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# Aquaculture drastically increases methane production by favoring acetoclastic rather than hydrogenotrophic methanogenesis in shrimp pond sediments

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## ABSTRACT

Emissions of methane (CH<sub>4</sub>), a major greenhouse gas, should be cut by at least 30% by 2030 according to the last Conference of the Parties, CoP26. Aquaculture pond is a major CH<sub>4</sub> emitter, yet the microbial mechanisms ruling methanogenesis by degradation of organic matter in sediments remain unclear. In particular, the respective roles of hydrogenotrophic and acetoclastic methanogenesis, and the impact of aquaculture farming practices are unknown. We studied methanogenesis in the surface sediments from a freshwater and an oligohaline pond before, during, and after shrimp farming. Hydrogenotrophic and acetoclastic contributions were distinguished by acetoclastic inhibition with methylfluoride (CH<sub>3</sub>F), and by <sup>13</sup>C-analysis of CO<sub>2</sub> and CH<sub>4</sub>. We also monitored the methanogenic community structure, dissolved organic carbon (DOC) levels, carbon to nitrogen (C/N) ratios, and humification indices derived from Fourier transform infrared spectroscopy. The results reveal that aquaculture farming practices increased methanogenesis rates, and these increases were explained by higher levels of DOC and lower C/N ratios during farming. Of the total methane produced, 51%–78% was by hydrogenotrophic methanogenesis. However, the total methane contribution from acetoclastic methanogenesis increased from approximately 22% before farming to approximately 45% during and after farming, with a decreasing isotope fractionation factor  $\alpha_c$  and an increasing relative abundance of *Methanosaeta* acetoclastic methanogen. All humification indices decreased during and after farming compared to before farming due to the input of polysaccharide-rich aquafeed. The close relationship between the humification indices and methanogenesis pathways indicates that the changes in sediment substrate quality drove the variation in the methanogenesis pathways. Increases in salinity decreased the methanogenesis rates but did not change the methanogenesis pathways. Overall, our findings reveal that aquaculture farming practices increase methanogenesis rates and favor acetoclastic over hydrogenotrophic methanogenesis, and that adjusting shrimp diets, increasing salinity, and removing residual aquafeed could reduce methanogenesis.

## 1. Introduction

Methane, a major greenhouse gas, has a sustained global warming

potential of 45 times higher than CO<sub>2</sub> over 100 years (Neubauer and Megonigal, 2015). Methane concentrations increased by approximately 156% from 1750 to 2019, with levels of CH<sub>4</sub> reaching a value of 1866.3

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$\pm 3.3$  ppb in 2019 (IPCC, 2021). Emissions of CH<sub>4</sub> should be cut by at least 30% by 2030 according to the last Conference of the Parties, CoP26 (European Union, 2021). Aquatic ecosystems (including lakes, reservoirs, and rivers) contributed 32%–58% of earth's total natural CH<sub>4</sub> emissions (Saunio et al., 2020). Small but numerous aquaculture ponds are essential components of aquatic ecosystems (Naylor et al., 2021). Coastal aquaculture ponds account for 50% of the global aquaculture ponds and are expected to increase 1.3-fold from 2010 to 2050 (Waite et al., 2014). Although coastal aquaculture ponds have been recently recognized as major anthropogenic sources of CH<sub>4</sub> (Cui et al., 2016; Song and Liu, 2015; Yang et al., 2018; Yang et al., 2020; Yuan et al., 2019), the underlying mechanisms of methanogenesis are unclear.

Methane is produced at the terminal step of organic matter degradation (Conrad et al., 2010b). Organic matter is fermented to acetate, H<sub>2</sub>, and CO<sub>2</sub>, which are converted to CH<sub>4</sub> by methanogenic archaea (Conrad et al., 2010b; Xiao et al., 2020a). Acetoclastic and hydrogenotrophic are the two main pathways of methanogenesis in low-salinity aquatic systems (Conrad, 2005):

- Acetoclastic methanogenesis: CH<sub>3</sub>COOH → CH<sub>4</sub> + CO<sub>2</sub>.
- Hydrogenotrophic methanogenesis: 4H<sub>2</sub> + CO<sub>2</sub> → CH<sub>4</sub> + H<sub>2</sub>O.

Indeed, methanogens have preference for either H<sub>2</sub> or acetate substrates (Conrad et al., 2007; Etminan et al., 2016). For instance, *Methanoregula*, *Methanobacterium*, *Methanospirillum*, *Methanolinea*, and *Methanogenium* prefer H<sub>2</sub>, whereas *Methanosarcina* prefer acetate (Liu and Whitman, 2008; Sakai et al., 2008). Some methanogens, for example, *Methanosarcina*, are mixotrophs, which can use both H<sub>2</sub> and acetate substrates (Penger et al., 2012). Nevertheless, two strategies allow us to experimentally distinguish the relative importance of two pathways: the addition of methylfluoride (CH<sub>3</sub>F) to inhibit acetoclastic methanogenesis, and measuring <sup>13</sup>C isotope ratios of CO<sub>2</sub> and CH<sub>4</sub> (Conrad et al., 2020; Xiao et al., 2020b). The isotope fractionation is valid because hydrogenotrophic methanogens produces lighter methane with the δ<sup>13</sup>CH<sub>4</sub> ranging from −110‰ to −60‰, whereas acetoclastic methanogenesis produces heavier methane with the δ<sup>13</sup>CH<sub>4</sub> ranging from −70‰ to −30‰ (Conrad, 2005; Hodgkins et al., 2014).

Methanogenesis is theoretically governed by stoichiometric equivalence, i.e., 67% of the CH<sub>4</sub> is produced by acetoclastic methanogenesis, whereas 33% comes from the reduction of CO<sub>2</sub> with H<sub>2</sub> (Conrad, 1999). Nonetheless, in sediments of lakes (Mandic-Mulec et al., 2012), rivers (Mach et al., 2015; Qin et al., 2020), coves (King et al., 1983), paddies (Ji et al., 2018), peatlands (Hines et al., 2008; Kotsyurbenko et al., 2004; Krohn et al., 2017), and wetlands (Li et al., 2018; Xiao et al., 2019), the contribution of acetoclastic methanogenesis to the total methanogenesis varied from 0.5% to 99%. The contribution of each methanogenesis pathway depends on the pH (Ye et al., 2012), temperature (Chen and Chang, 2020), and more importantly, organic matter quantity and quality of the sediments (Corbett et al., 2015; Liu et al., 2012; Hofmann et al., 2016).

Farming practices could significantly alter methanogenesis rates in aquaculture pond systems. Indeed, large temporal variations in CH<sub>4</sub> emission flux across farming periods have been reported in various aquaculture pond systems (Ma et al., 2018; Song and Liu, 2015; Tong et al., 2021; Yang et al., 2019). Yet, little is known about how farming practices affect methanogenesis pathways in aquaculture pond systems. Nevertheless, several studies have reported that farming practices can modify the organic matter quality and quantity of sediments (Ali et al., 2021; Mulat et al., 2016; Pusceddu et al., 2011; Sabu et al., 2022; Wu and Song, 2021). For instance, in grass carp (*Ctenopharyngodon idella*) aquaculture ponds, carp feeding increased sediment dissolved organic carbon (DOC) concentrations by 2–5 times (Chen et al., 2015). Furthermore, in a *Labeo victorinus* pond, the total nitrogen contents from the pre-farming to post-farming periods increased from 1.58 to 1.84 mg g<sup>−1</sup> (Magoudu et al., 2013). Additionally, in a shrimp (*Litopenaeus stylirostris*) pond in New Caledonia, from the initial stage to the last stage of shrimp farming, the protein content increased from 3.8 to 5.9

mg g<sup>−1</sup>, while the lipid content increased from 1.2 to 3.0 mg g<sup>−1</sup> (Pusceddu et al., 2011). Such changes in the substrate quality and quantity of the sediments could affect the accumulation of H<sub>2</sub>, CO<sub>2</sub>, and acetate and thus, change the methanogenesis pathway (Berberich et al., 2020; Holmes et al., 2015). Consequently, it is reasonable to expect that methanogenesis rates and pathways differ across aquaculture stages, although there is currently a paucity of actual field data to demonstrate this hypothesis.

Salinity is another important environmental driver affecting the rates of methanogenesis. In numerous coastal wetland studies, methanogenesis was shown to decrease with salinity (Luo et al., 2019a; Neubauer et al., 2013; Chambers et al., 2013), and similar results were also reported in aquaculture pond systems. For instance, a prior study suggested that the CH<sub>4</sub> emission fluxes in the ponds with a salinity of 1.9‰–3.4‰ were approximately 10 times higher than those of the ponds with a salinity of 6.1‰–15.1‰ (Yang et al., 2018). In algae-shellfish mariculture ponds, net CH<sub>4</sub> exchange was also found to decrease with increasing salinity (Zhang et al., 2022). Furthermore, studies have reported that methanogenic community composition varies significantly with changing salinity since different methanogen taxa have different salinity tolerances (Chen et al., 2020; Liu et al., 2016). For instance, *Methanomethylivorans* only can live in sediments with salinity below 1.7‰ (Bräuer et al., 2011), while *Methanomassiliococcus* and *Methanospirillum* can tolerate salinities of 10‰–15‰ (Dridi et al., 2012). A study in the lake sediments of the Tibetan Plateau has even suggested that the distribution patterns of hydrogenotrophic and acetotrophic methanogens are related to changes in salinity (Liu et al., 2016). Therefore, variation in salinity between ponds may affect methanogenesis pathways in coastal aquaculture pond systems. A more comprehensive understanding of the responses of methanogenesis pathways to salinity may help us to better understand methanogenesis mechanisms in aquaculture systems.

To bridge these knowledge gaps, we investigated methanogenesis before, during, and after farming, in a freshwater and an oligohaline pond in the Min River Estuary, East China Sea. To isolate the effects of aquaculture farming practices and salinity, the selected ponds were similar in terms of size, sediment texture, bulk density, and adopted the consistent aquaculture management practices for all three aquaculture farming stages (Table 1 and Fig. 1). We monitored the methanogenesis and distinguished the contribution of acetoclastic versus hydrogenotrophic methanogenesis by CH<sub>3</sub>F addition, measuring δ<sup>13</sup>CO<sub>2</sub> and δ<sup>13</sup>CH<sub>4</sub>, and investigating the methanogenic community structure. The specific goal of this study was to: (i) clarify how aquaculture farming practices affect rates and pathways of methanogenesis across three aquaculture farming stages; and (ii) identify whether methanogenesis metabolism differs between freshwater and oligohaline ponds. We hypothesized that (i) aquaculture farming practices increase the rates of methanogenesis and contribute to acetoclastic methanogenesis because of the introduction of polysaccharide-rich aquafeeds; (ii) oligohaline ponds have lower methanogenesis rates than freshwater ponds because of the salt stress.

**Table 1**

Location, pond size, sediment texture, bulk density, and maximum water depth (means  $\pm$  standard deviation, n = 3 biological replicates) of the freshwater and oligohaline ponds in the Min River Estuary, southeast China.

