

VERTICAL DISTRIBUTION OF ARCHAEOAL COMMUNITIES IN COLD SEEP SEDIMENTS FROM THE JIULONG METHANE REEF AREA IN THE SOUTH CHINA SEA

DISTRIBUIÇÃO VERTICAL DA COMUNIDADE ARCHAEOAL EM ZONA DE EMANAÇÃO FRIA NA ÁREA DO RECIFE DE METANO DE JIULONG, NO SUL DO MAR DA CHINA

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ABSTRACT: Jiulong methane reef, a large cold seep area on the continental slope of the South China Sea (SCS), was characterized by chemosynthetic fauna, bacterial mats, methane-derived carbonate structures, and a shallow depth of sulfate-methane transition zone (SMTZ) in gravity cores. To characterize the microbial diversity and variation in correlation to methane venting in the cold seep area, the archaeal community in sediments of the gravity Core CL11 was analyzed, using the Illumina MiSeq sequencing system. A total of 96,917 valid sequences were obtained and classified into 275 OTUs. Taxonomic analysis showed that all the archaeal sequences belonged to the phyla of *Crenarchaeota* (69.41%) and *Eurarchaeota* (25.26%). Vertical distribution of the archaeal communities showed that the Marine Benthic Group B (MBG-B) was the predominant group (58.92%) in the topmost sediment of the core, while percentages of the South African Gold Mine Euryarchaeotal Group (SAGMEG) sequences increased with depth. Our findings indicate that the archaeal community in the cold seep of Jiulong Methane Reef is unique and provides us new insights into the archaeal community composition of the cold seep area.

KEYWORDS: Depth. Diversity. Sulfate-methane Transition Zone.

INTRODUCTION

Cold seeps represent one of the most extreme marine conditions and are characterized by fluids seepage into surface sediments, causing elevated methane and/or sulfide concentrations over those of ambient seawater, thus forming oases of elevated microbial biomass and various faunal assemblages on the seafloor. The first cold seep ecosystem was discovered on the Florida Escarpment in the Gulf of Mexico (PAULL et al 1984). Since then, seeps have been discovered in other parts of the world's oceans. Research revealed that biota in the cold seeps rely on chemoautotrophy, especially those bacteria metabolizing methane and hydrogen sulfide for energy.

Previous studies showed that microbial communities often thrive at cold seep sites, which sustain distinctive geochemical and microbial processes. It was demonstrated that methanotrophic archaea carry out a process called anaerobic oxidation of methane (AOM), initiating the living processes of growth, reproduction and energy flow to make other organisms' existence possible. Considerable research of archaeal communities has been conducted in different typological cold seep areas around the world, such as Hydrate Ridge

(KNITTEL et al 2005), Nankai Trough (ARAKAWA et al 2006), Tropical Timor Sea (WASMUND et al 2009), Northern South China Sea (Northern SCS) (ZHANG et al 2012), and Sonora Margin (VIGNERON et al 2013). These studies showed that different cold seep areas harbored different archaeal communities. Phylogenetic analysis of archaeal community revealed that anaerobic methane oxidation group (ANMEs) and MBG-D were detected at Hydrate Ridge (KNITTEL et al 2005), whereas the archaeal phylotypes were mainly composed of Marine Group I (MGI), Miscellaneous Crenarchaeotic Group (MCG) and MBG-B in the tropical Timor Sea methane seep (WASMUND et al 2009). A report from Zhang et al. (2012) showed that MBG-B and Halobacteria were dominant archaeal groups in cold seep sediment from the Northern SCS, whereas ANMEs dominated the archaeal community in the Sonora Margin cold seep ecosystem (VIGNERON et al 2013). A significant differences in microbial activity among the different seep systems were observed when comparing the rates of AOM and sulfate reduction (SR). Even though high concentrations of methane and sulfate are available, the methanotrophic microbial activity and biomass

may be low for unknown reasons in some seep environments.

In this study, a gravity sediment core was collected from the southern slope of Jiulong methane reef in the SCS. The analyses of sedimentary microbial community by pyrosequencing allowed us to determine the archaeal diversity and downcore variation coupled to the environmental parameters.

