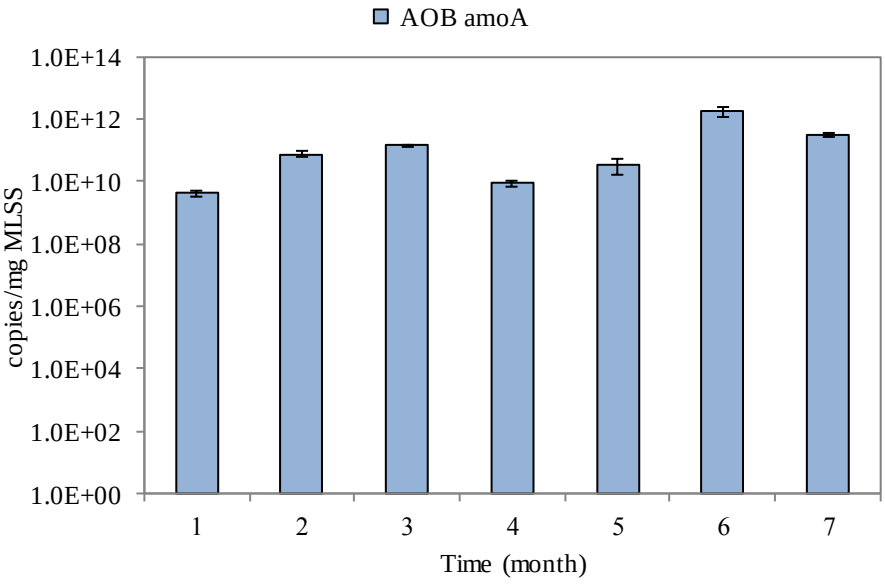


Figure 13Abundace of AOB *amoA* genes in NRII

a) copies ng genomic DNA^{-1} and b) copies mg$MLSS^{-1}$

During the monitoring periods, NRI and NRII were dominated by distinct groups of ammonia-oxidizing microorganisms. AOA *amoA* genes outnumbered AOB *amoA* genes in NRI, while only AOB *amoA* genes were detected in NRII. NRI and NRII were originally seeded with sludge collected from different WWTPs and were operated with different operating conditions that were believed to encourage the growth of AOA and AOB in different ways. NRI was seeded with sludge containing both AOA and AOB *amoA* genes and was operated with an inorganic medium containing various trace elements and vitamins which was previously used for culturing AOA The ammonia and nitrite concentrations in NRI was also maintained in a low range of $mgNL^{-1}$. Previous studies have used an ammonia concentration of ≤7 $mgNL^{-1}$ as the starting concentration for obtaining AOA cultures from environmental samples (Konneke et al. 2005b; Lehtovirta-Morley et al. 2016; Li et al. 2016). The seed sludge and operating conditions in NRI may have attributed to the higher abundance of AOA *amoA* genes than AOB *amoA* genes in NRI. On the other hand, NRII was seeded with sludge from the WWTP where only AOB, and no AOA, was detected. The inorganic medium used in NRII was minimal and the reactor was operated with higher ammonia and nitrite concentrations than NRI. The seed sludge operating conditions in NRII may have helped promote the domination of AOB in NRII.

4.1.1.3 Diversity of AOA and AOB amoA genes in nitrifying reactors

1. NRI

All AOA *amoA* gene sequences retrieved from NRI at months 1 and 6 were found to be specific to only the *Nitrososhaera* sister cluster which was a member within the group 1.1b *Thaumacheota* (Figure 14). The results indicated no change in AOA communities between months 1 and 6.

AOB *amoA* gene sequences were retrieved from each of month 1 and 6 samples and these sequences were found to belong to 3 AOB clusters: *Nitrosomonas europaea* cluster, *Nitrosomonas communis* cluster, and *Nitrosomonas oligotropha* cluster (Figure 15). The majority of the AOB *amoA* sequences changed from the Nitrosomonas oligotropha cluster at month 1 to the Nitrosomonas europaea cluster by month 6.

2. NRII

AOB *amoA* gene sequences analyzed for month 1 and 6 samples fell into 3 AOB clusters: *Nitrosomonas europaea* cluster, *Nitrosomonas communis* cluster, and *Nitrosomonas oligotropha* cluster (Figure 15).

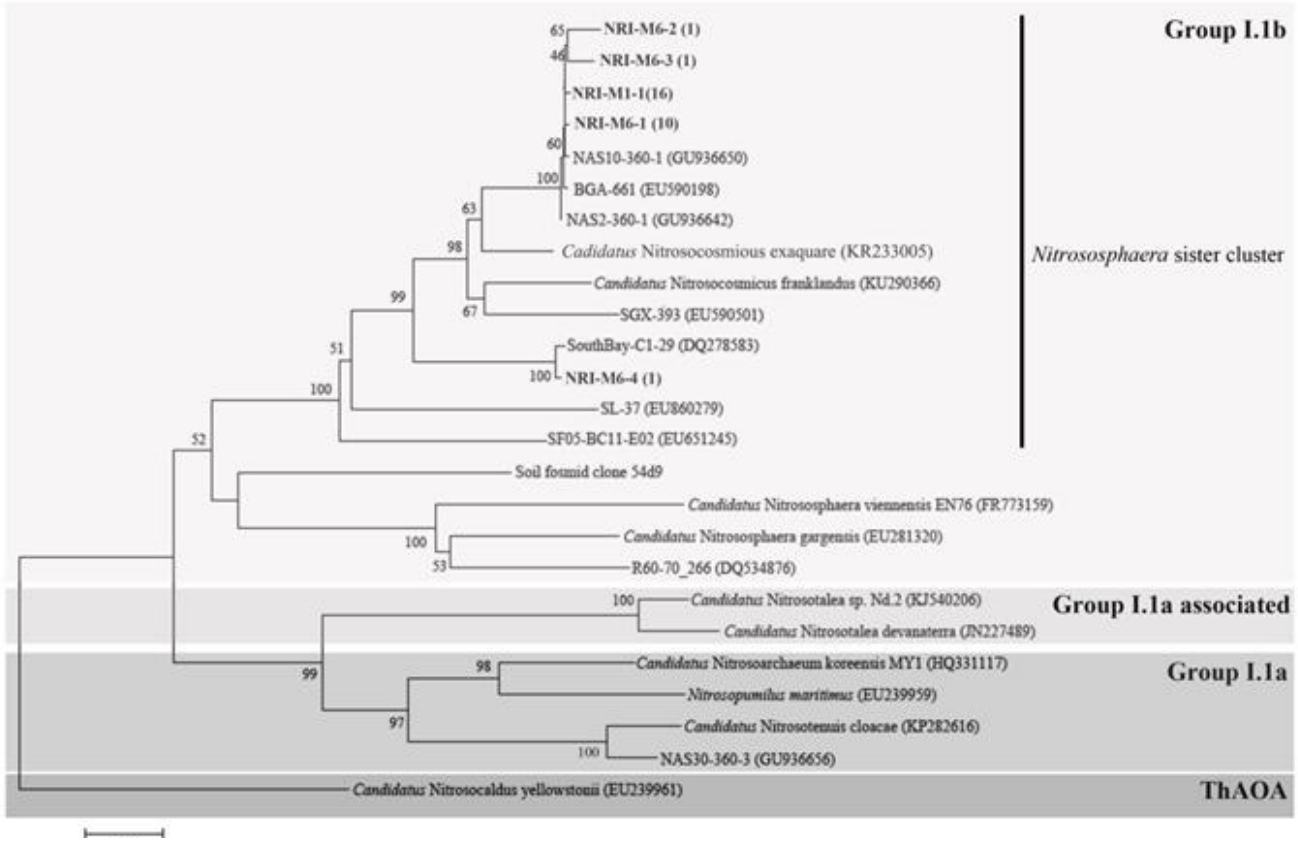


Figure 14 Phylogenetic tree computed based on sequences of AOA amoA genes. This study's sequences are shown in bold. The first and second abbreviations represent the reactors and the months the samples were collected. Bootstrap analysis was carried out with 1000 replicates and shown in the tree for values of >50 %

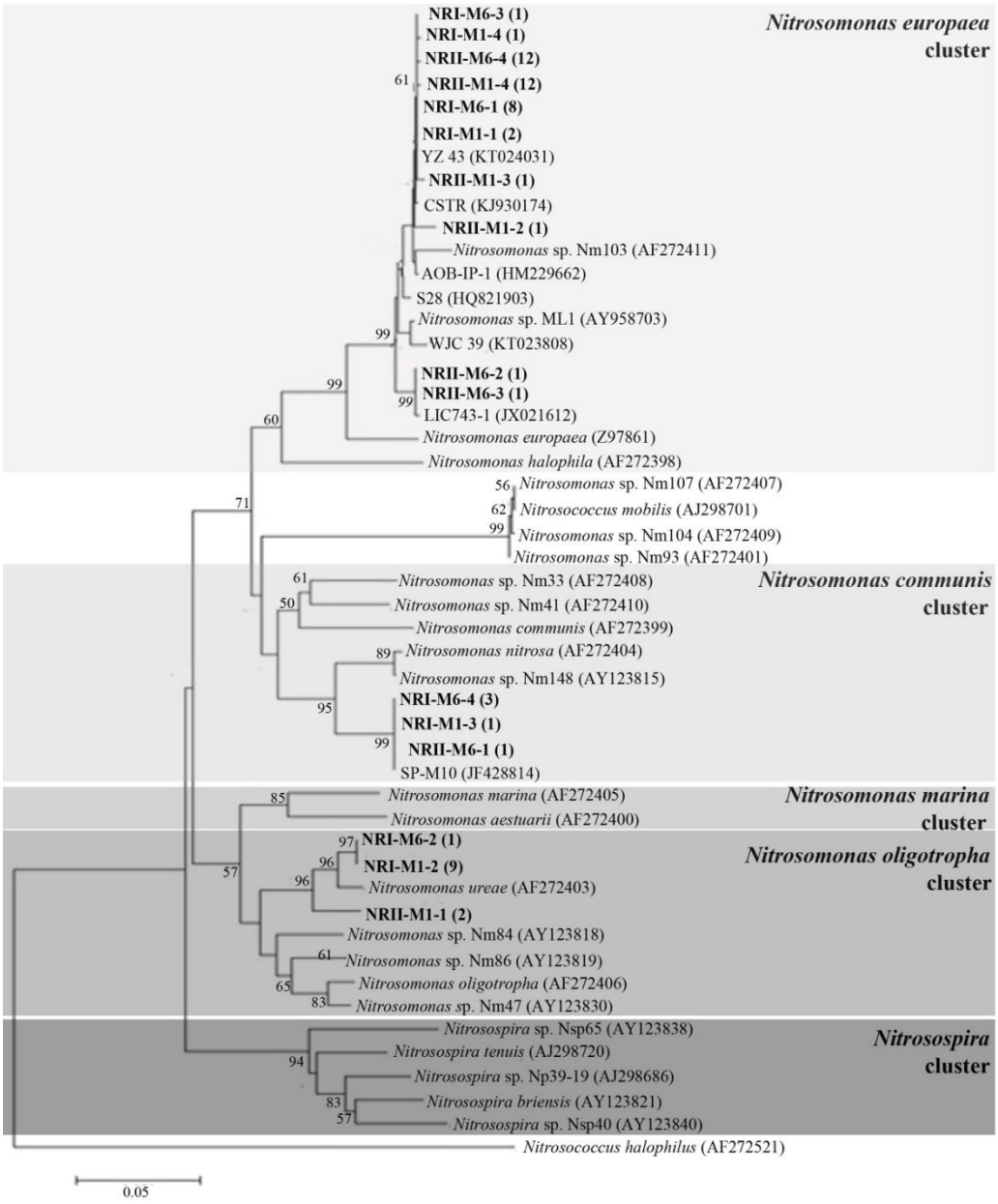


Figure 15Phylogenetic tree calculated based on sequences of AOB *amoA* genes. This study's sequences are shown in bold. The first and second abbreviations represent the reactors and the months the samples were collected. Bootstrap analysis was carried out with 1000 replicates and shown in the tree for values of >50 %

The phylogenetic analysis revealed that all AOA *amoA* gene sequences retrieved from NRI belonged to the *Nitrososhaera* sister cluster within Group 1.1b *Thaumacheota*. Closely related sequences to the sequences analyzed (with 98-99% sequence identities) were found in nitrifying reactors in our previous work (Sonthiphand and Limpiyakorn 2011). Presently, *Candidaus* Nitrosocosmicus franklandus and *Candidaus* Nitrosocosmicus exaquare, obtained from soil and a WWTP, respectively, are known as the two representatives for the *Nitrososhaera* sister cluster (Lehtovirta-Morley et al. 2016; Sauder et al. 2017). *Candidaus* Nitrosocosmicus franklandus has been obtained from the sandy loam soil of agricultural plots in Scotland (Lehtovirta-Morley et al. 2016) and *Candidaus* Nitrosocosmicus exaquare has recently been cultivated from rotating biological contactors of a municipal WWTP in Canada (Sauder et al. 2017). The AOA *amoA* gene sequences from our current study have a sequence identity of 90-95% to *Candidaus* Nitrosocosmicus exaquare and 89-91% to *Candidaus* Nitrosocosmicus franklandus.

AOB *amoA* gene sequences found in NRI and NRII belonged to the *Nitrosomonas europaea* cluster, *Nitrosomonas communis* cluster, and *Nitrosomonas oligotropha* cluster. Previously, AOB within these three clusters have been found in WWTPs and nitrifying reactors (Gao et al. 2013; Limpiyakorn et al. 2011; Sonthiphand and Limpiyakorn 2011).

4.1.1.4 Scanning electron microscopy (SEM)

1. NRI

SEM images taken at the end of the study (Figure 16) showed that some cells in NRI appeared in clusters as coccoid-shaped cells with a cell diameter of approximately 0.8 µm, and individual cells as rod-shaped cells with various cell lengths.

2. NRII

SEM images indicated that most cells appeared in rod shape with various cell lengths (Figure 17).

Scanning electron micrographs revealed that some cells from NRI were coccoidal with cell diameters of around 0.8 μm and that these cells tended to clump together. Similar types of cell morphology and formation have been reported for *Candidaus* Nitrosocosmicus exaquare, but with a larger cell diameter of 1.3 μm (Sauder et al. 2017). However, applying SEM to mixed culture systems is not possible to indicate that the coccoid cells appeared related to AOA.

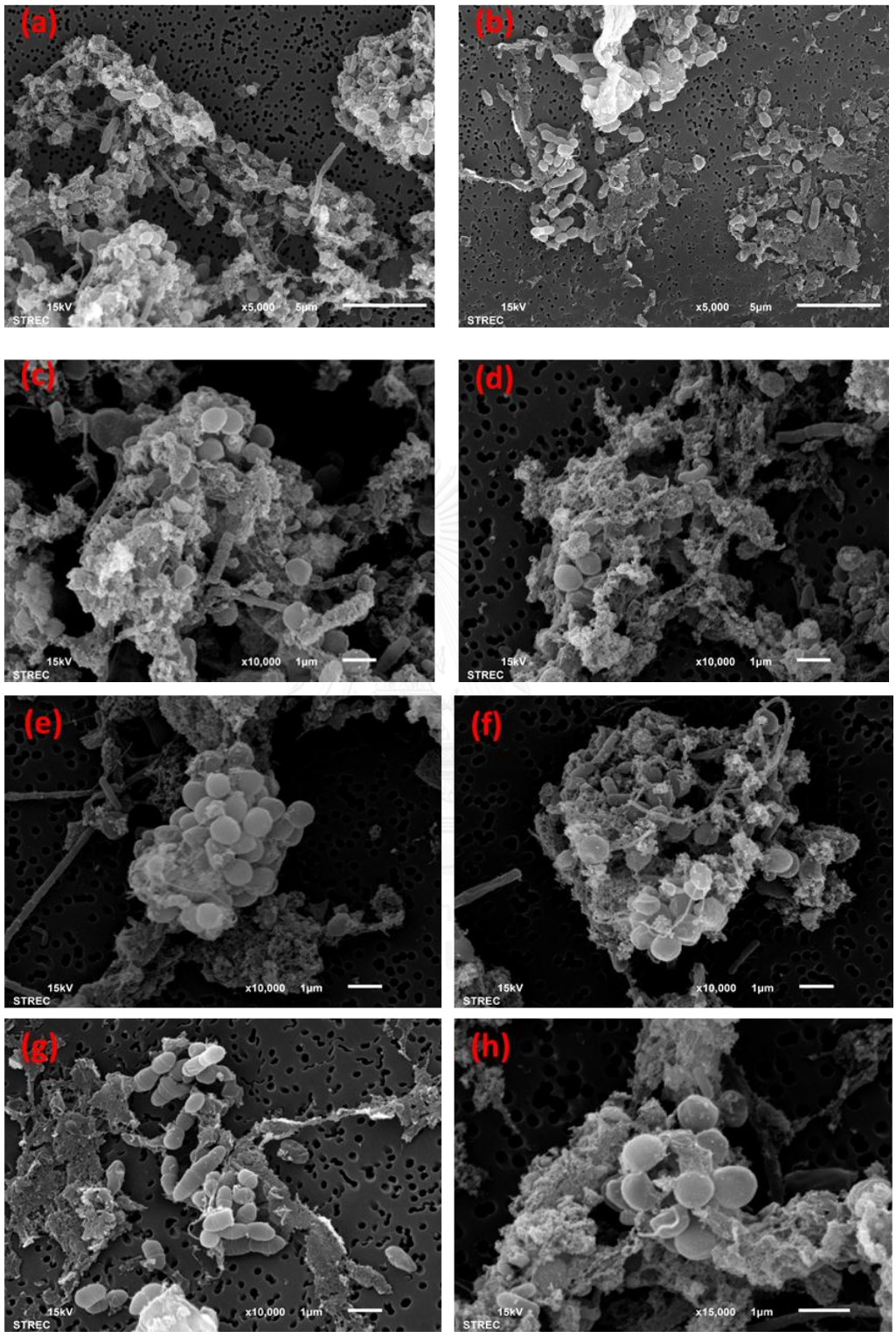


Figure 16 Scanning electron micrograph of NRI (a-b) scale bar 5 μM (c-h) scale bar 1 μM

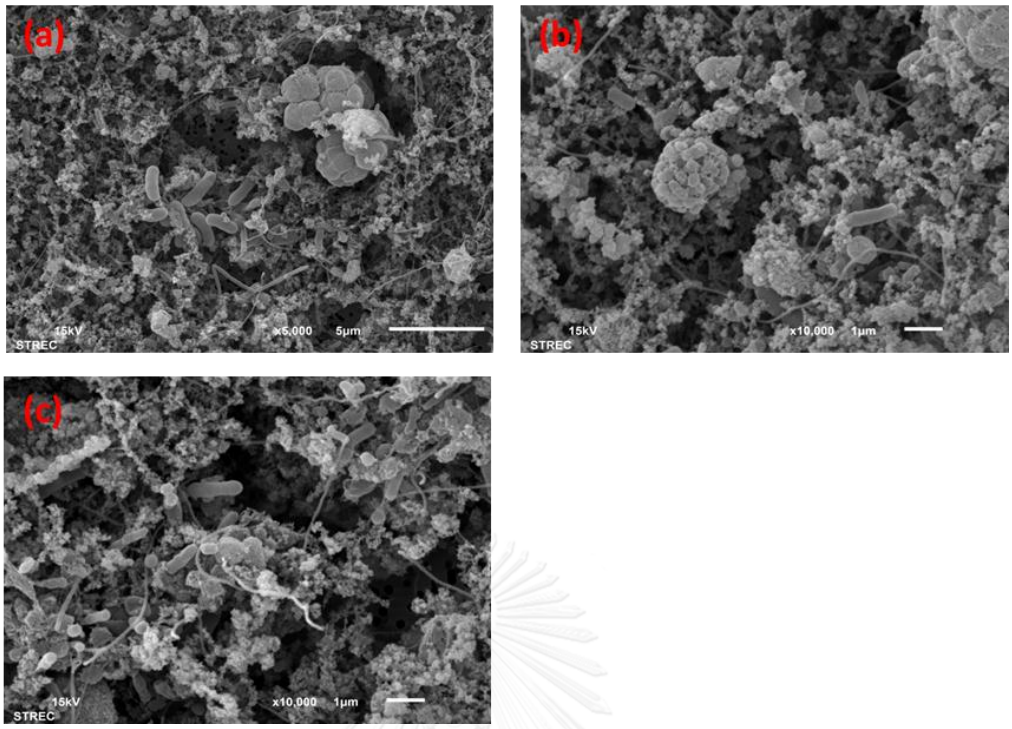


Figure 17 Scanning electron micrograph of NRII (a) scale bar 5 μM.
(b-c) scale bar 1 μM

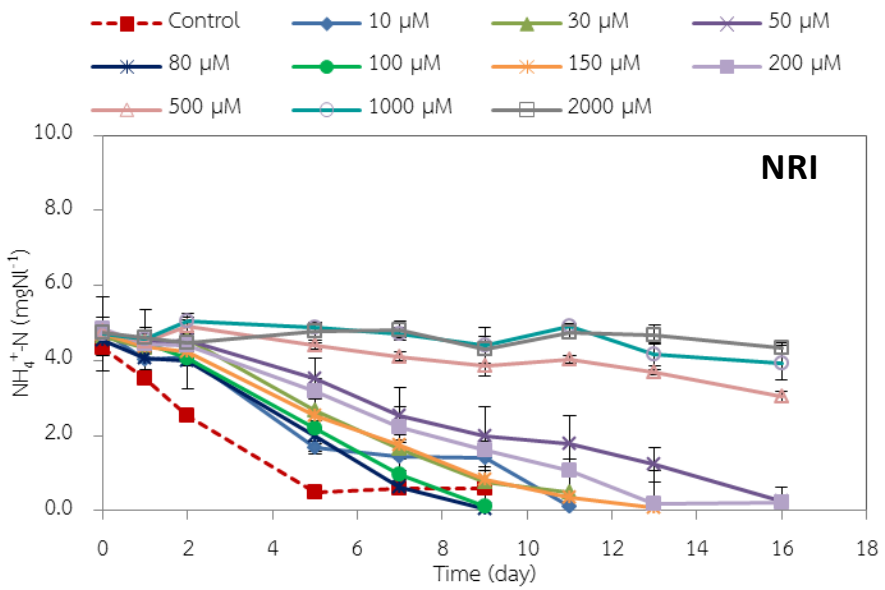
4.1.2 Part 2: Ammonia-oxidizing activity under the presence of ATU and PTIO

4.1.2.1 Ammonia-oxidizing activity under the presence of ATU

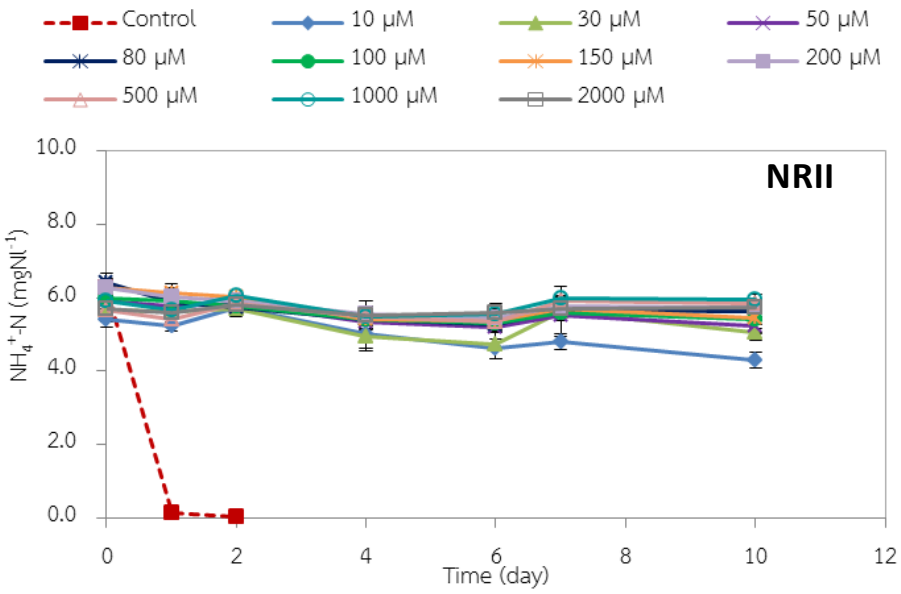
Tests of ammonia-oxidizing activity under the presence of ATU was performed on sludge taken from NRI at months 2 and 12 (Figure 18a and Figure 18c, respectively), and sludge taken from NRII at months 2 and 6 (Figure 18b and Figure 18d, respectively). For NRII sludge in which AOB was the only detectable ammonia oxidizers, ATU concentrations of ≥10 μM provided complete inhibition of ammonia oxidation (Figure 18b and Figure 18d). This behavior is different from NRI sludge where AOA were the predominant ammonia oxidizers. For NRI sludge, ATU concentrations of 10-200 μM were found to partially inhibit ammonia-oxidizing activity of the sludge (Figure 18a and Figure 18c) , and complete ammonia oxidizing inhibition occurred at ATU concentrations of 1000 and 2000 μM.

Figure 19 plots together percent inhibition of ammonia oxidation under various concentrations of ATU in a range of 10-2000 μM for NRI and NRII. The percent inhibition of ammonia oxidation was calculated by comparing to the control (without adding ATU) at the time the ammonia concentration in the control reached around 0 $mgNL^{-1}$. This results agreed with Figure 8a-d. Concentrations of ATU of ≥ 10 μM showed almost 100 % inhibition of ammonia oxidation for NRII. For NRI, partial inhibition of 20-80 % was found for ATU in a rage of 10-200 μM. Nearly complete inhibition with >90 % occurred at ATU concentrations of >500 μM.

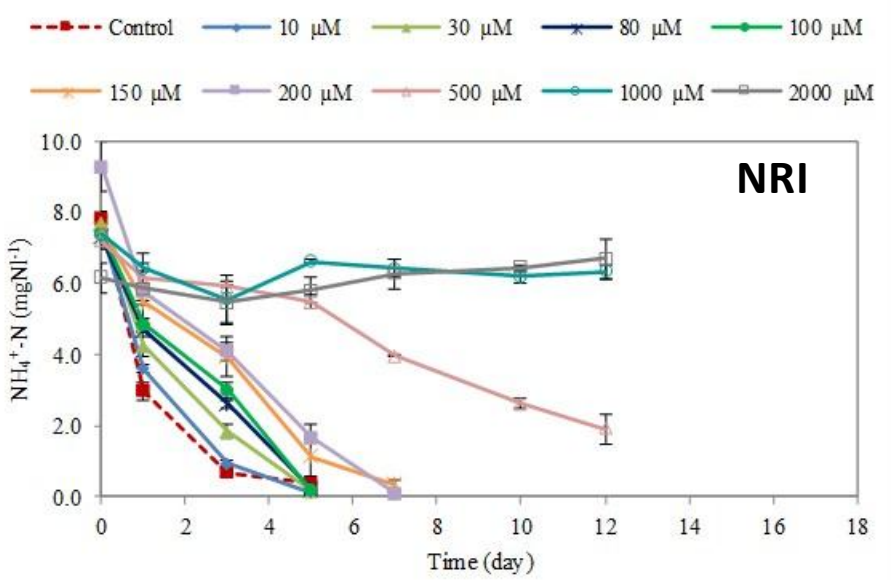
(a)


Control
10 µM
30 µM
50 µM
80 µM
100 µM
150 µM
200 µM
500 µM
1000 µM
2000 µM
NRI
NH_4^+-N (mgNl^{-1})
Time (day)

(b)


Control
10 µM
30 µM
50 µM
80 µM
100 µM
150 µM
200 µM
500 µM
1000 µM
2000 µM
NRII
NH_4^+-N (mgNl^{-1})
Time (day)

(c)



(d)

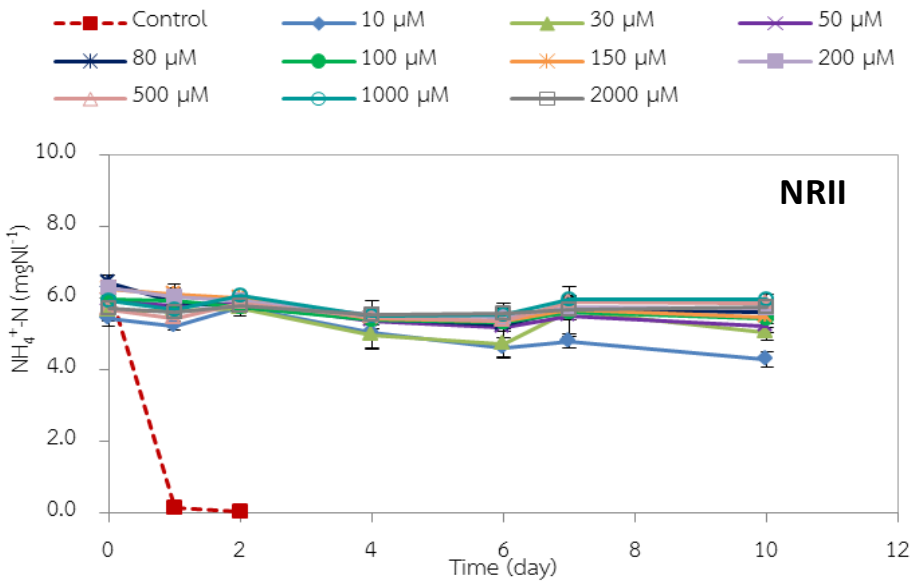


Figure 18 Change in ammonia concentrations under the presence of (a) ATU for NRI collected in month 2, (b) ATU for NRII collected in month 2, (c)) ATU for NRI collected in month 12, and (d) ATU for NRII collected in month 6

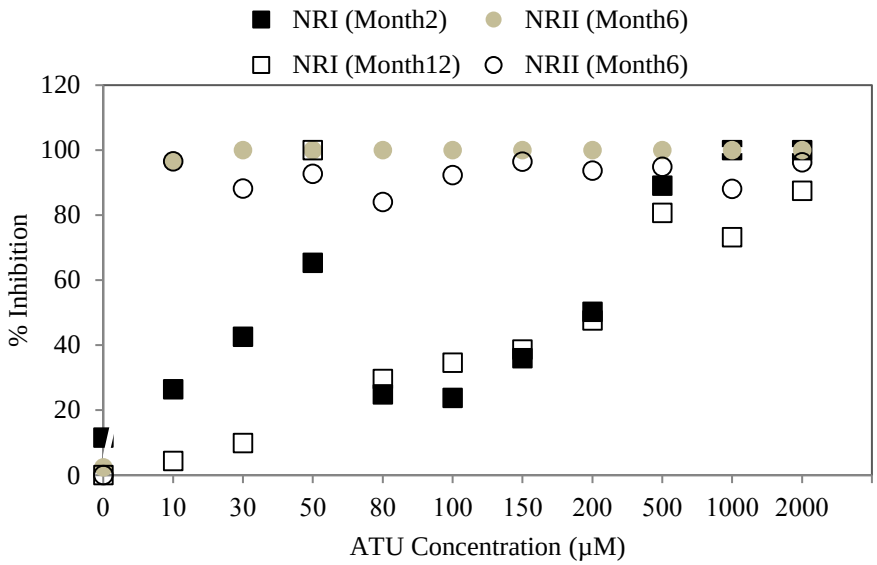
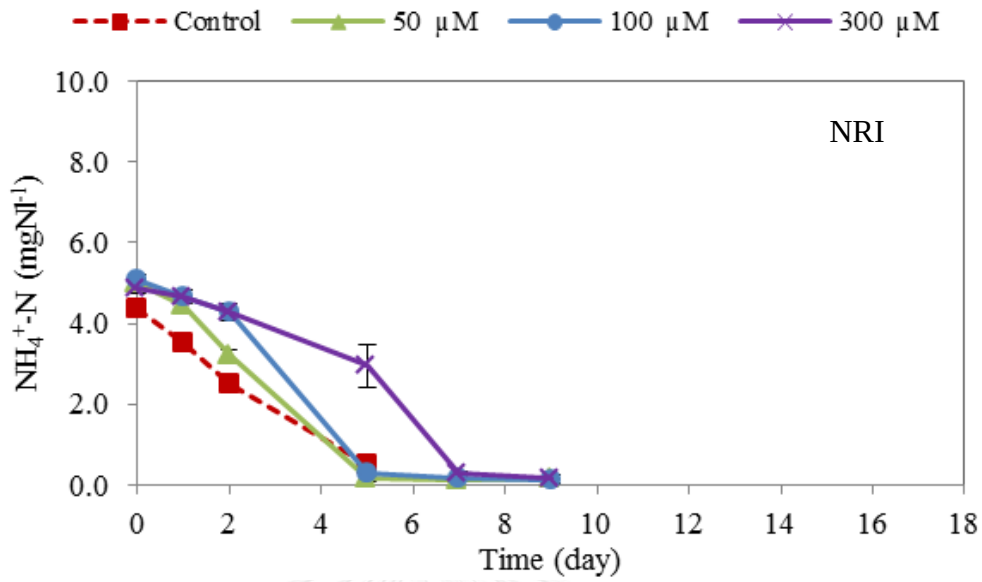


Figure 19 Percent inhibition of ammonia oxidation of NRI and NRII under the presence of ATU in a range of 10-2000 µM

4.1.2.2 Ammonia-oxidizing activity under the presence of PTIO

Tests of ammonia-oxidizing activity in the presence of PTIO was performed on both NRI and NRII sludge collected at months 2 and 3, respectively (Figure 20a and 20 b). For NRII sludge, no influence on ammonia-oxidizing activity was found on the three tested PTIO concentrations (Figure 20 b). For NRI sludge, PTIO concentrations at 50 and 100 µM showed no inhibition of ammonia oxidation, but for 300 µM, the ammonia-oxidizing activity was partially inhibited (Figure 20 a).

