amoA-encoding archaea and thaumarchaeol in the lakes on the northeastern Qinghai–Tibetan Plateau, China

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INTRODUCTION

Microbial oxidation of ammonia to nitrite, the first step in nitrification, plays an important role in the global nitrogen cycle. This biogeochemical process is mainly carried out by two groups of microorganisms: ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA; Nicol and Schleper, 2006), which share a highly divergent homolog of ammonia monoxygenase. The amoA gene, encoding the alpha subunit of ammonia monoxygenase, has been widely exploited as a molecular biomarker to study AOB and AOA distributions in various environments (Erguder et al., 2009), thus many studies proposed crenarchaeol as a characteristic lipid biomarker for studying AEA distribution in nature. All known ammonia-oxidizing archaea (AOA) belong to the phylum Thaumarchaeota within the domain Archaea. AOA possess the diagnostic amoA gene (encoding the alpha subunit of ammonia monooxygenase) and produce lipid biomarker thaumarchaeol. Although the abundance and diversity of amoA gene-encoding archaea (AEA) in freshwater lakes have been well-studied, little is known about AEA ecology in saline/hypersaline lakes. In this study, the distribution of the archaeal amoA gene and thaumarchaeol were investigated in nine Qinghai–Tibetan lakes with a salinity range from freshwater to salt-saturation. The results showed that the archaeal amoA gene was present in hypersaline lakes with salinity up to 160 g L−1. The archaeal amoA gene diversity in Tibetan lakes was different from those in other lakes worldwide, suggesting Tibetan lakes (high elevation, strong ultraviolet, and dry climate) may host a unique AEA population of different evolutionary origin from those in other lakes. Thaumarchaeol was present in all of the studied hypersaline lakes, even in those where no AEA amoA gene was observed. Future research is needed to determine the ecological function of AEA and possible sources of thaumarchaeol in the Qinghai–Tibetan hypersaline lakes.

Keywords: amoA gene, AEA, Thaumarchaeota, salinity, Qinghai–Tibetan lakes

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Originally, the AOA were thought to belong to the phylum *Crenarchaeota* on the basis of their 16S rRNA genes (Könneke et al., 2005). Subsequently, systematic comparison of 53 ribosomal proteins shared by Archaea, Eukarya, and the genomes of AOA isolates (i.e., *Nitrosopumilus maritimus* and *Nitrososphaera gargensis*) suggested that AOA belong to a separate phylum of the Archaea and should be classified as a new phylum proposed as *Thaumarchaeota* (Brochier-Armanet et al., 2008; Spang et al., 2010). In order to be consistent with the phylum *Thaumarchaeota*, crenarchaeol was renamed thaumarchaeol (Sinninghe Damsté et al., 2012). To date, thaumarchaeol has been detected in different environments, such as marine ecosystems (Sinninghe Damsté et al., 2002; Pitcher et al., 2011a), hot springs (Pearson et al., 2004, 2008; Zhang et al., 2006; Pitcher et al., 2009), and lakes (Cas-tateda and Schouten, 2011). To our knowledge, however, few studies have reported the presence and distribution of thaumarchaeol in hypersaline lakes where salinity is higher than that of seawater.

The Qinghai–Tibetan Plateau is the largest (2 × 10^6 km^2) and highest (average ∼ 4500 m a.s.l.) plateau on the Earth. It contains thousands of saline/hypersaline lakes, which possess a broad range of environmental gradients such as salinity (from 0.1 to 426.3 g L^-1^) and pH (5.4–10.2; Yang et al., 2004; Wu et al., 2006; Dong et al., 2010; Liu et al., 2012; Xiong et al., 2012). So the Qinghai–Tibetan lakes are ideal for assessing AEA diversity and community structure in response to environmental conditions (e.g., salinity). The objectives of this study were: (1) to investigate the abundance and diversity of AEA in Qinghai–Tibetan lakes with different salinities by using an integrated approach including lipids and amoA gene-based molecular analysis; (2) to assess how the AEA population correlates with environmental variables such as salinity and pH; and (3) to determine whether thaumarchaeol can be found above the seawater salinity and how AEA respond to salinity change from freshwater to hypersaline.

**MATERIALS AND METHODS**

**DESCRIPTION OF STUDY LAKES**

Nine lakes (Keluke Lake, Erhai Lake, Qinghai Lake, Tuosu Lake, Gahai Lake 1, Gahai Lake 2, Xiaochaidan Lake, Dongdabuxun Lake and Lake Chaka) on the Qinghai–Tibetan Plateau were selected for this study (Figure 1; Table 2). Keluke Lake is situated in the region of Delingha city. It has a surface area of 56.7 km^2^ with the maximum water depth of 13.3 m (Wang and Dou, 1998). Qinghai Lake is the largest saline lake in China, which is located in a structural intermontane depression at the northeastern corner of the Qinghai–Tibetan Plateau. It has an area of 4300 km^2^ and an average water depth of 19.2 m (Dong et al., 2006). Erhai Lake and Gahai Lake 1 are two daughter lakes of Qinghai Lake. Erhai Lake is a freshwater lake with a surface area of ∼ 5 km^2^ (Jiang et al., 2010). Gahai Lake 1 is a saline lake with a surface area of ∼ 47.2 km^2^ (Jiang et al., 2010). Tuosu Lake is located on the northeastern corner of the Qaidam Basin. It has an area of 4300 km^2^ and an average water depth of 19.2 m (Dong et al., 2006). Erhai Lake and Gahai Lake 1 are two daughter lakes of Qinghai Lake. Erhai Lake is a freshwater lake with a surface area of ∼ 5 km^2^ (Jiang et al., 2010). Gahai Lake 1 is a saline lake with a surface area of ∼ 47.2 km^2^ (Jiang et al., 2010). Tuosu Lake is located on the northeastern corner of the Qaidam Basin. It has an area of 165.9 km^2^ and the local average annual temperature is 2–4°C (Wang and Dou, 1998). Gahai Lake 2 is located on the northeastern edge of the Qaidam Basin. It has a surface area of 32 km^2^ with the maximum water depth of 13 m. The lake is situated in an arid climate system (100 mm of rainfall per year; Wang and Dou, 1998). Xiaochaidan Lake is a hypersaline lake located on the northern edge of the Qaidam Basin. It...
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Whatman GF/F glass fiber filters. The biomass-containing filters were collected into 50-mL Falcon tubes for aqueous geochemical analysis. For collection of suspended particulate matter (SPM), lake surface waters (4–20 L) were filtered through 0.7 μm polytetrafluoroethylene (PTFE) filter for archaeal total lipid analysis. The difference in yield of archaeal lipids between the hydrolyzed and non-hydrolyzed fractions is considered to be the archaeal polar lipids (PLs; Zhang et al., 2011). The thaumarchaeol was determined by using high performance liquid chromatography (HPLC)–atmospheric pressure chemical ionization (APCI)–mass spectrometry (MS) at Tongji University following a method slightly modified from previous studies (Hopmans et al., 2004; Schouten et al., 2007). An aliquot (5 μL) of sample was injected and separation was achieved with an Alltech Prevail Cyano Column (150 mm × 2.1 mm, 3 μm). The elution gradient was: isocratic (5 min) at 99% hexane/1% isopropanol followed by a linear gradient to 1.8% propanol in 45 min at a constant flow rate of 0.2 ml min⁻¹. Quantification was achieved by peak area integration of [M + H]⁺ ions in the extracted ion chromatogram in comparison with the C46 internal standard. The detection limit was 0.8 pg (Zhang et al., 2011).

