CHAPTER III

METHODOLOGY AND MATERIALS

3.1 Experimental framework

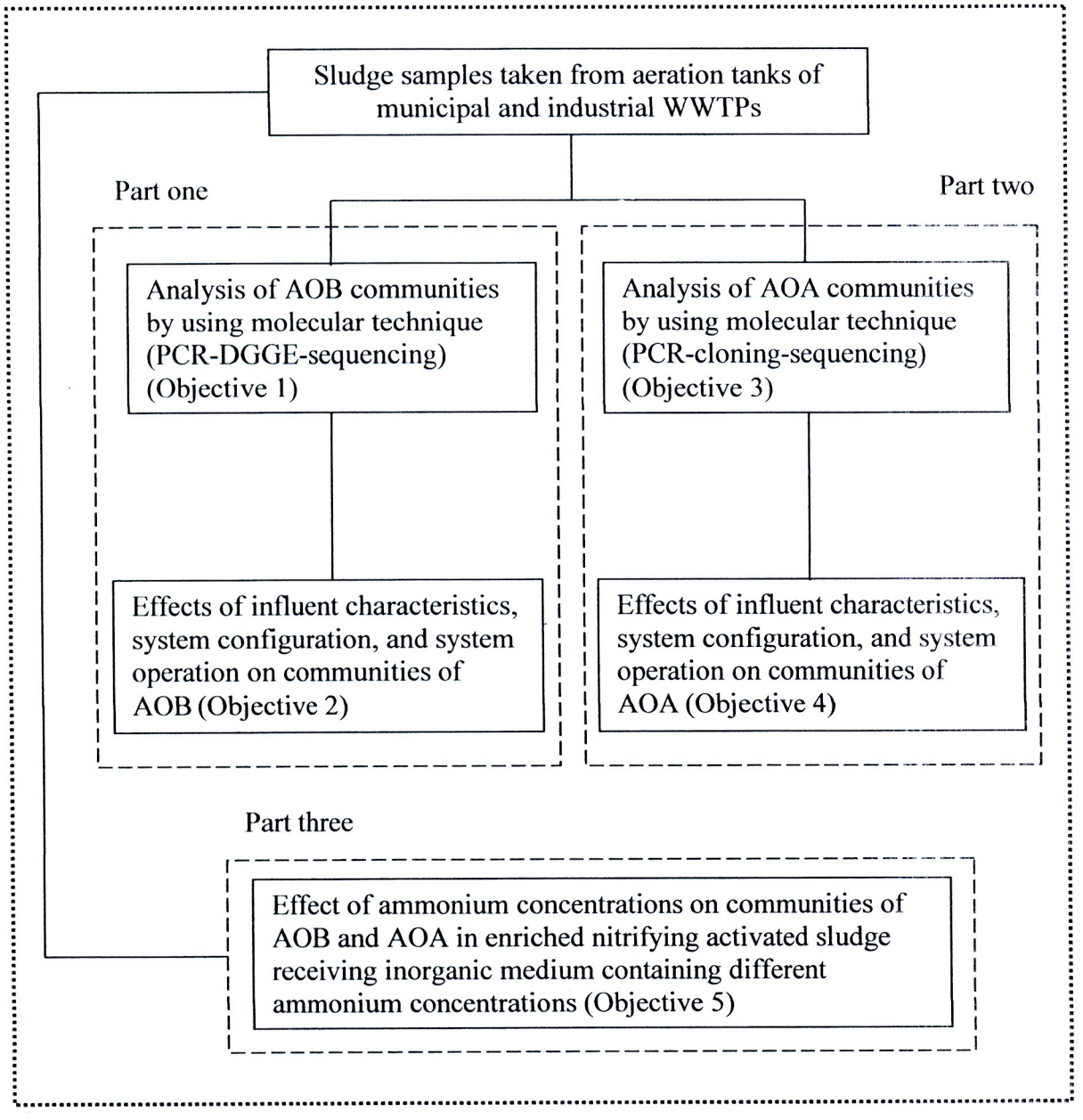

Figure 3.1 Experimental framework

This work was divided into three parts. The first part of this study focused on the study of communities of AOB in species level by using molecular technique (PCR-DGGE-sequencing) and analysis of influent characteristics, system configuration, and system operation on communities of AOB. The second part focused on the study of communities of AOA in species level by using molecular technique (PCR-cloning-sequencing) and analysis of influent characteristics, system configuration, and system operation on communities of AOA. The last part of this work was to investigate the effect of ammonium concentrations on communities of AOB and AOA in enriched nitrifying activated sludge (NAS) receiving inorganic medium containing different ammonium concentrations.

