Effect of salinity on the community structure and ecological functions of Archaea in Liaohe Estuary

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Abstract. Archaea plays an important role in biogeochemical processes. Using the quantitative polymerase chain reaction (qPCR) and Illumina high-throughput sequencing, eight sediment samples collected in Liaohe Estuary were used to investigate the effects of salinity gradient on the archaeal community structure and ecological functions. The results showed that archaeal abundance ranged from $5.1 \times 10^5$ to $1.3 \times 10^6$ copies/g. Archaeal community included the classes of Thaumarchaeota (69%), Euryarchaeota (29%), and Crenarchaeota (0.01%), respectively. Methanogens in the genera Methanococcoides, Methanoregula, and Methanoseta and Marine Group I, Candidatus Nitrososphaera, and Nitrososphaera within the ammonia-oxidizing archaea were abundant in freshwater sediments, whereas Marine Benthic Group B and Marine Benthic Group D were abundant in seawater sediments. The positive correlation was observed in between the abundances of Euryarchaeota and salinities ($P < 0.05$). Physico-chemical analysis in the sediments showed that the temperature and salinity were impacted on the abundance and distribution of archaea significantly ($P < 0.05$). In general, these results have broadened our understanding on the changes of the archaeal abundance and community structure in estuarine sediment along the salinity gradient.

1. Introduction

The domain of archaea represents the third line of evolutionary descent, which has separated from the domain of Bacteria and Eukarya. Members of the archaea have been linked to a variety of biogeochemical processes, such as ammonia oxidation, sulfate reduction, and methanogenesis [1]. Two ammonia-oxidizing archaea (AOA), Candidatus Nitrosoarchaeum limnia BG20 and SFB1 [2], were found in sediments from the San Francisco Bay Estuary, and they represent a clade of low salinity species. Marine Group I, a major group capable of aerobic ammonia oxidation, contains abundant groups of marine archaea [3]. Methanogens were found to be well adapted to high salinity but contributed little to overall anaerobic mineralization. Using hydrogen as a reductant, most methanogens (termed hydrogenotrophs) can reduce carbon dioxide to methane [4]. However, the community structure compositions of archaea are still unclear along the salinity gradients from the freshwater in the estuary to the ocean. And the effects of salinity on the community structure and ecological functions of archaea remain largely unknown.

Estuaries are ideal ecosystems to study the response of different microbial phylotypes to physico-chemical variations such as sharp salinity and nutrient gradients in the mixing regions with freshwater and saltwater. The ecological functions in the estuarine are largely affected by the special microbial conditions and the interactions among different microbial communities. The study of the effects of salinity on the archaeal community structure and ecological functions in estuarine sediment will provide insights into the biogeochemical processes and ecosystem functions in estuarine environments.
compositions [5], thus, understanding of how microbial community structure changes in response to physical-chemical variations is critical delineating their roles in the dynamic estuarine environment. However, the archaeal ecology is complicated in estuaries, and the relationship remains unclear between archaeal community structure/ecological function and the salinity gradient.

Liaohe Estuary is located in the southern part of the Liaohé Plain on the north coast of the Bohai Sea in China. To investigate the archaeal community and how ecological function might be influenced by salinity or others factors, eight sediment samples were collected along the salinity gradient from freshwater to seawater. The objective of this study was to describe the abundance and community composition of sediment-associated archaea in these samples and to assess potential ecological function of these archaea in the Liaohe Estuary using illumina Miseq sequencing of the archaeal 16S rRNA gene and quantitative PCR (qPCR) methods.

2. Material and methods

2.1. Collection of sediment

Eight surface sediment samples were collected from the Liaohe Estuary in October of Autumn, 2013, which was the normal water period (Figure 1). Water temperature, salinity, and pH were measured in situ using a YSI instrument. The pore water was filtered through 0.45 µm filters and stored in liquid nitrogen. The levels of nitrate as nitrogen (N-NO$_3^-$), ammonium as nitrogen (N-NH$_4^+$), phosphate as phosphorous (P-PO$_4^{3-}$), silicate as silica (Si-SiO$_2^{2-}$), total nitrogen (TN), and total phosphorous (TP) were measured in the laboratory using “The Specification for Marine Monitoring: Part 4: Seawater Analysis” [6].