	Ponds	
	Freshwater	Oligohaline
Latitude	26°03'03"N	26°01'32"N
Longitude	119°33'33"E	119°38'04"E
Pond size (hm <sup>2</sup> )	1.644	1.615
Sand (%)	15.2 $\pm$ 1.7	15.2 $\pm$ 2.8
Slit (%)	67.9 $\pm$ 2.2	66.9 $\pm$ 2.8
Clay (%)	16.9 $\pm$ 1.3	17.3 $\pm$ 0.9
Bulk density (g cm <sup>−3</sup> )	1.11 $\pm$ 0.23	1.20 $\pm$ 0.25
Maximum water depth (m)	1.51 $\pm$ 0.20	1.58 $\pm$ 0.17

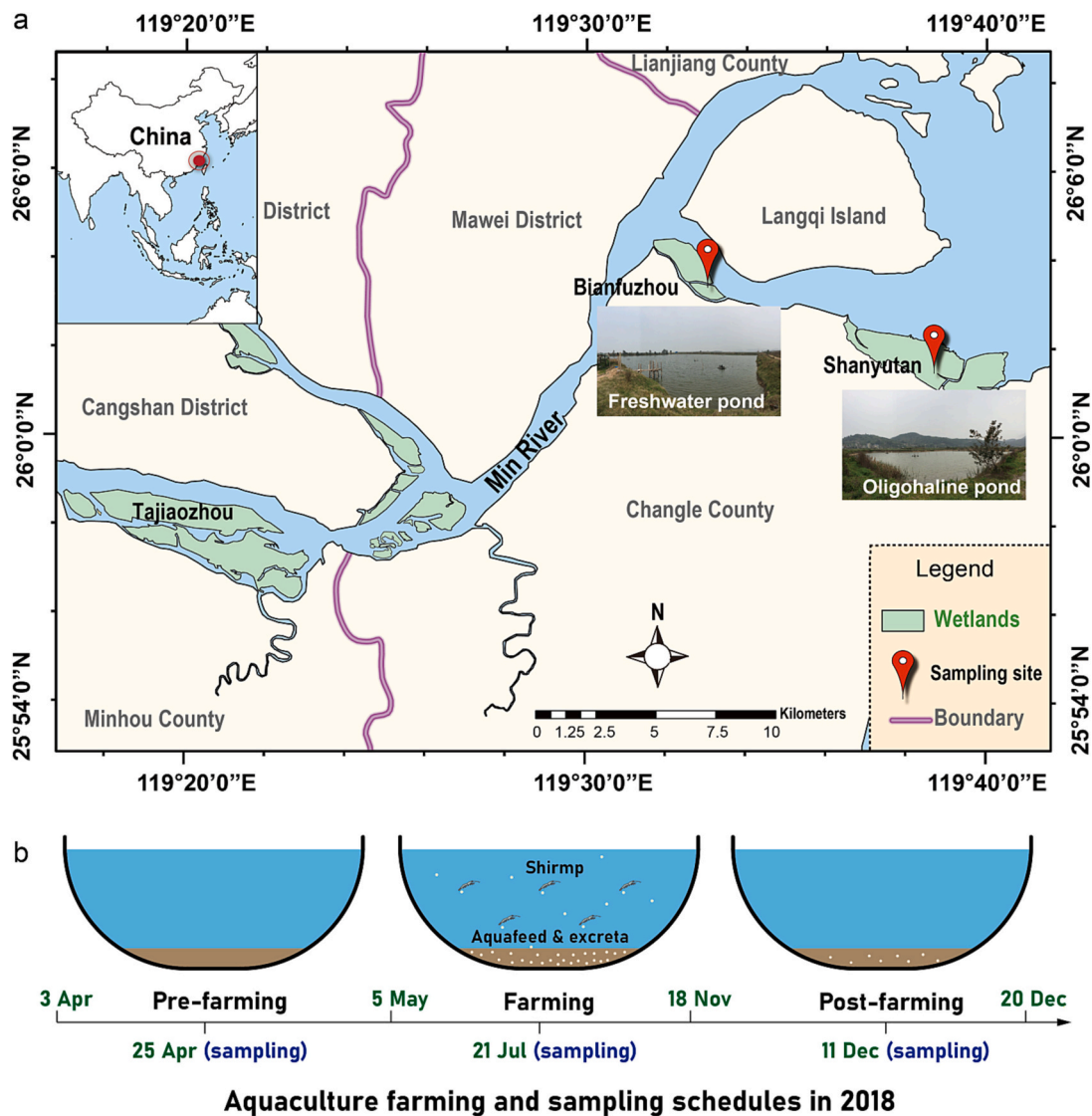


Fig. 1. (a) Location of the freshwater and the oligohaline ponds in the Min River Estuary, southeast China. (b) Aquaculture farming and sampling schedules across the aquaculture farming stages in 2018.

## 2. Materials and methods

### 2.1. Location and sampling strategy

The study was conducted in monoculture ponds of white shrimp, *Litopenaeus vannamei*, in the Min River Estuary, southeast China (26°00'N–26°05'N, 119°30'E–119°41'E). White shrimp accounted for 53% of total global crustacean production in 2016, and is one of the most important commercial species in the local coastal aquaculture industry (FAO, 2018). The climate in the study area is subtropical marine monsoon with an annual average temperature of 19.6 °C and an annual average precipitation of 1350 mm ([www.weather.com.cn](http://www.weather.com.cn)). We selected a freshwater pond, with salinity of 0.3‰–0.4‰, and an oligohaline pond, with salinity of 3.3‰–3.8‰, along the Min River Estuary (Fig. 1a). *L. vannamei* is a euryhaline shrimp species that could grow well in both ponds. The ponds were similar in size, maximum water depth, sediment texture, and bulk density (Table 1). Both ponds were converted from *Cyperus malaccensis* wetlands 7–9 years ago.

Aquaculture farming management was consistent for the two ponds during the experimental period from April to December 2018 (Fig. 1b). Specifically, during the pre-farming stage (April 3–May 4, 2018), the residual sludge from the previous harvest was removed mechanically

and water from nearby estuaries was introduced into the ponds. There was no water exchange during the farming and post-farming periods. Also, the maximum water depth of the two ponds ranged from  $1.51 \pm 0.20$  m to  $1.58 \pm 0.17$  m (Table 1). During the farming stage (May 5–November 17, 2018), the shrimp larvae were released into the ponds on May 5. The aquafeed was commercial aquatic feed pellets (including crude protein 31.1%, crude fat 6.8%, moisture 8.7%, ash 5.3%, and fiber 1.1%) and was added to the ponds twice a day. The feeding rates were 10–15, 40–50, and 30–40 kg ha<sup>-1</sup> d<sup>-1</sup> during the growth periods of the larvae, juveniles, and adults, respectively. On average, a total of ~3.0 t of aquafeed was added to each pond during the farming period. Then, the shrimps were harvested from November 12 to November 17, 2018. During the post-farming stage (November 18–December 20, 2018), the aquaculture wastewater remained in the ponds, and no more artificial disturbance occurred.

### 2.2. Sediment and porewater collection

In 2018, sediment sampling was conducted at the pre-farming stage (April 25), farming stage (July 21), and post-farming stage (December 11) in both shrimp ponds (Fig. 1b). During sediment sampling, PVC tubes (length: 10 cm; inner diameter: 10 cm) were randomly inserted

into the pond sediments. Each pond had three biological replicates within one stage. The sampling depth was 0–5 cm as this layer was the most impacted by aquaculture farming practices (Suárez-Abelenda et al., 2014; Tho et al., 2011). Sediments were bubbled using nitrogen ( $N_2$ ) and closed with silicone gaskets on both ends before being stored on ice and being transferred to the laboratory. Sediment samples were pooled, homogenized, and sieved (2 mm mesh size) in an anaerobic glove bag from AtmosBag, Sigma-Aldrich. Sediment subsamples for the molecular genetic analyses were immediately snap frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$ . The porewater samples for determining sulfate ( $SO_4^{2-}$ ), DOC, ammonia ( $NH_4^+$ ), and nitrate ( $NO_3^-$ ) concentrations were extracted by centrifuging the sediment subsamples for 6 min at 4000 rpm and were filtered through 0.45- $\mu\text{m}$  pore size membrane filters. Sediment and porewater subsamples were stored at  $4^\circ\text{C}$  and the analyses were completed within 7 days.

### 2.3. Sediment and porewater properties analyses

Sediment total organic carbon (TOC) and total nitrogen (TN) were measured using a CN-elemental analyzer with detection limits of 0.02  $\text{mg g}^{-1}$  (Vario MAX CN, Germany). Prior to performing the TOC measurements, sediments were leached with 10% HCl to remove inorganic carbon, washed with distilled water at pH 7, then dried at room temperature (Hou et al., 2013). The C/N ratio refers to the TOC versus the TN. Sediment temperature was measured by using a Thermo Fisher Scientific Temp 10 Series thermometer, with a detection accuracy of  $0.1^\circ\text{C}$ . Sediment pH was measured using a Thermo Fisher Scientific pH 450 pH meter, with a detection accuracy of 0.1. Porewater salinity was measured using a salinity meter (SALT 6+, Eutech instruments, Thermo Fisher Scientific), with a detection accuracy of 0.1‰. The DOC concentrations were analyzed using a TOC-VCPH analyzer from Shimadzu, with a detection limit of  $2\ \mu\text{mol L}^{-1}$ . Porewater  $SO_4^{2-}$  concentrations were analyzed in an ICS-2000 Dionex ion chromatograph (Thermo Fisher Scientific) with a detection limit of  $2\ \mu\text{mol L}^{-1}$ . Porewater  $NH_4^+$  and  $NO_3^-$  concentrations were analyzed by the sodium hypobromite and copper-cadmium reduction method, respectively (Zhang et al., 2014). Porewater  $NH_4^+$  and  $NO_3^-$  concentrations were determined using a San++ continuous flow analyzer (Skalar Analytical) with detection limits of  $2\ \mu\text{mol L}^{-1}$  and  $1\ \mu\text{mol L}^{-1}$ , respectively.

### 2.4. Fourier-transform infrared spectroscopy

The relative abundance of the different sediment C compound classes in the sediment powders were determined by attenuated total reflectance Fourier-transform infrared spectroscopy (ATR-FTIR) using a Nicolet iS10 instrument (Thermo Fisher Scientific). The spectra were acquired by averaging 32 scans at  $4\text{-cm}^{-1}$  resolution over 800–3500  $\text{cm}^{-1}$ . The spectra were corrected for the ATR to allow for differences in the depth of the beam penetration at different wavelengths, and then baseline was corrected using the instrument software. The humification indices were calculated using the ratio of the absorbance of aliphatics, aromatic esters, lignin and aromatics with the polysaccharides following the methodology described by Beer et al. (2008).

### 2.5. Incubation experiments

The incubation procedure followed the methods of Conrad et al. (2007). Approximately 20 g sediment subsamples were slurried and deoxygenated with in-situ filtered pondwater (1:2, w/w) in a nitrogen atmosphere, transferred into 175 mL sterile serum bottles, flushed with  $N_2$ , closed with butyl rubber stoppers, and then incubated at room temperature (20–23  $^\circ\text{C}$ ) in the dark. Methyl fluoride ( $CH_3F$ ) was added to the headspace of half of the bottles at 2% (vol/vol) of the bottle volume to inhibit acetoclastic methanogenesis. Gas samples were collected at 1–7 days intervals, depending on the  $CO_2$  and  $CH_4$  production rates. Before sampling, the bottles were vigorously shaken, and

the headspace pressure was measured using a pressure gauge (SSI technologies). Then, 0.5 mL of the headspace gas was injected into 20-mL airtight gas sampling bags that were pre-evacuated and filled with 10 mL helium gas, for the  $CO_2$  and  $CH_4$  analysis. After the gas was sampled, the bottles were backfilled with 0.5-mL  $N_2$  to re-establish normal atmospheric pressure. The concentrations of the  $CO_2$  and  $CH_4$  were determined using a GC-2010 Shimadzu gas chromatography equipped with a packed Porapak Q column and a flame ionization detector. Prior to the chromatography, the  $CO_2$  was converted to  $CH_4$  by reduction with  $H_2$  in a nickel reactor at  $350^\circ\text{C}$ . The  $CO_2$  and  $CH_4$  detection limits were 10 ppm and 0.3 ppm, respectively.

