Co-effects of silver nanoparticles and microplastics on nitrifying microorganisms from wastewater treatment plants and their activities

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ผลกระทบร่วมของอนุภาคซิลเวอร์นาโนและไมโครพลาสติกต่อจุลินทรีย์กลุ่มไนตริไฟอิงจากโรงบำบัดน้ำเสียและกิจกรรมการกำจัดไนโตรเจน

นายน้ำเพ็ชร เจียรนัยปยุกต์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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น้ำเพ็ชร เจียรนัยปยุกต์ : ผลกระทบร่วมของอนุภาคซิลเวอร์นาโนและไมโครพลาสติกต่อจุลินทรีย์กลุ่มไนตริไฟอิงจากโรงบำบัดน้ำเสียและกิจกรรมการกำจัดไนโตรเจน. (Co-effects of silver nanoparticles and microplastics on nitrifying microorganisms from wastewater treatment plants and their activities) อ.ที่ปรึกษาหลัก : รศ. ดร. ตะวัน ลิมปิยากร, อ.ที่ปรึกษาร่วม : ศ. ดร.เอกลักษณ์ คาน

อนุภาคซิลเวอร์นาโนและไมโครพลาสติกถูกจัดเป็นสารปนเปื้อนที่สามารถพบได้มากในน้ำเสียในปัจจุบัน โดยสารปนเปื้อนทั้งสองชนิดนี้มีสภาวะและการเคลื่อนที่ที่เหมือนกันในระบบบำบัดน้ำเสีย โดยจะสะสมในบ่อบำบัด ซึ่งเป็นที่อยู่อาศัยของจุลินทรีย์ออกซิไดซ์แอมโมเนีย เนื่องจากสารปนเปื้อนทั้งสองชนิดมีคุณสมบัติในการยับยั้งการเจริญของจุลินทรีย์ ทำให้จุลินทรีย์ออกซิไดซ์แอมโมเนียอาจถูกยับยั้งการเจริญเติบโตและยับยั้งกิจกรรมการออกซิไดซ์แอมโมเนียซึ่งอาจก่อให้เกิดผลกระทบต่อสิ่งแวดล้อมได้ ในการศึกษาครั้งนี้ จุลินทรีย์ออกซิไดซ์แอมโมเนีย 3 กลุ่ม ประกอบด้วย AOA, AOB และ comammox ถูกนำไปทดสอบผลกระทบของอนุภาคซิลเวอร์นาโนต่อกิจกรรมการออกซิไดซ์แอมโมเนีย จากผลการทดลองพบว่าไม่มีการยับยั้งกิจกรรมออกซิไดซ์แอมโมเนียเกิดขึ้นที่ความเข้มข้นของอนุภาคซิลเวอร์นาโน 0.1 มิลลิกรัมต่อลิตร ในขณะที่ความเข้มข้น 0.5 และ 1 มิลลิกรัมต่อลิตร อนุภาคซิลเวอร์นาโนสามารถยับยั้งกิจกรรมออกซิไดซ์แอมโมเนียได้บางส่วน และมีการยับยั้งกิจกรรมการออกซิไดซ์แอมโมเนียแบบสมบูรณ์ ที่ความเข้มข้น 2.5, 5 และ 10 มิลลิกรัมต่อลิตร จากการศึกษาการเปลี่ยนแปลงของประชากรจุลินทรีย์พบว่า อนุภาคซิลเวอร์นาโนสามารถยับยั้งการเจริญของ AOB และ comammox ถูกยับยั้งการเจริญที่ความเข้มข้นตั้งแต่ 2.5 และ 0.5 มิลลิกรัมต่อลิตรขึ้นไปตามลำดับ ในขณะที่ AOA ถูกยับยั้งที่ความเข้มข้น 0.5 มิลลิกรัมต่อลิตรขึ้นไปแต่ในจำนวนที่น้อยกว่าเมื่อเปรียบเทียบกับ AOB และ comammox จากนั้น จุลินทรีย์ออกซิไดซ์แอมโมเนียถูกนำไปทดสอบผลกระทบของอนุภาคซิลเวอร์นาโนและไมโครพลาสติกชนิดพีวีซีต่อกิจกรรมการออกซิไดซ์แอมโมเนีย ผลการทดลองพบว่า ไมโครพลาสติกชนิดพีวีซีไม่มีผลในการยับยั้งกิจกรรมการออกซิไดซ์แอมโมเนีย อย่างไรก็ตาม ในชุดการทดลองที่มีการนำโครพลาสติกชนิดพีวีซีไปเขย่าในอาหารเลี้ยงเชื้อ 7 วันก่อนการเติมอนุภาคซิลเวอร์นาโนและจุลินทรีย์ ใช้เวลาในการออกซิไดซ์แอมโมเนียน้อยกว่าชุดการทดลองอื่นที่มีการเติมอนุภาคซิลเวอร์นาโน ดังนั้นจึงอาจกล่าวได้ว่าไมโครพลาสติกชนิดพีวีซีที่ถูกนำไปเขย่าก่อน 7 วันอาจมีการเปลี่ยนแปลงคุณสมบัติที่อาจทำให้อนุภาคซิลเวอร์นาโนมีความเป็นพิษลดลงได้ นอกจากนี้ยังพบว่าไมโครพลาสติกชนิดพีวีซีไม่สามารถยับยั้งการเจริญของจุลินทรีย์ทั้ง 3 กลุ่มได้

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KEYWORD: Silver nanoparticles, Microplastics, Polyvinyl chloride, Ammonia oxidation, Ammonia oxidizing microorganisms, Ammonia oxidizing archaea, Ammonia oxidizing bacteria, Complete ammonia oxidizing bacteria

Nampetch Charanaipayuk : Co-effects of silver nanoparticles and microplastics on nitrifying microorganisms from wastewater treatment plants and their activities. Advisor: Assoc. Prof. TAWAN LIMPIYAKORN, Ph.D. Co-advisor: Prof. Eakalak Khan

Silver nanoparticles (AgNPs) and microplastics are emerging water contaminants of the decade. They share a similar fate and transport in wastewater treatment plants as they tend to accumulate in sludge of aeration tanks. Since both contaminants have negative effects on microbial growth, the ammonia-oxidizing microorganisms in the aeration tanks are at risk for inhibition and consequently nitrification process fails. This study investigated the effects of AgNPs and microplastics on ammonia-oxidizing activity and community. No inhibition of ammonia oxidation rate was observed at 0.1 mg/L AgNPs. Partial inhibition was found at 0.5 and 1 mg/L AgNPs, while complete inhibition occurred at higher concentrations of 2.5, 5, and 10 mg/L AgNPs. qPCR targeting AOA, AOB, and comammox *amoA* genes indicated that the numbers of the AOB *amoA* genes decreased when AgNPs were ≥2.5 mg/L while the comammox *amoA* genes dropped at ≥0.5 mg/L of AgNPs. Inhibition of AOA was found at AgNP concentrations above 0.5 mg/L but in substantially less compared to AOB and comammox. This study suggests that the three ammonia-oxidizing microorganisms have different responses to AgNP. The co-effect of AgNPs and PVC was studied in microcosms at concentrations of 0.5 mg/L and 500 mg/L, respectively. The results showed that the PVC microplastics had no inhibitory effects on the ammonia oxidation rate. Interestingly, the microcosms, in which the PVC was pre-shaken for 7 days before adding the sludge and AgNPs showed, a faster ammonia oxidation rate than the microcosms containing the sludge and AgNPs with and without fresh (non-shaken) PVC. This suggests that the pre-shaken PVC microplastics may reduce the toxicity of AgNPs. qPCR results indicated that PVC microplastics did not suppress AOA, AOB, and comammox.

Field of Study:	Hazardous Substance and Environmental Management	Student's Signature .....
Academic Year:	2022	Advisor's Signature .....
		Co-advisor's Signature .....

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Nampetch Charanaipayuk

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

Chapter 1
Introduction

1.1 Thesis title

English: Co-effects of silver nanoparticles and microplastics on nitrifying microorganisms from wastewater treatment plants and their activities

ภาษาไทย: ผลกระทบร่วมของอนุภาคซิลเวอร์นาโนและไมโครพลาสติกต่อจุลินทรีย์กลุ่มไนตริไฟอิงจากโรงบำบัดน้ำเสียและกิจกรรมการกำจัดไนโตรเจน

1.2 Keywords

Silver nanoparticles (AgNPs)

Microplastics

Polyvinyl chloride (PVC)

Ammonia oxidation

Ammonia oxidizing archaea (AOA)

Ammonia oxidizing bacteria (AOB)

Complete ammonia oxidizing bacteria (Comammox)

1.3 Introduction and background

Silver nanoparticles (AgNPs) are common nanoparticles as they have wide applications due to the broad-spectrum antimicrobial activity (Yang et al., 2014). AgNPs have been added to several consumer products; for example, detergents, medical products, and textiles, and used in environmental applications (Klasen, 2000; León-Silva et al., 2016; Massarsky et al., 2014). The global production of AgNPs was up to 550 metric tons annually in 2012 (Piccinno et al., 2012). Silver nanoparticles have been synthesized with various properties depending on functions and applications. Generally, AgNPs are coated with negatively charge capping agents, hydrophilic polymers, and starch (Vasileva et al., 2011; Zheng et al., 2017). AgNPs

can affect the microorganisms either heterotrophic or autotrophic. Yuan et al. (2013) found that AgNPs can inhibit *Nitrosomonas europaea*, which is an ammonia-oxidizing bacterium (AOB), by damaging cell wall. They reported that the toxicity of AgNPs is related to the concentration and released Ag^{+}. Zheng et al. (2017) discovered that AgNPs as well as Ag^{+} can inhibit the nitrification rate by suppressing ammonia monooxygenase gene (*amo*) and hydroxylamine oxidase gene (*hao*) which are the genes involved in the first step of nitrification. Because of AgNPs can inhibit the growth of microorganisms, they are additives in detergents and consumer products and consequently end up in municipal wastewater.

Microplastic is another contaminant that is regarded as one of the major environmental issues in the past decade. Normally, the size of microplastic is less than 5 mm and is made of polyethylene (PE), polypropylene (PP), polyvinylchloride (PVC), and other types of plastics (Carr et al., 2016). Microplastics are spreading into the water sources such as rivers (McCormick et al., 2014; Yonkos et al., 2014), oceans (Pabortsava & Lampitt, 2020), as well as wastewater treatment plants (Conley et al., 2019; Tagg et al., 2020). They exhibit potential to adversely impact aquatic life, human health, and microorganisms. Several studies indicate that several types of microplastic can decrease the nitrifying rate of the microorganisms. Song et al. (2020) reported that PVC can decrease the partial rates of AOB and nitrite-oxidizing bacteria (NOB), which play the main role in the nitrification process. They described that PVC released from microplastic is toxic to both types of nitrifying bacteria. Li et al. (2020) concluded that PE, polystyrene (PS), polyester (PES) have negative effects on the nitrification activity of activated sludge. However, they founded that the PP and PVC can promote the nitrification rate marginally.

Facing AgNPs is unavoidable for wastewater treatment plants (WWTPs). Municipal wastewater in Germany contained AgNPs up to 1.5 µg/L (Li et al., 2013). Shafer et al. (1998) revealed that 105 µg of total silver were detected in sewage. Not only AgNPs but also microplastics were found in municipal wastewater. Magnusson and NorÈn (2014) reported that one of the WWTPs in Sweden experienced microplastics in the influent up to 1,500 particles per liter. Fibers are a major form of these microplastics followed by fragments and flakes. Microplastics tend to accumulate in the sludge more than 1,600 particles per kilogram of wet weight (Magnusson & NorÈn, 2014). Ziajahromi et al. (2017) investigated microplastics in the effluent of WWTPs in Sydney, Australia. They concluded that most of the microplastic is in a fiber form of polyethylene terephthalate (PET) with a particle size range of 25 to 100 µm. Also, they found the irregular shape of PE at a similar amount to PET, whereas PS and PP were also found at trace levels (Ziajahromi et al., 2017). These studies suggested the co-occurrence of AgNPs and microplastics in municipal wastewater. Therefore, heterotrophic bacteria and/or nitrifying microorganisms that play important roles in wastewater treatment could be concurrently affected by the two contaminants.

Nitrifying microorganisms in WWTPs are a key microbial group for biological nitrogen removal from wastewater. Generally, nitrification is the main pathway used to convert ammonium (NH_4^+) to nitrite (NO_2^-) and then nitrate (NO_3^-) via biochemical oxidation. Nitrification can be divided into two steps, ammonia (NH_3) to NO_2^-, and NO_2^- to nitrate. The first step can be accomplished by ammonia-oxidizing archaea (AOA) and AOB, while the second step can be done by nitrite-oxidizing bacteria (NOB) such as *Nitrospira* group (Koch et al., 2015), *Nitrobacter* group (Sorokin et

al., 2012), and *Nitrotoga* group (Alawi et al., 2007). NH_3 can be converted into nitrate by a single microorganism from *the Nitrospira* group that is classified as commamox (Daims et al., 2015). Nitrifying organisms are very sensitive to environmental conditions such as pH, temperature, oxygen concentration, and toxic substances including heavy metals (Amatya et al., 2011; Kim et al., 2006; Stenstrom & Poduska, 1980; You et al., 2009). Therefore, co-synergistic effects of AgNPs and microplastics on nitrifying microorganisms are possible and worth an investigation.

Although AgNPs were reported to negatively impact the ammonia oxidation activity of nitrifying sludge, the understanding of this pollutant on nitrifying community has yet been clarified. In addition, how microplastics, as an emerging contaminant, influence ammonia oxidizing activity and community has yet extensively studied. Since these two contaminants have similar transport and fate in wastewater treatment plants, it is important to investigate the co-effect of AgNPs and microplastics on nitrifying activity and community.

1.4 Objectives

1.4.1 To evaluate the individual effects of AgNPs and PVC microplastics on ammonia oxidation activity and ammonia oxidizing microbial community.

1.4.2 To evaluate co-effect of AgNPs and PVC microplastics on ammonia oxidation activity and microbial community of ammonia oxidizing microorganisms.

1.5 Hypotheses

1.5.1 AgNPs suppress the growth of AOB and comammox greater than AOA. The rationale for this hypothesis is that since the cell wall of AOA is less permeable

than AOB and comammox it lessens the toxicity of AgNPs. (Valentine, 2007; Yin et al., 2018).

1.5.2 PVC microplastics reduce ammonia oxidation activity and inhibit AOB and comammox more than AOA. The rationale for this hypothesis is the same as that for the preceding hypothesis.

1.5.3 AgNPs together with PVC microplastics can reduce the ammonia oxidation activity of ammonia oxidizing microorganisms more than AgNPs or PVC microplastics alone. It is known that both contaminants show the negative effects on ammonia oxidation microorganisms so the severity of the effects might increase when they co-occur (Song et al., 2020; Zheng et al., 2017).

1.6 Scope of study

1.6.1 Two sludge samples were collected from industrial and municipal wastewater treatment plants, one sample for each plant type.

1.6.2 The sludge samples were cultivated in a bioreactor, supplied with inorganic medium mixing with 40 mgN/L of ammonium sulfate $(NH_4)_2SO_4$. The pH of the bioreactor was controlled at 7.4 using 0.2 M of sodium hydroxide (NaOH).

1.6.3 AgNPs were prepared in a yellow brown colloidal form with a size range of 5-20 nm. The concentrations of AgNPs in microcosms were 0.1, 0.5, 1, 2.5, 5, 10 mg/L based on preliminary experiments.

1.6.4 PVC microplastic was synthesized. The size of the synthesized microplastic was between 106-500 μm. The concentration of microplastics in microcosms was 500 mg/L based on previous work (Qin et al., 2020).

1.6.5 Microcosms were operated by adding sludge at 100 mg MLSS/L and supplied with inorganic medium and ammonium chloride (NH_4Cl) at 40 mgN/L. The pH of the microcosms was controlled at 7.5 with 25 mM final concentration of 3-(N-morpholino)-propanesulfonic acid (MOPS) (Zheng et al., 2017). The microcosms were incubated in the dark at 30°C and 200 rpm shaking.

1.6.6 Ammonia oxidation rate was determined based on change in NH_3 concentration in the microcosms per time.

1.6.7 The numbers of AOA, AOB, and comammox in bioreactor and microcosms were determined using quantitative polymerase chain reaction (qPCR).

1.7 Expected outcomes

1.7.1 To observe the survival of ammonia oxidizing microorganisms and predict ammonia oxidation activity under stress condition induced by AgNPs and microplastics in wastewater.

1.7.2 To determine the co-effects of AgNPs and microplastics and predict the toxicity of both contaminants to nitrifying microorganisms from wastewater origins.

Chapter 2

Literature review

2.1 Nitrogen cycle

Nitrogen is the essential element for every organism as it is a key structural component of proteins and nucleic acids (Holmes et al., 2019). It is the most abundant element in the atmospheric gas in a form nitrogen gas (N_2) (Hagemann et al., 2016). However, most organisms cannot use N_2 directly. The bioavailable nitrogen forms that most of organism can use for growth and survival are mainly NH_3 and NO_3^- (Galloway et al., 2008). Some microorganisms from archaea and bacteria domains called diazotrophs can convert N_2 to NH_3. Diazotrophs can live through both symbiosis and free living in terrestrial and marine environment (Breznak et al., 1973; Phillips, 1980). Normally, NH_3 in water and wastewater is generated from microbial activities as well as animal and human activities through urine excretion, use of fertilizer, body decomposition, food processing, and use of household cleaning products. High concentrations of NH_3 can be toxic to aquatic organisms and adversely affect ecosystem (Dasan & Edward, 2019). Therefore, NH_3 can be oxidized to nitrate by microorganisms via nitrification. Nitrate can return to atmosphere via denitrification to N_2. This process by denitrifiers is a main method to remove reactive nitrogen species in water and wastewater. Overall, the nitrogen cycle and involved genes and reactions are shown in Figure 2-1.

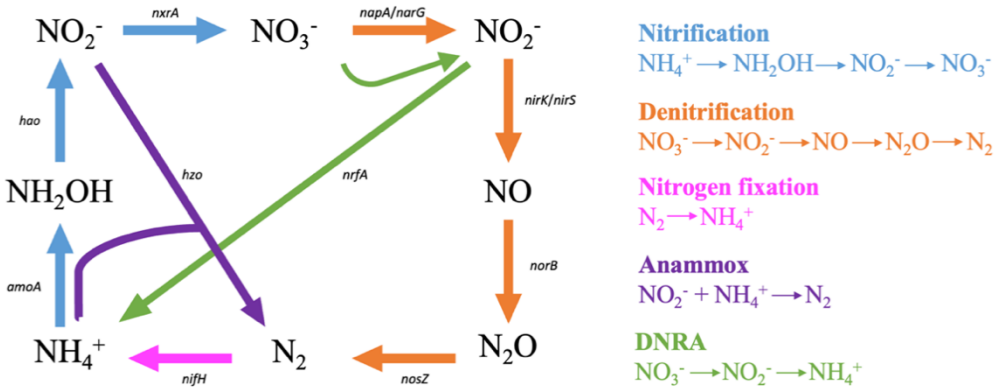


Figure 2-1 Nitrogen cycle with genes and reactions involved adapted from Hagemann et al. (2016)

2.1.1 Nitrogen fixation

Even though N_2 is a major gas in the atmosphere, most of organisms do not have an ability to fix nitrogen gas. Nitrogen fixation is the process to transform N_2 to reactive nitrogen by diazotrophs which are commonly known as N_2-fixing microorganisms (Francis et al., 2007). N_2-fixing microorganisms can be free-living microbes and symbiont with other organisms (Mus et al., 2016; Phillips, 1980; Vitousek et al., 2002). These microbes contain nitrogenase enzyme, encoded from *nif* gene, that reduce N_2 to NH_4^+, a nitrogen form which is more readily bioavailable for plants and other organisms (Reed et al., 2011). For the reason that nitrogenase is highly sensitive to oxygen, N_2 fixation usually takes place under O_2 limiting conditions (Vitousek et al., 2002).

2.1.2 Nitrification

Nitrification is a process driven by nitrifying microorganisms, in which NH_3 is converted to NO_3^- by oxidation reaction. Nitrification consists of two step processes: first, NH_3 or NH_4^+ is oxidized to NO_2^- (nitritation) driven by ammonia-oxidizing microorganisms and then NO_2^- is converted to NO_3^- (nitratation) driven by nitrite-

oxidizing microorganisms (Rodriguez-Caballero et al., 2013; Yu et al., 2018). Hydroxylamine (NH_2OH) is the intermediate product and NO_2^- is the end product of nitritation process (Hagemann et al., 2016). Ammonia monooxygenase (AMO) is the enzyme for NH_3 oxidizing activity and NH_2OH is the product of this enzyme. NH_2OH is turned into NO_2^- by the activity of hydroxylamine oxidoreductase (HAO) (Braker & Conrad, 2011). These two enzymes can be founded in ammonia oxidizing microorganisms. After nitritation completes, NO_2^- is further oxidized to NO_3^- (nitratation). Nitrite oxidoreductase (NXR) is the NO_2^- oxidizing enzyme, which is found in nitrite oxidizing microorganisms (Daims et al., 2016). During nitritation and nitratation, the nitrifying microorganisms turn carbon in the form of CO_2 to organic compounds which acquired energy (Yamanaka, 2008). The processes of nitritation and nitratation are shown in Figure 2-2. Although NO_3^- is a nitrogen source of plant and aerobic microorganisms, high concentration of NO_3^- can cause negative impact on environments such as eutrophication in surface waters (Schleper & Nicol, 2010). Nitrification is the main pathway used to remove nitrogen in wastewater treatment (Hagemann et al., 2016).