MATERIAL AND METHODS

Site description and sampling

Jiulong methane reef, located on the northern continental slope of the SCS, is the first cold seep area discovered by the joint Chinese-German RV Sonne cruise 177 in 2004 (SUESS et al

2005). It was characterized by chemosynthetic fauna and bacterial mats, methane-derived carbonate structures, and a very shallow depth of the SMTZ in gravity cores. Evidence of active cold vents in this area were reported (ZHANG et al 2012). The gravity Core CL11 (767 cm in length, water depth of 1607 mbsf) was collected from the southern slope of Jiulong methane reef during a cruise of the Chinese research vessel “Haiyang 4” for geological survey of gas hydrate in SCS in the summer of 2012 (Figure 1). The core was immediately cut into sections of length 30 cm onboard, and the topmost 3 cm interval of each section was cut and preserved in liquid nitrogen. The samples were kept frozen and at the end of the trip transported to the Geomicrobiology Laboratory of China University of Geosciences (Beijing) for microbial analyses.



Figure 1. Location of Core CL11 (marked by red star) in the northern SCS.

Geochemical and sedimentary analyses

Granulometric analysis of sediments was measured using a Mastersizer 2000 laser particle size analyzer following the manufacturer’s instructions in the laboratory of Guangzhou Marine Geological Survey. The sediment porosity was determined as Ma et al. (2013) described. Total organic carbon (TOC) content was determined according to the Jiao et al. (2015) protocol using a Multi N/C 2100 Analyzer. Headspace methane concentration was measured by a gas chromatography mass spectrometry as previously described Deng et al. (2006).

Microbial cell and archaeal DNA abundance analysis

Total microbial cells from the samples were enumerated using the method of acridine orange staining as previously described by Bottomley (1994). Archaeal 16S rRNA gene copies were subjected to quantitative PCR (qPCR) analysis using an ABI 7500 real-time PCR system (Applied

Biosystems, USA). The standards for archaeal 16S rRNA genes were prepared from the total community DNA amplified using primers of Arch21F/Arch958R (DELONG et al 1992). The amplicons were made into serial dilutions at 1:10 as the templates to generate a standard curve for total archaeal 16S rRNA genes. The plots of CT versus standard DNA concentration yielded the linear relationship with R^2 values greater than 0.99. PCR was performed in a total volume of 20 μ L mixture, which containing 10 μ L of SYBR Premix Ex Taq (TaKaRa, Japan), 1 μ L of DNA, 10 pmol of each primer, 10 pmol ROX Reference Dye II and appropriate volume of ddH₂O. PCR amplification were performed at 1 cycle of 95 °C for 30 s, followed by 40 cycles of 95°C for 5 s, 60°C for 34 s. Triplicates of each sample was performed qPCR to determine the 16S rRNA gene copies.

DNA isolation and PCR amplification

Seven sediment subsamples CL11-1 (17 cmbsf), CL11-8 (164 cmbsf), CL11-18 (364 cmbsf),

CL11-26 (524 cmbsf), CL11-32 (644 cmbsf), CL11-37 (744 cmbsf), CL11-38 (764 cmbsf) were selected and subjected to microbial community and geochemical analyses. The total community DNA was extracted from each sample using the Soil DNA Isolation Kit (MP) following the manufacturer's instruction. The extracted DNA was quantified by Nanodrop (Thermo Scientific, NanoDrop ND-1000) and visualized on a 1% agarose gel using ethidium bromide staining. The archaeal 16S rRNA genes were amplified by PCR (94 °C for 5 min, followed by 32 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 2 min and a final extension at 72 °C for 10 min) using primers of 751F and 1204R (BAKER et al 2003). PCR reactions were performed in triplicate. A total volume of 20 μ L mixture was containing 4 μ L of 5 \times FastPfu buffer, 2 μ L of 2.5 mM dNTPs, 0.8 μ L of each primer (5 μ M), 0.4 μ L of FastPfu polymerase, and 10ng of template DNA.

