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Methane production correlates positively with methanogens, sulfate-reducing bacteria and pore water acetate at an estuarine brackish-marsh landscape scale

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Abstract

Methane production is influenced by the abundance of methanogens and the availability of terminal substrates. Sulfate-reducing bacteria (SRB) also play an important role in the anaerobic decomposition of organic matter. However, the relationships between methane production and methanogen populations, pore water terminal substrates in estuarine brackish marshes are poorly characterized, and even to our knowledge, no published research has explored the relationship between methane production rate and abundance of SRB and pore water dimethyl sulfide (DMS) concentration. We investigated methane production rate, abundances of methanogens and SRB, concentrations of pore water terminal substrates and electron acceptors at a brackish marsh landscape dominated by *Phragmites australis*, *Cyperus malaccensis* and *Spatina alterniflora* marshes zones in the Min River estuary. The average rates of methane production at a soil depth of 30 cm in the three marsh zones were 0.142, 0.058 and 0.067 $\mu g\, g^{-1}\, d^{-1}$, respectively. The abundance of both methanogens and SRB in the soil of the *P. australis* marsh with highest soil organic carbon content was higher than in the *C. malaccensis* and *S. alterniflora* marshes. The abundance of methanogens and SRB in the three soil layers was statistically indistinguishable. Mean pore water DMS concentrations at a soil depth of 30 cm under the *S. alterniflora* marsh were higher than those in the *C. malaccensis* and *P. australis* marshes. Methane production rate increased with the abundance of both methanogens and SRB across three marsh zones together at the landscape scale, and also increased with the concentration of pore water acetate, but did not correlate with concentrations of pore water DMS and dissolved CO_2. Our results suggest that, provided that substrates are available in ample supply, methanogens can continue to produce methane regardless of whether SRB are prevalent in estuarine brackish marshes.

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1 Introduction

Methane (CH_4) is an important greenhouse gas responsible for approximately 20 % of radiative forcing (IPCC, 2007). Biogenic sources account for more than 70 % of total global CH_4 emissions, where the single largest source of methane is natural wetlands (IPCC, 2007). The availability of terminal substrates is fundamental in controlling methane production. Acetate is considered an important precursor for methane production in wetlands, and has been shown to accumulate transiently in some freshwater and marine sediment due to temporal separation of acetate production and consumption processes (Avery et al., 1999; Shannon and White, 1996). A number of researchers have studied the spatial and temporal variations in pore water acetate concentrations and acetate cycling in peatlands and marine sediments (Shannon and White, 1996; Wu et al., 1997; Ho et al., 2002). However, pore water acetate concentrations in tidal marshes, and their relationships with soil methane production rates are poorly characterized. Dimethyl sulfide (DMS) acts as a substrate for methane production in marine sediments (Oremland and Polcin, 1982; Giani et al., 1996; Sunnons et al., 1998; Lyimo et al., 2002), yet only few studies have determined DMS concentrations in the estuarine sediment pore water (Sørensen, 1988), and to our knowledge, no published research has determined pore water DMS concentrations in brackish marsh, and examined their relationship with the soil methane production rate.

Soil microbiological properties can directly control methane production in wetland ecosystems (Conrad et al., 1989). Although, there are several reports on the relationship between soil methane production and abundance of methanogens, the findings are inconsistent (Cadillo-Quiroz et al., 2006; Freitag and Prosser, 2009; Liu et al., 2011). Cadillo-Quiroz et al. (2006) examined methane production and methanogen populations at different depths in two peatlands, McLean bog dominated by *Sphagnum angustifolium* moss and *hamaedaphne calyculata* shrub and Chicago bog dominated by *Sphagnum fuscum*, and found that the variation in population of methanogens did not change potential methane production. Liu et al. (2011) concluded that methane

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production potential was not significantly related to methanogen populations in four selected natural wetlands together on the national scale across China (Liu et al., 2011). In contrast, Freitag and Prosser (2009) observed that methane production rate was significantly correlated with the mcrA transcript: gene ratio in a peatland in North Wales, UK. Sulfate-reducing bacteria (SRB) also play a significant role in carbon cycling in aquatic environments. SRB and methanogens coexist in sulfate-rich marine sediments and compete for common substrates such as acetate and hydrogen (Oremland and Polcin, 1982; Holmer and Kristensen, 1994). Sulfate reduction dominates over methane production because SRB have a higher affinity for substrates such as acetate and hydrogen (Nedwell and Banat, 1981). Although some studies have determined the abundance of SRB in marine sediments and tidal flats in recent years (Leloup et al., 2005, 2007, 2009; Wilms et al., 2007; Zeleke et al., 2013), no published research has determined the spatial distribution of pore water concentrations of DMS among different brackish marshes along a gradient from dam to sea, and revealed their relationships with the methane production rate.

This study investigated the soil methane production rate, the abundance of methanogens and SRB, the concentrations of pore water terminal substrates (acetate, dissolved CO_2 and DMS) and electron acceptors (Fe^{3+}, SO_4^{2-} and NO_3^-) at a brackish marsh landscape dominated by *Phragmites australis*, *Cyperus malaccensis* and *Spatina alterniflora* marsh zones in the Min River estuary. The objective was to (1) examine the spatial variations of methane production rates, methanogens and SRB, and pore water terminal substrates in three marshes zones; (2) understand the relationships between methane production rates and abundance of methanogens and SRB, pore water concentrations of terminal substrates and electron acceptors across three brackish marsh vegetation zones at a landscape scale, and also the differences among different vegetation types.

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2 Materials and methods

2.1 Site description

This work was conducted in the Shanyutan wetland, the largest tidal wetland area (ca. 3120 ha) in the Min River estuary, southeast China. The climate is relatively warm and wet, with a mean annual temperature of 19.6 °C and a mean annual precipitation of ca. 1350 mm (Tong et al., 2010). Tides are typically semi-diurnal tides on the diurnal scale in the Shanyutan wetland. The study site was located in the west section of the Shanyutan wetland, where the *P. australis* marsh zone, *C. malaccensis* Lam. var. *brevifolius* Bocklr. marsh zone and *S. alterniflora* marsh zone from the dam to the sea lie (Fig. 1), and their mean relative elevations are 1.5, 0.5 and 1.0 m, respectively. At the study site, there is normally between 10 and 150 cm of water level above the soil surface at tide, while on neap tide days, soil surface is probably exposed over the full 24 h cycle. The mean height of three macrophytes is approximately 2 m in summer. Soil texture is characterized by silt soil.

2.2 Soil and pore water and sampling

A sampling line transect crossing the three marsh zones was conducted, in the middle of the transect within each marsh zone (*P. australis*: 26°01′55″ N, 119°36′59″ W; *C. malaccensis*: 26°01′58″ N, 119°37′02″ W; and *S. alterniflora*: 26°02′01″ N, 119°37′04″ W), we established three quadrats (1 m × 1 m) at intervals of 5 m on a line parallel with the dam. On a neap tide day in May 2011, a series of PVC tubes (5 cm inner diameter) with different sampling depths of 0–5, 5–10, 10–15, 15–20, 20–25 and 25–30 cm were installed in the center of each quadrat, with 5 cm protruding above the sediment surface, and the top mouth of each tube was sealed tightly with a cover. After several days, soil and pore water samples were collected.