(a)



(b)

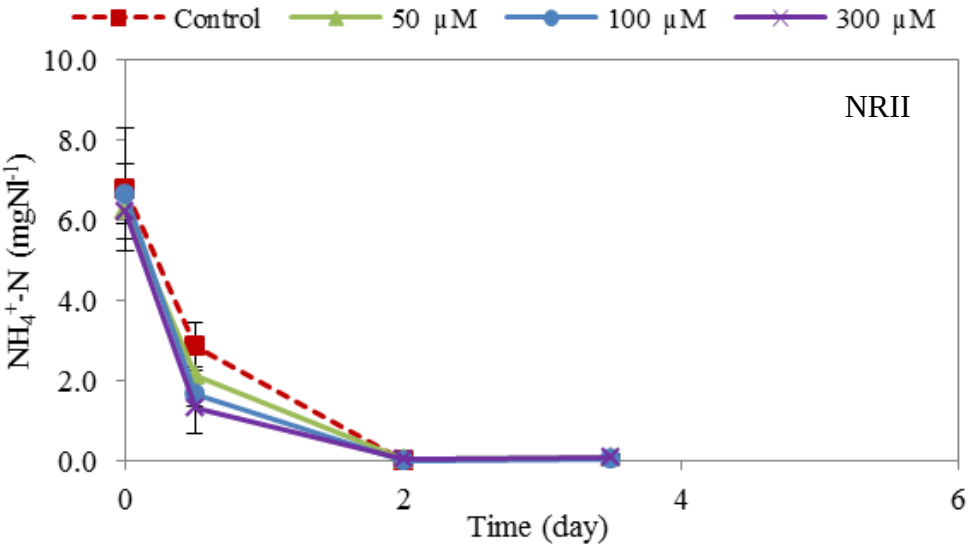
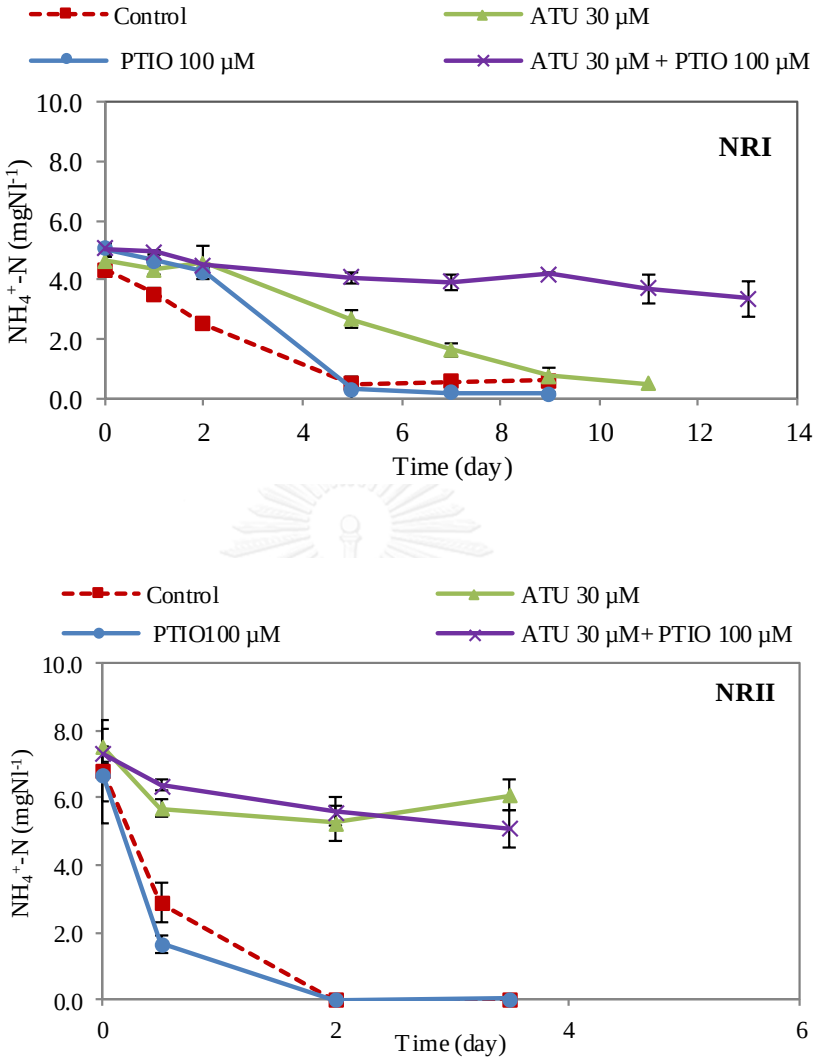


Figure 20 Change in ammonia concentrations under the presence of PTIO for (a) NRI collected in month 2, and (b) NRII collected in month 3

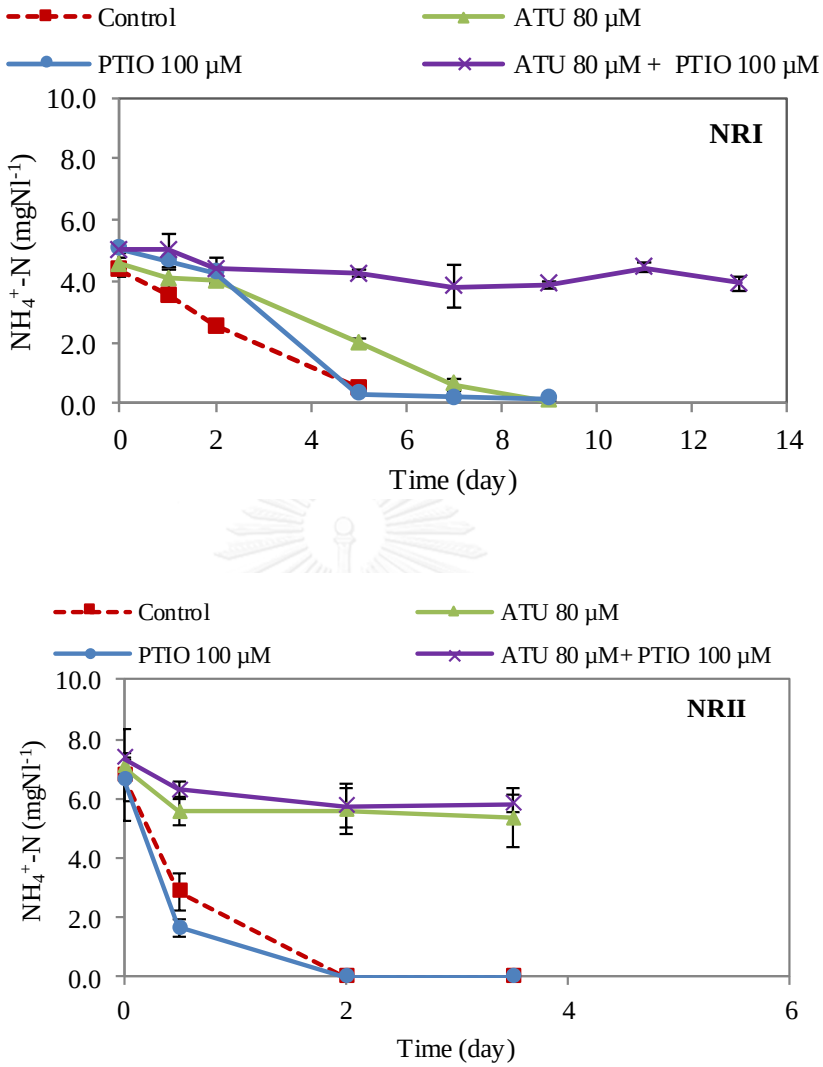
4.1.2.3 Ammonia-oxidizing activity under the presence of ATU and PTIO mixture

Tests of ammonia-oxidizing activity under the presence of an ATU and PTIO mixture was performed on NRI collected at month 2 and NRII sludge collected at month 3 (Figure 20 a-d). For NRII sludge, because ATU concentrations of ≥10 μM inhibited completely the ammonia-oxidizing activity of the sludge (Figure 19 b and Figure 20 d), adding ATU (30, 80, 150, and 2000 μM) together with PTIO (100 μM) (Figure 19 a, b, c, and d) showed no different results from when adding ATU at the corresponding concentrations. For NRI sludge where AOA and AOB coexisted, adding ATU (30, 80, 150 μM) together with PTIO (100 μM) completely inhibited the ammonia-oxidizing activity of the sludge, which was different from when adding only either one of ATU or PTIO at the corresponding concentrations which showed partial or no deterioration of the ammonia-oxidizing activity of the sludge (Figure 20a, b, and c).

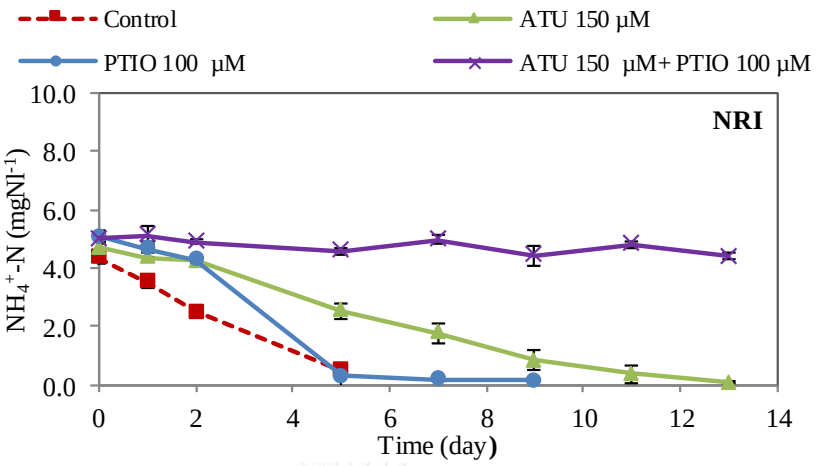
(a) ATU 30 µM + PTIO 100 µM

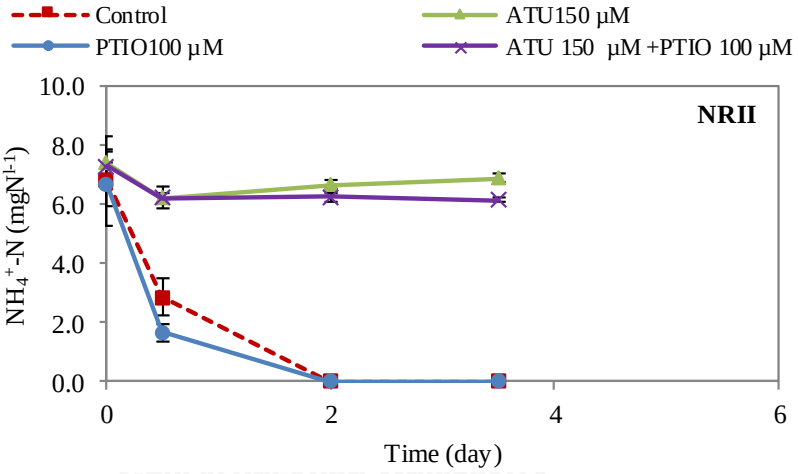


(b) ATU 80 µM + PTIO 100 µM



(c) ATU 150 µM + PTIO 100 µM





(d) ATU 150 µM + PTIO 100 µM

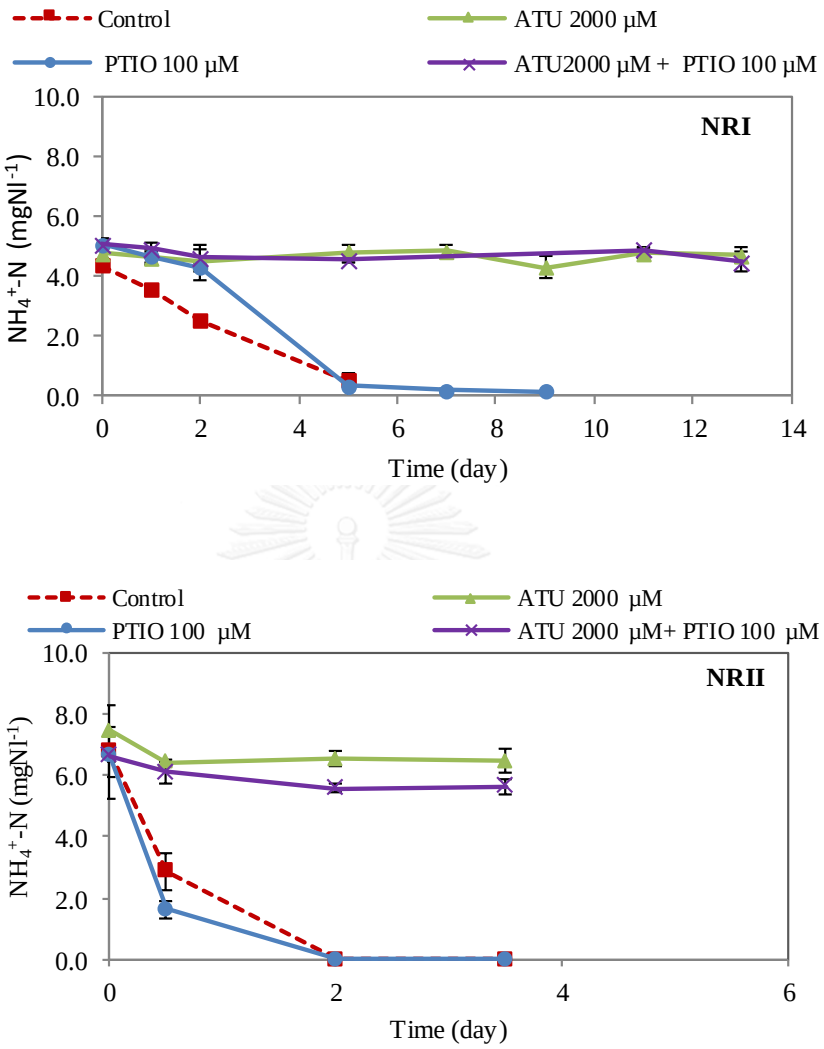


Figure 21 Change in ammonia concentrations under the presence of a mixture of different concentrations of ATU and a consistent concentration of PTIO for NRI and NRII collected in month 2 and 3, respectively.

ATU and PTIO were introduced in order to distinguish the ammonia-oxidizing activity of AOA and AOB in the NRI and NRII sludge. Because AOB were the major ammonia-oxidizing microorganisms in NRII, the ammonia-oxidizing activity of the sludge should be driven mainly by AOB. This was confirmed by the addition of ATU to the NRII sludge where the sludge's ammonia-oxidizing activity was completely inhibited at ATU concentrations of ≥10 µM. The similar range of ATU concentrations has previously

been reported to completely inhibit AOB in pure culture systems. For example, (Martens-Habbena et al. 2015) reported that the activity of *Nitrosomonas europaea, Nitrosomonas oligotropha, Nitrosomonas ureae, Nitrosomonas* cryotolerans, and *Nitrosospira multiformis* can be completely inhibited at ATU concentrations of >3.3 μM.

The ammonia-oxidizing ability of AOA is only slightly affected at the range of ATU concentrations that was reported to fully inhibit most AOB ammonia-oxidizing ability. ATU concentrations of <100 μM were reported to show no effect on the growth and activity of two AOA strains originated from WWTP samples: *Candidatus* Nitrosotenuis cloacae and *Candidatus* Nitrosocosmicus exaquare, respectively (Li et al. 2016; Sauder et al. 2017). In order to completely inhibit AOA, much higher ATU concentrations is required as compared to AOB. For example, *Candidatus* Nitrosotenuis cloacae and *Nitrosopumilus maritimus* strain HCA1 were completely inhibited at ATU concentrations of 700 and 1000 μM, respectively (Li et al. 2016; Martens-Habbena et al. 2015). By applying various concentrations of ATU to NRI sludge, our results demonstrated that the ammonia-oxidizing activity of the sludge was inhibited completely at ATU concentrations >500 μM. Because AOA were the predominant ammonia-oxidizing microorganisms in the NRI sludge, it can be implied that ATU concentrations >500 μM can completely inhibit the ammonia-oxidizing activity of AOA. This result agrees with previous studies (Li et al. 2016; Martens-Habbena et al. 2015). In addition, ATU concentrations between 10 and 200 μM were found to partially inhibit the ammonia-oxidizing activity of NRI sludge. Therefore, this lower range of ATU concentrations may be useful for observing the activity of AOA since the activity of AOB will cease within this ATU concentration range.

In our study, the application of PTIO at concentrations of 50, 100, and 300 μM to NRII sludge showed no inhibitory effects on the ammonia-oxidizing activity of the sludge. Previously, Martens-Habbena et al (2015) demonstrated that the ammonia-

oxidizing activities of *Nitrosomonas europaea, Nitrosomonas oligotropha, Nitrosomonas ureae, Nitrosomonas cryotolerans,* and *Nitrosospira multiformis* were unaffected by PTIO at the concentration of 100 μM. Our results confirm that AOB played the main role in the ammonia-oxidizing activity of NRII.

In contrast to AOB, AOA were found to be sensitive to PTIO. In a previous study, the activity of *Candidatus* Nitrosocosmicus exaquare was completely inhibited at PTIO concentrations of 100 μM (Sauder et al. 2017). PTIO at the concentration of 100 μM was also found to nearly completely inhibit *Nitrosopumilus maritimus* strain HCA1 and strain SCM1 (Martens-Habbena et al. 2015). Moreover, *Nitrososphaera viennensis* was completely inhibited at ≥50 μM PTIO (Shen et al. 2013). When PTIO was introduced to NRI sludge, the ammonia-oxidizing activity of the sludge was uninhibited at the PTIO concentrations of 50 and 100 μM, and was only partially inhibited at the concentration of 300 μM. Because AOB were also present in NRI sludge, the addition of PTIO alone cannot lead to the complete inhibition of the ammonia-oxidizing activity of the sludge. Therefore, ATU concentrations of 30, 80, 150 and 2000 μM were also applied together with 100 μM PTIO to NRI sludge. Results from the addition of both chemical inhibitors demonstrated complete inhibition of ammonia-oxidizing activity at all ATU concentrations when 100 μM of PTIO was present. This indicated that PTIO at the concentration of 100 μM completely inhibited the ammonia-oxidizing activity of AOA in NRI sludge since ATU concentrations of ≥10 μM were already shown to completely inhibit AOB. Regarding the contribution of AOA and AOB to ammonia oxidation in NRI, the results indicated that AOA contributed to the ammonia-oxidizing activity of the sludge as the activity still remained after adding ATU alone (30, 80, 150 μM) to the sludge and the overall activity disappeared after PTIO (100 μM) was added together with ATU (30, 80, 150 μM).

4.1.3 Part 3: Incorporation of ^{13}C-HCO_3^- by AOA and AOB during ammonia oxidation of NRI sludge under the absence and presence of ATU

DNA-SIP incubation was performed on NRI sludge collected in month 4. Two DNA-SIP incubations were conducted in parallel with ^{12}C-HCO_3^- and ^{13}C-HCO_3^- Together with the two incubations, an additional DNA-SIP incubation was carried out with ^{13}C-HCO_3^- and ATU at the concentration of 80 μM. This third incubation was performed to investigate ATU specificity at this concentration to selectively inhibit AOB, not AOA.

Ammonia, nitrite, and nitrate concentrations were monitored in the effluent water during the 21-day incubation period (Figure 22). The incubation with ^{13}C-HCO_3^- and ATU showed slight higher effluent ammonia concentrations than the incubations with ^{12}C-HCO_3^- and ^{13}C-HCO_3^- without ATU at the end of incubation period.

The numbers of AOA and AOB *amoA* genes were quantified by qPCR during the incubation period (Figure 23 a and b). At day 21 of incubation, AOA appeared in high numbers in all three incubations suggesting that AOA can grow and maintained their cells in these continuous-flow reactors, in which cells were allowed to be washed out, and in the presence of 80 μM of ATU. For AOB, *amoA* gene numbers were found in high numbers as a result of incubation with ^{12}C-HCO_3^- and ^{13}C-HCO_3^- without ATU. However, *amoA* gene numbers reduced on day 21 for the incubation with ^{13}C-HCO_3^- and ATU.

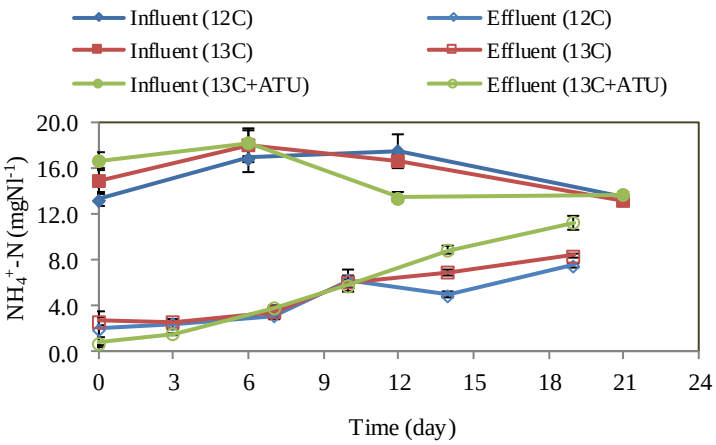
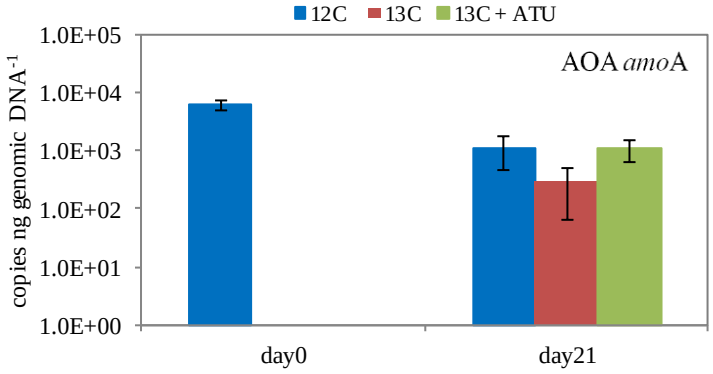


Figure 22 Change in ammonia concentrations during DNA-SIP incubation

(a)



(b)

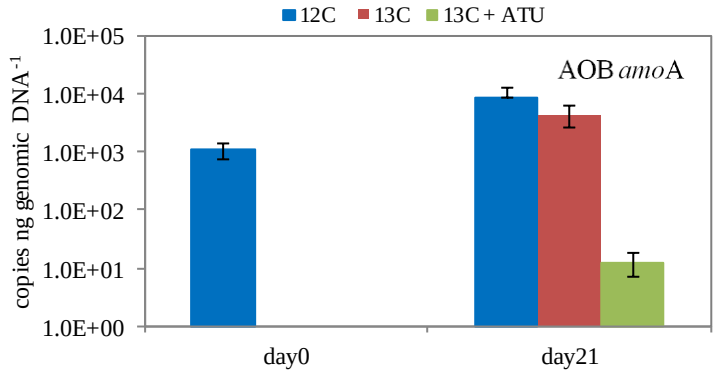
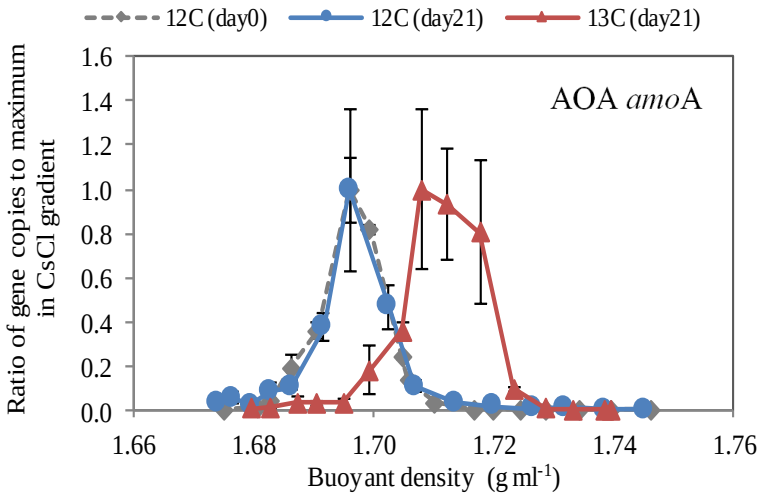


Figure 23 Change in (a) numbers of AOA *amoA* genes, and (b) numbers of AOB *amoA* genes during DNA-SIP incubation

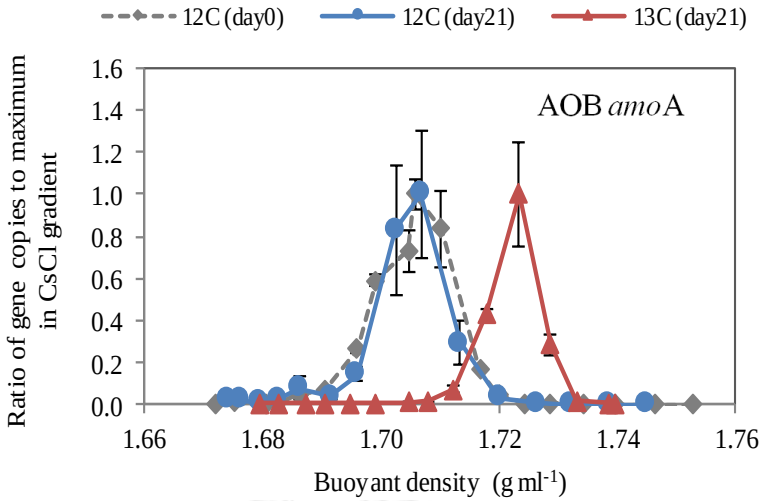
Figures 24 a and 22 b show the DNA-SIP profiles for AOA and AOB *amoA* genes in the incubation without ATU. The AOA *amoA* peak appeared at 1.6961 gmL^{-1} for the ^{12}C HCO_3^- incubation (day 21). However, the peak moved toward heavier fractions and arose at 1.7114 gmL^{-1} for the ^{13}C- HCO_3^- incubation. For AOB, similar results were observed. The AOB *amoA* gene peak shifted from 1.7070 gmL^{-1} to 1.7234 gmL^{-1} when the incubation changed from ^{12}C- HCO_3^- to ^{13}C-HCO_3^-.

AOA and AOB *amoA* genes on day 21 were quantified for each fraction of the ^{13}C- HCO_3^- with 80 µM ATU incubation. The DNA-SIP profile for AOA *amoA* genes is shown in Figure 24c. It should be noted that the profile for AOB *amoA* genes cannot be plotted because AOB *amoA* genes in every fraction were <LOD. This is because AOB cannot maintained high cell numbers in the reactor during the incubation with ATU as suggested in Figure 21b. The DNA-SIP peak for AOA *amoA* genes shifted toward heavier fractions, moving from 1.6961 gmL^{-1} to 1.7114 gmL^{-1}, when switching from the ^{12}C-HCO_3^- incubation to the ^{13}C- HCO_3^- with ATU incubation.

(a)



(b)



(c)

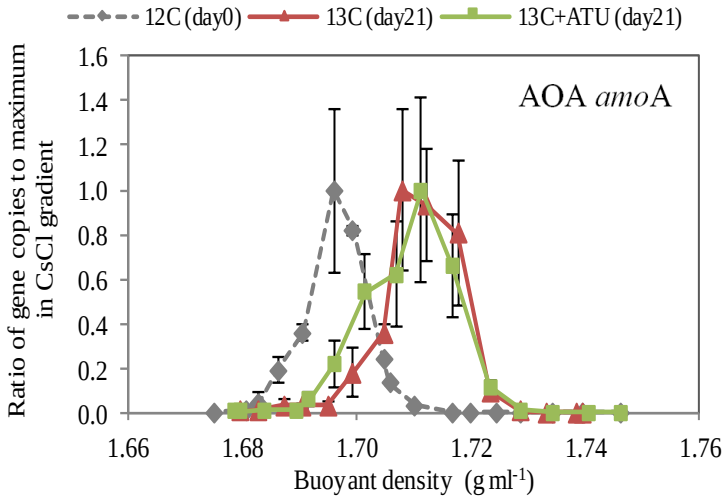


Figure 24 Change in the distribution pattern of (a) AOA *amoA* gene under the absence of ATU, (b) AOB *amoA* gene under the absence of ATU, and (c) AOA *amoA* gene under the presence of 80 μM of ATU

DNA-SIP was carried out on the NRI sludge and results demonstrated that AOA and AOB in the sludge incorporated the labelled ^{13}C compound into their *amoA* genes. This implies that AOA and AOB in NRI probably utilized inorganic carbons for cell synthesis during ammonia oxidation, and thus they may perform chemolithoautotrophy as a choice of their life. However, it must be noted that the downstream analysis was performed after 21 days of DNA-SIP incubation; therefore, the assimilation of the labelled ^{13}C caused by cross feeding can be possible. Nonetheless, the results from the 14-month monitoring supported that AOA and AOB in NRI should perform autotrophic ammonia oxidation because they were able to maintain their cells in NRI for a few years under conditions where ammonia was the only energy source and the media contained no organic. *Candidaus* Nitrosocosmicus exaquare, which was found to relate closely to the AOA in NRI, was previously reported to consume bicarbonate while oxidizing ammonia, but some organic compounds can also stimulate its growth (Sauder et al. 2017). The other AOA which originated from a WWTP, *Candidatus* Nitrosotenuis cloacae, was also found to perform autotrophic ammonia oxidation (Li et al. 2016).

