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Using the geneFISH to detect archaeal *amoA* genes in microbial mats and biofilms

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Abstract:

In present study, the geneFISH was used to detect archaeal *amoA* gene in microbial mats and MRC biofilms at different locations. To get this aim, clone libraries of archaeal 16S rRNA and *amoA* genes were constructed in different layers Sippewissett Salt Marsh microbial mats and Marine Resources Centre (MRC) biofilms collected at different locations were investigated. Community structures analysis indicated the MRC2 and MRC4 biofilms collected from MRC seawater inlet tubes form a special environment for Crenarchaea, where Crenarchaea as the dominated Archaea groups, while the microbial mats were dominated by Euyarchaea, and only few Crenarchaea were detected. The CARD-FISH counting indicated that only 5.7 to 15.6% and 3.5 to 26.2% of the total cells represented as the Archaea in microbial mats and MRC biofilms, respectively; while less than 1% and 9% of total cells represented as Crenarchaea in microbial mats and MRC biofilms. Based on the *amoA* genes clone libraries in MRC biofilms, two specific *amoA* genes targeted probes were designed for geneFISH. However, due to the low abundances of Crenarchaea and complex background in our research samples, only weak and potential signals of *amoA* gene probes were detected from MRC4 biofilms sample. Thus, based on these weak signals, the primary result of geneFISH indicated at least 25% of Crenarchaea harbored *amoA* gene, which might be catalyzing the oxidation of ammonia.

Key words: geneFISH, CARD-FISH, Community structures, abundances, Crenarchaea, archaeal-a*moA* genes

Introduction

Nitrification, a two-step process that includes the oxidation of ammonium via hydroxylamine to nitrite and then nitrate, is a key process in marine N cycling. The first and rate-limiting step, ammonia oxidation mediated by the ammonia monooxygenase enzyme (AMO), is executed by limited number of microbial groups, including aerobic chemoautotrophic bacteria and archaea (Francis et al. 2007). Ammonia oxidizing bacteria (AOB) include the β-proteobacteria *Nitrosomonas* and *Nitrosopiras*, and the γ-proteobacteria *Nitrosococcus* (Purkhold et al. 2000). Both, the 16S rRNA genes and *amoA* genes which encode the catalytic α-subunit of the AMO have been used in molecular studies of analyzing ammonia-oxidizing bacteria (AOB) in the environment (Kowalchuk and Stephen 2001; Prosser and Embley 2002). Ammonia oxidizing archaea (AOA) are considered as the Marine Group I *Crenarchaea* based on the similarity between the 16S rRNA and *amo*A genes from *Nitrosopumilus maritimus*, the first and only purified ammonia oxidizing archaeon from marine aquarium tank, with sequences retrieved from different seawater samples (Prosser and Nicol, 2008). According to the previous results, *Crenarchaea* are ubiquitously distributed in the planet, which have been considered as the most abundant archaea in the marine environments (DeLong 1992; Karner et al. 2001). Thus, AOA are proposed to make an important role, even a major role on the ocean nitrification (Karner et al. 2001). However, how many of the *Crenarchaea* are functional ammonia oxidizers, and their actual roles on nitrification, remain open questions.

Previous studies have quantified both AOA 16S rRNA and *amoA* genes in the environments, however, the relationships between these two genes in environmental samples were inferred indirectly. Recently, Moraru et al. (2010a) has developed the geneFISH – a protocol for linking functional gene presence with cell identity in environmental samples, the signals of which can be visualized at a single cell level. This protocol combines rRNA gene targeted catalyzed reporter deposition–fluorescence *in situ* hybridization (CARD-FISH) and *in situ* functional gene detection. Using this technique, it has been identified that at least 30% the marine *Crenarchaeota* in the Benguela upwelling system harbored the *amoA* genes, and the results provide stronger evidences for the important role of Crenarchaea on nitrification (Moraru et al. 2010a). However, more researches on different environments are needed to draw an overall conclusion.

In order to understand how many *Crenarchaea* with *amoA* genes in the complex environments, the geneFISH technique was used and the microbial mats (with different layers) and biofilms (with different growth conditions) were selected as my research samples, which have been proposed to have complex microbial community structures. To obtain this aim, the following research contents were carried out in this study (**Fig. 1**):

(1) Understanding the community structures of archaea in microbial mats and biofilms through clone libraries or T-RFLP analysis based on 16S rRNA genes.
(2) Uncovering the archaeal *amoA* genes diversity in microbial mats and biofilms by clone libraries analysis.
(3) Estimating the abundances of universal archaea and Crenarchaea in microbial mats and biofilms by CARD-FISH or qPCR.

(4) Estimating how many Crenarchaea in microbial mats and biofilms with *amoA* genes by geneFISH.

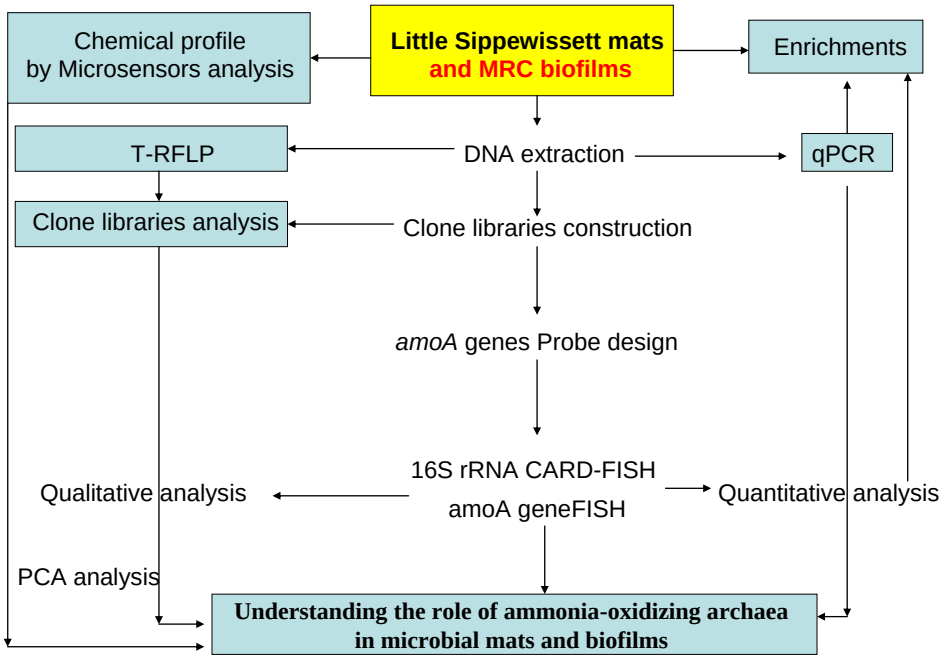


Fig. 1 Research strategy of present study

Materials and methods

Sample collection and chemical analyses

The microbial mat samples were collected from the Little Sippewissett Salt Marsh in Woods Hole on July 2, 2010. The mat samples were divided into five layers according to their characteristic macroscopic formations and chemical profiles. The first layer (0-2 mm) is a green layer of cyanobacteria, mostly of *Lyngbya* and *Oscillatoria* species. Below the green layer are pink and peach layers (2-5 mm) composed of purple sulfur bacteria. The third layer (5-10 mm) is the cohesive layer extended to 10 mm for the pink layer and the fourth layer, a layer of black sand can generally be observed from 10 mm to 20 mm below the mat surface. The fifth layer is 20 to 30 mm below the mat surface, mostly composing by the grey sand. The five different layers samples were labeled as Mat1 to Mat5 from top the bottom, respectively. Before the section of the mat samples, the oxygen and pH profiles were measured by microsensor (Unisense, Aarhus, Denmark), and data were showed in **figure 2.**

The biofilm samples were collected from the Marine Resource Centre of MBL on July 7, 2010. Two samples (MRC2 and MRC4) were collected from the surface of two inlet tubes which are used to filter seawater for aquaculture, and the third biofilms sample (MRC0) was collected from the surface of an outlet pipe. The two biofilms samples collected form inlet tubes were yellow and incompact, while sample collected from outlet pipe showed green and grey.