Soil cores were collected using steel soil samplers (d = 5 cm) in the center of each quadrat near the PVC pore water sampling tubes (within 5 cm). Two soil cores were col-

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lected at six depths of 0–5, 5–10, 10–15, 15–20, 20–25 and 25–30 cm in each quadrat. The first set of cores, for measuring the rate of methane production, were immediately placed into self-designed incubation chambers (constructed using transparent Plexiglas, inner diameter = 5 cm, height = 12 cm) and sealed with stoppers. The chambers were designed with some headspace (volume of chamber approximately 785 cm^3, volume of soil core 392.5 cm^3). The second set of cores, for measuring soil physical and chemical properties, were sealed in plastics bags. In addition, cores used for soil DNA extraction and quantitative real-time PCR were collected at three depths of 0–10, 10–20 and 20–30 cm in the three quadrats in each marsh zone; these soil cores were stored in sterilized serum bottles and kept on ice in coolers. All soil samples were transported to the laboratory within 6 h. Pore water samples were collected for each PVC tube in each quadrat (three replicates for each soil layer of each vegetation type). The pore water was sampled using 100 mL gas-tight glass syringes connected to a rubber hose and immediately placed into different containers. An aliquot of 25 mL pore water was transferred to a 25 mL glass vial, which was immediately placed in an ice box and stored in the dark for subsequent analysis of DMS levels. A further aliquot was placed into a 25 mL glass vial into which 0.1 mL nitric acid was added to preserve the sample for later determination of Fe^{3+} concentrations (Weston et al., 2006). Approximately 10 mL of pore water was removed and placed in a 18 mL vacuum glass vial for analysis of dissolved CO_2 content. The remaining pore water was used to determine acetate, SO_4^{2-} and NO_3^- concentrations. All pore water samples were transported to the laboratory within 6 h of being collected and were stored at 4 °C prior to analysis.

2.3 Soil and pore water analysis

Soil texture was determined using a Malvin Laser Particle Size Analyzer (Mastersizer-2000, UK). Soil pH was determined using an acidity meter (Orion 868, USA) with a soil-to-water ratio of 1 : 2.5 and soil conductivity was measured using a DDS-307 EC Meter (Hua Rui Bo Yuan S & T Co., Beijing) with a soil-to-water ratio of 1 : 5. Soil total nitrogen (TN) was measured using Kieldahl Azotometer (BUCHI-K-370, Switzerland).

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Soil organic carbon (SOC) content was determined by titration after wet combustion of soil in $H_2SO_4/K_2Cr_2O_7$ (Sorrell et al., 1997; Bai et al., 2005). Moisture content was determined from a 10 g soil sample dried at 100 °C for 24 h.

Acetate concentrations in pore water samples were determined using a gas chromatograph (GC-2010, Shimadzu, Japan) fitted with a flame ionization detector (FID) and RTX-WAX capillary column (30 m × 1 µm × 0.5 mm ID) (Wu et al., 1997; Ho et al., 2002). The column and detector temperatures were set at 135 °C and 250 °C, respectively, with nitrogen as the carrier gas at a flow rate of 23 mL min^{-1}, and air and H_2 for the FID at flow rates of 300 and 33 mL min^{-1}, respectively. DMS concentrations were analyzed with solid phase micro-extraction (SPME)-GC method (Jin et al., 2004). DMS was extracted via the SPME system as soon as the pore water sample arrived at the laboratory, and DMS concentrations were determined using a gas chromatograph (GC-2010, Shimadzu, Japan) fitted with a flame photometric detector (FPD) and RTX-WAX capillary column (30 m × 1 µm × 0.5 mm ID). The standard sample was prepared from analytical reagent DMS (Sigma Aldrich Co., USA). The column and detector temperatures were set at 80 °C and 220 °C, respectively, with nitrogen as the carrier gas at a flow rate of 60 mL min^{-1}, and air flow rates of 70 mL min^{-1}, respectively. Dissolved CO_2 concentrations were determined by the method developed by Ding et al. (2003) and Itoh et al. (2008). The headspace gas was withdrawn using a gas-tight syringe, and CO_2 concentration was determined using a gas chromatograph (GC-2010, Shimadzu, Japan) fitted with a FID detector (equipped with methanizer that converts CO_2 to CH_4). The column and detector temperatures were set to 45 °C and 20 °C, respectively, and nitrogen was used as the carrier gas at a flow rate of 20 mL min^{-1}. NO_3^- and SO_4^{2-} concentrations were measured by a flow injection analyzer (SKALAR San^{++}, the Netherlands). Fe and Fe^{2+} concentrations were determined using the standard ferrozine photometric method (Stookey, 1970), where Fe^{3+} was defined as the difference between total Fe and Fe^{2+} (Hyun et al., 2009).

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2.4 Measurement of methane production rate

The rate of methane production from soil was determined using a method from Wachinger et al. (2000). Soil cores in the chambers under anoxic incubation had intact undisturbed structures. Incubations were started by filling the chambers with N_2 gas for 10 min to replace all the oxygen (Wassmann et al., 1998). The soil cores were then incubated for 3 days at an in situ temperature of 20 °C. Gas samples (5 mL) were extracted using a syringe 4–5 times over the incubation period, where chambers were refilled with N_2 after each gas sampling until normal atmospheric pressure was re-established. CH_4 concentrations were also analyzed using a gas chromatograph (GC-2010, Shimadzu, Japan) fitted with a FID immediately following extraction. Methane production rates ($\mu g\, d^{-1}\, g^{-1}$(dw)) were calculated from the changes in gas concentrations in the chambers (Wassmann et al., 1998).

2.5 DNA extraction and real-time PCR

Total DNA was extracted from 0.25 g of each fresh marsh soil sample using the Power Soil DNA Extraction Kit (MoBio Laboratory, USA) according to the manufacturer's instructions. Briefly, 0.25 g fresh marsh soil was added to the PowerBead Tubes provided. Subsequently, the cells were lysed using a combination of detergents and mechanical disruption, and the released DNA was bound to a silica spin filter. The filter was washed and the DNA was recovered in Solution C6. The extracted DNA was evaluated on a 1 % agarose gel in 1× TAE buffer after staining with ethidium bromide. The concentration and purity of the extracted DNA were estimated by spectrophotometry (NanoDrop, USA).

Abundances of methanogenic archaea and SRB were determined by quantitative real-time PCR analysis of 16S rRNA and dsrA gene on a PCR Thermal Cycler Dice Real-Time System (Takara, Japan). Methanogenic archaea were quantified by SYBR Green I assays using the primer pairs 1106F (5′-TTWAGTCAGGCAACGAGC-3′) and 1378R (5′-TGTGCAAGGAGCAGGGAC-3′) (Watanabe et al., 2006, 2009). Each re-

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action mixture (25 µL) consisted of 12.5 µL SYBR *Premix Ex Taq* II (Takara, Japan), 1 µL each of 10 µM primer, 2 µL of DNA template (10 ng total), and 8.5 µL of sterilized distilled water. Quantitative PCR was carried out as follows: 30 s at 95 °C for initial denaturation; 40 cycles of 5 s at 95 °C, 30 s at 60 °C. SRB was quantified by SYBR Green I assays using the dsrA specific to the primer pairs DSR-1F+ (5′-ACSCACTGGAAGCACGGCGG-3′) and DSR-R (5′- GTGGMRCCGTGCAKRTTGG-3′) described by Kondo et al. (2004) and Leloup et al. (2007). The reaction mixture (25 µL) was 12.5 µL SYBR *Premix Ex Taq* II (Takara, Japan), 1 µL each of 10 µM primer, 2 µL of DNA template (20 ng total), and 8.5 µL of sterilized distilled water. Quantitative PCR was performed using a PCR Thermal Cycler Dice Real-Time System (Takara, Japan) as follows: 30 s at 95 °C for initial denaturation; and 45 cycles: 5 s at 95 °C, 30 s at 60 °C. For the preparation of methanogenic archaea 16S rRNA gene and SRB dsrA gene standards, 16S rRNA gene and dsrA gene were PCR amplified from extracted DNA with the primers 1106F/1378R and DSR-1F+/DSR-R, respectively, and then cloned into the pMD 19-T Vector (Takara, Japan). Plasmids from the proper insert clones of each target gene were extracted and used as standards for the calibration curve. SYBR Green I assays were performed with a melting curve analysis which was used to check the specificity of the products. Triplicates for standards and unknown templates were performed on a single plate. The results were analyzed using the Thermal Cycler Dice Real-Time System software (Takara, Japan).

