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Trophic strategy of diverse methanogens across a river-to-sea gradient[§]

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Methanogens are an important biogenic source of methane, especially in estuarine waters across a river-to-sea gradient. However, the diversity and trophic strategy of methanogens in this gradient are not clear. In this study, the diversity and trophic strategy of methanogens in sediments across the Yellow River (YR) to the Bohai Sea (BS) gradient were investigated by high-throughput sequencing based on the 16S rRNA gene. The results showed that the diversity of methanogens in sediments varied from multitrophic communities in YR samples to specific methylotrophic communities in BS samples. The methanogenic community in YR samples was dominated by Methanosarcina, while that of BS samples was dominated by methylotrophic Methanococcoides. The distinct methanogens suggested that the methanogenic community of BS sediments did not originate from YR sediment input. High-throughput sequencing of the *mcrA* gene revealed that active Methanococcoides dominated in the BS enrichment cultures with trimethylamine as the substrate, and methylotrophic Methanolobus dominated in the YR enrichment cultures, as detected to a limited amount in *in situ* sediment samples. Methanosarcina were also detected in this gradient sample. Furthermore, the same species of *Methanosarcina mazei*, which was widely distributed, was isolated from the area across a river-to-sea gradient by the culture-dependent method. In summary, our results showed that a distribution of diverse methanogens across a river-to-sea gradient may shed light on adaption strategies and survival mechanisms in methanogens.

Keywords: trophic strategy, methanogens, diversity, Bohai Sea (BS), river to sea gradient

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Introduction

Biomethane plays an important role in global warming and renewable energy (Cavicchioli, 2007). Furthermore, methanogenesis is a major terminal process of biogeochemical carbon cycle in ecosystems and energy production (Cavicchioli, 2007; Conrad, 2009). Methanogens are important biogenic sources of methane, which can be divided into seven orders: Methanopyrales, Methanococcales, Methanobacteriales, Methanomicrobiales, Methanosarcinales, Methanocellales and Methanomassiliicoccales (Großkopf *et al.*, 1998; Dridi *et al.*, 2012). Aside from Methanomassiliicoccales, all the methanogens have the functional gene *mcrA*, which is the key functional gene to detect the unexplored methanogens in circumstances (Lueders *et al.*, 2001; Lang *et al.*, 2014). Among these methanogens, there are three main pathways of methane production: hydrogenotrophic methanogenesis (H_2 reducing CO_2 to CH_4), methylotrophic methanogenesis (C1-methylated disproportion to CH_4 and CO_2) and acetoclastic methanogenesis (acetate disproportion into CH_4 and CO_2) (Cavicchioli, 2007; Conrad, 2009). Nowadays, a novel pathway is discovered that methanogens can accept electrons to reduce CO_2 to CH_4 via direct interspecies electron transfer (DIET) (Liu *et al.*, 2012). Methanogens are ubiquitous in various systems including soils (Liu and Conrad, 2010; Kantachote *et al.*, 2016; Heděnec *et al.*, 2017; Kim *et al.*, 2017; Sang *et al.*, 2017; Xiao *et al.*, 2017), sediments (Zeikus *et al.*, 1980; Mckay *et al.*, 2017), livestock (Moraes *et al.*, 2014; van Lingen *et al.*, 2017), digester (Ruf and Emmerling, 2017; Xing *et al.*, 2017) etc. However, limited researches about methanogenesis in marine areas (Li *et al.*, 2017), in which sulfate, ferric oxides are the major effect factors. But the methylotrophic pathway cannot be inhibited by the sulfate reduction, which sulfate reducing bacteria cannot use methyl (Purdy *et al.*, 2003). It was reported that trimethylamine concentration was an important factor in shaping the methanogenic archaea composition in a coastal salt marsh (Yuan *et al.*, 2014). Many researches have been reported the abundance of methanogens in marine and coastal region is relatively lower than other environments (Crutzen, 1991; Li *et al.*, 2008; Fan *et al.*, 2017). However, it is unknown what will occur when anthropogenic activities influence these areas, especially, with the development of fishery and industrialization (Waarde, 1988; Qu and Chen, 2009), for which trimethylamine is a byproduct that is input into the environment.