DNA-SIP was also performed on NRI sludge with the addition of 80 μM of ATU to confirm the effects of ATU at this concentration on AOA and AOB growth. AOB were washed out from the DNA-SIP incubation reactor in this trial, indicating that this concentration of ATU does deteriorate AOB growth. This result agrees with the above-mentioned inhibition study. Conversely, AOA were found to be unaffected by the presence of 80 μM ATU as they were found to still incorporate the labelled ^{13}C compound into their *amoA* genes during ammonia oxidation. These results confirmed that ATU at this concentration can be applied to observe AOA activity in NRI sludge. These results also reinforce the results of the inhibition experiments that AOA also contributed to ammonia oxidation in NRI.

4.1.4 Finding

AOA and AOB can be maintained for long periods and they both contribute to ammonia oxidation in nitrifying reactors. Both microorganisms incorporate inorganic carbons during ammonia oxidation, leading to the possibility of performing autotrophy as a choice of their life.

4.2 Experiment 2: Ammonia-oxidizing activity of sludge from full-scale wastewater treatment plants

Experiment 1 observed the effect of ATU (10-2000 μM) and PTIO (100 μM) concentrations on ammonia-oxidizing activity of AOA and AOB in two laboratory nitrifying reactors, NRI and NRII. The results indicated that ATU at concentrations of 10-200 μM inhibited completely the ammonia-oxidizing activity of AOB, but not AOA. Moreover, DNA-SIP confirmed that ATU at the concentration of 80 μM mainly inhibited growth of only AOB and did not have effect on AOA growth. Therefore, ATU at the same concentration range as used in Experiment 1 was applied to sludge from 5 full-scale WWTPs to observe the ammonia-oxidizing activity of AOA in the sludge. Criteria to select these 5 WWTPs was the numbers of AOA and AOB *amoA* genes in the sludge from the plants.

4.2.1 Numbers of AOA and AOB *amoA* genes in full-scale WWTPs

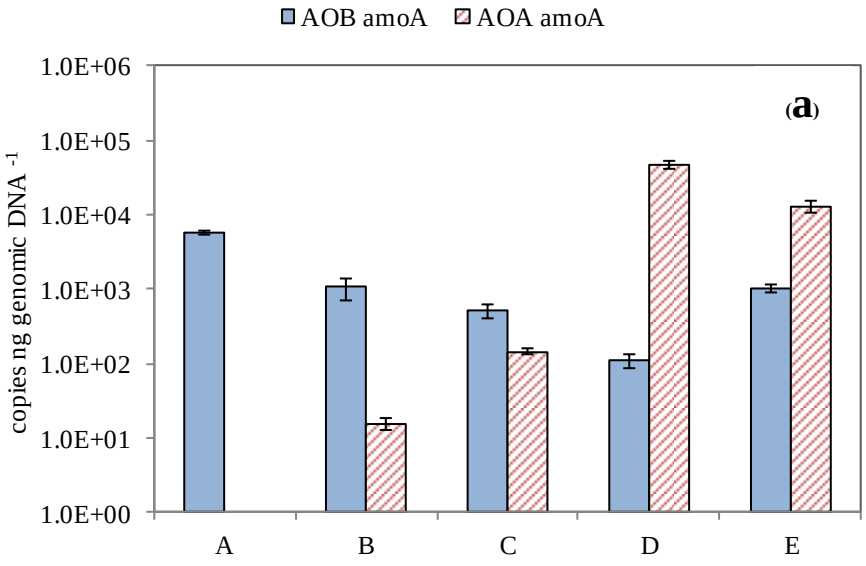
The numbers of AOA and AOB *amoA* genes were quantified by qPCR and were shown in Figure 25 and Figure 26. The results showed that the amounts of AOB and AOA *amoA* genes varied widely among the samples.

For sludge A, only AOB *amoA* genes were found. The number of AOB *amoA* genes was $5.90 \times 10^3 \pm 4.05 \times 10^2$ copies ng extracted DNA^{-1} (Figure 25). On the other hand, the number of AOA *amoA* genes in the sample was less than the limit of detection (LOD) of around 6.53 copies ng extracted DNA^{-1}. Agarose gel check of qPCR products indicated that no AOA existed in the sludge (Figure 27). Both AOB and AOA *amoA* genes existed in sludge B and C. The amounts of AOB *amoA* genes was $1.70 \times 10^3 \pm 3.4 \times 10^2$ and $5.20 \times 10^2 \pm 1.08 \times 10^2$ copies ng extracted DNA^{-1}, respectively (Figure 25). While AOA *amoA* gene numbers were $1.57 \times 10^1 \pm 3.23 \times 10^0$ and $1.46 \times 10^2 \pm 1.06 \times 10^1$ copies ng extracted DNA^{-1}, respectively. The AOB *amoA* genes were

found to be averagely 78.8 and 3.6 (for sludge B and C, respectively) outnumbered the AOA *amoA* genes indicating that AOB was the dominant ammonia-oxidizers in both sludge (Figure 25). For sludge D and E, AOA *amoA* genes outnumbered AOB *amoA* genes in both sludge. The numbers of AOA *amoA* genes in sludge D and E were $4.8 \times 10^4 \pm 6.18 \times 10^3$ and $1.30 \times 10^4 \pm 2.40 \times 10^3$ copies ng extracted DNA^{-1},respectively (Figure 26). AOB *amoA* genes were $1.12 \times 10^2 \pm 2.34 \times 10^1$ and $1.01 \times 10^3 \pm 1.24 \times 10^2$ copies ng extracted DNA^{-1}, respectively. The AOA *amoA* genes were found to be averagely 185.7 and 12.9 (for sludge D and E, respectively) outnumbered the AOB *amoA* genes (Figure 24).

The results demonstrated that among the WWTPs, the relative abundance of AOA and AOB *amoA* genes varied widely. For plant A, only AOB *amoA* genes were detectable. AOB *amoA* genes dominated AOA *amoA* genes in plants B and C. And, AOA *amoA* genes outnumbered AOB *amoA* genes in plants D and E. Thus far, how wastewater treatment system configuration and operation influence both microorganisms in WWTPs is still unclear. Li et al (2016) reported that AOA' works were still in conflict due to limited operational data available for analysis.

(a)



(b)

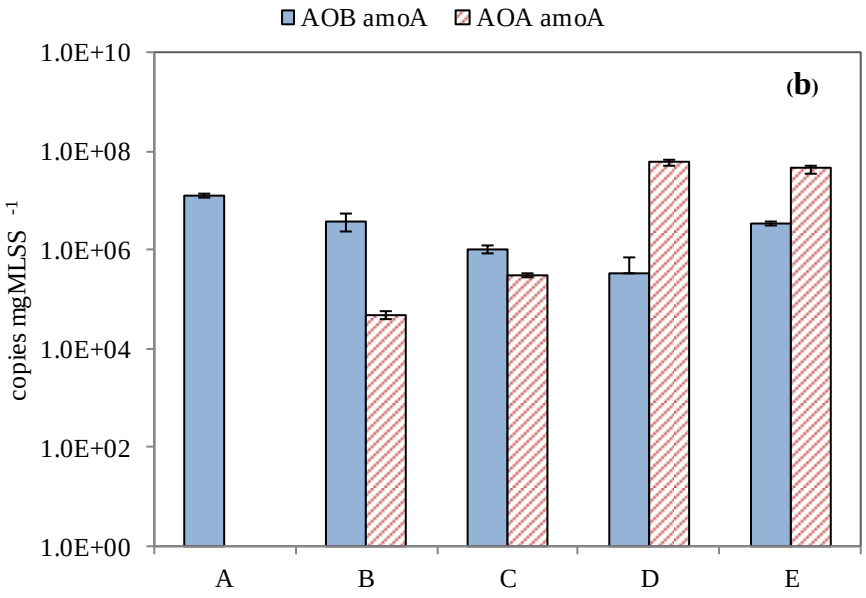


Figure 25Numbers of AOB and AOA *amoA* genes in full-scale WWTPs a) copies ng genomic DNA^{-1} and b) copies mgMLSS^{-1}

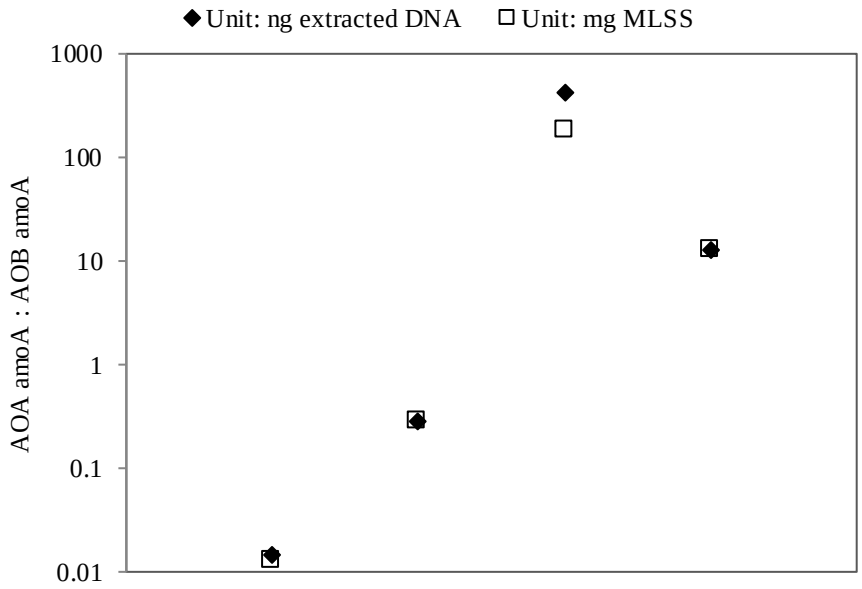


Figure 26 *Ratios of AOA and AOB amoA genes in full-scale WWTPs*

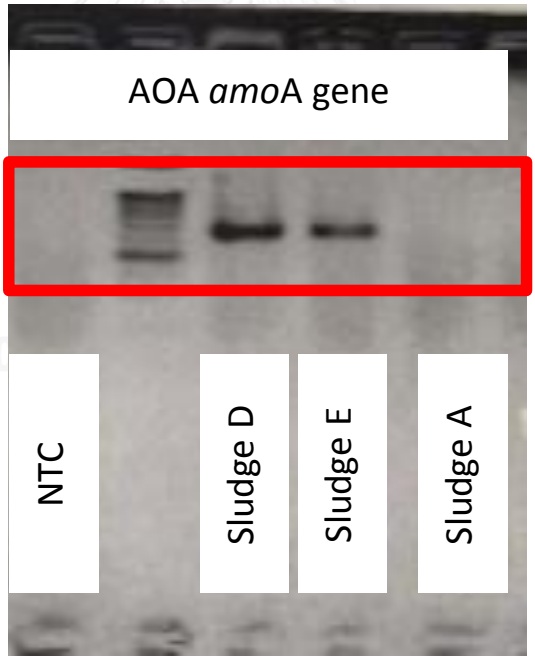


Figure 27 Agarose gel image of qPCR amplified products of AOA *amoA* genes

4.2.2 Ammonia-oxidizing activity under the presence of ATU

ATU in a range of 10-2000 μM was applied to the WWTP sludge. Figure 4-8 shows change in ammonia concentrations of sludge A-E under the presence of ATU.

For sludge A in which only AOB were detectable, the control without ATU showed rapid decrease of ammonia concentration that reached nearly 0 mgN L^{-1} within 1 day of the test (Figure 28). For ATU of 10 μM, ammonia concentration gradually decreased and reached nearly 0 mgN L^{-1} within 3 days. However, for ATU in a range of 10-50 μM, ammonia concentrations decreased and remained around 4 mgN L^{-1} at day 12. For the ATU concentration of >50μM, ammonia concentrations did not change during 12 days of the test. The results confirmed that ATU concentrations of > 50μM provided complete inhibition of ammonia-oxidizing activity of AOB.

Martens-Habbena et al (2015) reported that ammonia-oxidizing activity of AOB cultures*, Nitrosomonas europaea, Nitrosomonas oligotropha, Nitrosomonas ureae, Nitrosomonas cryotolerans* and *Nitrosospira multiformis,* can be inhibited completely at the ATU concentrations of >3.3 μM. Likewise to Experiment 1, no ammonia-oxidizing activity was found when applying ATU of > 10 μM to sludge from NRII in which only AOB were found. Comparing the ATU concentrations used to inhibited completely the ammonia-oxidizing activity of sludge A, AOB cultures, and NRI sludge, sludge A required higher ATU concentrations, which were >50μM, for complete inhibition. Daebeler et al (2015) applied ATU at a concentration of 100 μM to soil microcosms. The result showed no change on ammonia-oxidizing activity of AOB. They suggested that indigenous microorganisms in soil were be able to degrade the inhibitor leading to higher concentrations required for inhibition.

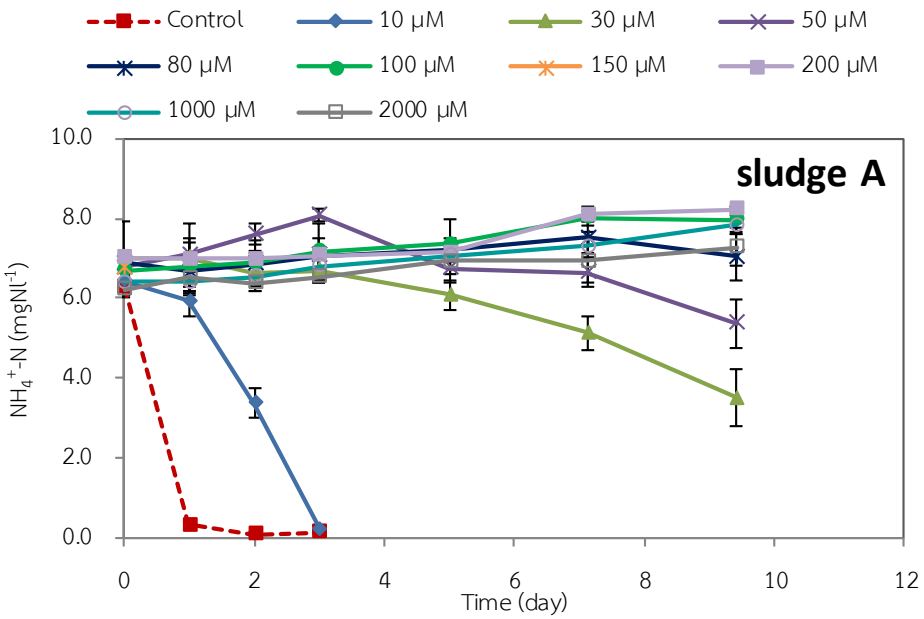


Figure 28Change in ammonia concentrations of sludge A under the presence of ATU

AOB *amoA* genes outnumbered AOA *amoA* genes in sludge B and C. For sludge B, ammonia concentration in the control without ATU reached nearly 0 $mgNl^{-1}$ within 12 day (Figure 29). The result showed that the ammonia-oxidizing activity of the sludge was lower than the other sludge. This might be derived from unknown incubation condition that deteriorate the activity or the sludge originally taken from the WWTP was less active due to some improper operation at the plant. ATU at concentrations of 10-2000 µM completely inhibited ammonia-oxidizing activity of the sludge. For sludge C, the control without ATU showed that ammonia concentration reached nearly 0 $mgNl^{-1}$ within approximately 10 day (Figure 30). ATU in a range of 10-500 µM showed slightly decreased in ammonia concentrations to 5 $mgNl^{-1}$ after 10 days. No ammonia concentration decreased at ATU >500 µM, the ammonia-oxidizing activity strongly inhibited the ammonia oxidizers. Similar to the above discussion for sludge A, sludge B and C tended to inhibited largely by ATU because AOB were the dominant ammonia-oxidizing microorganisms in the sludge.

For plants D and E, AOA was the predominant ammonia oxidizers in the sludge. Sludge D showed that ammonia concentration of the control without ATU decreased to nearly 0 $mgNl^{-1}$ within 2 day (Figure 31). With ATU of 10 µM, ammonia concentrations also reached nearly $mgNl^{-1}$ level but required longer period (8 days) as compared to the control. The range of ATU from 10 to 200 µM partially inhibited ammonia-oxidizing activity of the sludge. In addition, the inhibitory effect of the sludge depended on ATU concentrations; for example, higher inhibitory effect was observed at the ATU of 500 µM than 50 µM. With ATU of >1000 µM, the inhibition was nearly completed. For sludge E, in contrast, the same range of ATU concentrations completely inhibited the ammonia-oxidizing activity of sludge E. It must be noted that sludge E contained much lower proportion of AOA *amoA* genes to AOB *amoA* genes than sludge D (Figure 32). Therefore, sludge D was less sensitive to ATU than sludge E.

According to previous studies, inhibitory effect of ATU on ammonia oxidation in environmental samples including cattle manure compost (Oishi et al. 2012), soil (Jung et al., 2011; Lehtovirta-Morley et al., 2013), freshwater (Sonthiphand and Neufeld 2014) and volcanic grassland soil. Daebeler et al. (2015) suggested that ATU of 100 µM had high potential to inhibit ammonia oxidation of AOB. This ATU concentration had no effect on AOA.

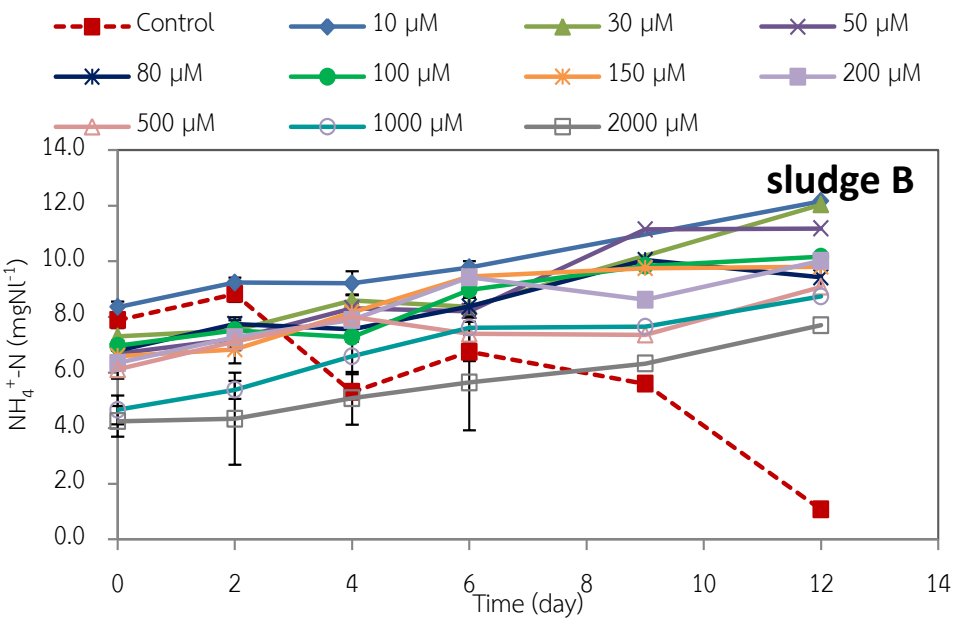


Figure 29 *Change in ammonia concentrations of sludge B*

under the presence of ATU

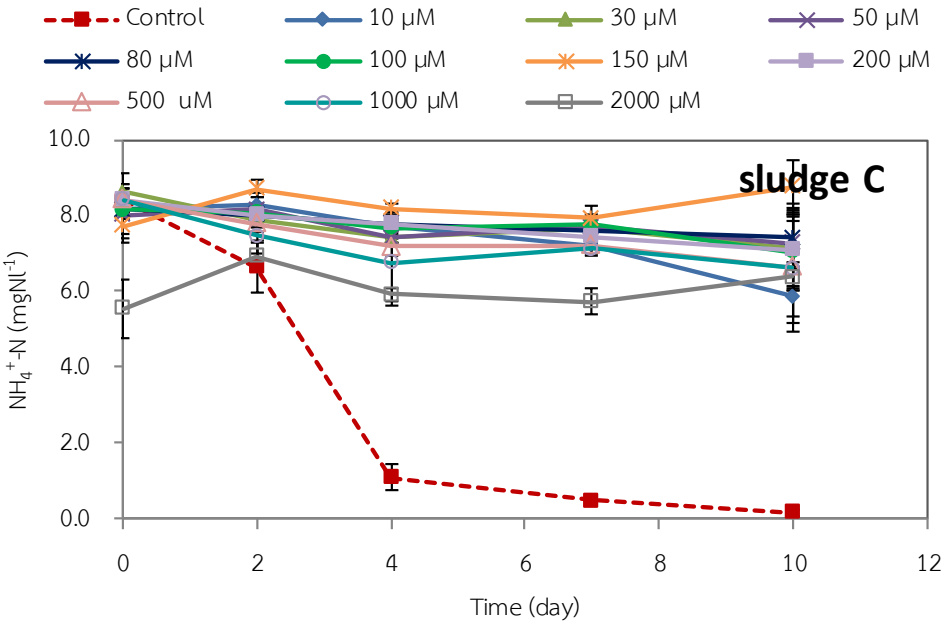


Figure 30 *Change in ammonia concentrations of sludge C*

under the presence of ATU

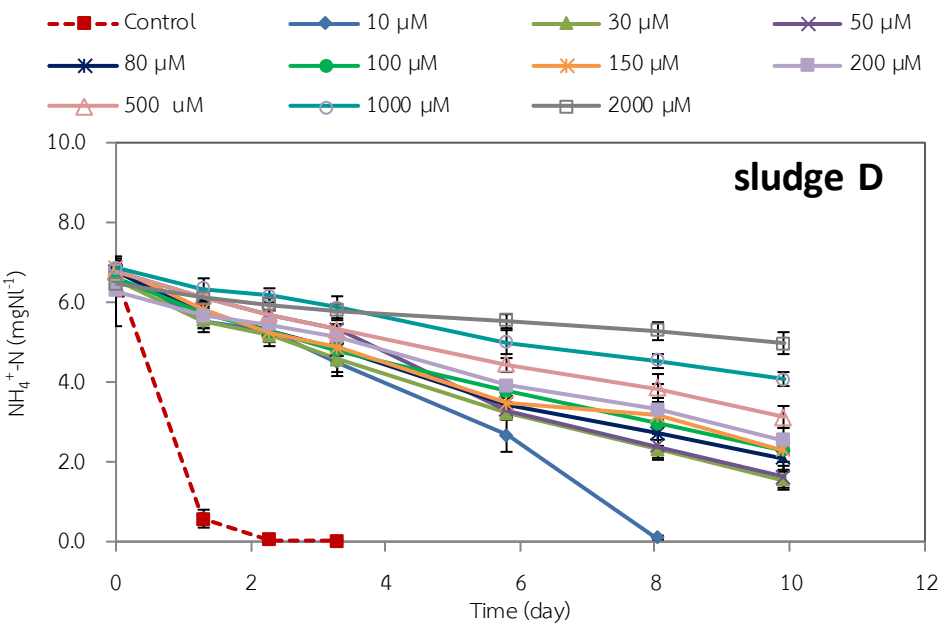


Figure 31 Change in ammonia concentrations of sludge D under the presence of ATU

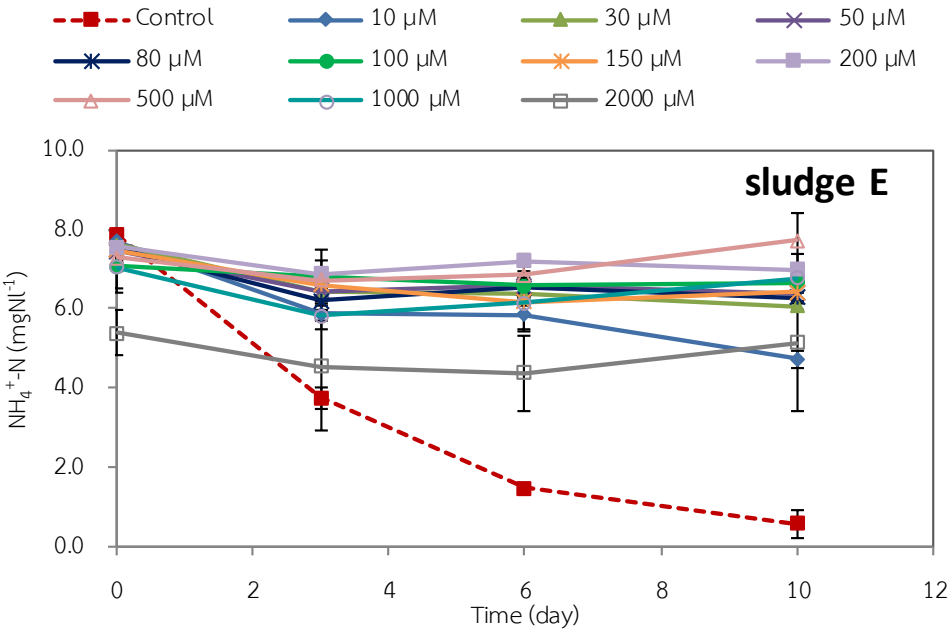


Figure 32 Change in ammonia concentrations of sludge E under the presence of ATU

4.2.3 Contribution of AOA and AOB to ammonia-oxidizing activity in full-scale WWTP sludge

Based on DNA-SIP results in Experiment 1, ATU at the concentration of 80 μM inhibited only the ammonia-oxidizing activity and growth of AOB. Therefore, Figure 33 plots percent inhibition of ammonia oxidation at the ATU concentration of 80 μM and percent AOB in the WWTP sludge. The percent inhibition of ammonia oxidation was calculated by comparing remaining ammonia concentrations in the tests to the control (without adding ATU) at a time an ammonia concentration of the control reached around 0 mgN L^{-1}.

Regarding the percent AOB in the sludge, sludge A contained the highest percentage (100%) followed by sludge B (98.75%), sludge C (78.04%), sludge E (7.20%) and sludge D (0.54%). The percent inhibition of ammonia oxidation was found to relate to the percent AOB in the sludge. Sludge A showed the highest percent inhibition (100%) followed by sludge B (100%), sludge C (92.57%), sludge E (82.25%) and sludge D (78.05%), respectively. The results demonstrated that sludge containing higher percent AOB had more ammonia oxidation inhibition effect.

For plants A, B, and C where AOB were the predominant ammonia-oxidizing microorganisms, AOB played the main role in ammonia-oxidizing activity as demonstrated by applying ATU to the sludge. For plants D and E, where AOA were found to outnumber AOB, AOB also played the main role in ammonia oxidation. AOA also involved in ammonia oxidation but contributed only around 20% as shown in Figure 33. The contribution of AOA to ammonia-oxidizing activity of the sludge is much less than what can be expected from the numbers appeared in the sludge. Previous studies demonstrated that some AOA cultures can maintain life apart from autotrophy because they can utilize organic carbons for cell synthesis as do for heterotrophs (Sauder et al. 2017; Tourna et al. 2011). Growth of some AOA can be stimulated by some organic compounds at low concentrations. For example, addition of 0.1 mM

pyruvate to *Nitrososphaera viennensis* lead to 12 times faster of their growth compared to *Nitrosopumilus maritimus* (Tourna et al. 2011). Sauder et al. (2017) found that the ammonia-oxidizing activity of *Candidatus* Nitrosocosmicus exaquare, which was recently isolated from a WWTP, was encourage by the addition of malate and succinate in inorganic carbon. These two studies indicated that both AOA are mixotrotrophy. It is possible that some organic compounds in plants D and E may stimulate growth of AOA in the system leading to high numbers of AOA in the plants. Previous studies on full-scale WWTPs also showed that AOA in their systems did not assimilate inorganic carbon for growth during ammonia oxidation. Mussmann et al. (2011) showed undetectable of $^{14}CO2$ fixation by AOA in actively nitrifying sludge from a refinery WWTP via FISH combined with microautoradiography. Furthermore, very weak signal of *in situ* transcription of archaeal *amoA* genes was detected after the addition of ammonia. The results indicated that AOA in the plant were unable to fix CO_2 and may not be able to uptake ammonia. Therefore, *amoA*-carrying groupI.1b *Thaumarchaeota* in this study were not obligate chemolithoautotrophs. Sauder et al. (2017) demonstrated that AOA related closely to *Candidatus* Nitrosocosmicus exaquare, that were found in biofilm samples from the WWTP in their studied, did not incorporate bicarbonate as observed by CARD-ISH-MAR. Therefore, they concluded that these AOA may incorporate nonbicarbonate as carbon sources.

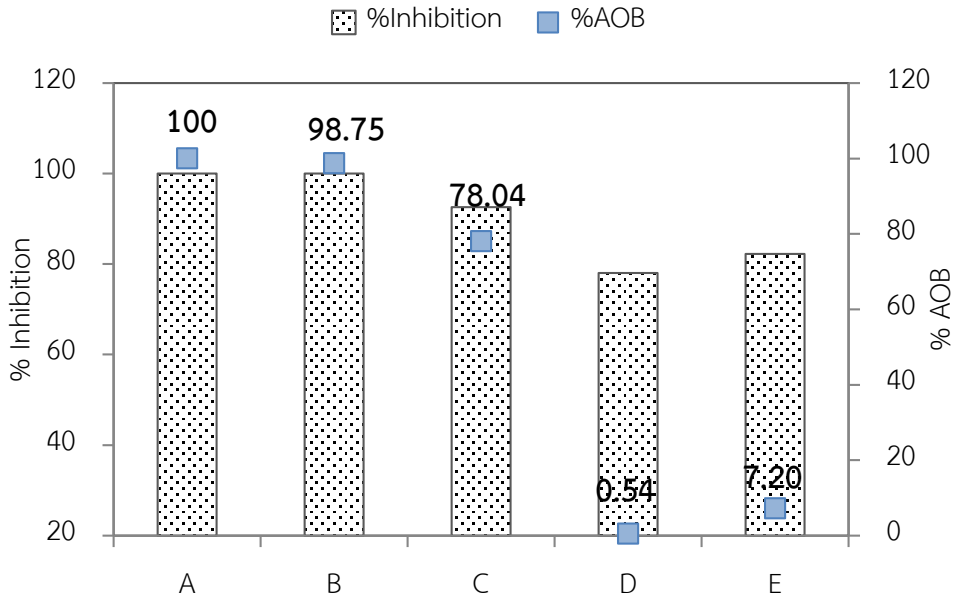


Figure 33 Percent inhibition of ammonia oxidation at the ATU concentration of 80 μM and percent AOB in WWTP sludge.

4.2.4 Findings

AOB played the main role in ammonia oxidation in all sludge. In the sludge that AOA outnumbered AOB, AOA involved around 20% of ammonia oxidation when ATU at the concentration of 80 μM was added.

4.3 Experiment 3: Effect of paranitrophenol (PNP) concentration on ammonia oxidation of sludge from nitrifying reactors

PNP is used in manufacturing processes of drug, pesticide, petroleum refining and leather products. Because of its frequent, widespread usage, PNP can be found in industrial wastewater and contaminated in groundwater and subsurface. For example, PNP can be detected in the final effluent from of a petroleum refining industry (ref). Moreover, this compound was also found in the effluent of municipal wastewater treatment plants; for example, in Sauget, Illinois, Los Angeles and California, at the average concentration of less than 10 μgL^{-1} (ref). According to toxicity of PNP, PNP contaminated in environment can cause adverse effect to living microorganisms and this compound can cause carcinogen and methanogens to the human. U.S.EPA listed PNP as a considering pollutant and restricted the concentration of PNP in natural water to be less than 10 ngL^{-1}. In addition, Industrial effluent should discharge monthly average concentration of PNP of lower than 162 μgL^{-1} (1.9 $mgL^{-1}Y^{-1}$).