![Figure 1. Map of Liaohe Estuary showing sample sites.](image-url)

2.2. DNA extraction and qPCR of archaea

Genomic DNA was extracted from 0.5 g of each sediment sample using a Rapid Soil DNA Isolation Kit. qPCR of the archaea in the sediment samples was conducted using the SYBR green method and an ABI 7500 real time PCR machine (Applied Biosystems, Foster City, CA, USA). The primers were Arch-519 (5'-CAGCCGCCGCGGTAA-3') and Arch-915 (5'-GTGCTCCCCCGCCAATTTC-3') [7]. All qPCRs were performed using the following reagents: 10 µL of 2×SYBR® MightyAmp™
(TaKaRa, Japan), 0.4 μL of ROX Reference Dye II, 0.6 μL of each primer, and 2.0 μL of template DNA. The thermocycling steps were set as follows: 95 °C for 30 s and 40 cycles of 95 °C for 5 s, 55 °C for 30 s, and 72 °C for 34 s. The last extension step was collected the fluorescence. The quantitation standard samples of purified plasmid DNA came from a cloned archaeal gene isolated from site 5. Each sample and standard was run in triplicate. PCR amplification efficiencies were 80.5%, and standard curve correlation coefficients ($R^2$) were > 0.99.

2.3. Illumina Miseq sequencing analysis of archaea gene amplicons

Archaeal 16S rRNA sequencing used Illumina Miseq platform. The primers were Arch-519 and Arch-915. The reads with sequencing adapters, N bases, poly bases, or low quality were filtered out using default parameters. The reads or quality filtered sequences were clustered into Operational Taxonomic Units (OTUs) with a 97% sequence identity threshold using Mothur software (V.1.33.3) (http://www.mothur.org/wiki/Main_Page). Sequence reads were compared with a SILVA database of known archaeal 16S rRNA genes and taxonomically assigned according to the Mothur classifier [8]. The nucleotide sequences obtained accession number were SRP100302, which deposited in the Sequence Read Archive of the National Center for Biotechnology Information.

2.4. Statistical analysis

Based on the number of OTU, alpha (ace, Chao, coverage, Shannon, Simpson) and beta estimates were calculated in Mothur. The rarefaction analysis of archaeal 16S rRNA gene sequences were conduct using R software (V3.0.3). According to the OTU classified, Neighbor-joining phylogenetic tree and the LDA score also were drawn by R software. The relationship between the distribution of archaeal phylotypes and environmental factors was explored using redundancy analysis (RDA) and Canoco software (V5.0).

3. Results

3.1. Physical and chemical properties of the sediment

Physical and chemical properties of the eight sediment samples in Liaohe Estuary were listed in Table 1. Salinity increased from 10.22‰ (site 3) to 26.11‰ (site 28). The pH was about 8.1 and did not differ greatly among sites. The temperature ranged from 15.90 to 18.22 °C. Nutrient-related parameters in the sediment varied along the Liaohe Estuary. The N-NO$_3^-$, P-PO$_4^{3-}$, Si-SiO$_3^{2-}$, TN, and TP values generally exhibited a similar pattern, ranging from high concentrations inshore (site 3) to low concentrations offshore (site 28). However, the N-NH$_4^+$ concentrations were the opposite. The reason may be due to the increase of suspended solids in estuarine sediments, which provide a suitable habitat for microorganisms. It shows that the adsorption capacity of the particles for ammonia nitrogen is increased, and the nitrification rate is accelerated, resulting in the phenomenon that the ammonia nitrogen content is low and the nitrate nitrogen content is high near the shore.

Table 1. Data of environmental factors in Liaohe Estuary.