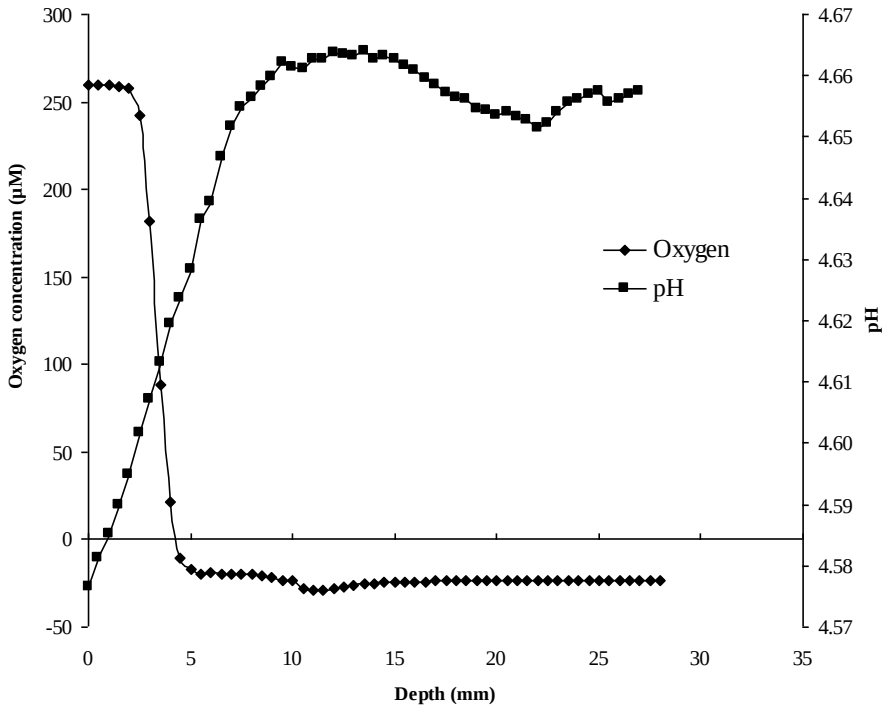


Fig.2 Profiles of Oxygen and pH measured in the Little Sippewissett microbial mats

DNA extraction, PCR amplification and cloning

Total genomic DNA of all fresh samples was extracted using the MO-Bio Power Soil DNA extraction kit (MoBio Laboratories, Solano Beach, CA). Archaeal *amoA* genes were amplified using primer sets Arch-*amoA*F and Arch-*amoA*R (**Table 1**). PCR reaction mixtures contained 1 µl DNA (30-50 ng μl^{-1}), 12.5 µl PCR pre-mix buffer, 1 µl of each forward and reverse primer (20 µM) and the final volume for each reaction was 25 µl. PCR reactions were performed as follows: 95 °C for 3 min; 35 cycles of 95 °C for 45 s, 53 °C for 1 min, followed by 72 °C for 1 min; and finally 72 °C for 7 min. For 16S rRNA genes of Archaea, the same PCR reaction mixtures were used with primers showed in table 1, and PCR reaction were carried out as following : 95 °C for 5 min; 30 cycles of 94 °C for 45 s, 57 °C for 1 min, followed by 72 °C for 2 min; and finally 72 °C for 7 min. PCR products were purified by DNA Gel Extraction Kit (Millipore, Berdford, MA) and cloned with TOPO TA Cloning Kit (Invitrogen Carlsbad, CA) following the manufacturer's instructions. Clones were sequenced with an automated sequencer at the Josephine Bay Paul Center Sequencing Facility (Marine Biological Laboratory, Woods Hole, MA).

Table 1 PCR primers and hybridization probes and conditions used in this study

Primers or probes	Sequence (5'-3')	Annealing or hybridization temp (°C)	Targeted group	References
Primers for cloning				
Arch-*amoA*F	STAATGGTCTGGCTTAGACG	50	Archaea-*amoA*	Francis et al. 2005
Arch-*amoA*R	GCGGCCATCCATCTGTATGT	50	Archaea-*amoA*	Francis et al. 2005
515f	GTGCCAGCAGCCGCGGTAA	57	Universal	Lane, 1985
1397r	GTGTGCAAGGRGCAGGGA	57	*Archaea*	Swan et al. 2010
Probes for CARD-FISH				
Arch915	GTGCTCCCCCGCCAATTCCT	46	*Archaea*	Stahl et al., 1991
Cren554	TTAGGCCCAATAATCMTCCT	46	*Crenarchaeota*	Massana et al. 1997
Primers for *amoA* gene probes synthesis				
amoA_MRC2_ F	CAGGTGACTATATCTTCTAC	54	MRC2	This study
amoA_MCR4_ F	CAGGAGACTACATCTTCTACT	54	MRC4	This study
amoA-MRC_R	TCAGGTTTACCATGTTGAAC	54	MRC2 and MRC4	This study

Phylogenetic and clone libraries analyses

Nucleotide sequences were screened for quality and then analyzed. For 16S rRNA genes, all sequences were aligned with the NAST alignment tool and checked the chimeras by the Bellerophon (version 3) at the Greengenes website (greengenes.lbl.gov) (DeSantis et al. 2006). The aligned, non-chimera sequences were classified using the classification tool at the Greengenes website (greengenes.lbl.gov). For *amoA* genes, DNA sequences were examined and edited by MEGA 4.0 software (Tamura et al. 2007). The aligned sequences were inputted the *amoA* gene database (Provided by Cristina Moraru in Max Planck Institute for Marine Microbiology) and translated into amino acids. Finally the phylogenetic trees for 16S rRNA and *amoA* genes were constructed by Neighbor-Joining method with bootstrap analysis under de ARB program (Ludwig et al. 2004).

To obtain views on the richness and diversity of archaea in microbial mats and biofilms, operational taxonomic unit (OTU) for community analysis were defined by 3% differences in nucleotide sequences, as determined by using the furthest neighbor algorithm in DOTUR (Schloss and Handelsman 2005). Shannon and Simpson indices for each sampling site were also generated by DOTUR. The community structures in microbial mats and biofilms samples were analyzed by the principal coordinates analysis (PCoA) and Jackknife Environment Clusters analysis in UniFrac (Lozupone et al. 2006).