NUCLEIC ACIDS EXTRACTION

DNA was extracted from biomass-containing filters and lake sediments by using FastDNA Pro soil-direct kits (MP Biomedicals, OH, USA) according to the manufacturer’s instructions. The amoA transcripts have been recovered from Qinghai Lake (Jiang et al., 2009b) and oceans (with salinity higher than Gahai Lake 1; Pitcher et al., 2011b). In order to test whether the amoA gene transcripts can be recovered from saline lakes (with salinity higher that of sea water), sediment samples from Gahai Lake 2 and Xiaochaidan Lake were selected for RNA extraction using FastRNA Pro soil-direct kits (MP Biomedical) according to the manufacturer’s protocol. DNA-based amoA gene PCR was not successful in Lake Chaka, so RNA was not extracted from the sediment of Lake Chaka. The extracted raw RNA was digested with the use of RNase-free DNase I (Takara, Japan). The DNase-digested RNA samples were checked for potential genomic DNA contamination by PCR amplification with the AOA-specific primer set (see “PCR Amplification and Phylogenetic Analysis”). The checked RNA samples were reverse-transcribed into cDNA using the Promega AMV reverse transcription system (Promega Corporation, Madison, WI, USA) as previously described (Yang et al., 2012). Double distilled water served as the template in negative controls for the cDNA synthesis and downstream PCRs (Jiang et al., 2010).

has a surface area of 71.5 km² with the maximum water depth of 0.69 m (Jiang et al., 2009a). Dongdabuxun Lake is a hypersaline lake located in an extremely arid climate region (average rainfall: 24.7 mm per year; Wang and Dou, 1998). It has a surface area of 184.0 km² with water depth of 0.36–1.02 m. Lake Chaka is a shallow lake with a high salinity of 32.5%. It has a surface area of ~104 km² with average water depth of 2–3 cm (Jiang et al., 2006).

FIELD MEASUREMENTS AND SAMPLE COLLECTION

Field work was conducted in August 2011. The pH values of the nine lakes were measured with a portable pH meter (PT-10, Ser
torius, Germany). Water chemistry (e.g., sulfide, ammonium, and
nitrate) was measured with a Hach colorimeter (model CEL 850, Hach Chemical Co., IA, USA). After field measurements, lake sur-
face water samples (250–500 mL) were filtered through 0.2 μm iso-
spore filters (Whatman, UK). The filtrate (∼40 mL) was col-
lected into 50-mL falcon tubes for aqueous geochemical analysis.

For clarity, these lake names are hereafter abbreviated as fol-
loows: KLKL-1-W, EHL-1-W, QHL-14-W, TSL-31-W, GHL1-32-W, GHL2-84-W, XCDL-160W, DBDBXL-308-W, and LCK-325-W for Keluke Lake, Erhai Lake, Qinghai Lake, Tsuoi Lake, Gahai Lake 1, Gahai Lake 2, Xiaochaidan Lake, Dongdabuxun Lake, and Lake Chaka, respectively. The numbers between “lake name” and “W” indicates salinity (g L⁻¹) of the lake. KLKL-1-S, EHL-1-
S, QHL-14-S, TSL-31-S, GHL1-32-S, GHL2-84-S, XCDL-160-S, DBDBXL-308-S, and LCK-325-S are used for the sediments of these lakes.

MEASUREMENTS OF WATER SALINITY

The concentrations of eight major ions: potassium, sodium, calcium, magnesium, chloride, sulfate, carbonate, and bicarbonate were analyzed in the laboratory according to the Manual of Analytical and Testing Department in the Institute of Salt Lakes, Chinese Academy of Science (Analytical Lab of the Qinghai Institute of Salt Lakes, Chinese Academy of Sciences, 1988). Salinity was calculated by summing the concentrations of these eight ions.