| Site | pH  | Water Temperature(°C) | Salinity (%) | N-NH$_4^+$ (ug/L) | P-PO$_4^{3-}$ (ug/L) | Si-SiO$_3^{2-}$ (mg/L) | N-NO$_3^-$ (ug/L) |
|------|-----|----------------------|--------------|-------------------|----------------------|------------------------|-------------------|
| 3    | 8.14| 15.95                | 10.22        | 19.45             | 503.61               | 2418.47                | 930.76            |
| 4    | 8.16| 15.90                | 11.31        | 30.53             | 474.01               | 2559.18                | 849.38            |
| 5    | 8.12| 16.13                | 15.53        | 24.42             | 466.9                | 1954.49                | 737.42            |
| 7    | 8.10| 16.22                | 18.68        | 20.94             | 468.09               | 1587.24                | 695.79            |
| 9    | 8.11| 16.39                | 20.19        | 28.25             | 453.88               | 1606.53                | 668.32            |
| 15   | 8.09| 16.48                | 21.97        | 15.69             | 455.06               | 1531.22                | 621.64            |
| 29   | 8.10| 18.12                | 25.06        | 37.27             | 12.54                | 440.85                 | 480.79            |
| 28   | 8.10| 18.22                | 26.11        | 40.86             | 14.35                | 370.99                 | 325.08            |
3.2. Quantification of archaea
qPCR results showed that the abundance of archaea ranged from $5.1 \times 10^5$ to $1.3 \times 10^6$ copies per gram of sediment. Sites 3, 4, and 5, which were located in the nearshore area had lower values of archaeal 16S rRNA (order $10^5$). While the offshore sites had higher values (reach to $10^6$).

3.3. Diversity of archaea
A total of 334,097 effective sequences were obtained in all samples. The normalized Shannon and Simpson indices were used to assess the sediment archaeal community diversity, and they ranged from 3.13 to 5.80 and from 0.02 to 0.26, respectively. The Shannon and Simpson index values offshore (sites 3, 4, 5, 7, 9, and 15) were lower than those inshore (sites 29 and 28).

3.4. Taxonomy and relative abundance of archaea present in the sediment samples

![Figure 2](image1.png)  
**Figure 2.** Proportions of phylum (a) and genus (b) information in different samples.

![Figure 3](image2.png)  
**Figure 3.** Evolutionary branch map and abundance histogram of archaea in the sediments of the Liaoh Estuary based on LEfSe analysis.

Three phyla, 5 classes, 7 orders, 8 families, and 25 genera were discovered. All samples were divided into three phyla: Thaumarchaeota (69%) and Euryarchaeota (29%), with a few Crenarchaeota (0.01%) and unclassified groups (Figure 2a). At the phylum level, Thaumarchaeota included mainly Marine Benthic Group B (3.23%) and Group C3 (5.2%), Marine Group I (49.62%), and Miscellaneous Crenarchaeotic Group (10.4%). Euryarchaeota mainly consisted of archaeal CCA47 (1.18%), Deep
Sea Euryarchaeotic Group (0.09%), Deep Sea Hydrothermal Vent Group 6 (19.9%), Marine Benthic Group D and DHVEG 1 (3.39%), and Marine Hydrothermal Vent Group (0.61%). Crenarchaeota accounted for only 0.01% of all archaea. At the class level, archaea included Thermoplasmata (6.7%), Halobacteria (21.4%), Methanomicrobia (0.08%), Methanobacteria (0.01%), and Thermoprotei (0.01%). At the genus level, most (83%) belonged to yet-uncultivated lineages (Figure 2b). This result shows that the majority of archaeal phylotypes were unidentified because of the lack of saturation of the OTU diversity of the samples.

A clear transition of archaeal community composition was observed from the low salt area (A group: sites 3, 4, 5, and 7) to the high salt area (B group: sites 9, 15, 29, and 28), as shown in the evolutionary branch map and abundance histogram of archaea (Figure 3). Methanobacteriaceae, Methanobacteriales, Methanobacteria, Methanoregulaceae, Methanomicrobiales, Candidatus Nitrosoarchaeum, and Candidatus Nitrosopumilus were the dominant archaea in the A group, whereas Halobacteriales uncultured archaeon CLEAR 24 was the dominant archaea in group B.

3.5. Physico-chemical control of the distribution of archaea

The relative abundance of Euryarchaeota was positively correlated with salinity ($P < 0.05$). In contrast, the relative abundance of Thaumarchaeota and Crenarchaeota was higher in freshwater than in saltwater sites. Of the Thaumarchaeota, Group C3 was positively correlated with salinity ($P < 0.05$), but the high number of Marine Group I were negatively correlated with salinity. The highest and lowest abundance of Marine Group I existed at the low salinity site (58.3%, site 3) and high salinity site (16.5%, site 28), respectively. Abundance of Candidatus Nitrosopumilus increased from the low to the high salinity site, but Candidatus Nitrososphaera was found mainly at the high salinity sites (sites 28 and 29) (Figure 4). Candidatus Nitrosoarchaeum and Cenarchaeum were only found at the low salinity sites and were present in low numbers.

