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Population dynamics of methanogens and methanotrophs along the salinity gradient in Pearl River Estuary: implications for methane metabolism

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Abstract

Methane, a major greenhouse gas, plays an important role in global carbon cycling and climate change. Methanogenesis is identified as an important process for methane formation in estuarine sediments. However, the metabolism of methane in the water columns of estuaries is not well understood. The goal of this research was to examine the dynamics in abundance and community composition of methanogens and methanotrophs, and to examine whether and how they take part in methane metabolism in the water columns from the lower Pearl River (freshwater) to the coastal South China Sea (seawater). Quantitative PCR (qPCR) and high-throughput sequencing results showed that the abundance of methanogens decreased with increasing salinity, suggesting that growth of these methanogens in the Pearl River Estuary may be influenced by high salinity. Also, the methane concentration in surface waters was lower than that in near-bottom waters at most sites, suggesting sediment methanogens are a likely source of methane. In the estuarine mixing zone, significantly high methane concentrations existed with the presence of salt-tolerant methanogens (e.g., *Methanomicrobiaceae*, *Methanocella*, *Methanosaeta* and *Methanobacterium*) and methanotrophs (e.g., *Methylocystis* and *Methylococcaceae*), which were found in brackish habitats. Furthermore, a number of methanotrophic OTUs (from *pmoA* gene sequence data) had specific positive correlations with methanogenic OTUs (from *mcrA* gene sequence data), and some of these methanogenic OTUs were correlated with concentrations of particulate organic carbon (POC). The results indicate that methanotrophs and methanogens may be intimately linked in methane metabolism attached with particles in estuarine waters.

Keywords Pearl River estuary · Methanogens · Methanotrophs · *mcrA* gene · *pmoA* gene

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Introduction

Methane, considered as a major greenhouse gas with ~20 times warming potential of carbon dioxide, has increased 2.5-fold since 1750 but the reasons for this increase are not as clear as for carbon dioxide (IPCC 2013). About 74% of the emitted methane is derived from biological methanogenesis (Whitman et al. 2006). Recent studies have identified estuarine and coastal areas to be an active source of microbial methane (Abril and Iversen 2002; Bange et al. 1998; Borges et al. 2016; Chen and Tseng 2006; Harley et al. 2015; Sun et al. 2013; Zhang et al. 2008; Zhou et al. 2009), which contributes ~7.4% of ocean methane production, although they represent only ~0.4% of the global ocean area (Bange et al. 1994, 1998).

Anaerobic methanogenesis is mainly performed by *Euryarchaeota*, although it has been reported in *Crenarchaeota* and other archaeal phyla (Evans et al. 2015; Liu and Whitman 2008; Lyu et al. 2018). Methanogens are classified into eight orders (*Methanobacteriales*, *Methanocellales*, *Methanococcales*, *Methanofastidiosales*, *Methanomassiliicoccales*, *Methanomicrobiales*, *Methanopyrales* and *Methanosarcinales*; Liu and Whitman et al. 2008; Thauer et al. 2008; Iino et al. 2013, Nobu et al. 2016). All methanogens require the final step of methane synthesis to be catalyzed by the methyl-coenzyme M reductase (*mcr*; Liu and Whitman 2008), and the alpha subunit of the *mcr* (*mcrA*) gene has been frequently used as a marker to trace the community of methanogens in ecosystems (Chaudhary et al. 2013; Hallam et al. 2003; Wilkins et al. 2015; Zhou et al. 2014).

Since microbial methanogenesis mainly occurs in anoxic environments, most estuarine studies of methanogens focused on sediments with limited oxygen penetration (Webster et al. 2015; Lazar et al. 2016; Rakowski et al. 2015; Roussel et al. 2009; Rocío et al. 2013; Sawicka and Brüchert 2017; She et al. 2016; Tong et al. 2017; Xie et al. 2014; Zeleke et al. 2013). However, recent studies showed that methanogenesis can occur within the micro-niches of particles residing in oxygenated water columns in various aquatic environments such as lakes and rivers (Bogard et al. 2014; Grossart et al. 2011; Hayden and Beman 2015; Hu et al. 2015, 2016; Smith et al. 2013). Some methanogens can tolerate high salinity and survive when exposed to oxygen. For example, *Methanosaeta* could tolerate oxygen exposure in epilimnion lake water (Huser et al. 1982; Smith and Ingram-Smith 2007). *Methanocella* and *Methanosarcina* could transcribe the *mcrA* and oxygen detoxifying catalase genes under oxic conditions in desert soils (Roey et al. 2011), and the different methanogens have different salinity tolerance: *Methanoregula* can stand salinity below 1.7‰ (Bräuer et al. 2011), *Methanomethylovorans* below 4‰ (Jiang et al. 2005; Lomans et al. 1999), *Methanomassiliicoccus* and *Methanospirillum* of 10~15‰ (Dridi et al. 2012; Iino et al. 2013; Zhou et al. 2014), *Methanocella* of 20‰ (Sakai and Imachi 2016), and *Methanobacterium*, *Methanosaeta* and *Methanosarcina* can grow over at 33‰ salinity (Ciulla et al. 1994; Mori and Harayama et al. 2011; Mori et al. 2012; Sowers and Gunsalus 1988).

A large proportion of methane is oxidized to CO_2 by methane-oxidizing bacteria (methanotrophs) during its release from surface sediment to the water column (Bastviken et al. 2004; Samad and Bertilsson 2017). Methanotrophs are mainly classified into two types. Type I includes *Crenothrix*, *Methylobacter*, *Methylocaldum*, *Methylococcus*, *Methylogaea, Methyloglobulus*, *Methylohalobius*, *Methylomagnum*, *Methylomarinovum*, *Methylomarinum*, *Methylomicrobium*, *Methylomonas*, *Methyloparacoccus*, *Methyloprofundus*, *Methylosarcina*, *Methylosoma*, *Methylosphaera*, *Methylothermus*, and *Methylovulum*. Type II includes *Methylocapsa*, *Methylocella*, *Methylocystis*, *Methyloferula*, and *Methylosinus* (Bowman 2016; Hanson and Hanson 1996; Jiang et al. 2010; Park and Lee 2013). Genes encoding particulate forms of methane monooxygenase (pMMO) are present in almost all methanotrophs (Theisen and Murrell 2005) and the alpha subunit of pMMO (*pmoA*) gene is commonly used as a biomarker for detecting methanotrophs (Luesken et al. 2011; McDonald and Murrell 1997; Zhou et al. 2014).

The Pearl River ranks as the 13th/14th largest river in the world and is the largest river discharging into the South China Sea (Chen et al. 2008). The increasing discharges of nutrients and wastes from nearby regions' anthropogenic activities make Pearl River Estuary (PRE) a hot spot for greenhouse gas production (Cai et al. 2004; Chen et al. 2008; Guo et al. 2009; Lin et al. 2016; Zhang et al. 2016a; Zhang et al. 2016b; Zhou et al. 2009). In the PRE, methane production was known to occur in freshwater and organic-enriched sediments (Chen et al. 2008; Zhou et al. 2009), where the *Methanobacteriales*, *Methanococcales*, *Methanomicrobiales*, and *Methanosarcinales* were found (Hu et al. 2016; Jiang et al. 2008, 2011; Jin et al. 2011; Liu et al. 2015; Wang et al. 2012; Xie et al. 2014; Zhou et al. 2014). Methane-oxidizing archaea were detected as a minor group in shallow sediments of the PRE (Chen et al. 2013). Novel phylotypes of denitrifying anaerobic methane-oxidizing bacteria (e.g., NC10) were also found, which differ from those in freshwater sediment (Chen et al. 2015).

A few studies have reported on the methanogens and methanotrophs in water along the salinity gradient in estuaries (Crump and Baross 2000; Dang and Jiao 2014; Hao et al. 2010; Liu et al. 2014, 2015; Xie et al. 2017; Wang et al. 2018; Zhang et al. 2006); however, the methane metabolism interplayed by methanogens and methanotrophs in estuarine waters is largely overlooked. The goal of this research was to examine the spatial dynamics in abundance and composition of methanogens and methanotrophs in estuarine waters and to assess if they could influence the methane metabolism in the PRE.