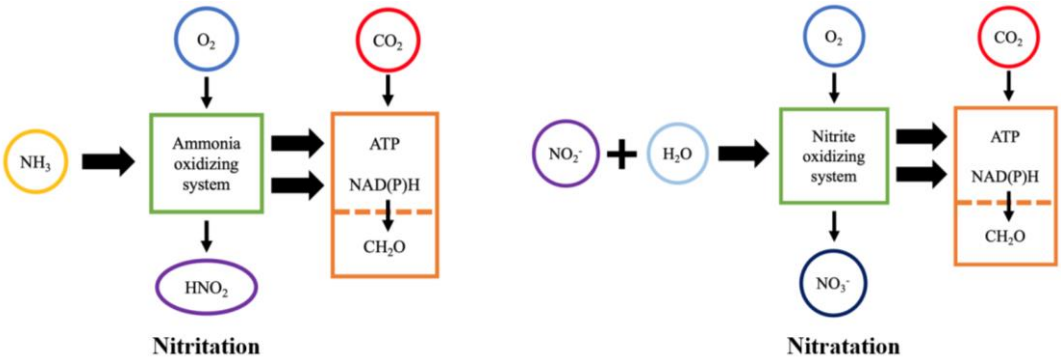


Figure 2-2 Processes of nitritation and nitratation adapted from Yamanaka (2008)

2.1.3 Denitrification

Denitrification is a process in nitrogen cycle which converts NO_3^- to N_2 (Francis et al., 2007), changing nitrogen in the bioavailable form to the gaseous form. This process is widely used in WWTPs to remove nitrogen to prevent the environmental effects such as eutrophication (Holmes et al., 2019). NO_2^-, nitric oxide (NO) and nitrous oxide (N_2O) are the intermediates of the process (Francis et al., 2007). Microorganisms containing the enzymes that can completely denitrify NO_3^- to N_2 are called true denitrifiers. However, some denitrifiers that lack of key enzymes, nitrite reductase (NiR), are called incomplete denitrifying microorganisms, will generate NO_2^- as a product (Holmes et al., 2019). Denitrifying bacteria utilize an organic compound together with NO_3^- and acquire N_2, CO_2, and energy as products. This process is called nitrate respiration (Yamanaka, 2008). The process of nitrate respiration is shown in Figure 2-3.

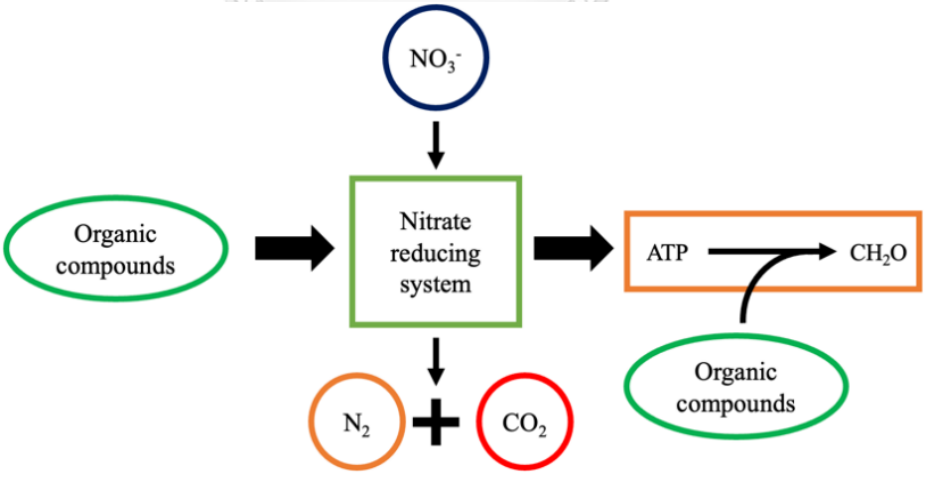


Figure 2-3 Process of nitrate respiration adapted from Yamanaka (2008)

2.2 Ammonia oxidizing microorganisms

2.2.1 Ammonia oxidizing archaea

AOA is a group of microorganisms having an ability to nitrify NH_3^+ to NO_2^- (Könneke et al., 2005). The cell volume of most AOA was smaller than AOB 10 to 100 times (Yin et al., 2018). The lipid membrane of archaea is completely different from that of bacteria (Brown & Doolittle, 1997). The phospholipid of AOA consists of glycerol with isoprenoid as a side chain and are linked to moiety glycerol with ether bond, instead of ester bond as found in bacteria. Moreover, the isoprenoid of AOA is linked to each other in the formation of monolayer (Schleper & Nicol, 2010). The main component of cellular lipid membrane is glycerol diakyl glycerol tetraethers (GDGTs) that may consist of multiple cyclopentane inside chain. The GDGTs of AOA is shown in Figure 2-4. These lipid membrane structures are less permeable compared to those of AOB. This infers that the ammonia oxidation rate, futile ion cycling, and level of energy maintenance of AOA are lower than those of AOB. However, these characteristics provide AOA with an ability to adapt better to the environment (Valentine, 2007; Yin et al., 2018). Martens-Habbena et al. (2009) have reported that one of the AOA, *Nitrosopumilus maritimus* SMA1, has a lower ammonia oxidation rate than AOB about by 10-fold. Ammonia oxygenase (AMO) is the key enzyme for ammonia oxidation in AOA. Nevertheless, there is a debate on a choice of substrate for archaeal AMO between NH_4^+ or NH_3 (Yin et al., 2018). HAO is the enzyme that turns hydroxylamine (NH_2OH), a product of AMO, into NO_2^- in AOB but has not yet identified in AOA (Schatteman et al., 2022; Schleper & Nicol, 2010).

There are several environmental factors that affect AOA growth. High NH_3 concentrations can suppress the growth of AOA (Gao et al., 2016; Sauder et al., 2012; Ye & Zhang, 2011). Hydrazine, a substrate of hydroxylamine dehydrogenase for AOB, can both induce and inhibit the ammonia oxidation rate of AOA (Schatteman et al., 2022). Oxygen concentration also affects the ammonia oxidation rate of the archaea. Under low oxygen concentrations, AOA tend to be higher in number compared to AOB due to their higher tolerance to adverse conditions (Yin et al., 2018). According to de la Torre et al. (2008), AOA were found in the deep-water region of the North Japan Sea at a temperature of 0.2°C, while Nakagawa et al. (2007) found AOA in a hot spring at 74°C in the Yellowstone National Park. AOA also show the ability to survive in acidic and alkaline conditions (He et al., 2007; Jiang et al., 2014; Xiao et al., 2017). *Candidatus* Nitrosotalea devanaterra showed an ability to adapt to pH variation (Gubry-Rangin et al., 2011).

Generally, municipal WWTPs have more AOB than AOA (Gao et al., 2014; Gao et al., 2013; Limpiyakorn et al., 2011). Nevertheless, some WWTPs have higher abundance of AOA than AOB (Bai et al., 2012; Limpiyakorn et al., 2013). These reports support that AOA may be founded widely in WWTPs, especially those operated under stress conditions, for example, low temperature or low DO (Yin et al., 2018). AOA can be divided into 5 clusters including *Nitrososphrera*, *Nitrosocosmicus*, *Nitrosocaldus*, *Nitrosotalea*, and *Nitrosopumilus* (Pester et al., 2012). Distinct habitats drive the occurrence of different of AOA groups (Figure 2-5). The studies of *amoA* gene revealed that AOA found in terrestrial and marine environments are placed in distinct lineages (Crump & Baross, 2000; Ochsenreiter et al., 2003; Schleper et al., 1997; Schleper & Nicol, 2010). In addition, the coincidence

of ammonia oxidizing activity of AOA and AOB is mostly found in soil environment. On the other hand, AOA in marine environment is more relevant to nitrification compared to AOB (Schleper & Nicol, 2010).

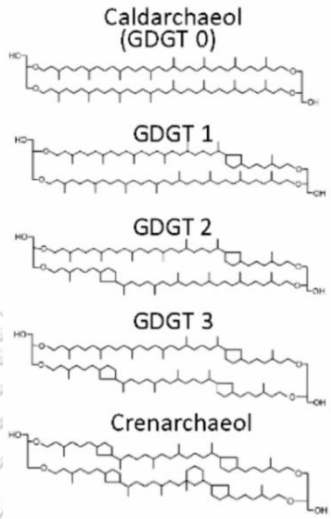


Figure 2-4 GDGTs found in archaeal lipid membrane (Schleper & Nicol, 2010)

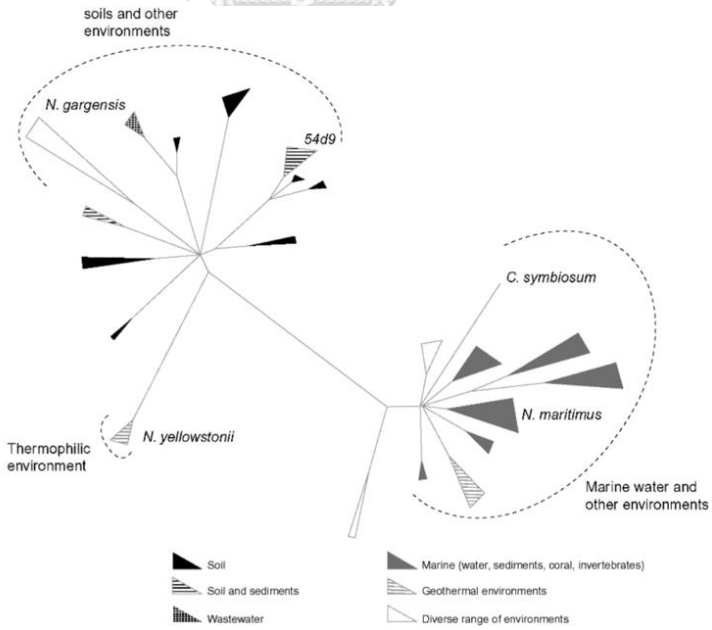


Figure 2-5 Phylogenetic tree of archaeal *amoA* gene-define lineage and their habitat (Schleper & Nicol, 2010)

2.2.2 Ammonia oxidizing bacteria

AOB is a group of chemolithoautotrophic bacteria having an ability to utilize NH_4^+ and carbon dioxide (CO_2) as nitrogen and carbon sources for growth (Koops & Pommerening-Röser, 2005). Most of them are from family *Nitrobacteraceae* which is classified into two phylogenetic groups. The first group is genus *Nitrosococcus* in the class of Gammaproteobacteria. The second group consists of genera *Nitrosospira* and *Nitrosomonas* in the class of Betaproteobacteria (Holmes et al., 2019; Koops & Pommerening-Röser, 2005). Most of Betaproteobacterial AOB are found in soil habitat and wastewater treatment systems while Gammeproteobacterial AOB are found in marine habitat and acidic wastewater (Lehtovirta-Morley, 2018). The phylogenetic tree of AOB is shown in Figure 2-6. AMO and HAO are the two key enzymes of AOB which play the main role in converting NH_3^+ to NO_2^- (Arp et al., 2002). Therefore, the 2 genes tied to AMO and HAO, *amo* and *hao*, were used as target genes to detect AOB. NH_3 acts as a substrate for bacterial AMO (Suzuki et al., 1974). AOBs live in various environments such as soils, rivers, freshwater, salt lake, soda lake, oceans, and WWTPs (French et al., 2012; Li et al., 2018; Limpiyakorn et al., 2011; Prosser & Nicol, 2012; Sorokin et al., 2001; Wang et al., 2017; Ward et al., 2000). AOBs that live in aquatic environment have been often observed to produce biofilm or floc (Avrahami et al., 2011; Fudala-Ksiazek et al., 2014; Soliman & Eldyasti, 2018), which is a survival strategy for AOB under unfavorable conditions.

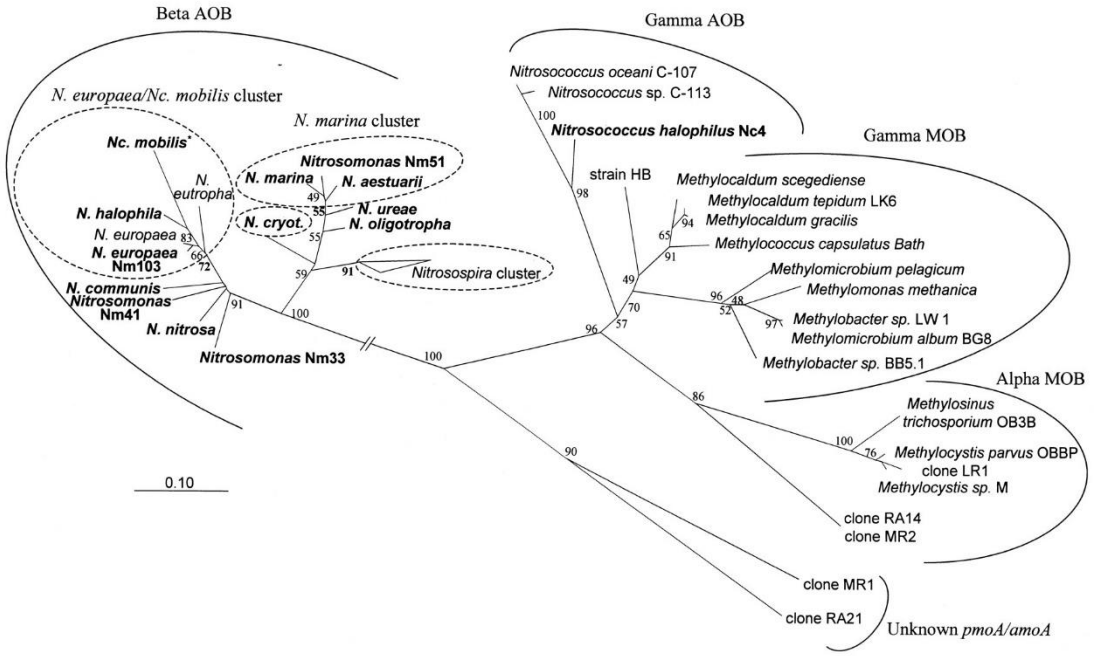


Figure 2-6 Phylogenetic tree of AOB and methane oxidizing bacteria (MOB) (Purkhold et al., 2000)

2.2.3 Comammox

Comammox is a group of bacteria having an ability to convert NH_3 to NO_3^- by themselves, unlike AOA and AOB that can only partially nitrify. For its discovery, comammox was isolated from a recirculation aquaculture system, where effluent ammonium concentration was lower than 100 µM. The bacteria were cultured in a hypoxic bioreactor with low nitrogen concentration for a year. An image from fluorescence in situ hybidrization reveals that comammox made up 15% of the community (van Kessel et al., 2015).

The DNA of an enriched nitrifying culture was extracted and analyzed for its genome. The result shows that the 2 comammox from the culture are from *Nitrospira* group, which is well known for nitrite oxidizing bacteria, but their genome contains the genes that are related to both first and second steps of nitrification. The genome of

comammox contains the full set of *amo* and *hao*. Moreover, the genome contains nitrite oxidoreductase gene (*nxr*) that is involved in nitrite oxidation activity. Interestingly, a phylogenetic tree reveals that the comammox AmoA protiens are novel and are rather identical to methane monooxygenase of *Crenothrix polyspora*, methane oxidizing bacteria, more than bacterial AmoA. These discoveries change the perspective of nitrogen cycle and effort to use comammox in WWTPs to remove NH_3 from wastewater (Daims et al., 2015; van Kessel et al., 2015). Different from AOB, comammox are well adaptable to low NH_3 habitats. However, the growth rate of comammox is lower than other ammonia oxidizers due to the pathway length under competitive condition (Lehtovirta-Morley, 2018).

2.3 Ammonia oxidation pathway

Ammonia monooxygenase (AMO) is the enzyme that plays an important role in the first step of ammonia oxidation in ammonia oxidizing microorganisms. AMO consists of 3 subunits including AmoA, AmoB, and AmoC that are encoded by *amo* gene. Different ammonia oxidizers have different structures of *amo* genes. Because *amo* is highly diverse, *amo* gene is used as a marker for ammonia oxidizers in the environment especially the *amoA* gene (Francis et al., 2005; Norton et al., 2002; Rotthauwe et al., 1997). In fact, AMO of AOA, AOB, and comammox are working in different conditions due to the different structures and physiology (Lehtovirta-Morley, 2018). Although NH_3 concentration and pH are the main factors, other environmental factors also affect to the diversity and abundance of ammonia oxidizing microorganisms (Bates et al., 2011; Lehtovirta-Morley, 2018; Martens-Habbena et al., 2009; Merbt et al., 2012).

2.3.1 AOA

AOA contain *amoA*, *amoB*, and *amoC* that encode the 3 subunits of AMO. AOA also contain *amoX* that encode the additional subunit (Kerou et al., 2016; Treusch et al., 2005). The achaeal *amo* gene arrangement varies depending on the species and may consist of multiple isolated *amo* gene copies. In addition, these genes may locate together or separately in different open reading frames (Lehtovirta-Morley, 2018). It is still unclear which is the substrate for archaeal AMO between NH_3 or NH_4^+. Martens-Habbena et al. (2009) revealed that the growth of AOA is higher than AOB under low NH_3 concentration. This implies that archaeal AMO favorably works under the condition that NH_3 is scarce. AOA can oxidize NH_3 at low concentrations because their AMO have high substrate affinity. In addition, the half saturation constant (K_m) of AOA is lower than AOB (Martens-Habbena et al., 2009; Prosser & Nicol, 2012). AOA do not have HAO and there is no clear conclusion on NH_2OH oxidizing pathway. Kerou et al. (2016) suggested that NH_2OH are oxidized by multicopper oxidase (MCO1), while Kozlowski et al. (2016) published that the copper containing proteins (CCPs) act as electron transporters for NH_2OH oxidation. The model of ammonia oxidation pathway for AOA is shown in Figure 2-7. AOA fix HCO_3^- as a carbon source via hydroxypropionate-hydroxybutyrate cycle (Figure 2-8) (Berg et al., 2007).

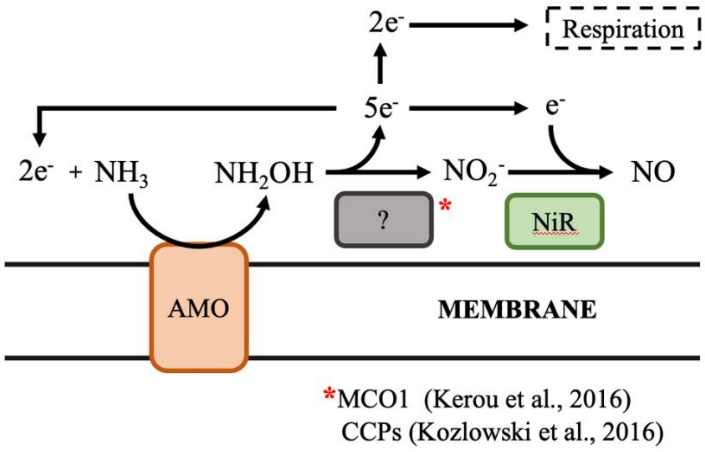


Figure 2-7 Archaeal ammonia oxidation pathway adapted from Lehtovirta-Morley (2018)

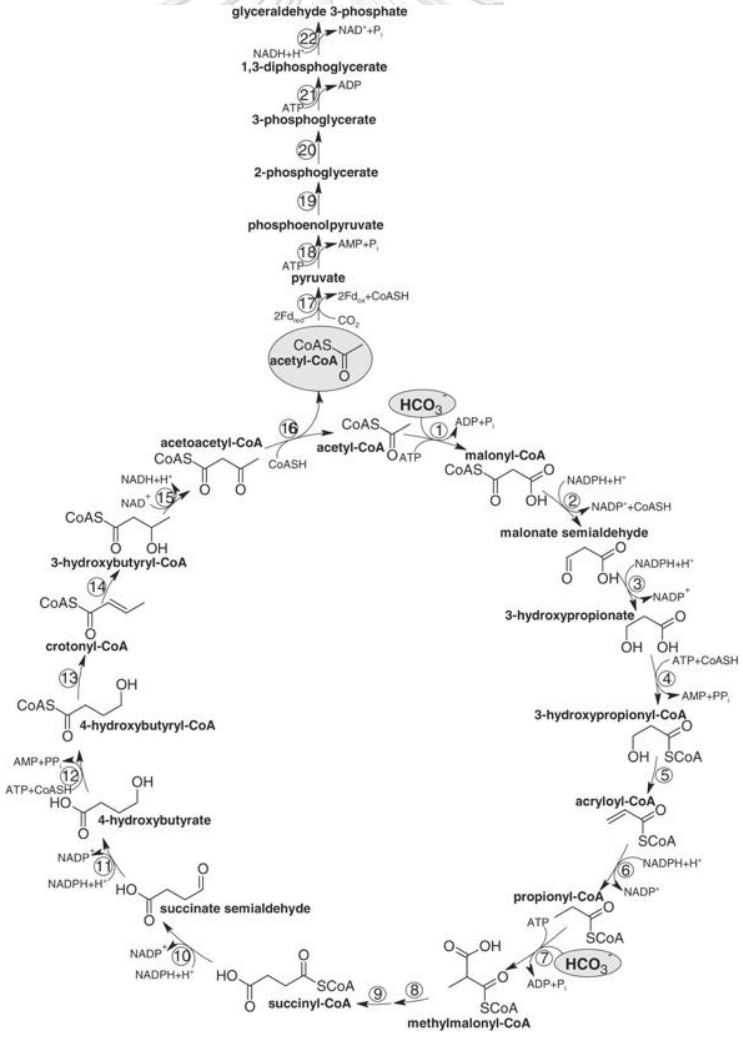


Figure 2-8 Hydroxypropionate-hydroxybutyrate cycle (Berg et al., 2007)

2.3.2 AOB

Bacterial AMO consists of 3 subunits that encoded from *amoA*, *amoB*, and *amoC*. The *amo* gene cluster organization of AOB is *amoCAB* that often contains multiple copies (Arp et al., 2007). Under acidic condition, most of ammonia exists as in NH_4^+. Therefore, AMO of AOB does not function under acidic condition since bacterial AMO substrate is NH_3. However, AOB can survive by hydrolyzing urea as a nitrogen source and promoting the biofilm formation (Burton & Prosser, 2001; De Boer et al., 1991). AOB have a large amount of an extensive cytoplasmic membrane structure that is in favor of ammonia oxidation (Fiencke & Bock, 2006). Since AMO is a membrane-bound protein, extensive membrane can increase the number of AMO in AOB. After bacterial AMO turns NH_3 to NH_2OH, HAO and nitrite reductase (NiR) oxidize NH_2OH to NO and NO_2^-, respectively. Although NiR can reduce NO_2^- to NO, it also oxidizes NO to NO_2^- (Wijma et al., 2004). However, Kozlowski et al. (2016) suggested that there are also some other oxidizing enzymes that convert NO to NO_2^- since *nirK* does not exist in *Nitrosomonas communis*. The bacterial ammonia oxidation pathway is shown in Figure 2-9. Unlike AOA, atmospheric CO_2 is fixed through the Calvin cycle for use as a carbon source for AOB (Lehtovirta-Morley, 2018).