The Bohai Sea (BS) is a continental sea of China that is shallow and semienclosed (with an approximately 18 m average depth and area of 78,000 km^2). The BS is surrounded by the Shandong, Hebei, Liaoning provinces and the Tianjin municipality (Hu *et al.*, 2016). The BS connects and exchanges seawater with the Yellow Sea (YS) through the Bohai Strait. Meanwhile, BS is being affected by water quality degenera-

tion, including hypoxia and eutrophication. Recently, increasing anthropogenic activities (e.g., industrial, agricultural, and municipal activities) have accelerated these problems in BS (Fang *et al*., 2010; Wang *et al*., 2015). As a result, the BS will provide a good example to understand the effect of human activity on methane emission in marine sediments. It is reported that the nearshore emits more methane than other areas (Li *et al*., 2010), but the role of methanogens is still unknown. It was reported that coastal areas have high potential CH_4 emission, possibly because tidal water accelerates cellulose degradation by increasing oxygen to provide substrates for methanogens (Arai *et al*., 2016). The BS regions and estuarine areas undergo drastic water input and include tidal flooding places. The four major rivers, including Liao He, Luan He, Hai He, and Yellow River (YR), absorb this action. The Yellow River, as the largest river of these rivers, carries a huge amount of sediment to the BS (Cauwet and Mackenzie, 1993), which significantly influences the offshore places. Aside from the carbon resource, other factors on methane production in marine sediment should be considered.

The effect of physico-chemical characteristics such as pH, salinity and sulfate from the river to the sea may shape microbial communities. Estuarine areas of transition between the land and sea have dynamic interactions and contain much organic carbon with huge greenhouse gases production potential (Hedges, 2002). However, the diversity and trophic strategy of methanogens in these areas in still unknown. Moreover, we have found that archaea communities in the sediments of some BS regions have a similar composition, but the potential of methane emission is higher in coastal areas than in other parts of the BS (Wang *et al*., 2017). As YR is the largest water body (4.23×10^{10} m^3/year), the sediment (1.006×10^9 t/year) and terrestrial organic carbon discharge in the surrounding rivers (Shi, 2014; Gao *et al*., 2016), is also significant. The region from YR to BS provides a good example to evaluate methanogen changes across the river to coastal areas and to the sea. Thus, we chose the YR among four rivers to explore the river impact across the river to sea accurately. At the same time, we selected the representative samples YR-1, YR-2, YR-3, M1, M5, M7, M9, E4, L3, and BHB2 for more detailed information about the archaea community and the differences among YR and other river coastal area in BS.

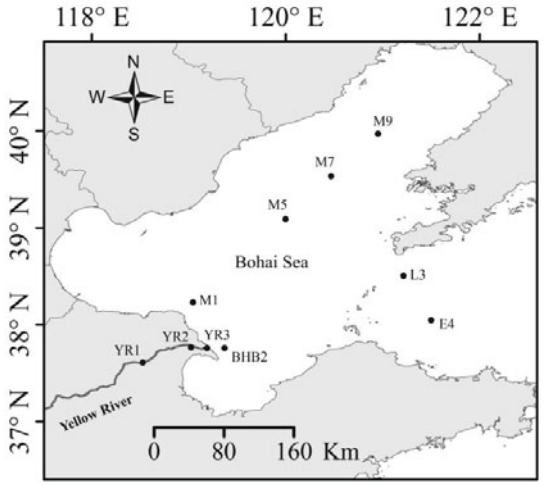

Fig. 1. Location of sampling points in Bohai Sea (BS) and Yellow River (YR).

Materials and Methods

Sediment samples

Surface sediment samples (0–20 cm) were collected from different sites of the Bohai Sea (BS) and the Yellow River (YR), China (Fig. 1). Samples were homogenized within 2 min, and samples were frozen at 4°C. The collections were immediately transported to the Yantai Institute of Coastal Zone Research in Yantai. A portion of the sediment samples were kept at -80°C for geochemical analysis (Supplementary data Table S1) or nucleic acids extraction, and another portion of sediments were stored at 4°C for enrichment cultures (Supplementary data Table S1).

Chemical analysis

The geochemical characteristics of samples were analyzed using standard methods as described previously (Wang *et al*., 2017). Total organic carbon (TOC), pH, SO_4^{2-}, and total nitrogen (TN) analyses were performed, as described previously (Wang *et al*., 2017). The inorganic nitrogen (NH_4^+-N, NO_3^--N, and NO_2^--N) in samples was extracted with 2 mol/L KCl at a 1:10 sediment: water (w/v) ratio and analyzed using an autoanalyzer (Autoanalyzer III, Seal). In addition, weak-acid-soluble Fe(II) and total reactive hydroxylamine-reducible iron were determined as described by Zheng *et al.* (2015). The gaseous samples (100 µl) were collected from enrichment cultures, and the concentrations of CH_4 were analyzed using gas chromatography (GC) 7820A (Agilent Technologies) equipped with a flame ionization detector (FID).