Material and methods

Sample collection

Surface (within 1 m water depth) and near-bottom (1 m above sediment) water samples were collected at 15 sites along a 148-km transect of PRE and its adjacent area (Fig. 1 and Table S1) during 9–20 November 2013 (dry season). Water samples were collected using Niskin bottles attached to a shipborne CTD (SeaBird 917). Water was transferred into 150-ml glass vials, which were immediately injected with 150 μl saturated $HgCl_2$ solution for methane concentration determination in the laboratory. DNA samples were collected by filtering 0.5–1.0 l of seawater using a 0.22 μm membrane (Millipore GSWP4700) filter on board the ship. The filtered liquid was transferred into a 50-ml centrifuge tube for nutrient analysis. For determination of particulate organic carbon and nitrogen (POC and PON), about 1 l of water was filtered through a pre-combusted (450 °C for 4 h) 0.7 μm glass microfiber membrane (Whatman™ 1825-025). After filtration, the filters were folded and wrapped in aluminum foil. All filters and nutrient samples were stored on dry ice on board and preserved at − 20 °C in the laboratory until further analyses.

Physicochemical analysis

Temperature, salinity, depth, and dissolved oxygen (DO) were measured by the CTD on board. Methane concentrations were measured using a gas chromatograph (Shimadzu GC 12A) equipped with a flame-ionization detector (FID) and 2-m Porapak Q column (80/100 mesh; Institute of Soil Science, Chinese Academy of Sciences). Ammonium was analyzed by the indophenol blue spectrophotometric method. Nitrite, nitrate, and phosphate were measured with a four-channel continuous-flow Technicon AutoAnalyzer 3 (AA3; Bran-Lube GmbH, Germany). POC and PON concentrations were measured following Kao et al. (2012). Briefly, the filters were freeze-dried and then acidified with 1 ml of 1 M HCl solution. All filters were dried at 60 °C for 48 h. The decarbonated samples were analyzed for POC and PON in a continuous-flow elemental analyzer (Elementar Vario PYRO cube). The precisions for both PON and POC were within 1% (Kao et al. 2012).

DNA extraction and quantitative PCR (qPCR)

Membranes were cut into pieces for DNA extraction using the Fast DNA SPIN Kit for Soil (MP Biomedical, OH, USA) following the manufacturer's protocol. The qPCR analyses were performed on a Real-time Thermal Cycler 5100 and analyzed by using PikoReal software 2.2. The reaction volume was 10 μl: 5 μl of SYBR Premix Ex Taq™ II (Takara, China), 0.4 μM of each primer, and 1 μl of template DNA. Thermal cycling consisted of initial denaturation at 95 °C for 30 s followed by 40 cycles of denaturation at 95 °C for 5 s, annealing at 55 °C for 30 s, and extension at 72 °C for 60 s.

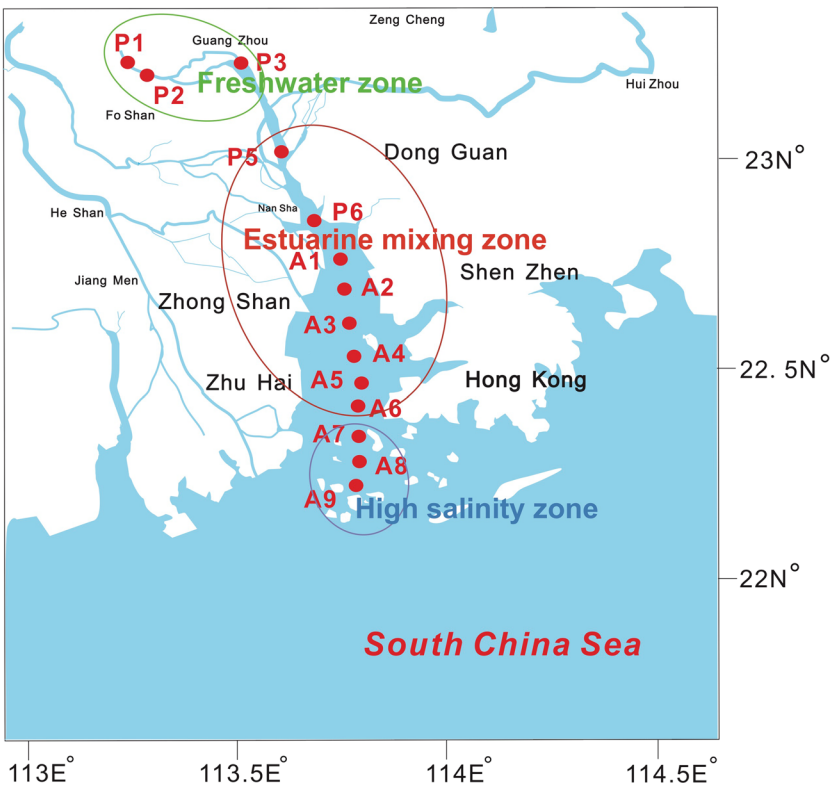

Fig. 1 Location map for sampling stations. The sites were grouped into three zones according to the salinity range: freshwater zone (salinity 0.2–0.3‰; sites P1, P2, and P3), estuarine mixing zone between freshwater and seawater (salinity 0.7–17.7‰; sites P5, P6, P7, A1, A2, A3, A4, A5, and A6), and high salinity zone within seawater (salinity 25.9–31.6‰; sites A7, A8, and A9)

The quantification standard was generated with a series of 10-fold diluted purified plasmid DNA from the cloned genes isolated from PRE water. The R^2 values of the standard curves were greater than 0.99.

The primers for the archaeal 16S rRNA gene were Arch344f: ACGGGGCGCAGCAGGCGCGA (Raskin et al. 1994) and Arch915r: GTGCTCCCCCGCCAATTCCT (Stahl 1991). Primers for the *mcrA* gene were MLf: 5'-GGTGGTGT MGGATTCACACARTAYGCWACAGC-3′ and MLr: 5'-TTCATTGCRTAGTTWGGRTAGTT-3′ (Luton et al. 2002). Primers for the bacterial 16S rRNA gene were Bac331F: 5'-TCCTACGGGAGGCAGCAGT-3′ (Takai and Horikoshi 2000) and Bac797R: 5'-GGACTACCAGGGTCTAATCC TGTT-3′ (Nadkarni et al. 2002), and primers for the *pmoA* gene were pmoA189f: 5'-GGNGACTGGGACTTCTGG-3′ and pmoA661r: 5'-CCGGMGCAACGTCYTTACC-3′ (Costello and Lidstrom 1999).

Illumina sequencing and bioinformatic analyses

Fourteen DNA samples from seven sites were sequenced with an Illumina HiSeq 2000 platform targeting the archaeal V5-V6 16S rRNA gene (Arc787F: 5'-ATTAGATA CCCSBGTAGTCC-3′ and Arc1059R: 5'-GCCATGCA CCWCCTCT-3′) and the bacterial V4-V5 16S rRNA gene (Bac515F: 5'-GTGCCAGCMGCCGCGG-3′ and Bac907R: 5'-CCGTCAATTCMTTTRAGTTT-3′; Shanghai Personalbio Biotechnology Co. Ltd., Shanghai, China; Yu et al. 2005). Water samples from seven sites (10 samples) were sequenced with a HiSeq-PE250/300 platform targeting the *mcrA* gene (Mlas-mod-F: 5'-GGYGGTGT MGGDTTCACMCARTA-3′ and mcrA-R: 5'-CGTTCATBGCGTAGTTVGGRTAGT-3′) and the *pmoA* gene (amplified *pmoA* gene via a seminested PCR approach using primers A189f and A682r: GAASGCNGAGAAGA ASGC for the first round, and the primers A189f, mb661r and 650r: ACGTCCTTACCGAAGGT systems as the second round; Guangdong Magigene Bioinformatics technology Co. Ltd., Guangzhou, China; Angel et al. 2011; Costello and Lidstrom1999; Holmes et al. 1995; Knief 2015).