2.6 Statistical analysis

All data were expressed on the basis of oven-dried soil. All statistical analyses were performed with SPSS for Windows 17.0. The effects of vegetation types, soil depth and their interaction on the concentrations of terminal substrates, electron acceptors, soil methane production rate, population of methanogens and SRB were examined by two-way ANOVA. Differences in soil properties, terminal substrate and electron acceptor concentrations, abundance of methanogens and SRB, and methane production rates in the three marsh zones, and differences in the above variables at different soil depths

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in each marsh zone were examined by a least-significant difference (LSD) test in one-way ANOVA. Regression analysis was used to test relationships between methane production rates and the abundance of methanogens and SRB, and concentrations of terminal substrates. When we conducted the correlation analysis between methane production rates and abundance of methanogens and SRB, because the abundances of methanogens and SRB were measured at every 10 cm depths while methane production rate were measured at every 5 cm depths, we first calculated the average values of methane production rates of 0–5 and 5–10 cm, 10–15 and 15–20 cm, 20–25 and 25–30 cm, respectively, and then conducted the correlation analysis.

3 Results

3.1 Soil properties

Soil vertical profile properties at each marsh zone are shown in Table 1. Mean value of soil pH gradually decreased from the dam to the sea; mean soil pH in the depth of 30 cm in the *P. australis* marsh was significantly lower than that of the other two marsh zones. Soil pH was statistically indistinguishable among different soil layers in the *C. malaccensis* and *S. alterniflora* marshes; however, soil pH in the 0–5 cm layer of the *P. australis* marsh was significantly higher than in the other two layers. Soil moisture in the *P. australis* marsh was also significantly higher than that in the other two marsh zones. Soil conductivity in the three marshes was below 1 mS cm^{-1}, indicating that they all belonged to the category of brackish marsh. The soil texture of the three marsh zones was similar, and characterized by silt making up about 60 %. SOC and TN at a soil depth of 30 cm in the *P. australis* marsh were significantly higher than in the soil beneath the *C. malaccensis* and *S.alterniflora* marshes, however the differences in different soil layers were not distinct in three marsh zones (Table 1).

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3.2 Pore water terminal substrates and electron acceptors

Vertical profiles of the concentrations of pore water terminal substrates and electron acceptors in the *P. australis*, *C. malaccensis* and *S. alterniflora* marsh zones are shown in Fig. 2. The concentrations of DMS, dissolved CO_2, SO_4^{2-} and Fe^{3+} varied with vegetation types, but the acetate and NO_3^- concentrations were statistically indistinguishable among the three marsh zones (Table 2). Acetate and SO_4^{2-}concentrations varied with soil depths, however, there was not a significant interaction between vegetation types and depths for all terminal substrates and electron acceptors (Table 2).

Acetate concentrations ranged from 90 to 850, 50 to 490 and 130 to 430 µM in the three marsh zones, while average values at a soil depth of 30 cm beneath the three marsh zones were 380, 190 and 260 µM. Dissolved CO_2 concentrations ranged from 270 to 670, 190 to 320 and 270 to 460 µM; average values at a depth of 30 cm beneath the three marsh zones were 450, 260 and 320 µM, while the dissolved CO_2 concentration in the *P. australis* marsh was significantly higher than that in the *C. malaccensis* marsh ($F(1,54) = 7.24$, $P < 0.001$) and *S. alterniflora* marsh ($F(1,54) = 4.679$, $P = 0.035$); the concentrations in the *C. malaccensis* and *S. alterniflora* marshes were statistically indistinguishable ($F(1,54) = 2.387$, $P = 0.128$). DMS concentrations ranged from 0.03 to 0.08, 0.02 to 0.07 and 0.07 to 0.72 µM; average values at a depth of 30 cm beneath the three marsh zones were 0.05, 0.03 and 0.47 µM, while the concentration in the *S. alterniflora* marsh was significantly higher than that in *C. malaccensis* marsh ($F(1,34) = 13.494$, $P = 0.001$) and *P. australis* marsh ($F(1,34) = 12.016$, $P = 0.001$); the concentration in the *P. australis* marsh was also significantly higher than that in the *C. malaccensis* marsh ($F(1,34) = 7.638$, $P = 0.009$). SO_4^{2-} ranged from 680 to 1360, 1180 to 1320 and 780 to 1220 µM in the three marsh zones, average values at a depth of 30 cm beneath the three marsh zones were 990, 1280 and 1110 µM; the concentration in the *P. australis* marsh was significantly lower than that in the *C. malaccensis* marsh ($F(1,34) = 9.319$, $P = 0.004$). NO_3^- concentrations ranged from 0.94 to 4.13, 0.35 to 1.27 and 0.19 to 1.85 µM, while in the three zones, average values at

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a depth of 30 cm in the three zones were 2.70, 0.81 and 0.96 μM. The concentration in the *P. australis* marsh was significantly higher than that in *C. malaccensis* marsh ($F(1,34) = 8.744$, $P = 0.006$). Fe^{3+} concentrations ranged from 0.08 to 2.05, 0.36 to 2.5 and 0.15 to 3.13 μM in the three marsh zones, while average values at the depth of 30 cm under the three marsh zones were 0.95, 1.08 and 2.07 μM.

3.3 Soil methane production rate

Vertical profiles of the methane production rates in the *P. australis*, *C. malaccensis* and *S. alterniflora* marsh zones are shown in Fig. 3. The soil methane production rates varied with vegetation types, and there was a significant interaction between vegetation type and depth for soil methane production rate (Table 3). Methane production rates ranged from 0.014 to 0.413, 0.024 to 0.078 and 0.025 to 0.091 $\mu g\, g^{-1}\, d^{-1}$. Average values at a depth of 30 cm beneath the three marsh zones were 0.142, 0.058 and 0.067 $\mu g\, g^{-1}\, d^{-1}$. Only the soil methane production rate in the *P. australis* marsh was significantly higher than that in the *C. malaccensis* marsh ($F(1,31) = 4.576$, $P = 0.040$), with the remaining rates being statistically indistinguishable.

3.4 Abundance of methanogens and SRB

Vertical distribution of the abundance of methanogens and SRB in the soils of *P. australis*, *C. malaccensis* and *S. alterniflora* marsh zones are shown in Fig. 4. The abundance of methanogens and SRB varied with vegetation types, and did not vary with depth, and there was a significant interaction between vegetation type and depth for the abundance of SRB (Table 3). The abundance of methanogens ranged from 7.79×10^7 to 7.50×10^8, 1.77×10^7 to 7.68×10^7 and 2.01×10^7 to 1.04×10^8 gene copies g^{-1} dry weight soil (dws) in the three marsh zones. The average abundance of 3.72×10^8 gene copies g^{-1} dws at a depth of 0–30 cm in the *P. australis* marsh was significantly higher than 3.34×10^7 gene copies g^{-1} dws in the *C. malaccensis* marsh ($F(1,16) = 20.66$, $P < 0.001$) and 5.73×10^7 gene copies g^{-1} dws in the *S. alterniflora*

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marsh ($F(1,16) = 17.37$, $P = 0.001$). The abundance of SRB ranged from 4.09×10^7 to 1.45×10^8, 1.24×10^7 to 5.65×10^7 and 8.41×10^6 to 2.99×10^7 dsrA copies g^{-1} dws in the three marsh zones. The average abundance of 8.54×10^7 dsrA copies g^{-1} dws at a depth of 0–30 cm in the *P. australis* marsh was significantly higher than 2.40×10^7 dsrA copies g^{-1} dws in the *C. malaccensis* marsh ($F(1,16) = 35.950$, $P < 0.001$) and 1.51×10^7 dsrA copies g^{-1} dws in *S. alterniflora* marsh ($F(1,16) = 24.273$, $P = 0.001$).

