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1. Title

pH-based ecological coherence of active canonical methanotrophs in paddy soils

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Abstract

Soil pH is considered one of the main determinants of the assembly of globally distributed microorganisms that catalyse the biogeochemical cycles of carbon and nitrogen. However, direct evidence for niche specialization of microorganisms in association with soil pH is still lacking. Using methane-oxidizing bacteria (methanotrophs) as a model system of carbon cycling, we show that pH is potentially the key driving force selecting for canonical gamma- (type I) and alpha- (type II) methanotrophs in rice paddy soils. DNA-based stable isotope probing (DNA-SIP) was combined with high-throughput sequencing to identify the taxonomic identities of active methanotrophs in physiochemically contrasting soils from 6 different paddy fields across China. Following microcosm amendment with $^{13}CH_4$, methane was primarily consumed by *Methylocystis*-affiliated type II methanotrophs in soils with a relatively low pH (5.44-6.10), whereas *Methylobacter*/*Methylosarcina*-affiliated type I methanotrophs dominated methane consumption in soils with a high pH (7.02-8.02). Consumption of $^{13}CH_4$ contributed 0.203% to 1.25% of soil organic carbon, but no significant difference was observed between high-pH and low-pH soils. The fertilization of ammonium nitrate resulted in no significant changes in the compositions of ^{13}C-labelled methanotrophs in the soils, although significant inhibition of methane oxidation activity was consistently observed in low-pH soils. Mantel analysis further validated soil pH, rather than other parameters tested, had significant correlation to the variation of active methanotrophic compositions across different rice paddy soils. These results suggest that soil pH might have played pivotal roles in mediating the niche differentiation of ecologically important aerobic methanotrophs in terrestrial ecosystems and imply the importance of such niche specialization in regulating methane emissions in paddy field under increasingly intensified input of anthropogenic N fertilizers.

Keywords: pH; Niche differentiation; Methanotrophs; Rice paddy; DNA stable isotope probing; High-throughput sequencing

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1 Introduction

Rice paddy fields are one of the major sources of the potent greenhouse gas methane, contributing to approximately 10-25% of global methane emission (Kögel-Knabner et al., 2010). Constantly produced through methanogenesis from the anaerobic compartment of inundated paddy fields, methane can diffuse into the oxic-anoxic interface of the soil and reach high concentrations of above 5 mM, which is approximately equivalent to 50,000 ppmv (Eller and Frenzel, 2001;Nouchi et al., 1990;Nouchi et al., 1994). It is estimated that up to 80% of methane gas is consumed by soil aerobic methanotrophs (i.e. methane-oxidizing bacteria, MOB) before being released into the atmosphere (Conrad and Rothfuss, 1991;Frenzel et al., 1992). Therefore, methanotrophs in rice paddy fields are considered a crucial biological filter attenuating potential methane emissions as well as an important contributor to the maintenance of soil organic carbon (C) because a substantial amount of CH_4-derived C was used for MOB grow and biomass synthesis (Bridgham et al., 2013).

Accumulating evidence has indicated that the specialized MOB clades designated as upland soil clusters α and γ (USCα and USCγ, respectively) have high CH_4 affinity and catalyse atmospheric methane oxidation in unsaturated soils (Holmes et al., 1999;Roslev and Iversen, 1999;Tveit et al., 2019;Knief et al., 2003), while the canonical *α*- and *γ*-*Proteobacterial* MOB (known as type II and type I methanotrophs) are considered to adapt better to high concentration methane and thus regulate the methane oxidation in ecosystems with constant methane productions, such as wetlands. Despite co-existence of type I and II methanotrophs in these ecosystems, their activity and contribution to methane oxidation vary largely depending on different environmental conditions and the predominant activity of either type I or type II methanotrophs has often been observed (Daebeler et al., 2014;Liebner and Wagner, 2007;He et al., 2012;Chen et al., 2008a;Lin et al., 2004). This might be due to some major physiological differences that exist between these two groups. For instance, type I methanotrophic strain is more competitive under relatively lower methane and higher oxygen concentrations compared to type II methanotrophs (Graham et al., 1993).

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Additionally, adaptation to slightly acidic pH values (growth optima 5.0 - 6.0) is characteristic for type IIb (*Methylocella* and *Methylocapsa*) and some *Methylocystis* strains (Dedysh et al., 2000;Dedysh et al., 2007;Belova et al., 2013). These and other physiological traits of type I and II methanotrophs may be important in partitioning their specialized niches in different ecosystems.

Increasing lines of evidence from ecological studies have suggested that soil pH, among other environmental variables, might be one of the most important determinant in the emergence and maintenance of microbial communities across a wide variety of environments (Tripathi et al., 2018;Lauber et al., 2009). Recent studies have provided compelling evidence for niche specialization of biogeochemically important guilds associated with pH variation and consequent distinct patterns of soil resource utilization. For example, the biogeographical distribution of ammonia-oxidizing oxidizers is more strongly associated with soil pH than other parameters tested in soils (Gubry-Rangin et al., 2011;Aigle et al., 2019), as is that of denitrifiers (Liu et al., 2010). It is implied that type I and II methanotrophs might also be selectively favoured under different pH conditions in natural wetland system, despite no systematic comparison has yet been made. Dominance of type II methanotrophs have been revealed in many natural acidic peatlands (Chen et al., 2008a;Dedysh, 2009;Kip et al., 2012;Chen et al., 2008b;Gupta et al., 2012), whereas in neutral-alkaline wetlands, type I methanotrophs appear to be more active (Lin et al., 2004;Gupta et al., 2012;Morris et al., 2002). The similar pattern, however, has not been obtained in anthropogenically flooded rice paddy fields, possibly due to lack of study on methanotrophic activity in such soils with a wide range of pH. In fact, type I methanotrophs were considered to dominantly catalyse methane at high concentration in the rice paddy soils (Ma et al., 2013;Qiu et al., 2008;Reim et al., 2012;Shrestha et al., 2008), while the activity of type II methanotrophs and their contribution to methane oxidation in rice soils remains unclear (Semrau et al., 2011;Leng et al., 2015). Since these findings all came from neutral-alkaline soils, it is necessary to investigate the active methanotrophs in more acidic soils, which likely have different community compositions (Shiau et al., 2018).