The original *mcrA* gene database (Yang et al. 2014) was modified by adding the novel *mcrA* genes from *Bathyarchaeota*, *Verstraetearchaeota* and *Syntrophoarchaeum*, and modified to QIIME format. Both *mcrA* and *pmoA* gene databases (Yang et al. 2014; Yang et al. 2016) were simplified (deleted redundant uncultured sequences and kept pure culture sequences in each genus). The raw sequence data were quality filtered using USEARCH and de novo OTU picking at 97% identity (Edgar 2013). The chimera sequences were removed according to a reference database (gold database for 16S rRNA gene, modified databases for *mcrA* and *pmoA* genes; Edgar 2013). The *mcrA* and *pmoA* OTU sequences after removing the chimeric sequences were translated by FrameBot tool (http://fungene.cme.msu.edu/FunGenePipeline/framebot/form.spr) for detecting and correcting the frameshift errors. After removing unknown amino acid sequences (identity below 40% in Fungene database or length shorter than 80 Amino Acid Score), the remaining original amino acid nucleotide sequences were used for further analysis (*mcrA* and *pmoA* genes). Taxonomies of representative OTUs for the 16S rRNA-, *mcrA*- and *pmoA* genes were assigned against the Silva132 16S rRNA- (Quast et al. 2013), *mcrA* gene- and *pmoA* gene databases using the RDP classifier implemented in QIIME1.9.1 with a bootstrap cutoff of 80% (Caporaso et al. 2010). All the taxonomies at the rank of OTUs were chosen to recalculate the proportion and clustered by QIIME1.9.1.

OTU representatives of *mcrA* and *pmoA* genes were translated into amino acid sequences, and their closest relatives were searched against the NCBI amino acid non-redundant (nr) database using BLASTp. Neighbor-joining phylogenetic trees based on OTU sequences (*mcrA* and *pmoA* genes were translated to amino acid sequences) in all samples and related sequences were built in MEGA7 to check the classification (Kumar et al. 2016). Alpha diversity was calculated in Mothur after the sequence numbers of each sample were normalized to an equal number (Schloss et al. 2009).

Statistical and ecological analyses

The analyses of similarities (ANOSIM) and nonmetric multidimensional scaling (NMDS) were carried out to delineate any differences among sequencing samples based on Bray-Curtis similarity matrix at the OTU level using the vegan package (Dixon 2003) in RStudio. Redundancy analysis (RDA) was also performed using the vegan package. Relationships between the qPCR data of the *mcrA*-, *pmoA*-, archaeal 16S rRNA-, and bacterial 16S rRNA genes and environmental variables (including methane, DO, salinity, temperature, distance, POC, PON and NO_2^-, NO_3^-, NH_4^+ and PO_4^{3-}) were analyzed using RDA and Pearson method in RStudio. Relationships between the methane-related organisms and environmental parameters were analyzed using Pearson method in RStudio, and the Pearson correlations having $P < 0.01$ and $R^2 > 0.64$ were visualized in Cytoscape 3.2.1 (Shannon et al. 2003).

Nucleotide sequence accession numbers

The GenBank Sequence Read Project accession numbers for the raw sequences are PRJNA558283, PRJNA558448, and PRJNA558454.

Results

Environmental variables

Three groups of water samples were defined according to the salinity range: freshwater zone (salinity: 0.2–0.3‰; sites P1, P2 and P3), estuarine mixing zone between freshwater and seawater (salinity: 0.7–17.7‰; sites P5, P6, P7, A1, A2, A3, A4, A5 and A6), and a high salinity zone within seawater (salinity: 25.9–31.6‰; sites A7, A8, and A9; Fig. 1). The water depths ranged from 3 to 6 m in the freshwater zone, 4–12 m in the estuarine mixing zone, and 4–22 m in the high salinity zone (Table S1). The concentration of DO was lowest in the freshwater zone (1.0–4.2 mg/l) followed by the estuarine mixing zone (3.3–6.4 mg/l) and the high salinity zone (6.2–6.6 mg/l). Among the four sites in the freshwater zone, DO was lower (< 2 mg/l) at P1 surface and P3 (both surface and near-bottom), and higher at sites P2 and P5 (> 3 mg/l; Table S1).

The methane concentrations decreased from freshwater to the estuarine mixing zone (8.75–0.16 μmol/l), increased again in the estuarine mixing zone (0.34–4.46 μmol/l), and decreased in the high salinity zone (0.28–0.05 μmol/l). In general, methane concentrations in surface water were lower than that in near-bottom water except for sites A1, A2, and A4 located in the estuarine mixing zone. Methane concentrations increased from site P5 (0.16–0.18 μmol/l) to sites A1 (3.13–3.11 μmol/l) and A2 (2.94–4.46 μmol/l), and dropped at site A3 (0.33–0.34 μmol/l; Fig. 2a and Table S1). All these concentrations were well above methane concentration in equilibrium with the atmosphere at 0.004 μmol/l (Joye et al. 2011).

The concentrations of NO_3^- (16.6–294.5 μmol/l) and POC (27.2–212.0 μmol/l) in the freshwater zone were higher than that in the high salinity zone. NH_4^+ concentration decreased downward from the freshwater zone (393.7–3.9 μmol/l), increased slightly in the estuarine mixing zone (3.4–16.9 μmol/l), and dropped in the high salinity zone (1.7–0.4 μmol/l; Table S2). PON (2.7–31.2 μmol/l) and NO_2^- (3.2–27.7 μmol/l) varied strongly, while the change in temperature remained moderate (20–22.8 °C). The concentration of PO_4^{3-} was low (0.8–2.4 μmol/l) throughout the PRE (Table S2).

Abundances of archaea and methanogens

The archaeal 16S rRNA gene copies ranged from 1.5×10^8 copies/l in the near-bottom seawater at site A8 to 2.9×10^9 copies/l in the near-bottom freshwater site P3, with an average value of $8.1 \pm 5.8 \times 10^8$ copies/l (Table S3).

The *mcrA* gene was used to reflect both methanogens and anaerobic methanotrophic archaea (ANME) (Lueders et al. 2001; Scheller et al. 2010). The archaeal 16S rRNA gene sequencing revealed the ANME group was extremely low in PRE (≤ 0.2%, Fig. 5a). The qPCR primers MLf/MLr could not quantify the *mcrA* gene in *Bathyarchaeota* (Evans et al. 2015; Mckay et al. 2017) and *Methanocella* (Checked with *mcrA* genes from *Methanocella conradii*-NC017034 and *Methanocella arvoryzae*-NC009464), indicating that the qPCR data of *mcrA* gene only represented the abundance of methanogens within *Euryarchaeota* (without *Methanocella*) in PRE waters.

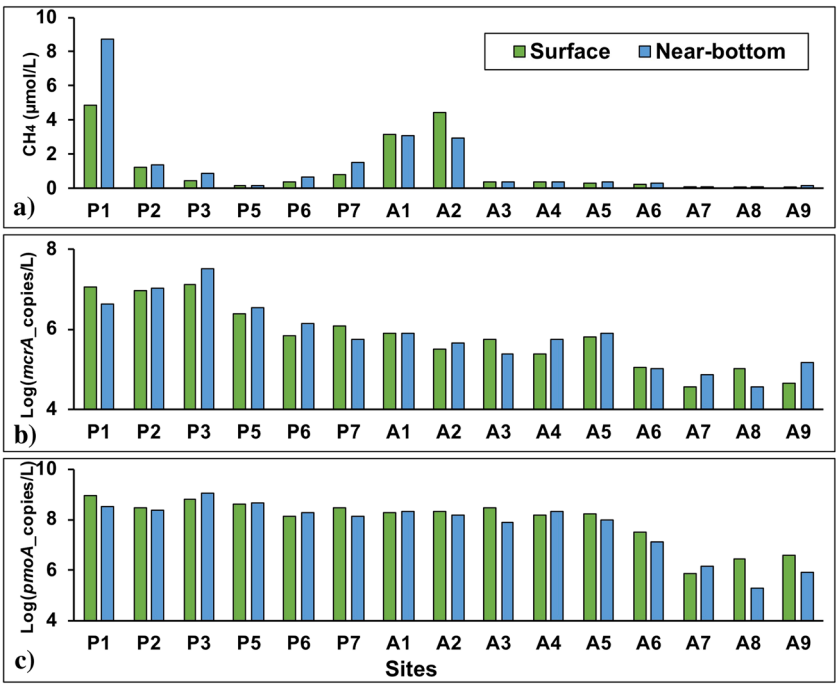

Fig. 2 Distribution of methane concentration (**a**), *mcrA* gene (methanogens) abundance (**b**), and *pmoA* gene (methanotrophs) abundance in PRE. The qPCR data were mean values from triplicate measurements. All error bars were below 0.1 standard deviation

Abundance of the *mcrA* gene in PRE water columns was 1.8 to 4.0 orders of magnitude lower than the total archaea and decreased significantly with increasing salinity in the three zones (analysis of variance, $P < 0.01$), dropping from 3.2×10^7 copies/l in the near-bottom water of freshwater site P3 to 3.5×10^4 copies/l in the near-bottom water of high salinity site A8, with an average value of $3.2 \pm 6.6 \times 10^7$ copies/l ($n = 30$; Fig. 2b and Table S3). Although methane concentration had a high value in the estuarine mixing zone (A1 and A2 sites; Fig. 2a and Table S1), the abundance of *mcrA* gene did not significantly increase ($3.1–8.2 \times 10^5$ copies/l) around sites P7 and A3 ($2.4–12.6 \times 10^5$ copies/l; Fig. 2b and Table S3).