THAUMARCHAEOL ANALYSIS

GF/F filters and sediments (~5 g per sample) were freeze-dried and extracted according to the procedures described previously (Wang et al., 2013). Briefly, samples were ultrasonically extracted three times using a single phase solvent mixture including MeOH, dichloromethane (DCM), and phosphate buffer (pH 7.4; 2:1:0.8, v/v/v) following a previous procedure (Bligh and Dyer, 1959). Samples were centrifuged (5 min, 2500 rpm) and the extract was collected into another tube. This procedure was repeated three times. DCM and phosphate buffer were added to the combined extract at 1:1:0.9 (v/v/v) to achieve phase separation, after which the bottom DCM phase (containing lipids) was collected into a 40 mL glass tube. The resulting aqueous phase was rinsed twice with DCM and all DCM fractions were collected into a glass tube. Subsequently, the DCM phase containing the total Bligh-Dyer extract (BDE) was dried under N₂. To quantify thaumarchaeol a known amount of a C46 internal standard (Huguet et al., 2006) was added to the BDE which was then dissolved in DCM. The resulting mixture was divided into two aliquots: one was dried under N₂, re-dissolved in hexane/isopropanol (99:1 v/v), and filtrated through a 0.45 μm polytetrafluoroethylene (PTFE) filter for analysis of archaeal core lipids (CLs); and the other was subject to acid hydrolysis, and the extracted organic phase was re-dissolved in hexane/isopropanol (99:1, v/v) and filtrated through a 0.45 μm PTFE filter for archaeal total lipid analysis. The difference in yield of archaeal lipids between the hydrolyzed and non-hydrolyzed fractions is considered to be the archaeal polar lipids (PLs; Zhang et al., 2011). The thaumarchaeol was determined by using high performance liquid chromatography (HPLC)–atmospheric pressure chemical ionization (APCI)–mass spectrometry (MS) at Tongji University following a method slightly modified from previous studies (Hopmans et al., 2004; Schouten et al., 2007). An aliquot (5 μL) of sample was injected and separation was achieved with an Alltech Prevail Cyano Column (150 mm × 2.1 mm, 3 μm). The elution gradient was: isocratic (5 min) at 99% hexane/1% isopropanol followed by a linear gradient to 1.8% propanol in 45 min at a constant flow rate of 0.2 ml min⁻¹. Quantification was achieved by peak area integration of [M + H]⁺ ions in the extracted ion chromatogram in comparison with the C46 internal standard. The detection limit was 0.8 pg (Zhang et al., 2011).

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QUANTITATIVE PCR

Quantitative PCR (qPCR) was used to determine the abundances of the archaeal 16S rRNA and amoA genes in the waters and sediments of the nine lakes with the primer sets of Arch349F (5’-GYY CAS CAG KCG MGA AW-3’) /Arch806R (5’-GGA CTG GAG CCT ATC AT-3’; Takai and Horikoshi, 2000), and Arch-amoAF (5’-TAAATGTCGTGGCTTACGAGC-3’)/Arch-amoAR (5’-GCGGCCATCCATCTGTATGTTAT-3’; Francis et al., 2005), respectively. qPCRs were performed in a reaction volume of 20 μL containing 10 μL of 2 × SYBR Premix Ex Taq™ (TaKaRa), 0.4 μM of each primer, 0.4 μL of ROX reference dye II (50×), and 1 μL of soil DNA. qPCRs were performed on an ABI7500 real-time PCR system (Applied Biosystems, Carlsbad, CA, USA). The qPCRs were performed with the following conditions: 95 °C for 30 s, followed by 40 cycles (5 s at 95 °C for denaturing, 34 s for annealing at 53 °C for the archaeal 16S rRNA and amoA genes, and 60 s at 72 °C). A dissociation stage was added to yield a dissociation curve after the cycling amplification step. Standard curves were obtained by using serial dilutions (10^2 to 10^8 copies) of plasmids (pGM-T) containing cloned archaeal 16S rRNA and amoA genes. The data were used to create standard curves correlating the Ct values with the archaeal 16S rRNA and amoA gene copy numbers. Linear plots (not shown) between the Ct value and log (copy numbers/reaction) were obtained with correlation coefficients of R² > 0.99. PCR efficiencies were 90–95%. The quality and length of the qPCR products were checked by dissociation curve analysis and 1% agarose gel electrophoresis. The qPCR results were expressed as gene copies per gram (copies g⁻¹) for sediments and gene copies per milliliter (copies mL⁻¹) for water samples.

ACMPLIFICATION AND PHYLOGENETIC ANALYSIS

Five lakes (Erlhai Lake, Gahai Lake 1, Gahai Lake 2, Xiaoachaidan Lake, and Lake Chaka) were selected for the AEA and Thaumarchaeot in Qinghai–Tibetan lakes. The appropriate bands were excised from the gel and excised DNA was purified with Agarose Gel DNA purification Kit (TaKaRa). The purified PCR products were ligated into the pGEM-T vector (Promega Inc.) and transformed into Escherichia coli JM109 competent cells (TaKaRa) according to the manufacturer’s instructions. The transformed cells were spread on Luria–Bertani plates containing 100 μg mL⁻¹ of ampicillin and 30 μg mL⁻¹ of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and cultivated overnight at 37°C.

Sixteen (eight for each primer set) DNA clone libraries were constructed from the following samples: EHL-1-W, GHL1-32-W, GHL2-84-W, XCDL-160-W, EHL-1-S, GHL1-32-S, GHL2-84-S, and XCDL-160-S. One DNA clone library (XCDL-160-SR) was constructed for the sediment from Xiaoachaidan Lake. Around 30–40 randomly selected clones per sample were analyzed for the insert amoA gene sequences. Positive clones were sequenced using M13F with the BigDye Terminator version 3.1 chemistry (Applied Biosystems, Foster City, CA, USA) on an ABI 3730 automated sequencer.

The obtained raw nucleotide sequences were checked and trimmed manually by using the BioEdit program³. The sequences of poor quality were removed from further analysis. The operational taxonomic units (OTUs) of the amoA gene clone sequences were determined based on a cutoff value of 98% by using nearest neighbor algorithm in the DOT UR program (Schloss and Handelsman, 2005). The saturation of the sampled clones from each amoA gene clone library was assessed by calculating the coverage (C) values as follows: C = 1 – (n/N), where n is the number of OTUs that occurred only once in the clone library and N is the total number of analyzed clones (Jiang et al., 2006).

One representative sequence was selected from each OTU for phylogenetic analysis. Closest references of the amoA gene were retrieved from the GenBank² using BLAST. Maximum likelihood trees were constructed from the amoA gene sequences obtained in this study and their references (Pester et al., 2012) with the use of the MEGA program version 5.0 (Tamura et al., 2011), and were assessed using 1000 bootstrap replications. The nucleotide sequences obtained in this study were deposited in the GenBank database under accession numbers JX488399–JX488453 and KF606897–KF606927.