PNP can also adversely affect microorganisms in environment. Bhatti et al (2002) reported that PNP at a concentration of 0.5mM (69.5 mgL^{-1}) was toxic to most microorganisms. Sverdrup et al (2002) reported that aromatic compounds can reduce soil nitrification activity; for example, nitrobenzene can inhibit nitrification in soil by acting as the ammonia monooxygenase's suicide substrate. To date, few studies provided knowledge on inhibitory effect of PNP on ammonia oxidation. Therefore, the threshold of PNP concentration that inhibits ammonia oxidizing is yet clearly clarified.

In Experiment 3, effect of PNP concentration on ammonia oxidation was studied with sludge from NRI and NRII. The sludge from NRI and NRII was first quantified for the numbers of AOA and AOB *amoA* genes by qPCR, characterized for microbial communities using Miseq (next generation sequencing), and analyzed for active nitrifying microorganisms using fluorescence in situ hybridization (FISH). Inhibitory effect on ammonia oxidation was test with PNP in a range of 1-400 mgL^{-1} under initial ammonia concentrations of 7, 14, and 70 $mgNL^{-1}$. Then, some selected PNP concentrations was used to observe cell viability and active nitrifying microorganisms after PNP exposure.

4.3.1 Nitrifying reactor

4.3.1.1 Numbers of AOA and AOB amoA genes in nitrifying reactors

Results from Experiment 1 indicated that AOA and AOB coexisted in NRI during the study period of 14 months. Only AOB could be detected in NRII during the study period of 7 months. Because Experiment 3 was conducted around 15 months after Experiment 1, the numbers of AOA and AOB *amoA* genes in NRI and NRII were analyzed again to confirm stability of cultures between the two experiments. Figure 34 shows the numbers of both microorganisms' *amo*A genes in the nitrifying reactors at the beginning of Experiment 3. The results showed that AOA and AOB still coexisted in NRII and the numbers of both microorganisms' *amo*A genes were comparable. AOB still the only ammonia-oxidizing microorganism detected in NRII. The results indicated no major change in *amoA* gene numbers of both microorganisms in both reactors between Experiments 1 and 3.

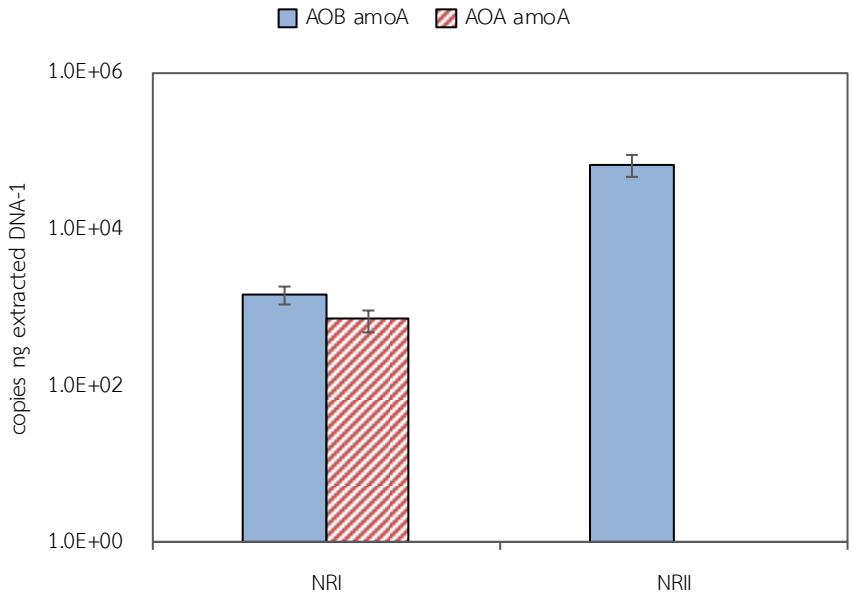


Figure 34 Numbers of AOA and AOB *amoA* genes in nitrifying reactors at the beginning of Experiment 3

4.3.1.2 Characterization of microbial communities in nitrifying reactors

Microbial communities in NRI and NRII were characterized by Miseq. The primer set used for PCR amplification was modified by Ding et al. (2015) to target 16S rRNA genes of microorganisms within both *Bacteria* and *Archaea* domains. In total, 416329 reads can be retrieved from NRI and NRII samples, 238277 and 178052 read for NRI and NRII, respectively. The analyzed sequences were calculated for an arrangement of operational taxonomic unit (OUT) at 97% similarity using SUMACLUST. After the construction of OTU, 21686 sequences per sample were randomly subsampled for analysis of microbial community by using QIME version 1.9.1 (Caporaso et al. 2010).

Figure 35 shows diversity of microorganisms in phylum level. *Nitrospirae* was the largest phylum found sharing 29.8% of the sequences analyzed for NRI. *Proteobacteria*, *Crenarchaeota*, and *Panctomycetes* shows 21.8%, 16%, and 12.5% of the sequence analyzed, respectively. Proteobacteria comprised 49.9% of the sequence analyzed in NRII, followed by Bacteroidetes (24.7%) and Acidobacteria (9.4%), respectively. Nitrospirae is a phylum of which nitrite-oxidizing bacteria, genus *Nitrospira* are members. β-AOB are placed in subphylum *β-Proteobacteria* within phylum *Proteobacteria*. AOA have been recently placed in phylum *Thaumacheota* which was split out from phylum *Crenarchaeota*. However, this analysis was performed based on Greengenes database. For this database, *Thaumacheota* may not classified as the other phylum yet.

Figure 36 shows diversity of microorganisms in genus level. *Nitrospir*a was dominant genus found in NRI (29.8%). *Nitrospir*a is known as nitrite-oxidizing bacteria who oxidize nitrite to nitrate under aerobic condition. Nitrite is a product of ammonia oxidation; therefore, it is not surprised to have this genus as the dominant genus in this ammonia-fed reactor. *Nitrospir*a is often found in systems where nitrite concentration is low (0.1–5 $mgNL^{-1}$; Whang et al, 2009). In NRI, no nitrite accumulation was observed. The second dominant genus in NRI was *Candidatus* Nitrososphaera (16%). As described in Experiment 1, all AOA *amoA* sequences retrieved from NRI felled within *Nitrososphaera* sister cluster. The Miseq result confirmed the presence of AOA of *Nitrososphaera* cluster in NRI. In addition, Miseq also confirmed that AOA of this cluster were the dominant ammonia-oxidizing microorganisms in NRI which

corresponded to the results of long-term monitoring of *amoA* genes of AOA and AOB in Experiment 1. Miseq suggested that AOB found in NRI was in genus Nitrosomonadaceae which was accounted for only 0.5% of the sequences analyzed. However, genus Nitrosomonadaceae was found to be dominant in NRII as it was accounted for 26.2% of the sequences analyzed. NRII was fed with high influent ammonia concentration of 420 $mgNl^{-1}$. This may lead to high abundance of AOB. Sequence of *Candidatus* Nitrososphaera was not found in NRII. The results were well fit with qPCR in Experiment 1 and 3 and AOA *amoA* gene sequence analysis in Experiment 1 that AOA *amoA* gene sequence cannot be detected in this reactor.

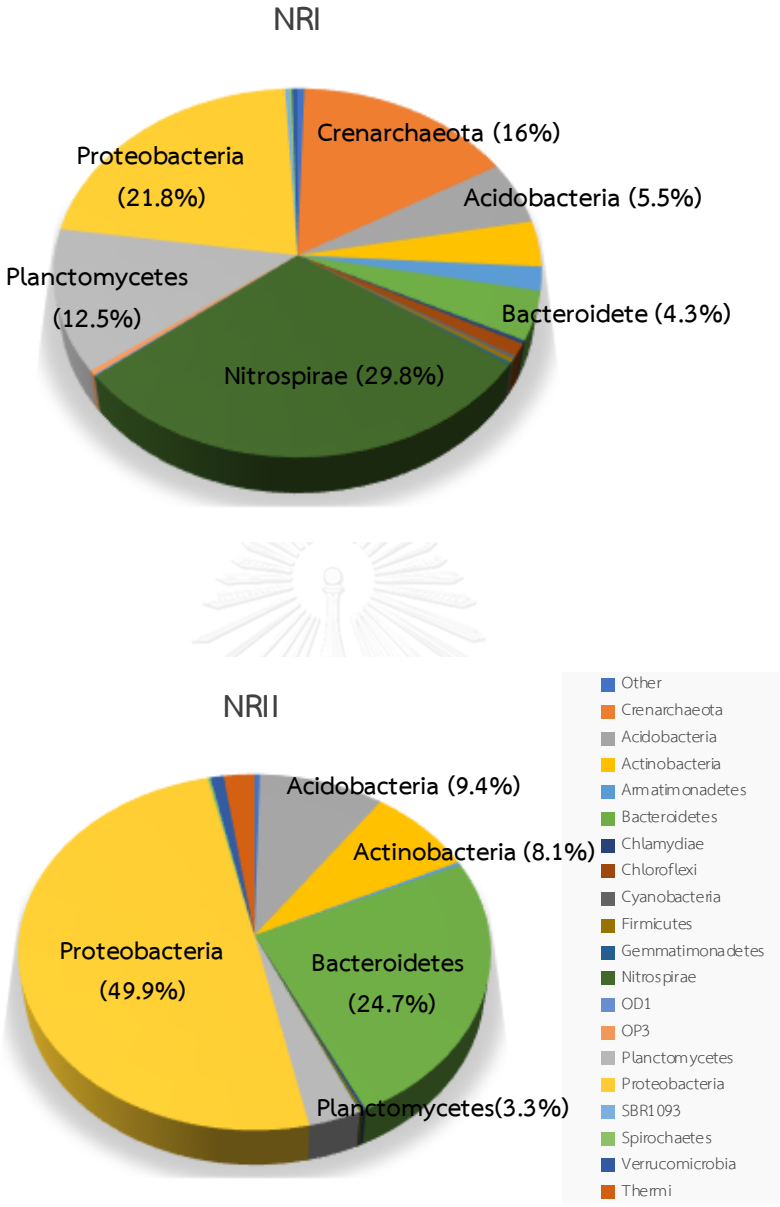


Figure 35 Diversity of microorganisms in Phylum level

(a) NRI and (b) NRII

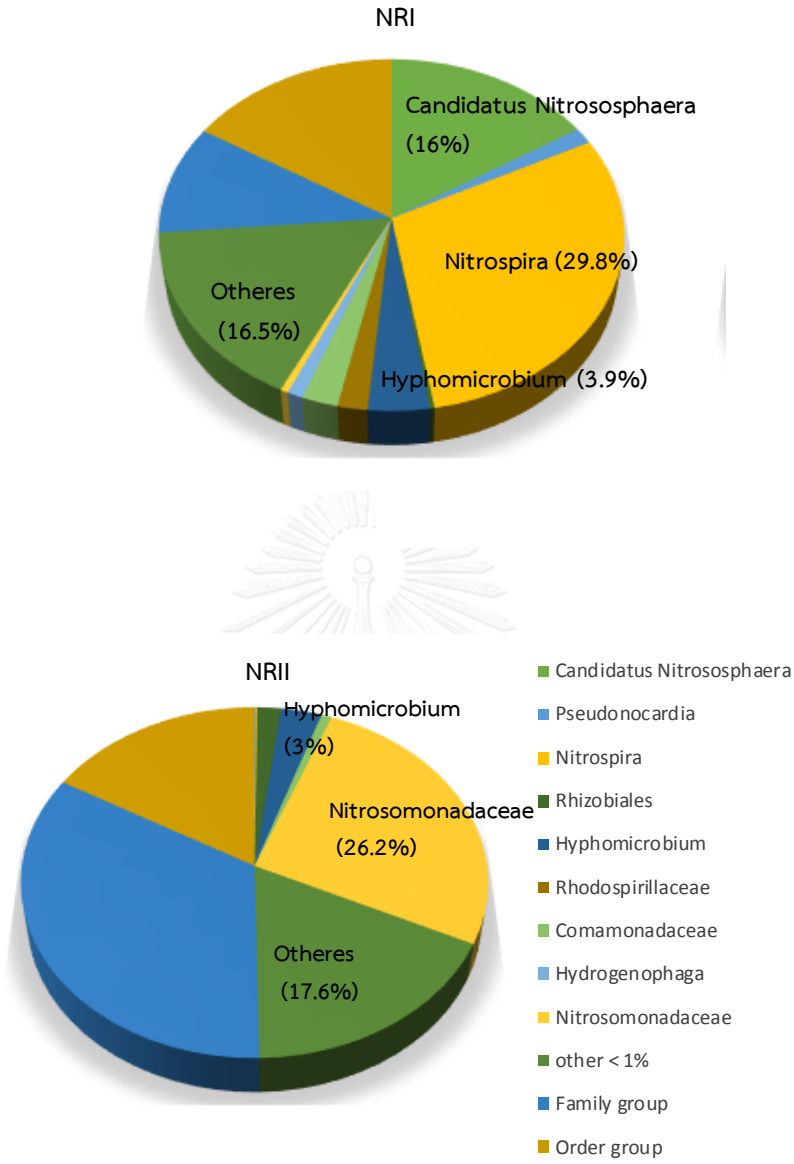


Figure 36 Diversity of microorganisms in genus level

(a) NRI and (b) NRII

4.3.1.3 Composition of active nitrifying microorganisms in NRII

A composition of active ammonia oxidizng bacteria and nitrite oxidizng bacteria in NRII were analyzed by FISH with 16S rRNA-targeted oligonucleotide probes (Figure 37). Examples of FISH-CLSM images are shown supplement C. DAPI staining total microorganisms was shown in blue, probe NSO190 hybridized AOB in green, probe Ntspa 662 hybridized with *Nitrobacter* in red and probe NIT3 hybridized with *Nitrospira* in red. The area from around 10 FISH-CLSM images of each target microorgsnism was calculated. Figure in 37 showing that AOB was 48 ± 12.7% and NOB including *Nitrobacter* and *Nitrospira* were 15 ± 9.9% and 13.8 ± 7.7%, respectively. This results clearly indicated that AOB was dominated active species in this sludge.

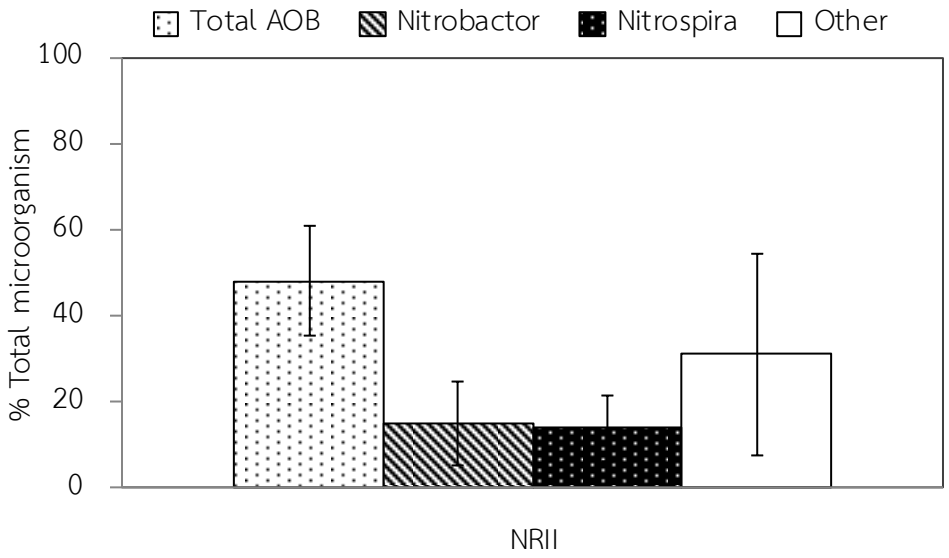


Figure 37 Composition of active microbial comminity in NRII

4.3.2 Effect of PNP concentration on ammonia-oxidizing activity of NRI sludge

Ammonia- oxidizing activity of NRI sludge was investigated at PNP concentrations ranging from 1 to 400 mgL^{-1} and initial ammonia concentration of 7 $mgNL^{-1}$ (Figure 38). Percent inhibition of ammonia oxidation was plotted as shown in Figure 39. A PNP concentration of 1 mgL^{-1} shows no inhibition of ammonia oxidation since the result indicated similar tendency to the control without PNP. Ammonia oxidation was partially inhibited at PNP concentrations of 5 and 10 mgL^{-1} which can be accounted as 91.21 ± 9.51% and 89.64 ± 9.19% inhibition, respectively. PNP concentrations from 50 mgL^{-1} exhibited nearly complete inhibition of ammonia oxidation. The percent inhibition was 95.72 ± 4.93%, 97.12 ± 2.51%, 100% and 100% for PAP concentrations of 50, 100, 200, and 400 mgl^{-1}, respectively.

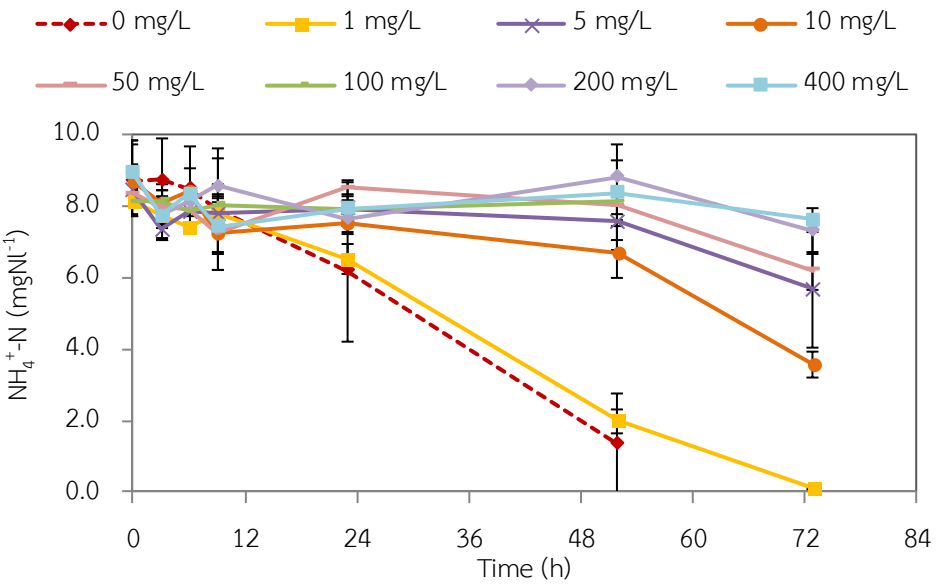


Figure 38 Change in ammonia concentrations under the presence of various PNP concentrations for NRI sludge at the initial ammonia concentration of 7 $mgNl^{-1}$

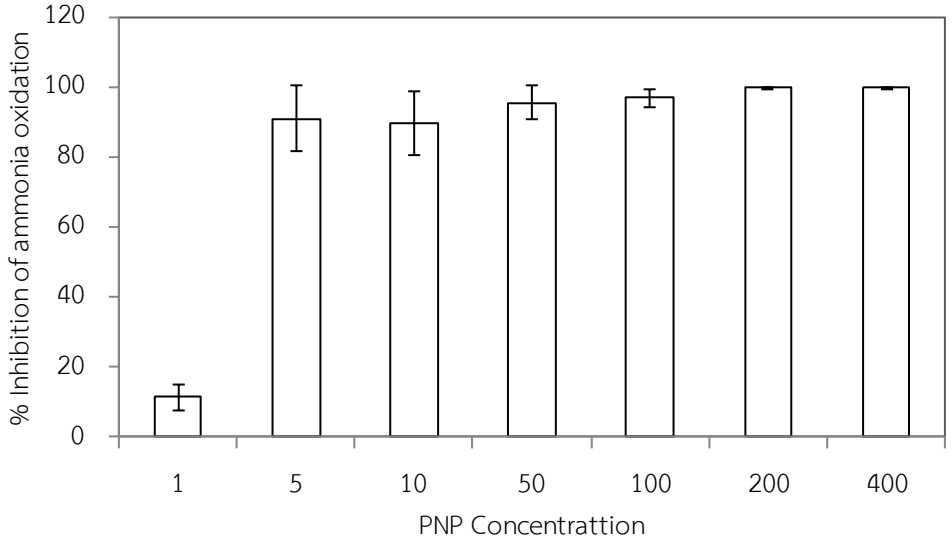


Figure 39 Percent inhibition of ammonia oxidation for NRI sludge at the initial ammonia concentration of 7 mgNl^{-1}

4.3.3 Effect of PNP concentration ammonia-oxidizing activity of NRII sludge

A PNP concentration range of 1-2000 mgL^{-1} was applied to NRII sludge for tests of ammonia-oxidizing activity. The initial ammonia concentration of the tests was varied at 7, 14 and 70 mgNL^{-1}.

4.3.3.1 Initial ammonia concentration of 7 mgNL^{-1}

Figure 40 presents change in ammonia concentration under the presence of PNP concentrations of 1-400 mgNL^{-1}at the initial ammonia concentration of 7 mgNL^{-1}. The percent inhibition was plotted as shown in Figure 41. The control with no PNP addition showed ammonia concentration reduction to nearly 0 mgNl^{-1} within 24 h. PNP concentrations of 1-10 shows similar ammonia-oxidizing activity to the control with percent inhibition of less than 20% as compared to the control. Partial inhibition of ammonia oxidation of 20-80% was found with PNP concentrations ranging from 5-50 mgL^{-1}. Nearly complete inhibition with more than 80% was found for PNP concentrations of 100-400 mgNL^{-1}.

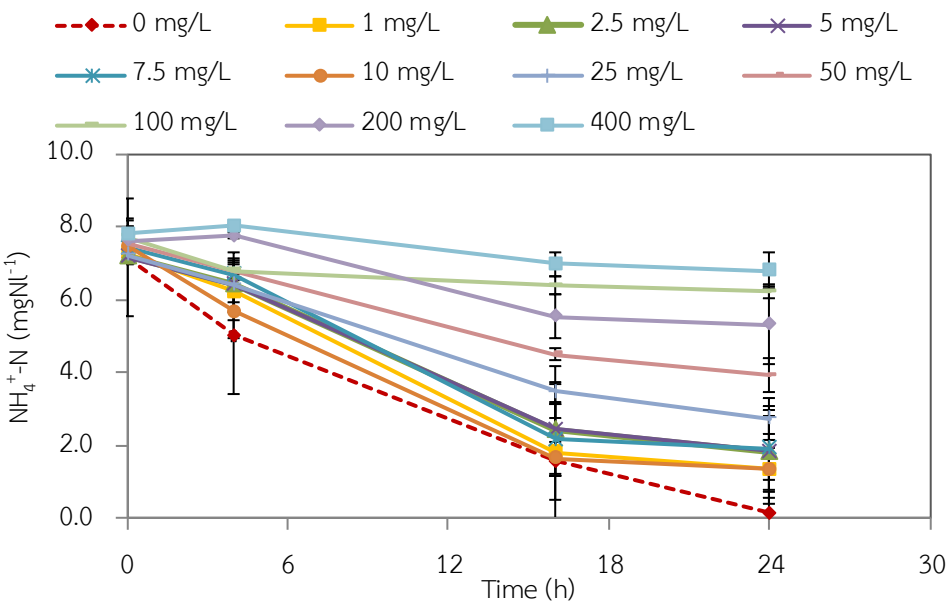


Figure 39 Change in ammonia concentrations under the presence of various PNP concentrations for NRII sludge at the initial ammonia concentration of 7 $mgNL^{-1}$

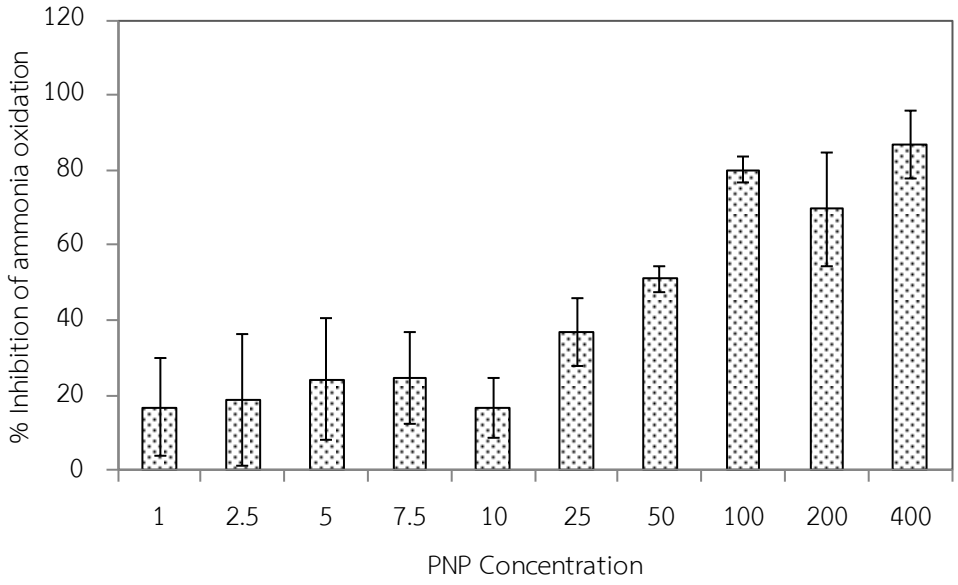


Figure 40 Percent inhibition of ammonia oxidation for NRI sludge at the initial ammonia concentration of 7 $mgNL^{-1}$

4.3.3.2 Initial ammonia concentration of 14 mgNL^{-1}

Figure 41 and Figure 42 show change in ammonia concentration and percent inhibition of ammonia oxidation, respectively, under various PNP concentrations at initial ammonia concentration of 14 mgNL^{-1}. PNP concentrations of 1 and 5 mgL^{-1} showed no effect on ammonia oxidation as the percent inhibition was 7.5 ± 14.63 % and 7.52 ± 15.91%, respectively. Partial inhibition of 34.87 ± 2.13% was found for PNP at 10 mgL^{-1}. Nearly complete inhibition with >81.55 % occurred at PNP concentrations of >50 mgL^{-1}.

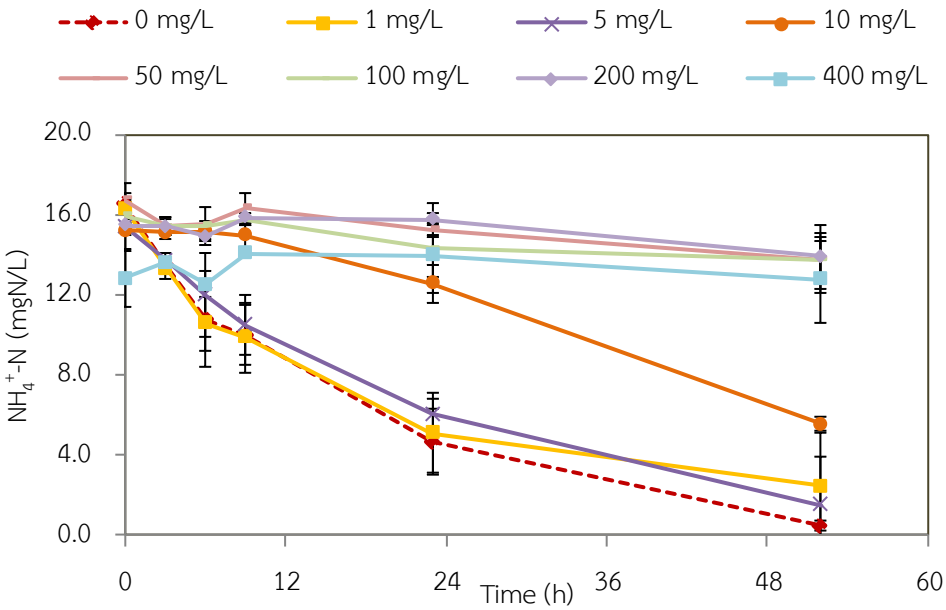


Figure 41 Change in ammonia concentrations under the presence of various PNP concentrations for NRII sludge at the initial ammonia concentration of 14 mgNL^{-1}

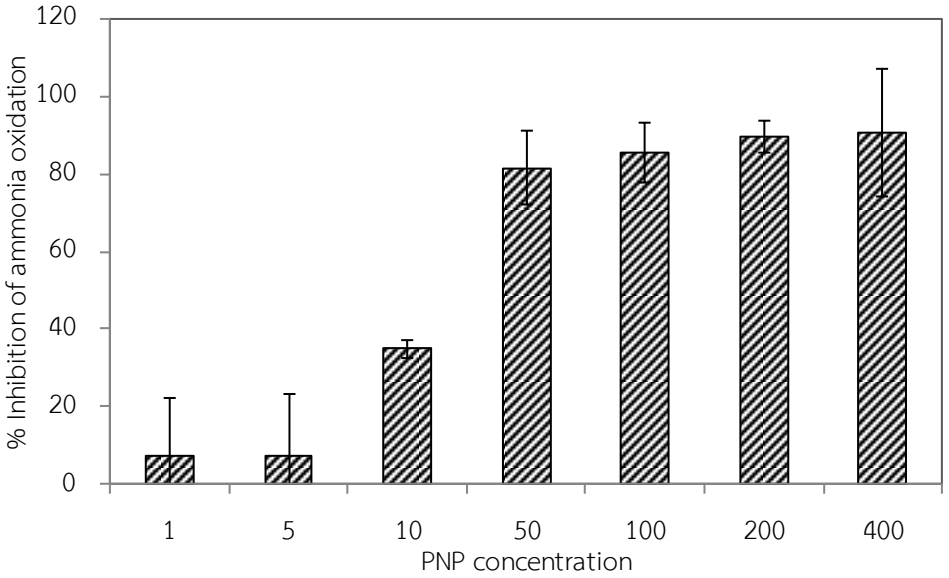


Figure 42 Percent inhibition of ammonia oxidation for NRII sludge at the initial ammonia concentration of 14 mgNL^{-1}

4.3.3.3 Initial ammonia concentration of 70 mgNL^{-1}

Figure 43 exhibits change in ammonia concentration at various PNP concentrations and initial ammonia concentration of 70 mgNL^{-1}. Figure 44 shows the percent inhibition of the tests. PNP concentrations of 5 and 10 mgL^{-1} demonstrated less than 20% inhibition of ammonia oxidation as compared to the control without PNP. This indicated that this range of PNP concentrations had slight effect on ammonia oxidation. More than 80% inhibition was found for PNP concentrations of >50 mgL^{-1}.