### 2.6. Isotope analysis

After 60 days of incubation, 15 mL of the headspace gas was sampled then overpressure injected into 12 mL pre-evacuated exetainer sample vials for the  $\delta^{13}\text{C}$  isotope analysis. The  $\delta^{13}\text{C}$  isotope analysis was performed using a GasBench-PreCon trace gas system coupled to a Delta V Plus IRMS from Thermo Scientific. Gas samples (0.5 mL) were used to determine the isotopic composition of the released  $CO_2$ . Analyses of the  $\delta^{13}\text{C}_{CH_4}$  used a Precon-Gas Bench-Isotope Ratio Mass Spectrometer (Precon-GB-IRMS) with gas samples (10 mL) injected into the Precon with a helium carrier gas. The  $^{13}\text{C}/^{12}\text{C}$  isotope ratios were expressed as  $\delta^{13}\text{C}$  relative to the international standards of Vienna Pee Dee Belemnite (V-PDB), where  $\delta^{13}\text{C} = (R_{\text{sample}} / R_{\text{standard}} - 1) \times 1000$ , and  $R_{\text{sample}}$  and  $R_{\text{standard}}$  are the  $^{13}\text{C}/^{12}\text{C}$  ratios in the sample and the PDB standard, respectively. Since the  $\delta^{13}\text{C}_{CH_4}$  also depends on  $\delta^{13}\text{C}_{CO_2}$ , the isotope fractionation factor,  $\alpha_c$ :  $(\delta^{13}\text{C}_{CO_2} + 10^3) / (\delta^{13}\text{C}_{CH_4} + 10^3)$  is calculated to distinguish the contributions of  $CH_4$  and  $CO_2$  (Whiticar, 1999).

### 2.7. Genetic analysis

DNA was extracted from 0.25 g of each sediment sample using the MOBIO Laboratories PowerSoil DNA Isolation Kit. Real-time quantitative polymerase chain reaction (qPCR) assays were used to assess the abundance of methanogenic groups using the specific primers mlas-mod-F (5'-GGYGGTGTMGDDTTACACMARTA-3') and mcrA-rev-R (5'-CGTTCATBGCCTAGTTVG GRTAGT-3') (Steinberg and Regan, 2008). The qPCR assays of the standards and samples were performed using the Thermo Fisher Scientific Applied Biosystems 7500 Real-Time PCR System. The reaction efficiencies for mcrA gene groups were 91.3%, with an  $r^2$  above 0.99. The results were reported as the number of gene copies per gram of dry sediment.

High-throughput amplicon sequencing was performed using an Miseq PE300 sequencing platform (Illumina Inc., CA, USA). For amplicon sequencing, the mcrA gene fragments were amplified using the primer pair mlas-mod-F and mcrA-rev-R (Steinberg and Regan, 2008). The qualified reads were separated using the sample-specific barcode sequences and trimmed using Illumina Analysis Pipeline version 2.6. The datasets were analyzed using QIIME1.8.0 (<http://qiime.org/>; Caporaso et al., 2012). A total of 470,017 high-quality Illumina sequence reads were obtained for the mcrA genes. Rarefaction analysis was used to rarefy the operational taxonomic unit (OTU) tables to count to 26,444 reads for mcrA gene sequences. This was the lowest sequencing depth obtained from a sample, and was therefore used as the threshold for rarefaction calculations (Gotelli and Colwell, 2001). The sequences were assigned to OTUs by referencing with a subset of the SILVA 128 database for mcrA genes.

### 2.8. Data analysis

All analyses were conducted using R software (version 4.0.3; R Core Team, 2013). We applied modified Gompertz models to fit the  $CO_2$  and  $CH_4$  accumulation curves (Zwietering et al., 1990):

$$M_t = M_0 \cdot \exp \left\{ - \exp \left[ \frac{R_{\max} \cdot e}{M_0} (\lambda - t) + 1 \right] \right\} \quad (1)$$

where  $M_t$  ( $\mu\text{g g}^{-1}$ ) denotes the cumulative methanogenesis per gram of dry sediment;  $M_0$  ( $\mu\text{g g}^{-1}$ ) denotes the maximum methanogenesis per gram of dry sediment by the end of incubation;  $t$  (d) denotes the incubation time;  $R_{\max}$  ( $\mu\text{g g}^{-1} \text{d}^{-1}$ ) denotes the maximum methanogenesis rates per gram of dry sediment;  $\lambda$  (d) denotes the time lag. The fitting results were determined by  $r^2$  with a significant level of  $p < 0.05$ . Model fitting used the 'nls' function. The rates of  $\text{CO}_2$  production,  $\text{CH}_4$  production, and hydrogenotrophic methanogenesis were equal to the  $R_{\max}$  that was derived from the accumulation curves of  $\text{CO}_2$ ,  $\text{CH}_4$ , and  $\text{CH}_4$  with an inhibitor, respectively. The rates of acetoclastic methanogenesis were calculated using the difference between  $\text{CH}_4$  production rates and hydrogenotrophic methanogenesis rates.

The contribution of the  $\text{CH}_4$  production rates to the total sum of the production rates of  $\text{CH}_4$  and  $\text{CO}_2$  (i.e.,  $f_{\text{CH}_4}$ ) was calculated as follows:

$$f_{\text{CH}_4} = \text{CH}_4 \text{ production rates} / (\text{CH}_4 + \text{CO}_2) \text{ production rates} \quad (2)$$

The contribution of acetoclastic methanogenesis to the total  $\text{CH}_4$  production rates (i.e.,  $f_{\text{acetoclastic}}$ ) was calculated as follows:

$$f_{\text{acetoclastic}} = \text{Acetoclastic methanogenesis rates} / \text{CH}_4 \text{ production rates} \quad (3)$$

Two-way analysis of variance (ANOVA) was used to explore the primary and interactive effects of farming practices and the salinity on the sediment and porewater properties,  $\text{CH}_4$  and  $\text{CO}_2$  production rates, hydrogenotrophic and acetoclastic methanogenesis rates,  $f_{\text{CH}_4}$ ,  $f_{\text{acetoclastic}}$ , and the abundance of *mcrA* genes at a significance level of  $p < 0.05$ . Prior to performing ANOVA, all datasets were analyzed to identify whether they met the assumptions of homogeneity (the Brown and Forsythe test) and normality (the Shapiro-Wilk test) and data was  $\log_{10}$ - or box-cox transformed if the transformation substantially improved the distribution. The ANOVA was calculated using the 'aov' function. Multiple comparisons across the three aquaculture farming stages were calculated using one-way ANOVAs followed by a Tukey's HSD post hoc test. Pairwise comparisons between the freshwater and oligohaline ponds were performed using the paired-sample *t*-tests. Pearson correlations between the methanogenesis rates and environmental variables were calculated using the 'cor' function with significance at  $p < 0.05$ . Linear regression models were used to examine the relationships between the humification indices and the  $f_{\text{acetoclastic}}$  using the 'lm' function

with significance at  $p < 0.05$ . The beta diversity of the methanogenic community composition was visualized using Nonmetric Multi-dimensional scaling analyses (NMDS) with a weighted Bray-Curtis distance. Dissimilarity tests were performed by two-way PerMANOVA (permutational multivariate analysis of variance) based on the Bray-Curtis distance. The NMDS and PerMANOVA were calculated using the 'vegan' package.

A hypothesized structural equation model was built (Fig. S2) to access the impact of aquaculture farming practices and salinity on rates and pathways of methanogenesis using 'lavaan' package (Rosseel, 2012). After the initial model was evaluated, we went through a process of adding/removing pathways and variables. The goodness of fit was assessed based on a chi-squared ( $\chi^2$ ) test, the Comparative Fit Index, and the Akaike Information Criterion values.

### 3. Results

#### 3.1. Sediment and porewater properties

Both the freshwater and oligohaline ponds possessed a higher sediment temperature (of approximately 30 °C) in the farming stage compared with the pre-farming stage (approximately 24 °C) and the post-farming stage (approximately 18 °C) ( $p < 0.001$ ; Table 2). The sediment pH was 7.6–8.1 and did not significantly vary between the two ponds or with the farming stages ( $p > 0.05$ ; Table 2). The freshwater pond samples had lower porewater salinity and sulfate concentrations than the oligohaline pond samples ( $p < 0.001$ ; Table 2). The porewater salinity and sulfate concentrations were similar in all three farming stages in each pond ( $p > 0.05$ ; Table 2). The TOC contents (range: 21.1–24.5  $\text{mg g}^{-1}$ ) were similar between the ponds and aquaculture farming stages ( $p > 0.05$ ; Table 2). The C/N ratios (range: 8.8–11.3) and the DOC concentrations (range: 8.8–19.9  $\text{mmol L}^{-1}$ ) did not vary between the ponds ( $p > 0.05$ ; Table 2). However, lower C/N ratios and higher DOC concentrations occurred in the farming and post-farming stages compared with the pre-farming stage ( $p < 0.01$ ; Table 2). The porewater  $\text{NH}_4^+$  concentrations ranged from 44.7 to 201.9  $\mu\text{mol L}^{-1}$  (Table 2), while the porewater  $\text{NO}_3^-$  concentrations ranged from 1.0 to 2.8  $\mu\text{mol L}^{-1}$  (Table 2). The porewater  $\text{NH}_4^+$  and  $\text{NO}_3^-$  concentrations were higher in the farming and post-farming stages compared with the pre-farming stage ( $p < 0.001$ ; Table 2).

**Table 2**

Sediment and porewater properties (means  $\pm$  standard deviation,  $n = 3$  biological replicates) across three aquaculture farming stages of the freshwater and oligohaline shrimp ponds in the Min River Estuary, southeast China.