CARD-FISH and geneFISH

Each sample (0.1 g) from microbial mats and biofilms were fixed in 4% paraformaldehyde (1 part 24% paraformaldehyde and 5 parts 1×PBS buffer) overnight at 4 °C. Fixed samples were washed three times with 1×PBS, with centrifugation at 16,000g for 10 min between washes, and stored in PBS/ethanol (1:1) at -20 °C until further

processing. Samples were dispersed by sonication at low power (level 2) for 90 s with a sonicator W-20 (MISONIX, Indianapolis, IN). 10 µl sonicated samples were mixed with 10 ml 1×PBS buffer and filtrated on 0.2 µm polycarbonate filter. After washing the filter with Mill-Q water and 1×PBS, the dried filters were embedded with 0.1% (wt/v, Biozym, USA; in Milli-Q water) agrose, washed with 96% EtOH and dried under air. To obtained a good permeabilization of cells on the filters, methods with proteinase K Proteinase K (0.2 µl ml^{-1}, 1.844 U mg^{-1}, 10.9 mg ml^{-1}; Fluka, Buchs, Switzerland), archromopeptides (60 U ml^{-1}) and HCl were used, respectively. The optimization procedures were following: after incubated the membranes in 0.01 M HCl for 10 min and 0.1 N HCl for 1 min at room temperature, Milli-Q water and 96% EtOH were used to wash the membranes for further hybridization. The details of CARD-FISH procedures were performed according to Moraru et al. (2010a). In brief, the samples were hybridized with the Cren554 probe (Table 1) to target the Marine Group I *Crenarchaeota,* or the Arch915 probe (Table 1) for universal archaea at 46 °C overnight under 0% and 30% of formamide; after washing step, filters incubated in substrate mix (1 part Cy3-tyraminde, 10 parts of amplification solution) for 45 min at 46 °C under dark. Then, the filters were incubated again with 1×PBS buffer for 15 min at room temperature, washed by Milli-Q water and 96% EtOH. The filter were mounded on glass slide with 4',6'-diamidino-2-phenylindol (DAPI) (1 µg ml^{-1}) after air dried the filters. All preparations were done in triplicate. The microscopic images of hybridized samples were recorded on a Zeiss Axio Imager.M1 (Carl Zeiss, Jena, Germany) using an AxioCam MRc camera and the AxioVision software (version 4.7).

For geneFISH, specific probes for *amoA* genes were needed to design. Based on the partial archaeal *amoA* gene sequences MRC biofilms samples (MRC2 and MRC4), two specific *amoA*-gene probes were designed by the PolyPro software (Moraru et al. 2010b). According to Moraru et al.(2010a), the designed probes were produced by incorporating Dig-dUTP into dsDNA via PCR (70 mM Dig-dUTP) with the PCR Dig Probe Synthesis Kit (Roche, cat. No.11636090910). For probes synthesis, PCR reaction mixture were prepared with the designed primers (**Table 1**), according to the manual of the manufacture. PCR reactions were performed as follows: 95 °C for 5 min; 28 cycles of 94 °C for 45 sec, 54 °C for 45 sec, followed by 72 °C for 1 min; and finally 72 °C for 7 min. The PCR products were column purified using the Gene Clean Turbo kit. The concentration was determined spectrophotometrically, using a Nano-Drop instrument (Fischer ThermoScientific). The whole procedure of geneFISH experiments were divided into three steps, including sample preparation, rRNA hybridization and DNA hybridization. The sample preparation and rRNA hybridization steps were the same as above, while for antibody binding step in the DNA hybridization, with and without sheep serum (50µ l/ml, Sigma, No. S3772-10ML) methods were used to get more confident results. The brief workflow of geneFISH experiment was showed in **figure 3** and further information could also be found in the previous papers (Moraru et al. 2010a; Moraru et al. 2010b).

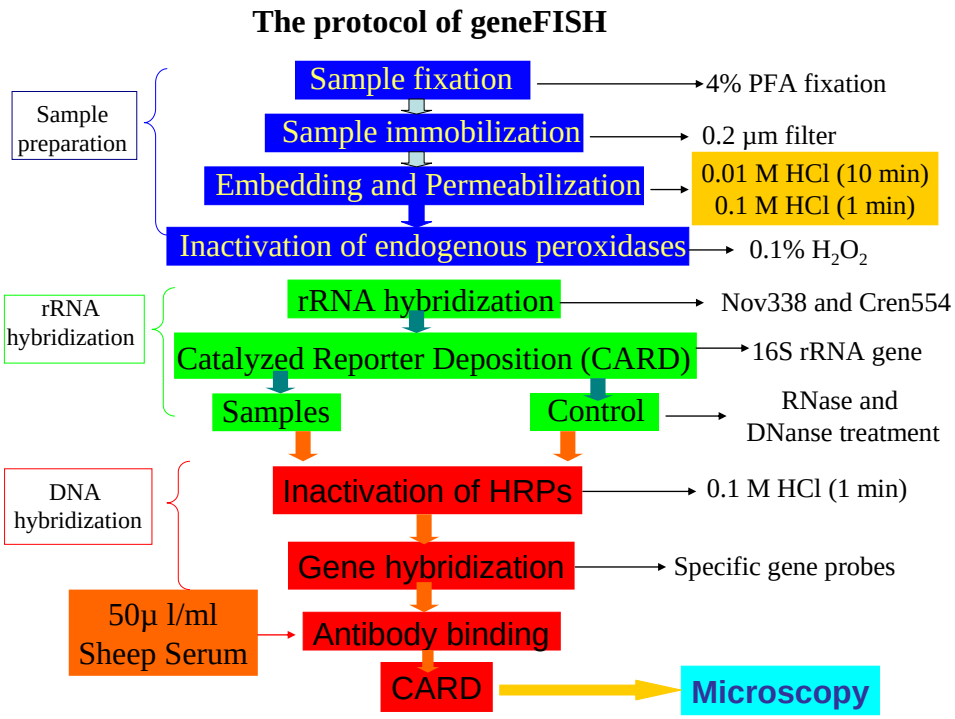


Fig.3 The protocol of geneFISH (modified from Moraru et al. 2010a)