The abundance of bacteria and methanotrophs

The bacterial 16S rRNA gene copies ranged from 9.2×10^8 copies/l in the near-bottom seawater at site A8 to 7.3×10^{10} copies/l in the surface freshwater site P1, with an average value of $1.8 \pm 1.6 \times 10^{10}$ copies/l. Total bacterial abundance decreased by approximately 15-fold ($n = 30$) from freshwater to high salinity zone (Table S3).

The *pmoA* gene is used to reflect methanotrophic bacterial abundance (McDonald and Murrell 1997; Kolb et al. 2003). The abundance of *pmoA* gene in PRE water columns was 2.2 to 4.6 orders of magnitude lower than the total bacteria and up to 2.1 orders of magnitude higher than the *mcrA* gene, dropping from 2.4×10^8 copies/l in the near-bottom water of freshwater site P3 to 4.0×10^4 copies/l in the near-bottom water of high salinity site A8, with an average value of $4.7 \pm 5.4 \times 10^7$ copies/l ($n = 30$; Fig. 2c and Table S3). Different from the *mcrA* gene that decreased fast with increasing salinity in the estuarine mixing zone (Fig. 2b), the *pmoA* gene had a high abundance ($4.6–23.5 \times 10^7$ copies/l) in the freshwater zone and remained relatively stable ($1.6–6.2 \times 10^7$ copies/l) in the estuarine mixing zone. It decreased substantially ($4.0–259.9 \times 10^4$ copies/l) only in the high salinity zone (Fig. 2c).

Microbial correlations with environmental factors

The abundance of *mcrA* gene positively correlated with *pmoA* gene ($R = 0.87$, $P < 0.05$). The *mcrA* gene was also positively associated with POC ($R = 0.79$, $P < 0.05$). Total archaeal 16S rRNA gene copies only had positive relationships with *mcrA* gene copies, NO_2^- and NO_3^- concentrations ($R = 0.45$, 0.41 and 0.37, respectively; all $P < 0.05$; Fig. 3 and Fig. S1a).

Methane concentration rose significantly from site P5 to site A2 in the upper estuarine mixing zone (Fig. 2a). Pearson analysis ($n = 10$) showed that the methane concentration was positively correlated with distance ($R = 0.95$, $P < 0.05$) and negatively with *mcrA* gene copies, POC and NO_3^- ($R = -0.67$, -0.73, and -0.64, respectively; all $P < 0.05$) in the upper estuarine mixing zone (Fig. S1b).

Diversity of the microbial communities

The 16S rRNA gene yielded a total of 1,126,032 high-quality sequences from 14 water samples. Of these, 811,559 came from the primers for *Archaea* and 314,473 for *Bacteria*. The

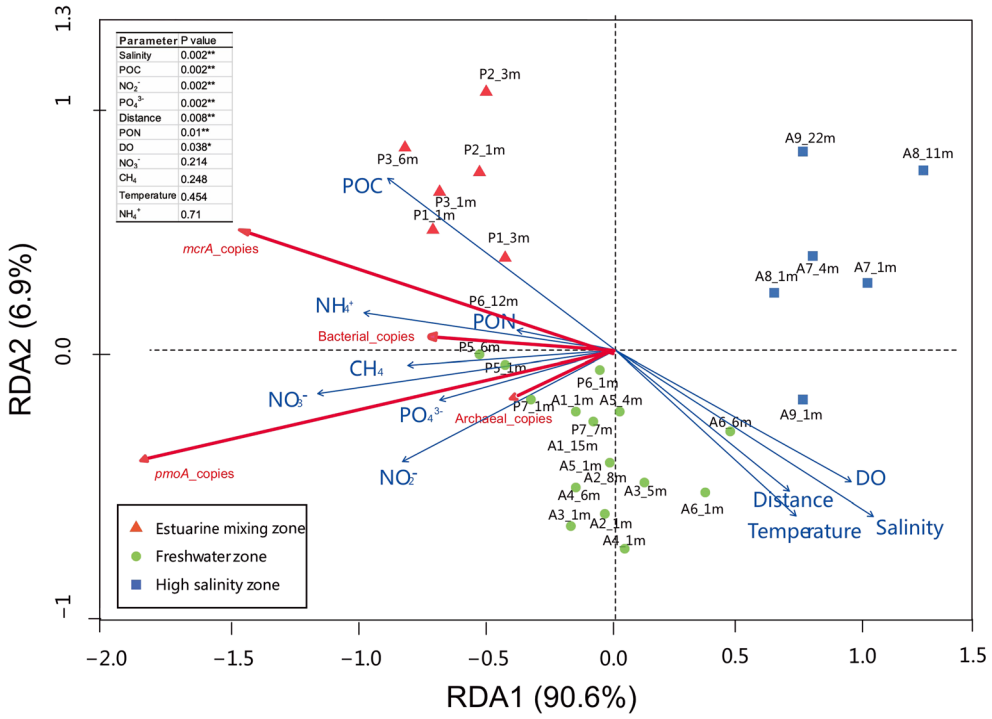

Fig. 3 Redundancy analysis (RDA) of the relationship between the qPCR data of *mcrA*, *pmoA*, archaeal 16S rRNA, and bacterial 16S rRNA genes and environmental variables (including CH_4, DO, salinity, temperature, POC, PON and NO_2^-, NO_3^-, NH_4^+, and PO_4^{3-}) in the PRE ($n = 30$)

total OTU (97% similarity) number was 2082 for archaeal 16S rRNA gene and 5192 for bacterial 16S rRNA gene, ranging between 171 and 1141, and between 398 and 1939, respectively (Tables S4a and S4b). The *mcrA* and *pmoA* genes yielded a total of 1,867,937 high-quality sequences from 10 water samples, with 513,499 sequences for the *mcrA* gene and 1,354,438 sequences for the *pmoA* gene. The yielded OTU (97% similarity) number was 1037 for the *mcrA* gene and 362 for the *pmoA* gene, ranging between 420 and 842, and between 190 and 293, respectively (Tables S4c and S4d).

Archaeal, bacterial, methanogenic (*mcrA* gene), and methanotrophic (*pmoA* gene) coverage ranged from 98.8 to 99.9%, indicating that most of the species in the samples were captured (Tables S4). The archaeal 16S rRNA gene Shannon values were 3.0–4.2 in the freshwater zone, 1.2–2.6 in the estuarine mixing zone, and 0.7–2.0 in the high salinity zone, indicating a significant loss of total archaeal diversity in the estuarine mixing zone (Table S4a). The *pmoA* gene Shannon values were 3.1–3.2 in the freshwater and low-salinity samples, and decreased to 2.6–2.9 in relative high-salinity samples in the estuary (Table S4d). Changes in the *mcrA* gene Shannon index, however, were small (between 3.5 and 4.2; Table S4c). Analyses of similarity confirmed that the freshwater-estuarine mixing zone groups were not significantly different for the *mcrA* gene ($R_{ANOSIM} = 0.13$, $P = 0.16$). However, the community structures of total archaea and bacteria, as well as methanotrophs, exhibited clear shifts in different salinity zones based on the NMDS analysis ($R_{ANOSIM} = 0.93$, $P < 0.01$, $R_{ANOSIM} = 0.54$, $P < 0.01$ and $R_{ANOSIM} = 0.62$, $P < 0.01$, respectively; Fig. 4 and Fig. S2).