Enrichment culture

The anaerobic estuarine enrichment medium (NH_4Cl 1.5 g/L, NaH_2PO_4 0.6 g/L, NaCl 11.7 g/L, $NaHCO_3$ 2.5 g/L, $CaCl_2{\cdot}2H_2O$ 1.0 g/L, KCl 0.1 g/L, $MnCl_2{\cdot}4H_2O$ 0.005 g/L, $Na_2MoO_4{\cdot}2H_2O$ 0.001 g/L, $MgCl_2{\cdot}6H_2O$ 5.3 g/L, $MgSO4{\cdot}7H_2O$ 0.1 g/L, Yeast Extract 0.05 g/L) was used to enrich methanogenic archaea in the YR and BS sediments, and 33 mmol/L trimethylamine served as the substrate. Before autoclaving, the medium was purged of O_2 in solution and headspace was sparged with N_2/CO_2 (80/20, v/v) for 30 and 15 min, respectively. The sludge sample was inoculated at 10% (w/v) into the culture medium after autoclaving. All cultures were incubated at 30°C in the dark without shaking under an atmosphere of N_2/CO_2 (80/20).

Isolation of methanogens

For methanogen isolation, samples were grown in mineral salts (MS) medium with 30 mmol/L trimethylamine. MS consisted of the following (Zehnder and Wuhrmann, 1977): Basic culture medium (0.5 g/L Tryptone, 0.5 g/L Soy peptone, 0.5 g/L L-cysteine·HCl, 46 ml 0.2 mol/L K_2HPO_4, 20 ml 0.2 mol/L KH_2PO_4); 20 ml Solution A (Mineral:Trace element:Vitamin

= 48:1:1); 20 ml Solution B (L-cysteine hydrochloride solution containing 80 g/L $NaHCO_3$:Na_2S = 49:1); and 1 ml (0.1%) resazurin. The medium and resazurin were allotted to anaerobic tubes and O_2 was eliminated with a gas combination of N_2/CO_2 (80/20), as shown above. Tubes were sealed and autoclaved for 20 min at 121°C. Before inoculation, Solution A and B were added to the tubes. Using the Hungate roll-tube technique, 10% inocula of the dilution (up to 10^{-9}) were injected into the MS medium with 2.0% agar (Bagnara *et al.*, 1985). Two weeks later, colonies were picked and transferred to anaerobic tubes containing 10 ml liquid medium individually using a disposable, sterilized inoculum needle in the Coy anaerobic chamber. To inhibit the growth of nonmethanogenic organisms, vancomycine (100 mg/L) was added. Methane-producing cultivation from a single colony was purified by the streak plate method till no contamination with nonmethanogenic bacteria remained.

Molecular analyses

The archaeal community of the selected BS and three YR samples was analyzed using high-throughput sequencing. The DNA of sediment samples was extracted using a FastDNA™ SPIN Kit for soil (MP Biomedicals) according to the manufacturer's protocol. Standard gel electrophoresis was used to determine the quality and concentrations of DNA extracts with a NanoDrop instrument (NanoDrop, Thermo Scientific). PCR was performed with a Taq DNA polymerase using primer Ar519f (CAGCCGCCGCGGTAA) and Ar915r (GTGCTCCCCCGCCAATTCCT) targeting the V4-V5 region of the 16S rRNA.

As methane production reached its plateau in each anaerobic enrichment culture, the total nucleotide acid was extracted using a bead-beating protocol (Shrestha *et al.*, 2009). In brief, cell lysis was homogenized with a FastPrep-24 instrument (MP Biomedicals). The extract was treated with gDNA Eraser (TaKaRa) to remove coextracted DNA. Reverse transcription was performed after RNA denaturation at 70°C for 10 min, followed by an incubation step at 37°C for 50 min according to the instructions of the PrimeScript™ RT reagent Kit (TaKaRa). To assess archaeal community composition, *mcrA* were amplified from the cDNA of enrichment cultures and sequenced by high-throughput sequencing.

The Quantitative Insights Into Microbial Ecology (QIIME) version 1.7.0 pipeline [(Caporaso *et al.*, 2010); http://www.qiime.org] was used to process raw sequencing data with the default parameters. Briefly, the representative sequences from each OTU were defined by 97% identity threshold level, after which chimeric and low quality reads were removed. Using the Ribosomal Database Project (RDP) classifier (Li *et al.*, 2016), the taxonomic classification of each OTU was assigned.

The average relative abundance (%) of the predominant genus-level taxonomy in each sample was assessed by comparing the assigned sequences number of a particular taxon to the total obtained sequences number. The computation of alpha diversity (Chao 1, Shannon and Simpson Index) was competed using the R program (http://www.r-project.org) with the vegan package. To clarify the differences in microbial community structure, beta diversity [unweighted UniFrac distances for principal coordinates analysis (PCoA)] was computed for a randomly chosen subset among samples.

Statistical analysis

Canonical correspondence analysis (CCA) with R and the package "CCA", were used to assess the correlation between archaeal community structure and environmental variables ($P < 0.05$).

