THESIS APPROVAL

GRADUATE SCHOOL, KASETSART UNIVERSITY

Master of Engineering (Environmental Engineering)

DEGREE

Environmental Engineering

FIELD

Environmental Engineering

DEPARTMENT

TITLE: Identification and Quantification of Bacteria and Archaea Responsible for Ammonia Oxidation in Different Activated Sludge System

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THESIS

IDENTIFICATION AND QUANTIFICATION OF BACTERIA AND ARCHAEA RESPONSIBLE FOR AMMONIA OXIDATION IN DIFFERENT ACTIVATED SLUDGE SYSTEM

NUTPORNNAPAT SINTHUSITH

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
Master of Engineering (Environmental Engineering)
Graduate School, Kasetsart University
2014

Nutpornnapat Sinthusith 2014: Identification and Quantification of Bacteria and Archaea Responsible for Ammonia Oxidation in Different Activated Sludge System. Master of Engineering (Environmental Engineering), Major Field: Environmental Engineering, Department of Environmental Engineering. Thesis Advisor: Associate Professor Pongsak Noophan, Ph.D. 49 pages.

Eutrophication is a serious problem in water bodies, which intensifies the significance of nitrogen removal in a cost-effective manner. Nitrification, the primary reaction of biological nitrogen removal, is the first choice in the standpoint of cost-effectiveness and broader applicability. Ammonia oxidation, the first step of nitrification, has been mediated by ammonia-oxidizing bacteria (AOB). However, recent studies have revealed some archaea, also known as ammonia oxidizing archaea (AOA) are capable of oxidizing ammonia under oligotrophic conditions. Such surprising discovery allows researchers to investigate their physiological and phylogenetical traits. This means that AOA are potentially important players in WWTPs to oxidize ammonium. Nonetheless, the information of AOA is still lacking. The aims of this research were to compare the abundance and sequences of bacteria and archaeal ammonia monooxygenase (*amoA*) genes in different wastewater treatment plants, which located in Thailand, Japan and United States of America (USA) and to survey significant parameters which could affect the abundance of *amoA* gene, responsible for ammonia oxidation to hydroxylamine which is known as the first oxidation step of biological nitrogen removal. Activated sludge samples were taken from three full-scale wastewater treatment plants. These samples were mainly analyzed by using molecular technique: quantitative real-time PCR (qPCR) and PCR-DGGE. The qPCR and PCR-DGGE were used to find the comparative abundance and identify sequences of AOB and AOA. The results from this study were found that the abundance and identify of AOB and AOA were elucidate in three different activated sludge systems. The number of AOA *amoA* genes was in every sample from Phuket, Thailand. The number of AOB *amoA* genes was the same number amount both Japan and USA. The main reason, AOA were found in Phuket wastewater treatment plant because there were high temperature, long retention time and low dissolved oxygen.

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Student's signature Thesis Advisor's signature

ACKNOWLEDGEMENTS

I would like to thank Associate Professor Pongsak Noophan, my major advisor, for giving me an opportunity to go to practice and study more technique from other Laboratories which are inside and outside country, for his guidance and patience. I also would like to thank Associate Professor Akihiko Terada for teaching the molecular technique, for his suggestion and guidance. Moreover, the special person whose I want to thank are Professor Chongrak Polprasert, Associate Professor Sanya Sirivithayapakorn, Ms.Nimaradee Boonapatcharoen, Ms.Maneerat Khemkhao and Ms.Chompoonut Auphimai (King Mongkut's University of Technology Thonburi), Ms.Areerat kunanopparat and Ms.Supaporn Phanwilai (Kasetsart University), I could not done my work if I did not have their help, guide, kindness and many things that they did for me.

I also would like to thank all of my many friends, especially Ms. Kanokporn Srisawan for helping me with my English, and people who I knew during my study in Thailand and Japan also. We shared every moment together and they helped me to pass the difficult situation.

I would like to thank my family for their love, support and giving me the motivation to go on and finish my work. I thank them for empowering me and thank for always being beside me.

Nutpornnapat Sinthusith

June 2014

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LIST OF ABBREVIATIONS

Λ	=	lambda
A_2O	=	the system which has aerobic t, anaerobic and anoxic tank
amoA	=	ammonia monooxygenase subunit A
amoB	=	ammonia monooxygenase subunit B
amoC	=	ammonia monooxygenase subunit C
AOA	=	ammonia-oxidizing archaea
AOB	=	ammonia-oxidizing bacteria
APS	=	ammonium persulfate
AS	=	activated sludge
BLAST	=	basic local alignment search tool
BOD	=	biochemical oxygen demand
DDBJ	=	DNA databank of Japan
dH_2O	=	distilled water
DNA	=	deoxyribonucleic acid
dNTP	=	deoxynucleotide-5'-triphosphate
DO	=	dissolved oxygen
EDTA	=	ethyl diamine tetraacetic acid
EMBL	=	european molecular biology laboratory
FASTA	=	program FAST-AII for search proteinand DNA sequence databases
F/M	=	food to microoganism Ratio
HRT	=	hydraulic retention time
K	=	kinetic value
MEGA	=	molecular evolutionary genetics analysis program
MLSS	=	mixed liquor suspended solids
NCBI	=	national center for biotechnology information
N. europaea	=	Nitrosomonas *europaea*
NH_4^+	=	ammonium
NO_2^-	=	nitrite
NO_3^-	=	nitrate
NOB	=	nitrite-oxidizing bacteria
PCR	=	polymerase chain reaction

LIST OF ABBREVIATIONS (Continued)

qPCR	=	quantitative real-time polymerase chain reaction
R	=	raw score
RNA	=	ribonucleic acid
SBR	=	sequencing batch reactor
SRT	=	sludge retention time
TAE Buffer	=	tris-acetic disodium EDTA
Temp.	=	temperature
TKN	=	total kejldahl nitrogen
TMED	=	N,N,N^4,N^4- tetramethylethylene-diamine
TN	=	total nitrogen
UV	=	ultra violet
WWTP	=	wastewater treatment plant

IDENTIFICATION AND QUANTIFICATION OF BACTERIA AND ARCHAEA RESPONSIBLE FOR AMMONIA OXIDATION IN DIFFERENT ACTIVATED SLUDGE SYSTEM

INTRODUCTION

Water, a natural occurring substance, is one of the most important needs in living of humans, animals and plants. Nowadays, the world is facing a water pollution, which caused mainly by a continuous growing of world population, industrialization and poor water use strategies. This condition not only affects the global environment but also health of living things including human. One substrate that caused the pollution in the water is nitrogen. Although nitrogen is an important substrate for plants and living creatures to grow, high quantity of nitrogen discharge into a water source such as a lake, river, lagoon etc. will cause effects to living things in the water and also be the pollution same with phenomenal of Eutrophication in the lake or Red tide in the coast. Nitrogen can be discharged into the water in many forms such as organic carbon, ammonia, nitrite and nitrate. All of these forms also affect to water pollution and for this reason, nitrogen from wastewater would be removed before discharge to the environment. Biological nitrogen removal (BNR) would be commended to use in order to remove nitrogen because there are many advantages when comparison with physical and chemical treatments. For example, BNR could be used less chemical, low energy consumption, and medium production of waste solids. Nitrification and denitrification process are interesting approaches to remove nitrogen because it has lower cost comparison with chemical treatment. However, the mechanisms of bacteria and archaea whose plays the important role to remove nitrogen still have some mysterious which waiting for discover.

In engineered systems such as wastewater treatment plants (WWTPs) and constructed wetland, nitrogen is biological removal by nitrification/denitrification or partial ammonia oxidation and anammox (Strous *et al.*, 1999). WWTPs employ bioreactors to prevent nitrogen discharges into the water bodies, avoiding occurrence of algae blooms and oxygen depletion (U.S. EPA., 1993). Nitrification, conversion of ammonia (NH_3) to nitrite (NO_2^-) by ammonia-oxidizing microorganisms and nitrite via nitrate (NO_3^-) by nitrite-oxidizing bacteria (NOB), is rate-limiting for nitrogen

removal (You *et al*., 2009), given WWTPs still an engineering challenge. Recently, progress of new molecular microbiological methods has paved the way for the remarkable discovery that autotrophic ammonia oxidation is not restricted by only the domain of bacteria such as aerobic ammonia-oxidizing bacteria (AOB) but also the domain of archaea within the kingdom *Crenarchaeota* in an aquatic system (Treusch *et al*., 2005; Tourna *et al*., 2008). *Candidatus Nitrosopumilus maritimus*, acquire energy for growth by aerobically oxidizing ammonia to nitrite and presents a similar growth rate and cell production of AOB under mesophilic conditions (Könneke *et al*., 2005). Biochemical and physiological studies on ammonia oxidizing archaea (AOA) have revealed the presence of putative genes for all three subunits (*amoA*, *amoB*, and *amoC*) of ammonia monooxygenase, the key enzyme for ammonia oxidation (Könneke *et al*., 2005), the maximum growth rate (e.g. 0.78 day^{-1}) comparable to that of AOB in WWTPs (You *et al*., 2009), and very high affinities for ammonia because of their low-permeability membranes and specific catabolic pathway (Valentine *et al*., 2007; Marten-Habbena *et al*., 2009). In WWTPs, the first time AOA were found in activated sludge sample: five of nine WWTPs in the US which nitrification was active (Park *et al*., 2006), two WWTPs in China (Zhang *et al*., 2009) and four municipal WWTPs in Thailand (Limpiyakorn *et al*., 2011). Although the recent discoveries on AOA shed light on potential AOA application to nitrogen removal in WWTPs, phylogenetic and physical information for the application to WWTPs are still elusive.

Given the context, the objectives of this study were to phylogenetically identify and quantify bacterial and archaeal functional genes encoding for ammonia oxidation (*amoA*) in activated sludge WWTPs which have employed different environmental and operational conditions. Three WWTPs, in Thailand, Japan and USA were subject for the comparison.

OBJECTIVES

1. To phylogenetically identify and quantify bacteria and archaea functional genes encoding for ammonia oxidation (*amoA*) in activated sludge WWTPs which have employed the same operational conditions.

2. To compare the amount and types of bacteria and archaea *amoA* genes in wastewater treatment system which has nitrogen removal process in Phuket, Thailand, Plum, Creek USA and Miyagi, Japan.

Hypothesis

1. In each full-scale wastewater treatment system which operated by the same system and conditions have different identity and quantity of bacterial and archaeal *amoA* genes.

2. Tempereture, dissolved oxygen (DO), ammonia concentration (NH_4^+), hydraulic retention times (HRT) and pH have effects to the abundance of bacterial and archaeal *amoA* genes in full-scale wastewater treatment systems.

LITERATURE REVIEW

Nowadays one problem which needs to be solve is the problem of wastewater because of the high growth of community and the development of industries. Wastewater can happens by all of human activities. So, the wastewater needs to be treat before discharge to the nature water source. The source of wastewater can separate in 3 types; municipal, industrial and agricultural wastewaters. In this study will focus on municipal wastewater which treated by activated sludge system.

The wastewater treatment process can separate into 2 types, There are aerobic and anaerobic processes, which used microorganism for organic compound degradation. In aerobic process, microorganism will use oxygen as an electron acceptor and to degrade organic compound to produce carbon dioxide and water. For anaerobic process, microorganism no need oxygen to degrade organic compound to produce carbon dioxide and methane, the anoxic condition also the same with anaerobic condition, but for anoxic condition or anaerobic denitrification will change nitrate to nitrogen gas by microorganism under the conditions which do not have free oxygen.

Activated Sludge System (AS)

Activated sludge is a biological wastewater treatment process which utilizes a fluidize to mix growth of microorganism under aerobic condition to remove the substrate in wastewater by microbial respiration and for synthesis of new microbial cells. The microorganism will condense together and settle down, the weight of sludge are heavier than the wastewater, and gas carbon dioxide will evaporate into the air. The main units of this system consist of aeration tanks which have an oxygen supply in the bioreactor basin, the final clarifier or solid-liquid separator, and the recycle sludge process (Department of industrial work and environmental engineering of Kasetsart University, 2011; Reynolds, T.D. and Richards, P.A., 1996). The suspended growth activated sludge system, was improved and modified.

1. Conventional activated sludge system has sedimentation tank and aeration tank. Hydraulic retention times in aeration tank were 4 to 8 hours and the recycle ratio (R/Q) were 0.25-1.0 (Reynolds, T.D. and Richards, P.A., 1996). The design of aeration tank should be the plug flow because the plug flow will inhibit the filamentous bacteria better than complete mixed system. The advantage of plug flow is about the constant volume of air through the tank and disadvantage of this system is cannot mixed the complete one (Department of industrial work and environmental engineering of Kasetsart University, 2011), see in Figure 1.