STATISTICAL ANALYSIS

Diversity indices were calculated by using DOTUR. LIBSHUFF analysis was performed to discern any similarity of the amoA gene composition among the samples according to the methods described previously (Jiang et al., 2006). Mantel test was performed to assess the correlations between amoA gene populations and environmental factors according to the procedures described previously (Yang et al., 2013). Briefly, the biotic matrices of amoA gene composition were constructed with the Bray–Curtis distance defined as follows: Bray–Curtis distance = 1 – d, where d refers to the Bray–Curtis similarity index, and the abiotic matrices of environmental variables were constructed using the Euclidean distance.

http://www.ncbi.nlm.nih.gov/biosamples/89349.html
http://www.ncbi.nlm.nih.gov
Cluster analysis was performed to compare differences in AEA communities between the studied Tibetan lakes and other lakes and saline environments worldwide (Francis et al., 2005; Dang et al., 2009; Herrmann et al., 2009; Jiang et al., 2009b; Kalanetra et al., 2009; Pouliot et al., 2009; Llirós et al., 2010; Wu et al., 2010; Auguet et al., 2011; Table 1). In order to avoid any bias resulting from different primers, those archaeal amoA gene sequences derived from the same primer set, Arch-amoAF/Arch-amoAR (Francis et al., 2005) were included in the cluster analysis. The combined amoA gene sequences (∼635 bp) were subjected to OTU identification using the DOTUR program (Schloss and Handelsman, 2005). The Jaccard similarity matrices were made and topology trees were constructed using the PAST software package3.

RESULTS

WATER CHEMISTRY OF THE STUDIED LAKES

The salinities of the investigated lakes were 0.7, 1.0, 14.2, 31.3, 31.9, 84.0, 160.4, 307.6, 325.0 g L$^{-1}$ for Keluke Lake, Erhai Lake, Qinghai Lake, Tuosu Lake, Gahai Lake 1, Gahai Lake 2, Xiaochaidan Lake, Dongdabuxun Lake, and Lake Chaka, respectively (Table 2). The pH was 7.0–9.4. The concentrations of sulfide ranged from 0.0 to 0.2 (mg L$^{-1}$; Table 2). The concentrations of major cations and anions of the investigated lake waters ranged as follows (mg L$^{-1}$): K$^+$ (6.5–2163.0), Na$^+$ (135.2–107460.3), Ca$^{2+}$ (17.7–1711.0), Mg$^{2+}$ (53.6–24700.0), SO$_4^{2-}$ (117.9–17099.8), Cl$^-$ (206.2–196231.9), CO$_3^{2-}$ (0.0–515.4), HCO$_3^-$ (149.8–824.1) NH$_4^+$ (0.4–1.2), and NO$_3^-$ (0.2–1.4, Table 2).

ABUNDANCE OF THAUMARCHAEOL

In the lake waters, thaumarchaeol concentration ranged from 0.0 to 0.3 ng L$^{-1}$ for CLs and from 0.0 to 0.5 ng L$^{-1}$ for PLs; in the lake sediments, thaumarchaeol concentrations ranged from 0.1 to 25.2 ng g$^{-1}$ for CLs and 0.5 to 37.5 ng g$^{-1}$ for PLs (Figure 2). The highest thaumarchaeol concentrations were observed in the sediment of Tuosu Lake (salinity: 31 g L$^{-1}$): 25.2 and 37.5 ng g$^{-1}$ for CLs and PLs, respectively (Figure 2).

ABUNDANCE OF ARCHAEAL 16S rRNA AND amoA GENES

Total archaeal 16S RNA gene abundance ranged from 3.86 × 10$^4$ to 4.15 × 10$^6$ copies mL$^{-1}$ and from 1.66 × 10$^5$ to

Table 1 | Summary of the AEA amoA gene sequences used for the comparison between this and other studies (the AEA amoA gene sequences were derived from the primer set of Francis et al., 2005), and the length of the amoA gene sequences was 635 bp.

| Location       | Lake name (sample type) | No. of clones or sequences | Reference          |
|----------------|------------------------|---------------------------|--------------------|
| Denmark        | Lake Hampen (sediment) | 306                       | Hermann et al. (2009) |
|                | Lake Karlsø (sediment) |                           |                    |
|                | Lake Grane Langsø (sediment) |                   |                    |
|                | Lake Søby (sediment) |                           |                    |
|                | Lake Almind (sediment) |                           |                    |
| Spain          | Lake Llebreta (water)  |                            | Auguet et al. (2011) |
|                | Lake Liton (water)    | Total 40 DGGE sequences   |                    |
|                | Lake Redi AT (water)  |                           |                    |
| Congo          | Lake Kivu (water)     | 14 DGGE bands            | Llirós et al. (2010)|
| Canada         | High Arctic lake A (water) |                   | Pouliot et al. (2009)|
|                | High Arctic lake C1 (water) |                      |                    |
| China          | Qinghai Lake water (QLW1-0) |                   | Jiang et al. (2009b) |
|                | Qinghai Lake sediment (QLS1-30) |                 |                    |
|                | Lake Taihu (sediment) | 106                       | Wu et al. (2010)    |
| Miscellaneous  | Antarctic coast (water) | 119                       | Kalanetra et al. (2009)|
|                | Arctic Ocean (water)  | 47                        | Francis et al. (2005)|
|                | Monterey Bay (water)  | 26                        |                    |
|                | The Eastern Tropical North Pacific (water) |                   |                    |
|                | Black Sea (water)     | 70                        |                    |
|                | Elkhorn Slough (sediment) |                   |                    |
|                | San Francisco Bay (sediment) |                |                    |
|                | Bahia del Tobin, Mexico (sediment) |               |                    |
|                | Huntington Beach, CA (sediment) |              |                    |
|                | Oak Ridge Josi | 27                        |                    |
|                | The deep-sea sediments of the tropical West Pacific continental margin (sediment) | 242 | Dang et al. (2009)|