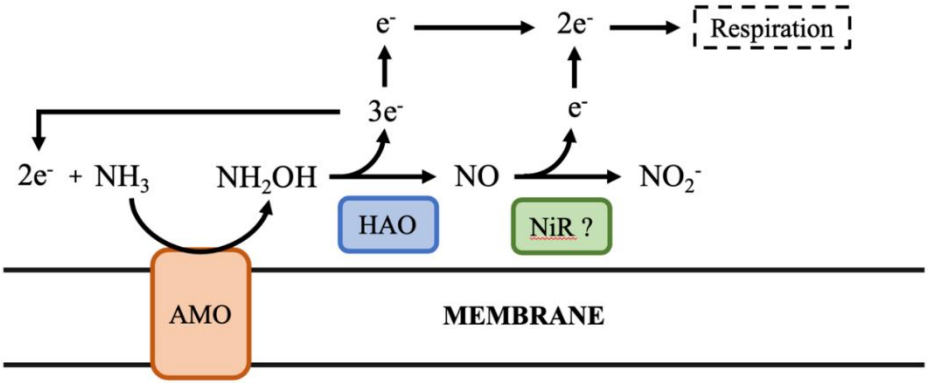


Figure 2-9 Bacterial ammonia oxidation pathway adapted from Lehtovirta-Morley (2018)

2.3.3 Comammox

Comammox *amo* gene includes *amoA*, *amoB*, and *amoC* like AOB. The comammox *amo* gene arrangement is *amoBAC* (van Kessel et al., 2015). Comammox also have *haoA* and *haoB* that encode HAO enzyme which turns NH_2OH to NO. In addition, *nxr* gene, which is found in *Nitrospir*a genera, also exists in comammox genome (van Kessel et al., 2015). Therefore, comammox can turn NH_3 to NH_2OH, NO_2^-, and NO_3^- by itself using AMO, HAO, and NXR enzymes, respectively. Comammox has an ability to survive under low NH_3 concentration like AOA as comammox AMO has a great substrate affinity and low K_m (Kits et al., 2017). This can increase the product yield. The growth rate of comammox is lower than AOA and AOB under low NH_3 concentration due to the pathway length (Costa et al., 2006). Comammox lacks an extensive cytoplasmic membrane, so most of their AMO is located in plasma membrane (Lehtovirta-Morley, 2018). Although the carbon source of comammox is the same as AOB, the reductive tricaboxylic acid cycle is the

pathway that comammox use to fix CO_2 from atmosphere (Berg et al., 2007). The comammox ammonia oxidation pathway is illustrated in Figure 2-10.

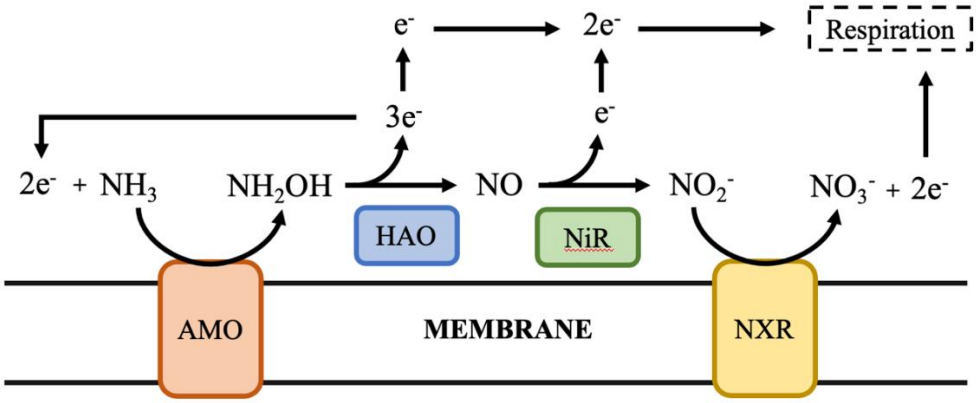


Figure 2-10 Comammox ammonia oxidation pathway adapted from Lehtovirta-Morley (2018)

2.4 Silver nanoparticles

AgNPs are nanoparticles that are widely produced during this decade. The global production of AgNPs is up to 550 tons per year (Piccinno et al., 2012). Pulit-Prociak and Banach (2016) estimated that the AgNPs production will reach more than 800 tons in 2025. AgNPs is produced in a large amount, larger than any other nanomaterials/nanoparticles due to their wide applications (Bouallegui et al., 2017; Katsumiti et al., 2015; Tiede et al., 2009). More than 80% were used as antimicrobial coatings for paints, cleaning agents, textiles, and medical equipment. Some of AgNPs were used in electronic devices and cosmetics. These applications lead to their potential release to water bodies resulting in environmental effects (Piccinno et al., 2012).

2.4.1 Fate and transport of AgNPs in WWTPs

Hedberg et al. (2014) evaluated the fate and transport of AgNPs and silver nitrate ($AgNO_3$) in a pilot wastewater treatment plant. They founded that most of AgNPs and $AgNO_3$ accumulated on the activated sludge. This implies that the nitrifying bacteria in wastewater treatment plants are exposed to AgNPs (Hedberg et al., 2014). Li et al. (2013) quantified AgNPs in municipal wastewater in Germany. Approximately 35% of 1.5 µg/L in the influent was removed by mechanical treatment such as screen and grit chamber. More than 72% of the left over AgNPs from mechanical treatment were reduced by biological treatment. Overall, more than 95% of AgNPs in wastewater were removed.

2.4.2 Quantities of AgNPs in WWTPs

AgNPs have been used as an antimicrobial agent in many commercial products, and municipal wastewater containing AgNPs is common. In 2013, the concentrations of AgNPs in the influent of nine municipal WWTPs in Germany were determined. Silver was analyzed by using graphite furnace atomic absorption spectrometry. In addition. the AgNPs were extracted by Ion Exchange Resin (IER) and Cloud Point Extraction (CPE) methods before analyzing them. The range of influent total silver concentration was 0.32 and 3.05 µg/L. The concentration of AgNPs in the influent was up to 1.5 µg/L associated with the total silver of 3.05 µg/L. Free Ag^+ concentration in the influent was calculated and the lowest and highest concentrations were 0.03 and 0.20 µg/L, respectively. However, AgNPs concentration decreased dramatically after treatment process. The ranges of effluent AgNPs were 2.2 to 9.4 ng/L based on the IER method, and 1.0 to 12.0 ng/L based on the CPE method (Li et al., 2013).

2.4.3 Transformation of AgNPs in water

The form of AgNPs is dependent on the chemistry of water. In natural freshwater, AgNPs remains stable (Li et al., 2022; Zou et al., 2017). Some of the natural organic matter (NOM) contents, such as humic acid, dissolve in natural freshwater. The NOM can maintain the stability of AgNPs via electrostatic and satirical promotion (Chen & Elimelech, 2007). In contrast, the form of AgNPs can be influenced by salinity, ionic strength, and dissolved organic matter (DOM). AgCl can be formed in saline water since AgNPs become unstable under high salinity condition. (Wimmer et al., 2020). High ionic strengths promote AgNPs aggregation by neutralizing the charge (Baalousha et al., 2013). Zou et al. (2017) reported that high DO interrupted the stability of AgNPs. Ag^+ is released from AgNPs in water by oxidation which is promoted by DO and H^+ (Liu & Hurt, 2010; Lok et al., 2007; Sotiriou & Pratsinis, 2010; Zou et al., 2017). Smaller sizes of AgNPs can release more Ag^+ due to more contact surface (Sotiriou & Pratsinis, 2010).

2.4.4 Effects of AgNPs on nitrifying microorganisms

Since AgNPs contain the broad-spectrum antimicrobial activity, they inhibit autotrophic microorganisms especially AOB (Beddow et al., 2016; Choi et al., 2008). In fact, AgNPs can severely suppress the growth of AOB. A previous study reported that 1 mg/L of 14 nm AgNPs can inhibit the nitrifying bacteria more than 86% (Choi et al., 2008). The toxicity of other silver species on nitrifying bacteria was also investigated. At the same concentration, Ag^+ and AgCl can inhibit the growth of nitrifiers about 40%. The nitrification rate was affected by both AgNPs and Ag^+ (Zheng et al., 2017). The inhibition mechanisms of AgNPs on nitrifying bacteria have been widely described. Choi et al. (2008) observed the effect of AgNPs on microbial

cell wall using electron microscopy, which revealed that AgNPs aggregated and attached to the cell wall or extracellular matrix. The accumulation of AgNPs may cause the production of reactive oxygen species in the cell (Choi et al., 2008). AgNPs also damaged the cell wall by interacting with sulfur-containing protein and phosphorus-containing elements resulting in the leakage of intracellular organelles of the bacteria (Yuan et al., 2013). Many researchers concluded that the toxicity of AgNPs is from Ag^+ that is released from the colloidal AgNPs (León-Silva et al., 2016; Radniecki et al., 2011; Zheng et al., 2017). The effects of AgNPs on nitrification related genes were investigated (Zheng et al., 2017). AgNPs suppressed the expressions of *amo* and *hao*, which are the genes involved in the first step of nitrification. Nevertheless, the inhibition of AgNPs on archaeal *amo* was lower than bacterial *amo* at 50 mg/L, whereas no significant effects were observed at 0.5 mg/L (Beddow et al., 2016).

2.5 Microplastics

Microplastic is plastic that its size is less than 5 mm and made of PE, PP, PVC, PES, and others (Carr et al., 2016; Mason et al., 2016). Microplastics can be divided into 2 types, primary and secondary (Figure 2-11). Primary microplastic is the plastic that is synthesized for use in personal care products; for example, microbeads in facial foam or toothpaste. Secondary microplastic is the plastic that disintegrated from the bigger size of plastic (Browne et al., 2011). Generally, microplastics in municipal wastewater are mostly fibers from laundry. Other shapes such as fragments, film, foam, and bead have been observed in lower amounts (Mason et al., 2016).

Figure 2-11 Primary and secondary microplastics

2.5.1 Fate and transport in WWTPs

Similar to AgNPs, microplastics are removed during wastewater treatment. Primary sedimentation removes most of the microplastics in wastewater (Raju et al., 2018). Other processes such as screening, secondary skimming, flotation, and filtration contribute to the removal as well (Carr et al., 2016; Stephenson & Stuetz, 2009; Talvitie et al., 2017). Microplastic fibers in municipal wastewater, are removed by primary sedimentation (Carr et al., 2016). In addition, they can accumulate and concentrate in the activated sludge (Habib et al., 1998). Biofilm can grow on microplastics in aeration tanks facilitating the settling of the combined entity (Hongprasith et al., 2020).

2.5.2 Quantity of microplastics in WWTPs

Microplastics in wastewater have been widely reported, especially municipal wastewater. The average numbers of microplastics in the influent, aeration tank, and effluent of municipal WWTPs in Bangkok, Thailand were 12.2, 138.2, and 2.0 particles/L, respectively (Hongprasith et al., 2020). Microplastics in the aeration tank were in mostly a fiber shape followed by unclassified shape, sheet, and fragment. The

microplastics were characterized using Fourier-transform infrared spectroscopy (FTIR). The FTIR results revealed that PES is the dominant type of microplastics in liquid phase (18%). The sludge phase contains polyacrylate up to 43%, PES at 29%, and PE at 7%. In addition, certain types of microplastics were observed in both the liquid phase and sludge phases; for example, dye particles (Hongprasith et al., 2020). Microplastics were detected more than 1,500 particles/L in the influent of municipal WWTPs. Fiber is the major shape followed by fragments and flakes (Magnusson & NorÈn, 2014). In WWTPs in Sydney, Australia, microplastics mostly in fiber shape of polyethylene terephthalate (PET) and a size range of 25 to 100 μm were found in the effluent. Irregular shape of PE was also observed at a nearly same amount of PET, whereas PS and PP were found in lower amounts. The concentration of microplastics in the final effluent was 0.21 to 1.5 particles/L (Ziajahromi et al., 2017).

2.5.3 Effects of microplastics on nitrifying microorganisms.

Several studies revealed that microplastics are toxic to nitrifiers (Li et al., 2020; Seeley et al., 2020; Song et al., 2020). However, there are some types of plastic that can promote the ammonia oxidizing activity. PES, which dominates in municipal wastewater, can improve the ammonia oxidation rate at concentrations higher than 5,000 particles/L (Li et al., 2020). PES may improve the ammonia oxidizing activity by promoting the biofilm formation of the nitrifying microorganisms (Paniagua-Michel et al., 2005). Other types of microplastics that can enhance the ammonia oxidation rate at high concentrations are such as PE and PS. Although these microplastics can promote ammonia oxidizing activity, they cause lower nitratation rates (Li et al., 2020). However, PLA and PUF positively affect both nitritation and nitratation, and in turn the overall nitrification rate. In addition, the abundance of

bacterial *amoA* also increases after incubation with these 2 types of microplastics. PVC is toxic to nitrifiers. The severity of PVC on ammonia oxidizing rate is related to the concentration. It is likely that PVC can inhibit ammonia oxidizing microorganisms by releasing toxic constituents (Li et al., 2020; Seeley et al., 2020; Song et al., 2020). Moreover, PVC causes lower bacterial *amoA* abundance (Seeley et al., 2020). Same as PVC, PP also decreases the ammonia oxidation rate at concentrations higher than 5,000 particles/L (Li et al., 2020).

2.5.4 Effects of microplastics and AgNPs on nitrifying microorganisms

Both AgNPs and microplastics in wastewater end up at WWTPs. Both contaminants can accumulate in biomass, which can promote the interactions between AgNPs and microplastics in a wastewater treatment system, especially in an aeration tank. Li et al. (2022) revealed that AgNPs agglomerated in the presence of DOM and/or ionic strength in brackish water. After that, the aggregated AgNPs are mostly captured on the PS surface while the AgNPs captured on PP and PE were in lower amounts. However, the aggregated AgNPs do not interact with microplastics in natural freshwater. The interactions of AgNPs and microplastic depend on the chemical structure of microplastic that influences the hydrophobicity and electrostatic property of AgNPs and microplastic. The results further show different levels of AgNPs aggregation on different types of microplastics. Niu et al. (2022) observed the effects of AgNPs and polymethyl methacrylate (PMMA) on the ammonia oxidation activity of biofilm sludge. They found that PMMA can adsorb larger AgNPs more than smaller AgNPs and AgNPs can adsorb on PMMA with biofilm better than PMMA without biofilm. AgNPs that accumulate on the biofilm can inhibit ammonia

oxidation activity and induce more production of lactic dehydrogenase and ROS, which are the substances produced by microorganisms under stress conditions.

Chapter 3
Methodology

3.1 Experimental framework

The experimental work is divided into 4 parts. In part 1, a nitrifying reactor was operated under specific conditions to produce a nitrifying consortium comprising AOB, AOA, and comammox, for subsequent experiments. Sludge was collected from a municipal WWTP and an industrial WWTP and used as a seed for the startup of the nitrifying reactor. Sludge samples from these two plants were analyzed by qPCR to ensure the existence of AOB, AOA, and comammox prior to applying the samples as a mixture to the reactor. The reactor was operated oligotrophically to promote all three ammonia oxidizers. In part 2, two types of contaminants, AgNPs and microplastics (PVC), were acquired. AgNPs were purchased from Nanoprime Technology (Bangkok, Thailand). Certain characteristics of the nanoparticles such as UV-vis spectra were provided by the supplier. Microplastics were synthesized by grinding PVC pipes. In part 3, microcosms were set up to investigate the impacts of the contaminants (both individual and combination) on overall ammonia oxidation rate and inhibitory effects of contaminants (AgNPs and/or PVC microplastic) on each group of ammonia oxidizers. Part 4 focused on the effects of AgNPs and PVC on ammonia oxidizers microbial community analyzed by qPCR technique with specific primers targeting each group of ammonia oxidizers. The entire experimental framework is shown in Figure 3-1.

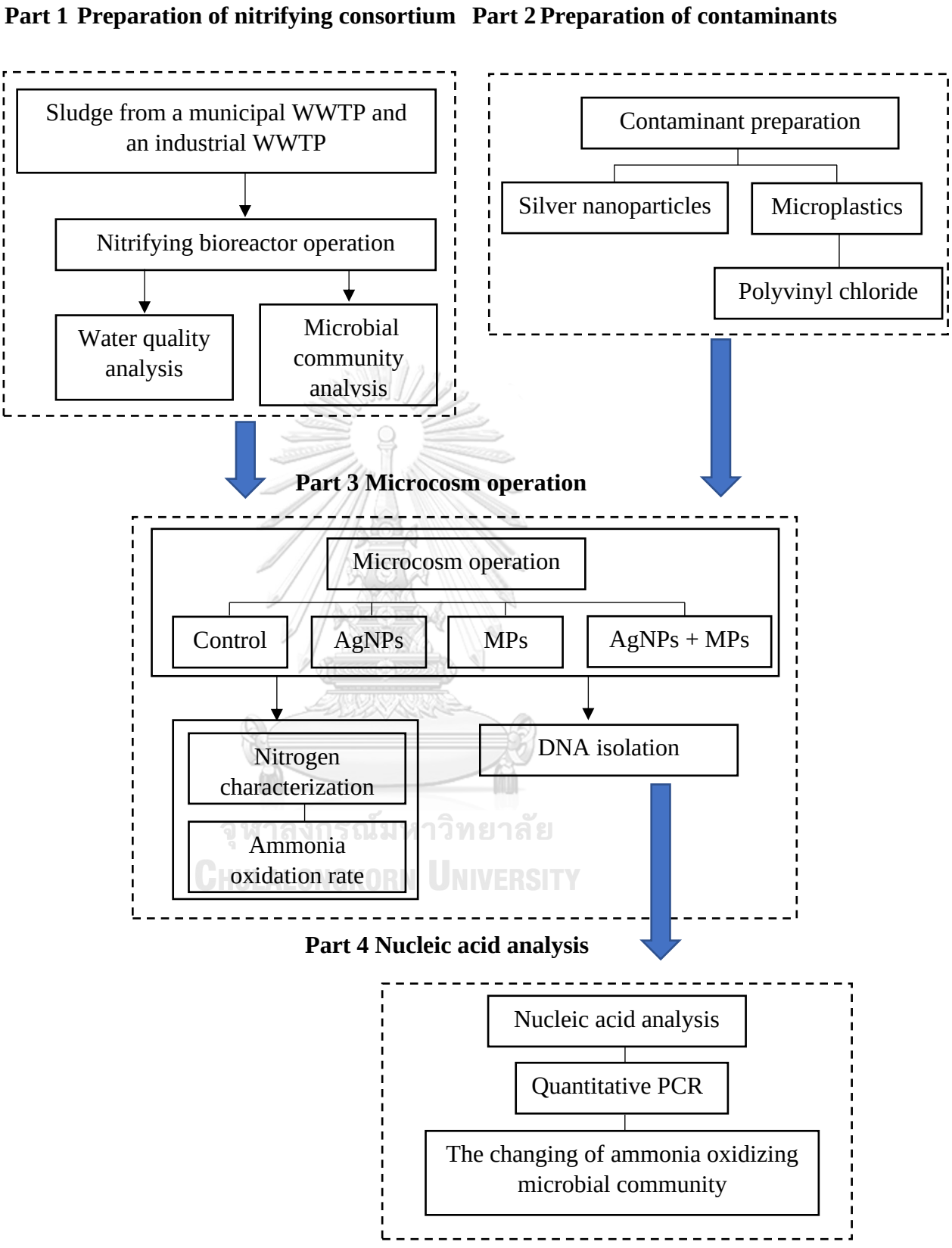


Figure 3-1 Experimental framework

3.2 Experimental methods

3.2.1 Seed sludge

Seed sludge for nitrifying bioreactor operation was collected from the aeration tanks of a municipal wastewater treatment plant and an industrial estate central treatment plant in Thailand. The sludge samples were stored at 4°C before being used. Sludge of 4 mg MLSS was used for DNA extraction and analyzed for nitrifying communities using qPCR. The samples from both plants were mixed at a ratio of 1:1 before applying to the reactor to obtain all three ammonia oxidizing microbial groups (AOA, AOB, and comammox) in the bioreactor. The seed sludge collection is shown in Figure 3-2.

Figure 3-2 Seed sludge collection from a wastewater treatment plant

3.2.2 Inorganic medium preparation

Inorganic medium was prepared to supply macro and microelements to the nitrifying microorganisms as the sole energy source according to Srithep et al. (2018). The medium was used for nitrifying bioreactor operation and microcosms operation by adding different nitrogen sources and pH buffer for both steps of experiments (nitrifying reactor and microcosms). The composition of the elements in the medium is shown in Tables 3-1 to 3-6. This medium was introduced for culturing AOA (Srithep et al., 2018), thus macro- and micro-elements in the media are expected to be sufficient for growth of all three types of ammonia oxidizers.

Table 3-1 Components of macro and microelements in 1 L of inorganic medium

Substance	Amount
NaCl	1.0 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
1M of $NaHCO_3$ solution	3 mL
Nonchelated trace element mixture	1 mL
Vitamin solution	1 mL
Thiamin solution	1 mL
Vitamin B12 solution	1 mL
Selenite tungstate solution	1 mL

Table 3-2 Components in 1L of nonchelated trace element mixture

Substance	Amount
HCl 7.7 M	12.5 mL
$FeSO_4 \cdot 7H_2O$	2.1 g
H_3BO_3	30 mg
$MnCl_2 \cdot 4H_2O$	100 mg
$CoCl_2 \cdot 6H_2O$	190 mg
$NiCl_2 \cdot 6H_2O$	24 mg
$CuCl_2 \cdot 2H_2O$	2 mg
$ZnSO_4 \cdot 7H_2O$	144 mg
$Na_2MoO_4 \cdot 2H_2O$	36 mg

Table 3-3 Components in 1L of vitamin solution

Substance	Amount
Na_2HPO_4 anhydrous	0.89729 g
$Na_2HPO_4 \cdot 2H_2O$	0.57401 g
4-aminobenzoic acid	0.24 g
D(+)-biotin	0.06 g
Nicotinic acid	0.6 g
Calcium D(+)-pantothenate	0.15 g
Pyridoxine dihydrochloride	0.45 g

Table 3-4 Components in 1L of thiamin solution

Substance	Amount
Na_2HPO_4 anhydrous	0.0012 g
$Na_2HPO_4 \cdot 2H_2O$	3.89891 g
Thiamine chloride hydrochloride	0.6 g

Table 3-5 Component in 1 L of vitamin B12 solution

Substance	Amount
Cyanocobalamin	0.3 g

Table 3-6 Components in 1L of selenite tungstate solution

Substance	Amount
NaOH	40 g
$Na_2SeO_3 \cdot 5H_2O$	0.6 g
$Na_2WO_4 \cdot 2H_2O$	0.8 g

3.2.3 Nitrifying bioreactor

A nitrifying bioreactor had an effective volume of 12 liters. The reactor was operated in a continuous mode without sludge recycling with a hydraulic retention time of 5 days. Feeding to the reactor was an inorganic medium containing 40 mgN/L of $(NH_4)_2SO_4$ as the nitrogen source to maintain ammonia oxidizing activity of the microorganisms. NaOH (0.2 M) was used to maintain pH in the reactor at a range of 7.4 – 7.8 through a feed pump connected to a pH controller (Hanna BL983329, Black

Stone®). Oxygen was supplied to the reactor using an electric air pump to maintain dissolved oxygen (DO) concentrations of 5-6 mg/L. The reactor was operated at room temperature (25-30°C) in dark for at least a month before its sludge is used in further experiments. During the operation, the effluent of the reactor was monitored for the nitrogen concentrations once a week. The sludge in bioreactor was collected (4 of mg MLSS weekly). The sludge was centrifuged and extracted for the DNA. The extracted DNA was analyzed for nitrifying community by qPCR. The nitrifying bioreactor is shown in Figure 3-3.