Sequencing and Bioinformatics

Amplicons were cut from 2% agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, U.S.) according to the manufacturer's instructions and quantified using QuantiFluor™ -ST (Promega, U.S.). The purified amplicons were pooled in equimolar and paired-end sequenced (2 \times 250) on an Illumina MiSeq platform (Shanghai, China) according to the standard protocols. The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database (Accession Number: PRJNA285416).

Raw fastq files were demultiplexed, quality-filtered using QIIME (version 1.17) with the following criteria: (i) The 250 bp reads were truncated at any site receiving an average quality score < 20 over a 10 bp sliding window, discarding the truncated reads that were shorter than 50bp. (ii) Exact barcode matching, 2 nucleotide mismatch in primer matching, reads containing ambiguous characters were removed. (iii) Only sequences that overlap longer than 10 bp were assembled according to their overlap sequence. Reads which could not be assembled were discarded. Operational Taxonomic Units (OTUs) were clustered with 97% similarity cutoff using UPARSE (version 7.1) and chimeric sequences were identified and removed using UCHIME. The phylogenetic affiliation of each 16S rRNA gene sequence was analyzed by RDP Classifier against the SILVA (SSU115) 16S rRNA database using confidence threshold of 70% (AMATO et al 2013). Program MOTHUR was used to calculate the Shannon and Simpson diversity indices, and the bias corrected Chao1 (SCHLOSS et al 2009).

RESULTS

Sedimentary parameters

The grain size analysis showed that sediments of Core CL11 are mainly composed of silt (68.49-78.53%), sand (0-2.06%) and clay (20.85-31.51%). A relatively higher percentage of sand and lower percentage of clay occurred in the upper interval (90-440 cmbsf) of the core. The sorting coefficient of sediments varied from 1.33 to 1.58, indicating that sediments grains were generally well-sorted (Figure 2).

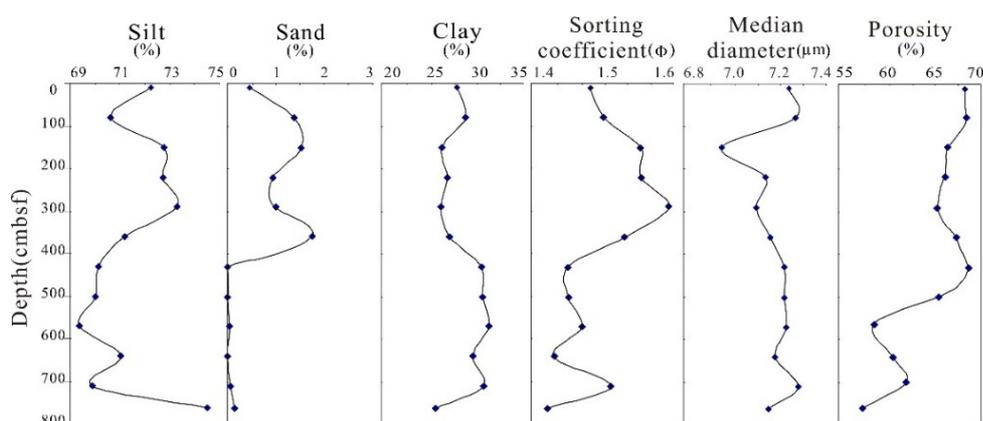


Figure 2. Downcore variation of sedimentary parameters in the Core CL11.

The sedimentary porosities were higher (>65%) above the depth of 550 cmbsf than that in the lower part of the core. The vertical variation of porosity was apparently correlated with the abundance of sand and clay in the sediments (Figure 2). This result agrees with Patrick et al. (2007),

which found that sediment porosity for shallow and unconsolidated sediments is mainly correlated to abundances of sand and coarser silt, and then to fractional-packing type of these coarse grains.