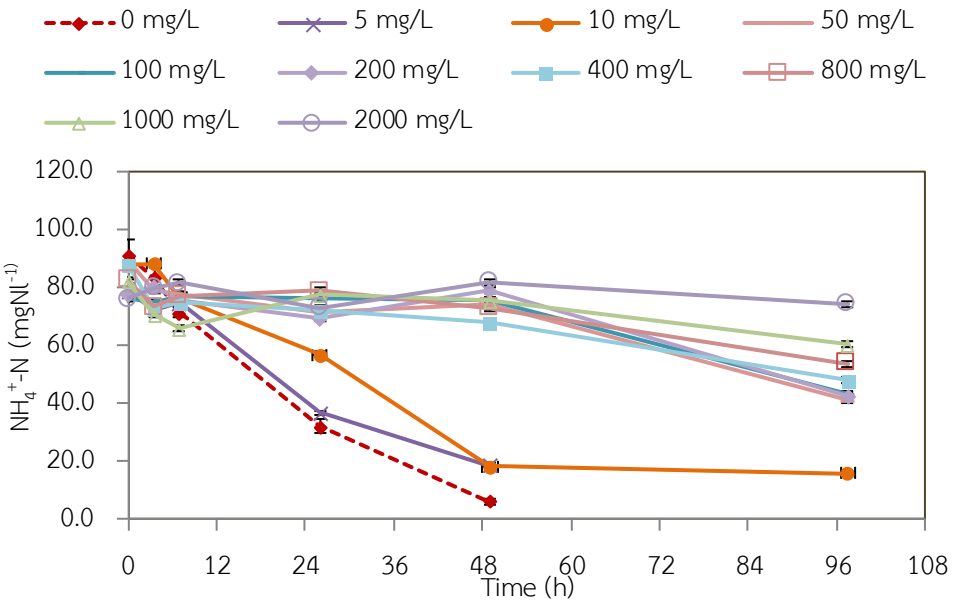


Figure 43 Change in ammonia concentrations under the presence of various PNP concentrations for NRII sludge at the initial ammonia concentration of 70 $mgNL^{-1}$

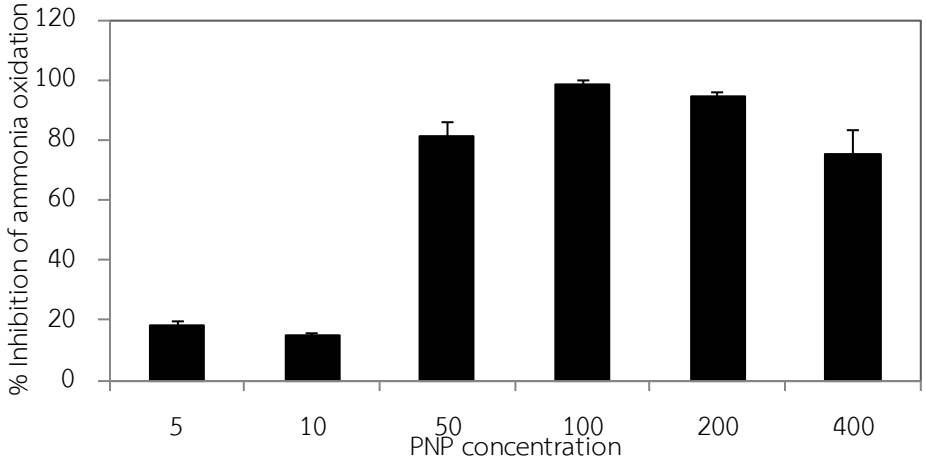


Figure 44 Percent inhibition of ammonia oxidation for NRII sludge at the initial ammonia concentration of 70 $mgNL^{-1}$

4.3.4 Percent inhibition and rate of ammonia oxidation of NRII sludge under various PNP and ammonia concentrations

Figure 45 plots together the percent inhibition of ammonia oxidation of NRII sludge at various concentrations of PNP (5-400 mgL^{-1}) and initial ammonia (7, 14 and 70 $mgNL^{-1}$). The results showed that PNP of 400 mgL^{-1} exhibited the highest inhibition of ammonia oxidation at all initial ammonia concentrations, follow by PNP of 200 mgL^{-1}, PNP of 100 mgL^{-1}, PNP of 50 mgL^{-1}, PNP of 10 mgL^{-1}, and PNP of 5 mgL^{-1}, respectively. Figure 46 presents the rate of ammonia oxidation under various PNP and initial ammonia concentrations. The results showed that rate of ammonia oxidation of the control without PNP was the highest followed by the rate for PNP at 5-50 mgL^{-1}, 100-200 mgL^{-1}.

Taken both together, the results strongly indicated that PNP inhibited ammonia oxidation in term of both amount and rate. The inhibitory effect was PNP concentration dependent. For percent inhibition, more than 80% inhibition was observed with PNP concentrations from 100 mgL^{-1}. Partial inhibition between 20-80% arose at PNP concentrations between 50 and 10 mgL^{-1}. When PNP concentrations were less than 5 mgL^{-1}, the percent inhibition was less than 20%. This is quite corresponded to the rate of ammonia oxidation. Sharp decline of ammonia oxidation rate can be observed at the PNP concentrations of 5-50 mgL^{-1}. No change in ammonia oxidation rate occurred at PNP concentrations of 100-200 mgL^{-1}. Volskay et al (1990) reported that the 50% effective concentration (EC_{50}) of PNP was 64 mgL^{-1}. Therefore, PNP concentration of ≥ 50 mgL^{-1} tended to inhibit ammonia oxidation more than at the concentration of ≤10 mgL^{-1}. Zhang et al. (2010) tested inhibitory effect of aromatic compounds, including PNP, on nitrification in soil samples. They found that this chemical could inhibit nitrification up to approximately 60% and 70% for the addition of 13.9 and 69.5 mg PNP kg^{-1}soil. In 2014, Guo et al. (2014) studied short-term effect of nitrophenol on WWTS sludge enriched for more than 200 days. The impact of exposure was tested by

adding PNP only one time. They found that biomass concentration decreased, sludge deflocculated, COD also increased, and ammonia concentration accumulated. The concentration of PNP profile was 6-34 mgL^{-1} along the test. The results indicated that PNP was able to inhibit ammonia- oxidizing activity and growth by affecting the population size of *Nitrosomonas* which was the main ammonia oxidizers in the system.

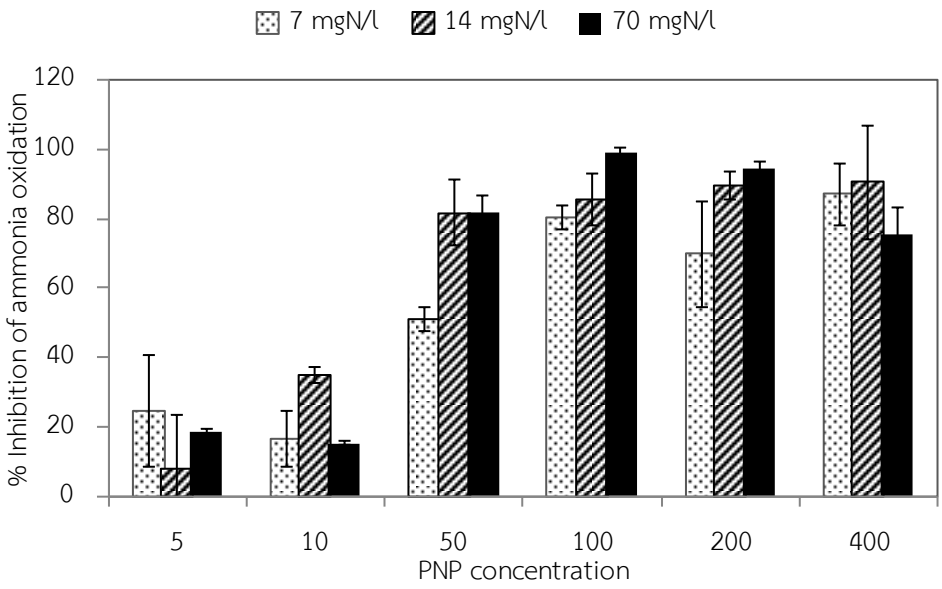


Figure 45 Percent inhibition of ammonia oxidation of NRII sludge at various PNP and initial ammonia concentrations

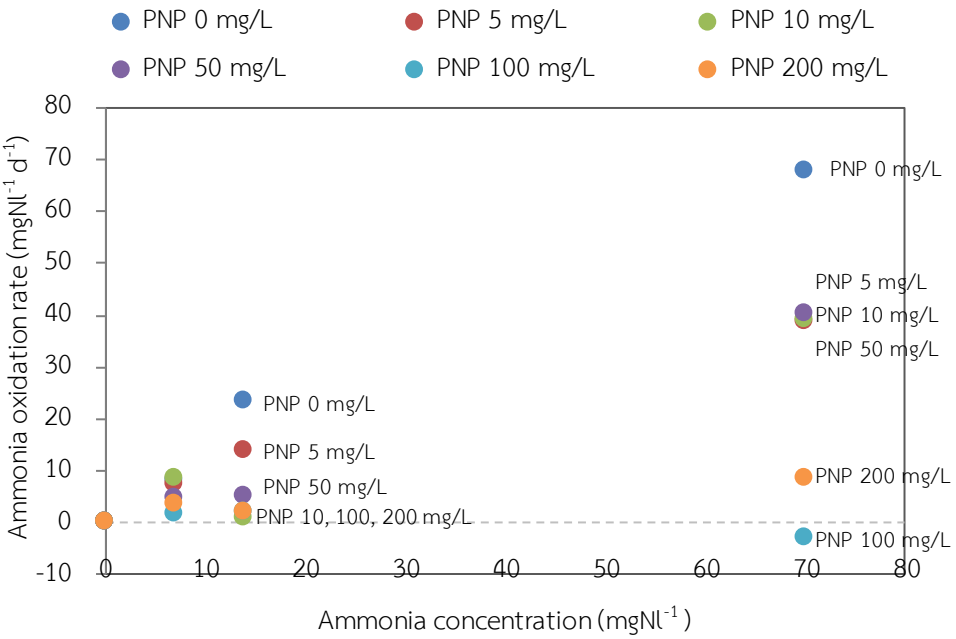


Figure 46 Ammonia oxidation rate of NRII sludge at various PNP and initial ammonia concentrations

4.3.5 Effect of PNP on bacterial cell viability and active nitrifying microorganisms in NRII sludge

Bacterial cell viability and active nitrifying microorganisms were observed after exposing NRII sludge to PNP at concentrations of 10 and 200 mgL^{-1}. The 10 and 200 mgL^{-1} PNP was selected for the tests because these PNP concentrations showed slight and full inhibition of ammonia oxidation in the earlier parts. An initial ammonia concentration selected for the tests was 70 $mgNL^{-1}$.

Figure 47 shows change in ammonia concentration during the tests. Comparing to the control with no PNP addition, ammonia–oxidizing activity slightly dropped in the 10 mgL^{-1} PNP test and the activity was largely inhibited at the PNP concertation of 200 mgL^{-1}.

Percent damage of cell membrane resulted from exposure to PNP at 48h was showed in Figure 48 and examples of CLSM images of membrane integrity of

bacterial cells was provided in Figure 49. According to Figure 48, the damage of membrane integrity of bacterial cells treated with PNP concentrations at 0, 10, and 200 mgL^{-1} approximately accounted 35%, 33% and 49%, respectively.

Figure 50 shows compositions of active nitrifying microorganisms exposed to PNP for 48h. FISH relies on 16S rRNA detection; therefore, the technique reflects availability of active microorganisms in a system. Figure 50 shows that percent total AOB tended to decrease from the control (60.1 ± 6%) after exposing the sludge to PNP at 10 mgL^{-1} (40.3 ± 12.3%), and 200 mgL^{-1} (35.5 ± 17.6%).

Taken together, the results indicated that exposing PNP to NRII sludge reduced the ammonia-oxidizing activity of the sludge. Active AOB and bacterial cells in the sludge also tended to reduce after exposing the sludge to PNP. However, the reduction of these two parameters may not be statistically meaningful as shown by Y bar errors. In addition, high portion of active AOB and bacterial cells were still available in the sludge. Therefore, ammonia-oxidizing activity may be able to recover after PNP was removed. Former studies indicated that PNP acted as the ammonium monooxygenase's suicide substrate so it could inhibit ammonia oxidation process (Sverdrup et al. 2002). However, the effect of PNP on cells is unclear.

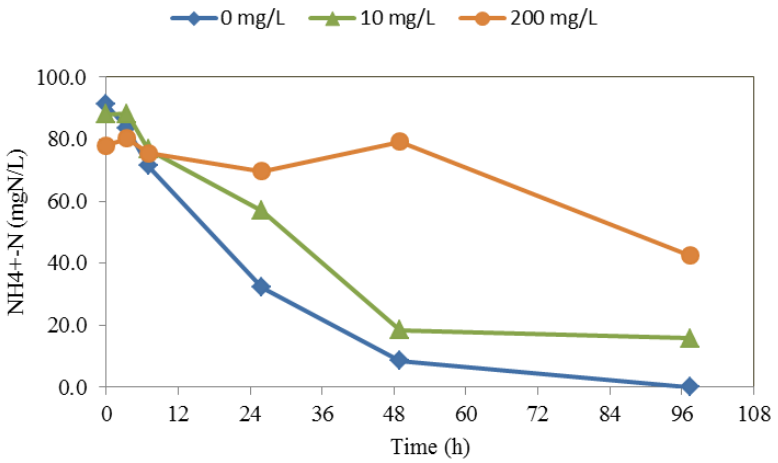


Figure **47** Change in ammonia concentrations during cell viability test

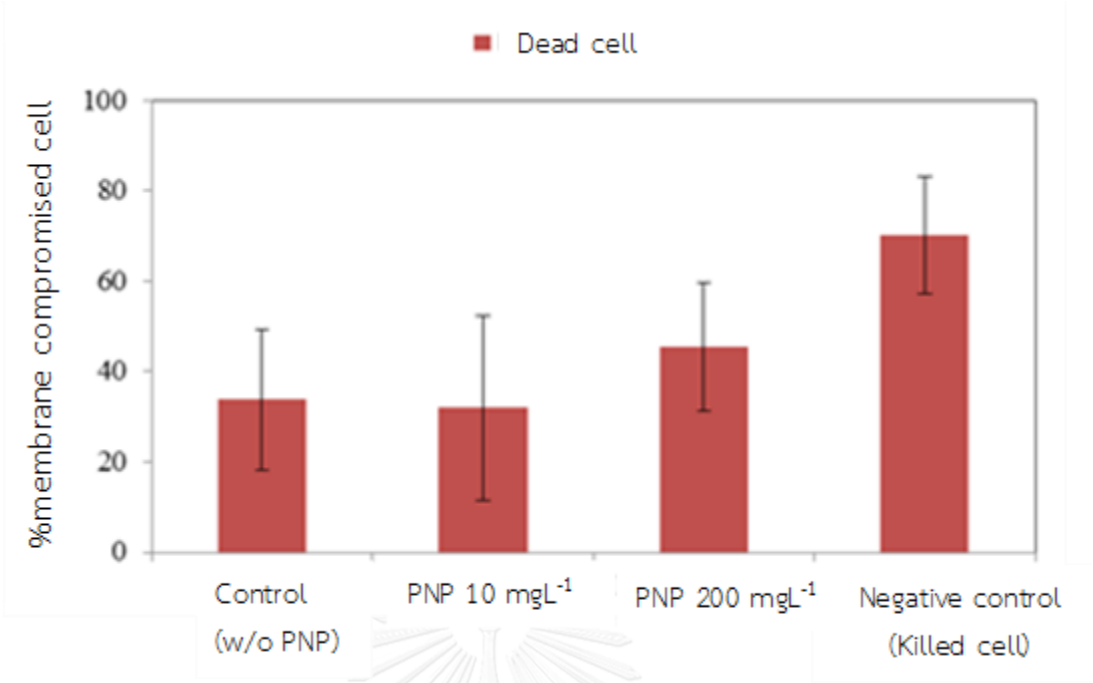


Figure 48 Percent damage of cell membrane resulted from exposure to PNP at 48h

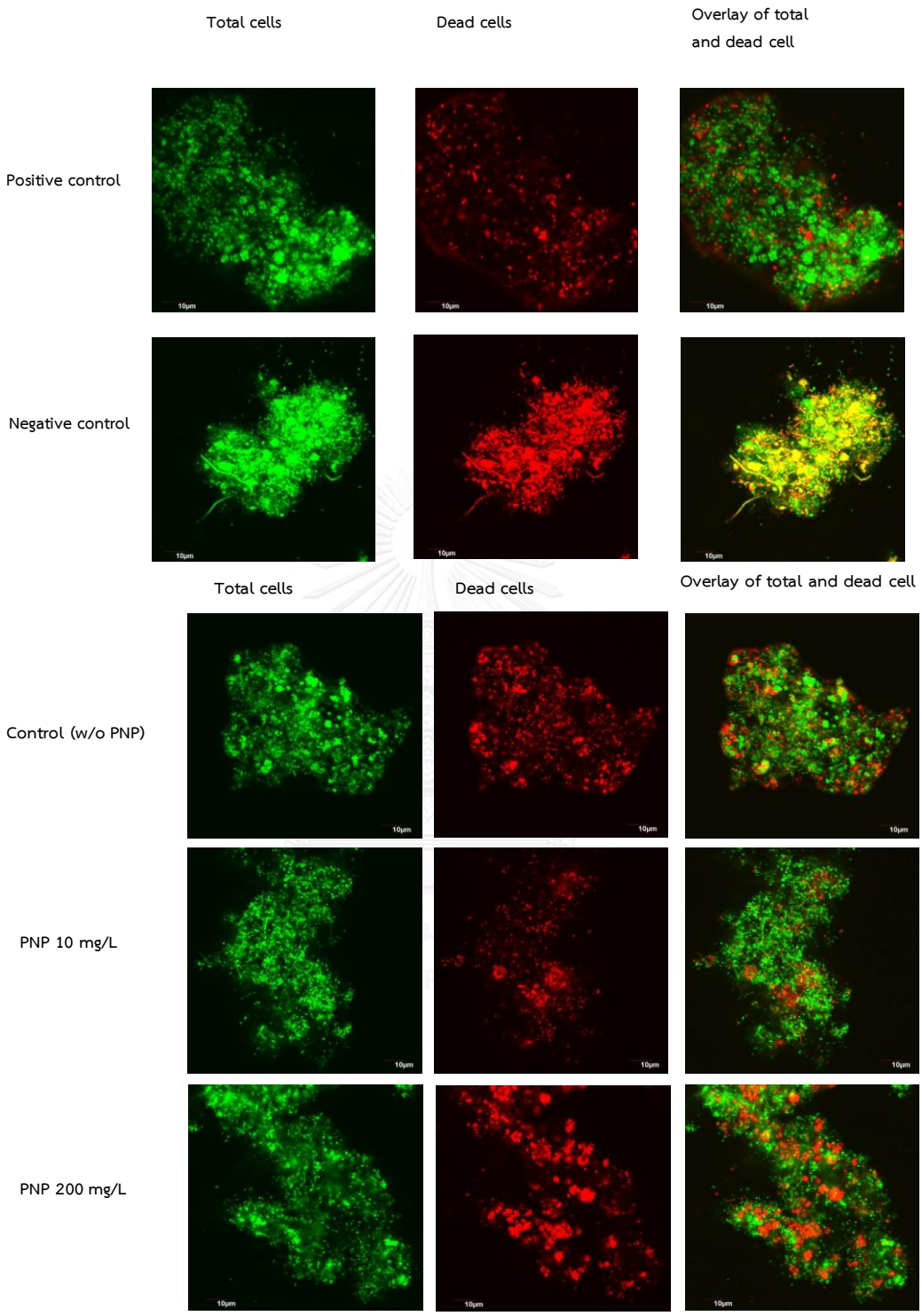


Figure 49 Example of CLSM images of membrane integrity of bacterial cells exposed to PNP at 48h

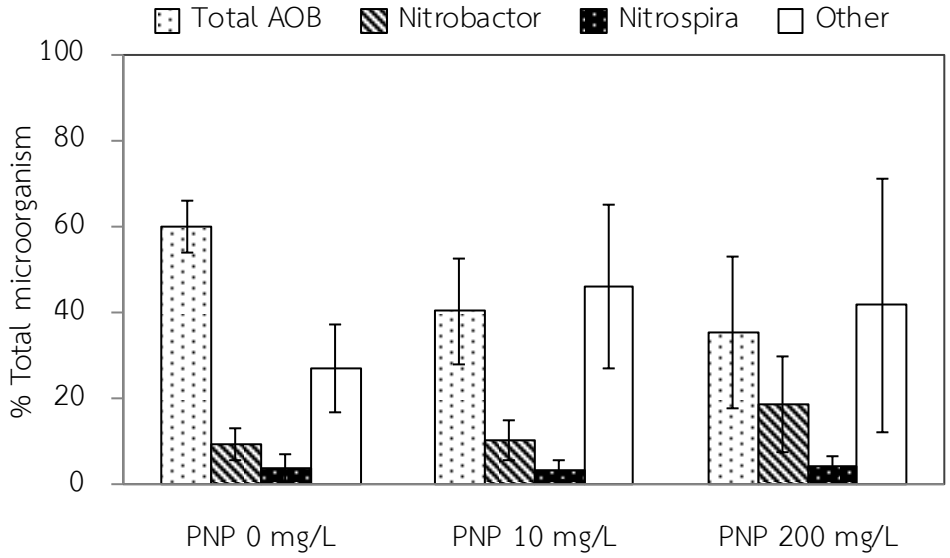


Figure 50 Composition of active nitrifying microorganisms exposed to PNP for 48h

4.3.6 Impact of PNP on population and cell structure of ammonia oxidizers in sludge NRI and NRII

Figure 54 shows percent inhibition of ammonia oxidation of NRI and NRII under various concentrations of PNP (1-400 mgL^{-1}). For NRI, the percent inhibition reached 80%, when PNP concentrations were higher than 5 mgL^{-1}, suggesting that ammonia-oxidizing activity was nearly completely inhibited from a low concentration range. The percent inhibition reached 80% level at PNP concentrations of higher than 100 mgL^{-1}.

The results indicated that NRI sludge was more sensitive to PNP than NRII sludge. Both reactors contained different proportion of AOA and AOB. AOA contributed around 67% of ammonia- oxidizing microorganisms in NRI, while no AOA can be detected in NRII. It may be implied that PNP was more toxic to AOA than AOB. However, until now no study on the effect of PNP on AOA had been available in literature. Differences in cell structure or ammonia oxidation partway may result in susceptibility of both microorganisms on PNP.

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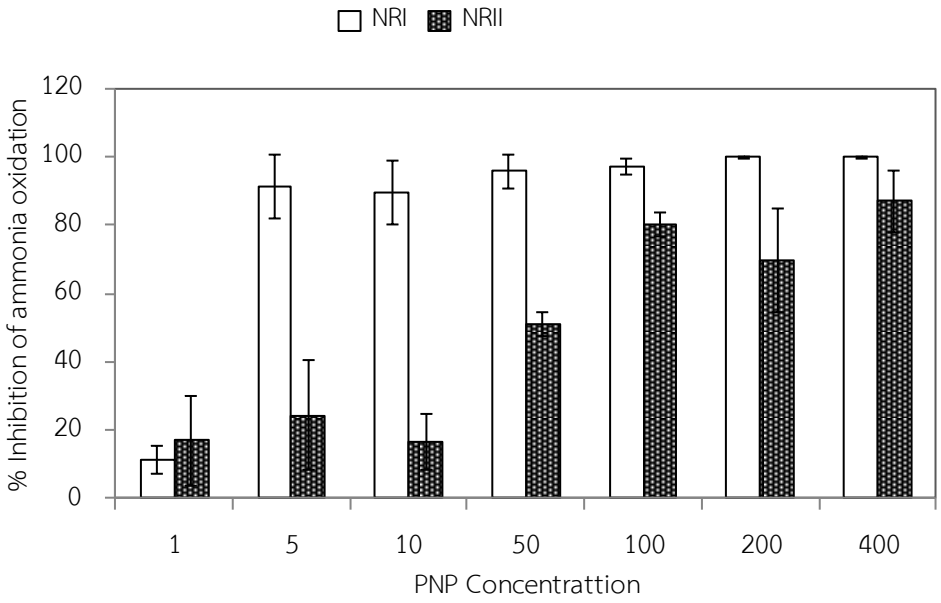

Figure 51 Comparison of percent inhibition of ammonia oxidation for NRI and NRII sludge at the initial ammonia concentration of 7 mgNL^{-1}

4.3.7 Finding

Our results demonstrated that the inhibitory threshold of PNP on ammonia oxidation in NRII. PNP at concentration of ≥ 50 completely inhibition of ammonia oxidation. Base on viability and active community analysis, showing that at low and high concentrations of PNP (10 mgL^{-1} and 200 mgL^{-1}, respectively) stop the activity of AOB, not kill cell.

Chapter V

CONCLUSION AND SUGGESTIONS

5.1 Conclusion

Two laboratory nitrifying reactors, NRI and NRII, were initiated by seed sludge taken from different WWTPs and were operated with different operating conditions leading to distinct proportions of ammonia-oxidizing microorganisms (ammonia oxidizing archaea; AOA and ammonia oxidizing bacteria; AOB) existing in the reactors. AOA *amoA* genes were on average 16 times outnumbering AOB *amoA* genes in NRI, while only AOB *amoA* genes were detectable in NRII. All AOA *amoA* gene sequences found in NRI related closely to the *Nitrososhaera* sister cluster within the Group 1.1b *Thaumacheota*. AOB *amoA* gene sequences retrieved from NRI and NRII belonged to the *Nitrosomonas europaea* cluster, *Nitrosomonas communis* cluster, and *Nitrosomonas oligotropha* cluster.

Study on inhibitory effect of various allylthiourea (ATU) concentrations (10-2000 µM) on ammonia-oxidizing activity of AOA and AOB in NRI and NRII revealed that ATU at the concentrations of 10-200 µM can be used to observe the ammonia-oxidizing activity of AOA in the sludge in which AOA and AOB coexisted. At this concentration range, ammonia-oxidizing activity of AOB was inhibited completely. Moreover, DNA Stable isotope probing (DNA-SIP) confirmed that ATU at the concentration of 80 µM mainly inhibited growth of only AOB, not AOA. 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO) at concentrations of up to 300 µM uninhibited ammonia-oxidizing activity of AOB. However, PTIO at the concentration of 100 µM completely inhibited the ammonia-oxidizing activity of AOA.

Regarding ATU and PTIO tests the results revealed that AOA an AOB contributed to ammonia oxidation in NRI sludge, while only the contribution of AOB was observed in NRII sludge. Appling DNA-SIP with ^{13}C-HCO_3^- to NRI sludge demonstrated the incorporation of ^{13}C into *amoA* genes of AOA and AOB. The results implied that AOA

and AOB can perform autotrophy during ammonia oxidation as a choice of their life. Taken together, the results from this part provide direct evidence demonstrating the contribution of AOA and AOB to ammonia oxidation in the nitrifying reactors.

ATU (10-2000 μM) was applied to observe the ammonia-oxidizing activity of AOA in 5 full-scale WWTP sludge. The 5 sludges possessed different ratios of AOA and AOB *amoA* gene numbers. Applying ATU to one WWTP sludge, in which AOB were the only detectable ammonia oxidizer, confirmed that ATU at concentrations >50 μM provided complete inhibition to ammonia oxidation of AOB. Regarding the ammonia-oxidizing activity, AOB played the main role in ammonia oxidation in all 5 sludge. In the sludge that AOA outnumbered AOB, AOA involved around 20% of ammonia oxidation when 80 μM ATU was added to the test.

Inhibitory effect of PNP (1-400 mgL^{-1}) on ammonia oxidation was studied with NRI and NRII sludge. The results demonstrated that NRI (≥ 5 mgL^{-1} PNP for complete inhibition) was more sensitive to PNP centration than NRII sludge (≥ 50 mgL^{-1} PNP for complete inhibition). The inhibitory threshold concentration of PNP was at 50 mgL^{-1} for complete inhibition of ammonia oxidation. Based on the analyses of bacterial cell viability and active nitrifying microorganisms, PNP at the concentrations of 10 and 200 mgL^{-1} tended to reduce bacterial cells and active AOB. However, the reduction of both parameters was not as much as expected from the results of ammonia-oxidizing activity tests.

5.2 Suggestions

5.2.1. Incorporation of $^{13}C\text{-}HCO_3^-$ into *amoA* genes of AOA and AOB in NRI sludge should be observed at less than 21 days of incubation to avoid cross feeding.

5.5.2. Contribution of AOA and AOB to ammonia oxidation in full-scale WWTPs should be confirmed using different *in situ* activity investigation techniques.