Methanogenic community composition

The archaeal 16S rRNA gene sequencing resulted in six orders of methanogens within the phylum *Euryarchaeota*: *Methanobacteriales*, *Methanocellales*, *Methanofastidiosales*, *Methanomassiliicoccales*, *Methanomicrobiales*, and *Methanosarcinales*, which included twelve genera *Methanobacterium*, *Methanobrevibacter*, *Methanocella*, *Methanocorpusculum*, *Methanoculleus*, *Methanofastidiosum*, *Methanolinea*, *Methanomethylovorans*, *Methanoregula*, *Methanosaeta*, *Methanosarcina*, and *Methanosphaera* (Fig. 5b).

Methanogens were the most dominant archaeal groups (63.9–90.6%) in the freshwater zone (P1 and P3 sites), decreased sharply (1.0–13.0%) in the estuarine mixing zone (P6, A1 and A2 sites), and became almost absent (≤ 0.1%) in the high salinity zone (A6 and A9 sites; Fig. 5a). The most abundant methanogens in freshwater were *Methanobacteriales* (10.2–36.6%), *Methanomicrobiales* (23.7–25.9%), and *Methanosarcinales* (19.6–26.4%), which decreased sharply along the salinity gradient in the estuarine mixing zone (total methanogens < 0.9% in A2 near-bottom) and were below detection limit in the high salinity zone (total methanogens < 0.5%; Fig. 5b). Also, the methanogenic proportion in total archaea was decreased, but still some genera were a relatively high proportion of methanogens in high salinity zone (e.g., *Methanocella*, *methanosaeta*, and *methanosarcina*; Fig. 5c). At the OTU level, *Methanosaeta*-OTU4 (9607) had a high proportion in the freshwater zone (12.82–16.69%) and widely distributed in the estuarine

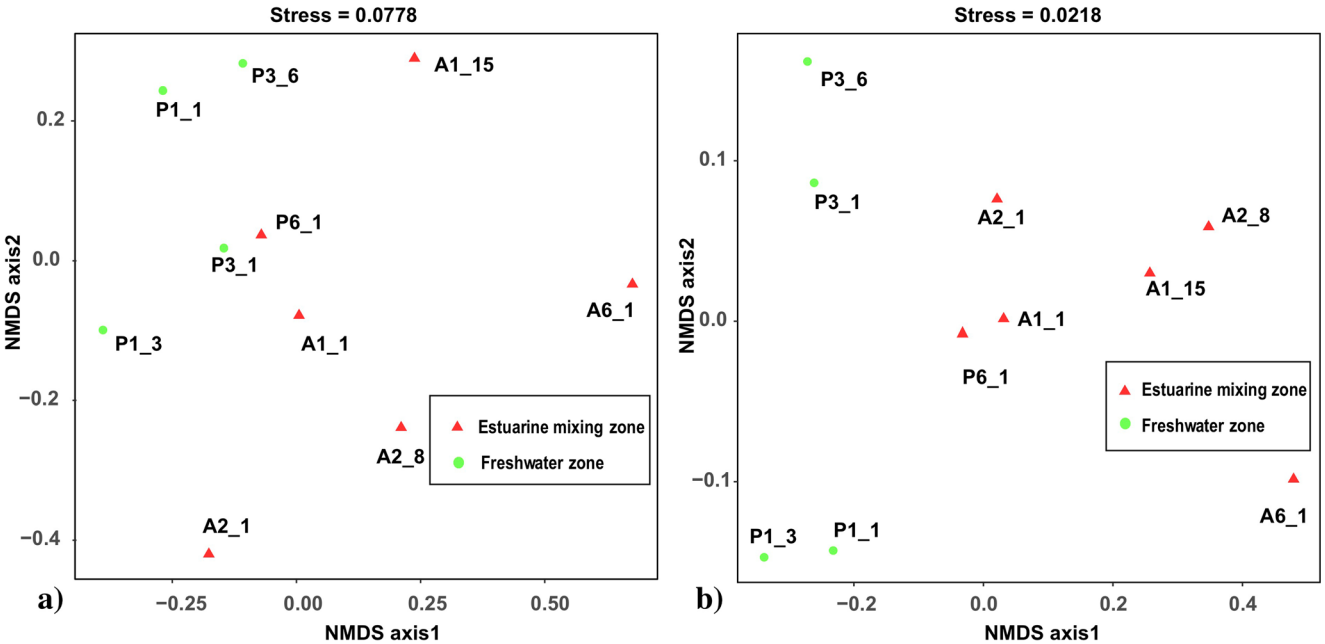

Fig. 4 Nonmetric multidimensional scaling (NMDS) analysis of the *mcrA* (**a**) and *pmoA* (**b**) retrieved communities at freshwater and estuarine mixing zones based on Bray-Curtis similarity matrix at the OTU level

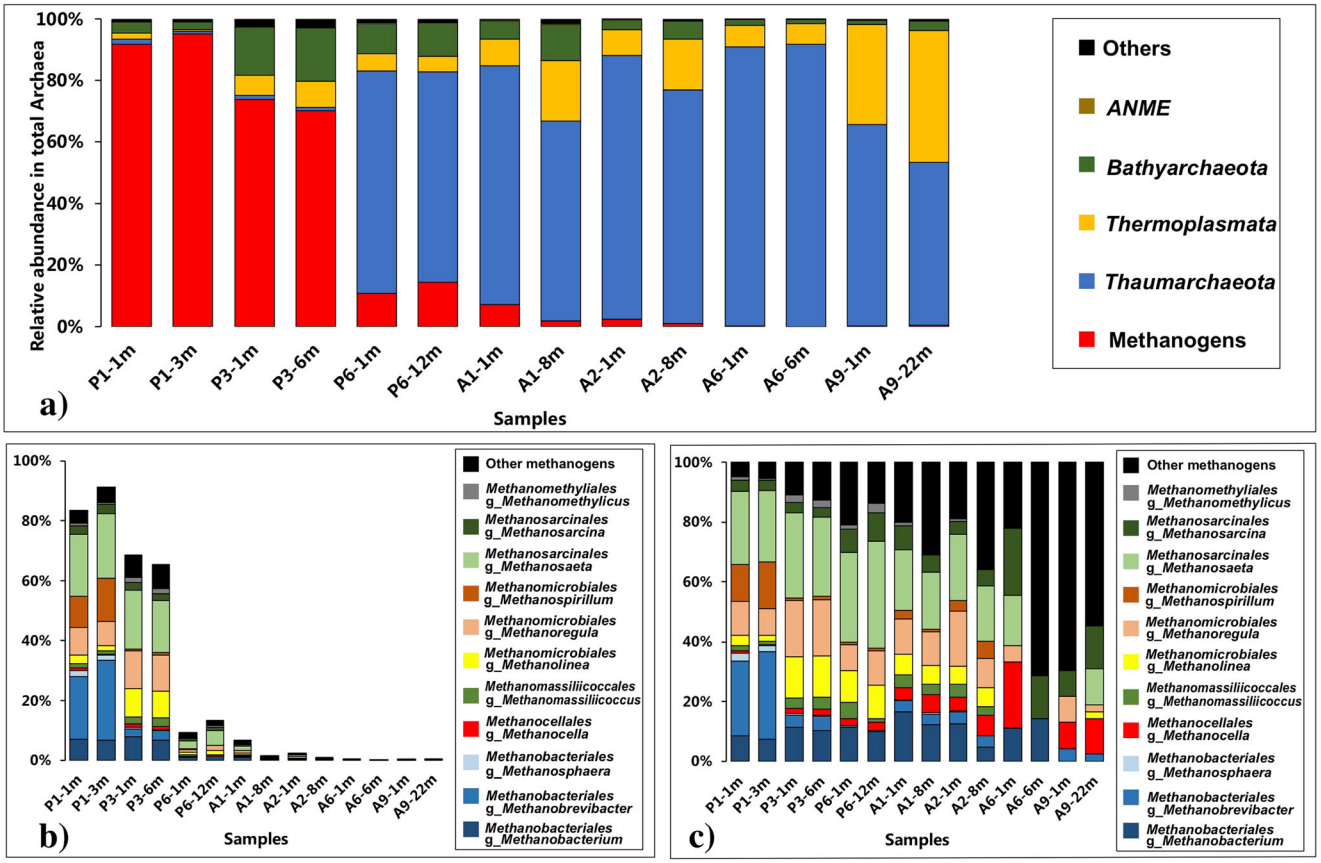

Fig. 5 Archaeal communities in different water samples: main groups in Archaea (**a**), methanogenic genera in total Archaea (**b**), and methanogenic genera relative proportions (**c**)

mixing zone (0.09–4.01%), but rarely observed in the high salinity water (≤ 0.02%). *Methanosarcina*-OTU40 (1908) was clustered with a reference sequence that existed in marine sediment of Nankai Trough basin (LC170394) in the phylogenetic tree. *Methanocella* OTU84 (304), OTU125 (185), and OTU403 (80) were observed in the freshwater and estuarine mixing zone (≤ 0.55%; Fig. S3).