	Ponds	Stages			Factor	Two-way ANOVA		
		Pre-farming	Farming	Post-farming		Stage	Pond	Interaction
Sediment temperature (°C)	Freshwater	23.6 $\pm$ 0.2B	30.4 $\pm$ 0.3A	18.2 $\pm$ 0.2C	<b>F</b>	<b>4628.839</b>	4.598	3.322
	Oligohaline	24.2 $\pm$ 0.2B	30.5 $\pm$ 0.2A	18.3 $\pm$ 0.3C	<b>P</b>	<b>&lt;0.001</b>	>0.05	>0.05
pH	Freshwater	8.0 $\pm$ 0.3A	7.6 $\pm$ 0.1A	7.9 $\pm$ 0.2A	<b>F</b>	2.803	0.115	0.234
	Oligohaline	8.1 $\pm$ 0.4A	7.8 $\pm$ 0.2A	7.9 $\pm$ 0.2A	<b>P</b>	>0.05	>0.05	>0.05
Salinity (‰)	Freshwater	0.4 $\pm$ 0.1A**	0.3 $\pm$ 0.0A**	0.3 $\pm$ 0.0A**	<b>F</b>	1.696	<b>907.837</b>	2.130
	Oligohaline	3.3 $\pm$ 0.3A	3.5 $\pm$ 0.2A	3.8 $\pm$ 0.3A	<b>P</b>	0.225	<b>&lt;0.001</b>	0.162
$\text{SO}_4^{2-}$ ( $\text{mmol L}^{-1}$ )	Freshwater	0.04 $\pm$ 0.01A**	0.03 $\pm$ 0.00A*	0.03 $\pm$ 0.01A**	<b>F</b>	0.542	<b>336.03</b>	0.308
	Oligohaline	0.42 $\pm$ 0.05A	0.38 $\pm$ 0.06A	0.41 $\pm$ 0.06A	<b>P</b>	0.595	<b>&lt;0.001</b>	0.740
TOC ( $\text{mg g}^{-1}$ )	Freshwater	22.0 $\pm$ 2.0A	22.7 $\pm$ 1.5A	23.8 $\pm$ 2.6A	<b>F</b>	1.514	0.004	0.646
	Oligohaline	21.1 $\pm$ 2.5A	24.5 $\pm$ 3.0A	23.1 $\pm$ 1.8A	<b>P</b>	>0.05	>0.05	>0.05
C/N ratio	Freshwater	11.3 $\pm$ 1.2A	9.0 $\pm$ 0.4B	9.9 $\pm$ 0.4B	<b>F</b>	<b>11.129</b>	1.456	0.176
	Oligohaline	10.6 $\pm$ 1.0A	8.8 $\pm$ 0.9B	9.4 $\pm$ 0.5B	<b>P</b>	<b>0.002</b>	>0.05	>0.05
DOC ( $\text{mmol L}^{-1}$ )	Freshwater	8.8 $\pm$ 0.6C	17.8 $\pm$ 2.2A	14.0 $\pm$ 1.6B	<b>F</b>	<b>54.820</b>	4.016	0.192
	Oligohaline	9.7 $\pm$ 1.3C	19.9 $\pm$ 1.4A	15.6 $\pm$ 2.0B	<b>P</b>	<b>&lt;0.001</b>	>0.05	>0.05
$\text{NH}_4^+$ ( $\mu\text{mol L}^{-1}$ )	Freshwater	44.7 $\pm$ 9.2B	141.0 $\pm$ 16.5A	173.3 $\pm$ 12.6A	<b>F</b>	<b>174.876</b>	4.676	1.452
	Oligohaline	47.9 $\pm$ 10.9B	150.2 $\pm$ 17.4A	201.9 $\pm$ 12.2A	<b>P</b>	<b>&lt;0.001</b>	>0.05	>0.05
$\text{NO}_3^-$ ( $\mu\text{mol L}^{-1}$ )	Freshwater	1.0 $\pm$ 0.2C	1.5 $\pm$ 0.3B	2.6 $\pm$ 0.2A	<b>F</b>	<b>48.180</b>	2.597	0.050
	Oligohaline	1.2 $\pm$ 0.2C	1.8 $\pm$ 0.4B	2.8 $\pm$ 0.4A	<b>P</b>	<b>&lt;0.001</b>	>0.05	>0.05

Different uppercase letters indicate significant differences across all three farming stages.

Asterisks denote significant difference between the ponds within a single stage (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ).

Bold letters denote significant differences at  $p < 0.05$ .

### 3.2. Chemical composition of organic matter

The main compounds in the sediment organic matter in ponds are polysaccharides, lignin, and aromatics (aromatic esters and aliphatics) (Fig. S1). All samples possessed similar compounds, but we observed changes in their relative abundances across three farming stages. The humification indices decreased from 0.04 to 0.13 before shrimp farming commenced to 0.01–0.05 during farming and 0.02–0.07 after farming ( $p < 0.001$ ; Table 3). The humification indices were not significantly different between the freshwater and oligohaline ponds ( $p > 0.05$ ; Table 3).

### 3.3. Production of CH<sub>4</sub> and CO<sub>2</sub> during sediment incubation

Incubating the pond sediments induced a rapid increase in the cumulative CH<sub>4</sub> and CO<sub>2</sub> concentrations during the first 16 days, then plateaued until day 60 (Fig. 2). The addition of CH<sub>3</sub>F (an inhibitor of acetoclastic methanogenesis) clearly inhibited the CH<sub>4</sub> production in both the freshwater and oligohaline sediments, while the CO<sub>2</sub> production was not significantly modified (Fig. 2). The CH<sub>4</sub> and CO<sub>2</sub> production curves fitted the modified Gompertz models with  $r^2$  coefficients above 0.85 ( $p < 0.001$ ) (Fig. 2 and Table S1).

Methanogenesis rates in the sediments ranged from 0.11 to 0.54  $\mu\text{g g}^{-1} \text{d}^{-1}$ , and the CO<sub>2</sub> production rates ranged from 6.2 to 29.5  $\mu\text{g g}^{-1} \text{d}^{-1}$ . Across the three aquaculture farming stages, the CH<sub>4</sub> and CO<sub>2</sub> production rates had higher levels in the farming and post-farming stages, when compared with the pre-farming stage ( $p < 0.001$ ; Fig. 3a and b). The  $f_{\text{CH}_4}$  was comparable in all three aquaculture farming stages ( $p > 0.05$ ; Fig. 3c). The freshwater pond had relatively higher CH<sub>4</sub> production rates but lower CO<sub>2</sub> production rates compared with the oligohaline pond ( $p < 0.01$ ; Fig. 3a and b), resulting in higher values of  $f_{\text{CH}_4}$  in the freshwater pond than the oligohaline pond ( $p < 0.001$ ; Fig. 3c).

The hydrogenotrophic methanogenesis rates ranged from 0.08 to 0.29  $\mu\text{g g}^{-1} \text{d}^{-1}$ , and the acetoclastic methanogenesis rates ranged from 0.02 to 0.26  $\mu\text{g g}^{-1} \text{d}^{-1}$  (Fig. 3d and e). Both the hydrogenotrophic and acetoclastic methanogenesis rates were higher in the farming and post-farming stages, compared with the rates in the pre-farming stage ( $p < 0.01$ ; Fig. 3d and e). Moreover, the  $f_{\text{acetoclastic}}$  were higher at the farming stages and post-farming stages than the pre-farming stages ( $p < 0.001$ ; Fig. 3f). Both the hydrogenotrophic and acetoclastic methanogenesis rates were higher in the freshwater pond than the oligohaline pond ( $p < 0.05$ ; Fig. 3d and e). However, the  $f_{\text{acetoclastic}}$  did not vary between the freshwater and oligohaline ponds ( $p > 0.05$ ; Fig. 3f).

### 3.4. Isotope analysis of CH<sub>4</sub> and CO<sub>2</sub>

In the pond sediments, the  $\delta^{13}\text{C}_{\text{CH}_4}$  ranged from  $-87\text{‰}$  to  $-72\text{‰}$ ,

whereas the  $\delta^{13}\text{C}_{\text{CO}_2}$  ranged from  $-21\text{‰}$  to  $-18\text{‰}$  (Fig. 4a and b). The  $\delta^{13}\text{C}_{\text{CH}_4}$  and  $\delta^{13}\text{C}_{\text{CO}_2}$  were not significantly different between the oligohaline and freshwater ponds ( $p > 0.05$ ; Fig. 4a and b). The  $\delta^{13}\text{C}_{\text{CH}_4}$  were lower prior to farming, compared with the farming and post-farming stages ( $p < 0.001$ ; Fig. 4a). The isotopic fractionation factors ( $\alpha_c$ ) were lower in the farming and post-farming stages (1.055–1.062), compared with pre-farming stage ( $p < 0.001$ ; 1.070–1.074) (Fig. 4c).

### 3.5. Methanogenic community abundance and composition

The abundance of *mcrA* gene in the sediments ranged from  $9.92 \times 10^6$  to  $12.03 \times 10^6$  copies  $\text{g}^{-1}$  and did not vary significantly between the farming stages and salinity levels ( $p > 0.05$ ; Fig. 5a). The methanogenic community clearly varied in the pre-farming, farming, and post-farming stages, according to the NMDS and two-way PerMANOVA analyses (Fig. 5b). The major methanogenic genera were *Methanoregula* (~21%), *Methanoseta* (~16%), *Methanobacterium* (~8%), *Methanospirillum* (~6%), *Methanosarcina* (~6%), *Methanolinea* (~3%), and *Methanococcoides* (~2%), *Methanogenium* (~2%), *Methanosphaera* (~2%), and *Methanocella* (~1%) (Fig. 5c). Other methanogenic genera accounted for less than ~33% of the *mcrA* gene. The relative abundance of *Methanoseta* was much higher during and after farming than before farming ( $p < 0.05$ ; Fig. 5c).

### 3.6. Relationships between the methanogenesis, organic substrate, humification indices, and methanogenic community composition

The methanogenesis rates increased with DOC concentrations ( $r = 0.77$ ;  $p < 0.001$ ;  $n = 18$ ), decreased with the C/N ratios ( $r = -0.69$ ;  $p < 0.01$ ;  $n = 18$ ). The *mcrA* gene abundance were not correlated with the methanogenesis rates ( $p > 0.05$ ;  $n = 18$ ). Changes in the humification indices were significantly associated with the  $f_{\text{acetoclastic}}$  ( $p < 0.001$ ; Figs. 6a to 6d).

The structural equation model suggested that aquaculture farming practices were related to the changes in the methanogenic community composition, while the latter were significantly linked to changes in the methanogenesis pathways (Fig. 7). Aquaculture farming practices also contributed to the changes in the methanogenesis rates (Fig. 7). Salinity was not related to the changes in the methanogenic community composition, whereas negatively related to changes in methanogenesis rates (Fig. 7).

## 4. Discussion

We analyzed the microbial production of methane and the underlying mechanisms in the sediments of a freshwater and an oligohaline pond before, during, and after shrimp farming. We focused on the effect of farming practices on methanogenesis and the respective contribution

**Table 3**

Humification indices derived from Fourier-transform infrared spectroscopy (FTIR; means  $\pm$  standard deviation,  $n = 3$  biological replicates) across three aquaculture farming stages of the freshwater and oligohaline shrimp ponds in the Min River Estuary, southeast China.

FTIR peaks used for humification index*	Ponds	Stages			Factor	Two-way ANOVA		
		Pre-farming	Farming	Post-farming		Stage	Pond	Interaction
1630/1000	Freshwater	0.11 $\pm$ 0.01A	0.04 $\pm$ 0.01B	0.07 $\pm$ 0.01B	<b>F</b>	<b>19.658</b>	0.044	0.436
	Oligohaline	0.11 $\pm$ 0.03A	0.05 $\pm$ 0.02B	0.06 $\pm$ 0.02B	<b>P</b>	<b>&lt;0.001</b>	>0.05	>0.05
1740/1000	Freshwater	0.04 $\pm$ 0.01A	0.01 $\pm$ 0.00B	0.02 $\pm$ 0.01B	<b>F</b>	<b>15.755</b>	0.226	0.204
	Oligohaline	0.05 $\pm$ 0.02A	0.01 $\pm$ 0.01B	0.02 $\pm$ 0.01B	<b>P</b>	<b>&lt;0.001</b>	>0.05	>0.05
2850/1000	Freshwater	0.10 $\pm$ 0.03A	0.02 $\pm$ 0.02B	0.04 $\pm$ 0.00B	<b>F</b>	<b>13.407</b>	0.005	0.652
	Oligohaline	0.08 $\pm$ 0.03A	0.03 $\pm$ 0.02B	0.05 $\pm$ 0.02B	<b>P</b>	<b>&lt;0.001</b>	>0.05	>0.05
2920/1000	Freshwater	0.13 $\pm$ 0.03A	0.02 $\pm$ 0.02B	0.06 $\pm$ 0.00B	<b>F</b>	<b>15.439</b>	0.107	0.356
	Oligohaline	0.12 $\pm$ 0.04A	0.04 $\pm$ 0.03B	0.06 $\pm$ 0.04B	<b>P</b>	<b>&lt;0.001</b>	>0.05	>0.05

\* FTIR peaks used to generate the humification indices: wavenumbers of lignin or aromatics ( $1630 \text{ cm}^{-1}$ ), aromatic esters ( $1740 \text{ cm}^{-1}$ ), and aliphatics ( $2920 \text{ cm}^{-1}$  and  $2850 \text{ cm}^{-1}$ ) with respect to polysaccharides ( $1000 \text{ cm}^{-1}$ ). References for the wavenumbers are Artz et al. (2008) and Parikh et al. (2014).