4 Discussion

4.1 Terminal substrates and electron acceptors

Acetate is an important intermediate in organic matter mineralization in both freshwater and marine sediments as well as soil (Sansone, 1986; Michelson et al., 1989). The average acetate concentration of three marshes was approximately 277 µM at a depth of 0–30 cm. The difference of acetate concentration in the three marsh zones was not significant, although SOC content in the *P. australis* marsh zone was higher than those in the other two marsh zones (Table 1). Duddleston et al. (2002) also found pore water concentrations of acetate to be approximately 100 µM in May, increasing rapidly to approximately 1000 µM when the water table rose to the surface in August in a northern Turnagain bog. It has generally been accepted that acetate concentration is relatively low in wetland soil/sediment. Pore water acetate concentrations in marine sediments have been measured within the range of 0.1 to 69 µM (Ansraek and Blackburn, 1980; Michelson et al., 1989; Shaw and McIntosh, 1990; Wu et al., 1997). The relatively higher acetate concentrations in present study and the study of Duddleston et al. (2002) suggest that soil pore water acetate concentrations within marshes and bogs may be higher than that in marine sediments because marsh and bog supply more freshly deposited organic matter.

Considering DMS is a highly volatile compound, we used a series of PVC tubes installed in soil to sample pore water, and did not use the centrifugation method. The

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average pore water concentration of DMS (0.47 µM) at 0–30 cm depth in the *S. alterniflora* marsh was higher than that in the *P. australis* (0.05 µM) and *C. malaccensis* (0.03 µM) marsh zones. This result may be explained by the conclusion reported by Dacey et al. (1987) that DMS may be released from physiological processes in the leaves of higher plants, mainly one species of *S. alterniflora*. Dacey et al. (1987) investigated DMSP concentrations in a variety of higher plants including *S. alterniflora, Phragmites communis, Spatina patens and Juncus gerardi*, and found that while DMSP levels were especially high in the tissues of *S. alterniflora* (80–200 µM g (dw)), concentrations no greater than 0.1 µM g (dw) were detected in the tissues of other marsh species. Although DMS is considered as terminal substrates of methane production in marine sediments (Oremland and Polcin, 1982; Giani et al., 1996; Lyimo et al., 2002), only Sørensen (1988) reported a seasonal variation in DMS concentrations in sediment pore waters, with the highest concentration of about 0.1 µM DMS observed within the upper 0–5 cm of the sediment in late summer in a Danish estuary. Compared with pore water concentration of DMS in the *S. alterniflora* marsh in our study, the DMS concentration in Sørensen' study was obviously lower.

Relationship between methane production rate and pore water acetate concentration within estuarine marsh ecosystems is poorly characterized. In our study, methane production rate increased linearly with the pore water concentration of acetate for the three vegetation zones together at the landscape scale (Fig. 5), however, it was not associated with concentrations of dissolved CO_2 and DMS at the landscape scale ($P > 0.05$, $n = 27$). The result indicated that the acetate fermentation path would explain more variation of methanogenesis than the methane production path via DMS in estuarine brackish marsh with lower salinity (< 1 mS cm^{-1}). When regression analysis was done for single vegetation zones, methane production rate only linearly increased with the pore water concentration of acetate in the *P. australis* marsh ($y = 0.329x + 0.039$, $R^2 = 0.535$, $P = 0.002$, $n = 9$), and did not correlate with the pore water concentration of DMS and dissolved CO_2 content in each marsh zone ($P > 0.05$, $n = 9$). In our study, acetate concentration explained only 26.2 % variation of methanogenesis. Avery

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et al. (1999, 2003) observed that acetate accumulation stimulated methane production in peatlands, being responsible for over 80 % of total methane production. Therefore, it can be speculated that acetate fermentation path would be more important in peatlands.

Average pore water SO_4^{2-} and NO_3^- concentrations at a depth of 0–30 cm beneath the three marsh zones were 1.13 mM and 1.49 µM, respectively. The SO_4^{2-} concentration in our study was lower than that (approximately 10 mM) detected in the creek-bank sediments of an intertidal site adjacent to the Dover Bluff in coastal Georgia and South Carolina, USA (Weston et al., 2006), and those (approximately 28 mM) in three oceanic dwarf mangrove habitats, Twin Cays, Belize (Lee et al., 2008). Pore water SO_4^{2-} concentrations have displayed an obvious seasonal variation in coastal wetlands (Koretsky et al., 2005). Our study site was relatively near the bank; May has relatively lower tides and relatively larger river runoff, which were probably responsible for the lower pore water SO_4^{2-} concentrations. In December 2007, SO_4^{2-} concentrations were 2.6 and 4.1 mM in pore waters at depths of 10 and 20 cm, respectively, in the *P. australis* marsh (Tong et al., 2010). SO_4^{2-}concentration in the *P. australis* marsh zone was lower than that in the *C. malaccensis* marsh zone, Fe^{3+} concentration in the *S. alterniflora* marsh was higher than that in the *P. australis* and *C. malaccensis* marsh zones, the reason is not clear. Higher pH value in the *S. alterniflora* marsh zone may be one reason causing the higher Fe^{3+} concentration, since Fe^{2+} is easy to be oxidized to Fe^{3+} in relatively higher pH condition. In our study, the rate of methane production was not associated with pore water concentrations of SO_4^{2-}, NO_3^- and Fe^{3+} for the three vegetation zones together at the landscape scale ($P > 0.05$, $n = 27$).

4.2 Abundances of methanogens and SRB

In Table 4 the abundance of both methanogens and SRB of 11 studies are presented with regard to the type of wetlands and their location. Table 4 indicates that latitude as well as temperature is not likely the key environmental factor controlling the abundance

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of both methanogens and SRB in wetlands on a global scale. In our study, the average abundance of both methanogens and SBR in the *P. australis* marsh was higher than in the *S. alterniflora* marsh; on the contrary, compared with the *P. australis* marsh, the *S. alterniflora* marsh had a higher abundance of both methanogens and SBR in the Yangzi River estuary (Zeleke et al., 2013) (Table 5). It is therefore possible that plant community type is not the key factor controlling the abundance of both methanogens and SRB in wetlands. Instead we suggest that both abundance of methanogens and SBR in wetlands is affected by the complex interactions between a number of abiotic and biotic factors.

The abundances of both methanogens and SBR in the *P. australis* marsh zone with highest SOC and NO_3^- contents were higher than those in the other two marsh zones. Populations of methanogens and SBR correlated with SOC and NO_3^- in the all three marsh zones together (Fig. 6). Liu et al. (2011) also reported that the population of methanogenic archaea in four wetlands correlated with SOC content and also with total nitrogen concentration. In our study, the abundance of methanogens increased linearly with the pore water acetate concentration ($y = 327.82x + 62.37$, $R^2 = 0.2389$, $P = 0.010$), however, it did not correlated with dissolved CO_2 concentration ($n = 27$, $R^2 = 0.1216$, $R^2 = 0.111$, $P = 0.097$); the abundance of SBR did not correlated with both acetate and DMS concentrations (acetate: $n = 27$, $R^2 = 0.083$, $P = 0.150$; DMS: $n = 27$, $R^2 = 0.073$, $P = 0.174$). The abundance of methanogens and SBR did not relate with the concentration of pore water electron acceptors (SO_4^{2-} and Fe^{3+}). Tong et al. (2011) reported above-ground living biomass ($1344.8 \pm 179.1\,g\,m^{-2}$) in the *S. alterniflora* marsh was significantly higher than that of the *P. australis* ($695.9 \pm 194.5\,g\,m^{-2}$) and *C. malaccensis* ($548.3 \pm 109.1\,g\,m^{-2}$), and the below-ground root biomass in soil depths of 0–30 cm was 752.1 ± 134.4, 1000.7 ± 144.0 and $837.5 \pm 117.5\,g\,m^{-2}$ in the *P. australis*, *C. malaccensis* and *S. alterniflora* marshes, respectively, in the study area in May; both plants above and below biomass did not seem to effect the abundance of both methanogens and SBR.

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There was no statistical difference in the abundance of methanogens in three soil depths (Fig. 4), which was consistent with the result that methanogens numbers did not strongly decline with depth in two peatlands (Cadillo-Quiroz et al., 2006). However, Liu et al. (2011) determined that the top soil layer had the highest population of methanogens in all wetlands except the Ruoergai peatland.