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In this study, we selected 6 rice paddy soils with a pH gradient ranging from 5.45 to 8.02 collected from 6 geographically different rice fields located in the main rice production areas across the south, east and middle of China, including Yu-Xi (YX), Ying-Tan (YT), Tao-Yuan (TY), Zi-Yang (ZY), Chang-Shu (CS) and Lei-Zhou (LZ) (Fig. S1) and used DNA-based stable isotope probing (DNA-SIP) to identify active methanotrophs under unfertilized situation and following a simulated fertilization. We predict distinct compositions of methanotrophic phylotypes of type I and type II in these soils which are associated with soil pH. Other environmental factors pertinent to methane oxidation were also tested to further elaborate the importance of soil pH in selection of active methanotrophic phylotypes in the rice paddies.

2 Material and methods

2.1. Site description and soil sampling

The soils were collected from 6 different rice fields located in the main rice production areas across the south, east and middle of China, including Yu-Xi (YX), Ying-Tan (YT), Tao-Yuan (TY), Zi-Yang (ZY), Chang-Shu (CS) and Lei-Zhou (LZ) (Fig. S1). All sites have a subtropical or tropical monsoon climate and a history of rice cultivation for >50 years. The fields usually receive annual fertilization of 250 to 350 kg N ha^{-1}. Soil sampling was performed at a 0-20-cm depth by mixing at least three randomly collected soil cores. The composite soil samples were air-dried as previously described (Noll et al., 2008;Mohanty et al., 2006;Murase and Frenzel, 2007a) and passed through a 2.0-mm-pore-size sieve before the construction of microcosms.

2.2. Soil physiochemical properties

The pH was assessed by a Mettler Toledo 320-S pH meter (Mettler Toledo Instruments, China) with a water-to-soil ratio of 2.5. Soil inorganic N (ammonium plus nitrate) was extracted from soil with a 2 M KCl solution and quantified using a Skalar San Plus segmented flow analyser (Skalar, The Netherlands). The soil organic matter (SOM) content was determined using the dichromate oxidation method. Total organic

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carbon (TOC) and total N (TN) were determined by a vario Max CN Element Analyzer (Elementar, Germany). The available soil copper content was determined using an OPTIMA 8000 inductively coupled plasma optical emission spectroscope (ICP-OES) (PerkinElmer, USA) after extraction with buffered 5 mM Diethylenetriaminepentaacetic acid (DTPA) solution. The soil oxidation capacity (OXC) was determined using the equation $5\times[NO_3^-]+2\times[Mn(IV)]+[Fe(III)]+8\times[SO_4^{2-}]$ as detailed previously (Zhang et al., 2009). The results of these properties were shown in Table 1.

2.3. Stable-isotope probing of methane-oxidizing bacteria

Three treatments were established in triplicate, including "Control" (under natural atmospheric condition), "$^{13}CH_4$" (incubated with 5% v/v $^{13}CH_4$ supplementation) and "$^{13}CH_4$+N" (incubated with 5% v/v $^{13}CH_4$ supplementation plus fertilization with NH_4NO_3). Soil equivalent to 6.0 g *d.w.s.* was incubated at a maximum water-holding capacity of approximately 60% and at 28°C in the dark in a 120-ml serum bottle sealed with a butyl stopper. For the "$^{13}CH_4$" and "$^{13}CH_4$+N" treatments, 6 ml of the headspace air in the bottles was replaced with the same volume of >99% pure $^{13}CH_4$ gas (Cambridge Isotope Laboratories, USA) to make an initial methane mixing ratio of approximately 5% in the headspace. For the "$^{13}CH_4$+N" treatment, NH_4NO_3 solution instead of distilled water was added to the soil microcosm in a dropwise manner to produce a supplement of 200 μg inorganic N g soil^{-1}. A 2-day pre-incubation was performed before applying ^{13}C-labelled methane and nitrogen fertilizer.

For the "$^{13}CH_4$" and "$^{13}CH_4$+N" treatments, the headspace methane mixing ratios were measured every two days by an Agilent 7890A Gas Chromatograph (Agilent Technologies, USA) to assess the rate of methane oxidation in SIP microcosms. Each microcosm incubation was completed when approximately 90% of the methane gas was consumed, i.e., the headspace methane concentration dropped to below 5,000 ppmv, or after 6 weeks if the headspace methane concentration was still higher than 5,000 ppmv. Soils were then collected and stored at -80°C for further analyses.