3http://folk.uio.no/ohammer/past/
| GPS location (N/E) | Keluke lake | Erhai lake | Qinghai lake | Tsou lake | Gahai lake 1 | Gahai lake 2 | Xiaochaidan lake | Dongdaibuxun lake | Lake Chaka |
|-------------------|-------------|------------|--------------|-----------|--------------|------------|----------------|------------------|------------|
| 37°18.7′ / 36°34.1′ | 38°38.0′ / 37°11.6′ | 36°58.1′ / 37°71′ | 37°28.1′ / 37°28.1′ | 36°46.0′ / 98°54.1′ | 100°44.3′ / 100°6.9′ | 100°35.9′ / 97°46.9′ | 95°26.2′ / 95°26.2′ | 95°4.8′ / 100°44.3′ | 96°54.1′ / 100°54.1′ |
| Salinity (g L\(^{-1}\)) | 0.7 | 1.0 | 1.42 | 31.3 | 31.9 | 84.0 | 160.4 | 3076 | 325.0 |
| pH | 8.8 | 9.4 | 9.1 | 8.8 | 8.9 | 8.4 | 70 | 78 |
| Sulfide (mg L\(^{-1}\)) | BDL | 0.1 | BDL | 0.1 | 0.2 | 0.0 |

| Concentration of major ions (mg L\(^{-1}\)) | Keluke lake | Erhai lake | Qinghai lake | Tsou lake | Gahai lake 1 | Gahai lake 2 | Xiaochaidan lake | Dongdaibuxun lake | Lake Chaka |
|-------------------|-------------|------------|--------------|-----------|--------------|------------|----------------|------------------|------------|
| K\(^+\) | 6.5 | 9.0 | 2.69 | 32.9 | 613.0 | 452.9 | 920.0 | 2163.0 | 2089.3 |
| Na\(^+\) | 135.2 | 218.8 | 3939.0 | 80570 | 9884.0 | 25770.0 | 54091.0 | 77990.0 | 103602.3 |
| Ca\(^{2+}\) | 38.5 | 20.0 | 177 | 32.4 | 22.8 | 392.0 | 625.0 | 17110 | 823.2 |
| Mg\(^{2+}\) | 53.6 | 69.5 | 824.1 | 21070 | 14670 | 40570.0 | 2988.7 | 24700.0 | 9740.8 |
| SO\(_4^{2-}\) | 140.4 | 1179 | 2188.0 | 6454.0 | 6058.0 | 10730.0 | 28735.2 | 4462.0 | 17039.8 |
| Cl\(^-\) | 206.2 | 2311.0 | 5625.7 | 13709.5 | 13004.7 | 41099.4 | 7263.4 | 1962319 | 167633.2 |
| CO\(_2\) | 0.0 | 48.6 | 405.0 | 418.0 | 515.4 | 113.9 | 388.1 | 40L | 80L |
| HCO\(_3\) | 149.8 | 26.55 | 854.1 | 7679 | 824.1 | 312.2 | 380.1 | 359.6 | 153.1 |
| NO\(_3\) | 0.5 | 0.2 | 0.4 | 0.4 | 0.5 | 0.6 | 0.6 | 1.1 | 1.4 |
| NH\(_3\) | 10.0 | 12.0 | 1.0 | 0.8 | 0.6 | 0.5 | 0.6 | 0.4 | 0.4 |
FIGURE 2 | The abundances (copies per gram of sediment or copies per milliliter of water) of total archaeal 16S rRNA and amoA genes and thaumarchaeol concentrations (nanogram per liter of water and nanogram per gram of sediment). Panels (A) and (B) are for waters and sediments, respectively.

2.33 × 10^9 copies g^{-1} for the waters and sediments, respectively (Figure 2). The amoA gene abundance ranged from 2.81 × 10^4 to 3.07 × 10^8 copies mL^{-1} and 7.34 × 10^4 to 4.13 × 10^6 copies g^{-1} for the waters and sediments, respectively (Figure 2). The highest water and sediment 16S rRNA archaeal abundances were observed in the water of Tuosu Lake (4.15 × 10^6 copies mL^{-1}) and the sediment of Qinghai Lake (2.33 × 10^9 copies g^{-1}); whereas the highest water and sediment archaeal amoA gene abundances were observed in the water of Gahai Lake 1 (3.07 × 10^8 copies mL^{-1}) and the sediment of Tuosu Lake (4.13 × 10^6 copies g^{-1}). In addition, the amoA gene abundance exhibited a significant correlation (CL: r = 0.879, P = 0.009; PL: r = 0.928, P = 0.003) with thaumarchaeol abundance for the sediment samples (except Lake Chaka, where no amoA gene abundance data were available; Figure 3).

AMO A GENE PHYLOGENETIC ANALYSIS
A total of 283 (187 and 96 from the two primer sets of Arch-amoaF/Arch-amoaR and CrenamoA23f/CrenamoA616r, respectively) amoA gene clone sequences were obtained (Table 3).
Table 3 | Ecological estimates of AEA amoA gene clone sequences amplified with primer sets of Arch-amoAF/Arch-amOAR (Francis et al., 2005) and CrenamoA23f/CrenamoA616r (Tourna et al., 2008; as indicated by the numbers before and after the backslashes, respectively).

| Clone libraries               | EHL-1-W | GHL-14-W* | GHL1-52-W | GHL2-94-W | XCDL-160-W | EHL1-5-S | GHL-14-S* | GHL1-32-S | GHL2-94-S | XCDL-160-S |
|-------------------------------|---------|-----------|-----------|-----------|------------|----------|-----------|-----------|-----------|------------|
| Library size (no. of clones)  | 24/12   | 19/ND     | 23/9      | 20/8      | 24/11      | 25/13    | 24/ND     | 24/12     | 23/16     | 24/15      |
| Coverage (%)                  | 73/93   | 100/ND    | 100/100   | 90/100    | 83/82      | 96/92    | 100/ND    | 100/100   | 87/87     | 79/93      |
| No. of observed OTUs (98% cutoff) | 12/5  | 3/ND      | 2/3       | 1/5       | 4/4        | 6/ND     | 2/1       | 8/6       | 10/6       |
| Chao 1                        | 19/05.5 | 3/03.0    | 7/36NA    | 4/05.5    | 4/04.0     | 2/06NA   | 9.5/03.0  | 15/06.5   | 5/04.0    |
| Simpson’s diversity index (D) | 0/10.2  | NA/ND     | 0/03.0    | 0/02.0    | 0/03.0     | NA/ND    | 0/02.0    | 0/10.2    | 1/03.0    |
| Shannon-Weaver’s diversity index (H) | 2.2/14 | 1.0/ND    | 1.0/11    | 1.8/ND    | 1.9/11     | 1.1/1.1 | 1.7/ND    | 0.5/ND    | 1.9/15     | 2.0/16     |