Figure 3-3 Nitrifying bioreactor

3.2.4 Mixed liquor suspended solids measurement

Mixed liquor suspended solids (MLSS) was measured to control the dry weight of sludge in the bioreactor. A 47mm glass fiber membrane filter disc (0.45 µm pore size) was dried at 105°C for at least 1 hour in a hot air oven and, then cooled down in a desiccator for 30 minutes. The weight of the disc was measured using a 4 decimal analytical balance (M_1). The disc was laid on a Buchner funnel connected

to an air suction pump. After that, 30 mL of well mixed activated sludge suspension from the bioreactor was filtered through the disc. The sludge was left on the disc. The disc was removed and dried in a hot air oven at 105°C for 1 hour. The disc was cooled down in a desiccator for 30 minutes. The weight of disc with sludge was measured (M_2) and the MLSS was calculated using the following equation.

$$\text{Mixed liquor suspended solids (MLSS)} = \frac{\text{M2-M1}}{\text{volume}} \text{ mg/L}$$

3.2.5 Nitrogen characterization

NH_3 and NO_2 concentrations were analyzed by a salicylate-hypochlorite method (Bower & Holm-Hansen, 1980) and a colorimetric method (APHA, 2005a), respectively, using a VICTOR X Multilabel Plate Reader (PerkinElmer, USA).Nitrate concentration was measured using the Ultraviolet Spectrophotometric Screening method (APHA, 2005b) using a GENESYS™ 10S UV-VIS spectrophotometer (Thermo Scientific, USA).

3.2.6 Silver nanoparticles

AgNPs (5,000 mg/L) coated with soluble starch in a colloidal form were purchased from Prime Nanotechnology Co., Ltd (catalog number Ag-106, Bangkok, Thailand). AgNPs was yellow-brown colloid with pH 6.0 and a particle size range of 5-20 nm. AgNPs were characterized using a UV-VIS spectroscopy. The wavelengths of max and half-ax absorbance are 401 and 64 nm, respectively. AgNPs were diluted to 0.1, 0.5, 1, 2.5, 5, and 10 mg/L with18Ω water to use as a contaminant in microcosms experiment. These concentrations were selected from a preliminary

experiment. The AgNPs obtained from Prime Nanotechnology Co., Ltd is shown in Figure 3-4.

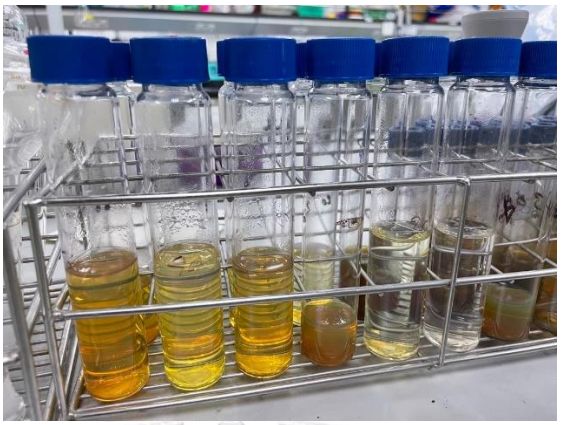


Figure 3-4 Silver nanoparticles

3.2.7 Microplastic preparation

Blue PVC water pipes were cracked into small pieces followed by grinding using a coffee bean grinder. The grinded plastic particles were separated according to their size by sieving. The sizes included >501 µm, 106-500 µm, 76-105 µm, and <75 µm. For the microcosms experiment, the sizes of the microplastics used were between 106 and 500 µm which is the dominant size in the sieving process. The PVC microplastic preparation is shown in Figure 3-5.

Figure 3-5 PVC microplastics preparation

3.2.8 Microcosm operation for selection of sludge concentration

Three sets of microcosms were prepared by adding sludge from the nitrifying bioreactor to 250 mL Erlenmeyer flasks containing 150 mL of an inorganic medium containing 40 mgN/L NH_4Cl as an energy source. The pH of the medium was adjusted to 7.5 with NaOH and maintained with 25 mM MOPS as pH buffer (Zheng et al., 2017). Sludge from the nitrifying bioreactor was added to the microcosms to achieve the final concentrations of 40, 60, and 100 mg MLSS/L. The microcosms were incubated for 7 days in the dark at 30°C at 200 rpm in an incubator shaker. Every 24 hours, 5 mL of the medium was taken to measure the nitrogen concentrations.

3.2.9 Microcosm operation for silver nano particles study

The 250 mL Erlenmayer flasks were prepared using 150 mL of inorganic medium. NH_4Cl was added at a concentration of 40 mgN/L, and the pH was maintained at 7.5 with 25 mM MOPS. The nitrifying sludge from the bioreactor was inoculated at a final concentration of 100 mg MLSS/L based on the sludge

concentration microcosm experiment described in the preceding subsection. Microcosms were shaken at 200 rpm and incubated at 30 °C in the dark. AgNPs were added at the final concentrations of 0, 0.1, 0.5, 1, 1.0, 2.5, 5, and 10 mg/L. Nitrogen concentration was measured every 24 hours and 4 mg MLSS of the sludge was collected on day 0 and day 5 to analyze for the microbial community. Each microcosm was prepared in triplicates.

3.2.10 Microcosm operation for the study of co-effect of silver nano particles and microplastics

The inorganic medium was prepared in an Erlenmeyer flask of 150 mL and supplied with NH_4Cl at a concentration of 40 mgN/L. The pH of the microcosms was maintained at 7.5 with 25 mM of MOPS and NaOH. The sludge was inoculated at a final concentration of 100 mg MLSS/L. The AgNPs and PVC microplastics were added at the final concentrations of 0.5 and 500 mg/L, respectively. All the microcosms experiments were triplicated. A positive control without the addition of any contaminants was included. There are 2 types of PVC that were added to microcosms. The first type is PVC without pre-shake that was added with AgNPs and sludge at the same time. The second type is the PVC that was pre-shaken for 7 days in the media before adding it along with AgNPs and sludge to eliminate the floating of microplastics. The microcosms were incubated in the dark at 200 rpm and 30ºC. All the experimented microcosms are illustrated in Figure 3-6 and Table 3-7.

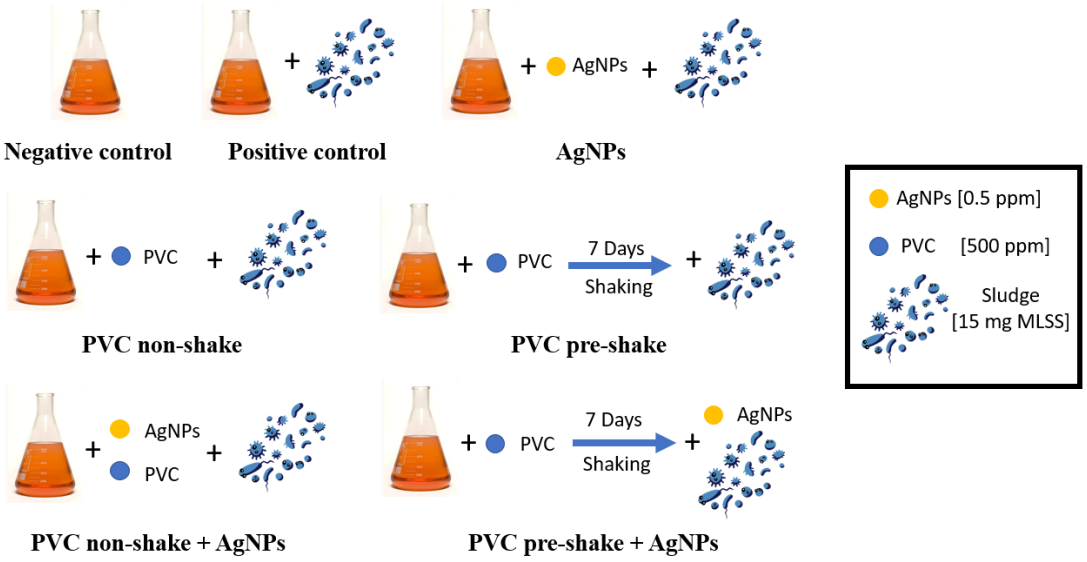


Figure 3-6 Microcosms sets for studying co-effects of AgNPs and PVC microplastics

Table 3-7 Microcosms sets for observing co-effect of AgNPs and PVC microplastics

Name	Sludge	AgNPs (0.5 mg/L)	PVC (500 mg/L)	Remark
Negative control				
Positive control	+			
AgNPs	+	+		
PVC non-shake	+		+	PVC, AgNPs, and sludge were added to the media at the same time
PVC non-shake + AgNPs	+	+	+	
PVC pre-shake	+		+	PVC was shaken for 7 days in the media before adding AgNPs and sludge
PVC pre-shake + AgNPs	+	+	+	

3.2.11 Genomic DNA extraction

Nitrifying sludge from the bioreactor (4 mg of MLSS) and microcosms (4 mg of MLSS) was collected to extract genomic DNA (gDNA). The sludge was centrifuged at 10,000 rpm for 20 minutes and the supernatant was removed. The centrifuged sludge was used to extract gDNA using a FastDNA™ Spin Kit for Soil (MP Biomedicals) following the manufacturer's protocol. The extracted DNA was

kept at -20°C for further analysis. DNA concentration and purity were analyzed by a Nanodrop™ Lite spectrophotometer (ThermoFisher, USA).

3.2.12 Quantitative polymerase chain reaction

The abundance of nitrifying microorganisms was analyzed by qPCR gDNA using specific primers targeting ammonia oxidizing involved genes and a CFX96™ Real-Time System (Bio-Rad, USA). qPCR was performed in triplicate using the primers listed in Table 3.8. In 20 µL of the reaction mixture, the composition included 10 µL of Luna® Universal PCR Master Mix (Biolabs, USA), 0.25 µM of forward and reverse primers, and 0.75 ng/µL of gDNA template. The qPCR thermal profiles are shown in Table 3.9. The specificity of qPCR amplification was determined by a dissociation curve and gel electrophoresis.

Table 3-8 Primers used for qPCR

Target gene	Primer	Sequence (5'-3')	Reference	Annealing temperature
Archaeal *amoA* gene	Arch-amoAF Arch-amoAR	STAATGGTCTGGCTTAGACG GCGGCCATCCATCTGTATGT	(Francis et al., 2005)	56°C
Bacterial *amoA* gene	amoA-1F amoA-2R	GGGGTTTCTACTGGTGGT CCCCTCKGSAAAGCTTCTTC	(Rotthauwe et al., 1997)	58°C
Comammox *amoA* gene	comamoA AF comamoA SR	AGGNGAYTGGGAYTTCTGG CCGVACATACATRAAGCCCAT	(Wang et al., 2018)	58°C

Table 3-9 qPCR thermal profile

Segment	Steps	temperature	Time	Cycle
1	Initial denaturation	95°C	10 min	1
2	Denaturation	95°C	30 sec	40
	annealing	Primer AT	1 min	
	extension	68°C	30 sec	
3	Denaturation	95°C	1 min	1
	Dissociation curve	AT-95°C	30 sec	
	Final extension	95°C	30 sec	

3.2.13 Statistical analysis

SPSS 28 was used for statistical analysis in this study. The *t*-test was performed by paired-samples analysis to test the significant differences of gene copy number at 95% confidence interval level.

Chapter 4

Results and discussion

4.1 Nitrifying bioreactor operation

The bioreactor was operated for several months after seeding with the sludge from the municipal wastewater treatment plant and the industrial wastewater treatment plant. The concentration of total ammonia nitrogen (TAN) in the inorganic medium, as the influent of the bioreactor, was 40 mgN/L. The influent flow rate was 2.6 L/day corresponding to hydraulic retention time (HRT) of the bioreactor of 4.7 days. During the operation, most of the TAN was converted to NO_3^-. The 99.41% of TAN was converted to NO_3^- (calculated using the average effluent NO_3^- divided by the average TAN in the influent). Therefore, the nitrifying sludge exhibited good activity for the oxidation of NH_3 to NO_2^- and NO_3^-. The nitrogen concentrations are presented in Figure 4-1.

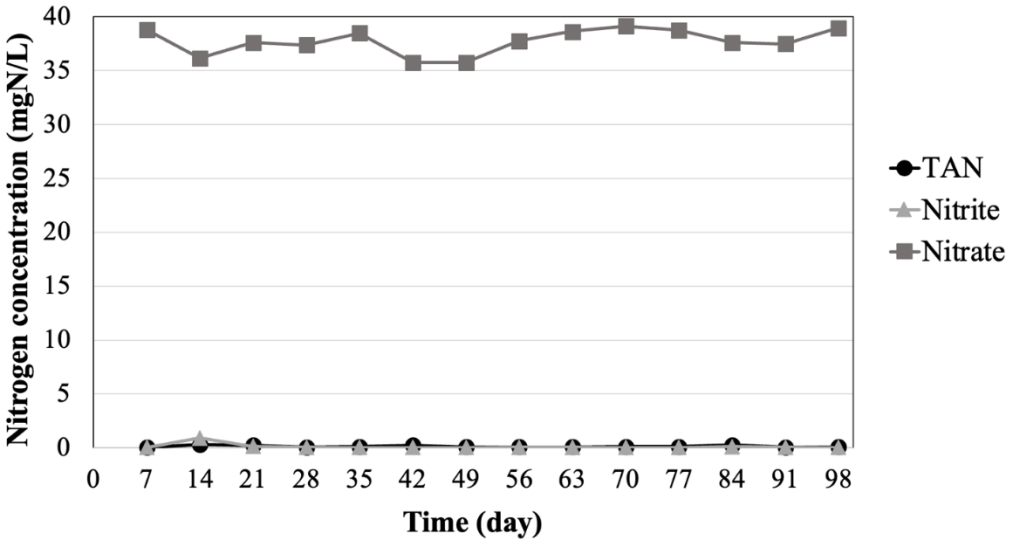


Figure 4-1 Nitrogen concentrations during bioreactor operation

The nitrifying microbial community was analyzed every two weeks using qPCR to monitor the numbers of ammonia oxidizing microorganisms' *amoA* genes. The error bars represent the standard deviation of triplicate analyses performed by qPCR (n=3). During the operation, the *amoA* gene copy numbers of AOA ranged from 2.38×10^4 to 1.29×10^5 copies/ng (Figure 4-2) while those of AOB ranged from 7.28×10^4 to 1.32×10^5 copies/ng (Figure 4-3). Comammox *amoA* numbers were from 5.39×10^4 to 1.01×10^5 copies/ng (Figure 4-4). The results suggest that the nitrifying sludge contained all three known ammonia oxidizing microbial groups with comparable *amoA* numbers and was ready to be used for subsequent experiments.

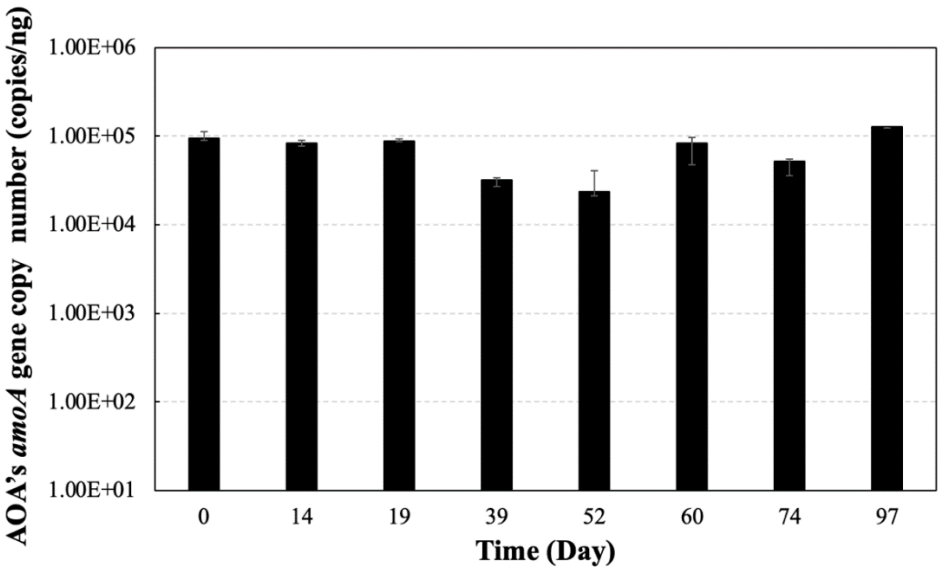


Figure 4-2 Numbers of ammonia oxidizing archaea (AOA) in the bioreactor during operation

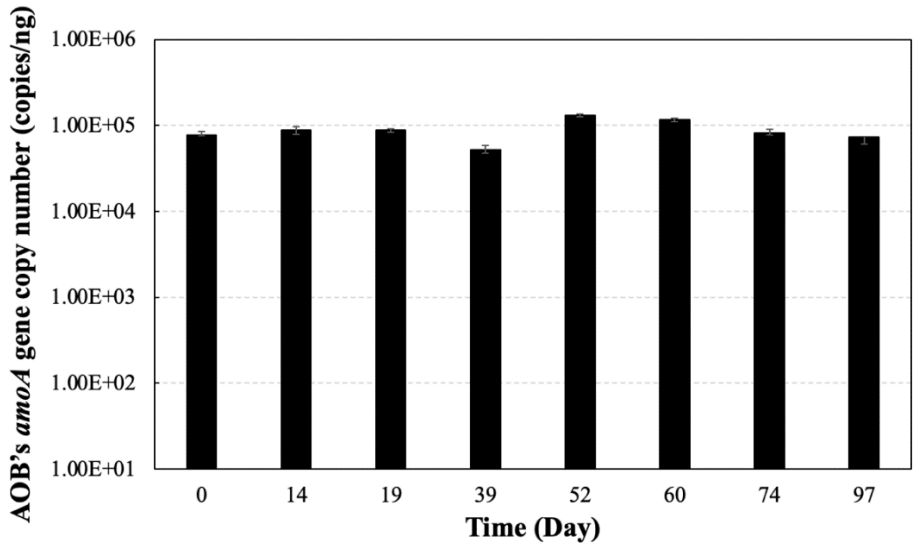


Figure 4-3 Numbers of ammonia oxidizing bacteria (AOB) in the bioreactor during operation

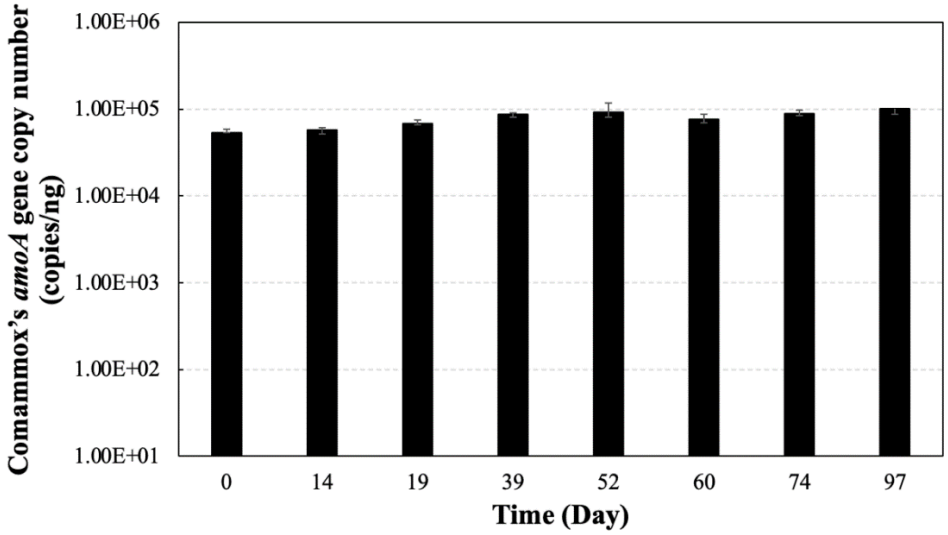


Figure 4-4 Numbers of complete ammonia oxidizing bacteria (comammox) in the bioreactor during operation

4.2 Microcosms for selecting sludge concentration

The nitrifying sludge was inoculated with at the final concentrations of 40, 60 and 100 mg MLSS/L in the microcosms to find the optimum MLSS concentration used for further experiments. High nitrifying sludge concentrations require less time for the conversion of TAN to NO_3^-. A lag phase of TAN oxidation appeared at the beginning at all sludge concentrations studied (Figure 4-5). In the negative control microcosms, TAN concentration remained at the same level during operation, indicating that there was no abiotic transformation of TAN to NO_3^-. At 40 mg MLSS/L sludge, 40 mgN/L TAN was consumed within 4 days. At 60 mg MLSS/L, it took 4 days to oxidize 40 mgN/L of TAN. However, the TAN concentration on day 3 was around 3.0 mgN/L which required several additional hours to be completely oxidized. Therefore, the sludge concentrations of 40 and 60 mg MLSS/L were not selected. The microcosms inoculated with 100 mg MLSS/L sludge took 3 days to completely convert the added TAN to NO_3^-. The accumulation of NO_2^- was detected in 40, 60, and 100 mg MLSS/L microcosms (8.5, 9.8, and 1.8 mgN/L respectively; Figure 4-6). The highest NO_2^- concentration was found on the first day of complete TAN depletion. The NO_3^- concentrations in the microcosms started at 5 mgN/L because of the interference by MOPS. At 40 and 60 mg MLSS/L, NO_3^- reached 40 mgN/L on day 4, whereas it was on day 3 for 100 mg MLSS/L microcosm (Figure 4-7).