Different uppercase letters indicate significant differences across all three farming stages.

Bold letters denote significant differences at  $p < 0.05$ .

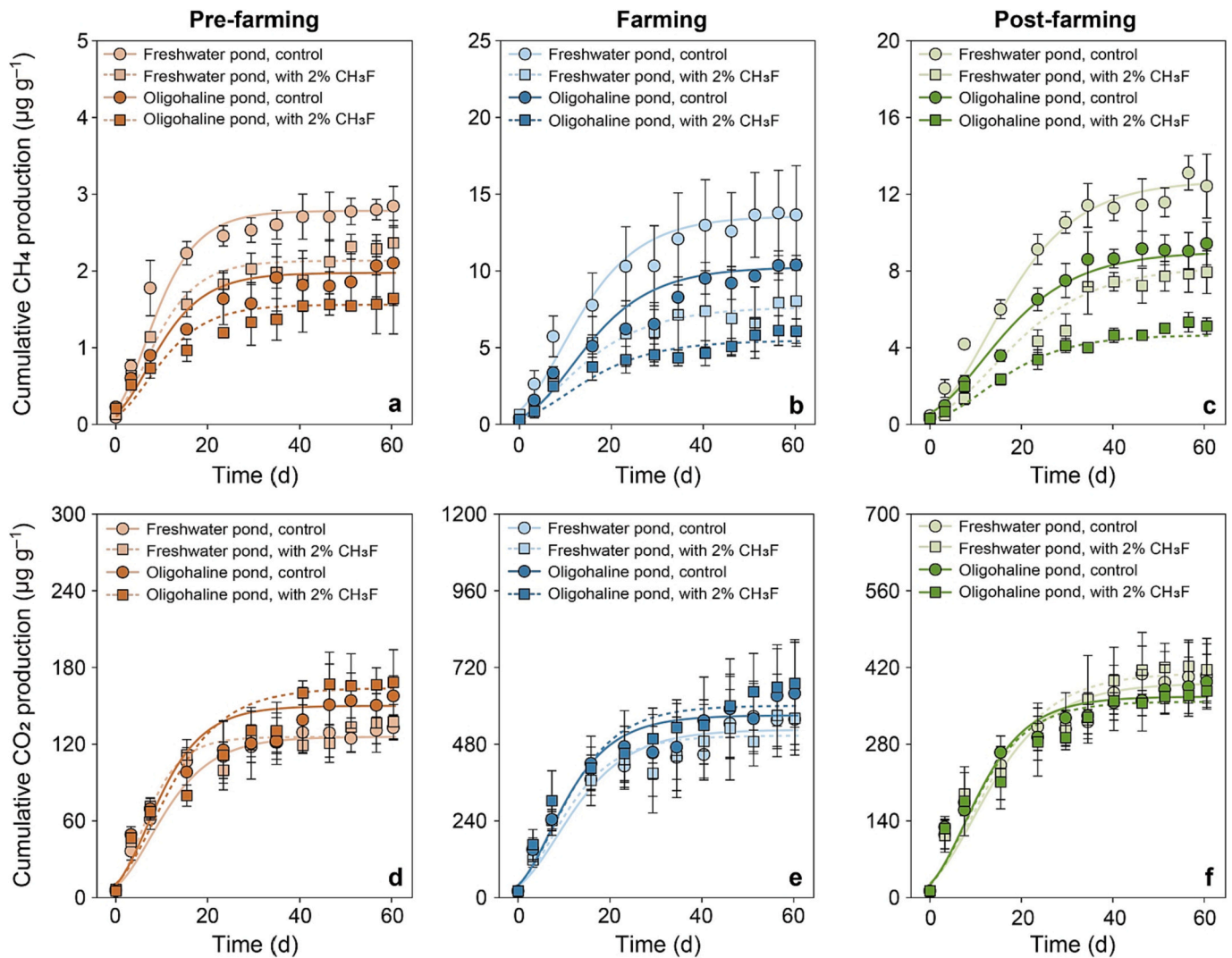


Fig. 2. Cumulative CH<sub>4</sub> and CO<sub>2</sub> production during incubation across three aquaculture farming stages in the sediments of a freshwater and an oligohaline shrimp pond. All data are mean  $\pm$  standard deviation ( $n = 3$  biological replicates). The lines represent the modified Gompertz model curves.

of acetoclastic and hydrogenotrophic methanogenesis.

#### 4.1. Effect of sediment organic substrate on methanogenesis

Shrimp aquaculture induced a sharp stimulation in the methanogenesis rates in the sediments at both the farming and post-farming stages compared to the pre-farming stage (Fig. 3a, supporting Hypothesis I). This finding is consistent with previous studies reporting that CH<sub>4</sub> emission fluxes raised during farming in aquaculture ponds (Ma et al., 2018; Song and Liu, 2015; Yang et al., 2018). The raised methanogenesis rates may be due to changes in the quantity and quality of the organic substrate.

The hypothesis that methanogenesis rates is affected by organic substrate quantity, is validated by our results showing that the DOC concentrations increased by an average of 82% during and after farming (Table 2), and were correlated with methanogenesis rates ( $r = 0.77$ ,  $p < 0.001$ ). The high aquafeed loads are typically hydrolyzed to DOC, which is a suitable substrate for methanogens (Olsen et al., 2014; Suhr et al., 2015). Moreover, the production of shrimp excreta is also likely to enrich the DOC in low molecular weight, easily assimilable organic compounds (Queiroz et al., 2019; Wang et al., 2021). Previous research reported similar correlations of methanogenesis rates and DOC concentrations in pristine ponds and paddies (Bertora et al., 2018;

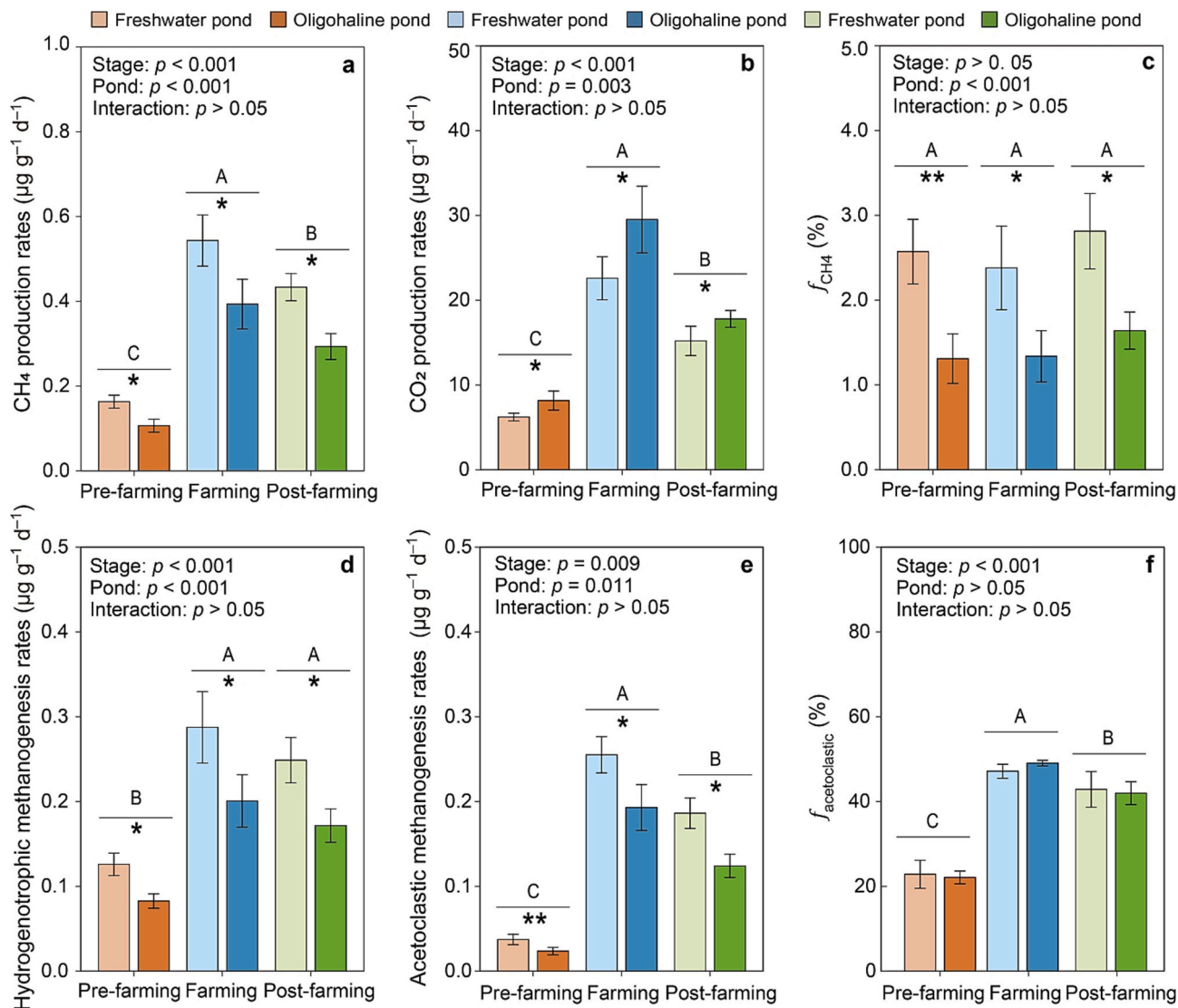
Holgerson, 2015).

The effect of the quality of the organic substrate on methanogenesis rates was demonstrated by a high C/N ratio before farming (10.6–11.3), which decreased during and after farming (8.9–9.6), suggesting an input of easily assimilable protein-like nutrients (Panigrahi et al., 2018). A C/N ratio higher than 10.0 is typical of recalcitrant compounds that are not available for benthic microbial metabolism (Baldi et al., 2010; Wei et al., 2022). Decreasing C/N ratios raise the N availability for methanogens and thus accelerate methanogenesis rates (Hou et al., 2022; Kim et al., 2015). Overall, aquaculture farming enhanced methanogenesis rates at the farming and post-farming stages relative to the pre-farming stages by changing the quantity and quality of the organic substrates for the methanogens.