Geochemical parameters and SMI determination

Downcore geochemical parameter analysis showed that TOC content slightly increased with the depth ranging from 0.8% to 1.2% along the Core CL11. This result was similar with Su et al. (2005) reported in the Dongsha area. They found that the TOC content was usually higher than 0.8% in sediments, based on a summary of TOC data from more than 150 sites in the SCS. The sedimental methane concentration was too low to be detected from the top of the core down to the depth of 650 cmbsf. However, a significantly increasing methane concentration was detected at the depths of 744 cmbsf

and 764 cmbsf (Figure 3a), indicating a methane seepage occurred below the sediment. A similar result was observed from a nearby located gravity Core HD196 (Figure 3b) (DENG et al 2006), in which a clear SMTZ was observed between the depths of 600 to 800 cmbsf with a sharp decrease in sulfate concentration and an increase in methane concentration. Although we did not assay the dissolved sulfate of pore water in this gravity core, we may infer that the sharply increase of CH_4 at the nearly bottom of the core is roughly representative of a sulfate-methane transition zone.

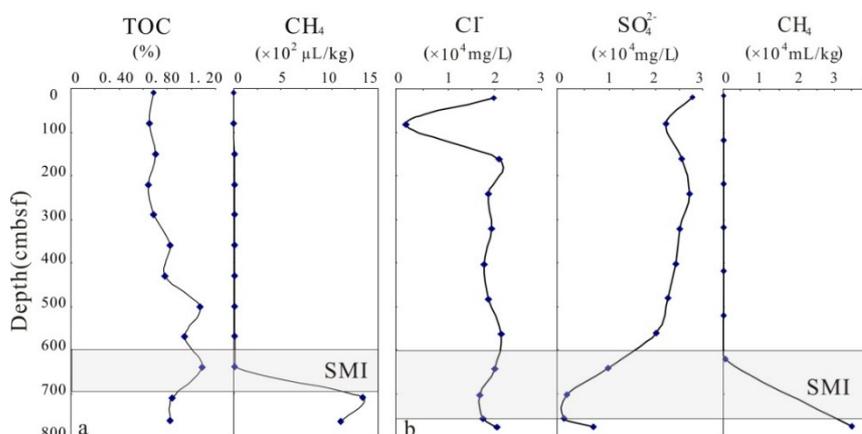


Figure 3. Downcore geochemical parameters of Core CL11 (a) and Core HD196 (b) (DENG et al 2006).

Vertical variation of microbial abundance

Downcore microbial analysis indicated that the microbial cell abundance varied from 2.13×10^7 to 10.47×10^7 cells/g (Figure 4). A relative higher abundance was observed at the depth of 17 cmbsf, 450 cmbsf and 740 cmbsf of the Core CL11. The high cell abundance at the surface sediment of the deep sea samples agreed with previous reports (ZHANG et al 2012). The higher abundance in the

middle and bottom intervals of the core is still unexplained, though it was inferred to be related to the specific environments. Total archaeal 16S rRNA gene copies estimated by qPCR varied from 1.64×10^7 to 3.57×10^7 copies/g and had a similar pattern as the microbial cell abundance (Figure 4). These data implied that archaea constituted a significant proportion of the microbial community.

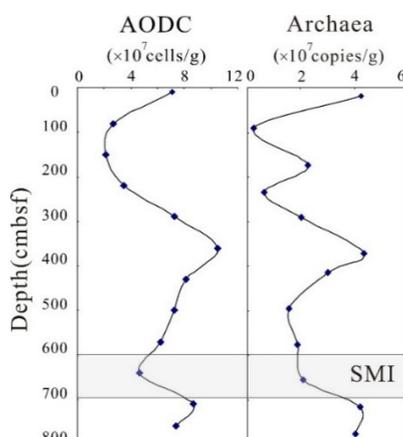


Figure 4. Downcore variation in abundance of microbial cell abundance and archaeal 16S rRNA gene copies in the Core CL11.

Sequencing analysis

A total of 101,115 reads were generated from seven samples. After filtering 96,917 high-quality sequences, nearly 95.85% of total reads were used for pyrosequencing analysis. Each library contained 9,996 to 18,676 valid reads (Table 1). A total of 275 OTUs were obtained with an average of OTUs ranging from 33 to 207 per sample based on $\geq 97\%$ nucleotide sequence identity between reads. The number of OTUs differed significantly between

the samples, with a higher number of OTUs detected in the upper core and decreasing numbers in the lower core. This result was also consistent with the alpha diversity analysis, which showed that the Shannon and Simpson indices varied with sediment depth, indicating a different alpha community and composition at different depths. Generally, a trend of decreasing diversity with sediment depths was apparent with rarefaction and OTUs number analyses.