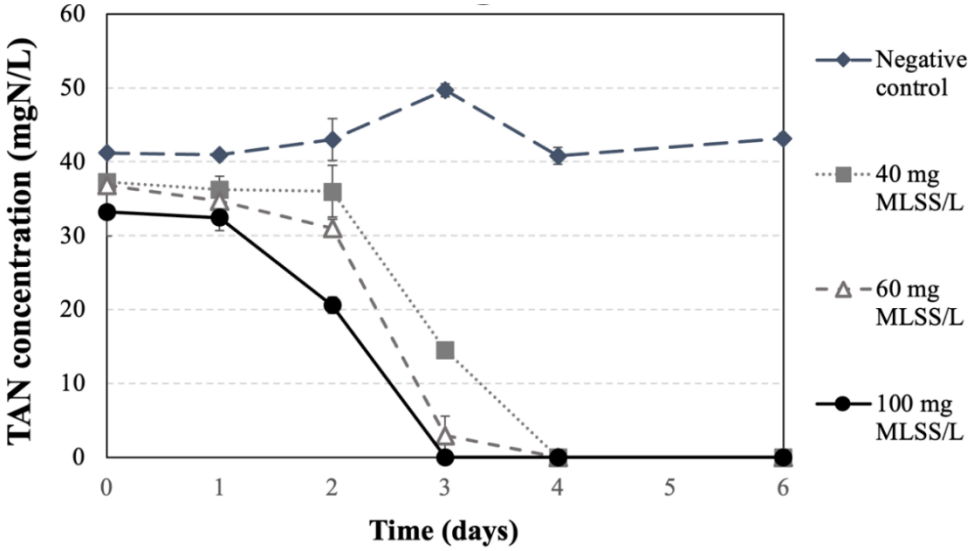


Figure 4-5 Total ammonia concentrations in microcosms for selecting sludge concentration

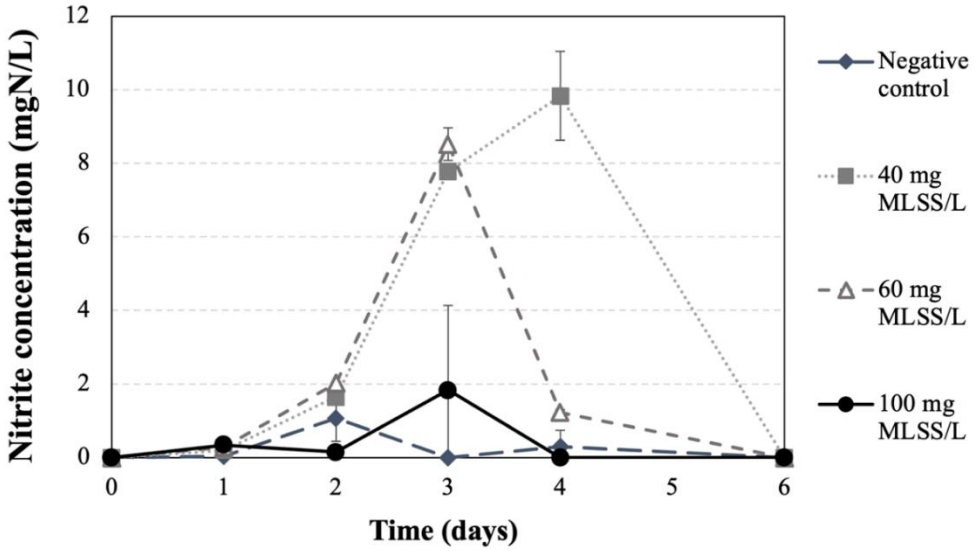


Figure 4-6 Nitrite concentrations in microcosms for selecting sludge concentration

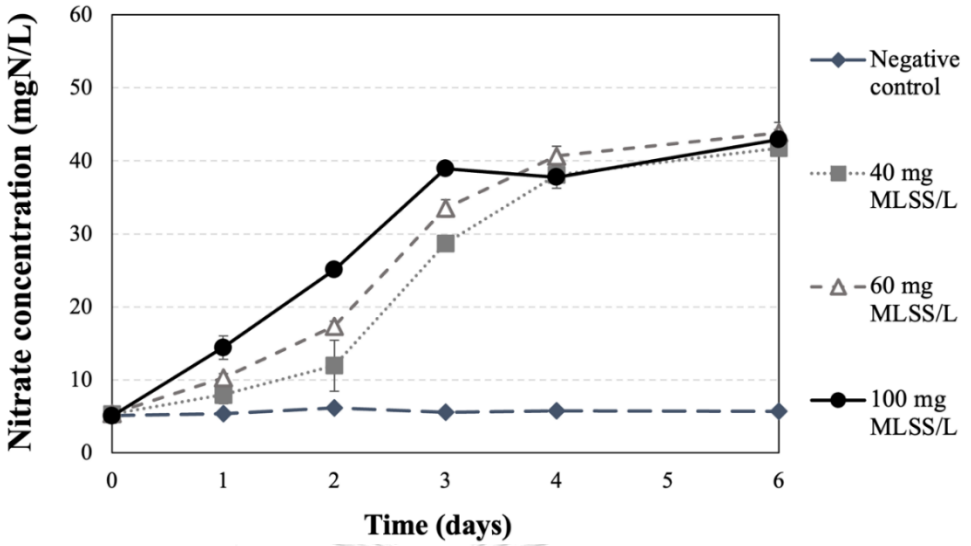


Figure 4-7 Nitrate concentrations in microcosms for selecting sludge concentration

Because of low NO_2^- accumulation and less time required than the other MLSS concentrations, 100 mg MLSS/L was selected as the sludge concentration for further microcosms experiments.

4.3 Effects of AgNPs on the activity and community of nitrifying sludge

Sludge was inoculated at 100 mg MLSS/L and mixed with AgNPs at 0, 0.1, 0.5, 1, 2.5, 5, and 10 mg/L. TAN was oxidized within 2 days for the positive control (Figure 4-8). No inhibitory effect on ammonia oxidation was observed for 0.1 mg/L AgNPs. Partial inhibitory effects were observed at 0.5 and 1 mg/L; at these two concentrations of AgNPs, more time was required for the conversion of TAN to NO_3^- compared to the lower concentrations. TAN was oxidized to NO_3^- within 5 and 10 days in the microcosms containing AgNPs at concentrations of 0.5 and 1 mg/L, respectively. At higher concentrations, AgNPs delivered complete inhibition of ammonia oxidation activity. The AgNPs concentration of 0.5 mg/L was selected for further microcosm experiments because the ammonia oxidation activity was not completely inhibited and the inhibition time was shorter than 1.0 mg/L AgNPs.

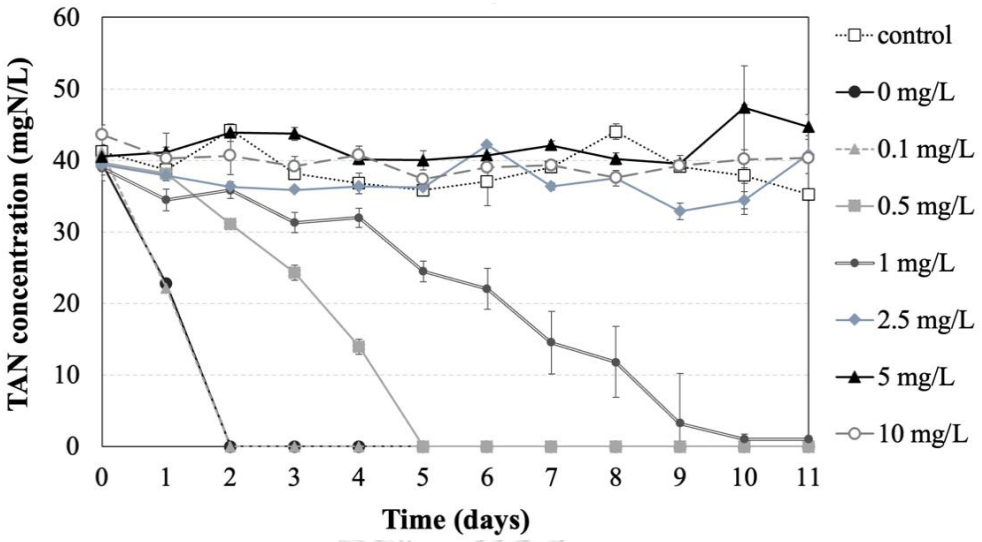


Figure 4-8 Total ammonia nitrogen concentrations in AgNPs microcosms

The NO_2^- concentrations in the microcosms are shown in Figure 4-9. The microcosms with 0 and 0.1 mg/L AgNPs had the highest NO_2^- accumulations during the microcosm incubation. At 0.1 mg/L AgNPs, 3.4 mgN/L NO_2^- was observed on day 2, whereas 2.7 mgN/L was detected in the microcosms without AgNPs. In the third place were the microcosms with 0.5 mg/L AgNPs, in which 1.0 mg/L NO_2^- was detected. Since AgNPs inhibited ammonia oxidation rate, NO_2^- was produced at low concentrations. Therefore, NO_2^- accumulated in low amount in the microcosms that contained high concentrations of AgNPs.

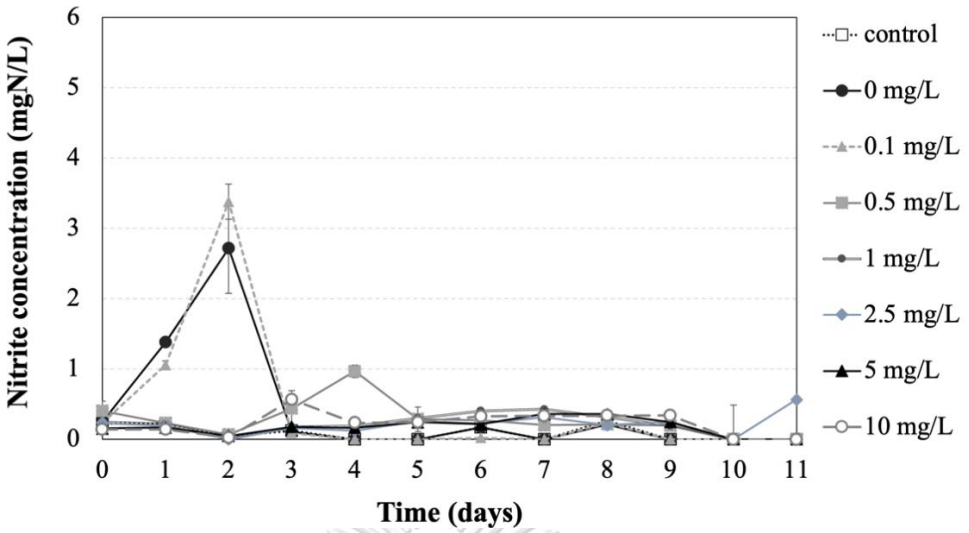


Figure 4-9 Nitrite concentrations in AgNPs microcosms

The NO_3^- concentration results confirmed the effects of AgNPs on ammonia oxidizing activity. The NO_3^- concentration profile in the microcosms is shown in Figure 4-10. Microcosms with 0 and 0.1 mg/L AgNPs reached 40 mgN/L NO_3^- on the second day, indicating that there was no inhibitory effect on ammonia oxidizing activity at 0.1 mg/L AgNPs. Partial inhibitions were observed at 0.5 and 1 mg/L AgNPs at which 40 mgN/L NO_3^- was reached on day 5 and day 10, respectively. At higher concentrations, a complete inhibition was confirmed as NO_3^- levels were always below 10.0 mgN/L during the operation.

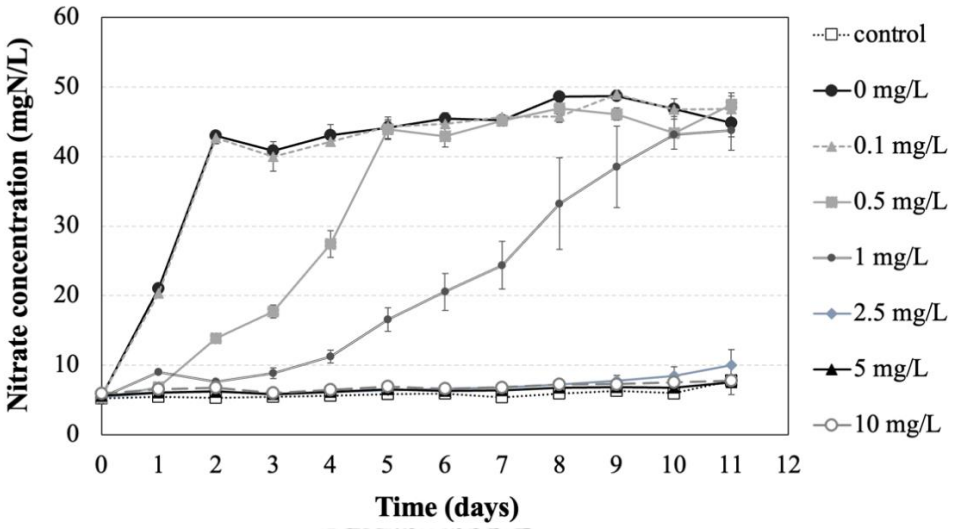


Figure 4-10 Nitrate concentrations in AgNPs microcosms

Specific initial ammonia oxidation rate ($q_{initial}$) was calculated by a linear regression model of TAN concentration over 2 days divided by the initial MLSS mass. At 0 and 0.1 mg/L AgNPs, the $q_{initial}$ was 0.20 and 0.21 mgN/mg MLSS/day, respectively. At 0.5 mg/L AgNPs, the $q_{initial}$ was 0.04 mgN/mg MLSS/day. The $q_{initial}$ was less than 0.01 mgN/mg MLSS/day at the higher concentrations of AgNPs (1, 2.5, 5, 10 mg/L) indicating nearly complete inhibition of the ammonia oxidizing activity (Table 4-1).

Table 4-1 Specific initial ammonia oxidation rate at different concentrations of AgNPs

AgNP concentration (mg/L)	Specific initial ammonia oxidation rate (day =2) (mgN/mg MLSS/day)	Overall ammonia oxidation inhibition
0	0.20	No inhibition
0.1	0.21	No inhibition
0.5	0.04	Partial inhibition
1	0.01	Partial inhibition
2.5	0.01	Complete inhibition
5	0	Complete inhibition
10	0.01	Complete inhibition

The initial ammonia oxidation rate was used to calculate the percent inhibition of ammonia oxidizing activity. It was calculated based on $q_{initial}$ at selected AgNPs concentration divided by $q_{initial}$ of 0 mg/L AgNPs. Microcosms consisting of 0.5 mg/L AgNPs was 78% inhibited, whereas 92% inhibition was observed at 1.0, 2.5, and 10 mg/L AgNPs, and 100% inhibition was observed at 5 mg/L AgNPs (Figure 4-11). This suggests that the adaptation of microorganisms occurs in the first phase of the operation of the microcosms containing 0.5 and 1 mg/L of AgNPs. Since the complete inhibitory effect occurred at 2.5, 5, and 10 mg/L, there may be errors in the TAN measurement resulting in the inhibition of the ammonia oxidation rate at 2.5 and 10 mg/L not being 100%.

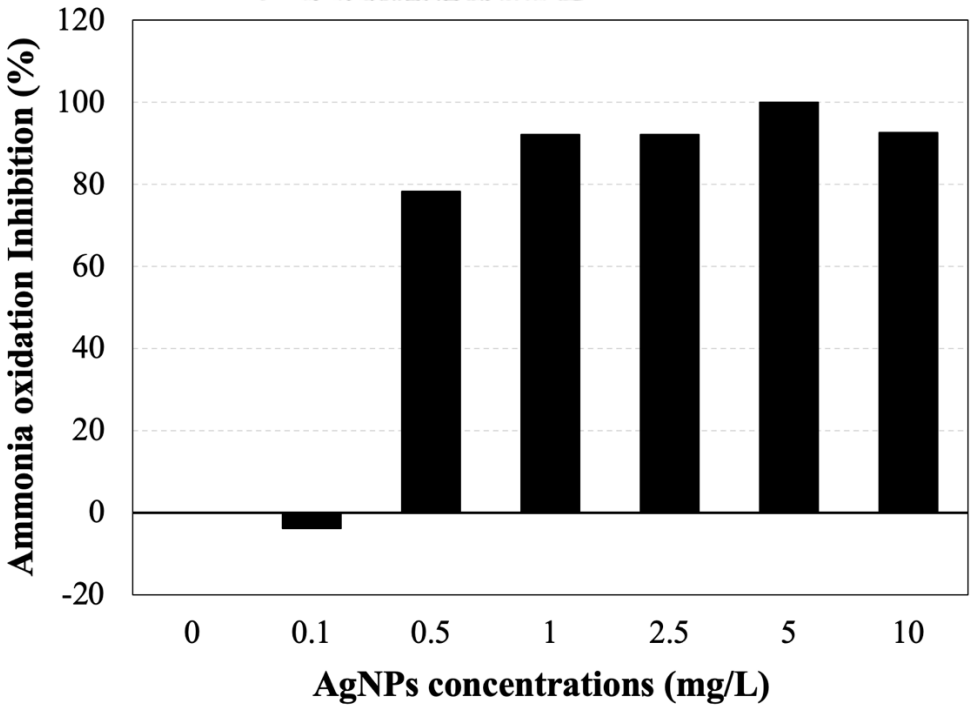


Figure 4-11 Ammonia oxidation inhibition during first 2 days of operation

Overall ammonia oxidation rate ($q_{overall}$) was calculated using a linear regression model of TAN concentration divided by time to NH_3 depletion. Microcosms with 0 and 0.1 mg/L of AgNP required 2 days for NH_3 depletion while at 0.5 and 1 mg/L AgNPs, 5 and 10 days were needed, respectively. The overall ammonia oxidation rates at different concentrations of AgNPs are shown in Table 4-2. The microcosm without AgNPs has $q_{overall}$ of 19.8 mgN/day. The microcosms with AgNPs at 0.1, 0.5, and 1 mg/L have $q_{overall}$ of 20.6, 7.9, 3.9 mgN/day, respectively. The $q_{overall}$ of other microcosms was 0 mgN/day since AgNPs delivered complete inhibition on ammonia oxidation activity.

Table 4-2 Overall ammonia oxidation rate at different concentrations of AgNPs

AgNP concentration (mg/L)	Overall ammonia oxidation rate (mgN/day)	Overall ammonia oxidation inhibition
0	19.8	No inhibition
0.1	20.6	No inhibition
0.5	7.9	Partial inhibition
1	3.9	Partial inhibition
2.5	0	Complete inhibition
5	0	Complete inhibition
10	0	Complete inhibition

The overall ammonia oxidation inhibition was calculated by dividing $q_{overall}$ at selected AgNPs concentration by $q_{overall}$ of 0 mg/L AgNPs. Thus, the inhibition was 0% at 0 mg/L AgNPs. The overall ammonia oxidation inhibitions at different AgNPs concentrations are shown in Figure 4-12. Microcosms added with AgNPs at 0.5 and 1 mgN/L were inhibited at 60% and 80%, respectively. This confirms that AgNPs induced the partial inhibition on ammonia oxidizing activity at these concentrations. The inhibition was 100% at higher AgNPs concentrations (2.5, 5, and 10 mg/L).

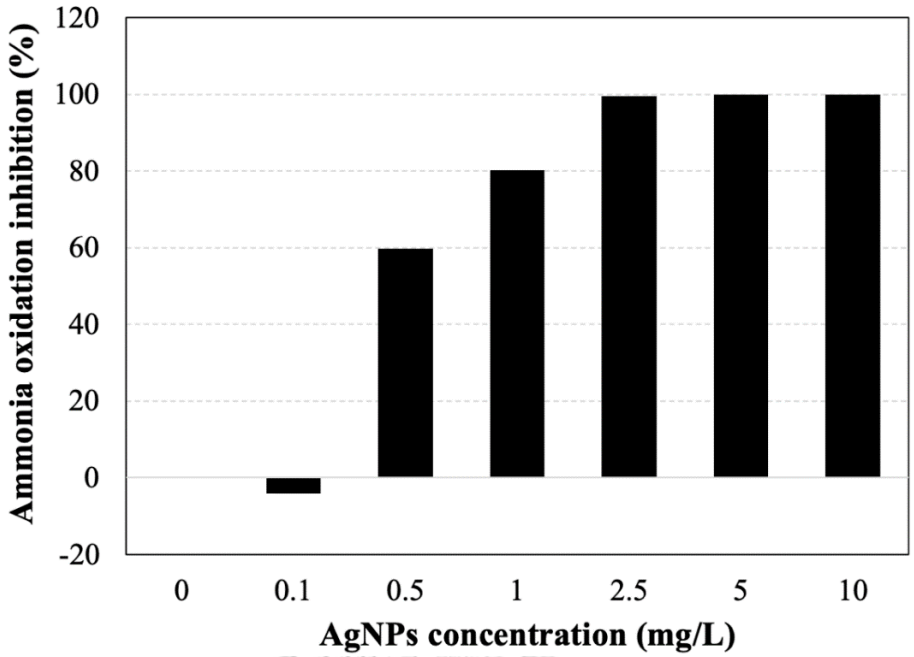


Figure 4-12 Overall ammonia oxidation inhibition

As ammonia oxidation is the first step of nitrification, the presence of AgNPs in wastewater may affect the overall nitrification rate in the wastewater treatment process by suppressing the ammonia oxidizing activity of nitrifying microorganisms. The microbial cells may be damaged by AgNPs, resulting in ammonia oxidation activity reduction. Choi et al. (2008) described that AgNPs attached to microbial membranes and caused the production of intracellular ROS. However, several researchers indicated that AgNPs caused membrane leakage, leading to loss of the ATP pool, H^+ moving force, and bacterial cell death (Lok et al., 2006; Morones et al., 2005; Sondi & Salopek-Sondi, 2004). Yuan et al. (2013) came to the same conclusion that AgNPs damaged the cell membrane, leading to the exit of intracellular organelles of *Nitrosomonas europaea*. These mechanisms decreased the ammonia oxidation rate and nitrification rate of nitrification sludge. The strength of AgNPs depends on the size, concentration, and other environmental factors (Zheng et al., 2017).

The effects of AgNPs on the nitrifying community were determined in these microcosm experiments using the qPCR with specific primers targeting the *amoA* of each ammonia oxidizers including AOA, AOB, and comammox. The amount of archaeal *amoA* in microcosms is shown in Figure 4-13. AOA was minimally affected by AgNPs. The number of archaeal *amoA* significantly decreased in the microcosms at AgNPs of 0.5, 1, and 10 mg/L. The decreases were not significant for AgNPs of 0, 2.5 and 5 mg/L, while for 0.1 mg/L, the number of *amoA* increased from 1.77×10^4 to 4.80×10^4 copies/ng. This implies that AOA was slightly inhibited by AgNPs at concentration from 0.5 mg/L on since the statistical analysis show the significant decrease at a confidence level of 95%.