Results and discussion

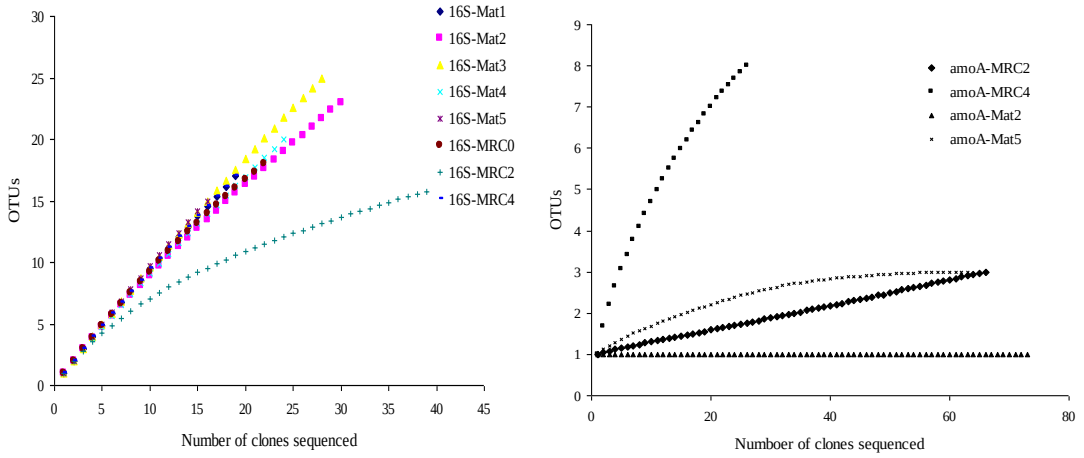
Clone libraries analysis

A total of 8 universal archaea 16S rRNA gene and 4 archaeal *amoA* gene clone libraries were constructed in this study, and details of characteristics for each clone library were summarized in the **table 2**, while the rarefaction curves for each clone library were shown in **figure 4**.

Based on the 16S rRNA gene clone library characteristics and rarefaction analysis, the OTU numbers would be higher if more clones were sequenced. The Archaea in microbial mats showed a relative higher diversity than that of MRC biofilms, while fewer Crenarchaea related clones were recovered from microbial mats. Using the RDP II classifier (http://rdp.cme.msu.edu/classifier/classifier.jsp), it is clearly shown that composition of Archaea in microbial mats and MRC biofilms were quite different (**Fig. 5**). In microbial mat samples, most of the recovered archaeal clones belonged to Euyarchaea and unclassified archaea, and only a few Crenarchaea related clones were found in the layer 2 and layer 5, which represented 3.7% and 10.3 % of total Archaea, respectively. However, Crenarchaea were detected in all MRC biofilm samples, where the Crenarchaea represented as the dominant group in MRC 2 and MRC4, composing about 70% and 73.5% of total Archaea.

Table 2 Characteristics of individual and combined clone libraries from microbial mats and MRC biofilms

Biomarker	Sample sites	No. of archaea clone	Number of Crenarchaea	No. of OTU	Shannon Index	Simpson Index	Chaol
16S rRNA	Mat1	19	0	17	2.799	0.006	52.0
	Mat2	27	1	26	3.014	0.025	80.0
	Mat3	29	3	25	3.165	0.011	151.5
	Mat4	25	0	20	2.889	0.025	96.5
	Mat5	18	0	16	2.686	0.008	60.5
	Total Mat	118	4	92	4.415	0.006	351.1
	MRC0	23	2	18	2.839	0.017	36.2
	MRC2	40	28	16	2.447	0.088	28.0
	MRC4	34	25	17	2.463	0.070	39.0
	Total biofilms	97	55	49	3.507	0.037	107.7
amoA	Mat1	-	-	-	-	-	-
	Mat2	73	-	1	0.000	1.000	1.0
	Mat3	-	-	-	-	-	-
	Mat4	-	-	-	-	-	-
	Mat5	63	-	3	0.331	0.848	3.0
	Total Mat	136	-	3	0.182	0.928	3
	MRC0	-	-	-	-	-	-
	MRC2	66	-	1	0.000	1.000	1.0
	MRC4	24	-	8	1.517	0.305	10.0
	Total biofilms	90	-	9	1.024	0.536	11



A B

Fig. 4 Rarefaction analysis of archaeal 16S rRNA (A) and *amoA* (B) genes recovered from microbial mats and MRC biofilms. Operational taxonomic units (OTUs) were defined by 97% nucleotide similarity based the calculation by DOTUR.

For archaea *amoA* genes, only four samples were amplified successfully by PCR, including 2 samples from microbial mats (Mat2 and Mat5) and 2 samples from MRC biofilms (MRC2 and MRC4). In addition, clone libraries and rarefaction analysis showed that the diversity of archaeal-*amoA* gene in the these samples was quite low, since only 1 to 8 OTUs were found based on 97% nucleotide sequences similarity (**Table 2**).

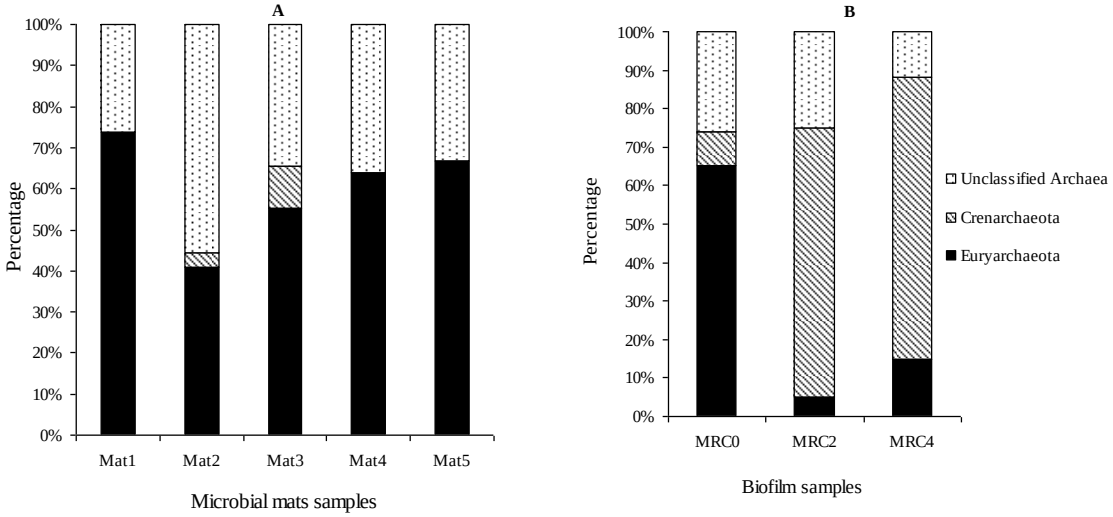


Fig. 5 Compositions of archaea communities in microbial mats (A) and MRC bioflims (B)

Phylogenetic analysis of archaeal 16S rRNA and *amoA* genes in microbial mats and biofilms

Fig.6 showed the phylogenetic relationship of archaeal 16S rRNA gene sequences recovered from microbial mats and biofilms. From 16S rRNA gene phylogeny, all sequences were divided into three major groups, unclassified archaea, Crenarchaea and Euyarchaea. For MRC biofilms, most of Crenarchaea were closely related to *Nitrosopumilus maritimus*, within the Marine Crenarchaea Group I; while for the microbial mats samples, the retrieved sequences were more related to Euyarchaea: Halobacteriales related clones, which were found in deep-sea hydrothermal vent. In addition, some sequences in microbial mats even formed a unique group which did not closely related to any other archaea sequences from databases. The results indicated that the microbial mats might harbor some new archaea groups, thus more research is needed.