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2.4. Soil ^{13}C-atom abundance assay

The synthesis of biomass carbon derived from ^{13}C-CH_4 was assessed by determination of ^{13}C-atom abundance in soil organic matter. Approximately 1.5 g of each frozen soil sample was vacuum freeze-dried using an Alpha 1-2 LDplus freeze dryer (Christ, Germany). The relative ^{13}C-atom ratio was assessed by a Flash 2000 elemental analyser coupled to a Delta V Advantage isotope ratio mass spectrometer (Thermo Scientific, USA), and the TOC content was then measured by a vario Max CN Element Analyzer (Elementar) using the desiccated soil samples.

2.5. DNA extraction and SIP gradient fractionation

Total DNA was extracted using 0.5 g of each soil by a FastDNA spin kit for soil (MP Biomedicals, USA) according to the manufacturer's instructions. The quantity and quality of DNA extracts were assessed using a NanoDrop ND-1000 UV-visible light spectrophotometer (NanoDrop Technologies, USA).

The isopycnic density gradient centrifugation was employed to ^{13}C-DNA from ^{12}C-DNA in the total DNA extract. In brief, approximately 2.5 μg of the extracted DNA was mixed with a CsCl solution to achieve a final volume of 5.5 ml with a CsCl buoyant density of 1.725 g ml^{-1} following ultracentrifugation at 177,000 g and 20°C for 44 h in a Vti65.2 vertical rotor (Beckman Coulter, USA). The DNA fractions for each sample were collected and measured for CsCl density as previously described (Zhao et al., 2015;Wang et al., 2015). The fractionated DNA was purified with 70% ethanol after polyethylene glycol (PEG) 6000 precipitation and dissolved in 30 μl of sterile water.

2.6. Real-time quantitative PCR of biomarker *pmoA* genes

To determine the changes in abundance of methanotrophic communities and to assess the $^{13}CH_4$ labelling of methanotrophs, the copy number of *pmoA* genes in the total DNA extracts as well as in the DNA gradient fractions (fractions 3-13) were determined by real-time quantitative PCR (qPCR) using a CFX96 Optical Real-Time detection system (Bio-Rad Laboratories, USA). The PCR primers A189f/mb661r were used (Holmes et al., 1995;Costello and Lidstrom, 1999) following the conditions shown

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in Table S1. The standards were generated using plasmid DNA from one representative clone containing bacterial *pmoA* genes, and a dilution series of standard template from 10^2 to 10^8 per assay was used. In addition, the total DNA extracts were diluted in a series to assess possible PCR inhibition by soil humic substances, and DNA extracts were diluted 20-fold for subsequent analysis. The amplification efficiencies ranged from 92% to 103%, with R^2 values of 0.994 to 0.999. Melting curve analysis and standard agarose gel electrophoresis were always performed at the end of a PCR run to verify the amplification specificity.

2.7. MiSeq sequencing of 16S rRNA and *pmoA* genes

Illumina MiSeq sequencing was employed to investigate the community shifts of methanotrophs in the soils. The total microbial communities were analysed in all soil microcosms using universal primers for 16S rRNA genes to investigate the proportional changes in methanotrophs relative to the total microbial communities in soils. In addition, the ^{13}C-DNA retrieved from "heavy" CsCl fractions (with a density of 1.738-1.740 g ml^{-1}) in $^{13}CH_4$-labelled microcosms was also subjected to amplicon-based sequencing targeting both the 16S rRNA and *pmoA* genes. The "light" DNA fractions (with a density of 1.719-1.726 g ml^{-1}) from the ^{12}C-control samples were also used for 16S rRNA gene sequencing to reveal the background microbial community compositions. The PCR primer pairs were 515F/907R (Stubner, 2002) for 16S rRNA genes and A189f/mb661r for *pmoA* genes, with each forward primer fused with a unique barcode sequence. The PCR primers and conditions are detailed in Table S1. The resulting PCR products were gel purified and combined in equimolar ratios in a single tube. The sequencing samples were prepared using a TruSeq DNA kit (Illumina, USA), and the purified library was diluted, denatured, re-diluted, and mixed with PhiX as described in the Illumina library preparation protocols. Paired-end sequencing (2×300 bp) was conducted using the Illumina MiSeq system (Illumina, USA).

2.8. Sequence data processing and deposition

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All raw sequence files were processed using the Quantitative Insights Into Microbial Ecology (QIIME) pipeline (Caporaso et al., 2010). Paired-end reads were first assembled using FLASH with a minimum overlap parameter value of 10 bp (Magoc and Salzberg, 2011). The quality control procedure removed reads with a quality score <20, mismatched primers and ambiguous bases. Chimeras were eliminated using USEARCH. For *pmoA* genes, putative frame-shifting reads were removed using the FRAMEBOT program (Wang et al., 2013). Subsequently, a total of 2,871,893 high-quality 16S rRNA and 421,696 *pmoA* sequences were retained for further analyses. The high-quality sequences were then clustered into operational taxonomic units (OTUs) at 97% (16S rRNA gene) or 93% (*pmoA* gene) sequence similarities by the UPARSE algorithm (Edgar, 2013). To cluster *pmoA* genes at 93% similarity, the "otu_radius_pct" option (default of 3) was changed to 7 when performing the "cluster_otus" command, and the id option (default of 0.97) was modified to 0.93 for the "usearch_global" command. The representative sequences of all 16S rRNA OTUs were taxonomically classified using the Ribosomal Database Project (RDP) classifier (Wang et al., 2007). For *pmoA* reads, representative OTU sequences were classified using a naïve classifier implemented with the mothur "classify.seq" command as described previously (Dumont et al., 2014). For the major ^{13}C-labelled *pmoA* OTUs retrieved from heavy CsCl fractions (containing ≥2% of *pmoA* gene sequences in at least one of the samples), a representative sequence was selected for phylogenetic analysis by comparison with known sequences from GenBank. A heatmap was constructed based on the relative abundances of the major ^{13}C-labelled *pmoA* OTUs across different microcosms by HemI version 1.0 (Deng et al., 2014), and hierarchical clustering of samples was performed with the calculated Pearson distance.