Table 1: Sequencing information and diversity estimates for each sample.

Sample	cmbfsf	Reads	OTUs	Coverage	Chao	Shannon	Simpson
CL11-1	17	18676	129	99.94	136(131, 157)	2.52(2.5, 2.54)	0.1744(0.1701, 0.1788)
CL11-8	164	16120	207	99.94	210(208, 219)	3.89(3.86, 3.91)	0.0401(0.0391, 0.0412)
CL11-18	364	10204	167	99.78	181(172, 205)	3.61(3.58, 3.64)	0.0534(0.0515, 0.0553)
CL11-26	524	13859	148	99.81	167(155, 198)	2.75(2.72, 2.78)	0.1386(0.1347, 0.1425)
CL11-32	644	17579	126	99.93	133(128, 154)	2.71(2.69, 2.74)	0.1335(0.1305, 0.1366)
CL11-37	744	10483	59	99.92	64(60, 82)	2.38(2.35, 2.4)	0.1549(0.1507, 0.1592)
CL11-38	764	9996	33	99.97	34(33, 44)	2.31(2.29, 2.33)	0.144(0.1404, 0.1476)

Composition of Archaeal Communities

Two phyla, *Crenarchaeota* and *Eurarchaeota*, were detected across sediment samples. Relative abundance of *Crenarchaeota* (69.41%) was nearly equal to the phylum of *Eurarchaeota* (25.26%). The retrieved crenarchaeal sequences were classified into four lineages, comprised of MBG-B (28.17%), MCG (23.36%), C3 (11.84%) and MGI (6.04%). Within the

phylum of *Euryarchaeota*, six lineages were detected, including SAGMEG (17.37%), *Thermoplasmatales* (3.65%), *Halobacteriales* (3.26%), MBG-E (0.06%), *Methanomicrobia* (0.91%), ANME-1 (0.01%) and unclassified sequences (5.33%). The two minor groups of *Methanomicrobia* and ANME-1 were thought to be related to methane oxidation in anaerobic conditions.

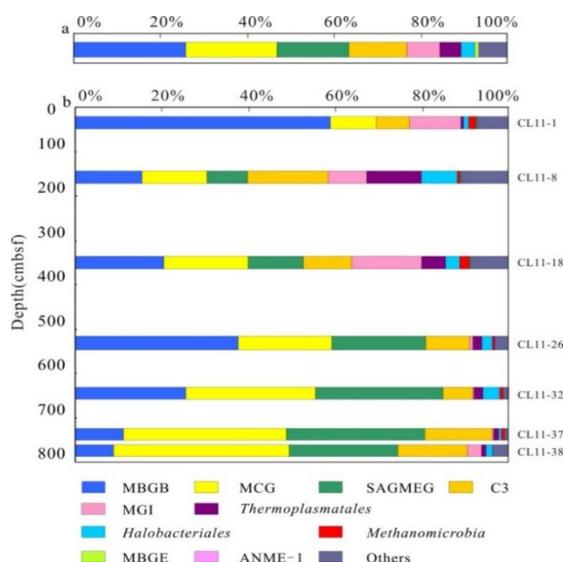


Figure 5. Relative abundance of taxonomic groups (a) and archaeal community compositions (b) in the sediments of Core CL11.

The depth profile of archaeal community showed clear community changes with depth (Figure 5b). Altogether, three lineages, MBG-B, MCG and SAGMEG, comprised 68.9% (66,777 sequences) of the archaeal communities, and these three were the dominant groups across all seven sediment depths. Among these, MBG-B occurred in the highest abundance, accounting for 58.9% (11,003 sequences) of the total sequences occurring in the topmost layer of the sediment, then MBG-B decreased significantly with depth. In contrast, the abundances of MCG and SAGMEG increased from 10% to 40% and 0 to 30% moving towards the downcore of CL11, respectively. However, the relative abundance of sequences corresponding to MGI was the dominant group from 17 to 364 cmbsf. The methane-oxidizing archaea are usually the dominant groups distributed in the sulfate-methane-transition zone (LEE et al 2013); however, for unknown reasons we only detected low abundances of ANME-1 in this zone.