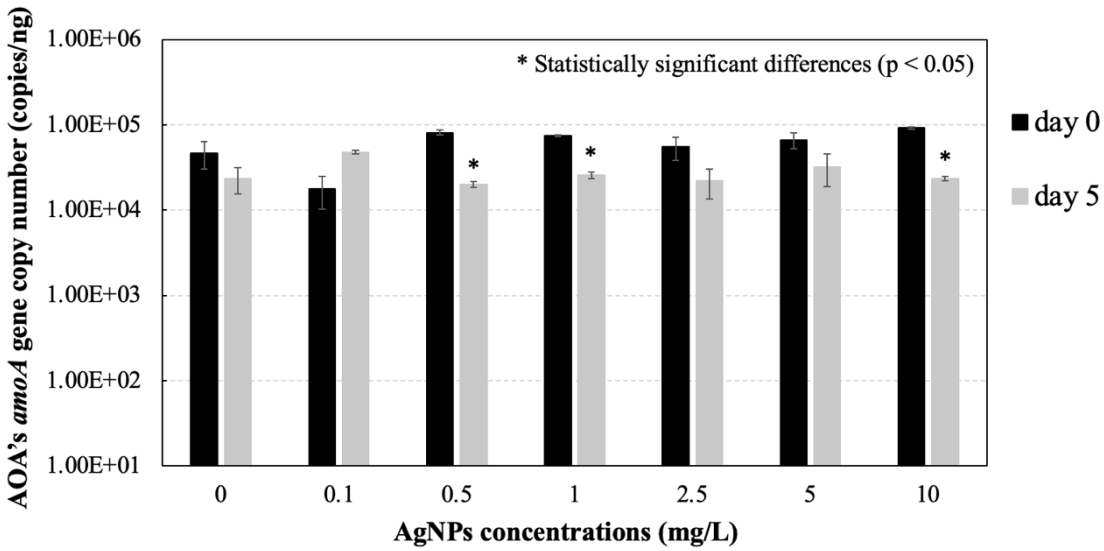


Figure 4-13 Numbers of ammonia oxidizing archaea (AOA) in AgNPs study microcosms

The amount of bacterial *amoA* in microcosms is shown in Figure 4-14. Bacterial *amoA* slightly increased in microcosms at 0, 0.1, 0.5, and 1 mg/L AgNPs. On the other hand, the copy numbers of bacterial *amoA* decreased at 2.5, 5, and 10 mg/L AgNPs in a concentration-dependent manner. This indicates that the AOB may be tolerant at AgNPs below 1.0 mg/L. Above this concentration, AOB can be deteriorated.

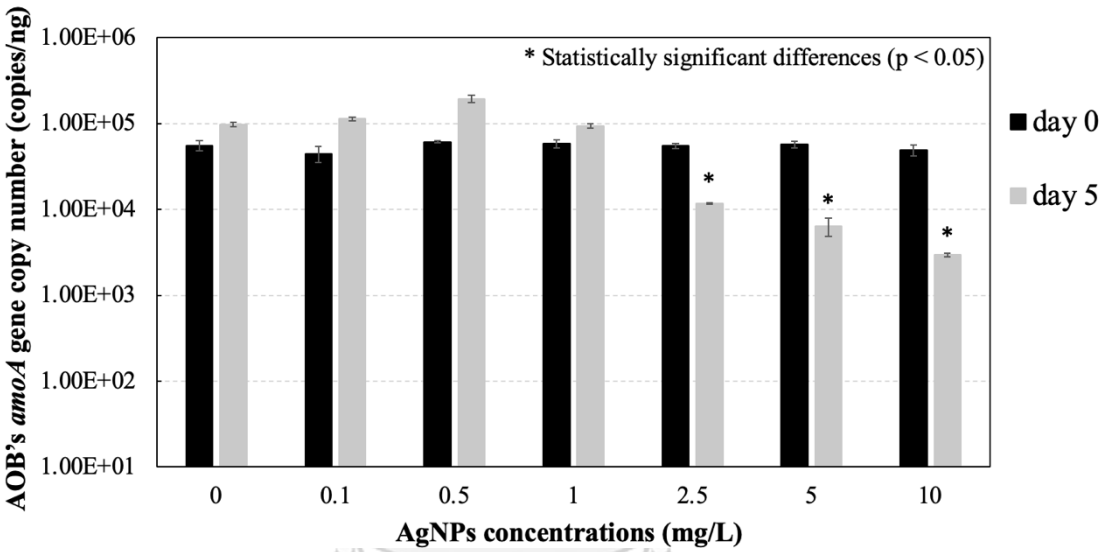


Figure 4-14 Numbers of ammonia oxidizing bacteria (AOB) in AgNPs study microcosm

The number of comammox *amoA* in microcosms is shown in Figure 4-15. Comammox tended to be more sensitive to AgNPs than AOB. At 0.5, 1, 2.5, 5, and 10 mg/L AgNPs, the comammox *amoA* decreased dramatically. The number of comammox *amoA* could be maintained only at 0.1 mg/L AgNPs.

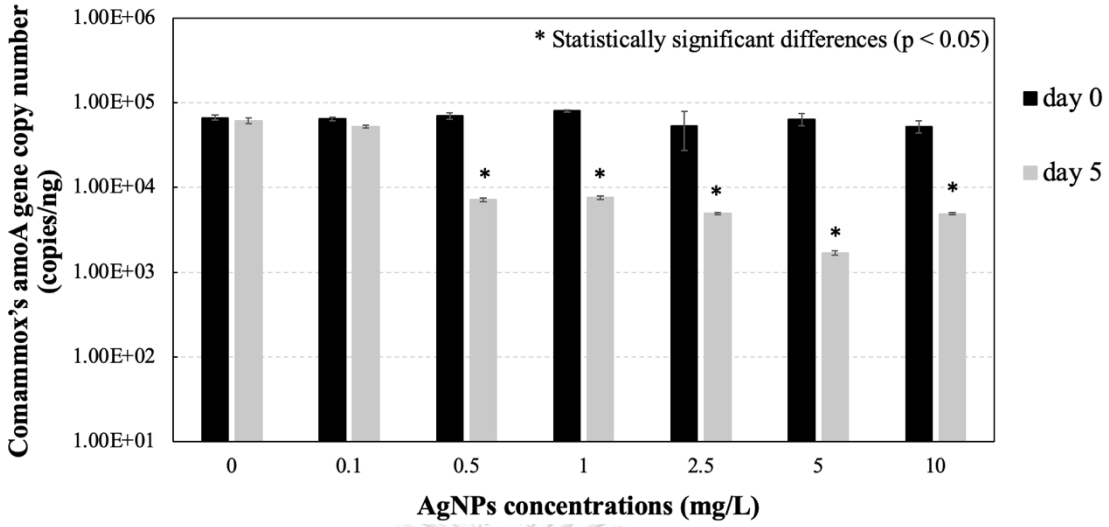


Figure 4-15 Numbers of comammox in AgNPs study microcosms

Beddow et al. (2016) discovered that the number of bacterial *amoA* decreased dramatically at 50 mg/L of AgNPs in low salinity sediments, while the change in archaeal *amoA* number was limited. However, at an AgNP concentration of 0.5 mg/L, there were no significant decreases in both archaeal and bacterial *amoA* genes. This is consistent with this study that the number of bacterial *amoA* decreased dramatically at the AgNP concentrations greater than 2.5 mg/L, while the number of archaeal *amoA* slightly decreased at AgNP concentrations higher than 0.5 mg/L. Although the result is clear that AgNPs decreased the number of bacterial *amoA*, it does mean that ammonia oxidizing activity also decreased. It is possible that the ammonia oxidizing activity could be made up by AOA, comammox or even survived AOB. Zheng et al. (2017) showed that comammox *amoA* expression was suppressed, but not completely, when the sludge was exposed to AgNPs, suggesting that comammox may dominate in NH_3 oxidation (Zheng et al., 2017). The finding by Zheng et al. (2017) contradicts the observation in this study that comammox was the most sensitive to AgNPs, as the number of comammox decreased dramatically at AgNPs concentration above of 0.5

mg/L. The meta-transcriptomics of genes involved in ammonia oxidation should be studied in the future to determine the major ammonia oxidizers that play important roles in ammonia oxidizing activity under the presence of AgNPs.

This portion of the experimental work confirmed the first hypothesis that AgNPs can suppress the growth of AOB and comammox more than AOA. The number of AOB *amoA* decreased at 2.5 mg/L AgNPs, while that of comammox *amoA* decreased at 0.5 mg/L. The number of AOA *amoA* decreased significantly at AgNPs 0.5 mg/L but far less than AOB *amoA* and comammox. This could be because the cell wall of AOA, which has lower permeability compared to bacterial cell wall. It would be interesting to study the response of the AOA lipid membrane to AgNPs to better understand the tolerance of AOA to them.

4.4 Co-effect of AgNPs and PVC microplastics on nitrifying sludge

AgNPs of 0.5 mg/L and PVC microplastic of 500 mg/L were spiked to the microcosms under different conditions. The microcosm sets that were pre-shaken for 7 days with PVC microplastics before adding nitrifying sludge maintain 40 mg/L of TAN at day 0 of incubation, indicating that PVC had no effect on TAN adsorption. The TAN concentration in the microcosms is shown in Figure 4-16. No reduction in TAN concentration in the negative control. All microcosms containing no AgNPs showed the same ammonia oxidation activity as the positive control, in which 40 mgN/L TAN was oxidized within 2 days. The microcosms with AgNPs oxidized the added TAN within 4 days, except for the microcosms that were pre-shaken with PVC, which took 3 days to convert TAN to NO_3^-.

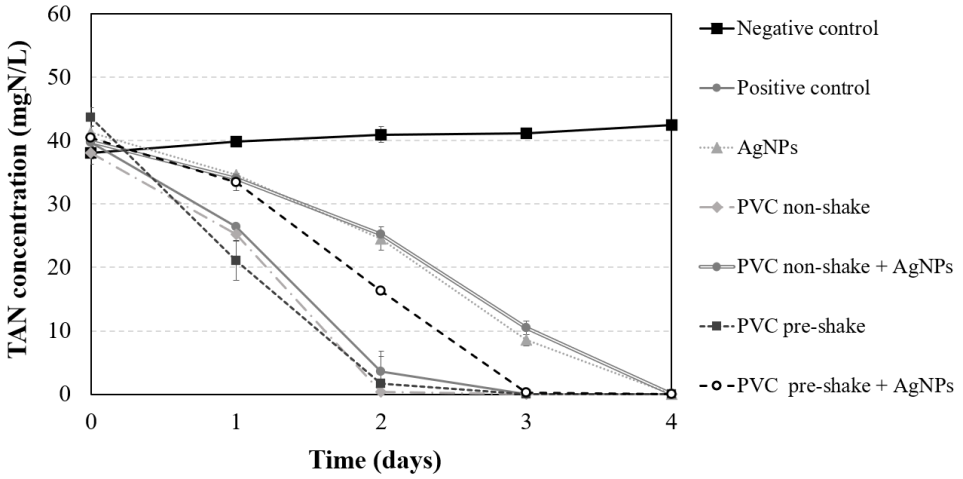


Figure 4-16 Total ammonia nitrogen concentrations in AgNPs and PVC study microcosms

The NO_2^- concentration in microcosms is shown in Figure 4-17. Accumulation of NO_2^- was observed in all microcosms. In the positive control, PVC without pre-shaking, pre-shaken PVC, and pre-shaken PVC with AgNPs, peaks of NO_2^- were observed on day 2 at the concentrations of 5.4, 5.7, 3.7, and 1.7 mgN/L, respectively. For the other sets with AgNPs, and AgNPs with nonpre-shaken PVC, the highest NO_2^- concentrations were 4.4 and 4.6 mgN/L, respectively, on day 3.

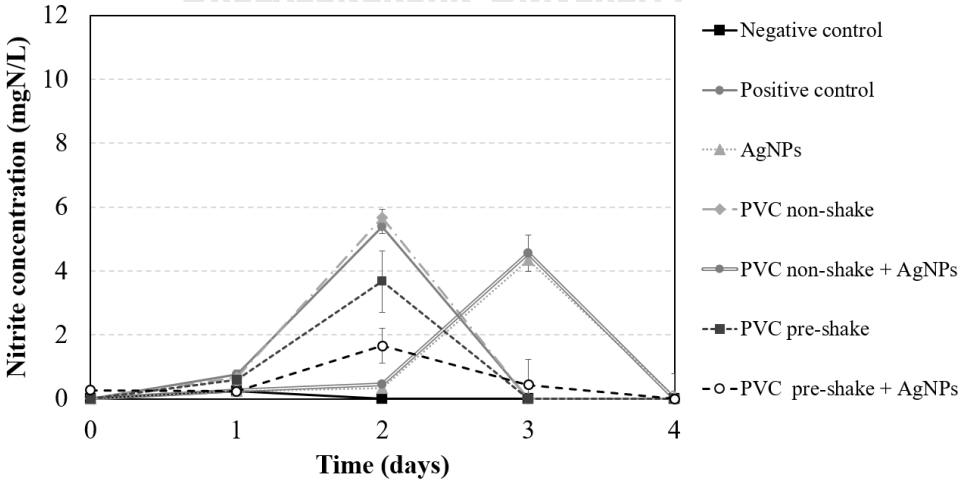


Figure 4-17 Nitrite concentrations in AgNPs and PVC study microcosms

The NO_3^- concentration in the microcosms is shown in Figure 4-18. NO_3^- concentration corresponds with TAN concentration reduction. Because the microcosms without AgNPs oxidized TAN within 2 days, the NO_3^- concentration reached 40 mg/L on day 2. The microcosms to which AgNPs were added took 4 days to reach 40 mg/L NO_3^-, except for the microcosms that PVC were pre-shaken that required 3 days.

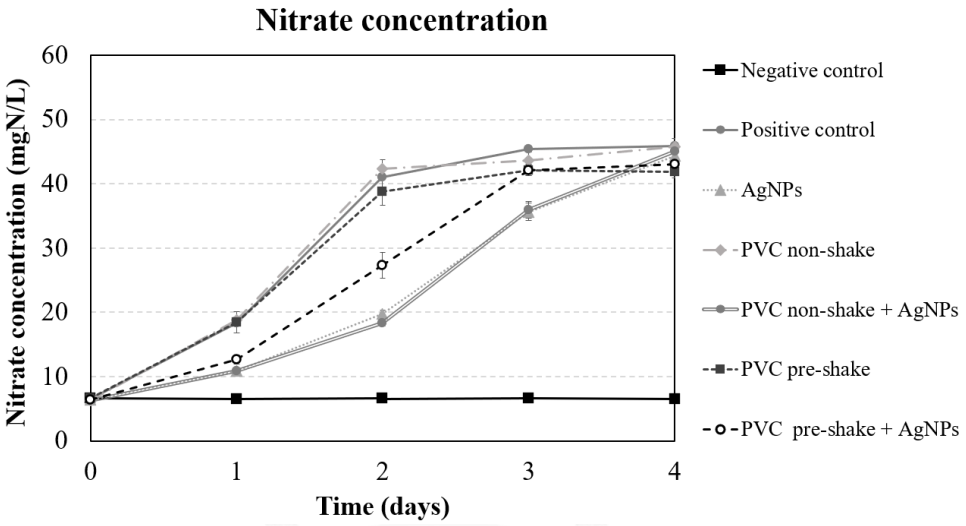


Figure 4-18 Nitrate concentrations in AgNPs and PVC study microcosms

From the study of Li et al. (2020), PVC was able to improve the ammonia oxidation rate at 1,000 particles/L, while higher concentrations such as 5,000 and 10,000 particles/L caused a slight inhibition. This study is consistent in showing that PVC has no negative effect on the ammonia oxidizing activity of nitrifying microorganisms. However, other studies have reported that PVC inhibits the ammonia oxidation rate. Seeley et al. (2020) revealed that the ammonia oxidation rate of sedimentary nitrifiers could be completely suppressed by PVC. Song et al. (2020) also concluded that PVC inhibited the ammonia oxidation rate in partial nitrification

reactors. This inhibition was concentration dependent, higher PVC concentrations exhibited higher toxicity via releasing of antimicrobial plasticizers. This is in contrast to the results of this study, where PVC showed no negative effect on ammonia oxidizing activity. In the study by Song et al. (2020), TAN at concentrations up to 1000 mgN/L was used. Since the TAN concentration was high, AOB were the main nitrifiers that play an important role in ammonia oxidation in the reactor. The PVC shows high toxicity to AOB and suppresses its activity (Song et al., 2020). The TAN concentration used in this study was quite low compared to the study of Song et. al. (2020). Therefore, other ammonia oxidizing microorganisms may be active in the microcosms containing PVC. It is very interesting that the properties of PVC may change during the 7-day pre-shaking leading to the quenching of the toxicity of AgNPs. Another possibility is that PVC microplastics may release chemical(s) that react with AgNPs that reduce the toxicity of AgNPs. From the study by Li et al. (2022) some types of microplastics such as PS tended to bind AgNPs on plastic surface, while other types such as PE and PP also have ability to capture AgNPs on their surfaces but in lower numbers compared to PS. Therefore, this part of the experiment work completely refutes the third hypothesis, as the pre-shaken PVC has the properties to reduce the toxicity of AgNPs and not to enhance the inhibition of ammonia oxidation activity. The properties of PVC should be analyzed to confirm the mechanisms of PVC for the reduction of the toxicity of AgNPs by chemical reaction or physical adsorption in the future.

The numbers of archaeal *amoA* in the microcosms are shown in Figure 4-19. The qPCR results show that the number of archaeal *amoA* significantly decreased in the microcosms treated with only AgNPs at 0.5 mg/L. This agrees with the finding from the earlier experiment that AOA was slightly inhibited by AgNPs. The results were similar for the microcosms treated with non-pre shaken PVC and AgNPs. However, the microcosms added with pre-shaken PVC did not show the decrease on archaeal *amoA* number. These results indicate that the PVC microplastics have no negative effect on AOA.

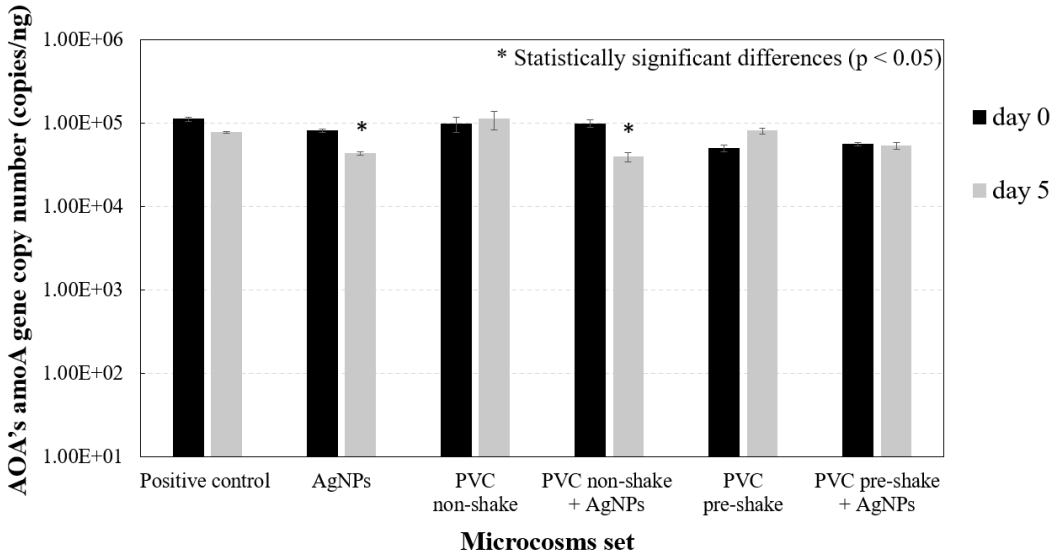


Figure 4-19 Numbers of AOA *amoA* in AgNPs and PVC study microcosms

The numbers of bacterial *amoA* in the microcosms are shown in Figure 4-20. The number of AOB increased in all microcosms including the AgNPs treated set. This proves that AOB was not affected by AgNPs at 0.5 mg/L. In addition, the PVC showed no inhibition on AOB.

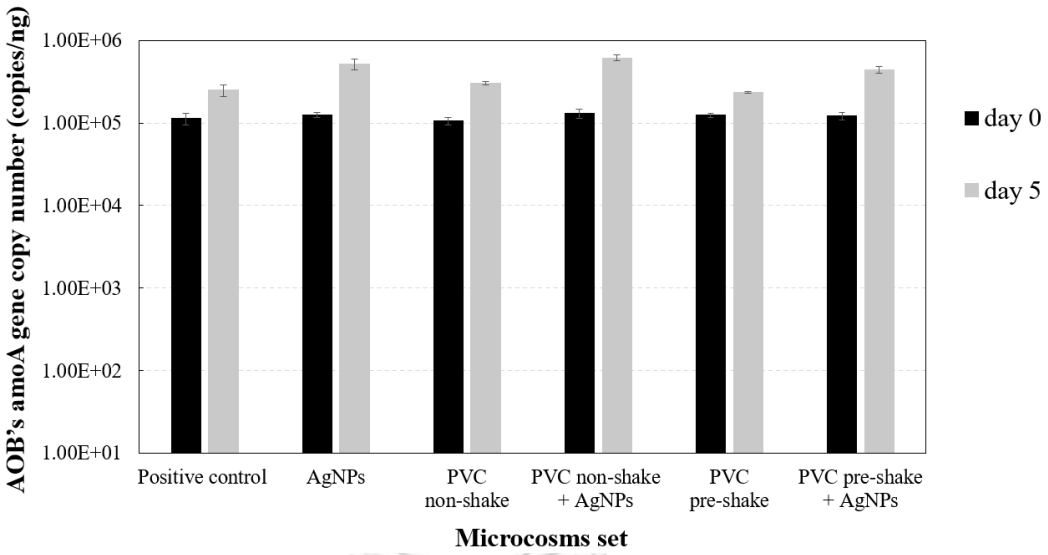


Figure 4-20 Numbers of AOB *amoA* in AgNPs and PVC study microcosms

All microcosms lacking AgNPs have the same numbers of comammox *amoA* at day 0 and day 5 of incubation. This indicates that the PVC did not inhibit comammox. In the microcosm containing only AgNPs at a concentration of 0.5 mg/L, the number of comammox *amoA* significantly decreased from 9.35×10^4 to 3.05×10^4 copies/ng, a decrease of 37%. The number of comammox *amoA* in the microcosms with PVC without pre-shaking and AgNPs decreased from 1.38×10^5 to 6.21×10^4, whereas that of pre-shaken PVC with AgNPs decreased from 1.66×10^5 to 6.26×10^4, corresponding to decreases of 45% and 37%, respectively. This is consistent with the previous experiments described above showing that 0.5 mg/L AgNPs can inhibit the growth of comammox. Thus, PVC was not able to inhibit the growth of comammox, but AgNPs were the main contaminant that affected comammox *amoA* number in these microcosms.