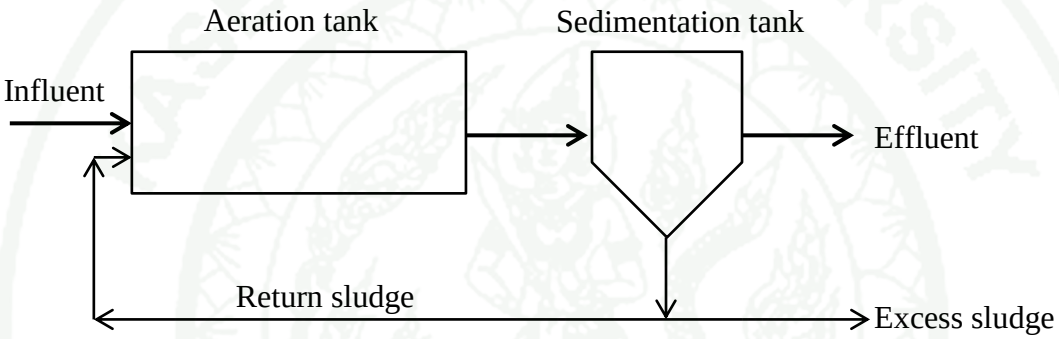

Figure 1 Conventional Activated Sludge

Source: Adapted from Department of industrial work and environmental engineering of Kasetsart University (2011).

2. Complete Mixed Activated Sludge. It was developed from conventional activated sludge. This system can give the equal air in every point of the tank and this system also apply to treat the wastewater which has toxic contaminated from industries, but the volume of toxic should be a few concentrated and do not have effect to the microorganism in the system (Department of industrial work and environmental engineering of Kasetsart University, 2011), see in Figure 2.

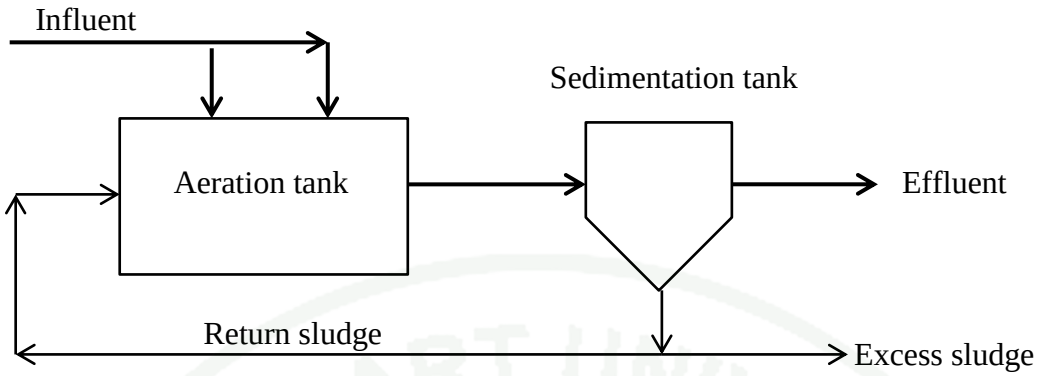

Figure 2 Complete Mixed Activated Sludge

Source: Adapted from Department of industrial work and environmental engineering of Kasetsart University (2011).

3. Extended Aeration Activated Sludge has longer hydraulic retention times more than other systems. The aim of this system is reducing the food to microbe ratio (F/M) to produce a few excess sludge should be 0.05 to 0.15 (Reynolds, T.D. and Richards, P.A., 1996). Sludge retention times around 15 to 30 days (Department of industrial work and environmental engineering of Kasetsart University, 2011), see in Figure 3.

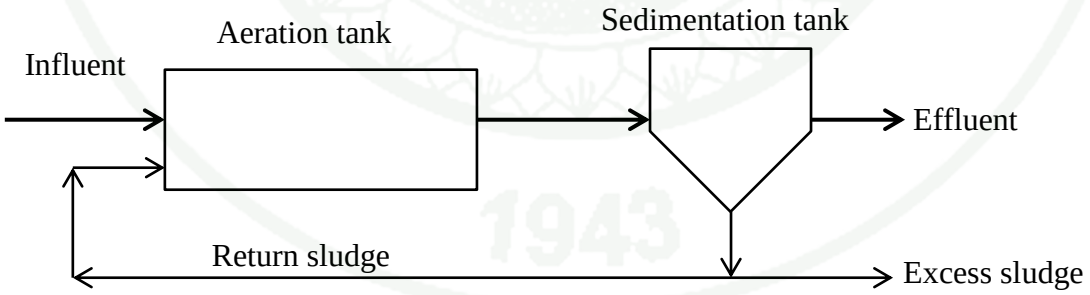

Figure 3 Extended Aeration Activated Sludge

Source: Adapted from Department of industrial work and environmental engineering of Kasetsart University (2011).

4. Oxidation Ditch is the system which has the shape of aeration tank like an ellipse. Because of the ellipse shape, the wastewater can flow around and can modify by adding the anoxic zone inside for nitrogen removal, see in Figure 4.

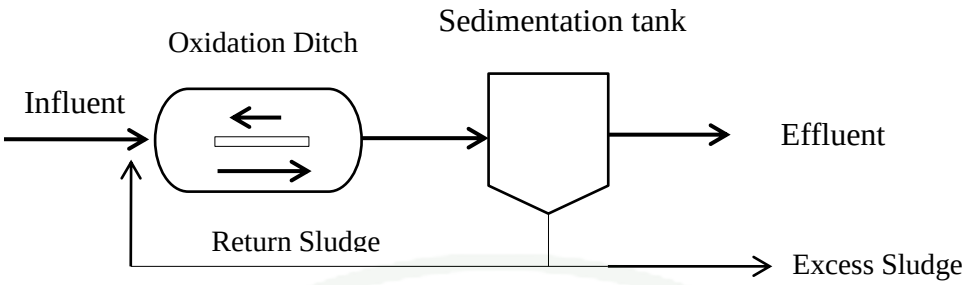

Figure 4 Oxidation Ditch

Source: Adapted from Department of industrial work and environmental engineering of Kasetsart University (2011).

5. Contact Stabilization Activated Sludge compound with stabilization tank and contact tank. Stabilization tank will take times around 2 to 4 hours for organic compound degradation inside the cells by adding the air (Department of industrial work and environmental engineering of Kasetsart University, 2011). The wastewater will flow into the contact tank, the retention times in this tank around 30 to 60 minutes (Department of industrial work and environmental engineering of Kasetsart University, 2011). Microorganisms which move from stabilization tank to contact tank will have high efficiency to degrade and adsorb the organic substrate into their cells. This system can operate under higher organic loading and the organic loading variation, see in Figure 5.

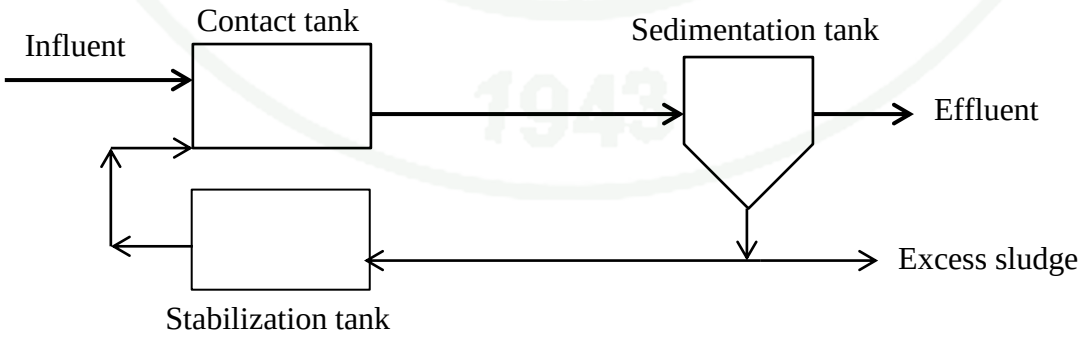

Figure 5 Contact Stabilization Activated Sludge

Source: Adapted from Department of industrial work and environmental engineering of Kasetsart University (2011).

Nitrogen and relations with wastewater

Nitrogen is the main substrate which important for plants, animals and human, play the role in terms of the main component in proteins and nucleic acid in the cell of living thing. Although in the atmosphere has 79% of nitrogen, but in the soils and water have few amount of the nitrogen. Status of nitrogen is very constant and not easy to transfer. So, microorganisms play the very important role for transfering nitrogen to ammonium under "Ammonification" process which transfers ammonia to nitrite via nitrate. Nitrogen cycle is shown in Figure 6. Most of municipal wastewater has organic nitrogen and ammonium which call "Total Kejldahl Nitrogen" or TKN. In aerobic conditions, bacteria can take oxygen to oxidize organic carbon to carbon dioxide and water, which organic carbon is electron donor and oxygen is the last electron acceptor. Organic nitrogen must pass ammonification process for change nitrogen to NH_4^+, such as ammonium chloride or ammonium sulfate or free ammonia (NH_3) which oxidized by nitrifying bacteria. Nitrogen in terms of organic nitrogen and ammonium, both will oxidize to nitrite via nitrate under aerobic condition by using oxygen as electron acceptor and ammonium as electron donor. The majority of estimates about the role of nitrification are based on measurements of key compounds within the nitrogen cycle. However, such measurements do not reflect the dynamic of nitrogen transformations, so the best is providing a net result. The nitrate levels are not indicative of nitrification levels owing to nitrate uptake by plants, microbial turnover, immobilization, and numerous other factors (Kowalchuk and Stephen, 2001).

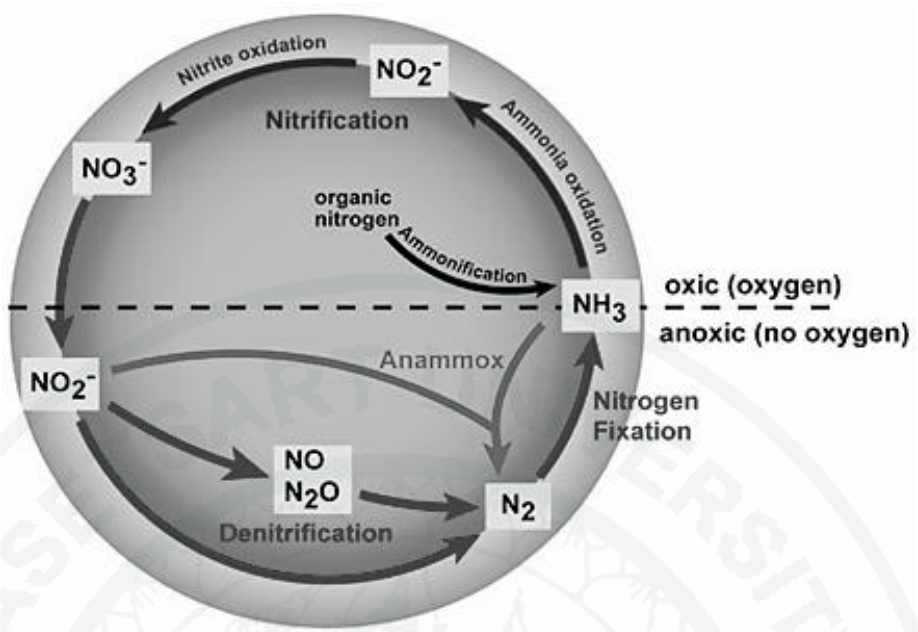

Figure 6 Nitrogen cycle

Source: Nature Education. (2010)

1. The importance of nitrogen.

1.1. Substrate for living things. Nitrogen is the main component of proteins and nucleic acid in cells. In the biological wastewater treatment process, nitrogen is necessary for the microorganisms in system for growth. In addition, nitrogen is the main substrate for algae to uptake for growth. If high amount of nitrogen was discharged into rivers the algae would cause the environmental problem, such as reduce the oxygen demand on water, odors etc., which calls "Eutrophication".

1.2. Water quality. Most of nitrogen presents in terms of organic nitrogen or ammonia nitrogen, which can transform to nitrite via nitrate underneath aerobic conditions. For the water that simply began to dirty, can find nitrogen in term of organic nitrogen and ammonia nitrogen. If nitrogen was founded in term of nitrate its mean that water was dirty for long times. Normally, organic nitrogen and ammonia always higher than a microorganism requirement for biological wastewater treatment process which microorganism needs oxygen 4.57 of nitrogen weight, if lack of oxygen the water can become to waste water.

1.3. Ammonia is an important molecule in the biogeochemical nitrogen cycle (Mancinelli and Mckay., 1988). The mainly of ammonia, which released into the environment is from the decay of organic matter or from agriculture by use fertilizer ammonia and serves as a nitrogen supply to plants and microorganisms. Ammonia will be toxic to fish, but fish can survive if ammonia transforms into ammonium ion which control of pH. If ammonia concentration equal 0.2 mg/l or higher, it will be dangerous for fish. The relation of pH and nitrogen transform is shown in Figure 7.