Phylogenetic analysis

Genomic DNA for the methanogenic isolate was extracted using a previously described procedure (Marmur, 1961). The 16S rRNA gene was amplified via PCR from extracted DNA with the primers Ar109f and Ar915r. The amplifications were purified and sequenced at Life Technologies. The sequence was checked using BLAST searches on the NCBI website

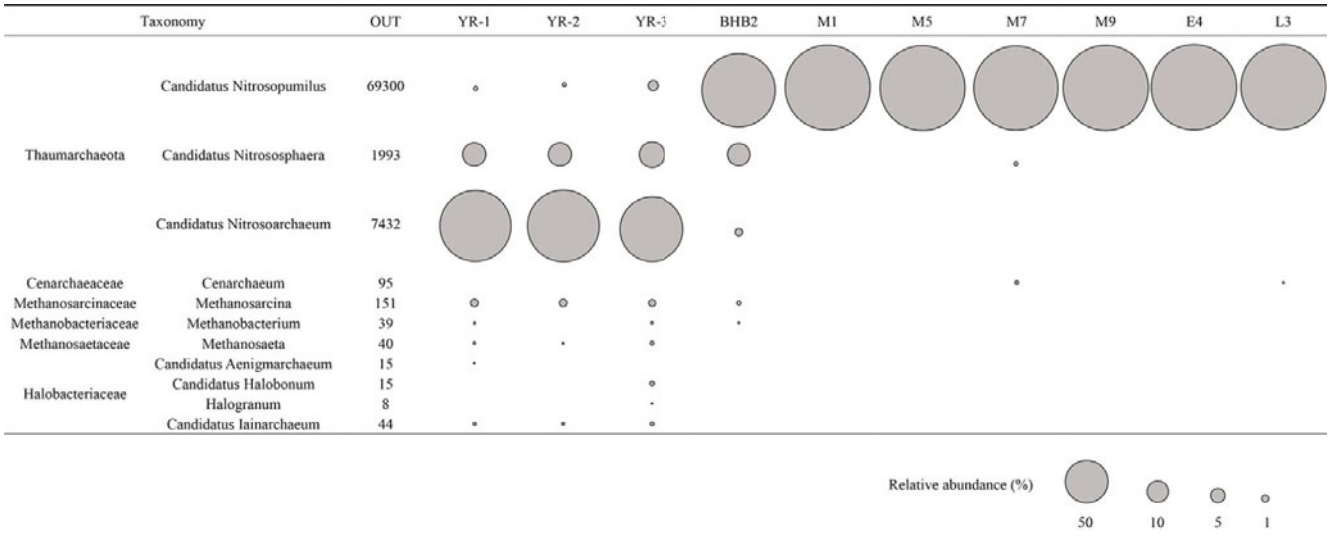

Fig. 2. Microbial community structure based on 16S rRNA gene amplicon sequencing. OTUs with maximum abundance > 0.2% in at least one sample are displayed. Bubble size represents relative abundance.

(http://www.ncbi.nlm.mih.gov). Phylogenetic analysis of the sequence and closely representative sequences were performed using the MEGA 7.0 (Tamura *et al*., 2013) software package with the neighbor-joining method.

Results

The diversity of methanogens from *in situ* sediments

The diversity of archaea was investigated via high-throughput sequencing based on 16S rRNA genes (Figs. 2 and 3). The dominant archaea in the BS samples was Candidatus Nitrosopumilus, and in YR was Candidatus Nitrosoarchaeum. Candidatus Nitrososphaera was present in YR samples and some BS samples, especially BHB2. In contrast to other archaea, methanogens in BHB2 was more similar to YR methanogens. The results showed that Methanosarcina is found in marine and river sediment samples. The YR samples were dominated by Methanobacterium, Methanosaeta, Methanobrevibacter, and Methanimicrococcus. The methanogens in coastal zone (BHB2) were similar to those in YR ones but contained methylotrophic Methanococcoides instead of Methanimicrococcus. This indicated that the microorganisms, especially methanogens in coastal sediments, are largely influenced by the river input. Interestingly, Methanococcoides were the main methanogens in BS sediment samples. These results indicated that the diversity of methanogens presented spatial variation from the YR to the estuary to the BS, with a reduction in trophic types. The mixed trophic Methanosarcina decline in the transition from water to sea also indicates that the freshwater environment is more suitable for these methanogens.