3.2 Measurement ammonia, nitrite, and nitrate

3.2.1 Measurement of ammonium

Sample was diluted with deionized water. 5 ml of dilution sample and 0.2 mL of phenol solution (Mix 11.1 mL liquefied phenol (≥89%) with 95% v/v ethyl alcohol to a final volume of 100 mL) were added and then mixed. 0.2 mL of sodium nitroprusside solution (0.5% w/v: dissolve 0.5 g of sodium nitropusside in 100 mL of deionized water), and 0.5 mL of oxidizing solution (Mix 100 mL alkaline citrate solution: dissolve 200 g of trisodium citrate and 10 g of sodium hydroxide in 1000 mL of deionized water with 25 mL of sodium hypochloride) were added into the tube. Sample was covered with plastic wrap or paraffin wrapper film and kept at room temperature in subdued light for at least 1 hr to develop color. Sample was measured for absorbance at 640 nm with UV visible spectrophotometers (Thermo Electron Corporation, Hexious α, Cambridge, UK) (Phenate method, Standard Method for the Examination of Water and Wastewater 20th edition).

3.2.2 Measurement of nitrite

Sample was diluted with deionized water. 5ml of diluted sample and 0.1mL of Sulphanilamide solution (dissolve 5 g of Sulphanilamide and 50 mL of hydrochloric in 500 mL) was added, and allowed to react 5 min, then 0.1 mL of NNED solution (dissolve 1 g of (N-(1-Naphthyl)-Ethylenediamine Dihydrochloride in

1000mL of de-ionized water) was added and allowed at room temperature in subdued light for at least 1 hr to develop color. Sample was measured for absorbance at 543 nm with UV visible spectrophotometers (Thermo Electron Corporation, Hexious α, Cambridge, UK) (Colorimetric method, Standard Method for the Examination of Water and Wastewater 20th edition).

3.2.3 Measurement of nitrate

Sample was diluted with deionized water. 5 mL of diluted sample was filtered and measured for absorbance at 220 nm to obtain NO_3^- reading and absorbance at 275 nm to determine interference due to dissolved organic matter with UV visible spectrophotometers (Thermo Electron Corporation, Hexious α, Cambridge, UK) (Ultraviolet Spectrophotometric Screening Method, Standard Method for the Examination of Water and Wastewater 20th edition).

3.3 Analysis of communities of ammonia-oxidizing bacteria and archaea

3.3.1 Sample preparation and DNA extraction

Sludge of approximately 2 mg of MLSS was transferred into a 1.7 ml eppendorf tube and centrifuged at 14,000 rpm for 10 min. The supernatant was removed, and the pellet was kept at -20 ^{0}C until analysis. DNA was extracted from samples using Fast-DNA SPIN kits for soil (QBiogene, Solon, Ohio, USA) according to the manufacturer's instructions. The product from DNA extraction was verified by electrophoresis in 2% agarose (Bio-Rad, Spain).