DISCUSSION

Microbial cellular abundance and affecting factors

Previous studies indicated that cell abundance determined with AODC could provide a broad overview of the distribution of microorganisms in the sediments (PARKES et al 2000). Total cell number ranged from 10^8 to 10^9 cells/cm³ in sediments near the surface, and declined with depth in the sediment column (D'HONDT et al 2004), but showed variation with the different environments. The bacterial cell abundance was estimated to range from 10^5 - 10^8 cells/g in sediments in continental slope or deep sea sediments from the SCS (ZHANG et al 2012; JIAO et al 2015; JIANG et al 2007). It was inferred that variations of microbial abundance in marine sediments might relate to concentrations of intact phospholipids and TOC (LIPP et al 2008). The microbial cellular abundance in sediments of Core CL11 was 10^7 cells/g, which was higher than both the 10^6 cells/g measured in abyssal (collected at about 3000 m water depth) cold seep sediment with 0.5% TOC of the northeastern SCS (ZHANG et al 2012). Our data is similar with the result from Qiongdongnan Basin, in which the cell number was 10^7 cells/g in sediments with 0.84 % to 1.2 % TOC (JIANG et al 2007). Downcore microbial cell abundance showed that relatively higher bacteria abundance was detected at depths of 350 cmbsf and 720 cmbsf, which is not consistent considering the TOC content. It was inferred that the bacterial cell abundance might be related to the porosity of

sediments and methane supply. With 2.06 % sand fraction at a depth of 350 cmbsf was measured. It can be inferred that sediment porosity increased with the increased sand fraction, resulting in a possible increase of fluid or energy supply in the sediments. Similar evidence was found in sediments at Integrated Ocean Drilling Site C0006, Nankai Trough, where cell abundance increased in the sand interval (about from 30 cmbsf to 75 mbsf) (EXPEDITION 316 SCIENTISTS), whereas TOC content was relatively low in those intervals. Methane is a very important factor in cold seep areas and plays a vital role in chemical cycling. Previous studies indicate strong correlations between downcore variations of microbial cellular abundance and methane concentrations in cold seep sediment, as well as in sediments containing gas hydrates (ZHANG et al 2012). The relative higher bacterial cell abundance at the depth of 700 cmbsf was inferred to be related with a higher methane concentration. However, a relative lower bacterial cell abundance was detected in SMTZ, which is inconsistent with higher methane-oxidizing microbial activity.

Archaeal community variation and geobiochemical reactions

The archaeal communities in cold seep sediments from the SCS were compared with those in other world oceans. A slight shift of community composition was observed, indicating that unique archaeal communities exist in various cold seeps around the world oceans. Halobacteria and MBG-B were dominant archaeal communities in the abyssal cold seep at a water depth of 3,000 mbsf in Core DSH-1 (ZHANG et al 2012). However, MGI and ANME archaeal groups were dominant in cold seep surface sediments from the Nankai Trough along the western margin of the Pacific Ocean (ARAKAWA et al 2006). In the Gulf of Mexico hydrocarbon seep, *Methanosarcinales*, ANME-1, *Thermoplasmatales* and *Methanomicrobiales* were found to be dominant (MILLS et al 2003), and ANME was the dominant group in the Sonora Margin cold seeps in Guaymas Basin (Gulf of California) (VIGNERON et al 2013).