Spatial variation and effect of environmental factors on microbial community

To analyze the spatial variation of microbial communities in different sediments, the main coordinate structure of the microbial community at 10 sites was analyzed by principal

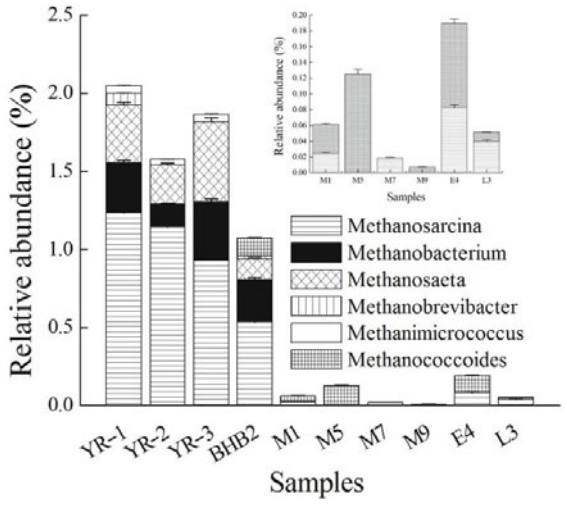

Fig. 3. Relative abundance of methanogens from *in situ* sediments of BS and YR.

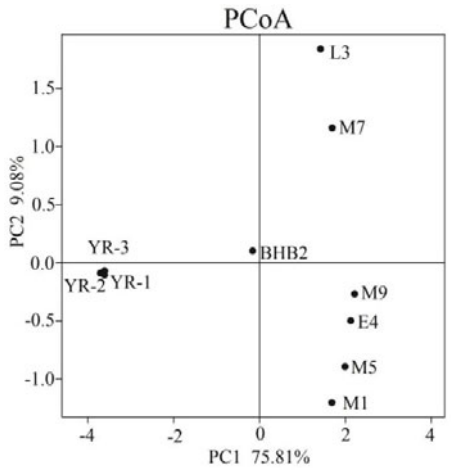

Fig. 4. Biplot of principal coordinate analysis (PCoA) of microbial community distribution of sediment samples. The scatterplot is of principal coordinate 1 (PC1) vs principal coordinate 2 (PC2). The percentage of variation in samples is described by plotted PCs as shown on the axes.

coordinates analysis (PCoA, Fig. 4). The results showed that the similarity of microbial communities among samples was not directly related changes in spatial location, especially for marine samples. The three YR (YR-1, YR-2, and YR-3) sediment samples clustered together and were significantly distant from that in the BS, except for BHB2, which was situated in the center and was surrounded by other BS sites. These results indicate potential methanogen diffusion along with sediment input processes, which originated from the YR to the coastal region and eventually diffused to other areas in BS. To analyze the effects of environment on this potential input process, the (CCA) method was tested to determine the relationship between the microbial community and environmental factors (Supplementary data Table S1), including pH, conductivity, SO_4^{2-}, NH_4-N^+, NO_3-N^-, Fe^{3+}, Fe^{2+}, and TOC in different sediments. The significant changes to environmental factors from water to sea, such as in salinity, pH and sulfate, significantly influenced the structure of archaeal communities in sediments from YR to BS (Table 1 and Fig. 5). This result indicated that these factors also influenced the reduction in diversity and transition of trophic types of methanogens in this input process from the river to the coastal zone to the marine environment. For methyl-type methanogens in all sediments, these methanogens in the sea may derive from river input.

Table 1. Pearson correlation between different environmental factors

	R[a]	*P*[b]
pH	1	
Salinity	**.800**	**.005**
Fe^{2+}	.494	.147
Fe^{3+}	.691	.027
NH_4-N^+	-.209	.563
NO_3-N^-	-.436	.208
SO_4^{2-}	**.816**	**.004**
TOC	.524	.120

[a] Pearson's correlation rate.
[b] *P*-value Pearson's correlation confidence.

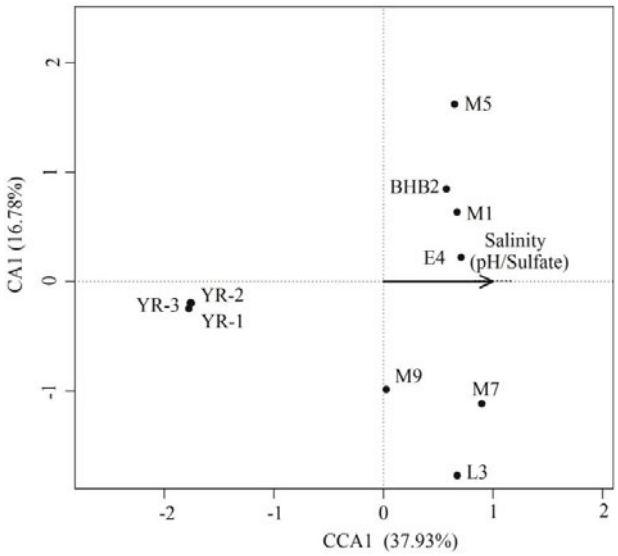

Fig. 5. Canonical correspondence analysis (CCA) of archaea community composition and environmental variables (arrows) from the YR and BS sediment samples. Environmental variables were selected based on significance as calculated from individual CCA results and variance inflation factors as calculated during CCA. The percentage of variation explained by each axis is shown. Ellipses in dotted line denote replicate samples from same site.