2.9. Statistical analysis

Mantel tests were used to test for significant correlations between methanotrophic community distance and different soil physiochemical properties, namely, pH, TOC, TN, C:N ratio, SOM, exchangeable inorganic N, soil copper content and OXC. The tests were performed in the *R* environment with the vegan package (Dixon, 2003).

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One-way analysis of variance with Tukey's *post hoc* test was used for comparisons among different treatments for each soil. An independent t-test was conducted to assess the possibility of significant differences between two groups. Analyses were conducted using the SPSS version 13.0 package for Windows (SPSS, Inc.). $P<0.05$ was regarded as statistically significant.

3 Results

3.1. Conversion of CH_4 to soil organic matter

During SIP microcosms incubation a fraction of ^{13}C-CH_4 were converted to soil organic matter (SOM) by MOB through cell biomass synthesis, and it was assessed as the changes in the ^{13}C-atom percent of the soil total organic carbon (TOC). The average background ^{13}C-atom abundance in soils under natural atmospheric condition was 1.08±0.01%, and all soils showed statistically significant increases in the ^{13}C-atom abundance of TOC up to 1.74±0.41% upon consumption of $^{13}CH_4$ (Fig. 1a). There was no significant difference in conversion ratio of $^{13}CH_4$ into soil organic matters between low-pH and high-pH soils, and fertilizing soils with inorganic nitrogen did not result in higher ^{13}C incorporation into organic matter than that in unfertilized soils (Table S2). Based on the changes in methane concentrations, soil organic carbon contents and ^{13}C-atom percent during incubation of SIP microcosms, it was theoretically estimated that 10.4-38.1% of ^{13}C-CH_4 was converted to soil organic carbon during the microcosm incubations (Table S2). And $^{13}CH_4$-derived carbon contributed 0.203-1.25% of total soil organic carbon after incubation (Table S2).

3.2. Methane oxidation rates

Assuming linear kinetics, the methane oxidation rates were 0.71-4.08 μmol CH_4 g *d.w.s.*$^{-1}$ day^{-1} in soils of YX, YT and TY (with a low pH) and 2.65-4.83 μmol CH_4 g *d.w.s.*$^{-1}$ day^{-1} in soils of ZY, CS and LZ (with a high pH) (Fig. 1b). Nitrogen fertilization led to significantly lower methane oxidation rates in low-pH soils, while varying effects were observed in high-pH soils (Fig. 1b). The changes in the concentrations of

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263 headspace methane in the microcosms also showed inhibition by inorganic nitrogen of
264 microbial methane oxidation during incubation of low-pH soils, leading to a prolonged
265 period for consumption of the same amount of methane, particularly for YT soil (Fig.
266 S2).

267 **3.3. Population dynamics of methane-oxidizing bacteria**

268 The absolute abundance of soil methanotrophs was estimated by qPCR using the
269 biomarker gene *pmoA* at the microcosm incubation endpoints. The consumption of
270 methane at high concentrations stimulated the growth of methanotrophs, represented
271 by 20.4- to 1,027-fold increases in the number of *pmoA* gene copies following
272 microcosm incubation in all six soils (Fig. 1c). Consistent with the dynamic changes in
273 methane oxidation rates, nitrogen fertilization had a similar impact on the abundance
274 of methanotrophic communities. Particularly, the *pmoA* gene abundances under
275 nitrogen fertilization were significantly lower than those in the unfertilized low-pH
276 soils (YX, YT and TY) (Fig. 1c).

277 Based on high-throughput sequencing of 16S rRNA genes, the percentages of type
278 I and type II MOB in the total microbial communities were calculated to track the
279 dynamic changes of methanotrophic communities during SIP incubations. The relative
280 abundances of type II methanotrophs increased significantly in methane-amended
281 microcosms with low-pH soils (YX, YT and TY) but did not change in the high-pH
282 soils of ZY, CS and LZ (Fig. 1d). For type I methanotrophic populations, the reversed
283 trend was observed. Specifically, type Ia methanotrophs showed an 8.0-16.9-fold
284 increase in high-pH soils of ZY, CS and LZ, while no change or only a minor increase
285 was observed in the low-pH soils (YX, YT and TY) following methane amendment
286 (Fig. 1e). High enrichment of type Ib methanotrophs was also observed in the ZY soil
287 (Fig. 1f).

288 **3.4. Stable isotope probing of active methane-oxidizing bacteria**

289 Following the isopycnic centrifugation of the total DNA extracted from $^{13}CH_4$-SIP
290 microcosms, real-time qPCR analysis of *pmoA* genes as a function of the buoyant

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density demonstrated active cell propagation and ^{13}C assimilation in all six soils fuelled by methane oxidation. A peak shift of relative abundances of *pmoA* genes towards heavy fractions was clearly observed in all soil microcosms amended with $^{13}CH_4$ compared to the control treatments (Fig. 2). The *pmoA* genes in the $^{13}CH_4$-amended microcosms were highly accumulated in the heavy DNA fractions with a buoyant density of approximately 1.735-1.745 g ml^{-1}, while the *pmoA* genes in the control treatments peaked only in the light DNA fractions with a buoyant density of 1.717-1.726 g ml^{-1}. Similar results were also obtained for SIP microcosms amended with inorganic nitrogen (Fig. 2). Notably, following N fertilization, the highest peak in the YT soil occurred in the light fraction, although the apparent labelling of *pmoA* gene-carrying methanotrophs was evidenced by increased abundances in the heavy fraction compared to control.