In our study, regression analysis showed that the rate of methane production linearly increased with the abundances of both methanogens and SRB for the three vegetation zones together at the landscape scale (Fig. 7). When regression analysis was carried out for single vegetation zone, methane production rate only linearly increased with the abundance of methanogens in the *C. malaccensis* marsh ($y = 2 \times 10^{-9}x - 0.008$, $R^2 = 0.6671$, $P = 0.007$, $n = 9$), and did not correlate with the abundance of SRB in each marsh zone ($P > 0.05$, $n = 9$). Freitag and Prosser (2009) observed that the rate of methane production correlated with the mcrA transcript : gene ratio in a peatland in North Wales, UK. Dubey et al. (2012) found a positive linear relationship between methane production potential and methanogenic population in tropical rice fields in India. Morrissey et al. (2013) also found that methanogen abundance showed a modest positive correlation to methane production rates. However, Liu et al. (2011) reported that methane production potential was not significantly related to methanogenic population in four wetlands on the national scale across China.

Sulfate-reducing bacteria outcompete methanogens for hydrogen, acetate, or both, but do not compete with methanogens for compounds like methanol, trimethylamine, or methionine, thereby allowing methanogenesis and sulfate reduction to operate simultaneously within anoxic, sulfate-containing sediments (Oremland and Polcin, 1982). Holmer and Kristensen (1994) proved that methanogens and SRB could coexist at high sulfate concentrations in sediments supplied with labile organic matter, and methane production rates of the same order of magnitude occurred even when sulfate was present in high concentrations (5–60 mM). Zeleke et al. (2013) even found that methanogens and SRB can coexist in the tidal *P. australis* marsh and *S. alterniflora* marsh (soil conductivity was $\sim 7\,\mathrm{mS\,cm^{-1}}$) of the Dongtan wetland in the Yangtze River

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estuary, China. In our study, we also found that methanogens and SRB can coexist and further their abundance can be linked (Fig. 8) in the brackish marsh (soil conductivity was below 1 mS cm^{-1} and the average pore water SO_4^{2-} concentration was only 1.13 mM).

5 Conclusions

Our data provides evidence that *S. alterniflora* marsh is a "special" habitat where pore water DMS concentration is relatively higher compared with other wetland habitats in estuarine and coastal areas. Methane production rate varies with different vegetation zones from the dam to the sea in the estuarine area. Methane production rate correlates linearly with the concentration of pore water acetate and the content of soil organic carbon across marsh zones together at the landscape scale, however, do not correlate with concentrations of pore water DMS and dissolved CO_2. The abundance of both methanogens and SRB in the soil of the *P. australis* marsh with the highest soil organic carbon and TN content, and NO_3^- concentration in pore water is higher than in the *C. malaccensis* and *S. alterniflora* marshes at a landscape scale, which indicates that soil organic carbon and/or nitrogen may control the abundance of both methanogens and SRB in wetlands. The abundance of both methanogens and SRB do not vary with soil depth. Methane production rate increased with the abundance of both methanogens and SRB across three marsh zones together at the landscape scale. Our results suggest that, provided that substrates are available in ample supply, methanogens can continue to produce methane regardless of whether SRB are prevalent in estuarine brackish marshes.

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Table 1. Soil profile properties of each sampling site.

Soil depth (cm)	pH	Moisture (%)	Conductivity ($mS\,cm^{-1}$)	Clay (%)	Silt (%)	Sand (%)	TN ($g\,kg^{-1}$)	SOC ($g\,kg^{-1}$)
P. australis								
0–5	5.71 ± 0.03^{a}	$50.33 \pm 0.86^{a,b}$	0.77 ± 0.02^{c}	12.65 ± 1.14^{a}	59.85 ± 2.93^{a}	27.51 ± 1.84^{a}	$0.81 \pm 0.01^{b,c}$	20.94 ± 0.57^{b}
5–10	5.37 ± 0.06^{b}	52.13 ± 0.67^{a}	$0.86 \pm 0.02^{a,b}$	9.13 ± 0.42^{a}	54.96 ± 9.07^{a}	35.91 ± 9.31^{a}	0.90 ± 0.02^{b}	$23.80 \pm 0.47^{a,b}$
10–15	5.34 ± 0.09^{b}	51.87 ± 1.63^{a}	0.89 ± 0.02^{a}	11.72 ± 0.79^{a}	64.62 ± 0.60^{a}	23.66 ± 1.04^{a}	1.03 ± 0.06^{a}	27.06 ± 2.62^{a}
15–20	5.37 ± 0.10^{b}	47.63 ± 1.73^{b}	$0.85 \pm 0.01^{a,b}$	11.42 ± 1.97^{a}	67.07 ± 4.23^{a}	21.50 ± 6.02^{a}	0.79 ± 0.04^{c}	21.52 ± 1.20^{b}
20–25	5.33 ± 0.08^{b}	43.17 ± 1.13^{c}	0.83 ± 0.01^{b}	11.86 ± 2.19^{a}	66.27 ± 2.53^{a}	21.86 ± 4.71^{a}	$0.75 \pm 0.02^{c,d}$	20.65 ± 0.74^{b}
25–30	5.27 ± 0.01^{b}	43.03 ± 1.20^{c}	$0.81 \pm 0.00^{b,c}$	10.85 ± 0.82^{a}	66.95 ± 2.73^{a}	22.20 ± 3.54^{a}	0.68 ± 0.02^{d}	19.96 ± 0.98^{b}
Average	5.40 ± 0.02^{e}	48.03 ± 1.69^{e}	0.83 ± 0.02^{e}	11.27 ± 0.49^{e}	63.29 ± 2.00^{e}	25.44 ± 2.28^{e}	0.83 ± 0.05^{e}	22.32 ± 0.60^{e}
C. malaccensis								
0–5	6.06 ± 0.13^{a}	41.63 ± 1.25^{a}	0.71 ± 0.01^{d}	$11.16 \pm 1.00^{a,b}$	62.55 ± 1.11^{a}	$26.28 \pm 2.01^{a,b}$	0.60 ± 0.02^{a}	$15.51 \pm 0.68^{a,b}$
5–10	6.07 ± 0.06^{a}	41.97 ± 1.29^{a}	$0.75 \pm 0.03^{c,d}$	8.85 ± 1.02^{b}	52.77 ± 7.67^{a}	38.38 ± 8.58^{a}	0.54 ± 0.01^{a}	$15.16 \pm 0.24^{a,b}$
10–15	5.96 ± 0.07^{a}	43.70 ± 1.17^{a}	$0.87 \pm 0.02^{a,b}$	12.69 ± 0.96^{a}	65.66 ± 3.18^{a}	21.65 ± 4.14^{b}	0.58 ± 0.04^{a}	14.97 ± 0.57^{b}
15–20	5.91 ± 0.02^{a}	42.67 ± 1.30^{a}	$0.82 \pm 0.02^{b,c}$	9.69 ± 1.02^{b}	57.49 ± 5.94^{a}	$32.83 \pm 5.25^{a,b}$	0.62 ± 0.03^{a}	16.902 ± 0.64^{a}
20–25	5.89 ± 0.08^{a}	44.80 ± 1.30^{a}	0.88 ± 0.02^{a}	9.81 ± 0.41^{b}	62.68 ± 2.49^{a}	$27.51 \pm 2.54^{a,b}$	0.58 ± 0.03^{a}	$15.60 \pm 0.64^{a,b}$
25–30	5.89 ± 0.10^{a}	45.60 ± 1.46^{a}	0.91 ± 0.01^{a}	$10.15 \pm 0.78^{a,b}$	62.64 ± 0.67^{a}	$27.21 \pm 2.38^{a,b}$	0.59 ± 0.00^{a}	14.66 ± 0.77^{b}
Average	5.96 ± 0.02^{f}	43.39 ± 0.65^{f}	0.82 ± 0.03^{e}	10.39 ± 0.55^{e}	60.63 ± 1.91^{e}	28.98 ± 2.38^{e}	0.59 ± 0.01^{f}	15.47 ± 0.20^{f}
S.alterniflora								
0–5	6.27 ± 0.12^{a}	$42.13 \pm 0.30^{c,d}$	0.54 ± 0.05^{c}	7.94 ± 0.24^{b}	55.93 ± 0.72^{a}	36.13 ± 0.95^{a}	0.64 ± 0.01^{a}	15.16 ± 1.04^{a}
5–10	6.25 ± 0.09^{a}	$41.60 \pm 0.47^{c,d}$	$0.64 \pm 0.05^{b,c}$	9.42 ± 0.38^{b}	56.98 ± 1.16^{a}	$33.60 \pm 1.36^{a,b}$	0.54 ± 0.01^{b}	15.03 ± 0.20^{a}
10–15	6.14 ± 0.06^{a}	41.30 ± 0.44^{d}	$0.75 \pm 0.04^{a,b}$	12.75 ± 1.04^{a}	60.38 ± 0.80^{a}	26.86 ± 0.38^{b}	0.53 ± 0.02^{b}	15.71 ± 0.48^{a}
15–20	6.10 ± 0.04^{a}	$42.83 \pm 0.55^{b,c}$	0.76 ± 0.03^{a}	12.24 ± 0.70^{a}	63.19 ± 0.02^{a}	24.57 ± 0.68^{b}	0.56 ± 0.01^{b}	14.92 ± 0.41^{a}
20–25	6.05 ± 0.04^{a}	44.70 ± 0.44^{a}	0.77 ± 0.03^{a}	13.05 ± 0.11^{a}	60.78 ± 2.40^{a}	26.17 ± 2.29^{b}	0.62 ± 0.02^{a}	16.88 ± 0.13^{a}
25–30	6.07 ± 0.06^{a}	$43.77 \pm 0.33^{a,b}$	$0.69 \pm 0.01^{a,b}$	9.58 ± 0.85^{b}	57.90 ± 4.87^{a}	$32.51 \pm 5.72^{a,b}$	0.61 ± 0.01^{a}	15.64 ± 1.08^{a}
Average	6.15 ± 0.02^{g}	42.72 ± 0.54^{f}	0.69 ± 0.04^{f}	10.83 ± 0.87^{e}	59.20 ± 1.11^{e}	29.97 ± 1.92^{e}	0.58 ± 0.02^{f}	15.56 ± 0.18^{f}