Methane production from enrichment cultures

To confirm the above hypothesis, YR-1 and M1 samples were cultured to observe microbial adaptability and variety as it varied with enrichment. Methane was produced from both the YR and BS sediments using trimethylamine, with 225% theoretical conversion to methane (Fig. 6). The lag phase of methane production was shorter in M1 samples from the BS than in the YR-1 samples from the YR. The initial time of methane production in the M1 site was 5 days and reached a maximum of 0.49 mmol (148.5% conversion rate) at 20 days, while the methane production of YR-1 samples accumulated at 10 days and reached 0.3 mmol (90.9% conversion rate) at 25 days. However, neither M1 nor YR sediments without added trimethylamine produced methane during this incubation (25 days). However, a shorter lag period indicated that methanogens in BS sediment samples were more suitable for methane production by trimethylamine in seawater than in YR. Ultimately, the YR produced methane, indicating that it contained methanogens capable of surviving the same treatment.

Active methanogens in enrichment culture

To verify the possibility of methanogens transfer from the river to the sea with sediments input, high-throughput sequencing based on the functional gene *mcrA* was used to analyze the diversity of archaea after enrichment of the M1 site and YR-1 samples (Fig. 7). Firstly there were very low relative abundances (< 0.05%) of Prevotella, Bacteroides, Faecalibacterium, and Lysobacter in both enriched sediments. The lower amount of Methanosarcina was found in both enriched samples compared to other methanogens. This indicated that Methanosarcina has strong environmental adaptability, but less than do Methanococcoides and Methanolobus in marine areas. Secondly, Methanococcoides dominated in the M1 enriched samples with higher relative abundance than was detected in *in situ* samples. However, Methanococcoides was not detected in the sediments of the YR after enrichment; exclusively methylotrophic methanogen was detected in the sediment of the BS, rather than the YR input. Finally, the dominant methylotrophic methanogens Methanolobus were also detected in the YR sediments after enrichment rather than in both BS and YR *in situ* sediment samples, indicating that it was not suited to the *in situ* environment.

The isolation of methanogens

To obtain the native methanogens from both the BS and the YR sediment, MS methanogenic medium was used for enrichment and isolation (Fig. 8). Four methanogens were isolated in MS methanogenic media with trimethylamine as the electron donor. Sequencing analysis of 16S rRNA gene indicated that all strains were affiliated with the species *Methanosarcina mazei* (KF29348) with 99–100% identity, and the closest cultivated relative is mesophilic archaeon *Methanosarcina mazei* strain Gö1 (JQ346757) (Pinto and Raskin, 2012).

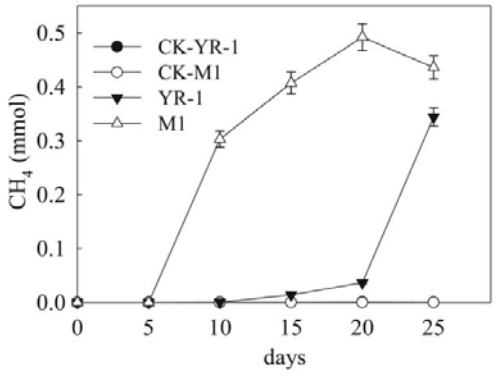

Fig. 6. Time-courses of methane production from seawater enrichment of YR sediment YR-1 and BS sediment M1 with trimethylamine as substrate. CK was M1 or YR-1 sediments without addition of trimethylamine.

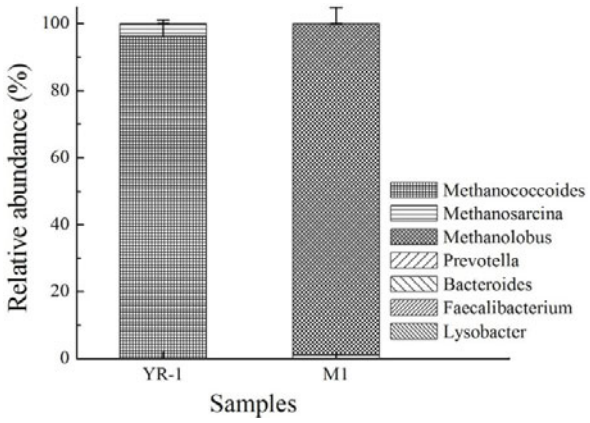

Fig. 7. Relative abundance of archaea from enrichment.