For *amoA* gene phylogeny, sequences were clearly divided into three groups, including MRC2, MRC4 and MAT groups. MRC2 group included all *amoA* sequences (66 sequences) from MRC2 and 1 sequence from MRC4, while the MRC4 group contained more than 30 sequences from MRC4 and 5 sequences from Mat5. Both of MRC2 and MRC4 groups were very closely related to *amoA* gene in *Nitrosopumilus maritimus*, which was consistent with the 16S rRNA gene phylogeny in the same sampling sites, indicating the MRC2 and MRC4 biofilms might harbor the Crenarchaea with *amoA* genes. Except five microbial mat a*moA* gene sequences clustered into MCR4 group, all other sequences from Mat2 and Mat5 grouped together, forming a new cluster which was closely related to the sequences recovered from West Pacific Continental Margin deep-ocean sediments and Vierra Marsh sediments.

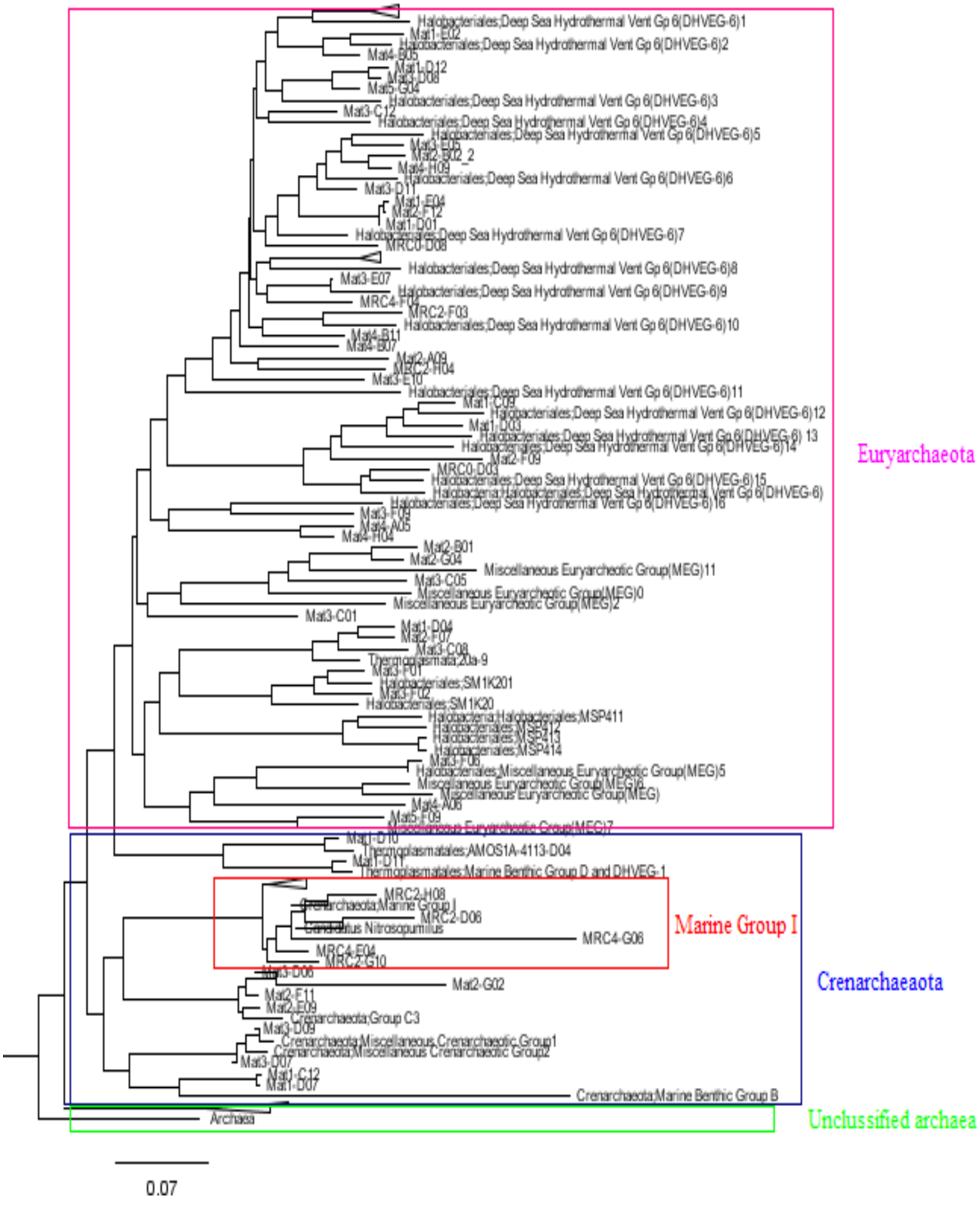


Fig. 6 Phylogenetic relationship of archaeal 16S rRNA gene sequences recovered from microbial mats and biofilms.

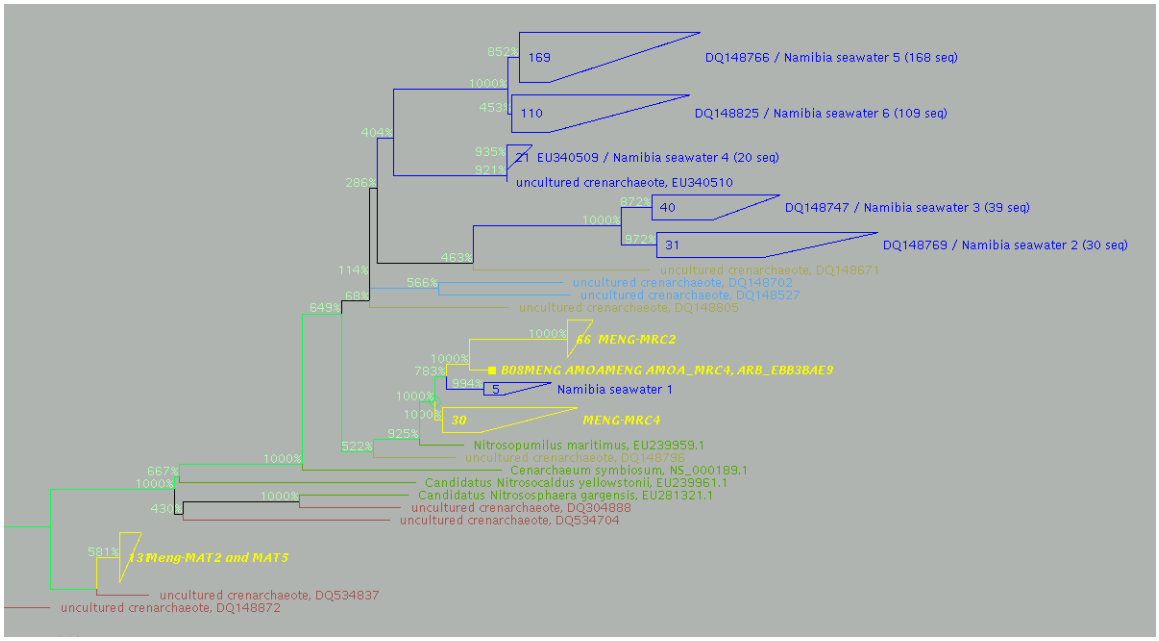
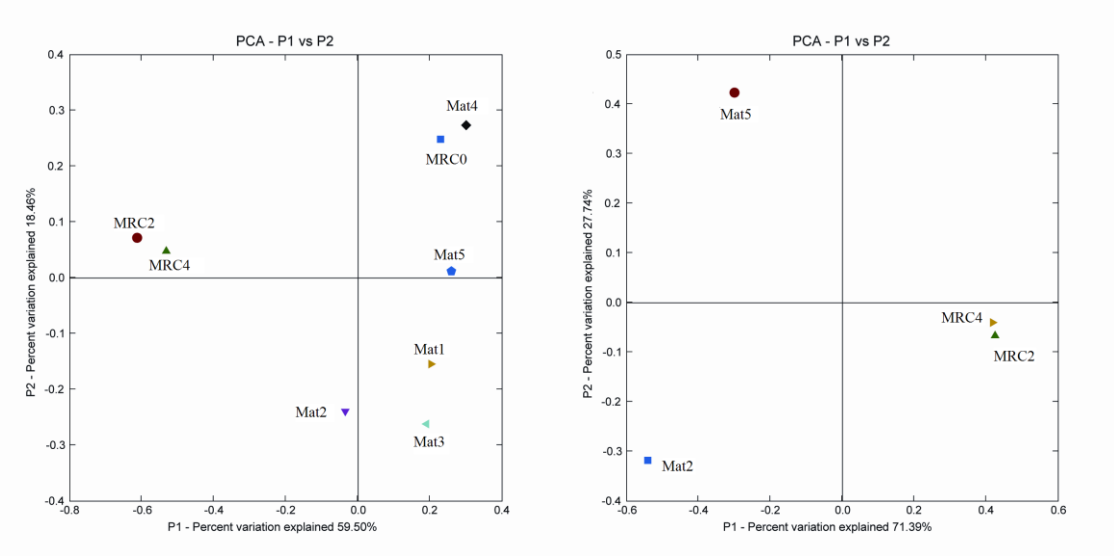