Values are means with standard error ($n = 3$).
Different superscript letters within the same column indicate significant differences at $P < 0.05$ within each plant marsh; different superscript letters between three averages in same column also indicate significant differences at $P < 0.05$ between three marsh zones.

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Table 2. Result of two-way ANOVA for the effects of vegetation types, soil depth and their interaction on the concentrations of terminal substrates and electron acceptors in the *P. australis* marsh, *C. malaccensis* and *S. alterniflora* marshes.

Factor	DF	Acetate	Dissolved CO_2	DMS	SO_4^{2-}	NO_3^-	Fe^{3+}
Vegetation type	2	$F = 1.937$	$F = 3.982^a$	$F = 3.932^a$	$F = 7.357^b$	$F = 2.470$	$F = 5.564^b$
Depth	5	$F = 2.966^a$	$F = 1.167$	$F = 1.189$	$F = 3.207^a$	$F = 0.881$	$F = 0.689$
Vegetation × depth	10	$F = 0.883$	$F = 1.303$	$F = 0.393$	$F = 1.509$	$F = 0.846$	$F = 0.570$

[a] $0.01 \leq P < 0.05$.
[b] $0.001 \leq P < 0.01$.
[c] $P < 0.001$.

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Table 3. Result of two-way ANOVA for the effects of vegetation types, soil depth and their interaction on methane concentration, methane production, population of methanogens and SRB in the *P. australis* marsh, *C. malaccensis* and *S. alterniflora* marshes.

Factor	DF	Methane production	DF	Population of methanogens	Population of SRB
Vegetation	2	$F = 3.482$[a]	2	$F = 25.411$[c]	$F = 59.238$[c]
Depth	5	$F = 1.289$	2	$F = 1.651$	$F = 2.321$
Vegetation × depth	10	$F = 2.476$[a]	4	$F = 2.701$	$F = 11.099$[c]

[a] $0.01 \leq P < 0.05$.
[b] $0.001 \leq P < 0.01$.
[c] $P < 0.001$.

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Table 4. Comparison of abundances of methanogens (MA) and SRB in various wetlands studies.

Location	Wetland types	Abundances of MA	Abundances of SRB	Primers targeted	References
West Siberia, Russia	Peat bog	0.5–0.9 × 10^7 cells g^{-1} fresh peat		Oligonucleotide probes used for FISH	Kotsyurbenko et al. (2004)
Migneint, UK	Acidic bog and calcareous fen	~ 1 × 10^7 cells g^{-1} soil		mcrA genes	Kim et al. (2008)
UK	Acidic transitional fen	3.45 × 10^4 to 7.95 × 10^5 copies g^{-1} soil		*mlas and mcrA-rev* genes	Steinberg and Regan (2009)
Aarhus Bay, France	Marine mudflat		2.9 × 10^6 to 4.8 × 10^8 cells cm^{-3} in sediment	dsrAB genes	Leloup et al. (2009)
Hunan Province, China	Paddy field		5.50 × 10^8 copy g^{-1} dws	dsrAB genes	Liu et al. (2009)
North Wales, UK	Peatland	4.8 × 10^8 gene copies g^{-1} soil		mcrA genes	Freitag and Prosser (2009)
Sanjiang Mire Wetland, Ruoergai Highland, Hongze and Poyang Lake, China	Freshwater marsh Peatland Lakeside marsh	1.07 × 10^9 to 8.29 × 10^9 cell g^{-1} dws		16S rRNA genes	Liu et al. (2011)
Eastern U.P., India	Rice field	4.88 × 10^5 and 1.40 × 10^6 gene copies g^{-1} dws		mcrA genes	Dubey et al. (2012)
Yangzi River estuary, China	*P. australis* marsh *S. alterniflora* marsh	2.4 × 10^5 gene copies g^{-1} dws 1.2 × 10^8 gene copies g^{-1} dws	5.99 × 10^6 gene copies g^{-1} dws 1.72 × 10^7 gene copies g^{-1} dws	mcrA genes for MA and dsrB gene for SRB	Zeleke et al. (2013)
Virginia, USA	Tidal freshwater marsh	1.2 9 × 10^9 gene copies g OM^{-1}		*mlas* and *mcrA-rev* genes	Morrissey et al. (2013)
Min River estuary, China	Brackish marsh	2.01 × 10^7 to 7.50 × 10^8 gene copies g^{-1} dws	8.41 × 10^6 to 1.45 × 10^8 gene copies g^{-1} dws	16S rRNA genes for MA and dsrA gene for SRB	This study

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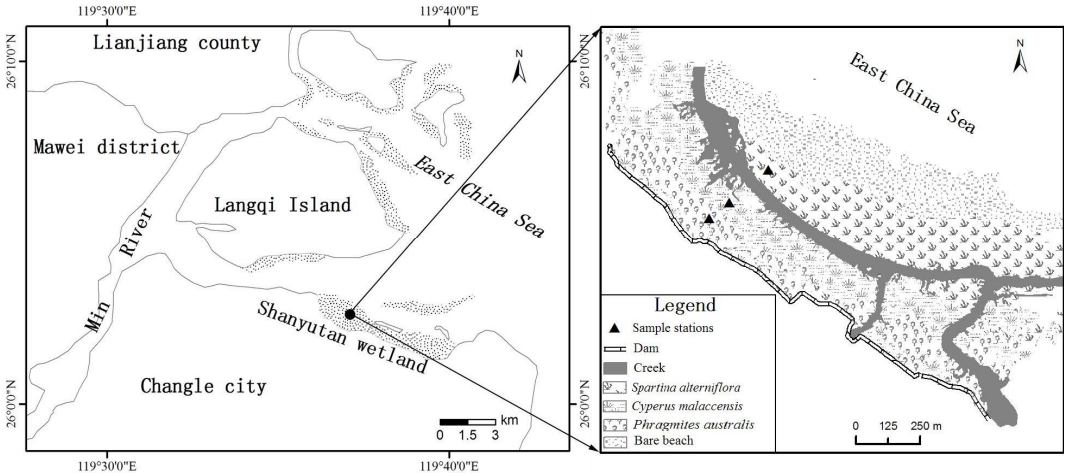

Fig. 1. Study area and sampling stations in the tidal marshes of the Min River estuary, southeast China.