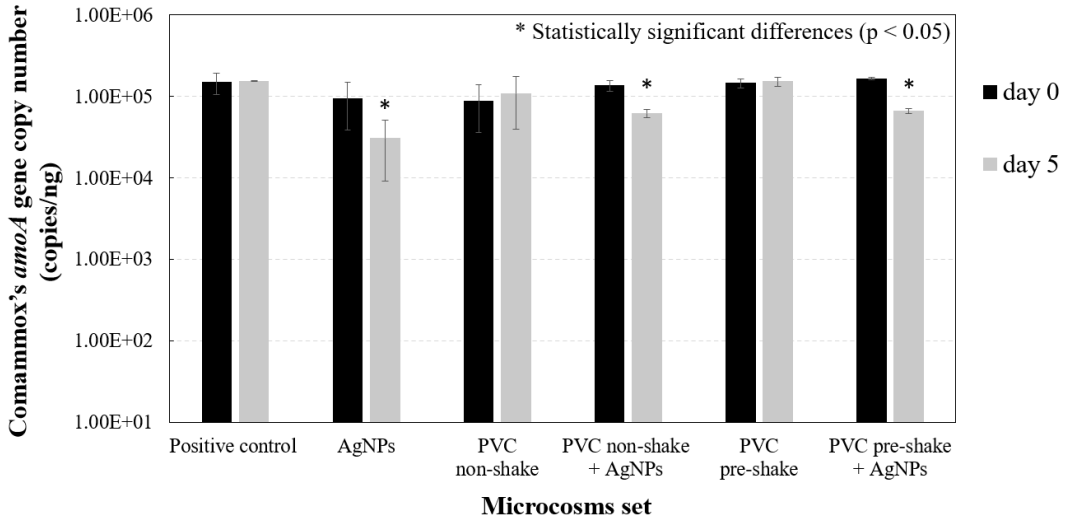


Figure 4-21 Numbers of comammox *amoA* in AgNPs and PVC study microcosms

PVC microplastics at a concentration of 500 mg/L exhibited no growth inhibitory effect on the three groups of ammonia oxidizing microorganisms: AOA, AOB and comammox. Some of the results from this study are in contradiction with other studies. Wang et al. (2021) showed that PVC microplastic had no effect on the growth of AOA and comammox but the number of AOB decreased at 100 mg/L PVC (Wang et al., 2021). Seeley et al. (2020) similarly concluded that PVC inhibited bacterial *amoA* abundance in saltwater sediment. The present study found that PVC at higher concentrations had no inhibitory effect on AOB. It is possible that the conditions of the experiments are different between the two studies. The size of PVC may affect to the toxicity. In the present study, the PVC was between 106-500 µm while it was 53-300 µm in (Seeley et al., 2020)'s study. The larger PVC used resulted in smaller adhesion areas. Chemical additives in the PVC are also likely different and consequently deliver different toxicity. The findings in the present study refute the second and third hypotheses that PVC has an inhibitory effect on ammonia oxidation

activity and growth of ammonia-oxidizing microorganisms. In fact, PVC did not show any effect on ammonia oxidation rate or growth of the microorganisms, but AgNPs are the main contaminants showing inhibitory effects. Future work should examine the effects on RNA expression level especially ammonia oxidation involved genes to elucidate the effects of PVC on the ammonia oxidizing activity of nitrifying microorganisms.

Chapter 5

Conclusions and recommendations for future work

5.1. Conclusions

5.1.1 Nitrifying bioreactor operation and selection of MLSS concentration for microcosm study

During the operation of the nitrifying bioreactor, ammonia oxidation and nitrification activities were satisfactory. AOA, AOB, and comammox *amoA* occurred at the similar ranges since the early day of the operation, indicating that the sludge originally had all the three groups of ammonia oxidizers. Nitrifying sludge from the bioreactor was inoculated at different final MLSS concentrations in the microcosms (40, 60, 100 mg MLSS/L). The 100 mg/L MLSS showed the most promising result and was selected for further experiments.

5.1.2 Effects of AgNPs on ammonia oxidizing microorganisms

Tests of ammonia oxidizing activity with varying AgNPs concentrations (0.1, 0.5, 1, 2.5, 5, 10 mg/L) indicated that the inhibition of AgNPs on ammonia oxidizing activity is concentration dependent. At 0.1 mg/L AgNP, there was no inhibition while partial inhibition was found at AgNP concentrations of 0.5 and 1 mg/L. The specific initial ammonia oxidation rate was calculated during the first 2 days of incubation. The initial rates at 0.5 and 1 mg/L AgNPs were 78% and 92% inhibited, respectively. At concentrations higher than 2.5 mg/L, AgNPs completely inhibited the ammonia oxidizing activity resulting in the inhibition of 100%. The overall ammonia oxidation rates at 0.5 and 1 mg/L AgNPs were 60 and 80% inhibited, respectively. Complete inhibition (100%) based on the overall ammonia oxidation rate was observed in the

microcosms with AgNPs above 2.5 mg/L. AgNPs slightly inhibited AOA at concentrations above 0.5 mg/L. AOB was inhibited by AgNPs concentrations ≥2.5 mg/L. Comammox showed the most sensitivity to AgNPs that their *amoA* gene numbers substantially decreased at the AgNPs concentrations above 0.5 mg/L. Overall, AOA was inhibited by AgNPs far less than AOB and comammox. The results suggested that different types of ammonia oxidizing microorganisms have different sensitivities to AgNPs. The results from this work agree with previous studies that found AgNPs inhibiting AOB but not AOA. The effects of AgNPs on comammox have been rarely studied.

5.1.3 Co-effect of AgNPs and PVC microplastics on ammonia oxidizing microorganisms

A series of microcosms was performed at an AgNPs concentration of 0.5 mg/L and a PVC microplastics concentration of 500 mg/L. The results showed that the PVC microplastics did not suppress the ammonia oxidizing activity of the sludge. The microcosms with AgNPs took longer time for TAN utilization. Regarding its negative effect on nitrifying community, PVC microplastics did not show any on all three groups of ammonia oxidizing microorganisms. This result contradicts with previous studies reporting that PVC microplastics inhibit AOB and comammox. It is possible that the size of PVC microplastics in this study is bigger resulting in less contact area. Since the toxicity of PVC microplastics can be due to the antimicrobial plasticizers present; a smaller contact area is likely release less plasticizers leading to less inhibition of ammonia-oxidizing microorganisms. On the other hands, the microcosms in which the PVC was pre-shaken for 7 days before adding to the sludge along with AgNPs showed a faster ammonia oxidation rate than other microcosms in

which the sludge was dosed with only AgNPs. This suggests that the pre-shaken PVC microplasctics may reduce the toxicity of AgNPs. Therefore, it is essential to investigate potential interactions and reactions between PVC and AgNPs in the future.

5.2 Recommendations for future work

5.2.1 Research point of view

5.2.1.1 Although previous studies have shown AOA to be tolerant to 50 mg/L AgNPs, the lethal dose of AgNPs on AOA growth should be investigated to better understand the mechanisms of AgNPs resistance.

5.2.1.2 Interactions of AgNPs and PVC microplastics should be examined to find the mechanisms that reduce the toxicity of AgNPs. Scanning electron microscopy and energy dispersive spectroscopy should be performed to confirm the capturing of AgNPs by PVC microplastics.

5.2.1.3 PVC microplastics concentration should be varied to evaluate its effects on the ammonia oxidizing activity and growth.

5.2.1.4 It is possible that there are some ammonia-oxidizing microorganisms that can survive but are not active under the presence of AgNPs and PVC microplastics together. Therefore, the RNA expression level should be studied to determine the exact effects of AgNPs and PVC microplastics on the growth and activity of ammonia oxidizing microorganisms.

5.2.2 Application point of view

5.2.2.1 Since AOA is tolerant to high concentrations of AgNPs, it may be beneficial to operate the wastewater treatment process under favorable AOA

conditions to improve nitrogen removal in wastewater treatment systems facing high levels of AgNPs. This topic should be further investigated.

5.2.2.2 Although PVC microplastics have been reported to be toxic to various organisms, ammonia-oxidizing microorganisms may not be subject to inhibitory effects by PVC. Therefore, the nitrification can continue despite the contamination of wastewater by PVC microplastics. The effects of PVC on denitrification should be examined to determine whether PVC could impact nitrogen removal in wastewater treatment.

Appendix A

Nitrogen concentrations in microcosms

Table 1 TAN concentrations in sludge concentration study microcosms

Day	Control (mgN/L)	40 mg MLSS/L (mgN/L)	60 mg MLSS/L (mgN/L)	100 mg MLSS/L (mgN/L)
0	41.2 ± 0.6	37.3 ± 1.0	36.8 ± 1.2	33.2 ± 3.3
1	40.1 ± 0.5	36.2 ± 1.8	34.7 ± 2.4	32.4 ± 1.7
2	43.0 ± 2.8	36.0 ± 3.5	31.0 ± 1.2	20.6 ± 1.1
3	49.7 ± 0.9	14.5 ± 0.5	2.9 ± 2.6	0.0 ± 0.1
4	40.8 ± 1.1	0.0 ± 0.1	0.0 ± 0.0	0.0 ± 0.1
6	43.2 ± 0.7	0.0 ± 0.0	0.0 ± 0.1	0.0 ± 0.0

Table 2 Nitrite concentrations in sludge concentration study microcosms

Day	Control (mgN/L)	40 mg MLSS/L (mgN/L)	60 mg MLSS/L (mgN/L)	100 mg MLSS/L (mgN/L)
0	0.0 ± 0.0	0.0 ± 0.2	0.0 ± 0.2	0.0 ± 0.0
1	0.0 ± 0.0	0.2 ± 0.1	0.3 ± 0.0	0.3 ± 0.0
2	1.1 ± 0.6	1.7 ± 0.1	2.0 ± 0.0	0.1 ± 0.2
3	0.0 ± 0.0	7.8 ± 0.0	8.5 ± 0.4	1.8 ± 2.3
4	0.3 ± 0.4	9.8 ± 1.2	1.2 ± 0.1	0.0 ± 0.1
6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

Table 3 Nitrate concentrations in sludge concentration study microcosms

Day	Control (mgN/L)	40 mg MLSS/L (mgN/L)	60 mg MLSS/L (mgN/L)	100 mg MLSS/L (mgN/L)
0	5.1 ± 0.1	5.3 ± 0.1	5.4 ± 0.2	5.1 ± 1.0
1	5.4 ± 0.0	7.9 ± 1.2	10.3 ± 0.6	14.4 ± 1.6
2	6.2 ± 0.4	11.9 ± 3.5	17.4 ± 0.7	25.1 ± 0.6
3	5.6 ± 0.0	28.7 ± 0.1	33.5 ± 1.2	38.9 ± 0.2
4	5.8 ± 0.1	38.1 ± 0.6	40.7 ± 1.4	37.8 ± 1.5
6	5.7 ± 0.3	41.7 ± 0.4	43.8 ± 1.5	42.9 ± 1.3

Table 4 TAN concentrations in AgNPs study microcosms

Day	AgNPs concentration (mg/L)							
	Control (mgN/L)	0 (mgN/L)	0.1 (mgN/L)	0.5 (mgN/L)	1.0 (mgN/L)	2.5 (mgN/L)	5.0 (mgN/L)	10.0 (mgN/L)
0	41.2 ± 0.8	39.5 ± 2.4	41.1 ± 0.5	39.7 ± 0.9	39.0 ± 0.0	39.4 ± 1.4	40.6 ± 0.1	43.6 ± 1.4
1	38.7 ± 1.5	22.7 ± 0.6	22.1 ± 0.2	38.2 ± 1.0	34.5 ± 1.5	37.9 ± 0.3	41.1 ± 2.7	40.3 ± 1.6
2	44.2 ± 0.6	0.0 ± 0.1	0.0 ± 0.1	31.1 ± 0.3	35.8 ± 1.2	36.3 ± 0.4	43.9 ± 1.2	40.6 ± 2.6
3	38.2 ± 0.6	0.0 ± 0.1	0.0 ± 0.0	24.3 ± 1.0	31.3 ± 1.4	35.9 ± 0.2	43.7 ± 0.9	39.2 ± 1.4
4	36.8 ± 1.4	0.0 ± 0.1	0.0 ± 0.1	13.9 ± 1.0	32.0 ± 1.4	36.4 ± 0.1	40.2 ± 0.8	40.8 ± 1.2
5	35.8 ± 0.8	0.0 ± 0.1	0.0 ± 0.	0.0 ± 0.1	24.5 ± 1.4	36.2 ± 1.1	40.0 ± 1.3	37.4 ± 0.4
6	37.1 ± 3.4	0.0 ± 0.1	0.0 ± 0.2	0.0 ± 0.0	22.0 ± 2.8	42.1 ± 0.3	40.7 ± 0.3	39.0 ± 0.2
7	39.0 ± 0.4	0.0 ± 0.0	0.0 ± 0.1	0.0 ± 0.0	14.5 ± 4.4	36.4 ± 0.5	42.1 ± 0.8	39.3 ± 0.5
8	44.0 ± 1.1	0.0 ± 0.3	0.0 ± 0.2	0.0 ± 0.1	11.8 ± 4.9	37.5 ± 1.1	40.2 ± 0.8	37.6 ± 0.6
9	39.1 ± 0.3	0.0 ± 0.1	0.0 ± 0.5	0.0 ± 0.0	3.2 ± 7.0	32.9 ± 1.1	39.5 ± 1.2	39.3 ± 0.6
10	37.9 ± 1.1	0.0 ± 0.1	0.0 ± 0.1	0.0 ± 0.0	1.0 ± 0.7	34.4 ± 1.2	47.4 ± 5.9	40.1 ± 7.7
11	35.2 ± 0.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.1	1.0 ± 0.1	40.8 ± 2.7	44.7 ± 1.8	40.3 ± 4.5

Table 5 Nitrite concentrations in AgNPs study microcosms

Day	AgNPs concentration (mg/L)							
	Control (mgN/L)	0 (mgN/L)	0.1 (mgN/L)	0.5 (mgN/L)	1.0 (mgN/L)	2.5 (mgN/L)	5.0 (mgN/L)	10.0 (mgN/L)
0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.4 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0
1	0.2 ± 0.0	1.4 ± 0.1	1.1 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0
2	0.0 ± 0.0	2.7 ± 0.6	3.4 ± 0.2	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
3	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.4 ± 0.3	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.6 ± 0.3
4	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.1	1.0 ± 0.1	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.1
5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.2	0.3 ± 0.0	0.3 ± 0.2	0.2 ± 0.0	0.2 ± 0.0
6	0.2 ± 0.2	0.2 ± 0.0	0.0 ± 0.0	0.3 ± 0.0	0.4 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.2
7	0.0 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.0	0.4 ± 0.1	0.3 ± 0.2	0.4 ± 0.0	0.3 ± 0.0
8	0.3 ± 1.1	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.6	0.2 ± 0.0	0.4 ± 0.0	0.3 ± 0.0
9	0.0 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.0	0.2 ± 0.2	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.2
10	0.0 ± 1.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 1.1	0.0 ± 0.8	0.0 ± 0.7	0.0 ± 0.8
11	0.0 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.1	0.0 ± 0.1	0.6 ± 2.2	0.0 ± 0.1	0.0 ± 0.8

Table 6 Nitrate concentrations in AgNPs study microcosms

Day	AgNPs concentration (mg/L)							
	Control (mgN/L)	0 (mgN/L)	0.1 (mgN/L)	0.5 (mgN/L)	1.0 (mgN/L)	2.5 (mgN/L)	5.0 (mgN/L)	10.0 (mgN/L)
0	5.2 ± 0.0	5.6 ± 0.1	5.6 ± 0.1	5.3 ± 0.1	5.4 ± 0.1	5.5 ± 0.1	5.6 ± 0.1	5.9 ± 0.1
1	5.5 ± 0.0	21.0 ± 0.3	20.4 ± 0.7	6.9 ± 0.1	9.0 ± 0.2	6.0 ± 0.1	6.1 ± 0.1	6.5 ± 0.1
2	5.3 ± 0.1	43.0 ± 0.3	42.7 ± 0.9	13.8 ± 0.6	7.6 ± 0.4	6.3 ± 0.0	6.2 ± 0.2	6.7 ± 0.2
3	5.5 ± 0.6	40.8 ± 0.8	40.0 ± 2.1	17.7 ± 0.9	8.9 ± 0.7	5.8 ± 0.1	5.9 ± 0.0	6.0 ± 0.3
4	5.6 ± 0.0	43.1 ± 1.6	42.2 ± 0.0	27.4 ± 1.9	11.3 ± 0.9	6.1 ± 0.1	6.2 ± 0.0	6.4 ± 0.2
5	5.8 ± 0.1	44.2 ± 1.5	44.3 ± 0.8	43.9 ± 1.4	16.6 ± 1.7	6.4 ± 0.1	6.6 ± 0.2	7.0 ± 0.2
6	5.9 ± 0.1	45.5 ± 0.8	44.7 ± 1.2	42.9 ± 1.6	20.6 ± 2.6	6.6 ± 0.2	6.3 ± 0.1	6.6 ± 0.1
7	5.4 ± 0.1	45.2 ± 0.8	45.7 ± 0.1	45.2 ± 0.2	24.4 ± 3.4	6.8 ± 0.2	6.4 ± 0.2	6.8 ± 0.1
8	5.9 ± 0.0	48.6 ± 0.6	45.8 ± 0.8	46.9 ± 0.9	33.2 ± 6.6	7.2 ± 0.4	6.8 ± 0.1	7.2 ± 0.1
9	6.3 ± 0.6	48.7 ± 0.4	48.9 ± 0.6	46.1 ± 0.9	48.5 ± 5.9	7.8 ± 0.8	6.9 ± 0.1	7.3 ± 0.1
10	6.0 ± 0.2	46.9 ± 1.5	46.8 ± 0.3	43.4 ± 2.3	43.2 ± 0.5	8.5 ± 1.3	6.8 ± 0.3	7.5 ± 0.4
11	7.8 ± 2.0	44.8 ± 3.9	46.9 ± 2.2	47.5 ± 0.8	43.8 ± 0.9	10.0 ± 2.2	7.5 ± 0.1	7.8 ± 0.2

Table 7 TAN concentrations in AgNPs study microcosms

day	Negative control (mgN/L)	Positive control (mgN/L)	AgNPs (mgN/L)	PVC non-shake (mgN/L)	PVC non-shake + AgNPs (mgN/L)	PVC pre-shake (mgN/L)	PVC pre-shake +AgNPs (mgN/L)
0	38.0 ± 1.8	39.7 ± 0.7	41.3 ± 1.0	38.1 ± 1.0	39.8 ± 0.5	43.7 ± 1.5	40.4 ± 0.9
1	39.9 ± 0.7	26.4 ± 0.4	34.6 ± 0.2	25.2 ± 1.0	34.0 ± 0.3	21.0 ± 3.1	33.4 ± 1.3
2	40.9 ± 1.3	3.6 ± 3.2	24.6 ± 1.9	0.4 ± 1.2	25.3 ± 0.3	1.6 ± 4.3	16.3 ± 0.5
3	41.1 ± 0.8	0.0 ± 0.0	8.5 ± 0.9	0.0 ± 0.0	10.5 ± 1.1	0.0 ± 0.0	0.2 ± 0.1
4	42.4 ± 0.8	0.0 ± 0.1	0.0 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.1	0.0 ± 0.1

Table 8 Nitrite concentrations in AgNPs study microcosms

day	Negative control (mgN/L)	Positive control (mgN/L)	AgNPs (mgN/L)	PVC non-shake (mgN/L)	PVC non-shake + AgNPs (mgN/L)	PVC pre-shake (mgN/L)	PVC pre-shake +AgNPs (mgN/L)
0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.3± 0.0
1	0.2 ± 0.0	0.8 ± 0.1	0.2 ± 0.0	0.7 ± 0.0	0.3 ± 0.0	0.6 ± 0.0	0.2 ± 0.0
2	0.0 ± 0.0	5.4 ± 0.2	0.3 ± 0.0	5.7 ± 0.3	0.5 ± 0.0	3.7 ± 1.0	1.7 ± 0.5
3	0.0 ± 0.0	0.0 ± 0.2	4.3 ± 0.1	0.0 ± 0.0	4.6 ± 0.6	0.0 ± 0.0	0.4 ± 0.8
4	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.2	0.0 ± 0.0	0.0 ± 0.5	0.0 ± 0.0	0.0 ± 0.8

Table 9 Nitrate concentrations in AgNPs study microcosms

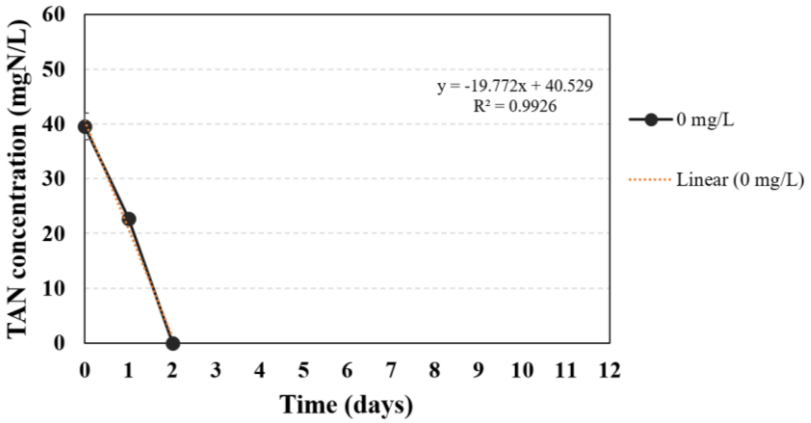
day	Negative control (mgN/L)	Positive control (mgN/L)	AgNPs (mgN/L)	PVC non-shake (mgN/L)	PVC non-shake + AgNPs (mgN/L)	PVC pre-shake (mgN/L)	PVC pre-shake +AgNPs (mgN/L)
0	6.7 ± 0.2	6.5 ± 0.1	6.3 ± 0.3	6.3 ± 0.0	6.3 ± 0.2	6.7 ± 0.1	6.4 ± 0.1
1	6.5 ± 0.1	18.3 ± 0.3	10.9 ± 0.2	18.8 ± 0.5	10.9 ± 0.5	18.5 ± 1.7	12.6 ± 0.7
2	6.6 ± 0.0	41.0 ± 1.5	19.7 ± 0.6	42.3 ± 1.5	18.4 ± 0.5	38.8 ± 2.2	27.3 ± 2.0
3	6.6 ± 0.1	45.4 ± 0.2	35.6 ± 1.3	43.7 ± 1.3	35.9 ± 1.3	42.1 ± 0.8	42.1 ± 0.9
4	6.5 ± 0.0	45.9 ± 1.2	44.3 ± 0.8	45.8 ± 0.5	45.1 ± 0.5	41.9 ± 0.9	43.0 ± 0.1