3.3.2 Analysis of ammonia-oxidizing bacteria communities

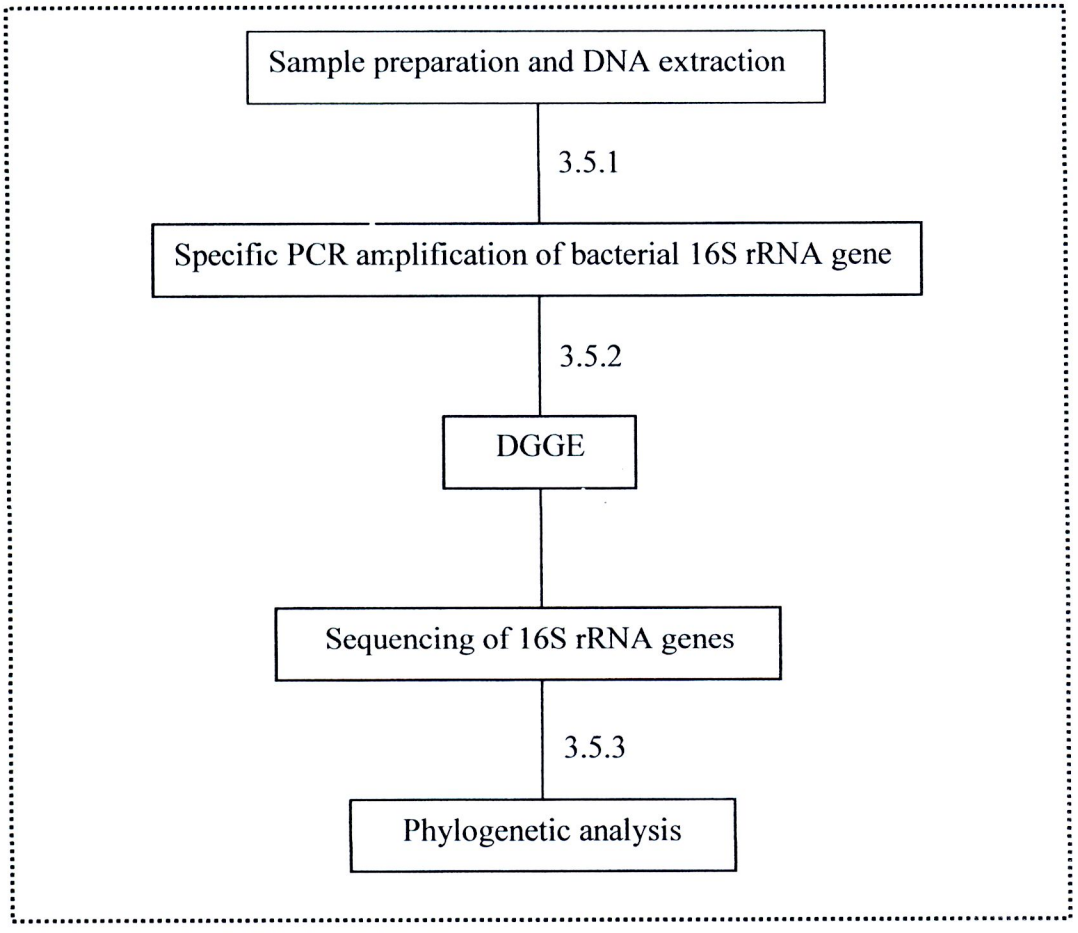

Figure 3.2 Experimental framework for analysis of ammonia-oxidizing bacteria communities

3.3.2.1 Polymerase chain reaction (PCR)

Primers CTO189f and CTO654r (Kowalchuck *et al.*, 1997) were used to amplify 465-bp of 16S rRNA gene fragment of *Betaproteobacteria*. The oligonucleotide sequences of the primers are shown in Table 3.1. Extracted DNA was PCR-amplified using the primer sets (the forward primer had a GC clamp) for 35 cycles in a 25 µl reaction volume. DNA eluted from bands excised from DGGE gels were amplified for 20–25 cycles using the primer set lacking the GC clamp in a 25 µl reaction volume. The PCR mixture was prepared using Takara DNA polymerase (TAKARA Inc, Tokyo, Japan) following the manufacturer's instructions. PCR amplification was performed in an Authorized thermal cycler (Biorad, USA) under

the conditions of 3 min at 94 ^{0}C followed by 35 cycles of 1 min at 94 ^{0}C, 1 min at 57 ^{0}C, and 1 min at 72 ^{0}C, followed by 10 mins final extension at 72 ^{0}C.