Among the Euryarchaeota, the abundance of CCA47 was positively correlated with salinity ($P < 0.05$). The Deep Sea Hydrothermal Vent Group 6 constituted a large proportion of the Euryarchaeota at all sites (11.6%–21.1%). Marine Benthic Group B and Marine Benthic Group D and DHVEG-1 mainly were present in the high salinity area. Natronomonas, Methanoregula, Methanimicrococcus, and Methanobacterium were absent in the relatively low salinity area (sites 3, 4, 5, and 7). The only Crenarchaeota found belonged to class Thermoprotei and were present at sites 3 and 5.
Salinity clearly affected the archaeal community composition. Sites 3, 4, and 7 were similar to sites 5, 9, and 15, which were located in the low salinity area, whereas the composition of the high salinity area (sites 28 and 29) was significantly different from the others (Figure 4). The freshwater group was characterized by *Candidatus Nitrosopumilus*, *Natronomonas*, *Thermoprotei*, and the other three methanogens, whereas the marine group was characterized by Group C3, *Halorielalis*, CCA47, Marine Benthic Group B, Marine Benthic Group D and DHVEG-1, Deep Sea Euryarchaeotic Group, Marine Hydrothermal Vent Group, Miscellaneous Crenarchaeotic Group, and Marine Benthic Group E. However, the abundance of *Candidatus Nitrosopumilus*, *Candidatus Nitrososphaera*, nitrogen-fixing archaea, and *Methanosaeta* decreased significantly ($P < 0.01$) with increasing salinity, indicating that the predominant ecological function of the archaea changed from nitrogen fixation and CO$_2$ fixation in the low salinity area to only ammonium oxidation in the high salinity area.

The RDA could explain 99.4% of the total variation (Figure 5). Only temperature and salinity had a significant ($P < 0.05$) impact on the distribution of archaea in Liaohe Estuary sediment, the composition of sediment archaeal communities in the Liaohe Estuary was significantly correlated with the tested parameters as follows: temperature > salinity > pH > Si-SiO$_2^{-}$ > N-NO$_3^{-}$ > P-PO$_4^{3-}$.

4. Discussions

The boundary between freshwater and marine environments is a transition barrier for archaea, and archaeal diversities in the estuaries are known to be affected by salinity [9]. In this study, we used Illumina Miseq high-throughput sequencing to explore the distribution of archaea in sediments along the salinity gradient of the Liaohe Estuary. The species composition was dominated by Thaumarchaeota and Euryarchaeota in each of the eight sites.

Thaumarchaeota present in the sediments mainly included Marine Benthic Group B, Marine Group I, Miscellaneous Crenarchaeotic Group, and Group C3. In the present study, the number of OTUs in the higher salinity area was higher than that at the other sites, and the high percentage of Marine Benthic Group B in the estuary was consistent with a previous finding of archaea in the sediment [10]. Most of the Marine Group I members are typically found in the sediment environment. Marine Group I members with unique ammonia monoxygenase subunit A genes are widely distributed in seawater and sediment, suggesting that they play an important role in the global nitrogen cycle. Marine Group I members tend to live in the oxidation zone of surface sediments, which is consistent with their aerobic metabolism and autotrophic way of life [11]. Marine Group I taxa were more abundant in the low salinity area than in the higher salinity waters. Incubation studies have confirmed the autotrophic capacity of Marine Group I members, suggesting that they are heterotrophic or mixotrophic [12]. The Miscellaneous Crenarchaeotic Group was less abundant than Marine Group I, and abundance was lower inshore than offshore in this study. Phylogenetic analysis has shown that the Miscellaneous Crenarchaeotic Group is widely distributed in the anaerobic sediment from shallow to deep marine sediments, where it is metabolically active [13]. Abundance of Group C3 in Liaohe Estuary sediment increased from low salinity to high salinity, which was consistent with results from the Pearl River Estuary [14].