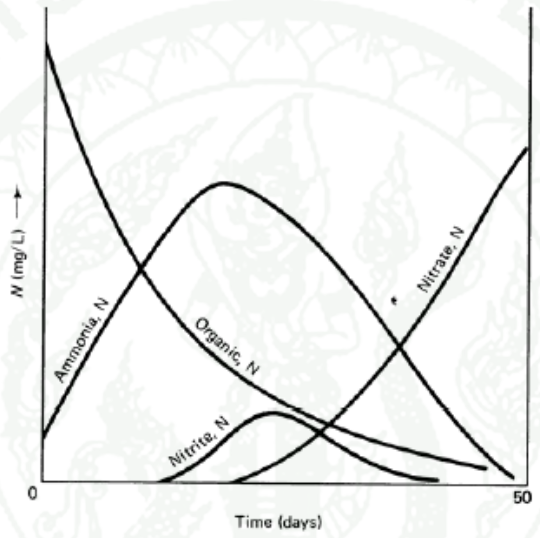

Figure 7 Transform of nitrogen

Source: King Mongkut's University of Technology Thonburi. (2013)

1.4. Oxidation of nitrogen in the water source. Oxidation of nitrogen happens by autotrophic nitrifying bacteria under aerobic condition. So, the wastewater which contains ammonia will have effects to reduce oxygen in the water source.

2. Nitrification and Denitrification Processes

Nitrification process has two parts. First is nitritation or nitrification, ammonia oxidizing bacteria (AOB), such as *N. europaea* and *N. oligocarbogenes* etc., which can be oxidized ammonium to nitrite. Second is nitration or nitratification, nitrite oxidizing bacteria (NOB) which can be oxidized nitrite to nitrate (Ward, B.B., 2011). However, wastewater treatment system always occurs un-completely nitrification because some

organic nitrogen cannot degradation. Parameters which have an effect to nitrification are: substrate, temperature, oxygen, pH, salinity, sludge age and bane. Denitrification is the process to reduce nitrite to nitrogen gas by microorganism. When nitrogen was transforming to nitrate, it can remove from system in two ways. First, assimilation denitrification, bacteria need nitrogen for protein synthesis. Second, dissimilatory denitrification, nitrifying bacteria play the role to both heterotroph and autotroph in anoxic condition. A very important role of conventional denitrification is the continued degradation of organic carbon in the absence of oxygen. Characteristics of the general physiology of nitrifiers, such as an obligate requirement for oxygen to oxidize ammonia, tolerance very low oxygen and sensitivity to inhibition of light were observed in natural systems and verified in culture.

Biological nitrogen removal

As known, nitrogen bacteria are autotroph which grow slower than heterotroph which use organic carbon as a nutrient. Autotroph can grow under the condition which less of organic carbon, then autotrophic bacteria will change the form of reduced nitrogen to nitrite via nitrate under the aerobic condition and then the heterotrophic bacteria will reduce nitrate to nitrogen gas or other gas, in this point is nitrogen removal.

The nitrogen removal system

1. Nitrogen removal system that has three tanks of sludge. This pattern has three processes. The first one is heterotroph's tank, this tank is only for growth of the heterotrophic bacteria under aerobic condition for removing organic carbon follow by 1st sedimentation tank. The second one is autotropic bacteria, this tank will grow only autotroph under aerobic condition for nitrification process and also follow by a 2nd sedimentation tank. The last one is the anaerobic tank for heterotrophic bacteria by work in terms of denitrification process and follow by a 3rd sedimentation tank. At the end of every tank usually have a sedimentation tank for return the sludge into each tank one by one. However, this system is closely a top ideal system, but it has many disadvantage which are constructed with high cost, need the complete mix between the air and wastewater, at the last tank need to add the outsource of organic carbon because of the last one cannot produce

enough sludge and this system need high energy to operate (Phannasawat, T., 2002), see in Figure 8.

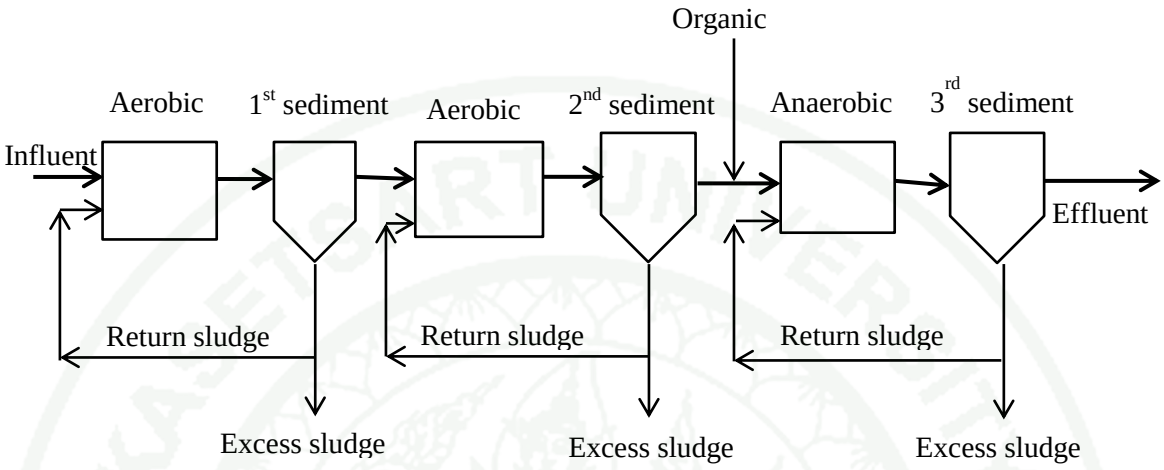

Figure 8 Remove nitrogen by 3 tanks of sludge

Source: Adapted from Phannasawat, T. (2002)

2. Post DN. This system has only two tanks which are aerobic and anaerobic tank. In the aerobic tank can grow both autotrophic and heterotrophic bacteria because the autotrophic and heterotrophic bacteria use the different carbon source as the substrate. By the way, in this aerobic tank should have enough oxygen and have longer sludge retention time. This system was designed by using 2 sets of processes which combine with sludge tank and sedimentation tank. The first step is aerobic tank, in this tank will oxidize both carbon and nitrogen and the high nitrate effluent from this tank will flow through the second one. The second tank which is an anaerobic tank will change nitrate to nitrogen gas (Phannasawat, T., 2002), see in Figure 9.

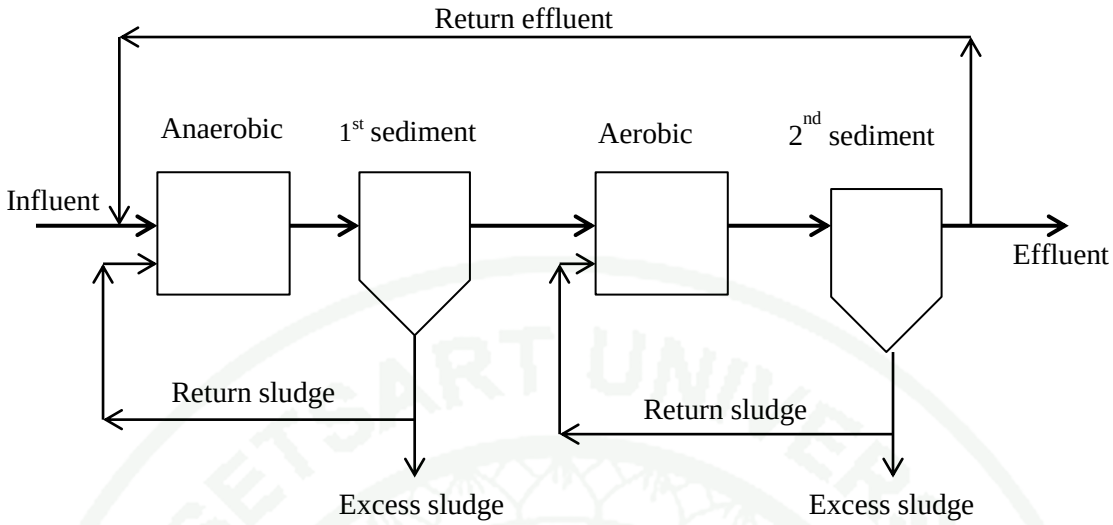

Figure 9 Post DN

Source: Adapted from Phannasawat, T. (2002)

3. Modified Ludzack-Ettinger (MLE) process. MLE is the most commonly used biological nutrient removal process in MBR. This system was designed by an aerobic tank after anoxic tank. The advantage of this system is no need to add the carbon which from outsourced, controlled pH by itself, need only small amount of air and the sludge settling well, higher MLSS in anoxic tank and simpler flow line (Phannasawat, T., 2002). The system is shown in Figure 10.

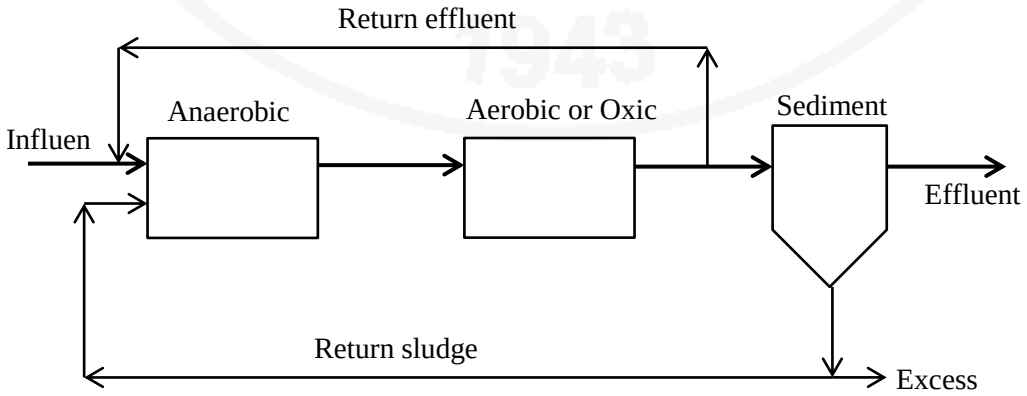

Figure 10 Modified Ludzack-Ettinger (MLE) process.

Source: Adapted from Phannasawat, T. (2002)

4. Sequencing Batch Reactor (SBR). This system can remove nitrogen by building the aerobic and anaerobic conditions in the same reactor by control sludge age, anoxic time, aerobic time, the volume of air, the level of wastewater in the reactor and sludge concentration (Department of industrial work and environmental engineering of Kasetsart University, 2011).

5. Oxidation Ditch. This system can be modified for nitrogen removal by control the volume of air. In the shape of oxidation ditch, it easy to separate aeration zone and anoxic zone, in aerobic zone will have high DO concentration by an air pump and the wastewater will flow through the part of anoxic zone by the strict way of oxidation ditch's shape. In the anoxic zone, DO concentration will reduce by heterotrophic bacteria and autotrophic bacteria. This system has high efficiency for nitrogen removal (Phannasawat, T., 2002), see in Figure 11.

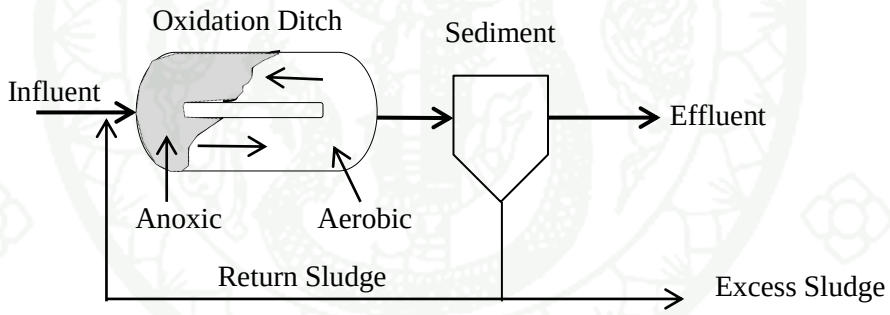

Figure 11 Oxidation Ditch

Source: Adapted from Phannasawat, T. (2002)

6. Orbal Sim-pre system is improved from oxidation ditch. It is a complete mix, looped reactor system. This system has many advantage such as process adaptability, can easily be expand for future load condition by adding on additional aerator to the existing channels or by adding on an additional channel to the existing channels, easy to modified to meet a wide assortment of influent conditions and effluent requirements, easy to maintenance, can operate easily because the characteristics of this system are a complete mix and able to operate under high MLSS concentrations. The last one is energy saving because this process requires less power to operate which means oxygen requirement (DO) for this system which is near zero. This system was designed by add 2 more loops inside the

oxidation ditch. Influent will flow through the external loop, anoxic zone, then flow to middle loop which is aerobic zone and the last loop is aerobic also (Siemens Water Technology, 2008; Phannasawat, T., 2002). This system can remove nitrogen more than 95 %, see in Figure 12.