Table 3.1 Specific primers for AOB used in this study

Primer	Nucleotide sequence (5'–3')
CTO189A/Bf	GGAGRAAAGCAGGGGATCG
CTO 189Cf	GGAGGAAAGTAGGGGATCG
CTO189A/Bf-GC	CGCCCGCCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGAGRAAA GCAGGGGATCG
CTO 189Cf-GC	CGCCCGCCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGAGGAAA GTAGGGGATCG
CTO 654r	CTAGCYTTGTAGTTTCAAACGC

3.3.2.2 Denaturing gradient gel electrophoreses (DGGE)

Denaturing gradient gel electrophoreses was performed according to the modification of a described method (Kurisu *et al.*, 2002). We use 8% polyacrylamide gels, and the urea–formamide denaturant gradient was 30–70%. Gels were run on the D Code system (Bio-Rad Laboratories, Hercules, CA, USA) for 16 h at 60 ^{0}C and 75 V. After electrophoresis, the gels are stained with Ethidium bromide (Amersham Biosciences, Munich, Germany) and analyzed by gel documentation (Dolphin-DOC Plus, NV, USA). Prominent bands were excised and dissolved in 30µl sterilized water. DNA was recovered from the gel by freeze–thawing three times.

3.3.2.3 Phylogenetic analysis

The completed 400-bp from 465-bp analyzed sequences were aligned with sequences from the SSU rRNA database (Antwerm, Belgium) using the ARB program package (Department of Microbiology, Technische Universitat Munchen, Munich, Germany; [http://www.arb-home.de]). Phylogenetic tree was constructed using the ARB program package. We added our 400-bp sequences into the distance tree, which was previously constructed based on comparison of 1000-bp sequences of all AOB, which are available in the SSU rRNA database, and some related non-AOB, which were used as outgroup sequences. Additionally, our 400-bp sequences and 400-bp sequences of described AOB species (Koops *et al.*, 2003) and some related non-AOB were calculated based on maximum parsimony, maximum likelihood, and

distance analyses using the external software provided in the ARB program package (Phylip DNAPARS, AxML, and Phylip Distance Method, respectively).