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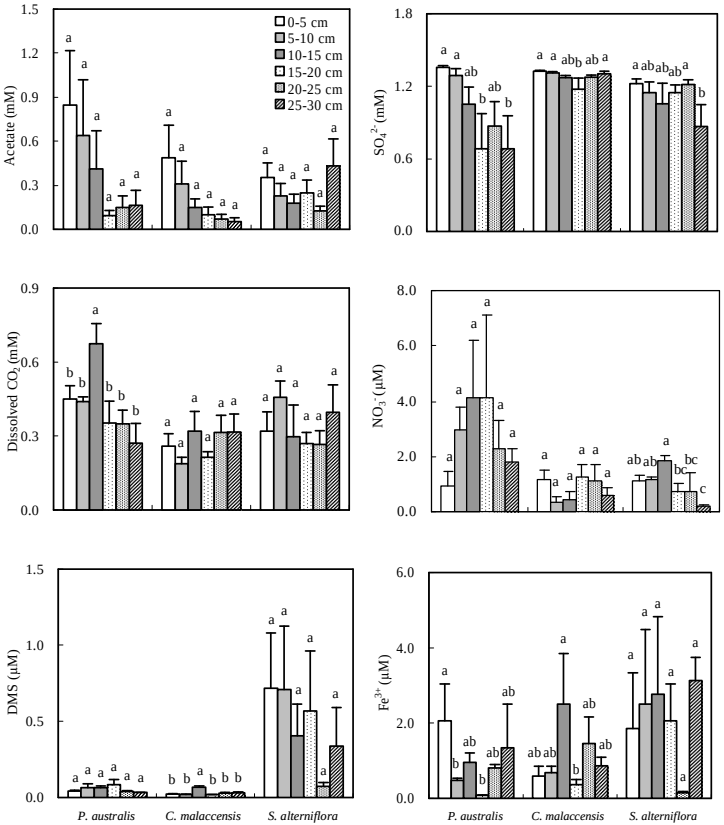

Fig. 2. Pore water concentrations of acetate, dissolved CO_2, DMS, SO_4^{2-}, NO_3^- and Fe^{3+} in vertical profile in the *P. australis*, *C. malaccensis* and *S. alterniflora* marsh zones, represented by mean ± standard error (n = 5 for dissolved CO_2; n = 3 for other variables). Different letters indicate significant differences at $P < 0.05$.

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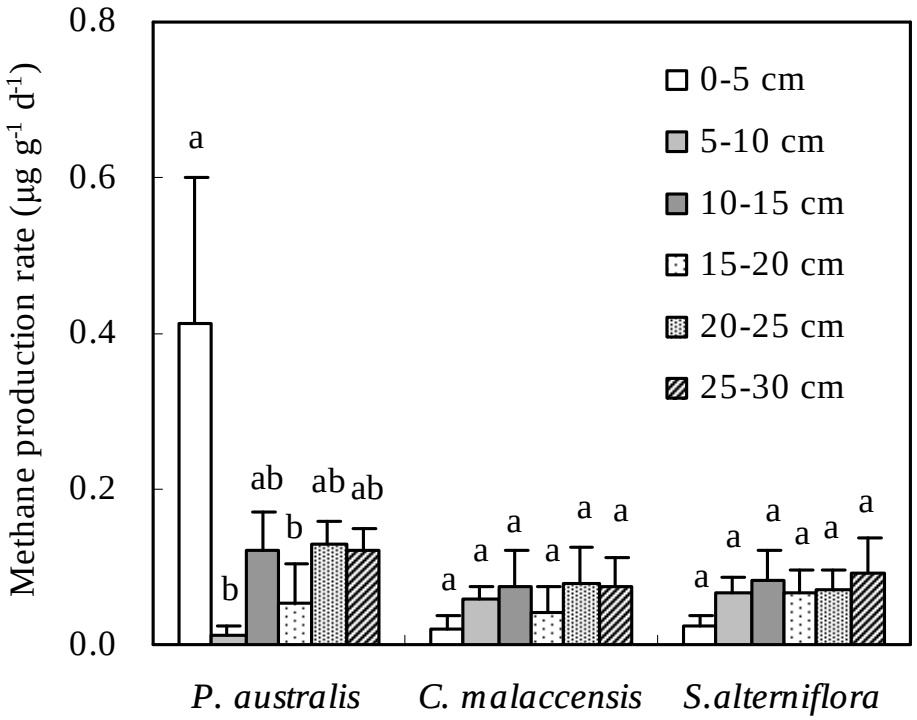

Fig. 3. Methane production rates in vertical profile in the *P. australis*, *C. malaccensis* and *S. alterniflora* zones, represented by mean ± standard error ($n = 5$). Different letters indicate significant differences at $P < 0.05$.

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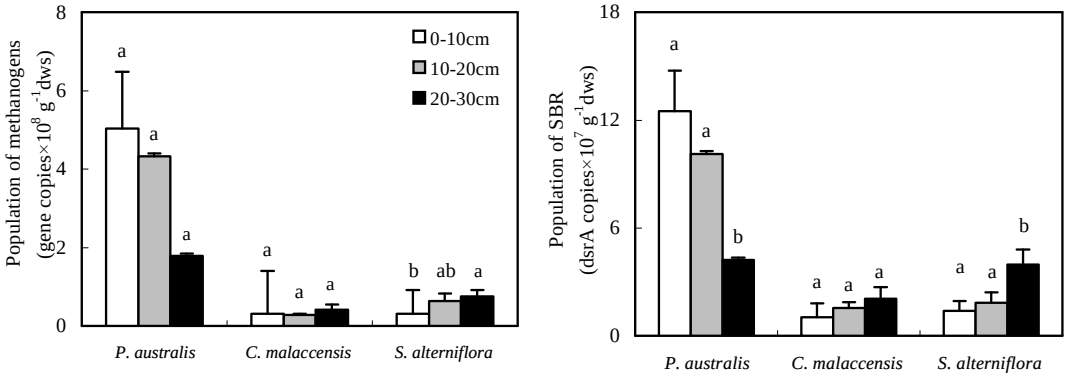

Fig. 4. Abundance of methanogenic archaea and SRB in vertical profile in the *P. australis*, *C. malaccensis* and *S. alterniflora* marsh zones based on 16S gene and dsrA copy numbers, respectively, represented by mean ± standard error (n = 3). Different letters indicate significant differences at $P < 0.05$.