*Clone libraries GHL-W and GHL-S corresponded to QL W1-0 and QLS-30 in Jiang et al. (2009b), respectively; ND, not determined; NA, not available.
The amoA gene clone sequences derived from the primer set of Arch-amoAF/Arch-amoAR were clustered into 55 OTUs: 12, 3, 7, and 9 for the water samples and 4, 2, 8, and 10 for the sediment samples of Erhai Lake, Gahai Lake 1, Gahai Lake 2, and Xiaochaidan Lake, respectively (Table 3). The coverage ranged from 72 to 100% for the amoA gene clone libraries (Table 3). Diversity indices were 2.0–19.0, 0.1–0.7, and 0.5–2.2 for Chao1, Simpson, and Shannon–Weaver, respectively (Lake 1, Gahai Lake 2, and Xiaochaidan Lake, respectively (and 4, 1, 6, and 6 for the sediment samples of Erhai Lake, Gahai Lake 1, Gahai Lake 2, and Xiaochaidan Lake, respectively (Table 3). The coverage ranged from 82 to 100% for the amoA gene clone libraries (Table 3). Diversity indices were 3.0–6.5, 0.2–0.4, and 1.1–1.6 for Chao1, Simpson, and Shannon–Weaver, respectively (Table 3). In comparison, the amoA gene diversity derived from CrenamoA23f/CrenamoA616r was lower than that from the Arch-amoAF/Arch-amoAR primer set. These amoA gene clone sequences fell into 31 OTUs: 5, 3, 1, and 5 for the water samples, and 4, 1, 6, and 6 for the sediment samples of Erhai Lake, Gahai Lake 1, Gahai Lake 2, and Xiaochaidan Lake, respectively (Table 3). The coverage ranged from 82 to 100% for the amoA gene clone libraries (Table 3). Diversity indices were 3.0–6.5, 0.2–0.4, and 1.1–1.6 for Chao1, Simpson, and Shannon–Weaver, respectively (Table 3). The LIBSHUFF analysis showed that thearchal amoA gene clone libraries were grouped into two separate clusters (one each for the waters and sediments, respectively, P-value < 0.01; Figure 4A).

The amoA gene clone sequences obtained from the waters were grouped into the Nitrosopumilus clusters (subcluster 1.1, 2.1, and 2.2, respectively). These amoA gene clone sequences were clustered into 14 OTUs (approximately 98% identity) to the clones retrieved from diverse environments, such as drinking water treatment plants (van der Wielen et al., 2009), Tibetan marsh wetland (unpublished), estuary sediments (Beman and Francis, 2006), Qinghai Lake sediments (Jiang et al., 2009b), and an AOA isolate Nitrosopumilus viennensis EN76 (Toursina et al., 2011).

For the sediment samples, the obtained amoA gene clone sequences were grouped into the Nitrosopumilus clusters (subcluster 1.1, 2.1, and 2.2, respectively) and a low-salinity cluster (Mosier and Francis, 2008; Figures 4B and 5A). The low-salinity cluster was the pre-dominant component (accounting for 62.6%) in the total water amoA gene clone sequences. The amoA gene clone sequences in the low-salinity cluster had high identity (~98%) with the clones from the San Francisco Bay estuary (Francis et al., 2005; Mosier and Francis, 2008) and low-salinity ammonia-oxidizing archaeon “Candidatus Nitrosoarchaeum limnia” (Blainey et al., 2011). The amoA gene clone sequences in the Nitrosopumilus cluster were closely related (~98% identity) to the clones retrieved from diverse environments, such as drinking water treatment plants (van der Wielen et al., 2009), Tibetan marsh wetland (unpublished), estuary sediments (Beman and Francis, 2006), Qinghai Lake sediments (Jiang et al., 2009b), and an AOA isolate Nitrosopumilus viennensis EN76 (Toursina et al., 2011).

In comparison, the amoA gene diversity derived from the water samples and 4, 2, 8, and 10 for the sediment samples of Erhai Lake, Gahai Lake 1, Gahai Lake 2, and Xiaochaidan Lake, respectively (Table 3). The coverage ranged from 82 to 100% for the amoA gene clone libraries (Table 3). Diversity indices were 2.0–19.0, 0.1–0.7, and 0.5–2.2 for Chao1, Simpson, and Shannon–Weaver, respectively (Lake 1, Gahai Lake 2, and Xiaochaidan Lake, respectively (Table 3). In comparison, the amoA gene diversity derived from CrenamoA23f/CrenamoA616r was lower than that from the Arch-amoAF/Arch-amoAR primer set. These amoA gene clone sequences fell into 31 OTUs: 5, 3, 1, and 5 for the water samples, and 4, 1, 6, and 6 for the sediment samples of Erhai Lake, Gahai Lake 1, Gahai Lake 2, and Xiaochaidan Lake, respectively (Table 3). The coverage ranged from 82 to 100% for the amoA gene clone libraries (Table 3). Diversity indices were 3.0–6.5, 0.2–0.4, and 1.1–1.6 for Chao1, Simpson, and Shannon–Weaver, respectively (Table 3). The LIBSHUFF analysis showed that thearchal amoA gene clone libraries were grouped into two separate clusters (one each for the waters and sediments, respectively, P-value < 0.01; Figure 4A).

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the San Francisco Bay sediment (Francis et al., 2005; Mosier and Francis, 2008) and drinking water treatment plants (van der Wijst et al., 2009). In addition, the cDNA-based AEA amoA gene clone sequences (n = 12) from Xiaoaihaidan Lake were grouped into one OTU and were affiliated with S_1.1 subcluster within Nitrososphaera (Figure S8).