Appendix B

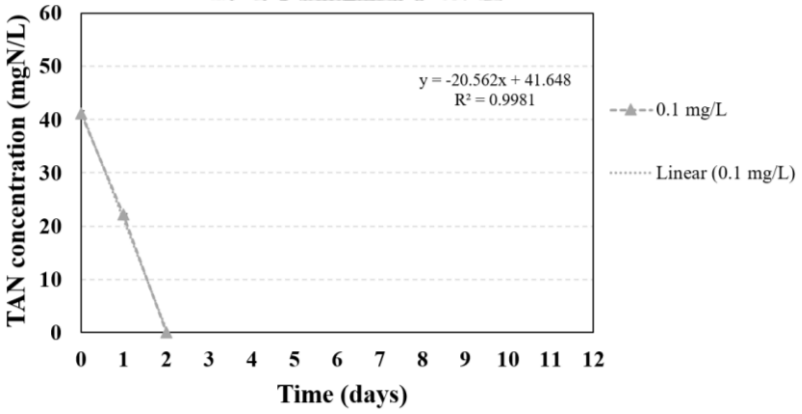
Linear regression for ammonia oxidation rate study

Figure 1 Linear regression on specific initial ammonia oxidation rate of AgNPs study microcosms

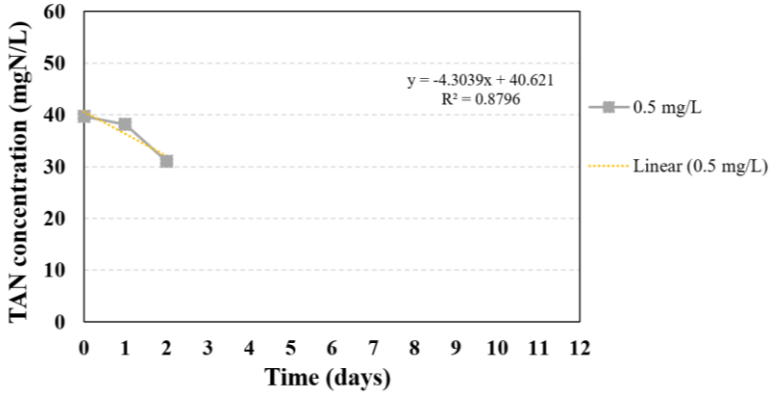
a) 0 mg/L



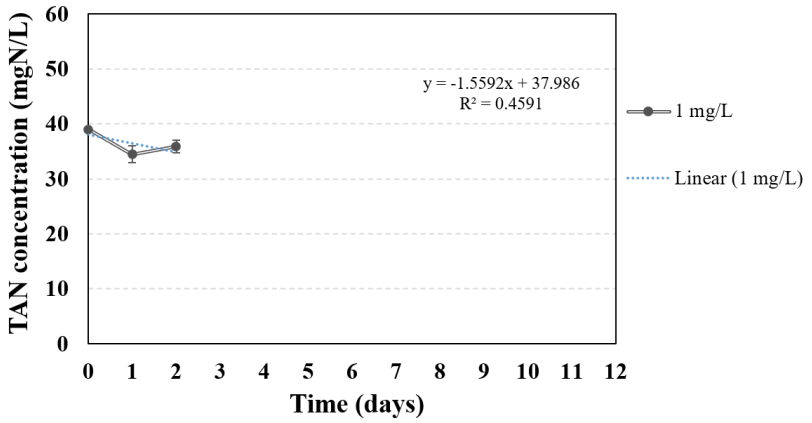
b) 0.1 mg/L



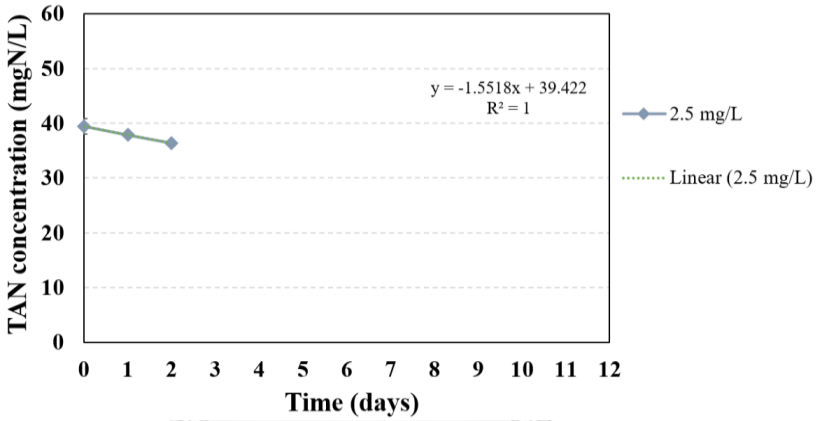
c) 0.5 mg/L



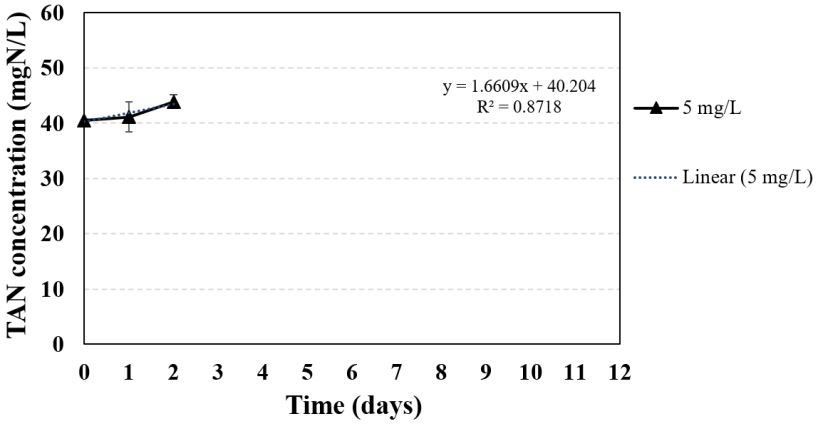
d) 1.0 mg/L



e) 2.5 mg/L



f) 5 mg/L



g) 10 mg/L

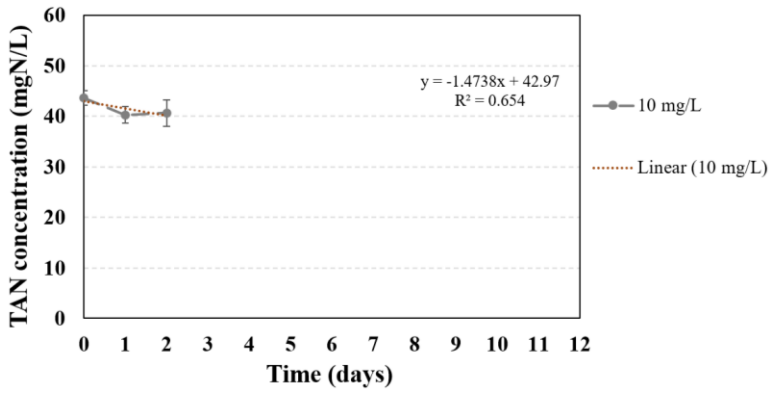
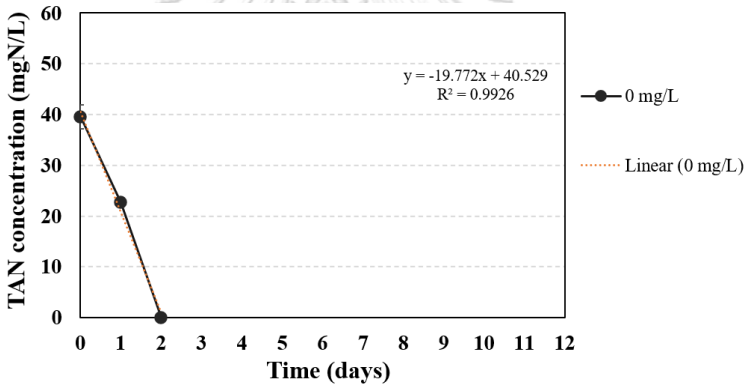
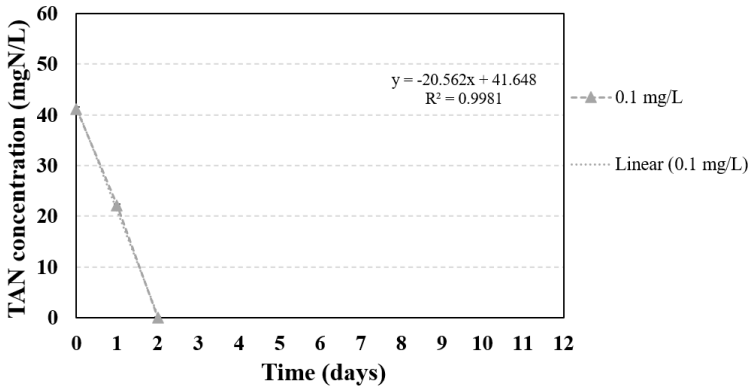


Figure 2 Linear regression on overall ammonia oxidation activity of AgNPs study microcosms

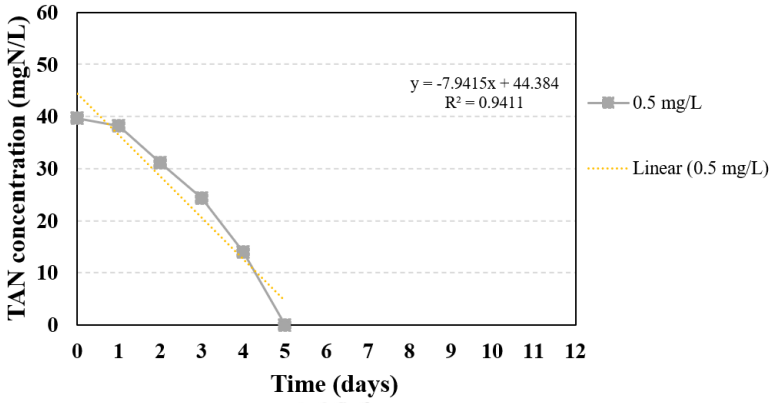
a) 0 mg/L



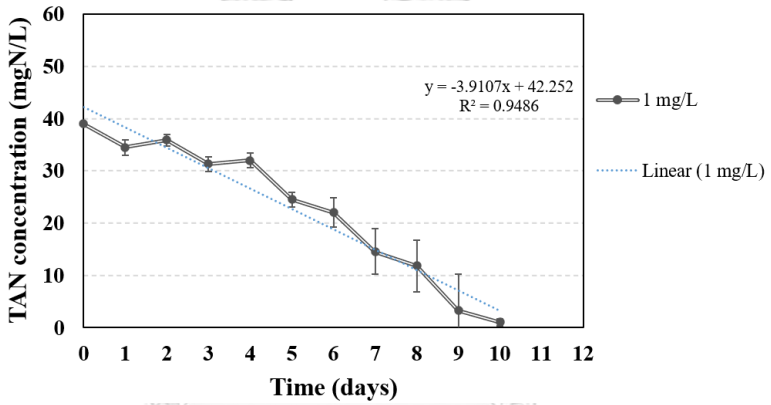
b) 0.1 mg/L



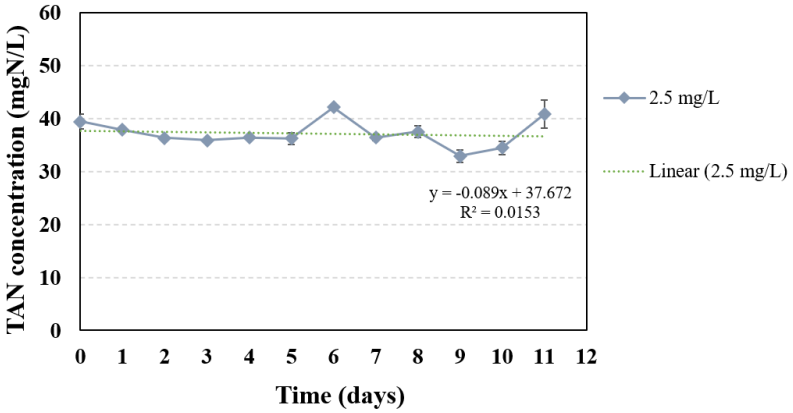
c) 0.5 mg/L



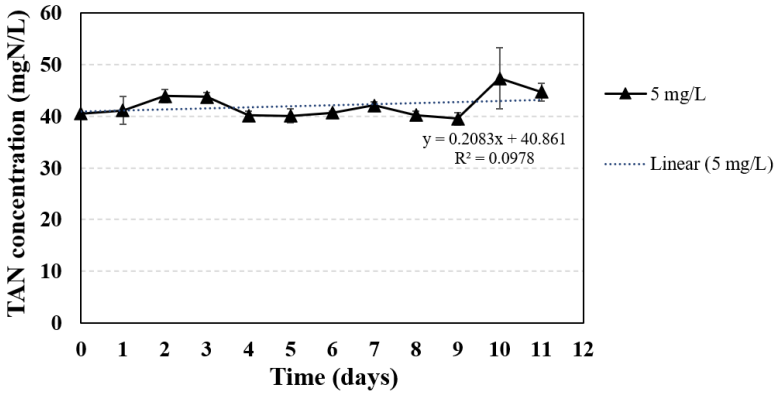
d) 1.0 mg/L



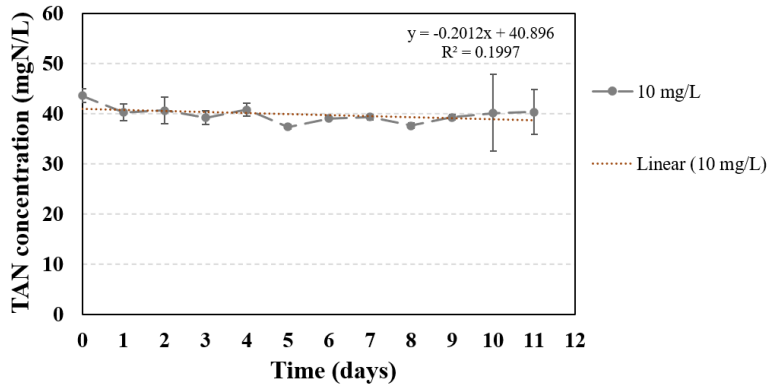
e) 2.5 mg/L



f) 5.0 mg/L



g) 10.0 mg/L



Appendix C

qPCR results

Table 1 Number of ammonia oxidizing microorganisms in bioreactor during operation

Operation day	AOA (copies/ng)	AOB (copies/ng)	Comammox (copies/ng)
0	$9.51x10^4 \pm 5.03x10^3$	$7.71x10^4 \pm 1.39x10^3$	$5.39x10^4 \pm 8.52x10^2$
14	$8.30x10^4 \pm 5.21x10^3$	$8.76x10^4 \pm 8.47x10^3$	$5.79x10^4 \pm 6.51x10^3$
19	$8.73x10^4 \pm 1.42x10^3$	$8.90x10^4 \pm 4.80x10^3$	$6.79x10^4 \pm 2.11x10^3$
39	$3.22x10^4 \pm 5.36x10^3$	$5.19x10^4 \pm 4.16x10^3$	$8.80x10^4 \pm 6.84x10^3$
52	$2.38x10^4 \pm 2.75x10^3$	$1.32x10^5 \pm 6.00x10^3$	$9.23x10^4 \pm 1.20x10^4$
60	$8.28x10^4 \pm 3.52x10^3$	$1.17x10^5 \pm 6.76x10^3$	$7.64x10^4 \pm 6.77x10^3$
74	$5.25x10^4 \pm 1.68x10^3$	$8.23x10^4 \pm 5.34x10^3$	$8.93x10^4 \pm 5.67x10^3$
97	$1.29x10^4 \pm 5.23x10^3$	$7.28x10^4 \pm 1.20x10^4$	$1.01x10^5 \pm 1.46x10^4$

Table 2 Number of ammonia oxidizing archaea in AgNPs studies microcosms

AgNPs concentration (mg/L)	Day 0 (copies/ng)	Day 5 (copies/ng)
0	$4.68x10^4 \pm 1.66x10^4$	$2.36x10^4 \pm 8.04x10^3$
0.1	$1.77x10^4 \pm 7.37x10^3$	$4.80x10^4 \pm 2.43x10^3$
0.5	$8.16x10^4 \pm 6.07x10^3$	$2.01x10^4 \pm 1.65x10^3$
1	$7.49x10^4 \pm 1.53x10^3$	$2.58x10^4 \pm 2.29x10^3$
2.5	$5.52x10^4 \pm 1.72x10^4$	$2.20x10^4 \pm 8.50x10^3$
5	$6.62x10^4 \pm 1.41x10^4$	$3.24x10^4 \pm 1.36x10^4$
10	$9.19x10^4 \pm 2.62x10^3$	$2.33x10^4 \pm 1.35x10^3$

Table 3 Number of ammonia oxidizing bacteria in AgNPs studies microcosms

AgNPs concentration (mg/L)	Day 0 (copies/ng)	Day 5 (copies/ng)
0	$5.55x10^4 \pm 7.45x10^3$	$9.84x10^4 \pm 5.57x10^3$
0.1	$5.48x10^4 \pm 9.32x10^3$	$1.14x10^5 \pm 5.73x10^3$
0.5	$6.13x10^4 \pm 2.02x10^3$	$1.95x10^5 \pm 1.90x10^4$
1	$5.82x10^4 \pm 6.47x10^3$	$9.41x10^4 \pm 5.11x10^3$
2.5	$5.50x10^4 \pm 4.08x10^3$	$1.17x10^4 \pm 2.66x10^2$
5	$5.71x10^4 \pm 4.58x10^3$	$6.40x10^3 \pm 1.55x10^3$
10	$4.93x10^4 \pm 6.92x10^3$	$2.97x10^3 \pm 1.49x10^2$

Table 4 Number of complete ammonia oxidizing bacteria in AgNPs studies microcosms

AgNPs concentration (mg/L)	Day 0 (copies/ng)	Day 5 (copies/ng)
0	$6.64x10^4 \pm 4.46x10^3$	$6.12x10^4 \pm 4.45x10^3$
0.1	$6.46x10^4 \pm 3.20x10^3$	$5.21x10^4 \pm 2.01x10^3$
0.5	$7.00x10^4 \pm 6.40x10^3$	$7.19x10^3 \pm 3.54x10^2$
1	$7.97x10^4 \pm 2.90x10^3$	$7.58x10^3 \pm 3.76x10^2$
2.5	$5.30x10^4 \pm 2.55x10^4$	$4.91x10^3 \pm 1.65x10^2$
5	$6.39x10^4 \pm 1.08x10^4$	$1.69x10^3 \pm 1.01x10^2$
10	$5.25x10^4 \pm 8.50x10^3$	$4.90x10^3 \pm 1.42x10^2$

Table 5 Number of ammonia oxidizing archaea in AgNPs and PVC studies microcosms

Microcosms	Day 0 (copies/ng)	Day 5 (copies/ng)
Positive control	$1.11x10^5 \pm 5.70x10^3$	$7.74x10^4 \pm 2.31x10^3$
AgNPs	$8.05x10^4 \pm 3.49x10^3$	$4.31x10^4 \pm 1.73x10^3$
PVC nonshake	$9.72x10^4 \pm 2.01x10^4$	$1.12x10^5 \pm 2.79x10^4$
PVC nonshake + AgNPs	$9.84x10^4 \pm 1.05x10^4$	$3.91x10^4 \pm 5.07x10^3$
PVC pre-shake	$4.98x10^4 \pm 4.15x10^3$	$8.08x10^4 \pm 6.70x10^3$
PVC pre-shake +AgNPs	$5.57x10^4 \pm 2.66x10^3$	$5.35x10^4 \pm 4.93x10^3$

Table 6 Number of ammonia oxidizing bacteria in AgNPs and PVC studies microcosms

Microcosms	Day 0 (copies/ng)	Day 5 (copies/ng)
Positive control	$1.13x10^5 \pm 1.81x10^4$	$2.50x10^5 \pm 3.99x10^4$
AgNPs	$1.26x10^5 \pm 9.16x10^3$	$5.18x10^5 \pm 7.53x10^4$
PVC nonshake	$1.06x10^5 \pm 1.15x10^4$	$3.01x10^5 \pm 1.52x10^4$
PVC nonshake + AgNPs	$1.31x10^5 \pm 1.66x10^4$	$6.16x10^5 \pm 4.68x10^4$
PVC pre-shake	$1.24x10^5 \pm 8.52x10^3$	$2.34x10^5 \pm 3.63x10^3$
PVC pre-shake +AgNPs	$1.22x10^5 \pm 1.36x10^4$	$4.42x10^5 \pm 4.56x10^4$

Table 7 Number of complete ammonia oxidizing bacteria in AgNPs and PVC studies microcosms

Microcosms	Day 0 (copies/ng)	Day 5 (copies/ng)
Positive control	$1.49x10^5 \pm 4.31x10^4$	$1.54x10^5 \pm 2.06x10^3$
AgNPs	$9.35x10^4 \pm 5.46x10^4$	$3.05x10^4 \pm 2.14x10^4$
PVC nonshake	$8.84x10^4 \pm 5.19x10^4$	$1.08x10^5 \pm 6.84x10^4$
PVC nonshake + AgNPs	$1.38x10^5 \pm 2.08x10^4$	$6.21x10^4 \pm 7.57x10^3$
PVC pre-shake	$1.45x10^5 \pm 1.87x10^4$	$1.53x10^5 \pm 1.93x10^4$
PVC pre-shake +AgNPs	$1.66x10^5 \pm 5.35x10^3$	$6.62x10^4 \pm 4.36x10^3$

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