High-throughput sequencing of the 16S rRNA genes in the heavy DNA fractions at the whole-community level further showed significant increase in relative abundance of methanotrophs in ^{13}C-labelled microcosms. The methanotroph-affiliated ^{13}C-16S rRNA genes accounted for 61.9 to 81.2% of the total microbial communities in the ^{13}C-DNA fractions, while in the control treatment, the background methanotrophs constituted only 3.1-7.2% of the total microbial communities (bottom columns of Fig. 2).

3.5. Linking soil physiochemical properties with active methanotrophs

Phylogenetic analysis of ^{13}C-labelled *pmoA* genes from heavy DNA fractions demonstrated that *Methylocystis* related type II organisms dominated the ^{13}C-labelled methanotrophs in the YX, YT and TY soils with low pH values (Fig. 3), which was confirmed by taxonomic classification of ^{13}C-labelled 16S rRNA gene classification (Fig. S3 and S4). In the high-pH soils (ZY, CS and LZ), ^{13}C-labelled methanotrophs were predominated by type Ia organisms. The ^{13}C-*pmoA* genes were related to the type Ia methanotroph *Methylobacter* sp. (Fig. 3), but the ^{13}C-labelled 16S rRNA genes suggested the methanotrophs might be closer to *Methylosarcina* sp. (Fig. S4). Notably, in the ZY soil, 17-30% of the ^{13}C-labelled sequences were phylogenetically related to

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the type Ib methanotroph *Methylocaldum* sp. (Fig. 3). The community compositions of the ^{13}C-labelled methanotrophs were deeply branched between high- and low-pH soils (Fig. 3).

Mantel tests showed that only the pH, out of all the eight soil characteristics tested, was significantly correlated with variation in the active methanotrophic communities (according to ^{13}C-labelled *pmoA* gene sequencing) between different soils (P<0.05) (Table S3). Regression analysis further revealed a significantly positive relationship between soil pH and the relative abundances of the primary ^{13}C-labelled type I methanotrophic cluster (OTU17), while pH was negatively related to the relative abundances of the dominant type II cluster (OTU32) under high methane concentrations (Fig. 4).

4 Discussion

Our results provide strong evidence for the important roles of pH-based selection of type I and type II methanotrophs in methane oxidation and assimilation occurring in paddy soils. In soils amended with $^{13}CH_4$ gas, the incorporation of ^{13}C into genomic DNA occurred in methanotrophic communities that directly utilized methane-derived carbon for growth. Therefore, the relative gene abundances of type I versus type II methanotrophs in ^{13}C-labelled DNA fractions can reflect their relative contributions to actual methane uptake and oxidation. In this study, the ratios of ^{13}C-labelled type II to type I methanotrophs in ZY, CS and LZ soils with high pH values were very low ranging from 0.002 to 0.014 (Table S4), suggesting that the type I methanotrophs in these three soils were more active than their type II counterparts. However, the ratios of ^{13}C-labelled type II to type I were 20.0 to 101 in the in the low-pH soils of YX, YT and TY, respectively (Table S4). Assuming that one cell contained 2 copies of *pmoA* genes (Kolb et al., 2003), type II methanotrophs could reach a cell-specific methane oxidation rate of 0.2-9.6 fmol CH_4 h^{-1} $cell^{-1}$ in these three soils (calculated in Table S4), being consistent with previous reports 0.2-15 fmol CH_4 h^{-1} $cell^{-1}$ obtained from both pure cultures and complex soils (Hanson and Hanson, 1996). These results thus suggest that

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aerobic methanotrophy was mostly sustained by the growth of type II methanotrophs in these acidic paddy soils.

Type II methanotrophs identified in low-pH soils were affiliated to *Methylocystis* based on ^{13}C-labelled *pmoA* genes (Fig. 3), which was also congruent with phylogenetic analysis of 16S rRNA genes (Fig. S4). The analysis of *pmoA* genes indicated that ^{13}C-methanotrophs in high-pH soils could be most closely related to *Methylobacter* of type Ia methanotrophs. However, phylogenetic analysis of the ^{13}C-labelled 16S rRNA genes suggested that in high-pH soils the dominant methanotrophs could cluster closely with *Methylosarcina* species (Fig. S4). We assume this discrepancy might be attributed to the lack of whole genome information in the present study, and the presence of phylogenetic incongruence between *pmoA* and 16S rRNA genes which could be better resolved with the increasing genome availability of methanotrophs in the future. Nevertheless, our results (both from *pmoA* and 16S rRNA genes) indicated that type Ia methanotrophs dominated methane oxidation activities in the high-pH paddy soils.

It is technically challenging for tracking the *in situ* activities of microbes, especially in ecosystems that are exposed to constantly fluctuating environments such as rice paddy fields. Agricultural management might influence the population dynamics of methanotrophs through irrigation, fertilization and plantation. In view of this variability, results based on microcosms cannot represent entirely the *in situ* conditions. However, the incubation of SIP microcosms was conducted under the same conditions, but the labelling of distinct methanotrophs indeed occurred, which might be the result of long-term ecological and evolutionary adaption of active methane oxidizers in paddy soils with contrasting physiochemical variables. Therefore, our results might reflect what is largely occurring under *in situ* inundated conditions, in which high methane emissions occur, particularly in regard to the relative activities of type I versus type II methanotrophs that were likely controlled by soil intrinsic biotic and abiotic factors.