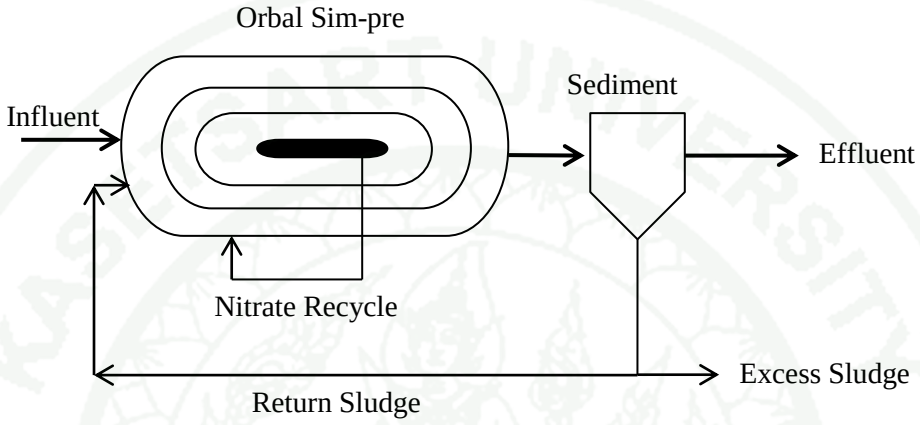

Figure 12 Orbal Sim-pre system

Source: Adapted from Siemens Water Technology. (2008)

7. Bio-denitro™ system. This system also developed from oxidation ditch and combined with SBR for nitrogen removal. The Bio-denitro™ consists of two oxidation ditch tanks which operated by sequentially alternating the flow and process conditions. So, do not need the balancing tank. The advantages of this system are minimum operating cost, flexible with the combines functional process design, highly adaptable operation, and high capacity volume. Each oxidation tank has aerator and a mixer, for control the aerobic or anoxic condition. This system start at oxidation ditch number 1 which receive a high nitrate concentration in influent, denitrification process are appear because of the organic carbon in the influent, now the first tank is anoxic condition. When operated with anoxic condition, it will open only the mixer for complete mix. Then the effluent which has low nitrogen and Biochemical oxygen demand (BOD) from the first tank will flow through the second tank. In tank II oxygen is introduced (aerobic conditions) leading to a biological degradation of the remaining organic matter and conversion of the wastewater flows from tank II to the sedimentation tank. After that, the second oxidation ditch will start by receiving the influent. The wastewater is lead to tank number II, which is aerobic conditions and oxygen is also introduced into tank I. This step is to reduce the content of ammonia in tank I before the

wastewater is discharged from this tank. The last steps correspond to two previous steps, except for the fact that the influent, effluent and the process condition are shuffled. Once this step is complete, the operation cycle will start the same loop again, see in Figure 13.

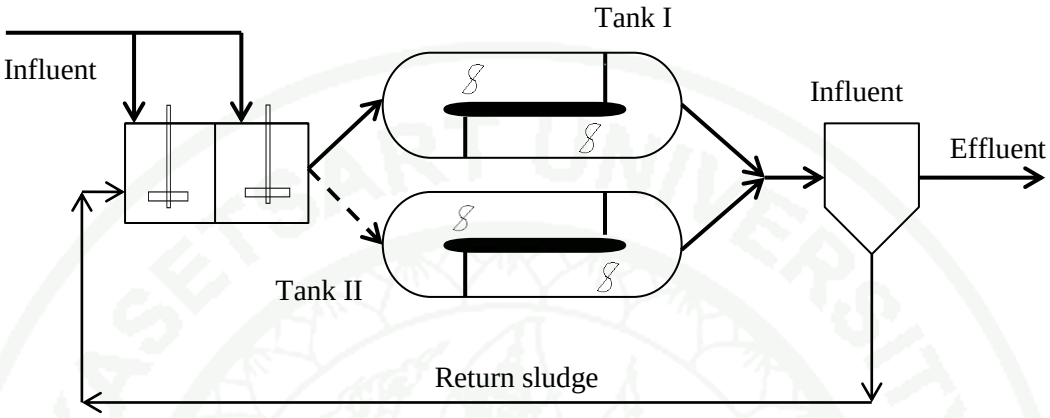

Figure 13 Bio-denitro™ system.

Source: Adapted from Krüger. (2009)

Ammonia oxidizing bacteria (AOB) and ammonia oxidizing archaea (AOA)

Cells of all living things have 2 types, which are prokaryote and eukaryote. Prokaryotic cells are single celled organisms and lack of nucleus. Prokaryotic cells are divided into the domains of bacteria and archaea. Plants, animals, protozoa and algae are eukaryotic. Eukaryotic cells contain a nucleus and organelles inside the membrane.

Bacteria and archaea are divided into prokaryotes. The shapes and size of archaea are similar to bacteria, but bacteria and archaea are differents because of archaea have some characteristic similar to eukaryotes such as non-sensitive to antibiotics, cell wall do not have peptidoglycan, have three RNA polymerases, etc. Archaea can survive under extreme conditions and harsh environment such as hot spring, salt lakes, and marine. The differences between bacteria and archaea are:

1. Archaea's cell wall does not contain peptidoglycan but bacteria's cell wall made of peptidoglycan.

2. Archaea's cell membrane uses ether linked lipid which different from bacteria. Bacteria's cell membrane uses ester linked lipids.

3. Bacteria have only one RNA polymerase, but archaea have three RNA polymerase likes eukaryotes

4. About growth and reproduction. Archaea reproduce asexually by the process of binary fission, budding and fragmentation. But eubacteria have the unique ability to form spores to remain dominant over years.

5. Archaea can survive under extreme conditions.

6. The base "Thymine" cannot be found in tRNA.

7. Methanogenesis is unique to archaea.

8. One important thing which distinguish archaea from bacteria is the adaptation to chronic energy stress, low permeability membrane and catabolic pathways (You *et al.,* 2009).

In environment, archaea and bacteria work together in terms of fermentation which contains organic compounds such as produce methane from degradation, bacteria will change organic compounds to acitic acids and hydrogen gas and archaea will change acitic acids and hydrogen gas to methane.

Ammonia oxidizing bacteria (AOB)

Ammonia oxidation is the first step to reduce ammonia to nitrate. Ammonia oxidation is through to be the rate-limiting step for nitrification in most systems, as nitrite is gently found to accumulate in the environment. Ammonia oxidizing bacteria are highly important for turnover of inorganic nitrogen in wastewater treatment plants and other ecosystems which oxidize ammonium to nitrite are predominantly chemolithoautotrophs including *Nitrosomonas*, *Nitrosospira* and *Nitrosococcus* (You *et al*., 2009). These bacteria obtain energy by reducing nitrogen for growth, carbon dioxide as a carbon source by the fixation of CO_2 via the Calvin cycle and oxygen as an electron acceptor. AOB were found in marine ecosystem, soils, oxygen-deficient water columns, anoxic sediments and also have been studied in extreme environments (Junier *et al.,* 2010). Ammonia oxidizing bacteria can survive under starvation conditions. In engineer systems, *Nitrosospira* and *Nitrosomonas* were appearing under low DO concentration (Park *et al*., 2002). In addition, some have an anaerobic metabolism (Bock *et al*., 1995; Mulder *et al*., 1995).

Ammonia oxidizing archaea (AOA)

The domain of *Archaea* within the kingdom *Crenarchaeota* was found in an aquatic system (Treusch *et al*., 2005; Tourna *et al*., 2008). *Candidatus Nitrosopumilus maritimus*, acquire energy for growth by aerobically oxidizing ammonia to nitrite and presents a similar growth rate and cell production of AOB under mesophilic conditions (Könneke *et al*., 2005). Biochemical and physiological studies on AOA have revealed the presence of putative archaeal ammonia monooxygenase, the enzyme for ammonia oxidation (Könneke *et al*., 2005), the maximum growth rate (e.g. 0.78 day^{-1}) comparable to that of AOB in WWTPs (You *et al*., 2009), and very high affinities for ammonia because of their low-permeability membranes and specific catabolic pathway (Valentine *et al*., 2007; Marten-Habbena *et al*., 2009). Although, the recent discoveries on AOA shed light on potential AOA application of nitrogen removal. AOA was found in the wastewater treatment plants (WWTPs) in the previous studies such as in five of nine activated sludge systems in United states (Park *et al*., 2006), from two WWTPs, which are the WWTPs in Hongkong, Chaina (Zhang *et al*., 2009) and municipal WWTPs in Thailand (Limpiyakorn *et al*., 2011). These AOA were present and play the role in removing nitrogen from wastewater.

Quantitative Real-time Polymerase Chain Reaction (qPCR)

Over the past several years, qPCR become leading tools for detection and quantification of DNA or RNA. This technique has many advantages such as ability to monitor the progress of the PCR reaction all times as it occurs, to accurately measure the amount of amplicon at each cycle, an increases dynamic range of detection and can amplify and quantify in the single tube, eliminating post-PCR manipulations. In the qPCR, the amount of DNA is measured after each cycle via fluorescent dyes that yield increasing fluorescent signal in direct proportion to the number of PCR product amplicons generated. Fluorescent reporters used in qPCR include double-stranded DNA-binding dyes, or dye molecule attached to PCR primers or probes that hybridize with PCR product during amplification. In qPCR has three major steps that make up each cycle in a qPCR reaction. Generally, set at 40 cycles for running reactions. First, denaturation, used high temperature to melt double –stranded DNA into single strands and loosen the secondary structure in single-stranded DNA. The highest temperature that DNA polymerase can withstand, which is typically used. The denaturation time can be increased if template GC content is high.

Second, annealing, the complementary sequences have an opportunity to hybridize during annealing, so the temperature which used is based on the calculated melting temperature of the primers. The last one is called extension, the activity of DNA polymerase is optimal at 70 to 72°C and primes extension occurs at rates of up to 100 bases per second. This step can be combined with the 60°C when the amplicant in qPCR is small.

Polymerase Chain Reaction (PCR)

This technique was used for increase the quantity of the target DNA in the sample. PCR is DNA synthesis by using enzyme polymerase again and again to increase the amount of target DNA. The target DNA was fixed by the primers. The point of these techniques is the code of base of DNA which wants to increase. PCR has 3 steps to increase the target DNA. First, melting the double–strand of DNA to single strand by using high temperature and then fastly decrease temperature. Second, annealing step, this step for letting the primers to catch the aim DNA and the last step is primer extension. This primer extension step is to change the temperature to the suitable one for the working of enzyme polymerase and add the enzyme in the reaction, this enzyme will synthesis DNA again after the primer finished. After finished all of 3 steps, DNA molecule will be double increase.

MATERIALS AND METHODS

Materials

1. SsoFast™ EvaGreen® Supermix (USA). Master Mix for quantitative Real-time polymerase chain reaction (qPCR).
2. Taq DNA Polymerase (QIAGEN, USA). Chemical for amplify DNA of bacterial and Archaeal *amoA* genes.
3. PowerBiofilm™ DNA Isolation Kit (MO BIO laboratories, USA)
4. FavorPrep™ Gel/PCR Purification Kit (Favorgen Biotech Corporation, Taiwan)
5. Agarose gel
6. Forward primers
   6.1. Arch-amoAF (5'- STAATGGTCTGGCTTAGACG -3')
   6.2. amoA 1F (5'- GGGGTTTCTACTGGTGGT -3')
   6.3. CTO 189A/Bf (5'- GGAGRAAAGCAGGGGATCG -3')
   6.4. CTO 189CF (5'- GGAGGAAAGTAGGGGATCG -3')
7. Reverse primers
   7.1. Arch-amoAR ((5'- GCGGCCATCCATCTGTATGT-3')
   7.2. amoA 2R (5'- CCCCTCKGSAAAGCCTTCTTC -3')
   7.3. CTO 654r (5'- CTAGCYTTGTAGTTTCAAACGC-3')
8. Water with molecular biology grade
9. Deionized Formamide
10. Urea
11. 40% Acrylamide
12. 50% TAE Buffer
13. 10% Ammonium Persulfate (APS)
14. N,N,N^4,N^4- Tetramethylethylene-diamine (TMED)
15. Dye solution
16. 10×Loading Buffer
17. DNA Ladder 100 bp
18. SYBR Gold
19. Denaturing Gradient Gel Electrophoresis (DGGE) machine.

20. CFX96 Touch™ (Bio Rad, USA). Thermal cycler for quantification and amplification.
21. Vortex
22. Centrifuge
23. Fume hood
24. Pipets and Pipet tips
25. Low DNA binding tubes
26. Micro centrifuge tube
27. NanoDrop Spectrophotometer (Thermo Fisher Scientific Inc., USA.)
28. FASTA program
29. BLAST program
30. MEGA program

Methods

1. Sample Preparation

Samples were collected from three different municipal WWTPs, consists of Phuket, Plum Creek and Miyagi WWTPs. These three WWTPs are activated sludge systems which have nutrient removal. Description of each WWTPs is described in the following topics. All samples were taken by grab sampling.