#### 4.2. Contribution of acetoclastic and hydrogenotrophic methanogenesis

Acetoclastic and hydrogenotrophic methanogenesis coexisted in the sediments before, during, and after shrimp farming, with a predominance of the hydrogenotrophic pathway by 51%–78% (Figs. 3d to 3f). The rates of the acetoclastic pathway increased by approximately 5.3 times in the sediments at both the farming and post-farming stages compared to the pre-farming stage (Fig. 3e, supporting Hypothesis I). Besides, the contribution of the acetoclastic pathway increased from





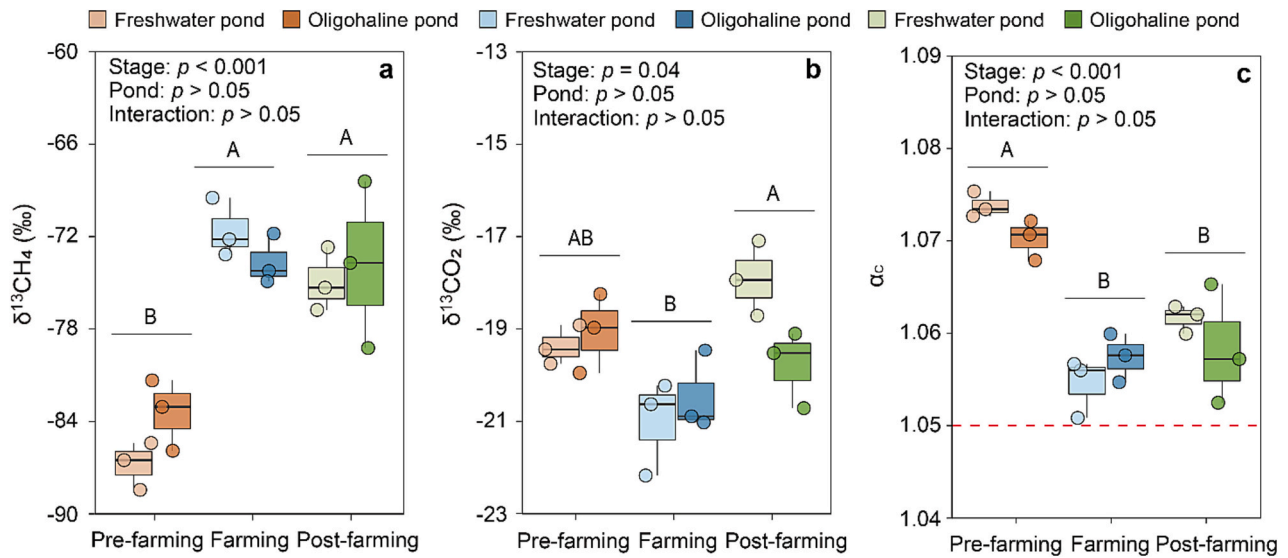
**Fig. 3.** CH<sub>4</sub> and CO<sub>2</sub> production rates (a and b),  $f_{\text{CH}_4}$  (c), hydrogenotrophic and acetoclastic methanogenesis rates (d and e), and  $f_{\text{acetoclastic}}$  (f) across three aquaculture farming stages in the sediments of a freshwater and an oligohaline shrimp pond. All data are the mean  $\pm$  standard deviation ( $n = 3$  biological replicates). Different uppercase letters indicate significant differences across the three aquaculture farming stages. Asterisks denote significant differences between the ponds within one stage (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ).  $f_{\text{CH}_4}$ : the contribution of CH<sub>4</sub> production rates to the sum production rates of CH<sub>4</sub> plus CO<sub>2</sub>.  $f_{\text{acetoclastic}}$ : the contribution of acetoclastic methanogenesis to the CH<sub>4</sub> production rates.

approximately 22% before farming to approximately 45% during and after farming (Fig. 3f). Therefore, aquaculture farming practices favor acetoclastic rather than hydrogenotrophic methanogenesis. The rise of acetoclastic methanogenesis was supported by a decrease in the isotopic fractionation  $\alpha_c$  from before farming (1.070–1.074) to during and after farming (1.055–1.062) (Fig. 4c). These values and their corresponding pathways agreed with previous literature (Holmes et al., 2014; Hornibrook et al., 2000). We also observed substantial changes in the composition of the methanogenic community during and after farming (Fig. 5b). In particular, the relative abundance of the acetoclastic methanogen, *Methanosaeta* increased from approximately 9% before farming to approximately 21% during and after farming, confirming the rise of acetoclastic methanogenesis (Fig. 5c).

The shift in the contributions of the different methanogenesis pathways are presumably due to changes in the chemical composition of sediment organic substrate. This is supported by the high association between methanogenesis pathways and the humification indices derived from FTIR (Figs. 6a to 6d). Before farming, the sediments contain relatively stable and recalcitrant organic matter (such as lignin, aromatics,

and aliphatics), which cause high humification indices (Table 3). The presence of lignin, aromatics, and aliphatics in sediments agrees with the historical conversion of these vegetated tidal wetlands to ponds as these wetlands are typically dominated by sedge species (Mitsch and Gosselink, 2015). When buried, the plant debris is partially decomposed, leaving lignin, aliphatics, and other complex compounds (Berberich et al., 2020; Gleixner, 2013; Lichtfouse, 1999; Martínez-García et al., 2018). These recalcitrant compounds can partly be oxidized to produce H<sub>2</sub> (Conrad et al., 2010a; Keller et al., 2004), thus favoring hydrogenotrophic methanogenesis.

During and after farming, the amounts of aquafeed were added to the ponds. Indeed, 30% of aquafeed is not captured by shrimps, and settles in the sediment (Soto, 2021). The aquafeed was mainly composed of peanut and bean dregs, which are rich in polysaccharides (Dawood et al., 2018). Consequently, the humification indices decreased during and after farming due to the high relative abundance of polysaccharides (Table 3). Rawoof et al. (2020) stated that polysaccharide hydrolysis produces glucose, fructose, and xylose, which are further biodegraded into acetate, thus stimulating acetoclastic methanogens. The stimulation



**Fig. 4.**  $\delta^{13}\text{CH}_4$  and  $\delta^{13}\text{CO}_2$  values (mean  $\pm$  standard deviation;  $n = 3$  biological replicates) of the sediment at the completion of a 60 d-incubation across three aquaculture farming stages of a freshwater and an oligohaline shrimp pond. Different uppercase letters indicate significant differences across the three aquaculture farming stages. Asterisks denote significant difference between ponds within a single stage (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ). The red line in panel c provides the separation between the relative importance of hydrogenotrophic (above) and acetoclastic (below) methanogenesis (Whiticar, 1999). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of acetoclastic methanogenesis by labile C has also been suggested in peat (Keller and Bridgman, 2007). Our previous study also showed acetoclastic methanogenesis was feasible when there were high concentrations of polysaccharides in anaerobic soils (Xiao et al., 2019). Overall, aquaculture farming favored acetoclastic over hydrogenotrophic methanogenesis, because of the input of polysaccharide-rich aquafeeds.

#### 4.3. Effect of salinity on methanogenesis

The methanogenesis rates were lower in the oligohaline pond than the freshwater pond (Fig. 3a). Salinity is known to control the methanogenic community by increasing ionic toxicity and altering the osmotic potential (Luo et al., 2019; Tully et al., 2019). Nonetheless, the abundance of the *mcrA* gene was not significantly different between the oligohaline and freshwater ponds (Fig. 5a). The results are probably due to the major methanogenic genera, e.g., *Methanoregula*, *Methanosaeta*, *Methanobacterium*, *Methanospirillum*, and *Methanosarcina* are all frequently encountered methanogenic taxa in estuaries where salinity is high (ranging from 5‰ to 20‰) (Chen et al., 2019; Liu et al., 2016; Tong et al., 2017). Therefore, salt stress is probably not the cause of the lower methanogenesis rates in the oligohaline pond relative to the freshwater pond (against Hypothesis II).

A more probable explanation is the sulfate reduction in the oligohaline pond yields a stronger Gibbs free energy compared to methanogenesis, and therefore inhibits methanogen activities (Herbert et al., 2015). This assumption is supported by the sulfate concentrations, these are approximately 10-times higher in the oligohaline pond compared to the freshwater pond (Table 2). Moreover, sulfate-reducing bacteria can oxidize  $\text{CH}_4$  to  $\text{CO}_2$  under anaerobic conditions (Rotaru and Thamdrup, 2016; Wilms et al., 2007), which could also explain the lower methanogenesis rates in the oligohaline pond.

While both hydrogenotrophic and acetoclastic pathways displayed lower methanogenesis rates in the oligohaline when compared to the freshwater pond (Fig. 3d), their respective contributions were similar (Fig. 3f). Similarly, the isotopic fractionation factor  $\alpha_c$  and the methanogenic community composition did not differ between the two ponds (Figs. 4c and 5b). These findings suggest salinity did not alter the partitioning of the methanogenesis pathways. A previous study suggested

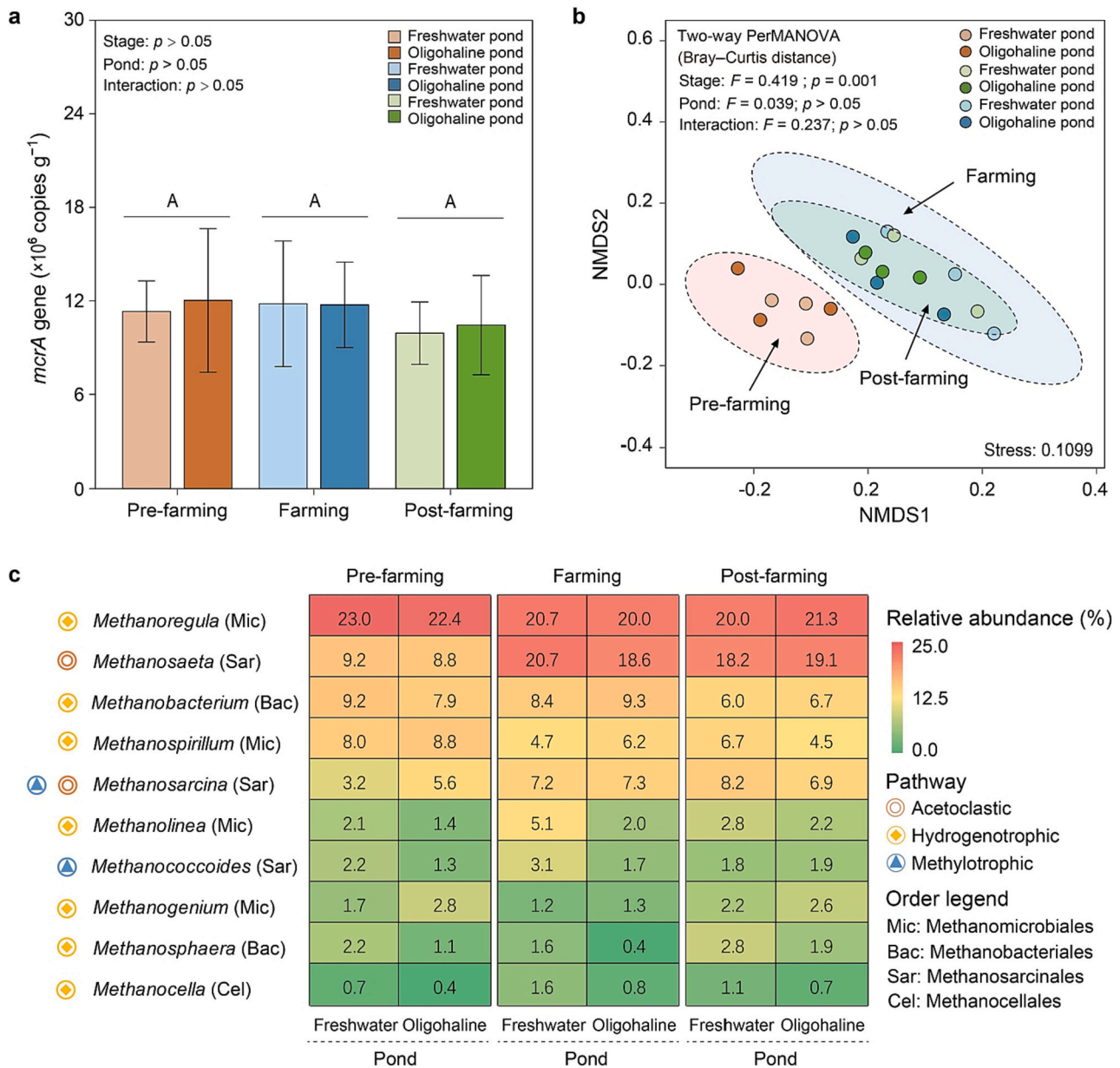
that salinity may change methanogenesis pathways due to the different relative salt-tolerances of the hydrogenotrophic and acetoclastic methanogens (Liu et al., 2016). However, the dominated hydrogenotrophic and acetoclastic methanogens were both tolerant to low-salinity as discussed before. Therefore, salinity differences between the ponds did not lead to different partitioning of methanogenesis pathways in the current study.