Archaeal community composition and distribution analyses showed that MBG-B was widely distributed across all the depths of the Core CL11. Previous studies showed that MBG-B occurred in a wide range of sampling sites and sediment types (TESKE et al 2008). Originally, MBG-B were detected in surface sediments on the continental slope and abyssal plain of the North Atlantic (VETRIANI et al 1999) and hydrothermal vent sites (TAKAI et al 1999), then in deep sea sediments in hydrate zones at

Nankai Trough and the Peruvian continental shelf (REED et al 2002; SØRENSEN et al 2006). MBG-B was also the dominant group in the hydrate-bearing sites of the Cascadia and Peru Margins; as well as in the sulfate reducing zone in shallow sediments above gas hydrates (INAGAKI et al 2006). It was suggested that MBG-B might play an important role in the biogeochemical processes of sulfate reduction and methane oxidation (BIDDLE et al 2006). Although there is limited knowledge about the metabolic functions of the MBG-B group, the recognition of microbial populations that consistently occur in the presence of methane hydrates probably serves as a starting point for defining their ecological and biogeochemical significance (INAGAKI et al 2006). Our data also showed that MCG and SAGMEG were also dominant groups, with both of them increasing in abundance with depth. MCG live in diverse habitats, including terrestrial and marine, hot and cold, surface and subsurface environments (BIDDLE et al 2006; KUBO et al 2012). Although this group was frequently observed to be abundant, diverse and widespread in marine sediments (KUBO et al 2012), it seems unlike the methanotrophs to utilize methane for energy. Biddle et al. (2006) suggested that the MCG dominated sediment horizons indicated that MCG utilized the buried organic carbon and likely a group of heterotrophic archaea to assimilate complex organic substrates. The archaeal group of SAGMEG was also another predominant group in the Core CL11, which slightly increased in abundance with the elevated methane concentration. Previous studies showed that SAGMEG appeared to be a metabolically active archaeal group in deep marine subsurface sediments (SØRENSEN et al 2006) and might prefer living in methane rich or gas hydrate zones, as it was commonly found in deep marine sediments containing methane hydrates in the Nankai Trough (REED et al

2002). The SMTZ is a general feature of marine sediments and is thought to be associated with microbial activities with anaerobic oxidation of methane coupled with sulfate reduction driven by bacteria. An analysis of the current data set for the 16S rRNA genes of the ANME groups indicates that their occurrence is limited to the upper few meters of subsurface sediments, as no genes have been retrieved from any deep biosphere core yet (KNITTEL et al 2005), which is very similar with our result obtained from the Core CL11.

CONCLUSIONS

The abundance of total bacterial cell number and the archaeal 16S rRNA gene copies were associated with either the sediment texture or methane concentration in the Core CL11.

The Illumina sequencing result showed the community was predominantly comprised of *Crenarchaeota* and *Eurarchaeota*. MBG-B, MCG and SAGMEG were the dominant groups across the seven sediment depths, and a slight shift of community composition was observed at increased depths.

The data in this study will add to the knowledge of the archaeal community composition and enhance our understanding of their possible roles in geochemical processes in the cold seep sediments of the northern SCS.

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RESUMO: O recife de metano de Jiulong, uma grande área de emissão fria no talude continental do Mar da China Meridional – SCS, foi caracterizado através da fauna quimiossintética, dos tapetes bacterianos, estruturas de carbonato derivadas do metano, e por perfis de sedimentos de pouca profundidade na zona de transição de sulfato-metano, SMTZ, coletados por gravity corer (testemunhador à gravidade). De modo a caracterizar a diversidade microbiana e a variação na correlação com a emissão fria de metano na área, a comunidade archaeal nos sedimentos do perfil CL11 foi analisada, usando o sistema de sequenciamento Illumina MiSeq. Um total de 96.917 sequências válidas foram obtidas e classificadas em 275 OTUs. As análises taxonômicas mostraram que todas as sequências archaeais pertenciam aos filos *Crenarchaeota* (69.41%) and *Eurarchaeota* (25.26%). A distribuição vertical das comunidades archaeal mostrou que o Grupo Bêntico Marinho B (Marine Benthic Group B, MBG-B) foi o grupo predominante (58,92%) no sedimento da extremidade superior do core, enquanto que porcentagens das sequências do South African Gold Mine *Euryarchaeotal* Group (SAGMEG) aumentaram com a profundidade. Os nossos resultados indicam que a comunidade archaeal na emissão fria do recife de metano de Jiulong é única e nos fornece novas perspectivas a respeito da composição da comunidade archaeal na área de emissão fria.

PALAVRAS-CHAVE: Profundidade. Diversidade. Zona de transição sulfato-metano.

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