3.3.3 Analysis of ammonia-oxidizing archaea communities

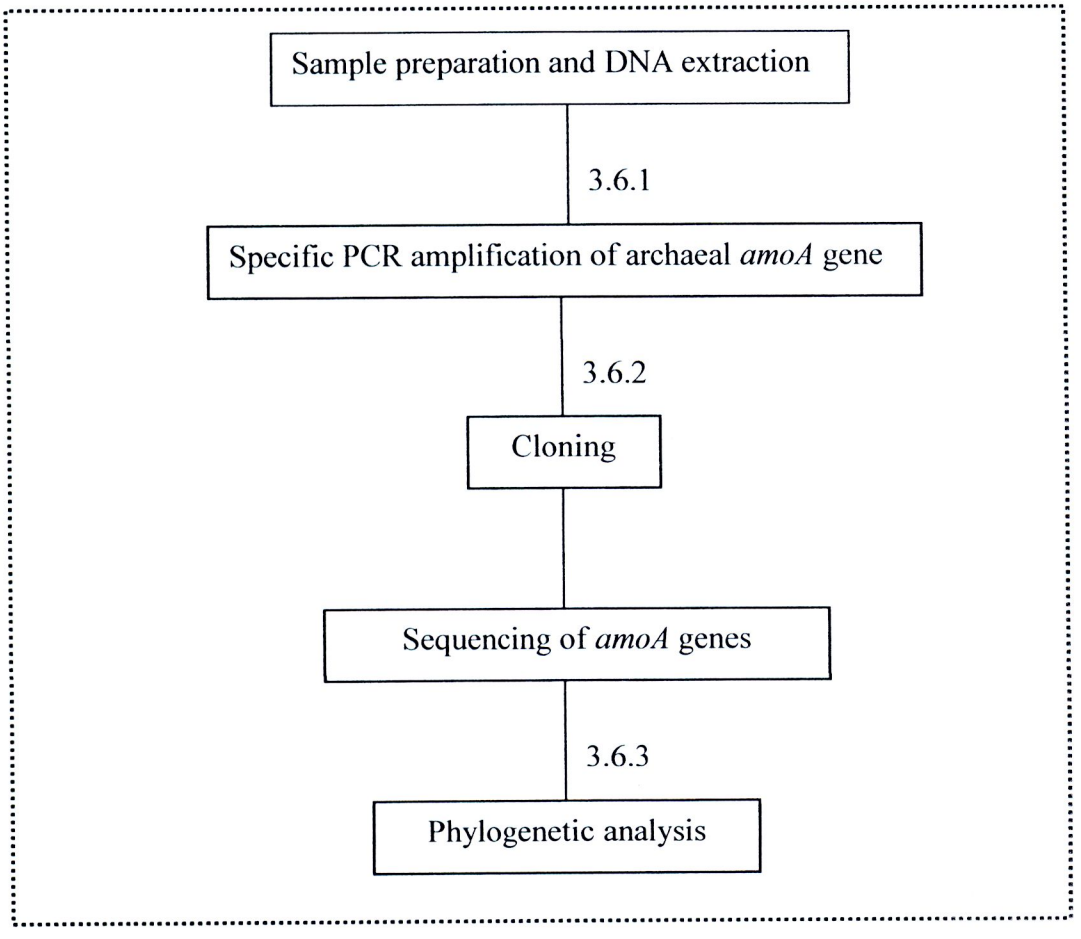

Figure 3.3 Experimental framework for analysis of ammonia oxidizing archaea communities

3.3.3.1 Polymerase chain reaction (PCR)

Primers Arch-amoAF and Arch-amoAR (Francis *et al.*, 2005) were used to amplify 635-bp of Archaeal *amoA* gene fragments. The PCR mixture was prepared using Takara DNA polymerase (TAKARA Inc, Tokyo, Japan) following the manufacturer's instructions. PCR amplification was performed in an Authorized thermal cycler (Biorad, USA) under the conditions of 3 min at 94 ^{0}C followed by 35

cycles of 1 min at 94 ^{0}C, 1 min at 57 ^{0}C, and 1 min at 72 ^{0}C, followed by 10 mins final extension at 72 ^{0}C.

Table 3.2 Specific primers of AOA used in this study

Primer	Nucleotide sequence (5'–3')
Arch-amoAF	STAATGGTCTGGCTTAGACG
Arch-amoAR	GCGGCCATCCATCTGTATGT

3.3.3.2 Cloning

Duplicate PCR products were pooled and purified by gel electrophoresis using a QIAEXII gel extraction kit (QIAGEN Inc., Valencia, CA). The purified PCR products were cloned using the pGEM-T Easy vector system (Promega, Madison, WI) following the manufacturer's instructions. For each sample, 10-20 clones were randomly selected for sequencing.

3.3.3.3 Phylogenetic analysis

Phylogenetic trees for AOA was constructed by tree different methods comprising of distance matrix, maximum parsimony, and maximum likelihood to confirm the grouping of AOA analyzed in this study and all major AOA reported in previous studies so far. Cluster was defined based on operational taxonomic units (OTUs) using the DOTUR program (Scholoss *et al.*, 2005). AOA clusters were defined based on OTUs using the DOTUR program. Any AOA sequences, showing >86% identity, were identified as the same AOA cluster.

3.4 Experiment Part I and II: Analysis of communities of ammonia-oxidizing bacteria and archaea in full-scale wastewater treatment plants (WWTPs)

3.4.1 Sample collection and description

Sludge sample were taken from 10 full-scale WWTPs, including 4 industrial WWTPs (I1-I4), 4 large municipal WWTPs (LM1-LM4), and 2 small municipal WWTPs (SM1 and SM4). All municipal WWTPs belong to Bangkok metropolitan administration (BMA). The WWTPs were selected basing on the difference in influent wastewater characteristics, system configuration, and system operation. The details were shown in Table 3.3.