The active methanotrophic compositions in the six paddy soils were strongly associated with only soil pH, based on tests on potential correlations with 8 key soil physiochemical properties. Specifically, rice paddy soils with higher type II

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methanotrophic activities were all acido-neutral (YX, YT and TY, with a pH of 5.44-6.10), while the more alkaline soils (ZY, CS and LZ, with a pH of 7.02-8.02) displayed stronger type I methanotrophic activity. Previous culture-dependent and ecophysiological studies, where type II methanotrophs were described as stress tolerators, provided strong support for low pH as the potential driving force for selection of type II over type I methanotrophs in paddy soils (Ho et al., 2013). Cultivated acidophilic or acid-tolerant methanotrophs are by far mostly type II strains which are phylogenetically close to the ^{13}C-methanotrophs retrieved in the low-pH soils in this study (Fig. 3). Our results are consistent with the observations from natural wetland systems, in which the activity of type II methanotrophs appeared to be more prevalent under low-pH than high-pH conditions (Chen et al., 2008a;Dedysh, 2009;Kip et al., 2012;Chen et al., 2008b;Gupta et al., 2012). It thus seems plausible that oxidation of methane at high concentrations by type II methanotrophs more frequently occurs in acidic than alkaline rice soils. However, the possibility cannot be ruled out that other untested abiotic and biotic variables might have stronger forces in shaping community structure of methanotrophs in rice paddy soils.

Our results demonstrated that chemical nitrogen fertilization did not alter the dominant community compositions of active methanotrophs (Fig. 3), implying that the growth of methanotrophs in these soils was not nitrogen-limited, or nitrogen availability was not the key factor for selection of distinct methanotrophs in rice soils. In this context, we speculate that N input could have stimulated plant growth and increased the levels of exudates and litter decomposition served as precursors for methanogenesis to enhance methane production. Despite no consistent pattern was observed with respect to the effects of N fertilization on methane oxidation rates in this study, it appears that N fertilization had an inhibitory effect on the methane oxidation rates in the low-pH soils, which were dominated by type II methanotrophic activity (Fig. 1b and Fig. S2). Consistently, the methanotrophic abundances under N fertilization situation were significantly lower than those in unfertilized low pH soils (Fig. 1c). These results agreed well with previous findings that high nitrogen input suppressed

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406 the activity and growth rates of type II methanotrophs from pure cultures (Graham et
407 al., 1993) and in complex soil samples (Noll et al., 2008;Bodelier et al., 2000;Mohanty
408 et al., 2006). Meanwhile it is noteworthy that contradictory results were obtained in
409 high-pH paddy soils. N fertilization stimulated the methane oxidation rate in only one
410 of the high-pH soils (LZ), which had a unique dominant type Ia cluster (OTU50, as
411 shown in Fig. 3) when compared to the other two high-pH soils (dominated by OTU17).
412 Our study therefore implied that the contradicted effects of nitrogen fertilization on
413 methane oxidation (inhibition or stimulation) frequently reported in different soils
414 (Bodelier et al., 2000;Zheng et al., 2014;Alam and Jia, 2012;Cai and Yan, 1999) might
415 be determined by the dominant methanotrophic phylotypes, but a larger-scale sampling
416 with activity-based molecular analysis (e.g. RNA or SIP based tools) is required to test
417 this hypothesis in the future.

418 The significant ^{13}C enrichment of soil organic carbon indicated that $^{13}CH_4$-derived
419 microbial biomass contributed significantly to the turnover of soil carbon. Meta-
420 analysis indicated microbial biomass represent 0.6-1.1% of total soil organic carbon
421 (Fierer et al., 2009), but it remained largely unknown about the contribution of methane-
422 driven microbial food web to soil fertility and quality (Murase and Frenzel, 2007b). Our
423 results showed no statistically significant difference in net soil ^{13}C input between low-
424 pH and high-pH soils during methane oxidation, although type I and type II
425 methanotrophs employed different strategies for carbon metabolisms (Trotsenko and
426 Murrell, 2008). For instance, 5-15% of cell biomass carbon in type I methanotrophs
427 could be derived from CO_2 (Trotsenko and Murrell, 2008), and recent study suggests
428 the proportion up to 60% in type II methanotrophs (Yang et al., 2013). It is noteworthy
429 that the fresh input of $^{13}CH_4$-derived biomass accounted for up to 1.25% of total organic
430 carbon in LZ soil (Table S2), implying that the amount of $^{13}CH_4$-C incorporated into
431 trophic networks comprised a substantial fraction of soil microbial biomass.
432 Quantitative assessment of soil microbial biomass pools and the relative contribution
433 of phylogenetically distinct methanotrophs to active carbon pool in soil would be

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essential for deciphering the underlying metabolism of methane oxidizers and their ecological and agricultural importance in paddy fields.

5 Conclusions

This study provides evidence for niche differentiation of type I and II methanotrophs strongly associated with soil pH variation. Low-pH could have likely selected for type II methanotrophs in paddy soils while type I was favoured in high-pH soils. The incorporation of CH_4-derived carbon into biomass contributed up to 1.25% of total organic carbon in paddy soil. The fresh input of new carbon from aerobic methanotrophy played a vital role in the turnover of soil microbial biomass and subsequent cycling of soil nutrients in support of agricultural sustainability. Nitrogen fertilization changed methane oxidation rates in five of the soils tested, but the composition of active methanotrophs was not significantly affected. These results provide a mechanistic basis for better understandings of community assembly mechanisms of ecologically important microbial guilds and their possible roles in agricultural sustainability.