1.1. Phuket municipal WWTP is shown in Figure 14. This wastewater treatment system is one of oxidation ditch, which well operated in Thailand. The location of this WWTP is Phuket Province. This plant was received all of municipal wastewater from Phuket Province and leachate from solid waste nearby. All wastewaters from household and leachate were flowed through the main collection pipe, which was connected to sump. Wastewater was collected and controlled the constant flow rate by sump, which similar to equalization tank. Then, wastewaters were flowed into oxidation ditch. There were two parts, anoxic and aerobic zone for remove biochemical oxygen demand (BOD) and nitrogen in terms of ammonium concentration in this oxidation ditch. Treated water were flowed to clarifier, oxidation pond and discharged to the river. Sludges from clarify were separated into two parts. First, sludge was returned to the system by return sludge line. Second, excess sludge was sent to sludge belt press before send to landfill. The flow rate of Phuket WWTP was 28,734.49 m^3/day, wastewater's temperature was around 29 to 30°C and pH was

controlled at 7 to 7.3 by pH controllers. Hydraulic retention time (HRT) and solid retention time (SRT) were 24 hours and 15 days, respectively. DO concentration in aerobic zone was 1.2 mg-O/L and in anoxic zone was 0.1mg-O/L. The BOD and ammonium removal efficiency were 97.86% and 45.15%, respectively. Samples were taken from oxidation ditch in the anoxic (a) and aerobic (b) zones on different months by grab sampling. Characteristics of this WWTP are shown in Table 1.

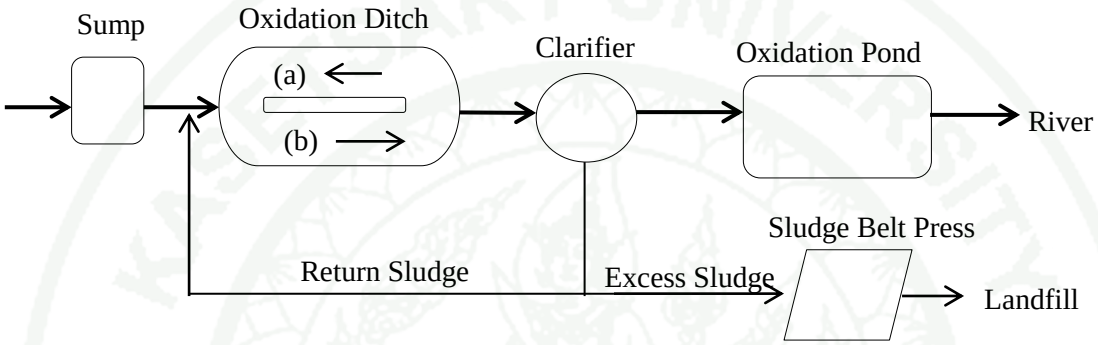

Figure 14 Phuket wastewater treatment plant.

1.2. Plum Creek wastewater treatment plant is shown in Figure 15. This wastewater treatment plant is oxidation ditch. The location of this WWTP is Colorado, USA. All of municipal wastewaters are flowed into this plant. The wastewaters are flowed through screener, grit chamber, primary sedimentation tank, and then flowed into oxidation ditch, respectively. The flow rate of Plum Creek WWTP was 24,000 m^3/day. Anaerobic chamber is installed in front of oxidation ditch. The oxidation ditch is contains aerobic and anoxic zones inside. Temperature of this plant was operated at 16°C, pH control were controlled at 7 by pH. HRT is 24 hours. Sludge samples were taken from aerobic (a) and anoxic (b) zones of oxidation ditch systems by grab sampling. After that, treated wastewaters are flowed to clarifier and flocculation tank. Alum was added into flocculation tank for separated effluent and sludges. After that, treated water are flowed to filtration, UV disinfection and discharge to the rivers. Sludges from clarifier are separated to return sludge line and excess sludge line. Excess sludge is sent to the sludge digester as an energy source for methane products. The BOD and ammonium removal efficiency were 98.91% and 97.91%, respectively. Others characteristics are shown in Table 1.

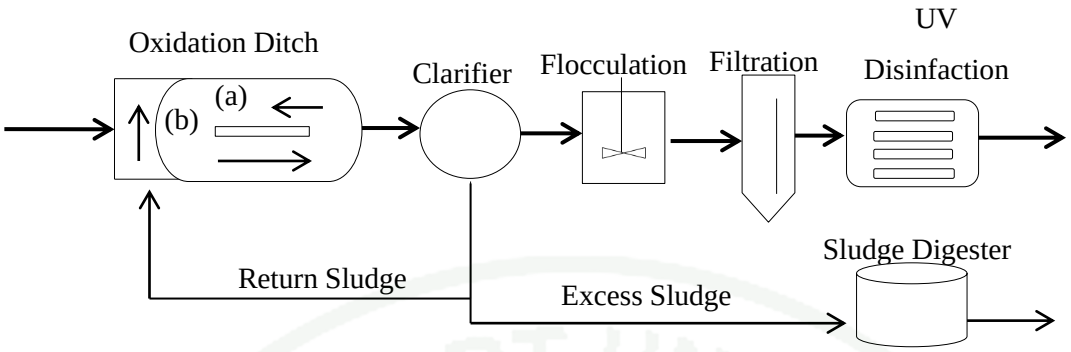

Figure 15 Plum Creek wastewater treatment plant.

1.3. Miyagi wastewater treatment plant is shown in Figure 16. This wastewater treatment is conventional activated sludge system, which modified for nitrogen and phosphorus removals. The location of this wastewater treatment plant is Tokyo, Japan. This plant was similar with normal conventional activated sludge, but the system was modified by installed anaerobic and anoxic zones before oxic zone. This WWTP could remove both nitrogen and phosphorus at the same time, this call A_2O system. The flow rate of Miyagi WWTP was 90,000 - 100,000 m^3/day. pH was controlled between 6 and 7. HRT and SRT were 8 and 6 days, respectively. The sludge samples were taken from aeration and anoxic tanks. BOD removal efficiencies in aerobic and anoxic tanks were 45.15% and 49.20%, respectively. Ammonium removal efficiencies in aerobic and anoxic tanks were 65.47% and 59.18%, respectively. Others characteristics are shown in Table 1.

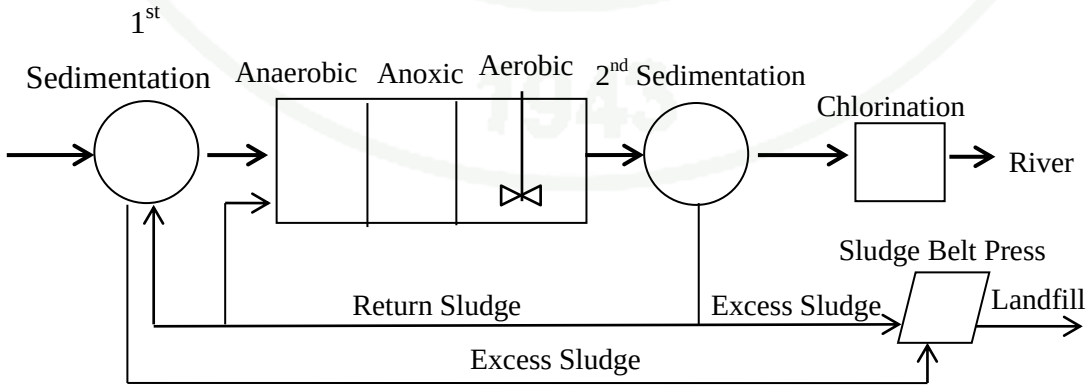

Figure 16 Miyagi wastewater treatment plant.

1.4. The characteristics of each WWTPs was from plant operators. Every parameter were approximate from three years in backward data, see in
Table 1.

Table 1 Characteristics of the WWTPs.

Parameter	**Unit**	**Wastewater treatment plants**				
		Thailand		**Japan**		**USA**
Treatment process		Oxidation Ditch		A_2O		Oxidation Ditch
		Aerobic	Anoxic	Conventional AS	Anoxic/ Oxic	
Flowrate	m^3/day	28,734		100,680	92,300	24,000
Temp.	◦C	30.20	29.80	22.10	21.80	16.00
pH		7.22	7.20	6.13	6.15	7.06
HRT	hours	24		8.3	7	24
SRT	days	15		5	5.8	N/A
DO	mg-O/l	1.20	0.10	2.37	1.40	0.46
BOD inf.	mg/l	174		110	110	336
BOD eff.	mg/l	4		8	2	4
NH_4^+ inf.	mg-N/l	15.88		15.90	15.90	35.80
NH_4^+ eff.	mg-N/l	8.71		5.49	6.49	0.75
NO_3^- eff.	mg-N/l	3.15		18.87	12.15	3.24
TN inf.	mg-N/l	25.33		26.50	24.70	N/A
MLSS	mg/l	7181		770	920	2330

Note: N/A, not available.

2. DNA Extraction

DNA was extracted from 1.5 mL of mixed liquor suspended solid from each activated sludge sample and centrifuged at 13,000 × g for 1 minute. Remove the supernatant and kept the pellet at -20◦C. DNA was extracted by using the PowerBiofilm™ DNA Isolation Kit (MO BIO laboratories, USA) followed by all of these steps. At first, take the stored samples from the refrigerator and remove excess liquid by using pipette tip. Add 350 µL of solution BF1, mix biofilm and solution, then transfer to the PowerBiofilm™ Bead Tube and add 100 µL of solution BF2, vortex to mix. Incubate at 65◦C for 5 minutes. After that, take PowerBiofilm™ Bead Tube to vortex at maximum speed for 10 minutes and centrifuge the tube at 13,000 × g for 1 minute at room temperature. Transfer the supernatant to a clean 2 mL Collection Tube. Add 100 µl of solution BF3 and vortex. Incubate at 4◦C for 5 minutes. Centrifuge at 13.000 × g for 1 minute at room temperature. Transfer the

supernatant to the clean tube and add 900 µL of solution BF4, then move to vortex to mix. Load supernatant into a Spin Filter and centrifuge at 13,000 × g for 1 minute. Discard the flow through and repeat again and again until empty of supernatant. Place the Spin Filter into the new Collection Tube. Add solution BF5, 650 µL, and centrifuge at 13,000 × g for 1 minute at room temperature. Then discard the flow through and add 650 µL of solution BF6 and centrifuge for 2 minutes. Place the Spin Filter into the new Collection Tube and add 100 µL of solution BF7 to the center of the filter. Centrifuge at 13,000 × g for 1 minute. DNA has now been in the flow through and keep at -20°C. Before extracting DNA, do not forget to wear the gloves and clean by 70% Alcohol every time. The summary process is shown in Figure 17.

3. Quantitative Real-time Polymerase chain reaction (qPCR)

Quantitative Real-Time PCR was performed with duplicate sets of extracted DNA to quantify the amount of bacteria and archaea *amoA* gene in each sample by using primers Arch-amoAF (5'- STA ATG GTC TGG CTT AGA CG -3') and Arch-amoAR (5'- GCG GCC ATC CAT CTG TAT GT -3') (Francis *et al*., 2005), primers amoA 1F (5'- GGG GTT TCT ACT GGT GGT -3') and amoA 2R (5'- CCC CTC KGS AAA GCC TTC TTC -3') (Rotthauwe *et al*., 1977) for archaea and bacteria *amoA* genes, respectively. The qPCR was run on a volume of 20 µL contained 10 µL of the qPCR master mix SsoFast™ EvaGreen® Supermix (USA), 1 µL of each primer (10 pM) and 5 µL of DNA template adjusted in 2 ng-DNA μL^{-1}. The qPCR mixes are shown in Table 2. In term of standard solutions of bacteria *amoA* genes was prepared by extracting DNA from *Nitrosomonas europaea* and amplified with primers amoA 1F-2R (Rotthauwe *et al*., 1977) and standard solution of archaea *amoA* genes was prepared by using *amoA* genes fragment of the archaea *amoA* clone AOA-S-4 (Limpiyakorn *et al*., 2011). The way to dilute products of standard solution is shown in Table 3. The initial high concentration of standard was diluted every 10 times until get the lowest concentration, which the qPCR machine can detect.