Euryarchaeota in the sediment samples mainly consisted of Deep Sea Hydrothermal Vent Group 6, Marine Benthic Group D and DHVEG 1, CCA47, Marine Hydrothermal Vent Group, and Deep Sea Euryarchaeotic Group. Deep Sea Hydrothermal Vent Group 6 constituted a large proportion of the total at all sites and mainly came from deep-sea sediments and others have reported a relatively high abundance of Deep Sea Hydrothermal Vent Group 6 in estuaries [15], suggesting that those archaeal groups are adaptable to the marine-freshwater boundary. Marine Benthic Group D taxa occur in surface sediments of many marine environments, but to date they have not been detected in seawater. Marine Benthic Group D can use receptors other than sulfate, such as Fe$^{3+}$, and Mn$^{4+}$ anaerobic methane oxidation has been confirmed. The 16S rRNA sequence of this group is most similar to that of methanogens (80% similarity), indicating that they play an important role in the methane cycle [16]. Marine Benthic Group D is an important evolutionary branch related to Thermoplasmatales, and it was first found on the Atlantic slope and the abyssal plain of New England. The group may live mainly in
deep sea sediments and is rarely found in seawater and nearshore sediments [17]. In this study, Marine Benthic Group D was present in lower abundance inshore than offshore. DHVEG are typically found around hydrothermal vents, and in the hydrothermal chimney environment, including at the Mid-Atlantic Ridge, East Pacific Rise, and Guaymas Basin [18]. Based on this environment, DHVEG taxa originally were thought to be thermophilic, acidophilic, and heterotrophic. However, new technology developed in recent years revealed that DHVEG are also found in the estuary [9]. Members of the Marine Hydrothermal Vent Group mainly occur in marine hydrothermal vent environments, but they have been found in other environments such as Pearl Estuary and Cascadia sediments [15]. CCA47 and Marine Benthic Group E related lineages reportedly prefer the marine habitat [19], and their abundances increased from the estuary to the deeper sites in this study.

The archaeal community is influenced by many factors, including water depth, the temperature of the sediments, dissolved oxygen content, sediment porosity, content of nutrients, and methane concentration. In general, as water depth increases, the abundance of bottom sediment microbes generally declines. Sedimentary dynamic changes in estuaries, such as the injection of terrigenous fresh water, also can impact the archaeal community structure. The mouth of an estuary is a transition zone between freshwater and seawater, thus the archaeal community structure in this setting has unique characteristics.

Cluster analysis showed that eight sediment samples clustered into two groups (Figure 4): one corresponded to low salinity sites (sites 3, 4, 5, 7, 9, and 15) and the other corresponded to high salinity sites (sites 28 and 29). The marine group was characterized by DHVEG-1, Marine Benthic Group B, and Candidatus Nitrosopumilus, and the freshwater group was characterized by Marine Group I, Thermoprotei, and the other methanogens. RDA further showed that salinity was the main factor impacting archaeal community structure. A lot of fresh water converges in the Liaohe Estuary and mixes with seawater, thereby decreasing salinity. At the same time, large amounts of terrigenous material are injected into the estuary by the coastal current, which greatly affects the salinity of the seabed sediment. Besides, salinity is an important factor in denitrification, as high salinity can increase osmotic pressure and cause metabolic changes in microorganisms [20] demonstrated that AOA were abundant in low salinity areas. In this study, Candidatus Nitrososphaera abundance was negatively correlated with salinity (P < 0.05). Candidatus Nitrosopumilus was present in high abundance at all study sites, which exhibited a significantly negative correlation with salinity (P < 0.01). In addition, archaeal community structure might reflect changes in the estuarine sedimentary dynamics. The nearshore area is more variable with low salinity and large flow, but the offshore area with high salinity varies much less and is a more stable environment. The analysis of archaeal ecological function in the Liaohe Estuary showed that methanogens played an important role in the nearshore low salinity area, but nitrification by azotobacters was the main ecological function. In low salinity offshore water, carbon and nitrogen fixation were important, but farther offshore in high salinity water nitrogen fixation was the main ecological function.

Acknowledgment
This work was supported by National Key R&D program of China [2017 YFC1404500], the Natural Science Foundation of China [No. 41676115], and Key Laboratory of Integrated Monitoring and Applied Technology for Marine Harmful Algal Blooms of State Oceanic Administration Open Foundation [MATHAB201815].

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