**Statistical Analysis**

The abundances of amoA gene and thaumarchaeol did not show any significant correlations with any of the measured environmental variables, such as pH, salinity, salinity-related ions, ammonium, nitrate, and sulfide (data not shown). No significant correlation was observed between the amoA gene composition (at the 98% similarity OTU level) and water chemistry (data not shown). The simple Mantel test showed no significant correlation between AEA composition and salinity at the 98% similarity OTU level (water samples: r = 0.110, P = 0.072; sediment samples: r = 0.587, P = 0.132).

Cluster analysis showed that the AEA communities in the lakes (including the saline/hypersaline lakes in this study and the freshwater lakes in other studies) within China were grouped into one cluster, separated from other freshwater lakes (except for the high Arctic Lake C1) around the world (e.g., Canada, Congo, Spain, Denmark; Figure 6). In addition, the AEA communities in Tibetan lakes exhibited little similarity to those in other saline habitats, such as the waters of Monterey Bay, the Eastern Tropical North Pacific, the Black Sea, Arctic Ocean, and Antarctic coasts (Figure 7A) and sediment samples from Elkhorn Slough, and Huntington Beach of California, Bahia del Tíchar of Mexico, San Francisco Bay, and the tropical West Pacific Continental Margin and a Oak Ridge soil (Figure 7B).

**Discussion**

**Occurrence of Thaumarchaeol in Hypersaline Qinghai-Tibetan Lakes**

Thaumarchaeol was observed from the waters and sediments of Gabai Lake2, Xiaoaihaidan Lake, and Lake Chaka (salinity: 84, 160, and 323 g.L⁻¹, respectively). Their salinities were much higher than those in other lakes where thaumarchaeol has been reported (Blaga et al., 2009; Sinninghe Damsté et al., 2009; Bechtel et al., 2010; Tierney et al., 2010; Pearson et al., 2011; Buckles et al., 2013). Many previous studies indicated that thaumarchaeol is a specific membrane lipid biomarker of Thaumarchaeota (Pearson et al., 2004; Zhang et al., 2006; Peiter et al., 2011; Pichler et al., 2011a; Sinninghe Damsté et al., 2012). So far, thaumarchaeol has not been discovered in other archaea suggesting that thaumarchaeol may be used as a characteristic tracer for Thaumarchaeota in the environment. A possible explanation for the occurrence of thaumarchaeol in such high-salinity lakes is that some AOA in Tibetan lakes may have adapted to higher salinity than that of seawater. This possibility was supported by the presence of archaeal amoA gene in these lakes, except for the Lake Chaka (323.0 g.L⁻¹) where the amoA gene could not be amplified. Indeed, the hypersaline Lake Chaka is optimal for halophilic Euryarchaeota but not for Crenarchaeota/Thaumarchaeota (Jiang et al., 2006, 2007; August et al., 2009). However, we observed higher PL thaumarchaeol concentration in the Lake Chaka sediment than in other hypersaline lakes. The inconsistency between amoA gene result and thaumarcheota data in Lake Chaka could be ascribed to the following possible reasons: (1) different detection limits between these two methods (qPCR vs. lipid biomarker); (2) preferential degradation of DNA/RNA relative to lipid (Castetada and Schouten, 2011; Schouten et al., 2013); PL-GDGTs may contain a number of GDGTs that have sugar or phosphate groups, and a majority of the PLs (especially glycolipids) in environmental samples might be a result of the selective preservation of fossil lipids (Schouten et al., 2010); (3) the PCR primers used in this study may be limited to amplify all AEA amoA genes. For example, the amoA genes from genus Nitrososphaera cannot be detected with the primers used in this study (de la Torre et al., 2008); (4) additional sources of thaumarcheota other than AEA; (5) transport of thaumarchaeota from surrounding soils by rain runoff and dust particles by wind, where amoA gene may have been preferentially degraded. Lake Chaka is a small and shallow lake that is surrounded by soil. When PL GDGTs from surrounding soil was washed into the lake, it can quickly deposit into sediment. GDGTs are difficult to degrade even under anoxic conditions (Kim et al., 2009) and thus they can be preserved in sediments for million years (Schouten et al., 2013). However, further investigation is required for the exact reasons for the presence of high GDGT in the Qinghai-Tibetan hypersaline lakes.

**AEa Community Composition in Qinghai-Tibetan Lakes and Their Response to Environmental Changes**

In recent years, there have been numerous studies to reveal AEA diversity and community composition in freshwater lakes and their relationships with environmental conditions (Buckles et al., 2010; Tierney et al., 2010; Pearson et al., 2011; Buckles et al., 2013). Many previous studies indicated that thaumarchaeol is a specific membrane lipid biomarker of Thaumarchaeota (Pearson et al., 2004; Zhang et al., 2006; Peiter et al., 2011; Pichler et al., 2011a; Sinninghe Damsté et al., 2012). So far, thaumarchaeol has not been discovered in other archaea suggesting that thaumarchaeol may be used as a characteristic tracer for Thaumarchaeota in the environment. A possible explanation for the occurrence of thaumarchaeol in such high-salinity lakes is that some AOA in Tibetan lakes may have adapted to higher salinity than that of seawater. This possibility was supported by the presence of archaeal amoA gene in these lakes, except for the Lake Chaka (323.0 g.L⁻¹) where the amoA gene could not be amplified. Indeed, the hypersaline Lake Chaka is optimal for halophilic Euryarchaeota but not for Crenarchaeota/Thaumarchaeota (Jiang et al., 2006, 2007; August et al., 2009). However, we observed higher PL thaumarchaeol concentration in the Lake Chaka sediment than in other hypersaline lakes. The inconsistency between amoA gene result and thaumarcheota data in Lake Chaka could be ascribed to the following possible reasons: (1) different detection limits between these two methods (qPCR vs. lipid biomarker); (2) preferential degradation of DNA/RNA relative to lipid (Castetada and Schouten, 2011; Schouten et al., 2013); PL-GDGTs may contain a number of GDGTs that have sugar or phosphate groups, and a majority of the PLs (especially glycolipids) in environmental samples might be a result of the selective preservation of fossil lipids (Schouten et al., 2010); (3) the PCR primers used in this study may be limited to amplify all AEA amoA genes. For example, the amoA genes from genus Nitrososphaera cannot be detected with the primers used in this study (de la Torre et al., 2008); (4) additional sources of thaumarcheota other than AEA; (5) transport of thaumarchaeota from surrounding soils by rain runoff and dust particles by wind, where amoA gene may have been preferentially degraded. Lake Chaka is a small and shallow lake that is surrounded by soil. When PL GDGTs from surrounding soil was washed into the lake, it can quickly deposit into sediment. GDGTs are difficult to degrade even under anoxic conditions (Kim et al., 2009) and thus they can be preserved in sediments for million years (Schouten et al., 2013). However, further investigation is required for the exact reasons for the presence of high GDGT in the Qinghai-Tibetan hypersaline lakes.