5.5.3. Maintaining life with different route apart from lithoautotrophy should be clarified for AOA in WWTPs.

5.5.4. Recovery of ammonia-oxidizing activity of sludge after exposed to PNP should be studied.

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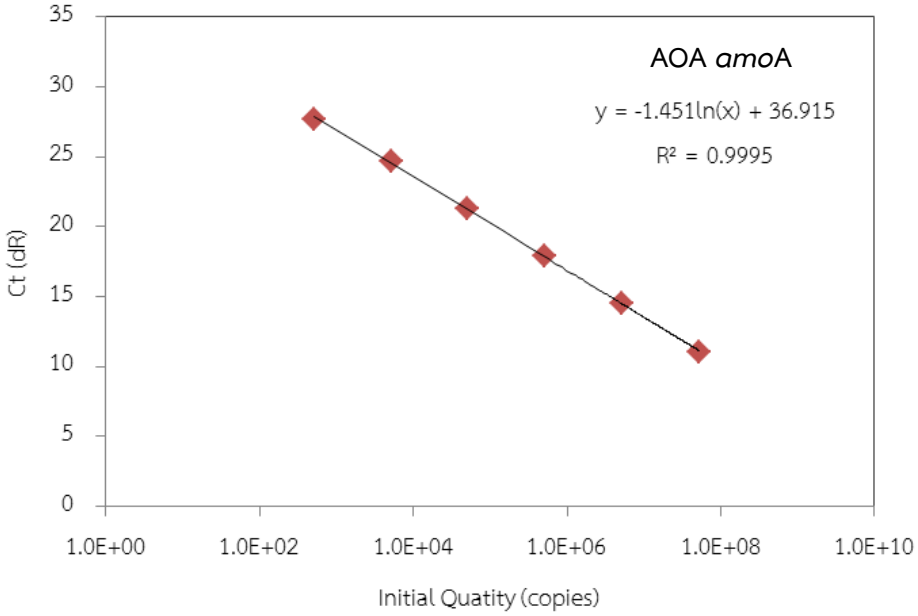
APPENDIX

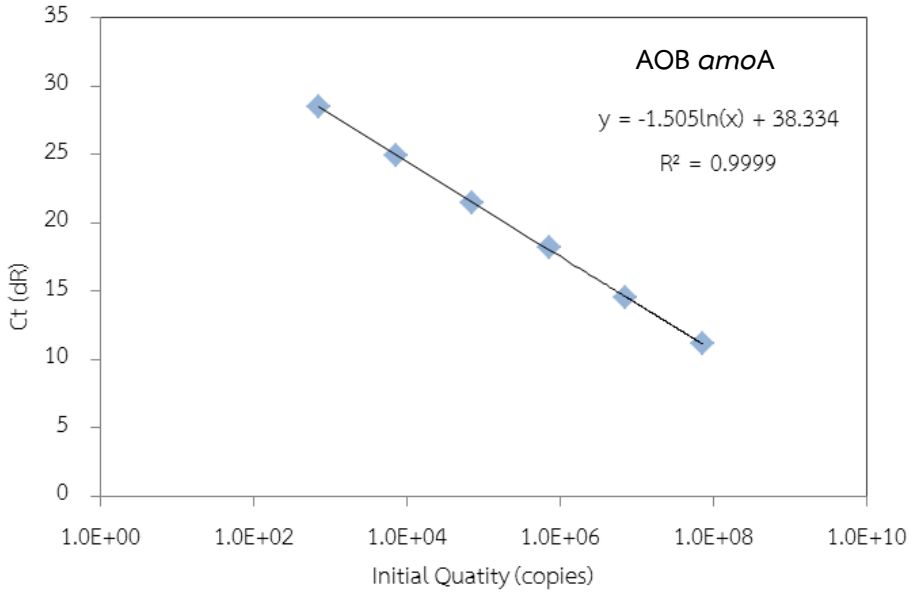
APPENDIX A

1. Examples of standard curves of AOA *amo*A genes and AOB *amo*A genes

(a) AOA *amoA* gene



(b) AOB *amoA* gene



2. Numbers of AOA *amo*A genes and AOB *amo*A genes in NRI and NRII (During 7-14 months of operation)

2.1 Numbers of AOA *amo*A genes and AOB *amo*A genes in NRI

Table 1 Numbers of AOA *amo*A genes and AOB *amo*A genes NRI (Unit: copies/ng genomic DNA)

Month	copies/ng genomic DNA			
	AOB *amoA*		AOA *amoA*	
	Average	SD	Average	SD
1	1.07E+02	3.58E+01	2.05E+03	6.09E+02
2	9.31E+01	3.43E+01	2.11E+03	4.16E+02
3	7.38E+01	1.79E+01	2.26E+03	6.99E+02
4	1.06E+03	3.43E+02	6.03E+03	1.03E+03
5	5.47E+02	1.98E+02	5.53E+02	9.34E+01
6	7.66E+02	4.45E+02	4.62E+03	1.34E+03
7	4.53E+02	1.37E+02	6.31E+02	3.46E+01
9	6.56E+02	1.30E+02	3.54E+03	6.95E+02
10	2.27E+02	4.14E+01	7.88E+03	1.17E+03
11	1.42E+02	1.77E+01	5.51E+03	3.04E+02
12	1.80E+02	1.57E+01	6.44E+03	5.39E+02
13	6.97E+02	1.98E+02	1.40E+03	2.69E+02
14	1.16E+03	1.39E+02	5.07E+03	5.08E+02

Table 2 Numbers of AOA *amo*A genes and AOB *amo*A genes NRI (Unit: copies/mg MLSS)

Month	copies/mg MLSS			
	AOB *amoA*		AOA *amoA*	
	Average	SD	Average	SD
1	2.17E+05	7.27E+04	4.28E+06	1.04E+06
2	1.96E+05	7.23E+04	4.43E+06	8.76E+05
3	1.31E+05	3.18E+04	4.02E+06	1.24E+06
4	1.17E+06	3.78E+05	6.64E+06	1.13E+06
5	4.04E+05	1.46E+05	4.07E+05	6.89E+04
6	7.74E+05	4.49E+05	4.66E+06	1.36E+06
7	2.76E+05	8.36E+04	3.85E+05	2.11E+04
9	1.52E+06	3.00E+05	8.20E+06	1.61E+06
10	5.92E+05	1.08E+05	2.05E+07	3.06E+06
11	1.37E+06	1.70E+05	5.28E+07	1.39E+07
12	1.19E+06	1.04E+05	4.25E+07	3.56E+06
13	4.99E+06	1.42E+06	1.00E+07	1.92E+06
14	2.36E+06	2.83E+05	1.03E+07	1.04E+06

2.2 Abundance of AOA *amo*A genes and AOB *amo*A genes in NRI

Table 3 Numbers of AOA *amo*A genes and AOB *amo*A genes in NRII (Unit: copies/ng genomic DNA)

Month	copies/ng genomic DNA			
	AOB *amoA*		AOA *amoA*	
	Average	SD	Average	SD
1	1.18E+05	2.75E+04	ND	
2	3.85E+05	6.56E+04	ND	
3	1.48E+05	1.62E+04	ND	
4	8.76E+04	2.08E+04	ND	
5	9.54E+04	4.88E+04	ND	
6	3.10E+05	9.75E+04	ND	
7	2.66E+05	5.40E+04	ND	

Table 4 Numbers of AOA *amo*A genes and AOB *amo*A genes in NRII (Unit: copies/mg MLSS)

Month	copies/mg MLSS			
	AOB *amoA*		AOA *amoA*	
	Average	SD	Average	SD
1	4.27E+09	9.93E+08	ND	
2	7.68E+10	1.31E+10	ND	
3	1.44E+11	1.58E+10	ND	
4	8.86E+09	2.10E+09	ND	
5	3.41E+10	1.75E+10	ND	
6	1.76E+12	5.53E+11	ND	
7	3.24E+11	6.56E+10	ND	

3. Representative sequences of AOA *amo*A genes and AOB *amo*A genes in NRI and NRII

3.1 Representative sequences of AOA *amo*A genes in NRI

>NR-I-M1-1

ACGTTGCTTACAATCAACGCAGGAGATTACATCTTCTATACTGACTGGGCGTGGACATCATT
TGTCGTATTTTCGATTTCTCAATCCACAATGCTTGTGGTTGGAGCTATATACTATATGTTGTT
TACAGGCGTTCCGGGTACTGCTACGTATTATGCAACTATAATGACTATATATACATGGGTTG
CCAAAGGTGCATGGTTTGCATTGGGATATCCATACGACTTTGTTGTGGTTCCTGTTTGGATA
CCATCGGCGATGTTGTTGGATTTGGCGTACTGGGCAACAAGGAGAAACAAACACGCGGCTA
TATTAATTGGTGGTGTGTTGGTAGGAATGTCGCTACCACTATTTAATATGATCAACTTGTTG
TTGGTTGCTGATCCCTTGGAAATGGCATTCAAGTATCCAAGACCCACTTTACCACCATACAT
GACTCCAATCGAACCTCAGGTAGGTAGGTTCTATAATAGTCCTGTTGCGCTAGGGGCCGGTG
CGGGAGCTGTGCTATGTGTTCCTATAGCGGCATTGGGTGCAAAACTCAA

>NRI-M6-1

ACGTTGCTTACAATCAACGCAGGAGATTACATCTTCTATACTGACTGGGCGTGGACATCATT
TGTCGTATTTTCGATTTCTCAATCCACAATGCTTGTGGTTGGAGCTATATACTATATGTTGTT
TACAGGCGTTCCGGGTACTGCTACGTATTATGCAACTATAATGACTATATATACATGGGTTG
CCAAAGGTGCATGGTTTGCATTGGGATATCCATACGACTTTGTTGTGGTTCCTGTTTGGATA
CCATCGGCGATGTTGTTGGATTTGACGTACTGGGCAACAAGGAGAAACAAACACGCGGCTAT
ATTAATTGGTGGTGTGTTGGTAGGAATGTCGCTACCACTATTTAATATGATCAACTTGTTGT
TGGTTGCTGATCCCTTGGAAATGGCATTCAAGTATCCAAGACCCACTTTACCACCATACATG
ACTCCAATCGAACCTCAGGTAGGTAAGTTCTATAATAGTCCTGTTGCGCTAGGGGCCGGTGC
GGGAGCTGTGCTATGTGTTCCTATAGCGGCATTGGGTGCAAAACTCAA

>NRI-M6-2

ACGTTGCTTACAATCAACGCAGGAGATTACATCTTCTATACTGACTGGGCGTGGACATCATT
TGTCGTATTTTCGATTTCTCAATCCACAATGCTTGCGGTTGGAGCTATATACTATATGTTGTT
TACAGGCGTTCCGGGTACTGCTACGTATTATGCAACTGTAATGACTATATATACATGGGTTG
CCAAAGGTGCATGGTTTGCATTGGGATATCCATACGACTTTGTTGTGGTTCCTGTTTGGATA

CCATCGGCGATGTTGTTGGATTTGGCGTACTGGGCAACAAGGAGAAACAAACACGCGGCTA
TATTAATTGGTGGTGTGTTGGTAGGAATGTCGCTACCACTATTTAATATGATCAACTTGTTG
TTGGTTGCTGATCCCTTGGAAATGGCATTCAAGTATCCAAGACCCACTTTACCACCATACAT
GACTCCAATCGAACCTCAGGTAGGTAAGTTCTATAATAGTCCTGTTGCGCTAGGGGCCGGTG
CGGGAGCTGTGCTATGTGTTCCGATATCGACATTGTGTGCAGTACTCAA

>NRI-M6-3
ACGTTGCTTACAATCAACGCAGGAGATTACATCTTCTATACTGACTGGGCGTGGACATCATT
TGTCGTATTTTCGATTTCTCAATCCACAATGCTTGTGGTTGGAGCTATATACTATATGTTGTT
TACAGGCGTTCCGGGTACTGCTACGTATTATGCAACTATAATGACTATATATACATGGGTTG
CCAAAGGTGCATGGTTTGCATTGGGATATCCATACGACTTTGTTGTGGTTCCTGTTTGGATA
CCATCGGCGATGTTGTTGGATTTGGCGTACTGGGCAACAAGGAGAAACAAACACGCGGCTA
TATTAATTGGTGGTGTGTTGGTAGGAATGTCGCTACCACTATTTAATATGATCAACTTGTTG
TTGGTTGCTGATCCCTTGGAAATGGCATTCAAGTATCCAAGACCCACTTTACCACCATACAT
GACTCCAATCGAACCTCAGGTAGGTAAGTTCTATAATAGTCCTGTTGCGCTAGGGGCCGGTG
CGGGAGCTGTGCTATGTGTTCCTATAGCGGAATTGAGCGCCAATCCCAA

>NRI-M6-4
ACGTTGCTTACAATCAACGCAGGAGATTACATCTTCTACACTGACTGGGCATGGACATCATT
TGTTGTATTCTCGATTTCTCAATCCACAATGCTTGTGGTAGGAGCTATATACTATATGTTGTT
TACAGGTGTTCCAGGCACTGCTACGTATTATGCGACTATAATGACGATATATACATGGGTTG
CAAAAGGAGCCTGGTTTGCATTGGGATACCCATACGACTTTGTCGTGGTCCCAGTTTGGATA
CCGTCTGCAATGCTATTGGATTTGGCGTACTGGGTAACAAGACGAAACAAACACGCAGCTAT
ATTAATTGGTGGTGTGCTAGTAGGAATGTCACTGCCACTATTTAATATGATTAATTTGTTGCT
GGTAGCAGACCCATTGGAAATGGCATTTAAGTATCCAAGACCGACACTACCTCCATACATGA
CTCCTATTGAACCACAGGTCGGAAAGTTCTATAACAGTCCTGTAGCGTTAGGAGCCGGTGCC
GGGGCGGTGCTTTGTGTGCCTATTGCAGCATTGGGTGCGAAACTCAA

3.2 Representative sequences of AOB *amo*A genes in NRI

>NRI-M1-1

TGCGCTGATGCTGGACTTCACGCTGTATCTGACACGCAACTGGCTGGTGACAGCTCTGGTTG
GAGGCGGATTCTTCGGTCTGCTGTTCTATCCGGGTAACTGGCCGATCTTTGGTCCAACGCAT
CTGCCAATCGTTGTAGAAGGAACACTGTTGTCGATGGCTGACTACATGGGCCATATGTATGT
TCGTACAGGTACACCCGAGTATGTTCGTCATATTGAGCAAGGTTCACTGCGTACCTTTGGTG
GTCATACCACAGTTATTGCAGCATTCTTCTCTGCGTTCGTATCAATGTTGATGTTCACCGTAT
GGTGGTATCTTGGAAAAGTTTACTGTACAGCCTTTTTCTACGTTAAAGGTAAAAGAGGTCGT
ATCGTACATCGCAATGATGTTACCGCA

>NRI-M1-2

GGTGCGTTGATGCTGGATACGATTTTATTATTGACGGGTAACTGGTTGGTAACCGCACTGTT
AGGTGGTGGATTCTGGGGTTTATTTTTCTATCCGGGCAACTGGCCTATTTTTGGTCCCACCC
ACTTACCGCTGGTTGTAGAAGGCGTATTGCTGTCAGTAGCTGACTACACAGGTTTTCTGTAT
GTACGTACAGGTACACCGGAATATGTTCGCCTGATTGAGCAAGGATCGCTGCGTACTTTTGG
TGGTCACACCACGGTTATTGCTGCGTTTTTCTCAGCCTTTGTATCAATGCTGATGTTCTGCG
TATGGTGGTACTTTGGCAAACTATACTGTACTGCTTTCTACTATGTTAAAGGAGAAAGAGGA
CGTATATCGATGAAGAATGACGTAACGG

>NRI-M1-3

TGCATTGATGTTGGATATCACGCTGTACTTGACCCGTAGCTGGTTGGTAACGGCACTGATTG
GCGGTGGCTTCTTTGGTCTGTTATTCTATCCAGGCAACTGGCCAATTTTTGGACCGACTCAC
TTGCCTGTCGTTGCAGAAGGCGTATTGCTTTCAATGGCAGACTACATGGGGCACCTTTATAT
CCGTACAGGTACACCTGAGTATGTGCGTTTGATTGAACAAGGATCGTTGCGTACCTTTGGTG
GTCATACCACGGTGATTGCTGCGTTCTTCTCAGCGTTTGTATCGATGCTGATGTTTGTTGTT
TGGTGGTACCTCGGTAAAGTCTATTGCACAGCCTTCTTCTACGTTAAAGGTAAAAGAGGCCG
TATTGTTAAAAAAGATGACGTTACTGCG

>NRI-M1-4

CTTCAGTGGTGTTCTGATGCTGGACTTCACGCTGTATCTGACACGCAACTGGCTGGTGACAG
CTCTGGTTGGAGGCGGATTCTTCGGTCTGCTGTCCTATCCGGGTAACTGGCCGATCTTTGGT
CCAACGCATCTGCCAATCGTTGTAGAAGGAACACTGTTGTCGATGGCTGACTACATGGGCCA
TATGTATGTTCGTACAGGTACACCCGAGTATGTTCGTCATATTGAGCAAGGTTCACTGCGTA

CCTTTGGTGGTCATACCACAGTTATTGCAGCATTCTTCTCTGCGTTCGTATCAATGTTGATG
TTCACCGTATGGTGGTATCTTGGAAAAGTTTACTGTACAGCCTTTTTCTACGTTAAAGGTAA
AAGAGGTCGTATCGTACATCGCAATGAT

>NRI-M6-1

GGTGCGCTGATGCTGGACTTCACGCTGTATCTGACACGCAACTGGCTGGTGACAGCTCTGGT
TGGAGGCGGATTCTTCGGTCTGCTGTTCTATCCGGGTAACTGGCCGATCTTTGGTCCAACGC
ATCTGCCAATCGTTGTAGAAGGAACACTGTTGTCGATGGCTGACTACATGGGCCATATGTAT
GTTCGTACAGGTACACCCGAGTATGTTCGTCATATTGAGCAAGGTTCACTGCGTACCTTTGG
TGGTCATACCACAGTTATTGCAGCATTCTTCTCTGCGTTCGTATCAATGTTGATGTTCACCG
TATGGTGGTATCTTGGAAAAGTTTACTGTACAGCCTTTTTCTACGTTAAAGGTAAAAGAGGT
CGTATCGTACATCGCAATGATGTTACCG

>NRI-M6-2

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TTACCGCTGGTTGTAGAAGGCGTATTGCTGTCAGTAGCTGACTACACAGGTTTTCTGTATGT
ACGTACAGGTACACCGGAATATGTTCGCCTGATTGAGCAAGGATCGCTGCGTACTTTTGGTG
GTCACACCACGGTTATTGCTGCGTTTTTCTCAGCCTTTGTATCAATGCTGATGTTCTGCGTA
TGGTGGTACTTTGGCAAACTATACTGTACTGCTTTCTACTATGTTAAAGGAGAAAGAGGACG
TATATCGATGAAGAATGACGTAACGGCA

>NRI-M6-3

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GGCGGATTCTTCGGTCTGCTGTTCTATCCGGGTAACTGGCCGATCTTTGGTCCAACGCATCT
GCCAATCGTTGTAGAAGGAACACTGTTGTCGATGGCTGACTACATGGGCCATATGTATGTTC
GTACAGGTACACCCGAGTATGTTCGTCATATTGAGCAAGGTTCACTGCGTACCTTTGGTGGT
CATACCACAGTTATTGCAGCATTCTTCTCTGCGTTCGTATCAATGTTGATGTTCACCGTATG
GTGGTATCTTGGAAAAGTTTACTGTACAGCCTTTTTCTACGTTAAAGGTAAAAGAGGTCGTA
TCGTACATCGCAATGATGTTACCGCATT

>NRI-M6-4

ATGTTGGATATCACGCTGTACTTGACCCGTAGCTGGTTGGTAACGGCACTGATTGGCGGTGG
CTTCTTTGGTCTGTTATTCTATCCAGGCAACTGGCCAATTTTTGGACCGACTCACTTGCCTG
TCGTTGCAGAAGGCGTATTGCTTTCAATGGCAGACTACATGGGGCACCTTTATATCCGTACA
GGTACACCTGAGTATGTGCGTTTGATTGAACAAGGATCGTTGCGTACCTTTGGTGGTCATAC
CACGGTGATTGCTGCGTTCTTCTCAGCGTTTGTATCGATGCTGATGTTTGTTGTTTGGTGGT
ACCTCGGTAAAGTCTATTGCACAGCCTTCTTCTACGTTAAAGGTAAAAGAGGCCGTATTGTT
AAAAAAGATGACGTTACTGCGTTTGGTG

3.3 Representative sequences of AOB *amo*A genes in NRII

>NRII-M1-1

GTGCGTTGATGCTGGACACAATTATGTTATTGACGGGTAACTGGCTGATAACCGCACTGTTA
GGTGGTGGATTCTGGGGATTATTTTTCTATCCAGGCAACTGGCCTATTTTTGGTCCAACCCA
CTTGCCTCTGGTTGTAGAAGGCGTGTTGCTGTCAGTAGCTGACTACACAGGTTTCTTGTATG
TGCGTACAGGTACACCGGAATATGTTCGCCTGATTGAGCAAGGATCGCTGCGTACTTTTGGT
GGCCACACCACGGTGATTGCCGCGTTCTTCTCAGCTTTTGTATCGATGTTGATGTTCTGTGT
ATGGTGGTACTTTGGCAAACTATACTGTACCGCTTTCTTCTATGTTAAAGGAGAAAGAGGAC
GTATATCGATGAAGAATGACGTAACGGC

> NRII-M1-2

GTGCGCTGATGCTGGACTTCACGCTGTATCTGACACGTAACTGGCTGGTGACAGCTCTGGTT
GGGGGCGGATTCTTTGGTCTGCTGTTCTACCCGGGTAGCTGGCCGATCTTTGGTCCAACGCA
TCTGCCAATCGTTGTAGAAGGAACACTGCTGTCGATGGCTGACTACATGGGCCATATGTATG
TTCGTACAGGTACACCCGAGTATGTTCGTCATATTGAGCAAGGTTCACTGCGTACCTTTGGT
GGTCATACCACAGTTATTGCAGCATTCTTCTCTGCGTTCGTATCAATGTTGATGTTCACCGT
ATGGTGGTATCTTGGAAAAGTTTACTGTACAGCCTTTTTCTACGTTAAAGGTAAAAGAGGTC
GTATCGTACATCGCAATGATGTTACCGC

> NRII-M1-3

GTGCGCTGATGCTGGACTTCACGCTGTATCTGACACGCAACTGGCTGGTGACAGCTCTGGTT
GGAGGCGGATTCTTCGGTCTGCTGTTCTATCCGGGTAACTGGCCGATCTTTGGTCCAACGCA

TCTGCCAATCGTCGTAGAAGGAACACTGTTGTCGATGGCTGACTACATGGGCCATATGTATG
TTCGTACAGGTACACCCGAGTATGTTCGTCATATTGAGCAAGGTTCACTGCGTACCTTTGAT
GGTCATACCACGGTTATTGCAGCATTCTTCTCTGCGTTCGTATCAATGTTGATGTTCACCGT
ATGGTGGTATCTTGGAAAAGTTTACTGTACAGCCTTTTTCTACGTTAAAGGTAAAAGAGGTC
GTATCGTACATCGCAATGATGTTACCGC

>NRII-M1-4
GTGCGCTGATGCTGGACTTCACGCTGTATCTGACACGCAACTGGCTGGTGACAGCTCTGGTT
GGAGGCGGATTCTTCGGTCTGCTGTTCTATCCGGGTAACTGGCCGATCTTTGGTCCAACGCA
TCTGCCAATCGTTGTAGAAGGAACACTGTTGTCGATGGCTGACTACATGGGCCATATGTATG
TTCGTACAGGTACACCCGAGTATGTTCGTCATATTGAGCAAGGTTCACTGCGTACCTTTGGT
GGTCATACCACAGTTATTGCAGCATTCTTCTCTGCGTTCGTATCAATGTTGATGTCCACCGT
ATGGTGGTATCTTGGAAAAGTTTACTGTACAGCCTTTTTCTACGTTAAAGGTAAAAGAGGTC
GTATCGTACATCGCAATGATGTTACCGC

> NRII-M6-1
GTGCATTGATGTTGGANATCACGCTGTACTTGACCCGTAGCTGGTTGGTAACGGCACTGATT
GGCGGTGGCTTCTTTGGTCTGTTATTCTATCCAGGCAACTGGCCAATTTTTGGACCGACTCA
CTTGCCTGTCGTTGCAGAAGGCGTATTGCTTTCAATGGCAGACTACATGGGGCACCTTTATA
TCCGTACAGGTACACCTGAGTATGTGCGTTTGATTGAACAAGGATCGTTGCGTACCTTTGGT
GGTCATACCACGGTGATTGCTGCGTTCTTCTCAGCGTTTGTATCGATGCTGATGTTTGTTGT
TTGGTGGTACCTCGGTAAAGTCTATTGCACAGCCTTCTTCTACGTTAAAGGTAAAAGAGGCC
GTATTGTTAAAAAAGATGACGTTACTGC

> NRII-M6-2
GTGCGCTGATGCTGGACTTCACGCTGTATCTGACACGTAACTGGCTGGTGACAGCTCTGGTT
GGGGGCGGATTCTTTGGTCTGCTGTTCTACCCGGGTAACTGGCCGATCTTTGGTCCAACGCA
TCTGCCAATCGTTGTAGAAGGAACACTGCTGTCGATGGCTGACTACATGGGCCATATGTATG
TTCGTACGGGTACACCTGAGTATGTTCGTCATATTGAGCAAGGTTCACTGCGTACCTTTGGT
GGACATACCACAGTTATTGCAGCGTTCTTCTCTGCATTCGTATCAATGTTGATGTTCACGGT

ATGGTGGTATCTTGGAAAAGTTTACTGTACAGCCTTTTTCTACGTTAAAGGTAAAAGAGGTC
GTATCGTACATCGCAATGATGTTACCGC

> NRII-M6-3

GTGCGCTGATGCTGGACTTCACGCTGTATCTGACACGTAACTGGCTGGTGACAGCTCTGGTT
GGGGGCGGATTCTTTGGTCTGCTGTTCTACCCGGGTAACTGGCCGATCTTTGGTCCAACGCA
TCTGCCAATCGTTGTAGAAGGAACACTGCTGTCGATGGCTGACTACATGGGCCATATGTATG
TTCGTACGGGTACACCTGAGTATGTTCGTCATATTGAGCAAGGTTCACTGCGTACCTTTGGT
GGACATACCACAGTTATTGCAGCGTTCTTCTCTGCATTCGTATCAATGTTGATGTTCACGGT
ATGGTGGTATCTTGGAAAAGTTTACTGTACAGCCTTTTTCTACGTTAAAGGTAAAAGAGGTC
GTATCGTACATCGCAATGATGTTACCGC

>NRII-M6-4

GTGCGCTGATGCTGGACTTCACGCTGTATCTGACACGCAACTGGCTGGTGACAGCTCTGGTT
GGAGGCGGATTCTTCGGTCTGCCGTTCTATCCGGGTAACTGGCCGATCTTTGGTCCAACGCA
TCTGCCAATCGTTGTAGAAGGAACACTGTTGTCGATGGCTGACTACATGGGCCATATGTATG
TTCGTACAGGTACACCCGAGTATGTTCGTCATATTGAGCAAGGTTCACTGCGTACCTTTGGT
GGTCATACCACAGTTATTGCAGCATTCTTCTCTGCGTTCGTATCAATGTTGATGTTCACCGT
ATGGTGGTATCTTGGAAAAGTTTACTGTACAGCCTTTTTCTACGTTAAAGGTAAAAGAGGTC
GTATCGTACATCGCAATGATGTTACCG

4. Ammonia oxidizing activity

4.1 Ammonia oxidizing activity of NRI under the presence of ATU

Table 5 Ammonia oxidizing activity in the control

Control (ATU 0 μM)					
Round 1 (Month2)			Round 2 (Month12)		
Time (day)	Average concentration ($mgNL^{-1}$)	SD	Time (day)	Average concentration ($mgNL^{-1}$)	SD
0	4.35	0.21	0	7.79	0.12
1	3.53	0.19	1	2.96	0.25
2	2.52	0.16	3	0.68	0.12
5	0.50	0.09	5	0.37	0.19
7	0.58	0.03	7	0.31	0.33
9	0.60	0.02	-	-	-

Table 6 Ammonia oxidizing activity under the presence of ATU of at 10 μM

ATU 10 μM					
Round 1 (Month2)			Round 2 (Month12)		
Time (day)	Average concentration ($mgNL^{-1}$)	SD	Time (day)	Average concentration ($mgNL^{-1}$)	SD
0	4.79	0.12	0	7.41	0.11
1	4.01	0.57	1	3.60	0.11
2	4.11	0.11	3	0.94	0.08
5	1.67	0.10	5	0.12	0.04
7	1.44	0.08	7	0.08	0.06
9	1.42	0.08	-	-	-
11	0.11	0.06	-	-	-

Table 7 Ammonia oxidizing activity under the presence of ATU at 30 μM

ATU 30 μM					
Round 1 (Month2)			Round 2 (Month12)		
Time (day)	Average concentration ($mgNL^{-1}$)	SD	Time (day)	Average concentration ($mgNL^{-1}$)	SD
0	4.63	0.14	0	7.67	0.32
1	4.34	0.13	1	4.22	0.29
2	4.58	0.56	3	1.82	0.20
5	2.68	0.30	5	0.16	0.04
7	1.65	0.23	7	0.19	0.12
9	0.76	0.30	-	-	-
11	0.48	0.68	-	-	-

Table 8 Ammonia oxidizing activity under the presence of ATU of 50 μM

ATU 50 μM					
Round 1 (Month2)			Round 2 (Month12)		
Time (day)	Average concentration ($mgNL^{-1}$)	SD	Time (day)	Average concentration ($mgNL^{-1}$)	SD
0	4.71	0.19	0	-	-
1	4.49	0.07	1	-	-
2	4.54	0.15	3	-	-
5	3.52	0.53	5	-	-
7	2.54	0.73	7	-	-
9	1.97	0.80	-	-	-
11	1.79	0.76	-	-	-
13	1.23	0.45	-	-	-
16	0.26	0.16	-	-	-

Table 9 Ammonia oxidizing activity under the presence of ATU at 80 μM

ATU 80 μM					
Round 1 (Month2)			Round 2 (Month12)		
Time (day)	Average concentration ($mgNL^{-1}$)	SD	Time (day)	Average concentration ($mgNL^{-1}$)	SD
0	4.56	0.19	0	7.28	0.22
1	4.07	0.31	1	4.68	0.10
2	4.00	0.10	3	2.60	0.11
5	2.00	0.15	5	0.19	0.10
7	0.64	0.19	7	-	-
9	0.07	0.05	0	-	-

Table 10 Ammonia oxidizing activity under the presence of ATU at 100 μM

ATU 100 μM					
Round 1(Month2)			Round 2 (Month12)		
Time (day)	Average concentration ($mgNL^{-1}$)	SD	Time (day)	Average concentration ($mgNL^{-1}$)	SD
0	4.78	0.13	0	7.40	0.42
1	4.48	0.25	1	4.83	0.16
2	4.05	0.13	3	2.98	0.24
5	2.18	0.03	5	0.17	0.08
7	0.97	0.11	7	-	-
9	0.13	0.05	0	-	-

Table 11 Ammonia oxidizing activity under the presence of ATU at 150 μM

ATU 150 μM					
Round 1(Month2)			Round 2 (Month12)		
Time (day)	Average concentration ($mgNL^{-1}$)	SD	Time (day)	Average concentration ($mgNL^{-1}$)	SD
0	4.71	0.22	0	7.53	0.06
1	4.37	0.16	1	5.45	0.47
2	4.25	0.10	3	3.98	0.56
5	2.53	0.25	5	1.12	0.91
7	1.76	0.31	7	0.33	0.12
9	0.85	0.33	10	0	0
11	0.37	0.30	-	-	-
13	0.08	0.05	-	-	-

Table 12 Ammonia oxidizing activity under the presence of ATU at 200 μM

ATU 200 μM					
Round 1 (Month2)			Round 2 (Month12)		
Time (day)	Average concentration ($mgNL^{-1}$)	SD	Time (day)	Average concentration ($mgNL^{-1}$)	SD
0	4.86	0.18	0	9.26	0.68
1	4.42	0.15	1	5.77	0.25
2	4.39	0.17	3	4.07	0.23
5	3.16	0.07	5	1.66	0.06
7	2.23	0.21	7	0.08	0.04
9	1.63	0.19	10	-	-
11	1.06	0.10	-	-	-
13	0.19	0.09	-	-	-
16	0.20	0.41	-	-	-

Table 13 Ammonia oxidizing activity under the presence of ATU at 500 μM

ATU 500 μM					
Round 1(Month2)			Round 2 (Month12)		
Time (day)	Average concentration ($mgNL^{-1}$)	SD	Time (day)	Average concentration ($mgNL^{-1}$)	SD
0	4.76	0.27	0	7.17	0.22
1	4.52	0.11	1	6.14	0.41
2	4.90	0.13	3	5.91	0.04
5	4.39	0.08	5	5.46	0.18
7	4.11	0.12	7	3.96	0.04
9	3.85	0.28	10	2.62	0.14
11	4.03	0.09	12	1.91	0.41
13	3.68	0.07	-	-	-
16	3.03	0.14	-	-	-

Table 14 Ammonia oxidizing activity under the presence of ATU at 1000 μM

ATU 1000 μM					
Round 1(Month2)			Round 2 (Month12)		
Time (day)	Average concentration ($mgNL^{-1}$)	SD	Time (day)	Average concentration ($mgNL^{-1}$)	SD
0	4.72	0.11	0	7.35	0.16
1	4.58	0.32	1	6.41	0.45
2	5.04	0.12	3	5.55	0.67
5	4.89	0.09	5	6.60	0.05
7	4.71	0.17	7	6.40	0.25
9	4.39	0.50	10	6.19	0.17
11	4.92	0.05	12	6.32	0.21
13	4.17	0.32	-	-	-
16	3.94	0.45	-	-	-

Table 15 Ammonia oxidizing activity under the presence of ATU at 2000 μM

ATU 2000 μM					
Round 1(Month2)			Round 2 (Month12)		
Time (day)	Average concentration ($mgNL^{-1}$)	SD	Time (day)	Average concentration ($mgNL^{-1}$)	SD
0	4.76	0.39	0	6.14	0.42
1	4.62	0.20	1	5.85	0.49
2	4.46	0.61	3	5.44	0.61
5	4.79	0.23	5	5.80	0.38
7	4.83	0.22	7	6.27	0.43
9	4.29	0.34	10	6.43	0.09
11	4.75	0.16	12	6.69	0.50
13	4.49	0.26	-	-	-
16	4.33	0.14	-	-	-

4.2 Ammonia oxidizing activity of NRI under the presence of PTIO

Table 16 Ammonia oxidizing activity under the presence of PTIO at 50 μM

Time (day	PTIO 50 μM	
	Average concentration ($mgNL^{-1}$)	SD
0	4.98	0.12
1	4.43	0.17
2	3.20	0.14
5	0.15	0.08
7	0.14	0.05
9	0.16	0.10

Table 17 Ammonia oxidizing activity under the presence PTIO at 100 μM

Time (day	PTIO 100 μM	
	Average concentration ($mgNL^{-1}$)	SD
0	5.07	0.19
1	4.66	0.26
2	4.28	0.13
5	0.31	0.42
7	0.19	0.06
9	0.14	0.03

Table 18 Ammonia oxidizing activity under the presence PTIO at 300 μM

Time (day	PTIO 300 μM	
	Average concentration ($mgNL^{-1}$)	SD
0	4.86	0.10
1	4.68	0.16
2	4.27	0.21
5	2.95	0.54
7	0.29	0.03
9	0.16	0.02

4.3 Ammonia oxidizing activity of NRI under the presence of mixture of ATU and PTIO

Table 19 Ammonia oxidizing activity of a mixture of ATU 30 μM and PTIO 100 μM and a mixture of ATU 80 μM and PTIO 100 μM