Methanogens were also classified based on the *mcrA* gene, which resulted in five orders (*Methanobacteriales*, *Methanocellales*, *Methanomassiliicoccales*, *Methanomicrobiales*, and *Methanosarcinales*) and 8 genera (*Methanobacterium*, *Methanoobrevibacter*, *Methanocella*, *Methanomassiliicoccus*, *Methanoregula*, *Methanosaeta*, *Methanosphaera*, and *Methanothermobacter*; Fig. 6a).

The *mcrA* gene-retrieved methanogenic genera were not all consistent with those retrieved based on the archaeal 16S rRNA gene (Figs. 5a and 6a), and the difference can be within the same samples (e.g., *Methanobacterium*, *Methanobrevibacter*, *Methanosphaera*, *Methanocella*, *Methanoregula*, *Methanosaeta*, *Methanosarcina*, and *Methanomassiliicoccus*; Figs. 5c and 6a). For example, there were 0.0–0.5% *Methanobrevibacter*, 8.3–92.4% *Methanobacterium*, and 1.6–37.4% *Methanocella* in *mcrA* gene revealed methanogen communities, while 0.0–29.5%, 4.9–16.7%, and 0.3–22.2% relative proportions of these methanogens, respectively, were retrieved using archaeal 16S rRNA gene (Figs. 5c and 6a). The low yet persistent presence of the *Methanosphaera* (< 0.9%; Fig. 5b) may indicate human activities because it is particularly found in human gut and wastewater digesters (Dridi et al. 2009; Toumi et al. 2015).

Methanotrophic community composition

The bacterial 16S rRNA gene sequencing produced methanotrophs whose proportions were relatively stable in the freshwater (1.8–2.3%) and estuarine mixing zone (1.1–2.8%) but decreased sharply in the high salinity zone (0.0–0.5%). In the estuarine mixing zone, a higher proportion was observed in the A1& A2 surface water (2.8–3.0%) than that in the near-bottom water (1.1–1.2%; Fig. 7b). The most abundant methanotrophic genus was *Methylocystis* (up to 2.3%), which had a high proportion in the freshwater (1.2–1.9%), and decreased in the estuarine mixing and high salinity zone (< 0.1% in A9 near-bottom). *Methylococcaceae* were the second dominant methanotrophs (0.0–0.8%), representing high relative proportion of methanotrophs in estuarine mixing- and high salinity zones (Fig. 7c).

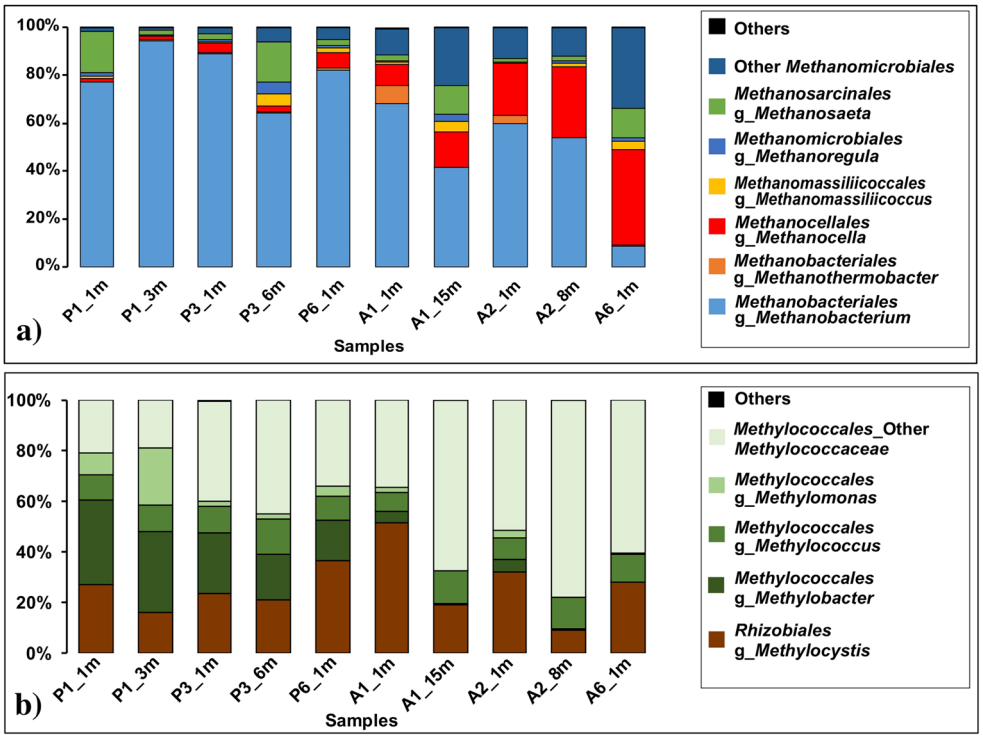

Fig. 6 Methanogenic genera retrieved using *mcrA* gene (**a**) and methanotrophic genera retrieved using *pmoA* gene (**b**) in different water samples

The methanotrophs in *α-Proteobacteria* contained three OTUs from bacterial 16S rRNA gene sequencing data: two *Methylocystis* and one *Rhizobiales*, which belonged to type II methanotrophs. *Methylocystis*-OTU105 (1605) was the most