#### 4.4. Implication for how aquaculture management can reduce methane emission in the future

First, we revealed the rise of acetoclastic versus hydrogenotrophic methanogenesis in shrimp farming. This rise is explained by the input of aquafeeds increasing the proportions of labile, microbially assimilable organic compounds, such as polysaccharides. Using this knowledge, we suggest changing shrimp diets, similarly to cow farming to better control and possibly reduce methane emissions (Hassanat et al., 2013; Marette and Millet, 2014). For instance, the addition of oilseeds (camelina and safflower) may not influence the growth of aquaculture products; however, these lipid-rich feeds have a toxic effect on the activities of archaea, which would further reduce methanogen abundance (Wang et al., 2017).

Second, our results reported that the lower rates of methanogenesis in the oligohaline pond are because of the inhibition of sulfate reduction and oxidation of  $\text{CH}_4$  to  $\text{CO}_2$  by sulfate in anaerobic conditions. Based on this conclusion, we suggested that freshwater shrimp aquaculture ponds would benefit from moderate salt levels. The addition of moderate salt (salinity = 2.5‰) into ponds had been applied in carp freshwater aquaculture systems in India, which was found to stimulate the growth of the fish and greatly suppress  $\text{CH}_4$  emissions (Naskar et al., 2020). *L. vannamei* is a euryhaline shrimp species, which could grow well in ponds with salinity of 0‰ to 30‰ (Jannathulla et al., 2017). Therefore, the addition of salt in the freshwater ponds would not influence the growth of the shrimps; however, it would greatly decrease the  $\text{CH}_4$  emissions.

Third, we have found that methanogenesis maintained high-levels even after farming. This was because the amounts of aquafeed residues deposited on the pond sediments. Therefore, an effective way to reduce methanogenesis in aquaculture ponds was to clean aquafeed



**Fig. 5.** Graphical representations of the methanogenic community structure variations in three aquaculture farming stages of a freshwater and an oligohaline shrimp pond. (a) The abundance of *mcrA* gene (mean  $\pm$  standard deviation,  $n = 3$  biological replicates). (b) The nonmetric multidimensional scaling (NMDS) plot of the methanogenic community composition ( $n = 3$  biological replicates). and (c) A heatmap demonstrating the relative abundance of methanogen genera. Different uppercase letters in panel a indicate significant differences across the three aquaculture farming stages.

residues (Chik et al., 2017). Once the farming activities are completed, the pond water should be drained and the aquafeed residues should be removed immediately to reduce the potential of methanogenesis.

## 5. Conclusions

This study reveals shrimp farming increases the acetoclastic methanogenesis when compared to hydrogenotrophic methanogenesis in a pond. This is due to the input of polysaccharide-rich aquafeed and shrimp excreta increasing the levels of fresh organic matter in the pond sediments. The methanogenesis rate is lower in the oligohaline pond than freshwater pond. Salinity did not change the relative composition of the methanogenic community or modify the respective contribution of the acetoclastic and hydrogenotrophic methanogenesis. In the future, methanogenesis in shrimp ponds could be reduced by changing shrimp diets, increasing salinity, and removing residual aquafeeds.

## Author contributions

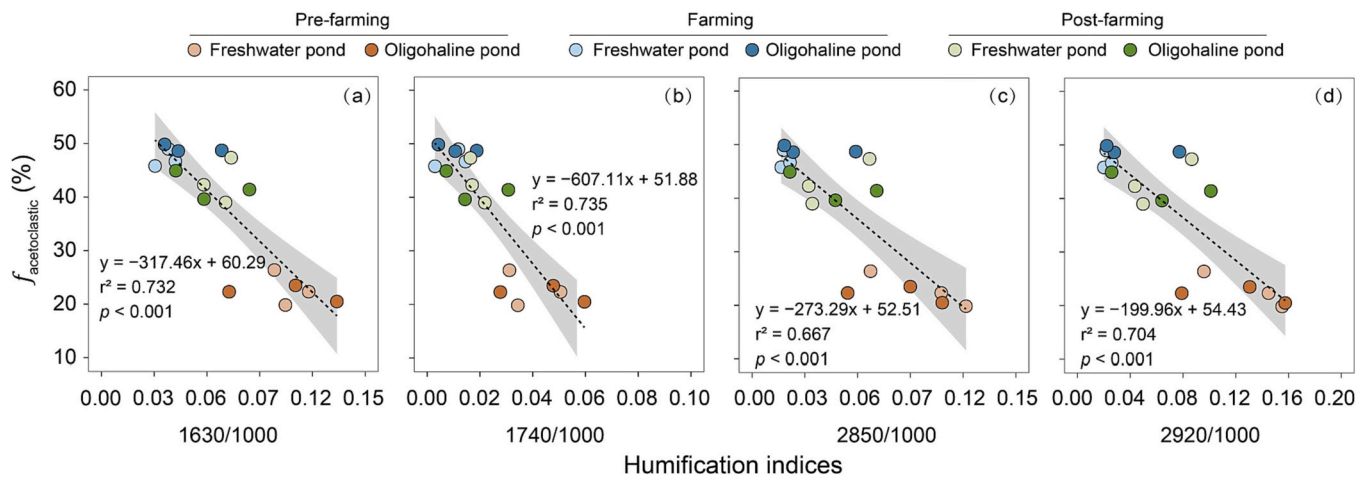
Luo and Xiao designed the experiment. Luo and Tan co-wrote the manuscript with substantial contributions from Lichtfouse. Tan, Zhang, Chen, Liu and Huang did the scientific analysis. All authors conducted the research and analyzed data. All authors edited the manuscript.

## Data availability statement

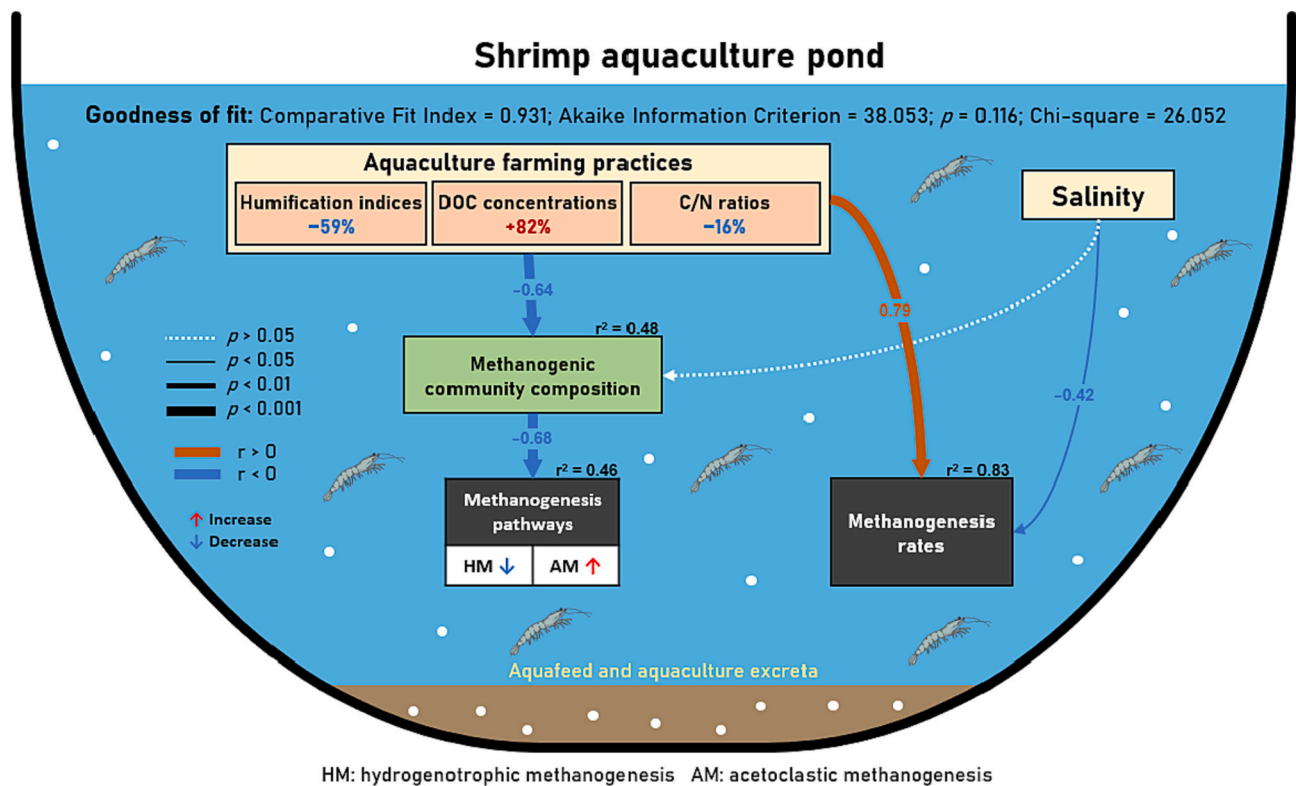
Data deposited in the figshare, <https://figshare.com/s/a081915a95654a76efd0>. Raw *mcrA* gene sequence reads for each sample are also available on NCBI Short Read Archive (SRA) under the BioProject number PRJNA532292.

## CRedit authorship contribution statement

**Ji Tan:** Investigation, Writing – original draft. **Eric Lichtfouse:**



**Fig. 6.** Relationships between humification indices and the  $f_{\text{acetoclastic}}$ . Humification indices are derived from Fourier-transform infrared spectroscopy.  $f_{\text{acetoclastic}}$ : the contribution of acetoclastic methanogenesis to the  $\text{CH}_4$  production rates.



**Fig. 7.** A structural equation modelling accessing the impact of aquaculture farming practices and salinity on rates and pathways of methanogenesis. Numbers on the arrows indicate the coefficients and the arrow width indicates their significance. Figures next to the variables indicate their explained variance ( $r^2$ ). DOC: dissolved organic carbon; HM: hydrogenotrophic methanogenesis; AM: acetoclastic methanogenesis.