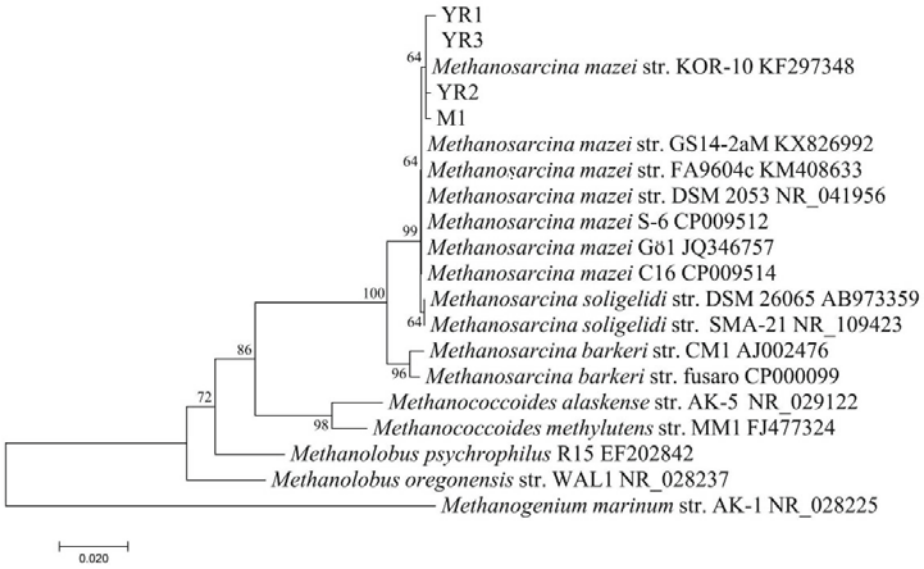

Fig. 8. Neighbor-joining phylogenetic tree of archaeal 16S DNA generated from a DNA extract of isolates. Sequences of isolates are shown with site name. Association with sequence of highest similarity in the database and GenBank accession number of reference sequence are indicated. Scale bars represents 2% sequence divergence. *Methanogenium marinum* strain AK-1 NR_028225 was selected as out-group.

Discussion

Methanogens produce methane and play significant roles in the biogeochemical carbon cycle. Methanogens are widely distributed in freshwater environments, coastal wetlands and marine sediments. As shown in Fig. 3, across river-to-sea gradients there was markedly reduced diversity and methanogenic type changing. At the genus level the diversity and trophic type of methanogens transferred from multitrophic methanogens such as the acetoclastic Methanosaeta (Jetten *et al*., 1992), hydrogenotrophic Methanobacterium (Smith *et al*., 1997), Methanobrevibacter (Miller *et al*., 1982), mixed trophic Methanosarcina (Elberson and Sowers, 1997) and methylotrophic or hydrogenotrophic Methanimicrococcus (Sprenger *et al*., 2000) into specific methylotrophic Methanococcoides (Kendall and Boone, 2006). Studies (Comte *et al*., 2014) have shown that marine-derived bacteria significantly diffused from river to sea with the growing pace of salinity, while freshwater-derived bacteria decreased. With the altered environment, microorganisms would actively regulate for adaptability, especially the trophic strategy. In this study, the relative abundance of freshwater-derived methanogens decreased or even disappeared, while the relative abundance of Methanococcoides increased across the river to sea gradient. As a result, the trophic strategy of diverse methanogens was altered by the *in situ* condition along the river input. Therefore, PCoA and CCA analyses were used to testify to this variation among these water and marine samples.

PCoA analysis revealed significant differences between the YR and the BS *in situ* sediment samples, while BHB2 as a coastal sample was located in the center, surrounded by other marine sediment samples. The BHB2 were closer to the YR samples but not closer than YR samples themselves, which means the water environment and input led to microorganismal community change along the water course to the sea. However, M5 and E4, which were further from BHB2 in space, were closer to the BHB2 community structure than M1, indicating that the direction of river input and ocean currents along the coast (Qiao *et al*., 2010) might be an important factor for microbial community structure. Hence, the environmental change, along with river input, created methanogen variation. Surprisingly, CCA analysis indicated that environmental factors, including pH, salinity and sulfate, which were caused by spatial extension from the river to the sea, were the important factor that caused the differences in community structure and the diversity of methanogens among these sediments. Zinder (1993) found that methanogens have good adaptability to salinity, so the sulfate following salinity change was the real reason for the large difference in methanogens in the BS and YR samples. Sulfate-reducing bacteria (SRB), which can compete with acetoclastic and hydrogenotrophic methanogens for substrates but not methyl compounds (Oremland and Polcin, 1982; Capone and Kiene, 1988), is grown on sulfate. Therefore, SRB in seawater inhibits other methanogenic pathways, except for methylotrophic methanogenesis (Oremland and Polcin, 1982). Thus, methylotrophic methanogen becomes the only organism in the BS environment. For this study, the methylotrophic methanogenic Methanococcoides were found in marine sediment samples but not in YR samples. The relative abundance of Methanococcoides was only limited by detection; it is not true that it does not exist in the YR environment (Li *et al*., 2018), due to substrate competition with Methanimicrococcus. YR carried a large amount of sediment and organic matter into the BS, especially in the coastal zone (Martin *et al*., 1993; Gao *et al*., 2016), the Methanococcoides might enter the sea, especially coastal area with this progress (Ticak *et al*., 2015). What is worse, the study of methanogen diversity with salinity in the coastal area of Min Jiang found that the diversity and activity of methanogens decreased with increasing salinity, while the input of organic matter can significantly inhibit this effect when salinity was low (Vizza *et al*., 2017). Based on these studies, it was reasonable to speculate that methanogens in BHB2 sediments might arise from YR input including Methanococcoides. In conclusion, sulfate and salinity increases led to changes in methanogens across the water to the sea, but whether these marine methanogens derive from water input is unknown. Hence, the enrichment