3.4.2 Analysis of communities of ammonia-oxidizing bacteria in full-scale WWTPs

See section 3.3.2

3.4.3 Analysis of communities of ammonia-oxidizing archaea in full-scale WWTPs

See section 3.3.3

Table 3.3 Influent and effluent characteristics, system configuration, and system operation of full-scale WWTPs

WWTP	Treatment Process	Design Flow (m^3/d)	Actual flow (m^3/d)	BOD in influent (mg/l)	BOD in effluent (mg/l)	NH_3 in influent (mg/l)	NH_3 in effluent (mg/l)	% NH_3 removal	DO conc. in effluent (mg/l)
I1[a]	AS	6,000	4,500	400	7	NA	NA	NA	NA
I2[a]	AS	6,000	5,000	3,150	11	443	14	96.84	3
I3[a]	AS	4,000	1,300	NA	NA	30	4	86.67	2
I4[a]	AS	8,000	6,500	299	4	199	15	92.46	4.44
LM1[b]	AS	200,000	124,282	35.20	6.6	8.75	1.41	83.89	6.33
LM2[b]	AS	40,000	28,107	57.99	13.76	6.53	2.90	55.59	5.94
LM3[b]	AS	157,000	124,423	32.43	3.84	7.08	0.43	93.93	6.24
LM4[b]	AS	30,000	20,961	54.22	5.35	NA	NA	NA	3.27
SM1[b]	AS	800	600	116.82	5.50	NA	NA	NA	3.50
SM4[b]	Aerated lagoon	1,500	1,188	69.82	10.64	NA	NA	NA	1.35

AS, activated sludge process; NA, data not available

[a] The values were the averages of the three months in before the sludge was collected.

[b] The values were the averages of the year 2007, providing on the website (http://dds.bma.go.th)

3.5 Experiment Part III: Effect of ammonium concentrations on communities of ammonia-oxidizing bacteria and archaea in enriched nitrifying activated sludge (NAS)