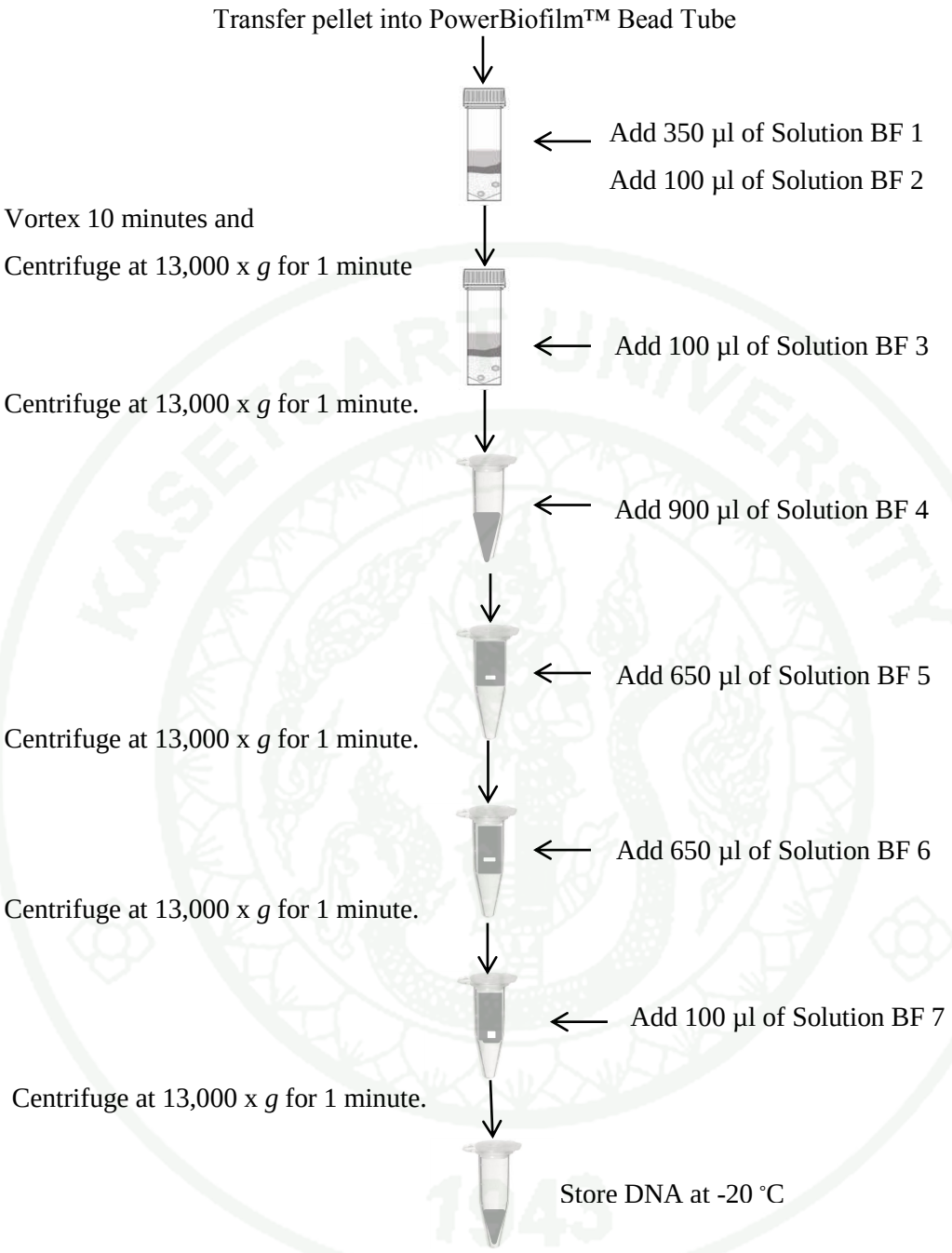

Figure 17 Protocal of PowerBiofilm™ DNA Isolation Kit.

Table 2 qPCR mixture.

No.	Mixer	µl / 1 reaction
1	Master mix	10
2	dH_2O	3
3	Forward primer	1
4	Reverse primer	1
5	DNA Template	5
	Total volume	20

Source: Adapted from Terada *et al.* (2010)

Table 3 Preparation of standard solution.

Dilution factor	Add. of template	Water to be added	Copies/5 µL
10	10	90	1.0×10^8
10	10	90	1.0×10^7
10	10	90	1.0×10^6
10	10	90	1.0×10^5
10	10	90	1.0×10^4
10	10	90	1.0×10^3
10	10	90	1.0×10^2

Source: Adapted from Rotthauwe *et al*. (1997)

Thermal conditions for 635-bp fragments of bacteria *amoA* genes were initiated by pre-heating at 94°C for 5 minutes, followed by 40 cycles of denaturation at 94°C for 1 minute, annealing at 56°C for 30 seconds, and elongation at 72°C for 30 seconds, with plate read for each cycle at 65°C for 5 seconds (Rotthauwe *et al*., 1977). For 495-bp of archaea *amoA* genes fragments, the qPCR conditions were using an initial enzyme activated step at 95°C for 5 minutes, followed by 40 cycles of denaturation at 94°C for 45 seconds, annealing at 56°C for1 minute, and elongation at 72°C for 1 minute, with plate read for each cycle at 65 °C for 5 seconds (Francis *et al*., 2005).

4. Polymerase chain reaction and Denaturing Gradient Gel Electrophoresis (PCR-DGGE)

For PCR-DGGE, the aim of this experiment to identify the types of bacteria and archaea *amoA* genes in each sample which from each point of each WWTP by using primers amoA 1F, amoA 2R-GC' and Arch-amoAF, Arch-amoAR-GC'. PCR primers were chosen to amplify the highly variable region of the 16s rDNA gene. The ammonia oxidizing bacteria (AOB) 16s rDNA gene was amplified by using primers CTO 189A/Bf (5'- GGA GRA AAG CAG GGG ATC G -3'), CTO 189 Cf (5'- GGA GGA AAG TAG GGG ATC G -3') and CTO 654r - GC' (5'-CTA GCY TTG TAG
TTT CAA ACG C -3') (Kowalchuck *et al*., 1997). The PCR mixture was prepared by using Taq DNA Polymerase (QIAGEN, USA), protocol is shown in Table 4.

Table 4 PCR mixtures.

Component	Final concentration	Volume (µl) / Reaction
10× CoralLoad PCR Buffer	1×	5
dNTP mix (10 mM of each)	200 µM of each dNTP	1
Forward primer	10 pM	0.5
Reverse primer	10 pM	0.5
Taq DNA Polymerase	2.5 units / reaction	0.25
RNAse free water	-	30.75
5× Q-Solution	1×	10
Template DNA	≤ 1 µg / reaction	2
Total reaction volume		50

Source: Adapted from protocol of Taq DNA Polymerase (QIAGEN, USA)

The PCR conditions were performed in a Thermal cycler. Condition for AOB were initiated by pre-heating at 93°C for 1 minute, followed by 35 cycles of denaturation at 92°C for 30 seconds, annealing at 57°C for 1 minute, elongation at 68°C for 45 seconds and cooling at 68 °C for 5 minutes (Kowalchuck *et al*., 1997). Bacteria *amoA* genes were amplified by using an initial enzyme activated step at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 56°C for 30 seconds, and elongation at 72°C for 30 seconds, with plate read for each cycle at 65°C for 5 seconds (Rotthauwe *et al*., 1977). For archaea *amoA* genes, PCR conditions were using an initial enzyme activated

step at 95°C for 5 minutes, followed by 34 cycles of denaturation at 94°C for 45 seconds, annealing at 56°C for 1 minute, and elongation at 72°C for 1 minute, with plate read for each cycle at 20°C for 5 seconds (Francis *et al*., 2005). Check the PCR products with 1% agarose gel by gel-electrophoresis, using 1×TAE buffer for run agarose gel in gel-electrophoresis. For the check, use a ladder lane with DNA Ladder 100 bp. Then, purified PCR products by FavorPrep™ Gel/PCR Purification Kit (Favorgen Biotech Corporation, Taiwan) and measure DNA concentration by NanoDrop Spectrophotometer (Thermo Fisher Scientific Inc., USA.).

Acrylamide gel was prepared follow by Table 5 and Figure 18, which 6.5% acrylamide for AOB and archaea *amoA* gene and 7.5% acrylamide for bacteria *amoA* gene.

Table 5 Preparation of acrylamide gel for DGGE gradient 30-55.

Component	**Final conc.**	**Stock 80%**		**Stock 0%**	
		6.5% Acrylamide	**7.5% Acrylamide**	**6.5% Acrylamide**	**7.5% Acrylamide**
40% Acrylamide	vary	2.4 ml	2.9 ml	2.1 ml	2.5 ml
50×TAE Buffer	1×	0.3 ml		3.9 ml	
dH_2O	-	Vary		vary	
Urea	5.6 M	5.05 g		-	
Formamide	32%	4.8 ml		-	
Total volume		15 ml		13 ml	

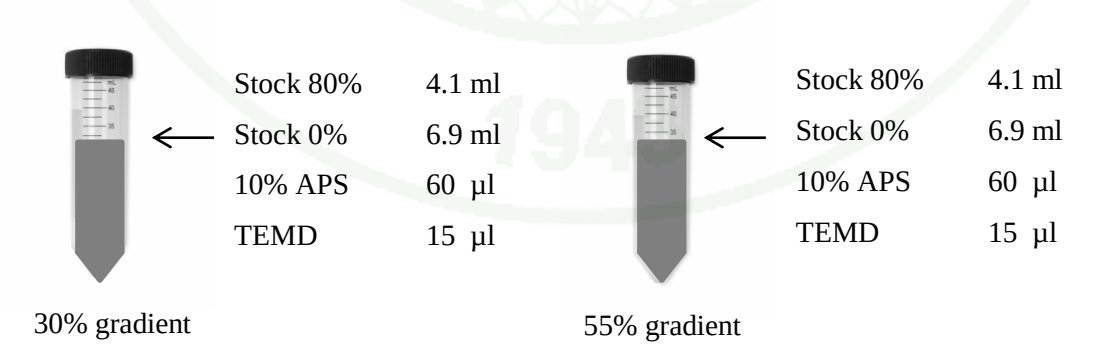

Figure 18 Acrylamide gel mixture

Mixed each sample with 10×Loading Buffer. Then, load each sample into each well of acrylamide gel. DGGE were run under conditions of 100 Volts, 60 mA for 16 hours in 1×TAE Buffer. After finished, took an acrylamide gel into SYBR Gold solution for 20 minutes. Cut bands as much as possible and eluted with 20 µL of dH_2O, stored at 4°C for 24 hours. After that, amplified each band again, purified each PCR products by using FavorPrep™ Gel/PCR Purification Kit (Favorgen Biotech Corporation, Taiwan) and sequencing, respectively. Nucleotide sequences were assembled and edited by using the FASTA program, version 35, and analyzed sequences by BLAST to identify the closest types of each band which appear in the sample. After that, create the phylogenetic tree by use MEGA program.

5. FASTA Program.

This FASTA is pronounced "FAST-A" using for search protein and DNA sequence databases. In this step, computers have become the tool in DNA analysis and protein sequence data. The FASTA program has many good points such as sensitive, fast, and readily available. These are the reasons why FASTA is the popular program for analysis the DNA and protein sequence data. FASTA is available as part of a package of programs that construct local and global sequence alignment. FASTA can compare either protein or DNA sequences. The DNA sequence comparison is suitable when comparing repeated sequence elements, transcription factor binding sites, or structural RNAs. The FASTA program work with many generally available formats. This study used the FASTA to analyze the purity of each sample and collected results which analyzed by using BLAST program. Figure 19 is the FASTA window which has 2 small windows inside, the first one is shown DNA sequence and second one is shown ABI chromatogram which the graph was showing about the purity of the results, in this picture the graph result can accept.

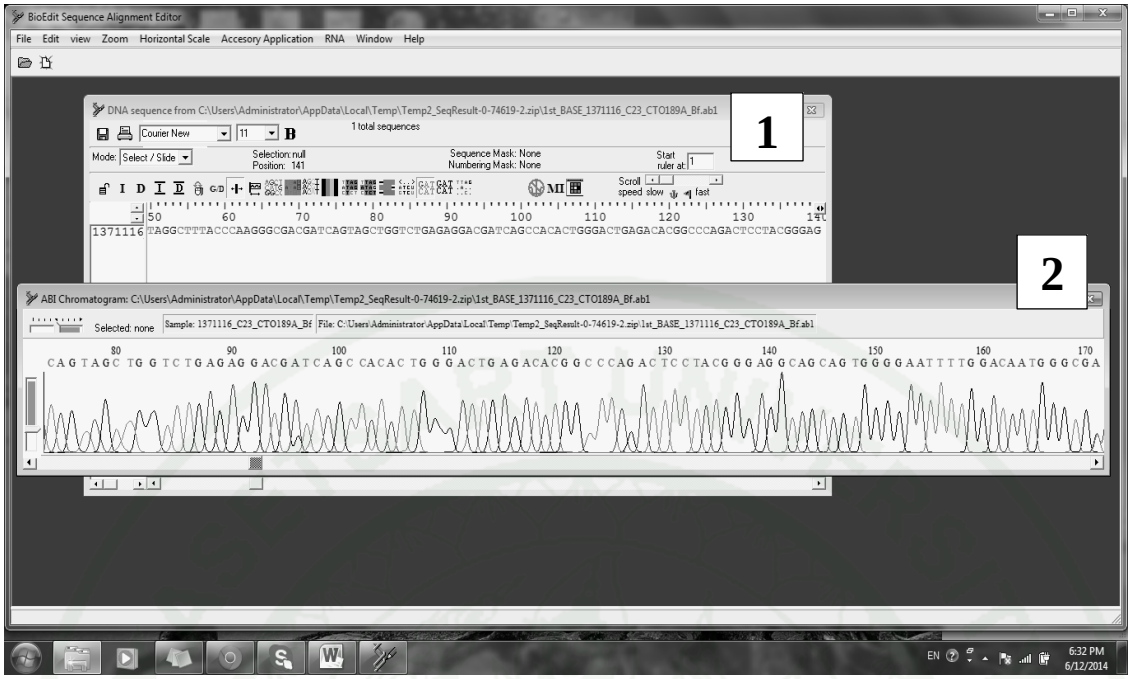

Figure 19 FASTA program.