culture was used to clarify this unknown.

Methane was produced from enrichment with methylamine as the substrate, indicating that the sediments contained methylotrophic methanogens and could survive in the seawater environment. The high-throughput results showed that in M1 samples only the relative abundance of methanogens changed after enrichment. In addition, the dominant methyltype methanogens appeared in enriched YR samples and were not found *in situ* in the sediment. Except for Methanosarcina, the others did not appear in the YR enriched sample, indicating that in comparing the BS there were many other methanogenesis pathways *in situ*. The results showed that Methanococcoides dominated in the M1 sample, as detected in the *in situ* samples, but with higher relative abundance. It was reported that Methanococcoides grew in a salinity-free environment in the laboratory (Ticak *et al*., 2015). Unfortunately, the genus was not found in YR-enriched samples, indicating that Methanococcoides is a methanogen unique to the *in situ* environment of BS, rather than YR sediment input. Furthermore, the dominant methylotrophic Methanolobus was also detected in the enriched YR sediment but not detected in the *in situ* samples. These results indicated that YR methanogens could not survive in the marine environment. Therefore, in addition to the obvious methanogens with YR sediment input, BHB2 methanogens were closer to the YR methanogens because fresh water inputs diluted various salt ions and the provision of abundant organic matter to help methanogens to survive (Vizza *et al*., 2017) and alleviate competition between SRB and methanogens in the environment (Maltby *et al*., 2016). Compared with enriched YR and BS sediments samples, Methanosarcina in the *in situ* YR and coastal samples had a higher relative abundance and indicated that it was affected slightly by seawater environmental factors in these *in situ* areas (Jetten *et al*., 1992). To gain a deeper understanding on the ecological significance of methanogens, pure cultures were used. Four isolates of *Methanosarcina mazei* were obtained from enrichments of YR and BS sediments, although the abundance of Methanosarcina in both enrichments was relatively lower than the other two methylotrophic methanogens, indicating better adaption. The relative abundance of Methanosarcina in the *in situ* environment was not obvious. However, due to its broad substrate use and ability to reduce CO_2 to methane with the reducing power generated by other microorganisms, it may actively participate in the carbon biogeochemical cycle such as through organic carbon mineralization, or interconversion between CH_4 and CO_2. In conclusion, the marine methanogens did not come from the YR input, and the YR has its own marine methylotrophic methanogens, namely, Methanolobus. Further research is required to determine how isolates of *Methanosarcina mazei* adapted to different environments, the pathways of methanogenesis in the river and sea sediments and why Methanolobus only appeared in the enriched sediment.

In summary, the diversity and trophic strategy of methanogens in the BS and YR sediments across a river-to-sea gradient were investigated by culture-independent molecular techniques based on 16S rRNA genes. Methylotrophic methanogens dominated in two regions; the YR sites were dominated by Methanosarcina, while the BS sites were dominated by Methanococcoides. The functional gene *mcrA* revealed that Methanococcoides and Methanolobus actively dominated in the enrichment cultures of the BS and YR sites with trimethylamine as the substrate, respectively. In addition, the same isolates of *Methanosarcina mazei* were isolated by culture-dependent methods from the enrichment of both regions with trimethylamine as the substrate, suggesting that *Methanosarcina mazei* might be an important native methane producer and could play a significant role in the carbon cycle of the BS and YR. These results may shed light on the adaption strategies and survival mechanisms of methanogens in a river-to-sea gradient of the interplay between sea and land. Further exploration is needed to gain additional information about the influence of YR on BS, such as the boundary and how the other two trophic gradients changed, as well as the difference between YR and other surrounding rivers.

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Conflict of Interest

The authors declare there are no conflict of interests.

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