Fig. 7 Phylogenetic tree of Archaeal *amoA* gene sequences recovered from different layers of microbial mats and biofilms.

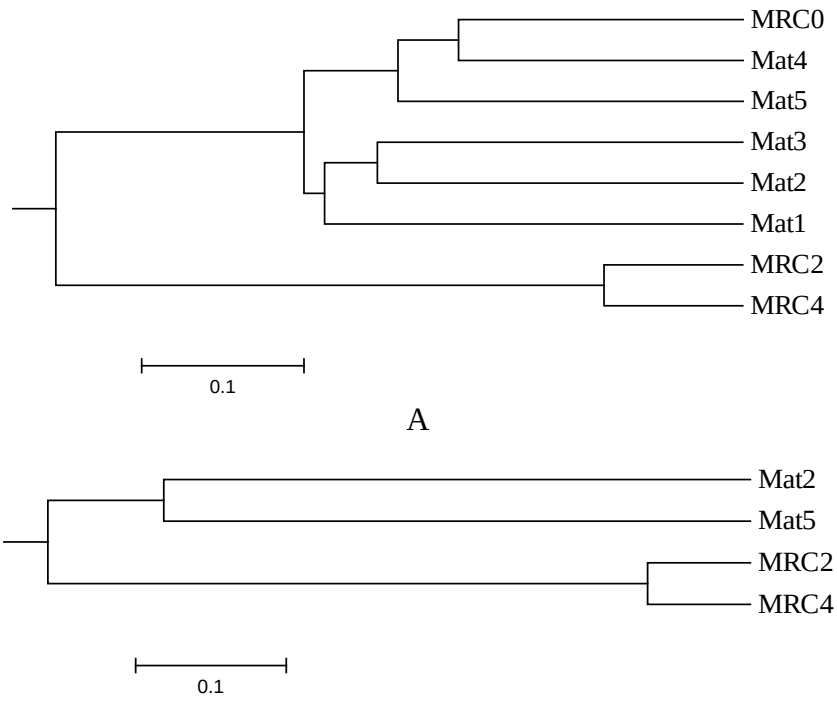
Comparisons of Archaea community structures in microbial mats and biofilms.

To get more clearly understanding the differences in archaeal community structures and *amoA* gene diversity in microbial mats and MRC biofilms, principal coordinate analysis (PCoA) and environment cluster analysis by UniFrac software were carried out. Figures 8 and 9 clearly showed that the Archaea in MRC2 and MRC4 have similar community structures, as showing in the PCoA plot and environment cluster analysis. All archaea sequences from different layers of microbial mats were not similar to that of MRC2 and MRC4, forming into another group based on the PCoA and environmental cluster analysis. However, it was interesting that the archaeal community in MRC0 biofilm was more similar to that of Mat4 than MRC2 and MRC4 biofilms. The results indicated the biofilms in MRC inlet tubes formed a very special environment for Archaea, in which the Crenarchaea is the dominant group, while the biofilm MRC0 collected from seawater outlet was more similar to microbial mats samples. For *amoA* gene diversity, the biofilm samples from MRC2 and MRC4 also grouped together, separating from that of microbial mat samples. Thus, based on the comparison of community structures of 16S rRNA and *amoA* genes, it is clear shown that the inlet biofilms of MRC2 and MRC4 provide a very special environment for Crenarchaea.



A B

Fig. 8 Principal coordinate plot by UniFrac analyses of the 16S rRNA (A) and *amoA* gene (B) sequences from microbial mat and biofilms.



A

B

Fig. 9 Environment cluster analysis the diversity communities of Archaeal 16S rRNA and *amoA* genes

Archaea and Crenarchaea abundance and distribution in microbial mats and biofilms by CARD-FISH

After understanding the community structures of microbial mats and MRC biofilms, CARD-FISH were used to estimate the relative abundance of Archaea and Crenarchaea in all research samples (Fig.10). According to the CARD-FISH counting, I found that the total cell number in microbial mats ranged from 4.7×10^8 to 20.2×10^8 cells/gram (wet weight), showing a decrease trend from the top layer to the bottom layer; while the total cell in MRC biofilms samples ranged 12.6×10^8 to 28.3×10^8 cells/gram (wet weight), which was significantly higher than that of microbial mats. For Archaea abundances, only 5.7 to 15.6% and 3.5 to 26.2% of the total cells represented as the Archaea in microbial mats and MRC biofilms, respectively. But for the relative abundance of Crenarchaea group in total cells, proportions were even smaller, as less than 1% and 9% of total cells in microbial mats and MRC biofilms represented as Crenarchaea.