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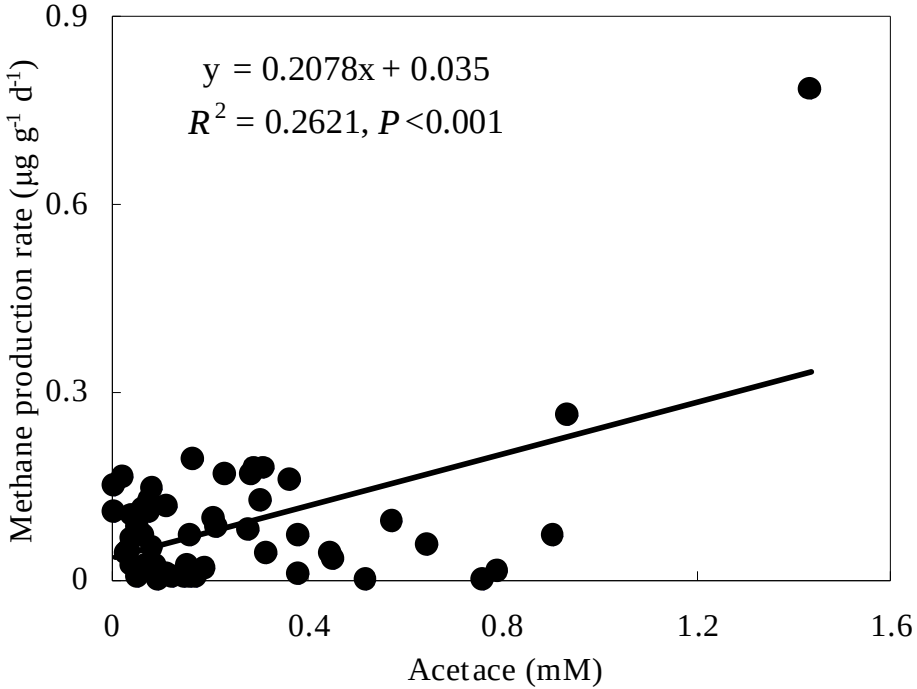

Fig. 5. Correlation between soil methane production rate and pore water acetate concentrations for three marsh zones together on the landscape scale (n = 27).

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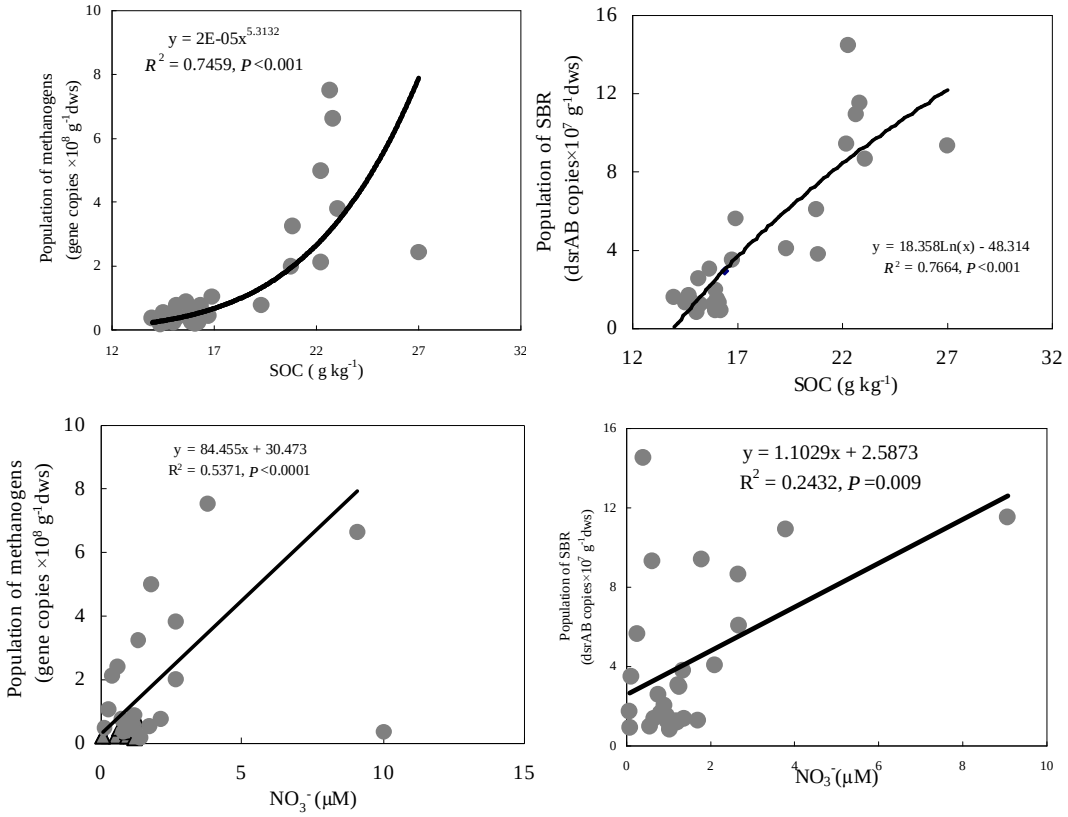

Fig. 6. Correlation between populations of methanogens and SBR and soil organic carbon (SOC) and pore water NO_3^- for three marsh zones together on the landscape scale (n = 27).

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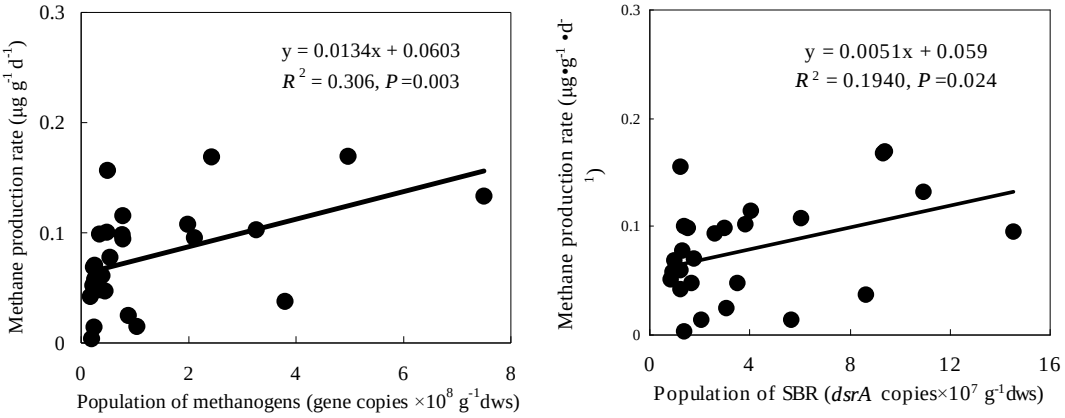

Fig. 7. Correlation between soil methane production rate and population of methanogens and SRB in three marsh zones on the landscape scale (n = 27).

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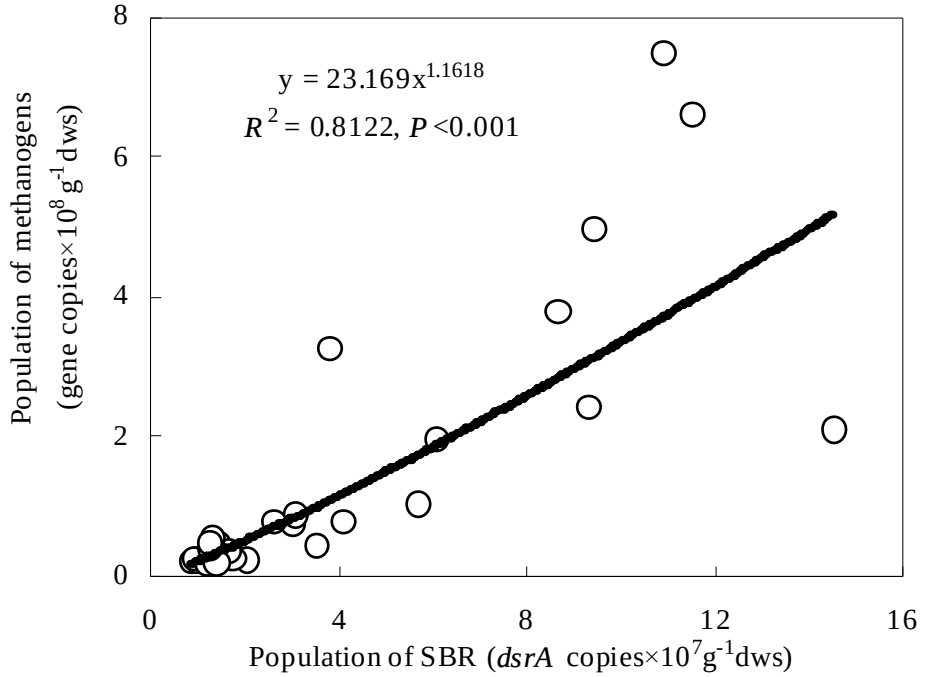

Fig. 8. Correlation between populations of methanogens and SRB in three marsh zones.

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