6. BLAST

NCBI is the National Center for Biotechnology Information and BLAST is Basic Local Alignment Search Tool. NCBI collects a molecular biological public database and developed software tools for the user for analyzing their genomic data. For BLAST, can use in the BLAST website homepage. The sequence can be DNA, RNA, or an amino acid chain. The program will compare the sequence which the user submitted with the sequence in the database of the GenBank. GenBank is part of the International Nucleotide Sequence Database Collaboration, which consists of three organizations which always exchange the data daily. There are DNA DataBank of Japan (DDBJ), the European Molecular Biology Laboratory (EMBL), and GenBank at NCBI. The BLAST results will show the score and e-value of the alignment. The score will show the similarity between the sequence from the user and found sequence. In nucleotide BLAST, the score is calculated by using the raw score (R), lambda (λ) and kinetic value (K) followed by the equation 1 (Davidson College., 2008).

$$\text{Score (S)} = [\lambda \bullet R - \ln(K)] / \ln(2) \quad \ldots(1)$$

The e-value represents the amount of alignments you would expect to find by chance that have the same score as the alignment you are looking at. The e-value would not more than 0.05 for a good sign. E-value is calculated by equation 2 (Davidson College., 2008).

$$\text{e-value} = \text{query length} \times \text{length of the database} \times 2^{-S} \quad \ldots(2)$$

The input sequences are in FASTA or GenBank format and the output and delivered in a variety of formats such as FASTA, XML, ASN. 1, INSDSeq XML, ect.

7. Molecular Evolutionary Genetics Analysis Version 6.0 or MEGA6

This advanced version of the MEGA software was announced by Tamura *et al*. (2013). The MEGA software was improve continuously, now MEGA6.0 is the latest version, aim of this software is inferring the evolutionary patterns of genes, genome, and species over time by comparing the sequences of DNA and protein analysis. The MEGA software can be downloaded from www.megasoftware.net/reltime (Tamura *et al*., 2013). The MEGA can be used for larger sequences number, faster, shows the good performance in computer simulations, estimating divergence times, estimating rates of molecular evolutions and inferring ancestral sequences. The special things of MEGA 6.0 are adding the time tree system which based on the Real-time method for estimating the relative and absolute times of divergence for all branching points in a phylogeny, using the EvoD server for exploring the functional impact of non-synonymous single nucleotide variants, etc.

RESULTS AND DISCUSSIONS

From the experiments can separate the results into two parts: qPCR and PCR-DGGE.

1. Quantitative Real-time PCR results

The qPCR results from this study were found that the abundance of AOA and AOB *amoA* genes varied considerably among the samples (Table 1). Results from this study were found that the sample which from Japan and USA, the qPCR measurement could not be detected the archaea *amoA* gene because the numbers of AOA *amoA* gene in Japan and USA were lower than the detection limit as low as 1.00×10^2 copies/ng-DNA. On the other hand, the number of AOB *amoA* gene were three or four orders of magnitude higher than AOA *amoA* genes (Conventional AS: $3.69\times10^4 \pm 1.50\times10^3$, Anoxic/Oxic: $3.04\times10^3 \pm 2.19\times10^3$ in Japan and Oxic: $1.82\times10^4 \pm 5.57\times10^3$, Anoxic: $2.22\times10^4 \pm 6.42\times10^2$ in USA). Ammonia oxidation was conducted by AOB, but not AOA. However, the opposite trend was observed in the samples from Thailand, which located in Phuket's perch: the number of AOA *amoA* gene exceeded AOB *amoA* gene in every sample. Limpiyakorn *et al*., (2011) found AOA occurred in 4 municipal WWTPs in Thailand, but could not find in 3 industrial WWTPs by Real-time PCR. Park *et al.* (2006) also mentioned that AOA were presented in activated sludge and abundant in aerobic sludge. From gene sequence analysis indicates that AOA are related to methanogens in activated sludge and have low activities under anoxic conditions (You *et al.*, 2009). AOA could grow artificial aerobic condition with higher abundance in the moderate municipal wastewater (Bai *et al*., 2012). These results indicate that ammonia oxidation might not only be mediated by both AOB and AOA.

Table 6 Abundances of AOB and AOA *amoA* genes in activated sludge from full-scale WWTPs**.**

Sample	Gene copy number [copy/ng-DNA]	
	Bacteria *amoA* gene	**Archaea *amoA* gene**
Conventional AS, Japan	$3.69\times10^{4} \pm 1.50\times10^{3}$	N/A
Anoxic/Oxic, Japan	$3.04\times10^{3} \pm 2.19\times10^{3}$	N/A
Aerobic (6/2013), Thailand	$1.47\times10^{2}\pm1.23\times10^{1}$	$1.11\times10^{3} \pm 3.02\times10^{1}$
Anoxic(6/2013), Thailand	$1.19\times10^{2}\pm1.58\times10^{1}$	$8.62\times10^{2} \pm 1.48\times10^{2}$
Aerobic(27/2/2013), Thailand	$1.76\times10^{2}\pm1.56\times10^{1}$	$1.35\times10^{3} \pm 5.70\times10^{1}$
Anoxic(27/2/2013), Thailand	$2.68\times10^{2}\pm3.06\times10^{1}$	$2.20\times10^{3} \pm 6.37\times10^{1}$
Aerobic(19/12/2012), Thailand	$6.35\times10^{1}\pm2.3$	$1.54\times10^{3} \pm 3.04\times10^{1}$
Anoxic(19/12/2012), Thailand	7.52 ± 2.15	$2.69\times10^{1} \pm 1.39\times10^{1}$
Aerobic(18/1/2013), Thailand	$1.04\times10^{2}\pm7.89$	$2.35\times10^{3} \pm 7.34\times10^{1}$
Anoxic(18/1/2013), Thailand	$2.39\times10^{1}\pm5.4$	$2.99\times10^{2} \pm 1.95\times10^{1}$
Oxic1, USA.	$1.36\times10^{3}\pm3.68\times10^{1}$	N/A
Oxic2, USA.	$2.63\times10^{4}\pm3.18\times10^{3}$	N/A
Oxic3, USA.	$2.71\times10^{4}\pm1.35\times10^{4}$	N/A
Anoxic1, USA.	$1.88\times10^{4}\pm7.26\times10^{2}$	N/A
Anoxic2, USA.	$2.79\times10^{4}\pm5.50\times10^{2}$	N/A
Anoxic3, USA.	$2.00\times10^{4}\pm6.52\times10^{2}$	N/A

Note: N/A, non-detection limits.

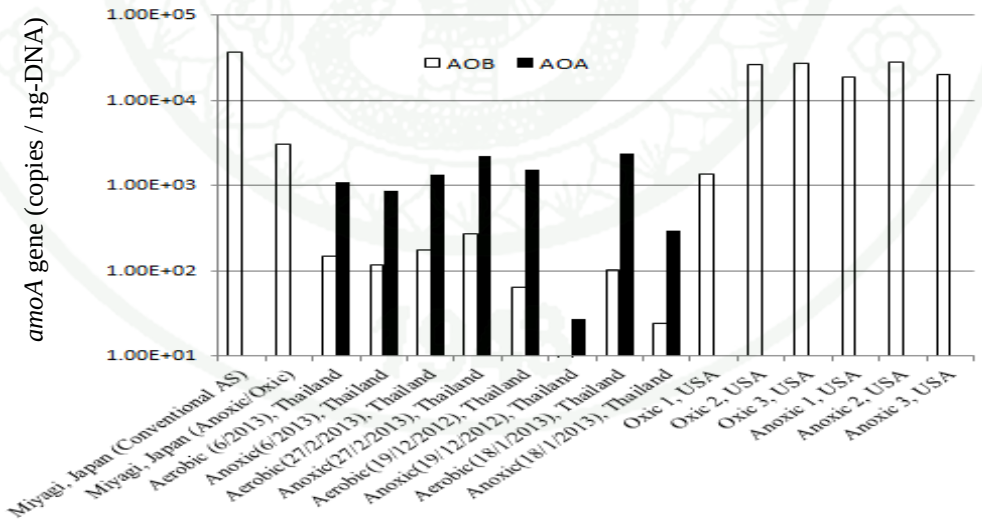

Figure 20 Abundances of AOB and AOA *amoA* genes in activated sludge from full scale WWTPs

2. PCR-DGGE results

PCR-DGGE results exhibited that every sample has the identical band derived from the same AOB *amoA* genes which all of the samples have the same pattern (Figure 21).

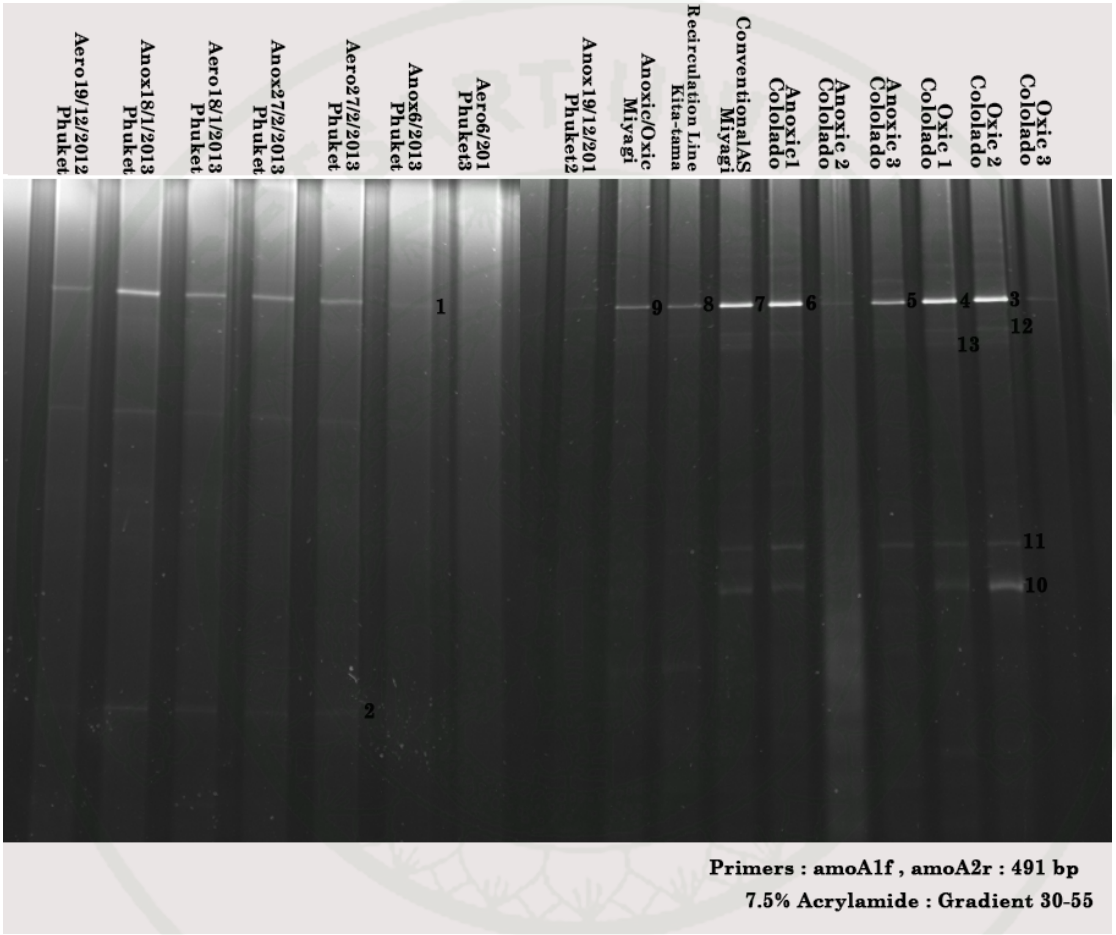

Figure 21 DGGE analysis of bacteria *amoA* gene in each sample.

The gene of sample's name: WWTP USA-B4, WWTP USA-B6, WWTP USA-B3 (3 OTUs), WWTP USA-B13, WWTP USA-B1 and WWTP Japan-B8 are close to uncultured bacterium *amoA* gene (FJ423002), which are found in full-scale municipal wastewater treatment plants by T-RFLP analysis of the diversity of ammonia-oxidizing bacteria (Wang and Wen, 2008), with phylogenetic identity by 99%. The sample: WWTP USA-B12 is closest to uncultured ammonia-oxidizing bacterium (KC967918), which are found in an influence in wastewater bioreactor which has the different inhibitors on the ammonia-oxidizing bacteria and archaea (Gao *et al*., 2013). The WWTP Thailand-B2 and WWTP USA-B11 were close to *Nitrosomonas aestuarii* (AF272404). This result is

confirmed by the study of Purkhold *et al*. (2000), which study about a phylogeny of all recognized species of ammonia-oxidizing based on comparative 16S rRNA and *amoA* sequence analysis. The closest of WWTP USA-B10 was uncultured ammonium-oxidizing bacterium (JF936545), which this sequence was found in the Dutch soils by Pereira *et* al. (2011). The relationships among bacteria *amoA* sequences from WWTP in Thailand, Japan and USA are shown in Figure 15.