	ATU 30 μM + PTIO 100 μM		ATU 80 μM + PTIO 100 μM	
Time (day	Average concentration ($mgNL^{-1}$)	SD	Average concentration ($mgNL^{-1}$)	SD
0	5.07	0.12	5.03	0.09
1	4.95	0.09	5.01	0.52
2	4.48	0.04	4.39	0.37
5	4.07	0.22	4.25	0.10
7	3.92	0.28	3.83	0.71
9	4.21	0.07	3.88	0.09
11	3.70	0.50	4.45	0.19
13	3.37	0.58	3.91	0.26

Table 20 Ammonia oxidizing activity of a mixture of ATU 150 μM and PTIO 100 μM and a mixture of ATU 2000 μM and PTIO 100 μM

	ATU 150 μM + PTIO 100 μM		ATU 2000 μM + PTIO 100 μM	
Time (day	Average concentration ($mgNL^{-1}$)	SD	Average concentration ($mgNL^{-1}$)	SD
0	5.02	0.20	5.06	0.13
1	5.13	0.29	4.92	0.18
2	4.89	0.06	4.61	0.31
5	4.57	0.08	4.54	0.08
7	4.95	0.15	5.43	0.45
9	4.43	0.32	5.28	0.19
11	4.80	0.12	4.88	0.07
13	4.41	0.13	4.47	0.35

4.4 Ammonia oxidizing activity of NRII under the presence of ATU

Table 21 Ammonia oxidizing activity of the control

Control (ATU 0 μM)					
Round 1 (Month2)			Round 1 (Month6)		
Time (day)	Average concentration ($mgNL^{-1}$)	SD	Time (day)	Average concentration ($mgNL^{-1}$)	SD
0	6.28	0.23	0	6.28	0..23
1	0.15	0.06	1	0.13	0.08
2	0.03	0.01	2	0.04	0.02

Table 22 Ammonia oxidizing activity under the presence ATU at 10 μM

ATU 10 μM					
Round 1(Month2)			Round 1(Month6)		
Time (day)	Average concentration ($mgNL^{-1}$)	SD	Time (day)	Average concentration ($mgNL^{-1}$)	SD
0	5.41	0.21	0	5.41	0.21
1	5.22	0.13	1	5.23	0.13
2	5.75	0.14	2	6.26	0.84
4	5.02	0.41	4	5.12	0.43
6	4.61	0.27	6	4.70	0.28
7	4.80	0.20	7	4.96	0.21
10	4.29	0.22	10	4.44	0.22

Table 23 Ammonia oxidizing activity under the presence ATU at 30 μM

ATU 30 μM					
Round 1(Month2)			Round 1(Month6)		
Time (day)	Average concentration ($mgNL^{-1}$)	SD	Time (day)	Average concentration ($mgNL^{-1}$)	SD
0	5.64	0.19	0	6.37	0.16
1	5.64	0.35	1	5.64	0.35
2	5.71	0.12	2	5.82	0.17
4	4.95	0.39	4	4.99	0.35
6	4.73	0.40	6	4.68	0.63
7	5.62	0.69	7	5.60	0.39
10	5.05	0.23	10	5.22	0.24

Table 24 Ammonia oxidizing activity under the presence ATU at 50 μM

ATU 50 μM					
Round 1(Month2)			Round 1(Month6)		
Time (day)	Average concentration ($mgNL^{-1}$)	SD	Time (day)	Average concentration ($mgNL^{-1}$)	SD
0	5.93	0.36	0	6.76	0.24
1	5.78	0.27	1	6.28	1.51
2	5.86	0.10	2	6.05	0.10
4	5.34	0.21	4	5.48	0.18
6	5.18	0.12	6	5.28	0.14
7	5.50	0.11	7	5.60	0.12
10	5.21	0.18	10	5.29	0.18

Table 25 Ammonia oxidizing activity under the presence ATU at 80 μM

ATU 80 μM					
Round 1(Month2)			Round 1(Month6)		
Time (day)	Average concentration ($mgNL^{-1}$)	SD	Time (day)	Average concentration ($mgNL^{-1}$)	SD
0	6.42	0.24	0	7.04	0.62
1	5.88	0.26	1	5.94	0.24
2	5.73	0.16	2	5.93	0.16
4	5.42	0.12	4	5.53	0.12
6	5.25	0.18	6	4.86	1.79
7	5.66	0.12	7	5.87	0.12
10	5.62	0.13	10	5.81	0.13

Table 26 Ammonia oxidizing activity under the presence ATU at 100 μM

ATU 100 μM					
Round 1(Month2)			Round 1(Month6)		
Time (day)	Average concentration ($mgNL^{-1}$)	SD	Time (day)	Average concentration ($mgNL^{-1}$)	SD
0	5.97	0.26	0	6.41	0.91
1	5.92	0.37	1	5.93	0.31
2	5.75	0.27	2	6.00	0.28
4	5.40	0.25	4	5.51	0.27
6	5.31	0.25	6	5.54	0.23
7	5.59	0.07	7	5.78	0.08
10	5.42	0.26	10	5.61	0.27

Table 27 Ammonia oxidizing activity under the presence ATU at 150 μM

ATU 150 μM					
Round 1(Month2)			Round 1(Month6)		
Time (day)	Average concentration ($mgNL^{-1}$)	SD	Time (day)	Average concentration ($mgNL^{-1}$)	SD
0	6.26	0.24	0	6.42	0.17
1	6.12	0.27	1	6.20	0.29
2	6.01	0.11	2	6.25	0.08
4	5.42	0.10	4	5.53	0.11
6	5.36	0.16	6	5.40	0.25
7	5.69	0.10	7	5.88	0.10
10	5.44	0.42	10	5.63	0.43

Table 28 Ammonia oxidizing activity under the presence ATU at 200 μM

ATU 200 μM					
Round 1(Month2)			Round 1(Month6)		
Time (day)	Average concentration ($mgNL^{-1}$)	SD	Time (day)	Average concentration ($mgNL^{-1}$)	SD
0	6.26	0.22	0	6.45	0.22
1	6.03	0.22	1	6.05	0.24
2	5.92	0.14	2	6.12	0.15
4	5.55	0.13	4	5.66	0.13
6	5.45	0.25	6	5.67	0.40
7	5.75	0.23	7	5.95	0.24
10	5.72	0.13	10	5.92	0.14

Table 29 Ammonia oxidizing activity under the presence ATU at 500 μM

ATU 500 μM					
Round 1(Month2)			Round 1(Month6)		
Time (day)	Average concentration ($mgNL^{-1}$)	SD	Time (day)	Average concentration ($mgNL^{-1}$)	SD
0	5.67	0.29	0	5.67	0.36
1	5.43	0.16	1	5.39	0.18
2	5.85	0.18	2	6.05	0.19
4	5.48	0.14	4	5.75	0.54
6	5.34	0.19	6	5.36	0.21
7	5.90	0.11	7	6.11	0.11
10	5.85	0.10	10	6.05	0.11

Table 30 Ammonia oxidizing activity under the presence ATU at 1000 μM

ATU 1000 μM					
Round 1(Month2)			Round 1(Month6)		
Time (day)	Average concentration ($mgNL^{-1}$)	SD	Time (day)	Average concentration ($mgNL^{-1}$)	SD
0	5.92	0.11	0	5.95	0.08
1	5.67	0.17	1	5.26	1.24
2	6.05	0.08	2	6.26	0.08
4	5.49	0.26	4	5.61	0.27
6	5.54	0.10	6	5.65	0.11
7	5.96	0.10	7	6.17	0.10
10	5.96	0.14	10	6.17	0.15

Table 31 Ammonia oxidizing activity under the presence ATU at 2000 μM

ATU 2000 μM					
Round 1(Month2)			Round 1(Month6)		
Time (day)	Average concentration ($mgNL^{-1}$)	SD	Time (day)	Average concentration ($mgNL^{-1}$)	SD
0	5.69	0.34	0	5.76	0.38
1	5.60	0.39	1	5.55	0.32
2	5.79	0.18	2	5.99	0.19
4	5.53	0.38	4	5.56	0.27
6	5.58	0.26	6	5.69	0.28
7	5.69	0.18	7	5.87	0.19
10	5.74	0.12	10	5.93	0.14

4.5 Ammonia oxidizing activity of NRII under the presence of PTIO

Table 32 Ammonia oxidizing activity under the presence PTIO at 50 μM

Time (day	PTIO 50 μM	
	Average concentration ($mgNL^{-1}$)	SD
0	6.25	0.33
0.5	2.12	0.23
2	0.02	0.04
3.5	0.06	0.07

Table 33 Ammonia oxidizing activity under the presence PTIO at 100 μM

Time (day	PTIO 100 μM	
	Average concentration ($mgNL^{-1}$)	SD
0	6.66	0.73
0.5	1.65	0.28
2	0.00	0.00
3.5	0.02	0.03

Table 34 Ammonia oxidizing activity under the presence PTIO at 300 μM

Time (day	PTIO 300 μM	
	Average concentration ($mgNL^{-1}$)	SD
0	6.23	0.69
0.5	1.29	0.63
2	0.02	0.01
3.5	0.07	0.09

4.4 Ammonia oxidizing activity of NRII under the presence of mixtures of ATU and PTIO

Table 35 Ammonia oxidizing activity of the control

Time (day	Control	
	Average concentration ($mgNL^{-1}$)	SD
0	6.79	1.53
0.5	2.87	0.60
2	0.00	0.00

Table 36 Ammonia oxidizing activity of ATU 30 μM and a mixture of ATU 30 μM and PTIO 100 μM

Time (day	ATU 30 μM		ATU 30 μM + PTIO 100 μM	
	Average concentration ($mgNL^{-1}$)	SD	Average concentration ($mgNL^{-1}$)	SD
0	7.53	0.50	7.32	0.24
0.5	5.69	0.28	6.38	0.15
2	5.25	0.54	5.59	0.42
3.5	6.09	0.44	5.09	0.57

Table 37 Ammonia oxidizing activity of ATU 80 μM and a mixture of ATU 80 μM and PTIO 100 μM

Time (day	ATU 80 μM		ATU 80 μM + PTIO 100 μM	
	Average concentration ($mgNL^{-1}$)	SD	Average concentration ($mgNL^{-1}$)	SD
0	7.06	0.25	7.37	0.13
0.5	5.55	0.47	6.31	0.29
2	5.59	0.78	5.76	0.73
3.5	5.35	0.98	5.84	0.28

Table 38 Ammonia oxidizing activity of ATU 150 μM and a mixture of ATU 150 μM and PTIO 100 μM

Time (day	ATU 150 μM		ATU 150 μM + PTIO 100 μM	
	Average concentration ($mgNL^{-1}$)	SD	Average concentration ($mgNL^{-1}$)	SD
0	7.40	0.37	7.30	0.56
0.5	6.20	0.16	6.20	0.37
2	6.66	0.13	6.25	0.14
3.5	6.87	0.15	6.14	0.08

Table 39 Ammonia oxidizing activity of ATU 2000 μM and a mixture of ATU 2000 μM and PTIO 100 μM

Time (day	ATU 2000 μM		ATU 2000 μM + PTIO 100 μM	
	Average concentration ($mgNL^{-1}$)	SD	Average concentration ($mgNL^{-1}$)	SD
0	7.44	0.16	6.65	0.22
0.5	6.42	0.12	6.11	0.36
2	6.55	0.28	5.59	0.12
3.5	5.47	0.39	5.65	0.26

4.6 Percent of inhibitory on ammonia oxidation activity

Table 40 Percent of inhibitory on ammonia oxidation activity

ATU	Round 1		Round 2	
	NRI	NRII	NRI	NRII
0	11.55	2.41	0	0
10	26.46	96.75	4.40	96.52
30	42.56	100.00	9.90	88.2
50	65.32	100.00	100.00	92.75
80	24.82	100.00	29.60	84.1
100	23.80	100.00	34.63	92.34
150	35.95	100.00	38.67	96.47
200	50.30	100.00	47.63	93.66
500	89.09	100.00	80.70	94.9
1000	100.00	100.00	73.18	88.08
2000	100.00	100.00	87.54	96.22

5. DNA-SIP

5.1 Ammonia concentrations in influent and effluent

Table 41 Ammonia concentrations in influent during DNA-SIP incubation of NRI

Day	Ammonia concentration in influent					
	^{12}C		^{13}C		^{13}C+ATU	
	Average concentration ($mgNL^{-1}$)	SD	Average concentration ($mgNL^{-1}$)	SD	Average concentration ($mgNL^{-1}$)	SD
0	13.29	0.57	14.91	1.13	16.66	0.79
6	16.88	1.20	18.04	1.48	18.22	1.14
12	17.55	1.41	16.64	0.59	13.47	0.37
21	13.42	0.07	13.18	0.12	13.67	0.20

Table 42 Ammonia concentrations in effluent during DNA-SIP incubation of NRI

Day	Ammonia concentration in effluent					
	^{12}C		^{13}C		^{13}C+ATU	
	Average concentration ($mgNL^{-1}$)	SD	Average concentration ($mgNL^{-1}$)	SD	Average concentration ($mgNL^{-1}$)	SD
0	2.01	1.49	2.73	0.35	0.84	0.43
3	2.42	0.37	2.63	0.20	0.58	1.70
7	3.17	0.33	3.47	0.48	3.87	0.15
10	6.23	1.02	6.07	0.50	5.86	0.51
14	4.95	0.24	6.94	0.27	8.88	0.35
19	7.53	0.20	8.39	0.24	11.27	0.66

5.2 Numbers of AOA *amo*A genes and AOB *amo*A genes

Table 43 Numbers of AOA *amo*A genes

day	AOA copies/ng genomic DNA					
	^{12}C		^{13}C		^{13}C+ATU	
	Average	SD	Average	SD	Average	SD
0	1.06E+03	3.43E+02	-	-	-	-
21	8.81E+03	4.37E+02	4.36E+03	1.74E+03	1.26E+01	5.54E+00

Table 44 Numbers of AOB *amo*A genes

day	AOB copies/ng genomic DNA					
	^{12}C		^{13}C		^{13}C+ATU	
	Average	SD	Average	SD	Average	SD
0	6.03E+03	1.03E+03	-	-	-	-
21	1.11E+03	6.51E+02	2.89E+02	2.27E+02	1.07E+03	4.56E+02

5.3 Proportions of AOA *amoA* genes

Table 45 Proportions of AOA *amoA* genes at day 0

Fraction Number	density (g/mL)	Proportion	
		average	SD
4	1.7463	0.000	0.000
5	1.7398	0.000	0.000
6	1.7343	0.000	0.000
7	1.7288	0.000	0.000
8	1.7245	0.000	0.000
9	1.7201	0.000	0.000
10	1.7168	0.002	0.000
11	1.7103	0.035	0.004
12	1.7059	0.135	0.003
13	1.7048	0.237	0.017
14	1.6993	0.817	0.366
15	1.6961	1.000	0.037
16	1.6906	0.358	0.060
17	1.6862	0.193	0.048
18	1.6829	0.042	0.002
19	1.6808	0.010	0.001
20	1.6753	0.003	0.001

Table 46 Proportions of AOA *amoA* genes (^{12}C) at day21

Fraction Number	density (g/mL)	Proportion	
		average	SD
3	1.7452	0.003	0.002
4	1.7387	0.004	0.003
5	1.7321	0.011	0.014
6	1.7267	0.007	0.005
7	1.7201	0.020	0.004
8	1.7135	0.032	0.003
9	1.7070	0.108	0.023
10	1.7026	0.467	0.100
11	1.6961	1.000	0.147
12	1.6917	0.374	0.061
13	1.6862	0.099	0.009
14	1.6829	0.085	0.035
15	1.6797	0.021	0.021
16	1.6764	0.054	0.027
17	1.6742	0.035	0.012

Table 47 Proportions of AOA *amoA* genes (^{13}C) at day21

Fraction Number	density (g/mL)	Proportion	
		average	SD
3	1.7398	0.002	0.001
4	1.7387	0.003	0.001
5	1.7332	0.003	0.001
6	1.7288	0.005	0.004
7	1.7234	0.094	0.009
8	1.7179	0.806	0.326
9	1.7124	0.929	0.252
10	1.7081	1.000	0.362
11	1.7048	0.360	0.035
12	1.6993	0.182	0.111
13	1.6950	0.035	0.021
14	1.6906	0.033	0.011
15	1.6873	0.035	0.029
16	1.6829	0.012	0.005
17	1.6797	0.007	0.002

Table 48 Proportions of AOA *amo*A genes (^{13}C+ATU) at day21

Fraction Number	density (g/mL)	Proportion	
		average	SD
3	1.7463	0.003	0.000
4	1.7409	0.002	0.000
5	1.7343	0.002	0.000
6	1.7288	0.012	0.005
7	1.7234	0.117	0.033
8	1.7168	0.661	0.233
9	1.7114	1.000	0.412
10	1.7070	0.622	0.239
11	1.7015	0.544	0.170
12	1.6961	0.217	0.103
13	1.6917	0.062	0.035
14	1.6895	0.014	0.007
15	1.6840	0.010	0.005
16	1.6797	0.008	0.005
17	1.6786	0.006	0.003

5.4 Proportions of AOB *amoA* genes

Table 49 Proportions of AOB *amoA* genes at day0

Fraction Number	density (g/mL)	Proportion	
		average	SD
3	1.7529	0.000	0.000
4	1.7463	0.000	0.000
5	1.7398	0.001	0.001
6	1.7343	0.001	0.001
7	1.7288	0.002	0.001
8	1.7245	0.005	0.001
9	1.7201	0.043	0.003
10	1.7168	0.163	0.013
11	1.7103	0.837	0.179
12	1.7059	1.000	0.068
13	1.7048	0.730	0.099
14	1.6993	0.591	0.026
15	1.6961	0.263	0.017
16	1.6906	0.065	0.007
17	1.6862	0.047	0.011
18	1.6829	0.012	0.002
19	1.6808	0.006	0.001
20	1.6753	0.010	0.011
21	1.6720	0.006	0.001

Table 50 Proportions of AOB *amoA* genes (^{12}C) at day 21

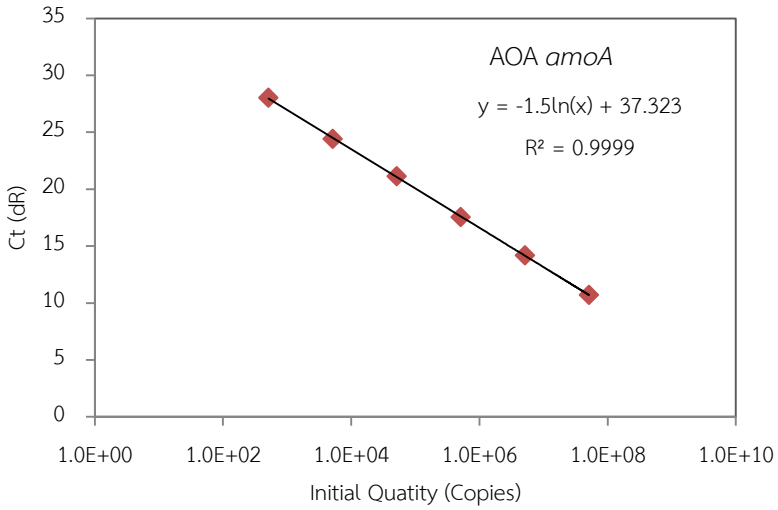
Fraction Number	density (g/mL)	Proportion	
		average	SD
3	1.7452	0.002	0.000
4	1.7387	0.000	0.000
5	1.7321	0.001	0.000
6	1.7267	0.003	0.000
7	1.7201	0.033	0.007
8	1.7135	0.294	0.110
9	1.7070	1.000	0.305
10	1.7026	0.830	0.307
11	1.6961	0.144	0.035
12	1.6917	0.036	0.013
13	1.6862	0.074	0.055
14	1.6829	0.024	0.013
15	1.6797	0.012	0.008
16	1.6764	0.020	0.005
17	1.6742	0.019	0.012

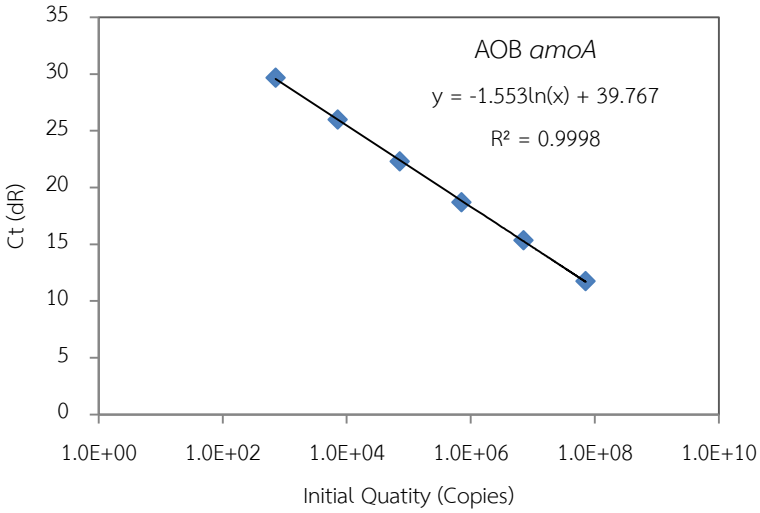
Table 51 Proportions of AOB *amo*A genes (^{13}C) at day 21

Fraction Number	density (g/mL)	Proportion	
		average	SD
3	1.7398	0.001	0.000
4	1.7387	0.003	0.000
5	1.7332	0.014	0.002
6	1.7288	0.285	0.048
7	1.7234	1.000	0.244
8	1.7179	0.430	0.030
9	1.7124	0.070	0.019
10	1.7081	0.016	0.002
11	1.7048	0.009	0.001
12	1.6993	0.005	0.000
13	1.6950	0.004	0.001
14	1.6906	0.002	0.000
15	1.6873	0.002	0.000
16	1.6829	0.003	0.001
17	1.6797	0.002	0.000

Appendix B

1. Examples of standard curves of AOA *amoA* genes and AOB *amoA* genes





2. Numbers of AOA *amo*A genes and AOB *amoA* genes in sludge A-E

Table 1 Numbers of AOA *amo*A genes and AOB *amo*A genes (Unit: copies/ng genomic DNA)

Sludge	copies/ng exteacted DNA				
	AOA *amo*A		AOB *amo*A		AOA: AOB
	average	SD	average	SD	
A	ND	-	5.90E+03	4.05E+02	0
B	1.57E+01	3.23E+00	1.07E+03	3.40E+02	0.01
C	1.46E+02	1.06E+01	5.20E+02	1.08E+02	0.28
D	4.80E+04	6.18E+03	1.12E+02	2.34E+01	185.73
E	1.30E+04	2.40E+03	1.01E+03	1.24E+02	12.88

Table 2 Numbers of AOA *amo*A genes and AOB *amo*A genes (Unit: copies/mgMLSS)

Sludge	copies/ mgMLSS				
	AOA *amo*A		AOB *amo*A		AOA:AOB
	average	SD	average	SD	
A	ND	-	1.27E+07	8.70E+05	-
B	4.86E+04	9.96E+03	3.83E+06	1.50E+06	0.01
C	2.99E+05	2.17E+04	1.06E+06	2.22E+05	0.28
D	6.07E+07	7.81E+06	3.27E+05	3.99E+05	428.18
E	4.46E+07	8.21E+06	3.46E+06	4.23E+05	12.88

3. Ammonia oxidizing activity

3.1 Ammonia oxidizing activity of sludge A under the present of ATU

Table 3 Ammonia oxidizing activity in the control and under the presence of ATU at 10 μM

Control (ATU 0 μM)			ATU 10 μM		
Time (day)	Average concentration ($mgNL^{-1}$)	SD	Time (day)	Average concentration ($mgNL^{-1}$)	SD
0	6.27	0.22	0	6.44	0.30
1	0.33	0.07	1	5.93	0.38
2	0.09	0.06	3	3.37	0.38
3	0.13	0.06	3	0.22	0.10

Table 4 Ammonia oxidizing activity under the presence of ATU at 30 and 50 μM

ATU 30 μM			ATU 50 μM		
Time (day)	Average concentration ($mgNL^{-1}$)	SD	Time (day)	Average concentration ($mgNL^{-1}$)	SD
0	7.03	0.92	0	6.78	0.30
1	7.03	0.86	1	7.09	0.42
2	6.61	0.26	2	7.60	0.25
3	6.71	0.28	3	8.07	0.15
5	6.08	0.37	5	6.75	0.34
7	5.14	0.43	7	6.64	0.37
10	3.50	0.72	10	5.36	0.61

Table 5 Ammonia oxidizing activity under the presence of ATU at 80 and 100 μM

ATU 80 μM			ATU 100 μM		
Time (day)	Average concentration ($mgNL^{-1}$)	SD	Time (day)	Average concentration ($mgNL^{-1}$)	SD
0	6.87	0.33	0	6.70	0.44
1	6.66	0.22	1	6.79	0.62
2	6.87	0.32	2	6.88	0.59
3	7.07	0.45	3	7.18	0.71
5	7.21	0.29	5	7.35	0.61
7	7.51	0.40	7	8.01	0.17
10	7.05	0.62	10	7.96	0.34

Table 6 Ammonia oxidizing activity under the presence of ATU at 150 and 200 μM

ATU 150 μM			ATU 200 μM		
Time (day)	Average concentration ($mgNL^{-1}$)	SD	Time (day)	Average concentration ($mgNL^{-1}$)	SD
0	6.77	0.30	0	7.02	0.55
1	6.92	0.36	1	6.99	0.84
2	7.53	0.95	2	6.99	0.17
3	7.78	0.60	3	7.06	0.23
5	7.52	0.50	5	7.15	0.24
7	8.10	0.71	7	8.11	0.19
10	8.35	0.42	10	8.22	0.31

Table 7 Ammonia oxidizing activity under the presence of ATU at 1000 and 2000 μM

ATU 1000 μM			ATU 2000 μM		
Time (day)	Average concentration ($mgNL^{-1}$)	SD	Time (day)	Average concentration ($mgNL^{-1}$)	SD
0	6.39	0.23	0	6.22	0.22
1	6.39	0.31	1	6.52	0.40
2	6.55	0.36	2	6.39	0.21
3	6.80	0.39	3	6.55	0.17
5	7.07	0.33	5	6.95	0.34
7	7.31	0.32	7	6.94	0.55
10	7.84	0.24	10	7.29	0.45

3.2 Ammonia oxidizing activity of sludge B and C under the presence of ATU

Table 8 Ammonia oxidizing activity of the control

Control					
Sludge B			Sludge C		
Time (day)	Average concentration ($mgNL^{-1}$)	SD	Time (day)	Average concentration ($mgNL^{-1}$)	SD
0	7.88	0.16	0	8.38	0.44
2	8.82	0.25	2	6.65	0.66
4	5.30	0.45	4	1.09	0.35
6	6.75	0.42	7	0.48	0.02
9	5.59	1.24	10	0.16	0.07
12	1.07	0.20	-	-	-

Table 9 Ammonia oxidizing activity under the presence of ATU at 10 μM

ATU 10 μM					
Sludge B			Sludge C		
Time (day)	Average concentration ($mgNL^{-1}$)	SD	Time (day)	Average concentration ($mgNL^{-1}$)	SD
0	8.35	2.16	0	8.16	0.67
2	9.24	2.56	2	8.28	0.34
4	9.21	0.39	4	7.72	0.14
6	9.78	1.95	7	7.22	0.14
9	14.40	0.37	10	5.88	0.91
12	12.16	0.92	-	-	-

Table 10 Ammonia oxidizing activity under the presence of ATU at 30 μM

ATU 30 μM					
Sludge B			Sludge C		
Time (day)	Average concentration ($mgNL^{-1}$)	SD	Time (day)	Average concentration ($mgNL^{-1}$)	SD
0	7.30	0.26	0	8.63	0.50
2	7.51	0.76	2	7.89	0.18
4	8.59	0.12	4	7.41	0.12
6	8.36	0.40	7	7.67	0.35
9	12.57	0.60	10	7.17	1.13
12	12.04	0.61	-	-	-

Table 11 Ammonia oxidizing activity under the presence of ATU at 50 μM

ATU 50 μM					
Sludge B			Sludge C		
Time (day)	Average concentration ($mgNL^{-1}$)	SD	Time (day)	Average concentration ($mgNL^{-1}$)	SD
0	6.68	0.27	0	8.03	0.60
2	7.21	0.41	2	8.16	0.31
4	8.31	0.25	4	7.43	0.02
6	8.21	0.64	7	7.69	0.28
9	11.14	0.33	10	7.28	0.60
12	11.18	0.31	-	-	-

Table 12 Ammonia oxidizing activity under the presence of ATU at 80 μM

ATU 80 μM					
Sludge B			Sludge C		
Time (day)	Average concentration ($mgNL^{-1}$)	SD	Time (day)	Average concentration ($mgNL^{-1}$)	SD
0	6.78	0.22	0	8.15	0.24
2	7.74	1.16	2	8.02	0.29
4	7.55	0.53	4	7.78	0.13
6	8.37	0.14	7	7.58	0.08
9	10.04	0.85	10	7.41	0.78
12	9.42	0.43	-	-	-

Table 13 Ammonia oxidizing activity under the presence of ATU at 100 μM

ATU 100 μM					
Sludge B			Sludge C		
Time (day)	Average concentration ($mgNL^{-1}$)	SD	Time (day)	Average concentration ($mgNL^{-1}$)	SD
0	6.96	0.45	0	8.15	0.24
2	7.52	0.04	2	8.07	0.08
4	7.28	0.45	4	7.68	0.40
6	8.97	0.94	7	7.76	0.15
9	9.83	1.02	10	7.04	1.03
12	10.16	0.24	-	-	-

Table 14 Ammonia oxidizing activity under the presence of ATU at 150 μM

ATU 150 μM					
Sludge B			Sludge C		
Time (day)	Average concentration ($mgNL^{-1}$)	SD	Time (day)	Average concentration ($mgNL^{-1}$)	SD
0	6.59	0.36	0	7.72	0.43
2	6.83	0.41	2	8.72	0.21
4	8.16	0.02	4	8.18	0.13
6	9.46	1.33	7	7.92	0.36
9	9.75	0.43	10	8.75	0.71
12	9.81	0.47	-	-	-

Table 15 Ammonia oxidizing activity under the presence of ATU at 200 μM

ATU 200 μM					
Sludge B			Sludge C		
Time (day)	Average concentration ($mgNL^{-1}$)	SD	Time (day)	Average concentration ($mgNL^{-1}$)	SD
0	6.34	0.55	0	8.41	0.31
2	7.27	0.07	2	8.03	0.09
4	7.89	1.05	4	7.80	0.19
6	9.42	1.19	7	7.44	0.15
9	8.61	1.24	10	7.11	1.08
12	10.02	0.36	-	-	-

Table 16 Ammonia oxidizing activity under the presence of ATU at 500 μM

ATU 500 μM					
Sludge B			Sludge C		
Time (day)	Average concentration ($mgNL^{-1}$)	SD	Time (day)	Average concentration ($mgNL^{-1}$)	SD
0	6.11	0.77	0	8.41	0.31
2	7.11	0.30	2	7.80	0.21
4	8.00	0.15	4	7.17	0.24
6	7.39	0.90	7	7.22	0.12
9	7.35	0.59	10	6.64	1.32
12	9.08	0.29	-	-	-