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FIGURE 5 | Maximum likelihood tree (partial sequences, 635 or 629 bp) showing the phylogenetic relationships of the amoA gene clone sequences obtained in this study to their closely related sequences from the GenBank database. One representative clone type within each OTU is shown, and the number of clones within each OTU is shown in parentheses. If there is only one clone sequence within a given OTU, the number “1” is omitted. The sequences from this study are bolded, and they are coded as follows for the example of XCDL-S-AOA-17: amoA sequences of clone no. 17 from the Xiaochaidan Lake sediment. Clone libraries QHL-14-W and QHL-14-S (Continued)
FIGURE 5 | Continued
were corresponded to QUV1.0 and QLS-30 in Jiang et al. (2009b), respectively. The "R" symbol in some clone names denotes RNA-based (cDNA) clones. The underlined clone sequences were derived from the CrenamoA23f/CrenamoA616r primer set. The classification system of Pester et al. (2012) was employed. The letters "S" and "U" in the cluster names indicated "subcluster" and "unclassified." The scale bars indicate the Jukes–Cantor distances. Bootstrap values of (1000 replicates) >50% are shown. The bacterial amoA gene from Nitrosococcus oceani was used as outgroup. Panels (A) and (B) are for waters and sediments, respectively.
FIGURE 6 | Jaccard similarity-based cluster analysis of the amoA gene communities in different lakes worldwide. The archaeal amoA gene sequences obtained in this study were combined with those previously reported for Qinghai Lake (Jiang et al., 2009b), Lake Taihu (Wu et al., 2010), high arctic lake (Pouliot et al., 2009), Lake Kivu (Llirós et al., 2010), Spanish lakes (Auguet et al., 2011), and Danish lakes (Herrmann et al., 2009). The numbers of clones from each location are given in Table 1. “W” and “S” indicate water and sediment samples, respectively.

study were highly similar to one other, suggesting that unique environmental conditions (high elevation, strong ultraviolet and dry climate) of the Qinghai–Tibet Plateau may have limited the AEA community diversity in these lakes to a low level.

Many studies have suggested that AOA (Fernández-Guerra and Casamayor, 2012) ecological niches are affected by various environmental factors, such as dissolved oxygen (DO), temperature, and salinity (see review by Erguder et al., 2009 and references therein). In this study, salinity and other water chemistry did not have significant correlations with AEA community diversity. However, the difference was observed in the AEA communities between waters and sediments (Figure 3), suggesting that the AEA in the sediments were native, and they were not derived from the water column. This observed difference between water and sediment was consistent with previous studies (Francis et al., 2005; Beman and Francis, 2006; Jiang et al., 2009b) and may be ascribed to the fact that water and sediment are different habitats with different environmental conditions. However, with limited data, it is not realistic to identify which factor accounts for the observed difference in the amoA gene communities between water and sediments.

Furthermore, the AEA community composition in the studied Qinghai–Tibetan lakes was different from those in other lakes (Figure 6) and saline environments (Figure 7) worldwide, suggesting that the Qinghai–Tibetan lakes are a unique habitat (e.g., high elevation, strong UV exposure, and dry climate) and thus AEA in these lakes may possess different evolutionary history than their counterparts in other ecosystems.
FIGURE 7 | Jaccard similarity-based cluster analysis of the amoA gene communities in different habitats worldwide. Panels (A) and (B) are for water and sediment/soil samples, respectively. (A) The archaeal amoA gene sequences obtained in this study were compared with those previously reported in the waters of Monterey Bay, the Eastern Tropical North Pacific, and the Black Sea (Francis et al., 2005), and Arctic Ocean and Antarctic coasts (Kalanetra et al., 2009).

(B) The archaeal amoA gene sequences obtained in this study were compared with those previously reported in the sediments from Elkhorn Slough, and Huntington Beach of California, Bahía del Tóbari of Mexico, and the San Francisco Bay (Francis et al., 2005), the tropical West Pacific Continental Margin (Dong et al., 2009), and an Oak Ridge soil (Francis et al., 2005). The numbers of clones from each location are given in Table 1.

COMPARISON BETWEEN THE TWO PRIMER SETS FOR AEA amoA GENES

The primer set of CrenamoA23F/CrenamoA616r was originally designed by Tourna et al. (2008) on the basis of the soil fosmid 54d9 (Treusch et al., 2005) and the Sargasso Sea data set (Ven ter et al., 2004). Subsequently it has been used to successfully amplify AOA amoA genes from soil samples (Tourna et al., 2008; Hallin et al., 2009; Stopnišek et al., 2010; Yao et al., 2011; Zhang et al., 2012). However, in this study higher AEA amoA gene diversity was obtained with the use of the primer set of Arch-amoAF/Arch-amoAR (Francis et al., 2005). Furthermore, non-singleton OTUs derived from the CrenamoA23F/CrenamoA616r primer set were already present within the clone libraries constructed from the Arch-amoAF/Arch-amoAR primer set (Figure 5). These lines of evidence suggest that the primer set of Arch-amoAF/Arch-amoAR is more appropriate than CrenamoA23F/CrenamoA616r in characterizing the AEA diversity in the Qinghai–Tibetan lakes.

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