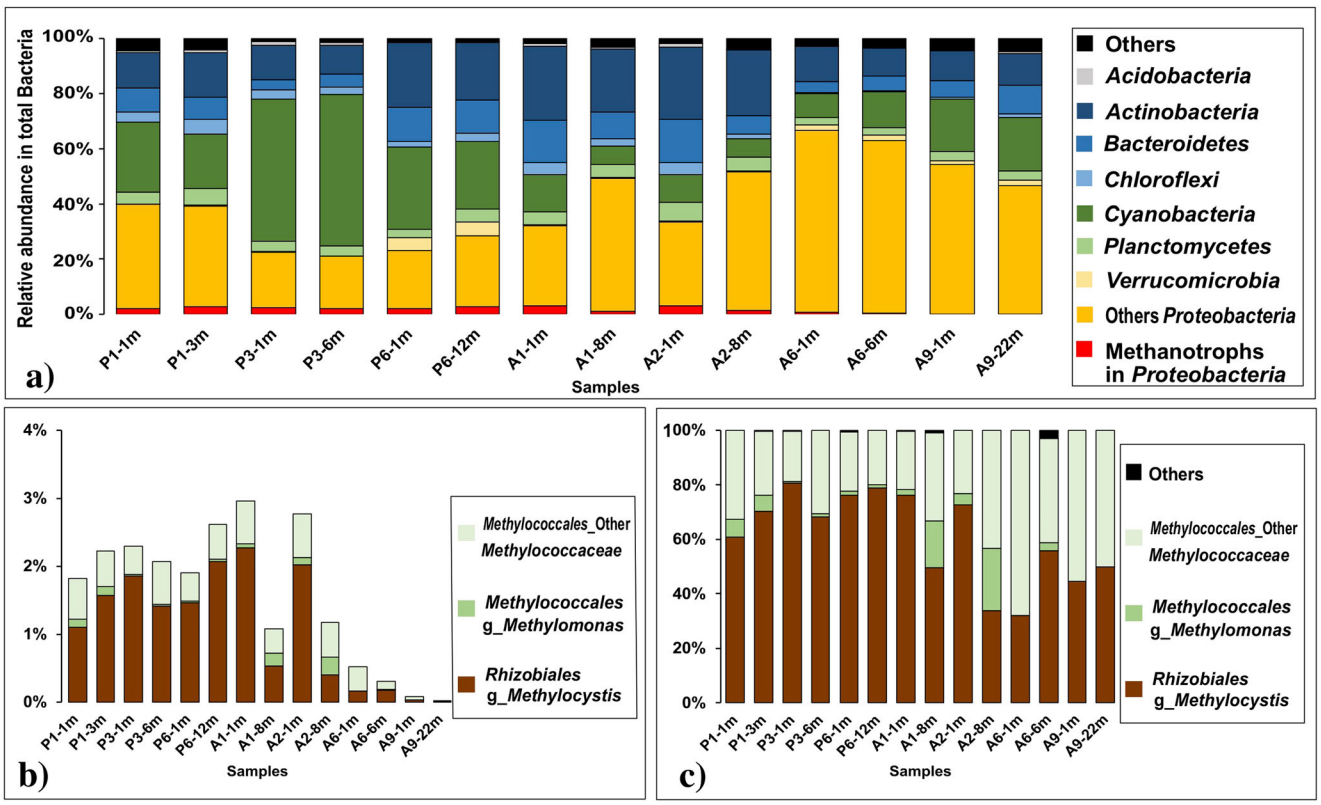

Fig. 7 Bacterial communities in different water samples: main groups in bacteria (**a**), methanotrophic genera in total bacteria (**b**), and methanotrophic genera relative proportions (**c**)

abundant methanotrophic OTU, had high proportions in freshwater and estuarine mixing zone (1.11–1.75% and 0.40–2.26%, respectively), and decreased in the high salinity zone (0.01–0.18%; Fig. S3b). Type I methanotrophs in *γ-Proteobacteria* contained fourteen OTUs: three *Methylomonas* and eleven other *Methylococcaceae*. Most of the type I OTUs were only found in freshwater and estuarine mixing zone with low proportion (< 0.18%), while the *Methylococcaceae*-OTU207 was found in the high salinity zone at low abundance (0.01–0.30%; Fig. S3b).

The *pmoA* gene-retrieved methanotroph community transitioned along the salinity gradient in PRE waters based on NMDS and ANOSIM analysis (Figs. 4b and 6b). *Methylococcales-Methylococcaceae* were the most dominant methanotrophs (48.4–91.1%) in the PRE waters, including *Methylobacter*, *Methylococcus*, *Methylomonas*, and other *Methylococcaceae*. *Methylobacter*, and *Methylomonas* had higher proportions in freshwater (18.2–33.6% and 1.9–22.8%, respectively) than in the estuarine mixing zone (0.3–16.3% and 0.2–3.9%, respectively), while *Methylococcus* had relative stable proportions (8.2–13.9%) in the estuary. Other OTUs in the *Methylococcaceae* were the major methanotrophs in all waters and rising in estuarine mixing zone (18.6–77.9%). *Rhizobiales-Methylocystis*, the second dominant methanotrophs varied in relative abundance between 8.9 and 51.6%, which had relatively higher proportions in surface (23.6–51.6%) than in near-bottom (8.9–20.9%) water samples (Fig. 4b).

Positive relationship between methane and methanogens or methanotrophs

To explore the possible relationship between the methane-related organisms (retrieved in 16S rRNA genes data) and environmental parameters, the co-occurrence network between the selected methanogenic and methanotrophic OTUs was constructed ($n = 14$, Pearson's correlation's P value < 0.05; Fig. S5). Fourteen methanogenic OTUs (e.g., *Methanobrevibacter*-OTU11, *Methanospirillum*-OTU23, *Methanosaeta*-OTU483, *Methanosphaera*-OTU49, and *Methanobacteriaceae*-OTU3044) revealed a positive relationship with methane concentration in PRE. Those OTUs (except OTU686) had a positive relationship with POC, which accounted for 33% of total methanogenic sequences. Only two methanotrophic OTUs (*Methyloparacoccus*-OTU371 and *Methylomonas*-OTU1540) were found to have a positive relationship with methane concentration, accounting for 8% of total methanotrophic sequences. POC and salinity were the hubs with the highest connectivity to methanogenic and methanotrophic nodes (Fig. S7).

The co-occurrence network was also constructed ($n = 10$, Pearson's correlation $R^2 > 0.64$ and $P < 0.01$) between the top 40 *mcrA*- and *pmoA* genes OTUs and environmental parameters (POC, salinity and methane concentration; Fig. 8). A similar relationship was found with the network from 16S rRNA genes data (Fig. S7). Low proportion methanotrophic nodes (< 3% in total *pmoA* gene reads) had positive relationships with methane concentration (Fig. 8). Six *Methanobacterium* OTUs (OTU7, OTU37, OTU 49, OTU95, OTU107, and OTU2912, take 10.7% *mcrA* sequences) and two *methylococcaceae* OTUs (OTU1 and OTU4, take 13.0% *pmoA* sequences) had a positive relationship with POC. Eight *methylococaceae* OTUs (OTU7, OTU8, OTU23, OTU29, OTU33, OTU34, OTU38, and OTU205, take 25.2% *pmoA* sequences) and two methanogenic OTUs (*Methanomicrobia*-OTU20 and *Methanocella*-OTU21, take 4.7% *mcrA* sequences) had a positive correlation with salinity. Four *Methanobacterium* OTUs (OTU22, OTU34, OTU 42, and OTU2281, take 6.7% *mcrA* sequences) and three methanotrophic OTUs (*methylococaceae*-OTU1, *methylococaceae*-OTUs290, and *Methylocystis*-OTU24, take 12.3% *pmoA* sequences) had negative relationships with salinity. Most methanotrophic OTUs in the network (take 55.3% *pmoA* sequences) also had a positive relation with methanogenic OTUs, some methanogenic OTUs (e.g., OTU 7, OTU20, OTU21, OTU22, OTU34, OTU37, OTU95, and OTU2912, comprised 19.6% *mcrA* sequences) were the hubs for methanotrophs, while environmental parameters (POC and salinity) were the hubs for most methanogenic OTUs (Fig. 8).

Discussion

The sensitivity of methanogenic *Euryarchaeota* to salinity change

Salinity is an important physiological parameter affecting methanogens (Liu and Boone 1991; Pattnaik et al. 2000; Schmitt et al. 2019; Xie et al. 2014). A few methanogens, however, have colonized niches in high salinity settings, such as saline lakes and marine habitats (Shlimon et al. 2004; Mori and Harayama 2011; Mori et al. 2012; Wen et al. 2017; Enzmann et al. 2018). The *mcrA* gene abundance showed a significant decrease (Fig. 2b), affected by salinity (Fig. 3). The methanogenic proportion in total archaea also decreased along the salinity gradient (Fig. 5a), indicating that many methanogens were inhibited by increasing salinity in PRE.

The methanogenic community shifted in PRE (from archaeal 16S rRNA gene sequences; Fig. S5). For example, *Methanoregula*-OTU12 & OTU44 only had high populations in the freshwater zone and were not detected in estuarine mixing and high salinity zones (Fig. S2a); *Methanomassiliicoccus*-OTU144 & OTU213 and *Methanomethylovorans*-OTU76 expanded from freshwater to the estuarine mixing zones but not to the high salinity zone (from archaeal 16S rRNA gene sequencing data; Fig. S2a).