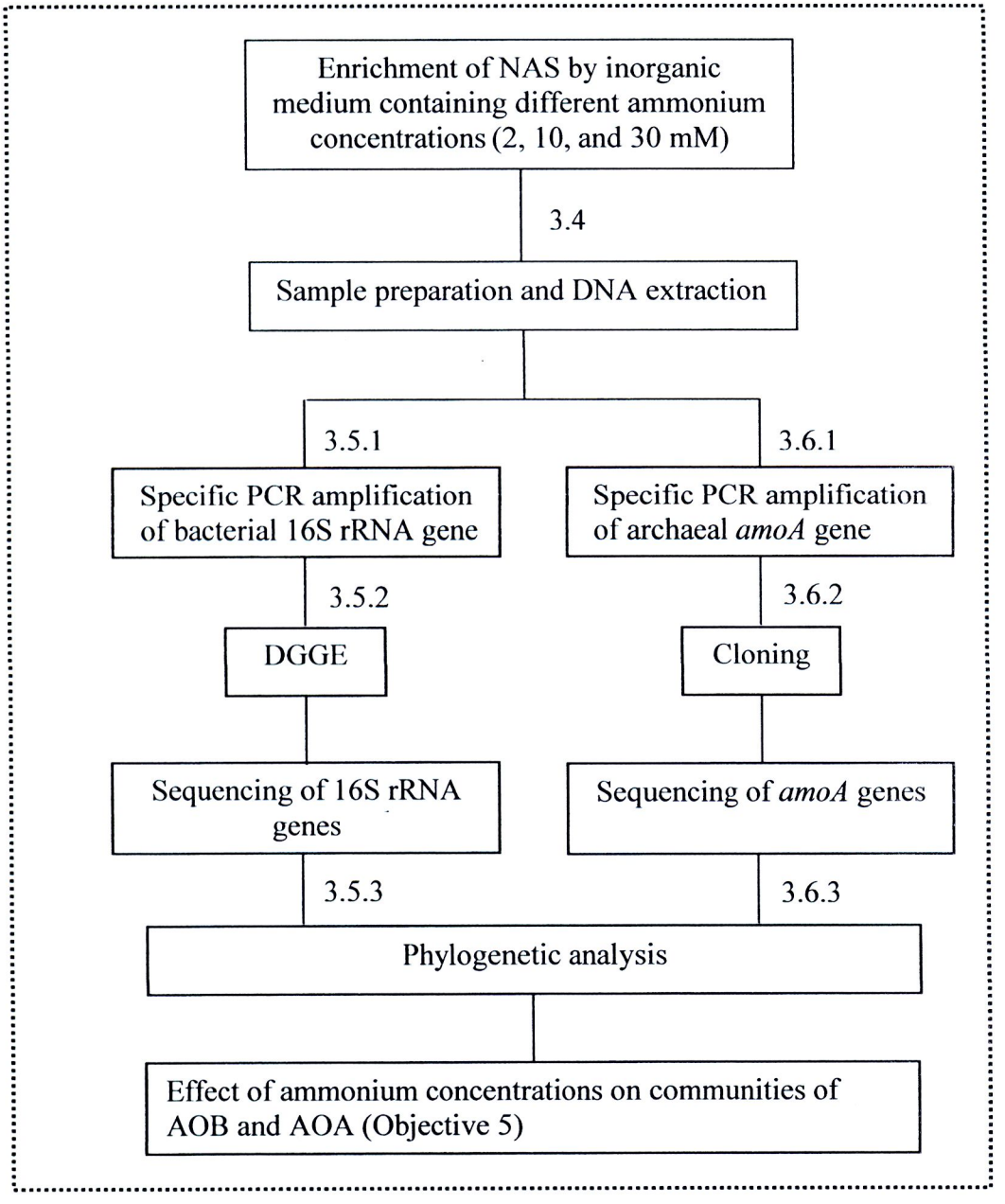

Figure 3.4 Experimental framework for analyzing the effect of ammonium concentrations on communities of AOB and AOA in enriched NAS

3.5.1 Seed sludge

Seed sludge was taken from a sludge buffer tank of a Chong Nonsi municipal wastewater treatment plant. This system is Cyclic Activated Sludge System (CASS) which is modified from Sequencing Batch Reactor (SBR) and can receive up to 200,000 m^3/day. On the day of sampling, biological oxygen demand (BOD) in the influent was 40 mg/ l, whereas ammonium concentration was 13 mg N/l. BOD and ammonium removal efficiencies of this system were 92.5 % and 84.6 %, respectively. Nitrite concentration in the aeration tank was 0.01 mg N/l, and pH was controlled around 6-7. Mixed-liquor suspended solids (MLSS) concentration on the day of sampling was 9385 mg/l.

3.5.2 Medium for enriching nitrifying activated sludge

The inorganic medium for enriching NAS contained $(NH_4)_2SO_4$, 40 mg of $MgSO_4 \cdot 7H_2O$, 40 mg of $CaCl_2 \cdot 2H_2O$, 200 mg of KH_2PO_4, 1 mg of $FeSO_4 \cdot 7H_2O$, 0.1 mg of $Na_2Mo_4O_4 \cdot 2H_2O$, 0.2 mg of $MnCl_2 \cdot 4H_2O$, 0.02 mg of $CuSO_4 \cdot 5H_2O$, 0.1 mg of $ZnSO_4 \cdot 7H_2O$, and 0.002 mg of $CoCl_2 \cdot 6H_2O$ per liter (Limpiyakorn *et al.*, 2007). $NaHCO_3$ was added to achieve 2 mg bicarbonate (HCO_3^-) per 1 mg of ammonium added. pH was adjusted to around 7.5 - 8.0 using 40 g/l $NaHCO_3$.

3.5.3 Enrichment of nitrifying activated sludge by inorganic medium containing different ammonium concentrations (2, 10, and 30 mM)

Sludge taken from the municipal wastewater treatment system was enriched in three laboratory-scale continuous flow reactors without sludge recycling introduced with inorganic medium containing three different ammonium concentrations: 2, 10 and 30 mM NH_4^+-N (28, 140, and 420 mg N/l, respectively). Total volume of each reactor was 4 l, with an effective volume of 2 l. To obtain the optimum condition for AOB growth, temperature was kept at 30 ^{0}C, DO concentration was controlled at around 2 mg/l, pH was maintained in a range of 7.5 - 8.0 using 1 N HCl and 1 N NaOH, and mixing was provided at rotating speed of 300 rpm. Inorganic medium was introduced into all reactors at a fixed dilution rate of 0.01 hr^{-1} (Limpiyakorn *et al.*, 2007).

3.5.4 Analysis of communities of ammonia-oxidizing bacteria in seed sludge and enriched NAS

See section 3.3.2

3.5.5 Analysis of communities of ammonia-oxidizing archaea in seed sludge and enriched NAS

See section 3.3.3