Data availability. The raw Illumina sequencing data have been deposited in the European Nucleotide Archive (ENA) under Ac. No. PRJEB37235 for 16S rRNA genes and PRJEB40045 for pmoA gene sequences. The sequences of 13C-labelled pmoA OTUs were deposited to GenBank with accession numbers MK613983-MK613993 and MK621911-MK621913.

Author contribution. JZ and ZJ designed the experiments and JZ carried them out. YC assisted the bioinformatic analyses. JZ and ZJ prepared the manuscript with contributions from all co-authors.

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

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661 Table 1 The sampling locations and chemical characteristics of the rice paddy soils

662 tested

Site	Location	pH	SOM (g kg^{-1})	TOC (g kg^{-1})	TN (g kg^{-1})	C/N ratio	Inorganic N (mg kg^{-1})	Cu (mg kg^{-1})	OXC (mmol kg^{-1})
Yu-Xi	N 24°17'	5.44±	27.6±	15.9±	1.74±	9.2±	85.1±	1.35±	18.2±
(YX)	E 102°15'	0.03^{e}	0.1^{d}	0.4^{d}	0.00^{d}	0.2^{b}	1.9^{d}	0.01^{c}	0.2^{b}
Ying-Tan	N 28°23'	5.68±	19.0±	13.1±	1.38±	9.5±	204.7±	1.09±	11.2±
(YT)	E 116°82'	0.01^{d}	0.1^{f}	0.4^{e}	0.02^{e}	0.3^{b}	1.9^{b}	0.01^{d}	0.1^{c}
Tao-Yuan	N 28°55'	6.10±	39.5±	23.1±	3.15±	7.3±	2356.0	1.52±	6.3±
(TY)	E 111°27'	0.02^{c}	0.2^{b}	0.1^{c}	0.02^{a}	0.1^{c}	±0.7^{a}	0.02^{b}	0.1^{d}
Zi-Yang	N 30°05'	8.02±	23.2±	29.9±	1.93±	15.5±	237.8±	0.52±	85.7±
(ZY)	E 104°34'	0.02^{a}	0.1^{c}	0.1^{a}	0.02^{c}	0.1^{a}	0.8^{a}	0.00^{e}	2.3^{a}
Chang-	N 31°33'	8.02±	44.7±	27.7±	2.90±	9.6±	95.5±	2.07±	19.3±
Shu (CS)	E 120°42'	0.01^{a}	0.3^{a}	0.3^{b}	0.03^{b}	0.2^{b}	1.2^{c}	0.02^{a}	0.2^{b}
Lei-Zhou	N 20°33'	7.02±	19.5±	13.4±	1.48±	9.1±	86.7±	0.38±	19.4±
(LZ)	E 110°04'	0.02^{b}	0.1^{e}	0.0^{e}	0.13^{e}	0.8^{b}	0.6^{d}	0.00^{f}	0.2^{b}

663 Abbreviations: SOM, soil organic matter; TOC, total organic carbon; OXC, soil oxidation capacity
664 Different letters (a-f) in each row of chemical properties indicate a significant difference between
665 soils (P<0.05).
666

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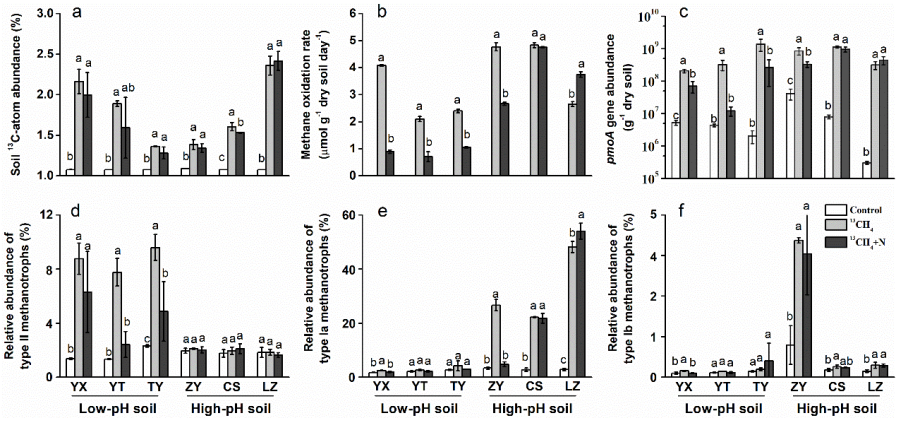
667 **Figure 1**



668

669 Figure 1 Changes in soil ^{13}C-atom abundances, methane oxidation rates, community
670 sizes and compositions of methanotrophs following incubation in soil microcosms. (a)
671 Soil ^{13}C-atom abundance was measured to assess methane assimilation in soil
672 microcosms amended with 5% $^{13}CH_4$. (b) The soil methane oxidation rate was
673 compared between soil microcosms incubated with or without NH_4NO_3 fertilization. (c)
674 The *pmoA* gene copy numbers of methanotrophs were estimated using real-time qPCR.
675 Illumina sequencing targeting 16S rRNA genes was performed at the whole microbial
676 community level in microcosms, and the relative abundance of type II (d), type Ia (e)
677 and type Ib (f) methanotrophs was expressed as the ratio of affiliated gene reads to the
678 total 16S rRNA gene reads in each microcosm. "Control" indicates soil under natural
679 atmospheric condition. "$^{13}CH_4$" and "$^{13}CH_4$+N" refer to soil microcosms incubated with
680 5% v/v $^{13}CH_4$ without and with extra NH_4NO_3 fertilization, respectively. The
681 designations below the X axis represent the soil sampling sites of rice paddy fields. All
682 treatments were conducted in triplicate. The error bars represent the standard errors of
683 the mean of the triplicate microcosms. Different letters above the columns indicate a
684 significant difference between different treatments in a given soil (P<0.05).