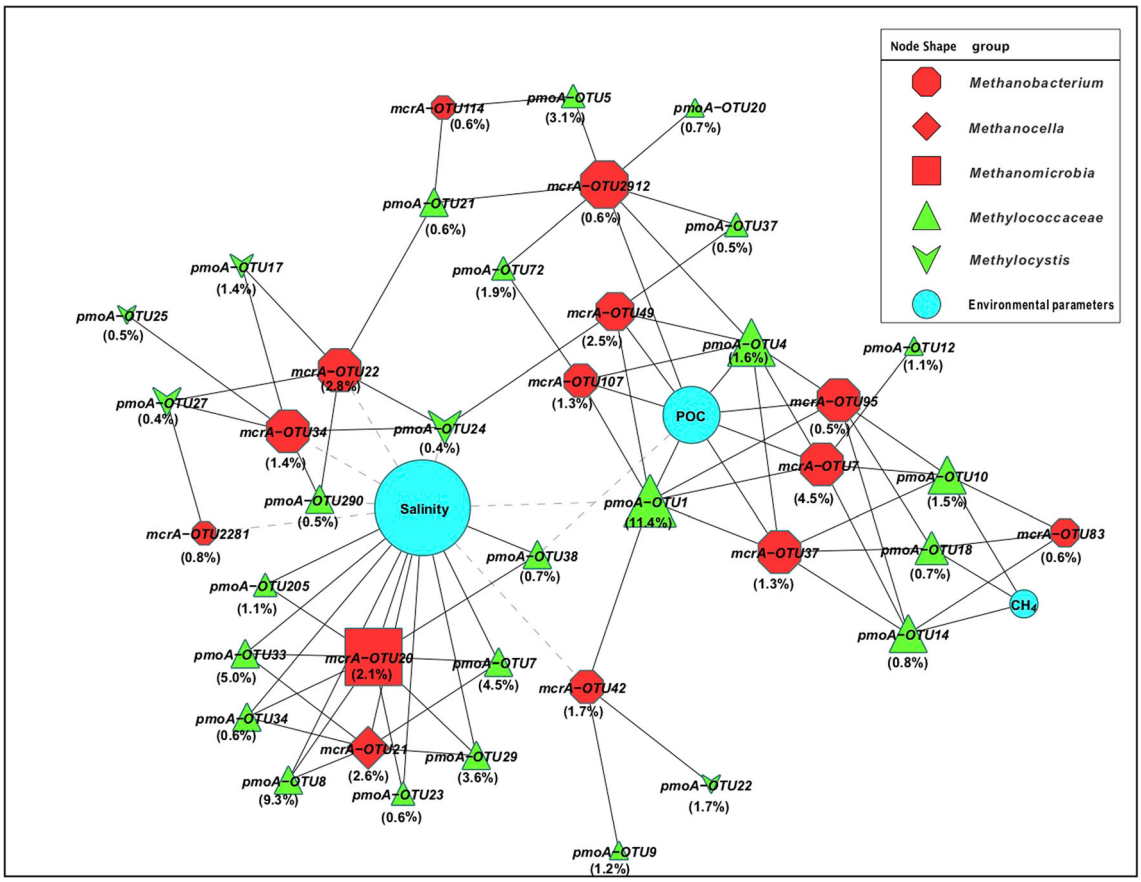

Fig. 8 Network analysis of *mcrA* and *pmoA* OTUs and environmental variables (including CH_4, salinity, and POC) in PRE ($n = 10$, $R^2 > 0.64$, and $P < 0.01$). The showed OTUs had significant correlations with CH_4, salinity, and POC. Solid lines indicate positive relationships and dashed lines negative relationships. The size of each node shows the number of connections with OTUs

The significant difference between archaeal 16S rRNA gene- and *mcrA* gene-retrieved genera proportion in total methanogens (Figs. 5c and 6a) may reveal activity changes in the estuary.

Some salt-tolerant methanogens (e.g., *Methanomicrobiaceae*, *Methanocella*, *Methanosaeta* and *Methanobacterium*; Liu and Whitman 2008; Mori and Harayama 2011; Mori et al. 2012; Lee et al. 2013; Joshi et al. 2018) were found to live in estuarine mixing and even high salinity zones (Fig. 6a and Table. S5). The methanogens in the water columns were different from those in surface sediment where *Methanomicrobia* was most abundant (Xie et al. 2014). The salinity-inhibition of methane production is suggested to coincide with reduced methanogenic population size (Pattnaik et al. 2000). Especially, *Methanobacterium*, *Methanocella*, and *Methanosaeta* had high proportions in the estuarine mixing zone (Fig. 6a), indicating they may be active and producing methane in PRE. Overall, our results showed that salinity was the most influential effect on the abundance of methanogens (Fig. 3 and Fig. S1a) and the relative high salinity acted as an ecological barrier for most of the terrestrial methanogens to diversify in the estuarine mixing and marine environments.

Salinity boundary was also found in methanotrophic communities (Fig. 4b). *Methylocystis* is an obligate type I methanotroph, utilizing only methane and methanol as sole carbon and energy sources (Bowman 2016; Dedysh et al. 2007). *Methylocystis* in a relatively high proportion (Fig. 7b) occurred only when salinity was below 7‰ (completely inhibited growth occurring at ~ 8‰ salinity; Dedysh et al. 2007). In this study, *Methylocystis* in the surface water (salinity 3.9–4.7‰) was higher than the near-bottom (salinity 15.3–15.8‰) in A1 and A2 sites (Figs. 6b and 7b), suggesting salinity constraint on its distribution. *Methylococcaceae* of type I methanotrophs were the major methanotrophs widely distributed in the high salinity PRE waters in this study (Figs. 6b and 7b), consistent with the finding that they are actively present in marine habitats (Bowman 2016; Paul et al. 2017).

Methanogens and methanotrophs taking part in methane metabolism

Methanogenesis was thought to mainly occur in sediments and the contribution of estuarine water columns was usually overlooked (Chen and Tseng 2006; Chen et al. 2013; Grossart et al. 2011; Zhou et al. 2009). In this study, methane

concentrations were lower in surface waters than in the near-bottom waters, which was consistent with previous studies (Zhou et al. 2009), indicating that in situ biological production in water was low relative to release from sediment or input from the upper area in the PRE (Fig. 2a). High methane and low DO concentrations were found in the freshwater zone (Fig. 2a and Table S1), in accordance with the higher abundance and proportion of methanogens. Collectively, methane in the estuary primarily originates from either input of methane-rich freshwater or methanogenic degradation in organic-enriched sediments (Xie et al. 2014; Zhou et al. 2009).

In the upper estuarine mixing sites A1 and A2, the concentration of methane was significantly higher than the nearby sites (especially than the upper sites), and the surface water exhibited higher methane concentrations than the near-bottom (Fig. 2a), which was not observed in previous studies (Chen et al. 2009; Zhou et al. 2009). *Methanocellales-Methanocella* were the dominant methanogens and increasing in this area (retrieved by *mcrA* gene; Fig. 6a). Although the *mcrA* gene abundance was not increased significantly in this area (qPCR primers cannot amplify the *mcrA* gene in *Methanocella,* which underestimated methanogenic abundance), some salt-tolerant methanogens (e.g., *Methanocella* and other *Methanomicrobia*) were found to have positive correlations with salinity (Fig. 8), implying that they can live and may have the potential to take part in methanogenesis in the water column of the estuarine mixing zone.

Methanotrophs of *Methylococcaceae* and *Methylocystis* may be active and consume methane in the PRE. *Methylococcaceae* only grow at methane concentrations above ~ 0.04 μM (Knief and Dunfield 2005), which is consistent with the methane concentration range of 0.05–8.75 μM in PRE waters. *Methylocystis* has two pMMO for different affinities of methane (> 0.01 μM), which provides efficiency utilizing various methane concentrations in estuarine waters (Baani and Liesack 2008). The abundance of methanotrophs had a positive relationship with methanogens and POC in PRE water (Fig. 3 and Fig. S1a). With high abundance and ubiquity in estuarine waters, methanotrophs may take part in methane metabolism attached to particles in PRE waters. Since some methanogenic OTUs were the hubs for methanotrophs, and POC and salinity were the hubs for most methanogenic OTUs (Fig. 8 and Fig. S7), particulate organic matter may serve as micro-niches for methanogens and methanotrophs to co-exist, which is constrained by salinity variation in PRE.

In conclusion, we describe here the changes in the abundance and communities of methanogens and methanotrophs along the salinity gradient in PRE. Results shows that the majority of terrestrial methanogens were inhibited by high salinity; some salt-tolerant methanogens, however, may be capable of growth and contribute to methane production toward high salinity in PRE waters. The POC appeared to be important for the biological formation of methane in the water columns. Some methanotrophs may depend on in situ methanogenesis and consume methane in close association with particle-associated methanogens in PRE waters. This study extends our knowledge of the distributional characteristics and environmental controls of methanogens and methanotrophs in subtropical estuaries, promoting our understanding of the roles of methane-metabolizing organisms in the estuarine carbon cycle.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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