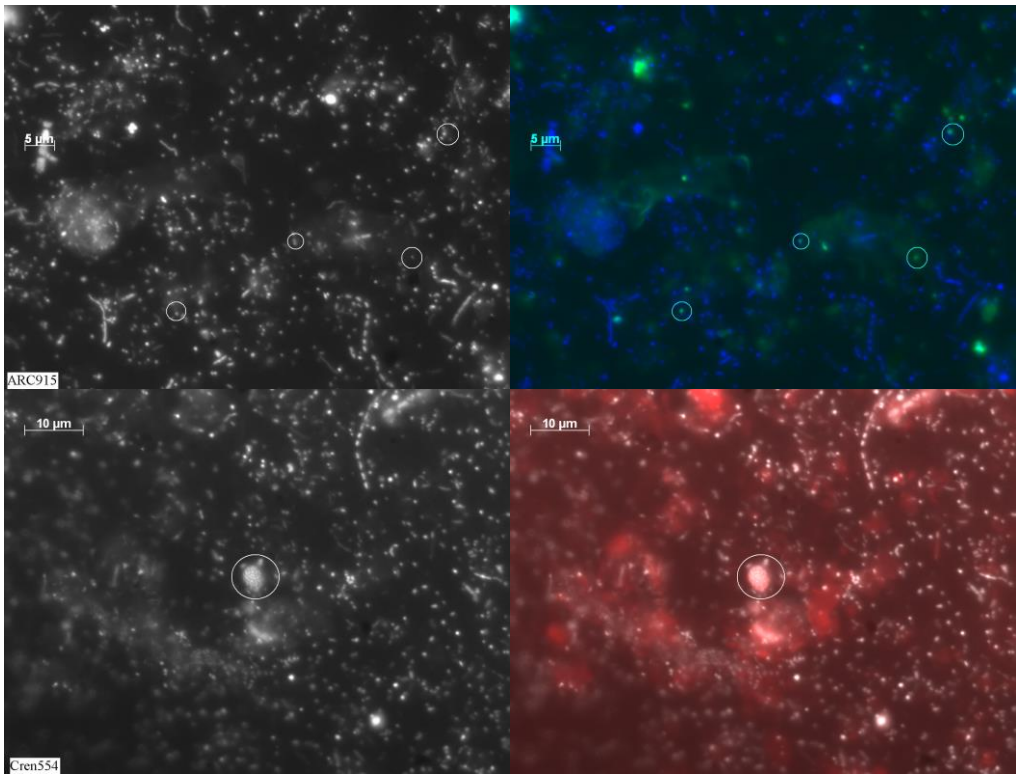
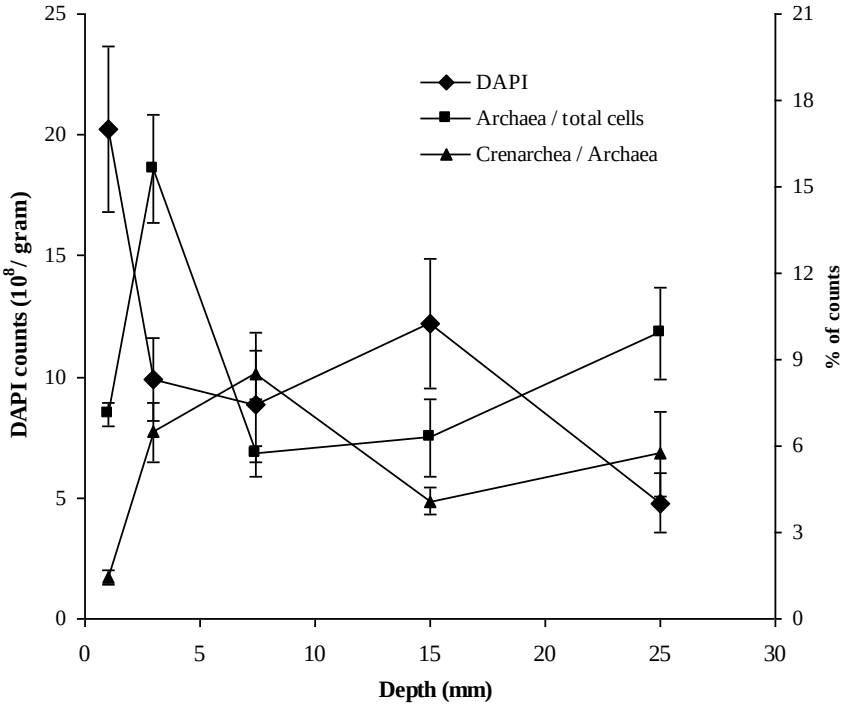
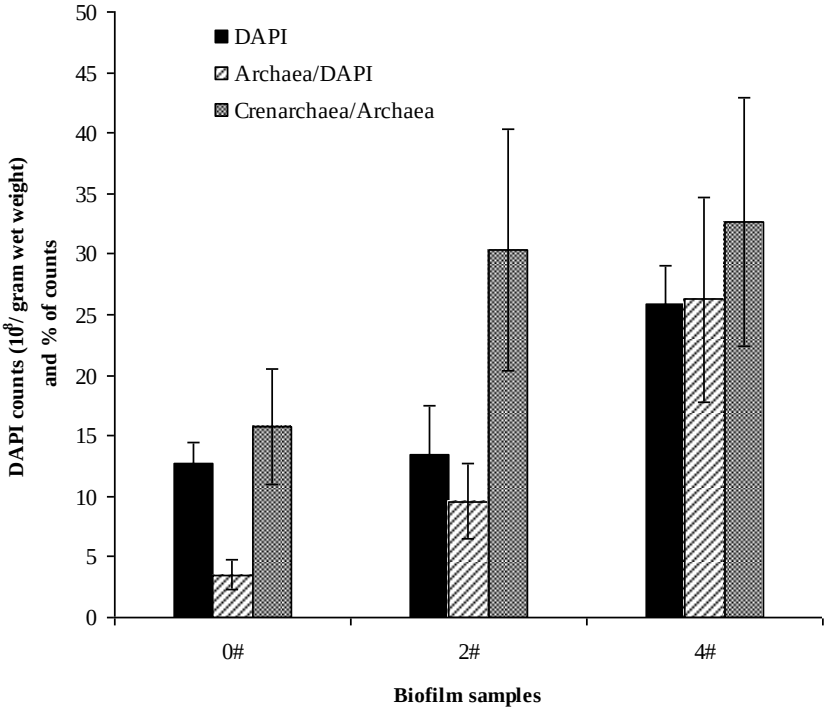


Fig. 10 Photomicrographs of CARD–FISH for universal archaea (Arc915) and Crenarchaea (Cren554)



A



B

Fig. 11 Relative abundances of Archaea and Crenarchaea in microbial mats (A) and MRC biofilms (B) determined by CARD-FISH counting

geneFISH

Although the Crenarchaea abundance in the microbial mats and MRC biofilms was relatively low, samples from MRC2 and MRC4, where Crenarchaea was the dominated group, were selected for the geneFISH in order to get more confident results. Thus, two specific *amoA* gene probes (351 nt, appendix 1) were designed based the *amoA* gene clone libraries of MRC2 and MRC4. According to the geneFISH experimental protocols introduced in materials and methods (also showed in fig. 3), I only got some potential signals of geneFISH probes from samples which sheep serum was added at antibody binding step (Fig. 12), but without adding sheep serum, I could not identify any signal from our probes due to the strong background. The weak geneFISH probe signals were possibly due to the low abundances of Crenarchaea in the research samples and complex microbial community structures in biofilms which brought a very high fluorescence background. Thus, in future experiments, the elimination of background should be taken more consideration, such as more sheep serum may be needed in the antibody binding step. Based on the weak geneFISH signals, the primary estimation of *amoA* gene abundances indicated that at least more than 25% of Crenarchaea in MRC4 biofilms harbored *amoA* gene, which might be catalyzing the oxidation of ammonia.