Writing – review & editing. **Min Luo**: Conceptualization, Methodology, Writing – review & editing, Supervision, Project administration. **Feng-feng Tan**: Investigation, Formal analysis. **Changwei Zhang**: Investigation, Formal analysis. **Jiafang Huang**: Investigation, Formal analysis. **Leilei Xiao**: Conceptualization, Methodology.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

#### Data availability

Data deposited in the figshare, <https://figshare.com/s/a081915a95654a76efd0>. Raw mcrA gene sequence reads are also available on NCBI Short Read Archive (SRA) under the BioProject number PRJNA532292.

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## Appendix A. Supplementary data

Supplementary data to this article can be found below and online at <https://doi.org/10.1016/j.aquaculture.2022.738999>.

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## Aquaculture drastically increases methane production by favoring acetoclastic rather than hydrogenotrophic methanogenesis in shrimp pond sediments

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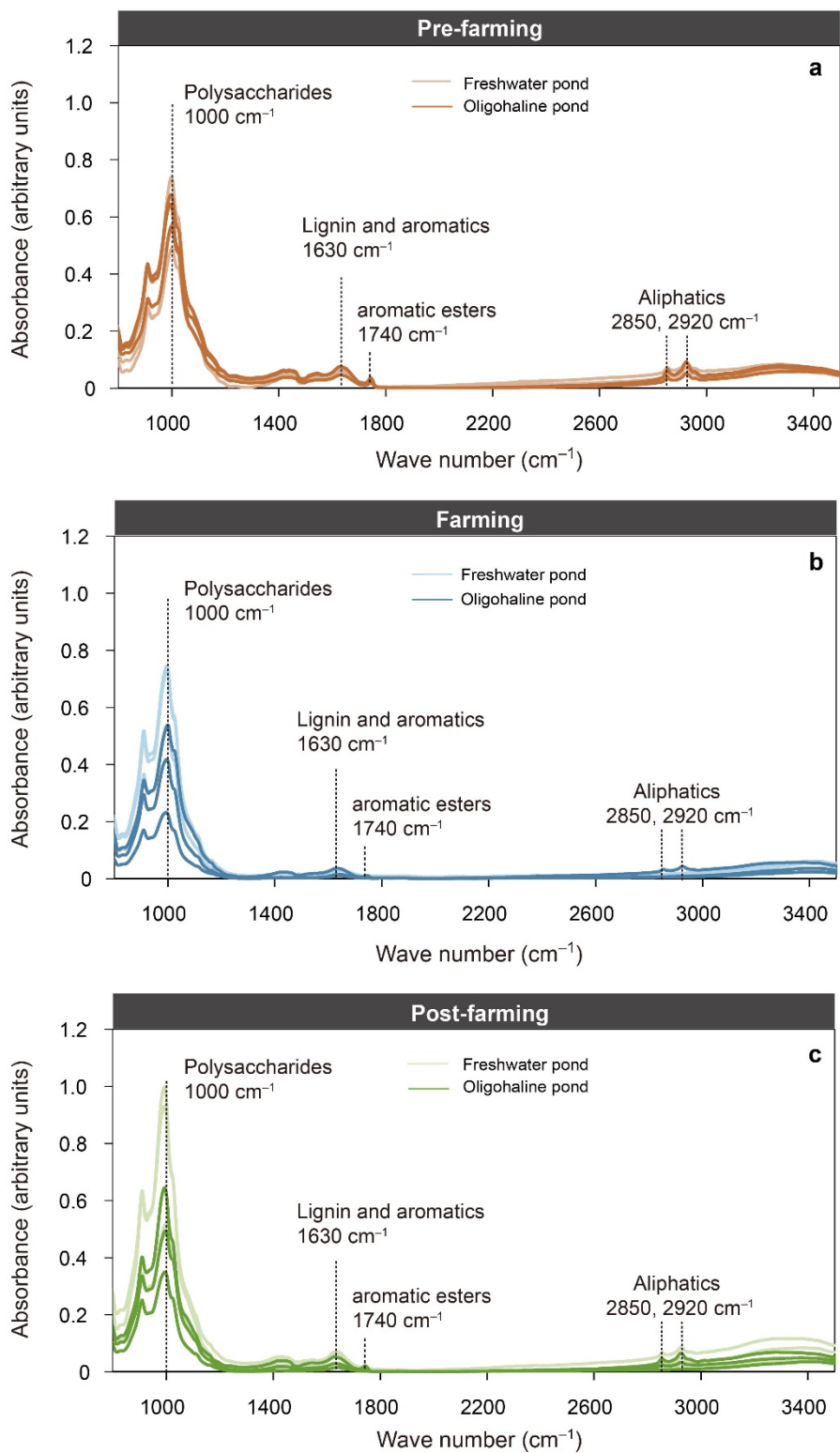
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**Table S1** CO<sub>2</sub> and CH<sub>4</sub> production parameters (means ± standard deviation; n = 3) based on the modified Gompertz model (Zwietering et al., 1990) of the freshwater and oligohaline ponds in the Min River Estuary, southeast China.

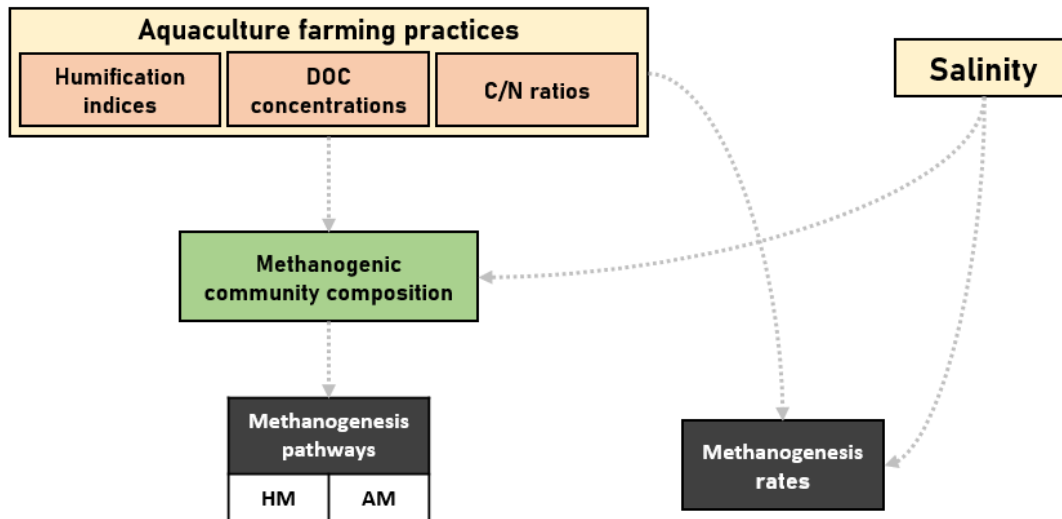
Stages	Ponds	Incubation	M(t) ( $\mu\text{g}\cdot\text{g}^{-1}$ )	$\lambda$ (d)	R <sub>max</sub> ( $\mu\text{g}\cdot\text{g}^{-1}\cdot\text{d}^{-1}$ )	RMSE ( $\mu\text{g}\cdot\text{g}^{-1}$ )	p	R <sup>2</sup>
<b>CH<sub>4</sub> production</b>								
Pre-farming	Freshwater	Control	2.78±0.12	0.00±0.00	0.16±0.02	0.23±0.02	<0.001	0.95
		With CH <sub>3</sub> F	2.13±0.01	0.00±0.00	0.13±0.01	0.22±0.03	<0.001	0.93
	Oligohaline	Control	1.98±0.31	0.04±0.05	0.11±0.01	0.23±0.05	<0.001	0.98
		With CH <sub>3</sub> F	1.56±0.29	1.68±0.80	0.08±0.01	0.24±0.07	<0.001	0.91
Farming	Freshwater	Control	13.58±3.10	0.04±0.06	0.54±0.06	1.07±0.07	<0.001	0.94
		With CH <sub>3</sub> F	7.66±3.07	0.01±0.06	0.29±0.04	0.71±0.22	<0.001	0.91
	Oligohaline	Control	10.26±0.35	1.68±0.80	0.39±0.06	1.06±0.13	<0.001	0.98
		With CH <sub>3</sub> F	5.47±0.82	0.20±0.34	0.39±0.03	0.58±0.17	<0.001	0.97
Post-farming	Freshwater	Control	12.71±1.83	0.07±0.07	0.44±0.03	0.74±0.13	<0.001	0.96
		With CH <sub>3</sub> F	8.22±0.60	0.00±0.00	0.25±0.03	1.08±0.33	<0.001	0.94
	Oligohaline	Control	9.02±1.09	1.83±1.62	0.30±0.03	0.66±0.49	<0.001	0.98
		With CH <sub>3</sub> F	4.66±0.78	1.63±1.55	0.17±0.02	0.72±0.21	<0.001	0.94
<b>CO<sub>2</sub> production</b>								
Pre-farming	Freshwater	Control	125.8±9.7	0.00±0.00	6.2±0.5	108.4±8.2	<0.001	0.85
		With CH <sub>3</sub> F	125.7±11.4	0.00±0.00	9.3±0.9	110.1±9.9	<0.001	0.89
	Oligohaline	Control	150.0±16.8	0.00±0.00	8.2±1.1	120.3±18.4	<0.001	0.87
		With CH <sub>3</sub> F	164.0±19.7	0.00±0.00	7.3±1.2	128.1±14.2	<0.001	0.86
Farming	Freshwater	Control	525.3±104.4	0.00±0.00	22.6±2.5	430.8±72.2	<0.001	0.91
		With CH <sub>3</sub> F	507.1±119.6	0.00±0.00	25.8±2.6	422.6±90.4	<0.001	0.92
	Oligohaline	Control	570.4±144.5	0.00±0.00	29.5±3.9	474.0±111.0	<0.001	0.92
		With CH <sub>3</sub> F	600.7±92.9	0.00±0.00	29.0±8.5	496.7±88.0	<0.001	0.90
Post-farming	Freshwater	Control	391.3±58.8	0.00±0.00	15.2±1.7	314.1±44.1	<0.001	0.87
		With CH <sub>3</sub> F	410.5±43.7	0.00±0.00	16.2±3.7	325.1±43.8	<0.001	0.92
	Oligohaline	Control	366.8±53.0	0.00±0.00	17.8±1.0	305.4±38.0	<0.001	0.98
		With CH <sub>3</sub> F	357.7±17.6	0.00±0.00	17.6±2.5	293.7±23.3	<0.001	0.95

M<sub>t</sub> ( $\mu\text{g}\cdot\text{g}^{-1}$ ): the cumulative methane production per gram of dry sediment; R<sub>max</sub> ( $\mu\text{g}\cdot\text{g}^{-1}\cdot\text{d}^{-1}$ ): the maximum methane production rates per gram of dry sediment;  $\lambda$  (d): time lag; RMSE ( $\mu\text{g}\cdot\text{g}^{-1}$ ): root mean square error.





**Figure S1** Fourier transform infrared (FTIR) spectroscopy within the region of 800 to 3500 cm<sup>-1</sup> of the sediments of the freshwater and oligohaline ponds in the Min River Estuary, southeast China. References for wavenumbers according to Artz et al. (2007) and Parikh et al. (2014).



**Figure S2** Hypothesized path model structure to evaluate the effects of aquaculture farming practices and salinity on rates and pathways of methanogenesis. DOC: dissolved organic carbon; HM: hydrogenotrophic methanogenesis; AM: acetoclastic methanogenesis.

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