The specific goal of WWTPs was an ammonia/ammonium treatment efficiency by using nitrification and to retain nitrifying biomass. *Nitrosomonas/Nitrosospira* species appear to dominate the natural and engineered system through the survey of 16S rRNA and *amoA* gene sequences from both cultures and environmental clones (You *et al.,* 2009; Withby *et al.,* 1999; Nold *et al.,* 2000; Park *et al.,* 2002). Norton (2011) assumed that WWTPs which have high level of ammonium usually have *N. europaea* and *N.eutropha. N. europaea* and *N.eutropha* could survive under high ammonium concentration in high nitrification rate system.

In concordance with the qPCR result, the DGGE band for AOA *amoA* gene appear in the sample from Thailand only, the patterns of all samples were similar (Figure 16). The closet gene (identity 99%) was uncultured *crenarchaeote amoA* gene (JX524541) which were found in a zero water exchange shrimp pond sediment (Aparajitha *et al*., 2012). The phylogenetic tree is shown in Figure 17. Additionally, as show in Table 1, the Phuket WWTP was operated with long retention time (HRT 24 hours and SRT 15 days) and low DO concentration (lower than 1.2 mg-O/l) under high temperature. These results were similar to the results from the study of Park *et al*. (2006).

Park *et al*. (2006) found AOA at low DO concentration (DO < 2 mg-O/L) and high ammonium concentration in 5 of 9 WWTPs in USA. The results of Park *et al.* (2006) were indicated that the presences of AOA appear to be depended upon the retention time (SRT > 15 days and HRT > 24 hours) and DO concentration, which were the factors to enrich AOA in WWTPs. The key factor, which had affected to the abundance of AOA and AOB, were wastewater characteristics. Especially, DO concentration and wastewater temperature and AOA could grow in aerobic environments, particularly in domestic wastewater, with high abundance (Bai *et al.*, 2012). On the other hand, the results of Limpiyakorn *et al.* (2011) were shown that all of Thailand municipal wastewater treatments,

which were found AOA in 4 municipal WWTPs in Thailand, were operated under low ammonium levels (< 11 mg-N/l).

Temperature was another parameter which could be affected to the abundance of AOA *amoA* genes in Thailand's WWTP. Urakawa *et al.* (2007) implied the temperature might be a key factor in the diversity and population structures of AOA and AOB in the aquarium biofiltration system. The diversity of both AOA and AOB in the aquarium were lower under cold water (5.5 ◦C). Reportedly, low temperature was likely an important factor controlling the growth and diversity of AOA in a WWTP. Essentially, reduces the diversity of AOA. Moreover, the activities of AOA might be higher than AOB. Erguder *et al.* (2009) also detected AOA *amoA* genes at very low (down to 0.2◦C) to high (up to 97◦C) temperatures.

The results from this study were implied that AOA might play the roles in the nitrification process under various conditions. Given these, longer SRTs in Thailand allowed AOA to grow, potentially leading to their out competition with AOB. For the WWTP in USA, the characteristics of wastewater were similar to Thailand's WWTP such as HRT (24 hours) and DO concentration (0.46 mg-O/L), which should have AOA, but cannot detect in this study. Furthermore, SRT and TN of WWTP in USA were unknown. So, the exact parameters which had affected to the abundance of AOA in USA's WWTP remain unclear. Even though, AOA could find in Phuket's WWTP, but AOB still played the dominant role in terms of nitrogen removal because of the high nitrogen removal efficiency in the WWTPs

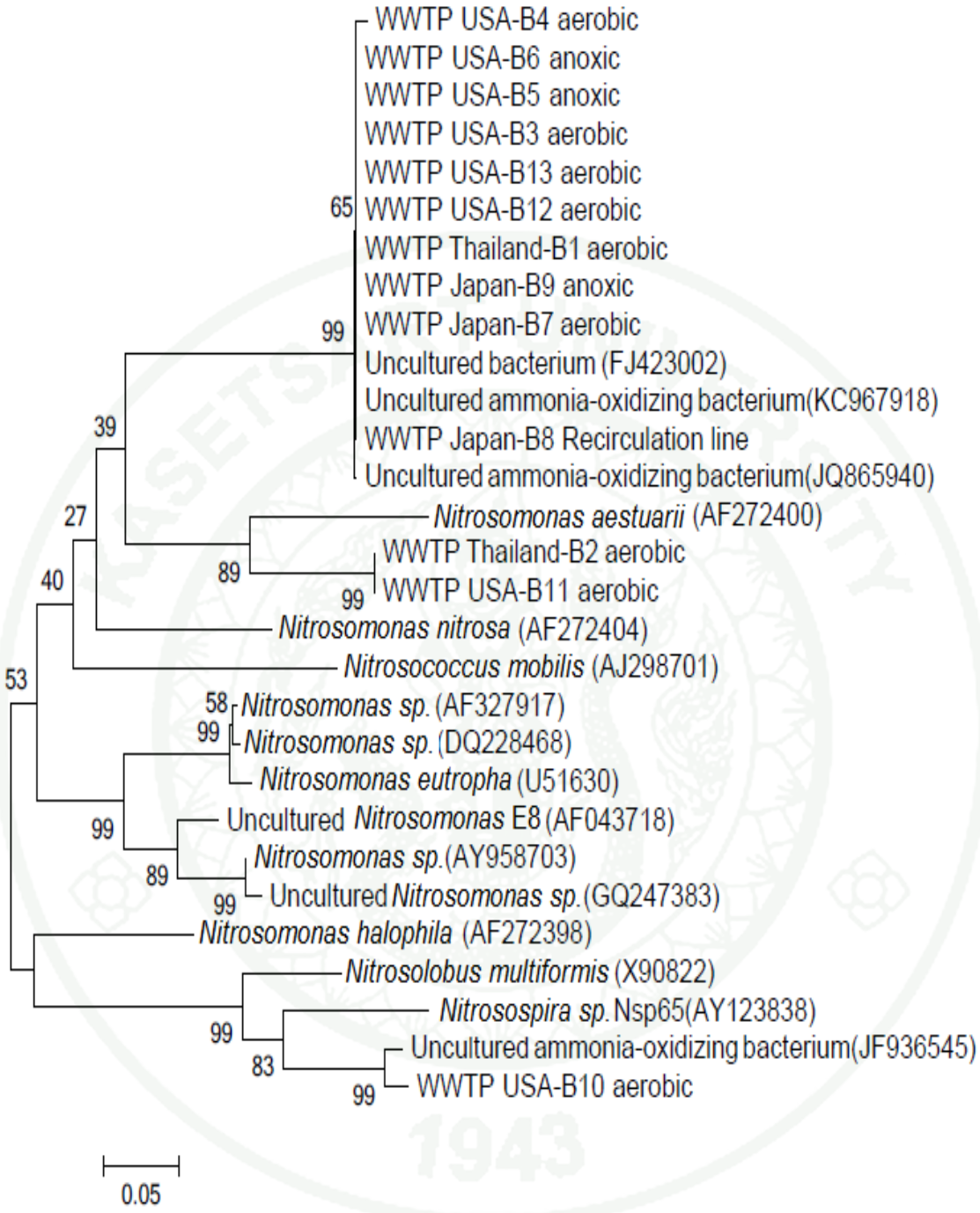

Figure 22 A neighbor-joining tree showing phylogenetic tree relationships among bacteria *amoA* sequences from WWTPs including Thailand (aerobic zone: B2 and anoxic zone B1), Japan(conventional activated sludge: B7 and anoxic/oxic zone: B9) and USA (oxic zone: B3, B4, B10, B11, B12,B13 and anoxic zone: B5). Bootstrap values are indicated at branch points.

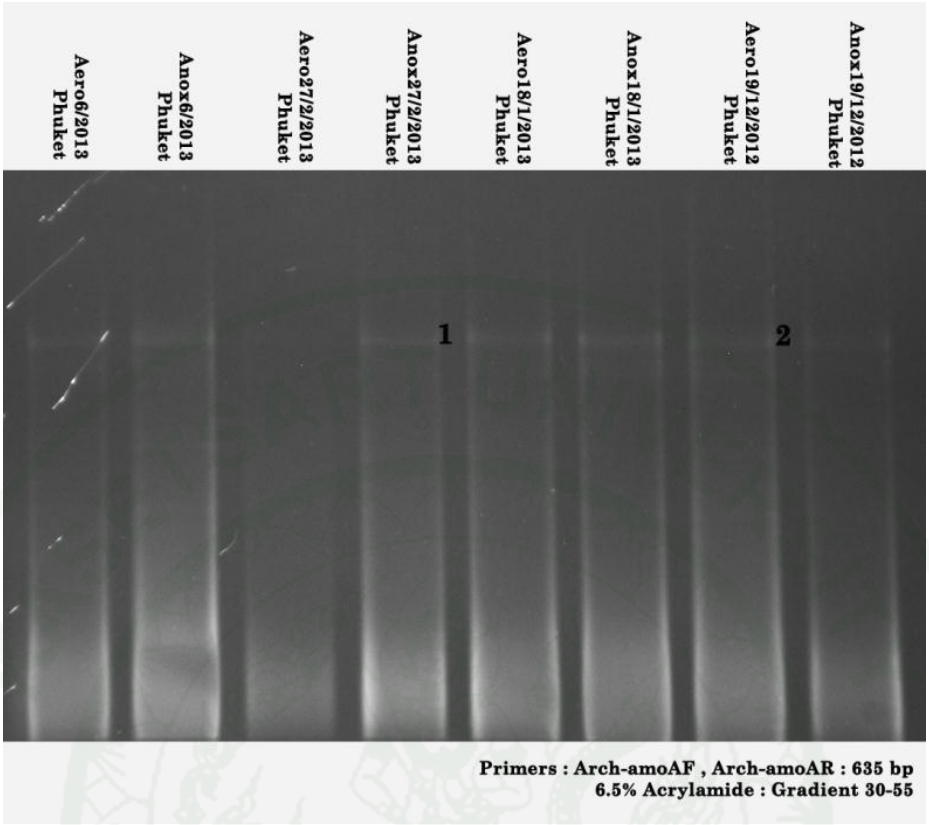

Figure 23 DGGE analysis of archaea *amoA* gene in each sample.

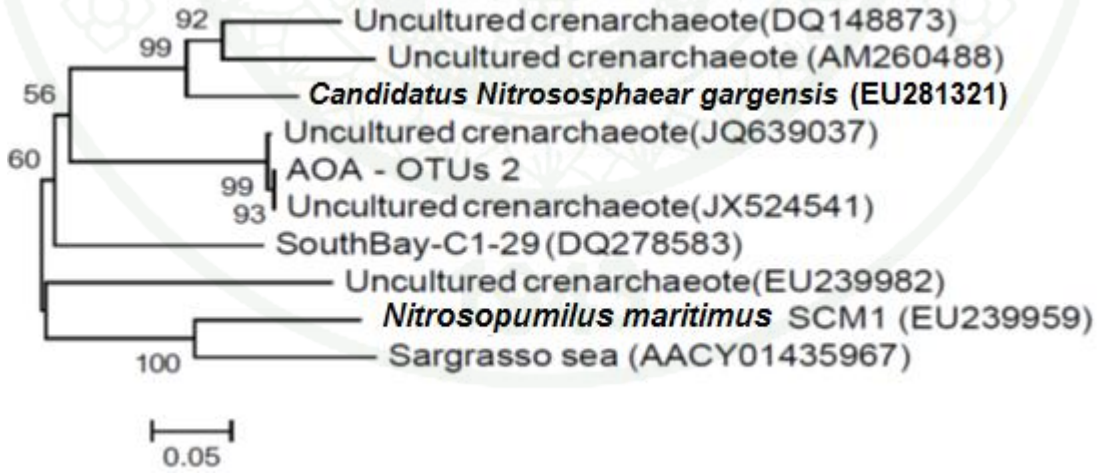

Figure 24 A neighbor-joining tree showing phylogenetic tree relationships among archaea *amoA* sequences from WWTPs including Thailand. Bootstrap values (>50%) are indicated at branch points.

CONCLUSIONS AND RECOMMENDATIONS

Conclusions

Only Phuket's WWTP was detected the abundance of AOA *amoA* genes higher than AOB *amoA* genes. The main reasons of this study, high temperature, HRT, SRT and low DO concentration were postulated on the main keys to find AOA *amoA* genes in the Phuket's WWTP. Predominant AOA and AOB were affiliated with the known ammonia oxidizing microorganisms, which were found in marine, natural and engineered system, respectively. Although physiological experiments of activated sludge warrants further study, different influent compositions and operational conditions may allow for the growth of AOA and contribution to nitrification, which have been underestimated or neglected.

Recommendations

1. Further study should significantly into the role of AOA and AOB, how do they work together and the specific condition for AOA and AOB to growth by operating only pure culture of AOA under the different conditions in a reactor.

2. Physiological properties of AOA and AOB of the samples in Thailand warrant future studies.

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