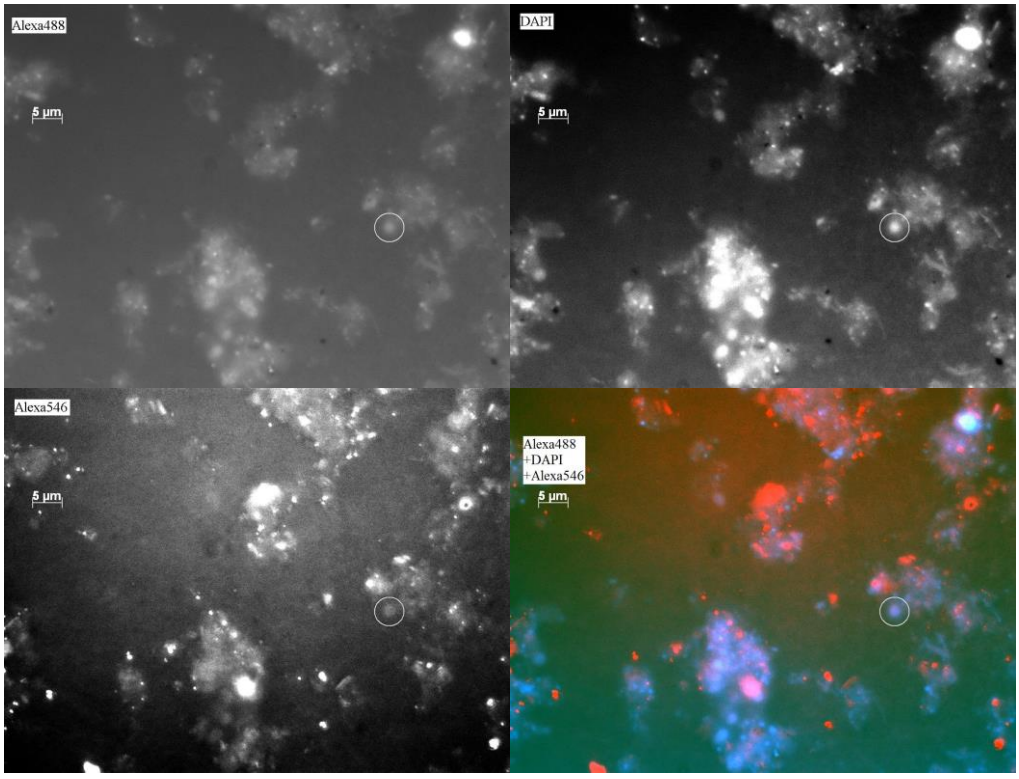


Fig.12 GeneFISH on the biofilms (MRC4)

Conclusions

1. Community analysis and CARD-FISH counting indicated lower Crenarchaea abundances in microbial mats than MRC biofilms.
2. The *amoA* genes diversity in microbial mats and MRC biofilms was relative low, but closely related to *amoA* gene in *Nitrosopumilus maritimus.*
3. Weak signals could be detected from amoA gene probe by geneFISH, and primary results indicated at least 25% of Crenarchaea in MRC biofilms harbored the *amoA* genes.

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References

DeLong EF (1992) Archaea in coastal marine environments. Proc Natl Acad Sci U S A 89:5685-5689

DeSantis TZ, Jr. et al. (2006) NAST: a multiple sequence alignment server for comparative analysis of 16S rRNA genes. Nucleic Acids Res 34:W394-399

Francis CA, Beman JM, Kuypers MM (2007) New processes and players in the nitrogen cycle: the microbial ecology of anaerobic and archaeal ammonia oxidation. ISME J 1:19-27

Karner MB, DeLong EF, Karl DM (2001) Archaeal dominance in the mesopelagic zone of the Pacific Ocean. Nature 409:507-510

Kowalchuk GA, Stephen JR (2001) Ammonia-oxidizing bacteria: a model for molecular microbial ecology. Annu Rev Microbiol 55:485-529

Lozupone C, Hamady M, Knight R (2006) UniFrac--an online tool for comparing microbial community diversity in a phylogenetic context. BMC Bioinformatics 7:371

Ludwig W et al. (2004) ARB: a software environment for sequence data. Nucleic Acids Res 32:1363-1371

Massana R, Murray AE, Preston CM, DeLong EF (1997) Vertical distribution and phylogenetic characterization of marine planktonic Archaea in the Santa Barbara Channel. Appl Environ Microbiol 63:50-56

Moraru C, Lam P, Fuchs BM, Kuypers MM, Amann R (2010a) GeneFISH - an in situ technique for linking gene presence and cell identity in environmental microorganisms. Environ Microbiol

Moraru C, Moraru G, Fuchs BM, Amann R (2010b) Concepts and software for a rational design of polynucleotide probes. Environ Microbiol Rep

Prosser JI, Embley TM (2002) Cultivation-based and molecular approaches to characterisation of terrestrial and aquatic nitrifiers. Antonie Van Leeuwenhoek 81:165-179

Purkhold U, Pommerening-Roser A, Juretschko S, Schmid MC, Koops HP, Wagner M (2000) Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and amoA sequence analysis: implications for molecular diversity surveys. Appl Environ Microbiol 66:5368-5382

Schloss PD, Handelsman J (2005) Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. Appl Environ Microbiol 71:1501-1506

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596-1599

Appendix

The two specific amoA-gene probes for MRC biofilms

> B10_MENG_MRC2
CAGGTGACTATATCTTCTACACTGACTGGGCTTGGACTTCGTACACGGTATTC
TCAATATCGCAAACGTTGATGCTTATAGTAGGTGCCACATATTATCTGACATT
TACTGGCGTTCCAGGCACGGCAACGTACTACGCTCTTATTATGACAGTATACA
CATGGATAGCAAAAGCCGCACGGTTTTCACTAGGATATCCATATGACTTCATT
GTAACTCCAGTTTGGCTCCCATCGGCGATGCTGTTGGATTTAGTTTACTGGGC
AACAAAGAAGAACAAACACTCTTTGATTCTGTTTGGAGGTGTGCTGGTAGGA
ATGTCTTTACCATTGTTCAACATGGTAAACCTGA

> B10_MENG _MRC4
CAGGAGACTACATCTTCTACACTGACTGGGCTTGGACTTCGTACACGGTATTT
TCAATATCGCAGACGTTGATGCTAATTGTAGGTGCTACATATTACCTAACATT
TACTGGCGTTCCAGGCACAGCAACGTACTACGCTCTAATTATGACAGTATAC
ACATGGATAGCAAAAGGCGCATGGTTTTCGCTAGGATATCCATATGACTTCA
TTGTAACTCCAGTTTGGCTTCCATCAGCAATGCTGTTGGACTTGGTCTACTGG
GCAACAAAGAAGAACAAGCACTCCTTGATACTGTTGGCGGCGTACTGGTAGG
AATGTCATTACCATTGTTCAACATGGTAAACCTGA