Table 17 Ammonia oxidizing activity under the presence of ATU at 1000 μM

ATU 1000 μM					
Sludge B			Sludge C		
Time (day)	Average concentration ($mgNL^{-1}$)	SD	Time (day)	Average concentration ($mgNL^{-1}$)	SD
0	4.66	0.18	0	8.41	0.31
2	5.38	0.76	2	7.46	0.44
4	6.58	0.59	4	6.76	1.14
6	7.61	0.18	7	7.13	0.14
9	7.65	0.70	10	6.62	1.42
12	8.73	0.43	-	-	-

Table 18 Ammonia oxidizing activity under the presence of ATU at 2000 μM

ATU 2000 μM					
Sludge B			Sludge C		
Time (day)	Average concentration ($mgNL^{-1}$)	SD	Time (day)	Average concentration ($mgNL^{-1}$)	SD
0	4.25	0.16	0	5.56	0.78
2	4.34	0.26	2	6.93	0.39
4	5.08	0.56	4	5.91	0.19
6	5.64	2.02	7	5.74	0.33
9	6.32	0.19	10	6.37	0.25
12	7.70	0.76	-	-	-

3.3 Ammonia oxidizing activity of sludge D and E under the presence of ATU

Table 19 Ammonia oxidizing activity of the control

Control					
Sludge D			Sludge E		
Time (day)	Average concentration ($mgNL^{-1}$)	SD	Time (day)	Average concentration ($mgNL^{-1}$)	SD
0	6.73	0.27	0	7.87	0.17
1.3	0.57	0.24	3	3.74	0.28
2.3	0.05	0.03	6	1.47	0.09
3.3	0.03	0.03	10	0.56	0.33

Table 20 Ammonia oxidizing activity under the presence of ATU at 10 μM

ATU 10 μM					
Sludge D			Sludge E		
Time (day)	Average concentration ($mgNL^{-1}$)	SD	Time (day)	Average concentration ($mgNL^{-1}$)	SD
0	6.87	0.23	0	7.70	0.20
1.3	5.53	0.14	3	5.88	0.17
2.3	5.28	0.36	6	5.85	0.43
3.3	4.50	0.22	10	4.72	0.23
5.8	2.68	0.44	-	-	-
8	0.10	0.06	-	-	-
9.9	0.17	0.07	-	-	-

Table 21 Ammonia oxidizing activity under the presence of ATU at 30 μM

ATU 30 μM					
Sludge D			Sludge E		
Time (day)	Average concentration ($mgNL^{-1}$)	SD	Time (day)	Average concentration ($mgNL^{-1}$)	SD
0	6.58	0.21	0	7.61	0.42
1.3	5.56	0.20	3	6.43	0.19
2.3	5.17	0.05	6	6.38	0.22
3.3	4.58	0.41	10	6.06	0.09
5.8	3.23	0.14	-	-	-
8	2.33	0.21	-	-	-
9.9	1.55	0.23	-	-	-

Table 22 Ammonia oxidizing activity under the presence of ATU at 50 μM

ATU 50 μM					
Sludge D			Sludge E		
Time (day)	Average concentration ($mgNL^{-1}$)	SD	Time (day)	Average concentration ($mgNL^{-1}$)	SD
0	6.79	0.19	0	7.44	0.23
1.3	6.15	0.11	3	6.45	0.20
2.3	5.69	0.12	6	6.58	0.16
3.3	5.32	0.15	10	6.40	0.24
5.8	3.31	0.22	-	-	-
8	2.41	0.33	-	-	-
9.9	1.62	0.27	-	-	-

Table 23 Ammonia oxidizing activity under the presence of ATU at 80 μM

ATU 80 μM					
Sludge D			Sludge E		
Time (day)	Average concentration ($mgNL^{-1}$)	SD	Time (day)	Average concentration ($mgNL^{-1}$)	SD
0	6.78	0.13	0	7.52	0.37
1.3	5.81	0.21	3	6.21	0.24
2.3	5.31	0.18	6	6.54	0.44
3.3	4.83	0.15	10	6.28	0.33
5.8	3.46	0.20	-	-	-
8	2.72	0.35	-	-	-
9.9	2.09	0.26	-	-	-

Table 24 Ammonia oxidizing activity under the presence of ATU at 100 μM

ATU 100 μM					
Sludge D			Sludge E		
Time (day)	Average concentration ($mgNL^{-1}$)	SD	Time (day)	Average concentration ($mgNL^{-1}$)	SD
0	6.65	0.31	0	7.07	0.64
1.3	5.72	0.24	3	6.80	0.13
2.3	5.29	0.17	6	6.57	0.16
3.3	4.80	0.21	10	6.67	0.28
5.8	3.77	0.14	-	-	-
8	2.97	0.11	-	-	-
9.9	2.30	0.30	-	-	-

Table 25 Ammonia oxidizing activity under the presence of ATU at 150 μM

ATU 150 μM					
Sludge D			Sludge E		
Time (day)	Average concentration ($mgNL^{-1}$)	SD	Time (day)	Average concentration ($mgNL^{-1}$)	SD
0	6.88	0.06	0	7.49	0.20
1.3	5.83	0.04	3	6.60	0.03
2.3	5.26	0.27	6	6.18	0.07
3.3	4.89	0.13	10	6.41	0.20
5.8	3.48	0.18	-	-	-
8	3.18	0.79	-	-	-
9.9	2.31	0.31	-	-	-

Table 26 Ammonia oxidizing activity under the presence of ATU at 200 μM

ATU 200 μM					
Sludge D			Sludge E		
Time (day)	Average concentration ($mgNL^{-1}$)	SD	Time (day)	Average concentration ($mgNL^{-1}$)	SD
0	6.32	0.88	0	7.56	0.28
1.3	5.68	0.41	3	6.87	0.36
2.3	5.45	0.24	6	7.20	0.15
3.3	5.15	0.03	10	6.98	0.41
5.8	3.93	0.11	-	-	-
8	3.33	0.30	-	-	-
9.9	2.54	0.33	-	-	-

Table 27 Ammonia oxidizing activity under the presence of ATU at 500 μM

ATU 500 μM					
Sludge D			Sludge E		
Time (day)	Average concentration ($mgNL^{-1}$)	SD	Time (day)	Average concentration ($mgNL^{-1}$)	SD
0	6.79	0.19	0	7.29	0.33
1.3	6.15	0.11	3	6.71	0.77
2.3	5.69	0.12	6	6.85	0.58
3.3	5.32	0.15	10	7.74	0.69
5.8	4.45	0.20	-	-	-
8	3.86	0.34	-	-	-
9.9	3.13	0.28	-	-	-

Table 28 Ammonia oxidizing activity under the presence of ATU at 1000 μM

ATU 1000 μM					
Sludge D			Sludge E		
Time (day)	Average concentration ($mgNL^{-1}$)	SD	Time (day)	Average concentration ($mgNL^{-1}$)	SD
0	6.88	0.22	0	7.04	0.52
1.3	6.35	0.25	3	5.82	0.33
2.3	6.18	0.17	6	6.14	0.64
3.3	5.88	0.29	10	6.76	0.22
5.8	5.00	0.30	-	-	-
8	4.54	0.18	-	-	-
9.9	4.08	0.17	-	-	-

Table 29 Ammonia oxidizing activity under the presence of ATU at 2000 μM

ATU 2000 μM					
Sludge D			Sludge E		
Time (day)	Average concentration ($mgNL^{-1}$)	SD	Time (day)	Average concentration ($mgNL^{-1}$)	SD
0	6.48	0.30	0	5.39	0.55
1.3	6.13	0.20	3	4.55	1.65
2.3	5.96	0.15	6	4.38	0.95
3.3	5.80	0.17	10	5.13	1.72
5.8	5.55	0.18	-	-	-
8	5.31	0.23	-	-	-
9.9	4.98	0.28	-	-	-

3.4 Relationship between percent inhibition of ammonia oxidation and percent AOB

Table 30 Percent inhibition of ammonia oxidation at the ATU concentration of 80 μM and percent AOB in WWTP sludge

Sludge sample	%AOB	% inhibition of ammonia oxidation
Sludge A	100	100
Sludge B	98.75	100
Sludge C	78.04	92.57
Sludge D	0.54	78.05
Sludge E	7.20	82.25

Appendix C

1 Ammonia oxidizing activity under the presence of various PNP concentrations of NRI sludge

1.1 Initial ammonia concentration of 7 $mgNL^{-1}$

Table 1 Ammonia oxidizing activity under the presence of PNP at 0 and 1 mgL^{-1}

Ammonia concentration 7 $mgNL^{-1}$				
Time (h)	Control		PNP 1 mgL^{-1}	
	Average concentration ($mgNL^{-1}$)	SD	Average concentration ($mgNL^{-1}$)	SD
0	8.71	1.00	8.12	0.43
3	8.74	1.12	7.76	0.37
6	8.48	1.16	7.43	0.05
9	7.91	1.72	7.78	0.34
23	6.19	1.98	6.50	0.42
52	1.36	1.38	1.98	0.35

Table 2 Ammonia oxidizing activity under the presence of PNP at 5 and 10 mgL^{-1}

Ammonia concentration 7 $mgNL^{-1}$				
Time (h)	PNP 5 mgL^{-1}		PNP 10 mgL^{-1}	
	Average concentration ($mgNL^{-1}$)	SD	Average concentration ($mgNL^{-1}$)	SD
0	8.43	0.46	8.68	0.51
3	7.34	0.24	8.05	0.55
6	7.83	0.48	8.39	0.66
9	7.79	0.45	7.24	0.55
23	7.93	0.71	7.52	0.24
52	7.57	0.81	6.71	0.74

Table 3 Ammonia oxidizing activity under the presence of PNP at 50 and 100 mgL^{-1}

Ammonia concentration 7 mgNL^{-1}				
Time (h)	PNP 50 mgL^{-1}		PNP 100 mgL^{-1}	
	Average concentration (mgNL^{-1})	SD	Average concentration (mgNL^{-1})	SD
0	8.34	0.07	8.13	0.37
3	7.98	0.26	8.14	0.11
6	7.88	0.41	7.85	0.61
9	7.25	1.06	8.03	0.59
23	8.51	0.19	7.88	0.05
52	8.00	0.41	8.13	0.37

Table 4 Ammonia oxidizing activity under the presence of PNP at 200 and 400 mgL^{-1}

Ammonia concentration 7 $mgNL^{-1}$				
Time (h)	PNP 200 mgL^{-1}		PNP 400 mgL^{-1}	
	Average concentration ($mgNL^{-1}$)	SD	Average concentration ($mgNL^{-1}$)	SD
0	8.94	0.88	8.94	0.88
3	7.74	0.70	7.74	0.70
6	8.12	0.25	8.34	0.08
9	8.57	0.78	7.43	0.79
23	7.65	0.21	7.93	0.33
52	8.81	0.49	8.38	1.34

2 Ammonia oxidizing activity under the presence of various PNP concentrations of NRII sludge

2.1 Initial ammonia concentration of 7 mgNL^{-1}

Table 5 Ammonia oxidizing activity under the presence of PNP at 0 and 1 mgL^{-1}

Ammonia concentration 7 mgNL^{-1}				
Time (h)	Control		PNP 1 mgL^{-1}	
	Average concentration (mgNL^{-1})	SD	Average concentration (mgNL^{-1})	SD
0	7.16	0.27	7.28	0.17
4	5.00	1.41	6.21	0.78
16	1.56	0.94	1.82	1.33
24	0.13	0.08	1.34	0.96

Table 6 Ammonia oxidizing activity under the presence of PNP at 2.5 and 5 mgL^{-1}

Ammonia concentration 7 mgNL^{-1}				
Time (h)	PNP 2.5 mgL^{-1}		PNP 5 mgL^{-1}	
	Average concentration (mgNL^{-1})	SD	Average concentration (mgNL^{-1})	SD
0	7.22	0.10	7.17	0.13
4	6.44	0.45	6.40	0.76
16	2.07	1.30	2.45	1.25
24	1.48	1.28	1.83	1.12

Table 7 Ammonia oxidizing activity under the presence of PNP at 7.5 and 10 mgL^{-1}

Ammonia concentration 7 $mgNL^{-1}$				
Time (h)	PNP 7.5 mgL^{-1}		PNP 10 mgL^{-1}	
	Average concentration ($mgNL^{-1}$)	SD	Average concentration ($mgNL^{-1}$)	SD
0	7.42	0.06	7.49	0.14
4	6.67	0.35	5.67	0.70
16	2.16	1.00	1.64	0.49
24	1.91	0.88	1.34	0.57

Table 8 Ammonia oxidizing activity under the presence of PNP at 25 and 50 mgL^{-1}

Ammonia concentration 7 $mgNL^{-1}$				
Time (h)	PNP 25 mgL^{-1}		PNP 50 mgL^{-1}	
	Average concentration ($mgNL^{-1}$)	SD	Average concentration ($mgNL^{-1}$)	SD
0	7.22	0.20	7.52	0.47
4	6.42	0.28	6.77	0.51
16	3.46	0.69	4.49	0.18
24	2.73	0.56	3.91	0.46

Table 9 Ammonia oxidizing activity under the presence of PNP at 100 and 200 mgL^{-1}

Ammonia concentration 7 $mgNL^{-1}$				
Time (h)	PNP 100 mgL^{-1}		PNP 200 mgL^{-1}	
	Average concentration ($mgNL^{-1}$)	SD	Average concentration ($mgNL^{-1}$)	SD
0	7.73	0.47	7.61	0.62
4	6.79	0.26	7.75	0.08
16	6.39	0.26	5.54	0.61
24	6.21	0.18	5.32	1.07

Table 10 Ammonia oxidizing activity under the presence of PNP at 400 mgL^{-1}

Ammonia concentration 7 $mgNL^{-1}$		
Time (h)	PNP 400 mgL^{-1}	
	Average concentration ($mgNL^{-1}$)	SD
0	7.80	0.22
4	8.01	0.05
16	6.99	0.33
24	6.80	0.50

3.2.2 Initial ammonia concentration of 14 mgNL^{-1}

Table 11 Ammonia oxidizing activity under the presence of PNP at 0 and 1 mgL^{-1}

Ammonia concentration 14 mgNL^{-1}				
Time (h)	Control		PNP 1 mgL^{-1}	
	Average concentration (mgNL^{-1})	SD	Average concentration (mgNL^{-1})	SD
0	16.60	0.98	16.33	0.49
3	13.48	0.39	13.31	0.52
6	10.78	1.57	10.58	2.18
9	10.00	1.47	9.90	1.74
23	4.64	1.64	5.12	1.99
52	0.45	0.26	1.62	2.36

Table 12 Ammonia oxidizing activity under the presence of PNP at 5 and 10 mgL^{-1}

Ammonia concentration 14 $mgNL^{-1}$				
Time (h)	PNP 5 mgL^{-1}		PNP 10 mgL^{-1}	
	Average concentration ($mgNL^{-1}$)	SD	Average concentration ($mgNL^{-1}$)	SD
0	15.43	0.15	15.22	0.89
3	13.72	0.36	15.13	0.37
6	11.98	2.10	15.18	0.35
9	10.50	1.50	14.96	0.56
23	6.06	0.76	12.60	0.94
52	1.53	2.37	5.57	0.37

Table 13 Ammonia oxidizing activity under the presence of PNP at 50 and 100 mgL^{-1}

Ammonia concentration 14 $mgNL^{-1}$				
Time (h)	PNP 50 mgL^{-1}		PNP 100 mgL^{-1}	
	Average concentration ($mgNL^{-1}$)	SD	Average concentration ($mgNL^{-1}$)	SD
0	16.79	0.27	15.98	0.76
3	15.44	0.43	15.43	0.40
6	15.52	0.18	15.46	0.95
9	16.36	0.76	15.71	0.21
23	15.27	0.35	14.34	2.28
52	13.79	1.69	13.73	1.38

Table 14 Ammonia oxidizing activity under the presence of PNP at 200 and 400 mgL^{-1}

Ammonia concentration 14 $mgNL^{-1}$				
Time (h)	PNP 200 mgL^{-1}		PNP 400 mgL^{-1}	
	Average concentration ($mgNL^{-1}$)	SD	Average concentration ($mgNL^{-1}$)	SD
0	15.46	0.47	12.83	1.37
3	15.44	0.47	13.64	0.17
6	14.94	0.27	12.54	0.65
9	15.86	0.28	14.07	0.19
23	15.77	0.30	13.98	1.08
52	13.91	0.78	12.78	2.13

2.3 Initial ammonia concentration of 70 $mgNL^{-1}$

Table 15 Ammonia oxidizing activity under the presence of PNP at 0 and 5 mgL^{-1}

Ammonia concentration 70 $mgNL^{-1}$				
Time (h)	Control		PNP 5mgL^{-1}	
	Average concentration ($mgNL^{-1}$)	SD	Average concentration ($mgNL^{-1}$)	SD
0	91.27	5.50	78.97	3.45
3.5	83.41	4.13	72.43	2.20
7	71.49	1.92	74.91	0.28
26	32.15	2.58	36.77	5.57
49	6.15	0.97	18.77	0.05

Table 3.16 Ammonia oxidizing activity under the presence of PNP at 10 and 50 mgL^{-1}

Ammonia concentration 70 $mgNL^{-1}$				
Time (h)	PNP 10 mgL^{-1}		PNP 50 mgL^{-1}	
	Average concentration ($mgNL^{-1}$)	SD	Average concentration ($mgNL^{-1}$)	SD
0	88.12	3.49	89.49	2.39
3.5	88.17	6.85	78.88	2.55
7	76.71	2.21	77.77	2.61
26	56.99	5.01	71.89	2.38
49	18.42	0.19	74.06	3.90

Table 17 Ammonia oxidizing activity under the presence of PNP at 100 and 200 mgL^{-1}

Ammonia concentration 70 $mgNL^{-1}$				
Time (h)	PNP 100 mgL^{-1}		PNP 200 mgL^{-1}	
	Average concentration ($mgNL^{-1}$)	SD	Average concentration ($mgNL^{-1}$)	SD
0	76.21	2.41	78.01	5.05
3.5	75.35	7.17	80.24	2.34
7	77.10	2.51	75.53	0.69
26	76.09	3.81	69.68	4.72
49	75.77	2.48	79.20	4.10

Table 18 Ammonia oxidizing activity under the presence of PNP at 400 and 800 mgL^{-1}

Ammonia concentration 70 $mgNL^{-1}$				
Time (h)	PNP 400 mgL^{-1}		PNP 800 mgL^{-1}	
	Average concentration ($mgNL^{-1}$)	SD	Average concentration ($mgNL^{-1}$)	SD
0	88.07	1.19	82.86	9.49
3.5	72.74	9.27	73.34	2.48
7	75.32	3.44	77.20	3.31
26	72.03	8.37	79.08	3.65
49	68.03	6.95	73.21	1.96

Table 19 Ammonia oxidizing activity under the presence of PNP at 1000 and 2000 mgL^{-1}

Ammonia concentration 70 $mgNL^{-1}$				
Time (h)	PNP 1000 mgL^{-1}		PNP 2000 mgL^{-1}	
	Average concentration ($mgNL^{-1}$)	SD	Average concentration ($mgNL^{-1}$)	SD
0	82.21	8.54	76.22	0.71
3.5	70.73	0.65	80.15	1.76
7	65.92	3.25	81.67	1.33
26	77.61	11.44	73.26	6.03
49	75.64	5.44	82.13	3.72

2.4 Percent inhibition of ammonia oxidation of NRI and NRII sludge at various PNP and initial ammonia concentrations

Table 20 Percent inhibition of ammonia oxidation of NRI and NRII sludge at various PNP and initial ammonia concentrations

PNP	NRI		NRII					
	7 $mgNL^{-1}$		7 $mgNL^{-1}$		14 $mgNL^{-1}$		70 $mgNL^{-1}$	
	Average	SD	Average	SD	Average	SD	Average	SD
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1	11.37	3.93	16.92	13.06	7.50	14.63	-	-
2.5	-	-	18.93	17.55	-	-	-	-
5	-	-	24.33	16.09	7.52	15.91	18.25	1.19
7.5	-	-	24.45	12.18	-	-	-	-
10	89.64	9.17	16.50	8.05	34.87	2.13	15.15	0.72
25	-	-	36.81	9.13	-	-	-	-
50	95.72	4.93	51.02	3.31	81.55	9.42	81.53	4.89
100	97.12	2.51	80.14	3.38	85.55	7.57	98.97	1.35
200	100.00	0.00	69.77	15.11	89.73	4.07	94.62	1.70
400	100.00	0.00	87.01	9.00	90.46	16.52	75.54	7.74

2.5 Ammonia oxidation rate of NRII sludge at various PNP and initial ammonia concentrations

Table 21 Ammonia oxidation rate of NRII sludge at various PNP and initial ammonia concentrations

Concentration of PNP (mgL^{-1})	Ammonia oxidation rate ($mgN\ L^{-1}\ d^{-1}$)		
	7 $mgNL^{-1}$	14 $mgNL^{-1}$	70 $mgNL^{-1}$
0	8.05	23.26	67.82
5	7.27	13.79	38.62
10	8.61	0.69	39.13
50	4.55	5.11	40.17
100	1.73	2.07	-3.05
200	3.41	2.06	8.52

2.6 Composition of active nitrifying microorganisms in NRII sludge before and after exposing to PNP for 48 h

Table 22 Composition of active nitrifying microorganisms in NRII sludge before and after NRII sludge exposing to PNP for 48 h

Sample	Total AOB		*Nitrobacter*		*Nitrospira*		Other microbes	
	Average	SD	Average	SD	Average	SD	Average	SD
NRII	48.0	12.7	15.0	9.9	13.8	7.7	31.0	23.7
PNP 0 mgL^{-1}	60.1	6.0	9.4	3.9	3.9	3.3	26.8	10.2
PNP 10 mgL^{-1}	40.3	12.3	10.3	4.6	3.4	2.4	46.0	19.0
PNP 200 mgL^{-1}	35.5	17.6	18.5	11.1	4.2	2.5	41.8	29.6

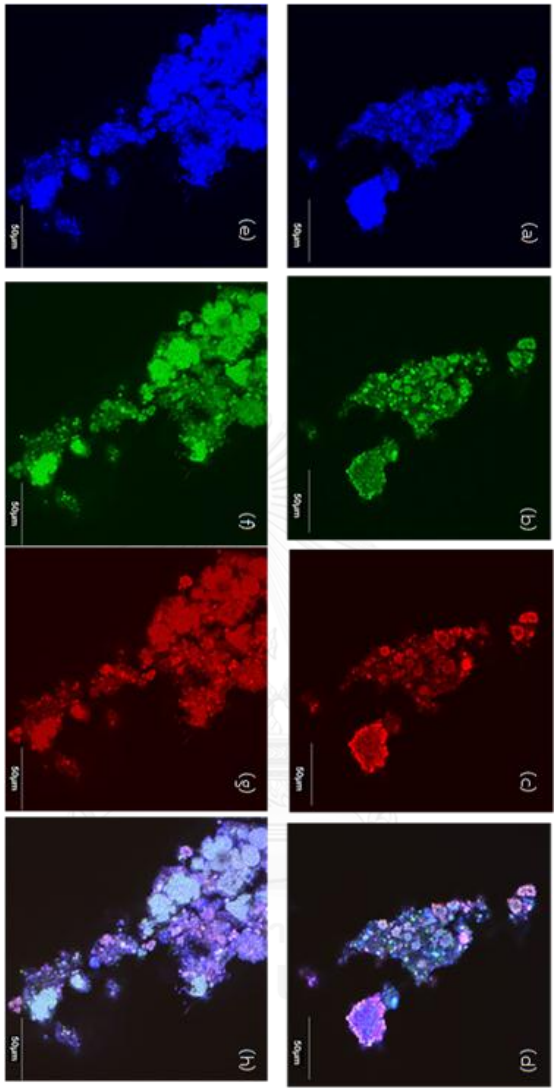


Figure 3.1 FISH signal for NRII. (Light sensitivity and threshold were adjusted in all images) First row showed FISH signal of (a) total microorganisms, (b) AOB, (c) *Nitrobacter* and (d) combination of all microorganisms, AOB and *Nitrobacter*. Second row showed FISH signal of (e) total microorganisms, (f) AOB, (g) *Nitrospira* and (h) combination of all microorganisms, AOB and *Nitrospira*.

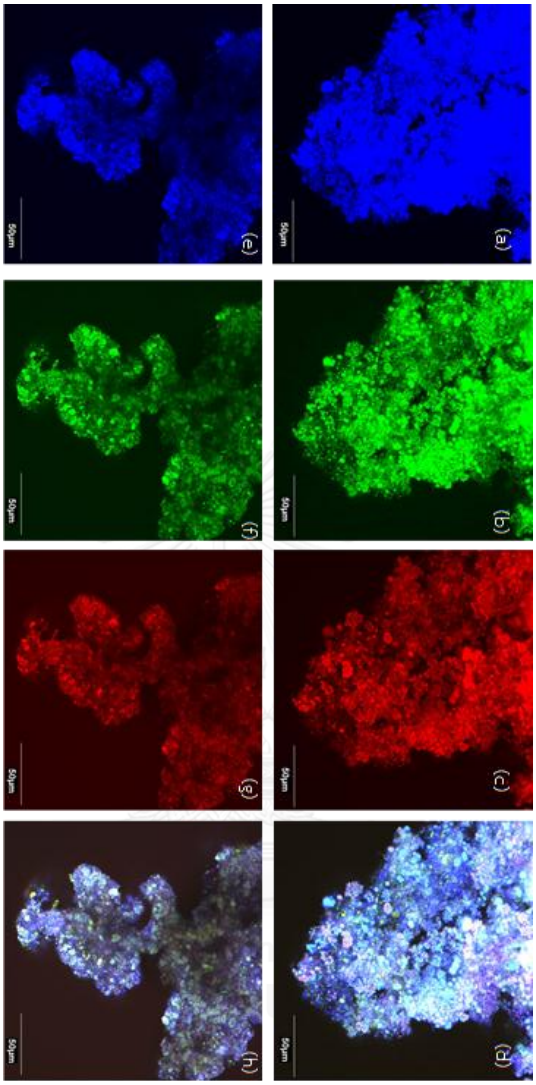


Figure 3.2 FISH signal for control (PNP 0 mgL^{-1}). (Light sensitivity and threshold were adjusted in all images)

First row showed FISH signal of (a) total microorganisms, (b) AOB, (c) *Nitrobacter* and (d) combination of all microorganisms, AOB and *Nitrobacter*.

Second row showed FISH signal of (e) total microorganisms, (f) AOB, (g) *Nitrospira* and (h) combination of all microorganisms, AOB and *Nitrospira*.

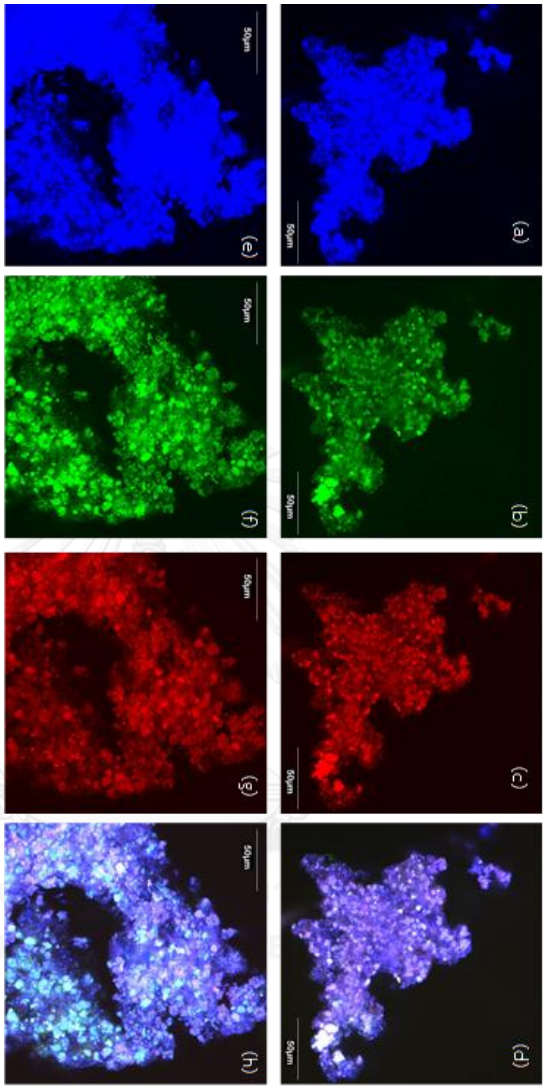


Figure 3.3 FISH signal in a test exposed to 10 mgL-1 of PNP. (Light sensitivity and threshold were adjusted in all images)

First row showed FISH signal of (a) total microorganisms, (b) AOB, (c) Nitrobacter and (d) combination of all microorganisms, AOB and Nitrobacter.

Second row showed FISH signal of (e) total microorganisms, (f) AOB, (g) Nitrospira and (h) combination of all microorganisms, AOB and Nitrospira.

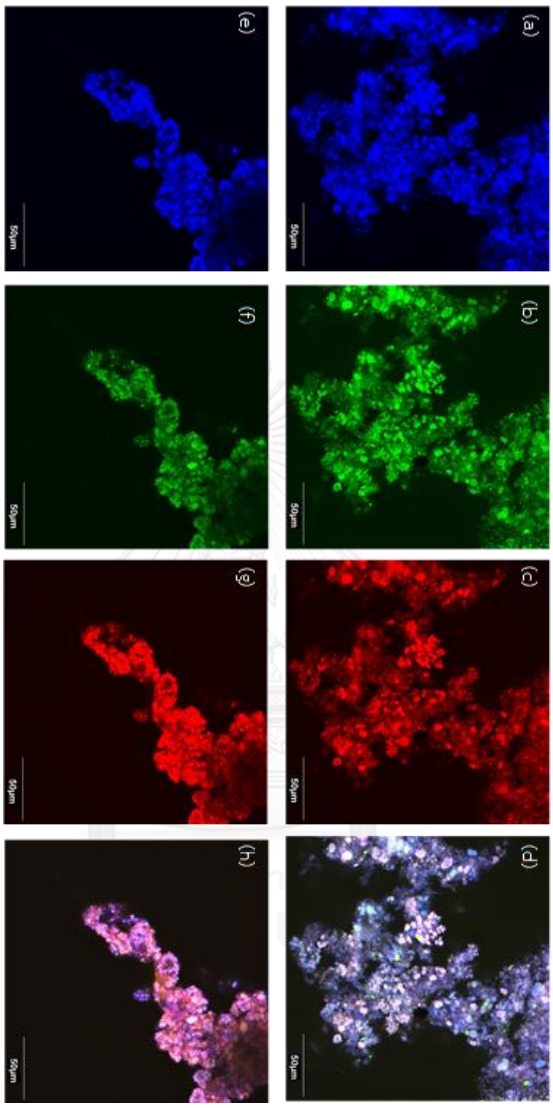


Figure 3.4 FISH signal in a test exposed to 200 mgL-1 of PNP. (Light sensitivity and threshold were adjusted in all images)

First row showed FISH signal of (a) total microorganisms, (b) AOB, (c) Nitrobacter and (d) combination of all microorganisms, AOB and Nitrobacter.

Second row showed FISH signal of (e) total microorganisms, (f) AOB, (g) Nitrospira and (h) combination of all microorganisms, AOB and Nitrospira

VITA

Name Papitchaya Srithep

Date of Birth February 22, 1985

Place of Birth Suratthani, Thailand

Institution Attended

1. Faculty of Environment and Resource Studies, Mahidol University, Bangkok.

B.Sc. (Environmental Science and Technology)

2. Faculty of Engineering, Chulalongkorn University, Bangkok.

M.Eng. (Environmental Engineering)

3. International Programs in Hazardous Substances and Environmental Management, Graduate School, Chulalongkorn University, Bangkok.

Ph.D. (Environmental Management)

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