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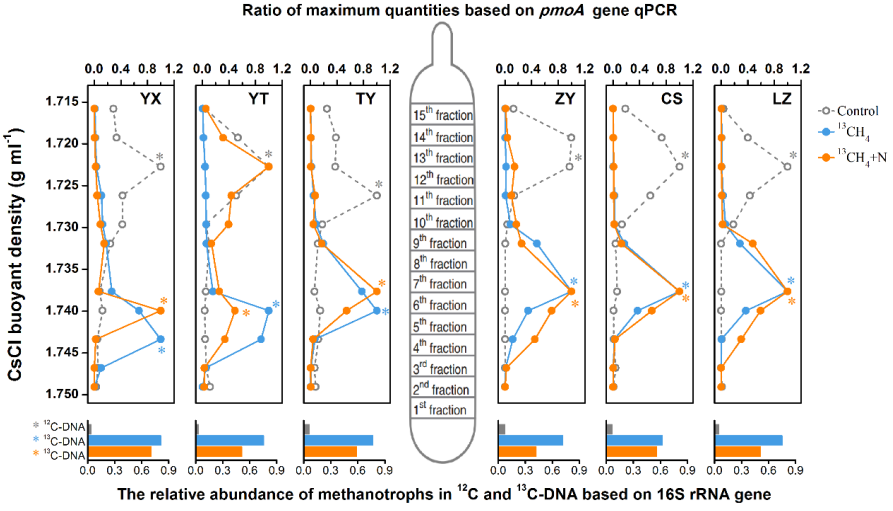

686

687 Figure 2 The enrichment of ^{13}C-labelled methanotrophs based on qPCR of *pmoA* and
688 sequencing of 16S rRNA genes. The quantitative distribution of *pmoA* genes across
689 the entire buoyant density gradient of the DNA fractions from soil microcosms
690 incubated with $^{13}CH_4$ compared to the controls. The normalized data are the ratios of
691 the gene copy number for each DNA gradient to the maximum number for each
692 treatment. The columns beneath display the relative abundance of methanotroph-
693 affiliated reads in all 16S rRNA genes in the ^{12}C-DNA from the control and ^{13}C-DNA
694 from $^{13}CH_4$-amended soil microcosms, respectively. * represents the DNA fractions
695 selected for Illumina sequencing.

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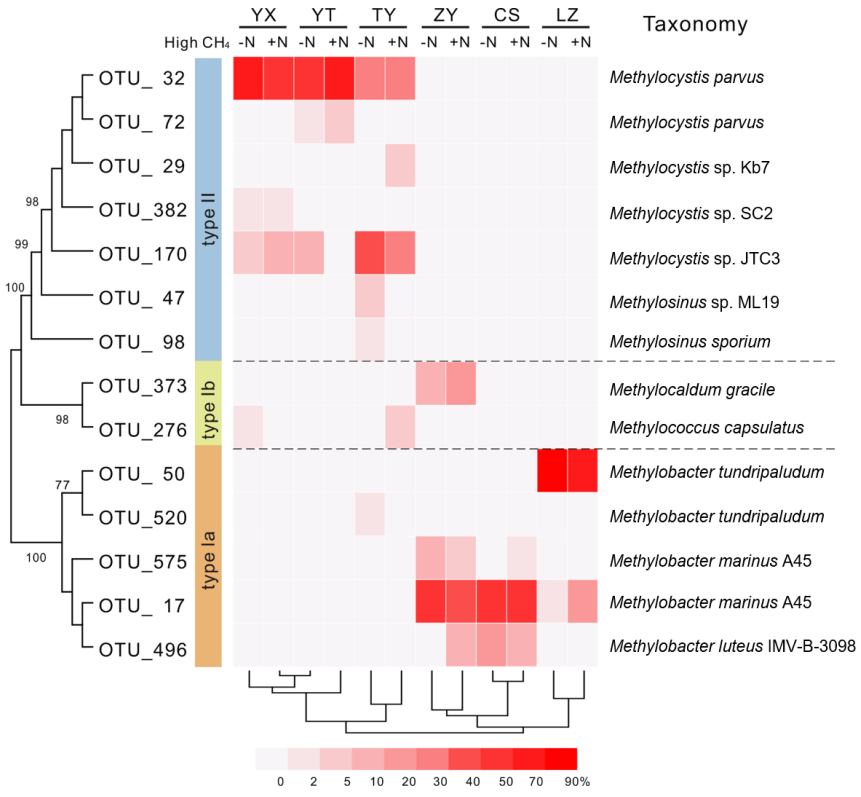

696

697 Figure 3 Heat map of relative abundances of major ^{13}C-labelled methanotrophic
698 OTUs based on *pmoA* gene sequencing. "-N" and "+N" indicate $^{13}CH_4$-amended
699 microcosms without and with nitrogenous fertilization, respectively. Hierarchical
700 clustering of samples was performed, and phylogenetic relations between different
701 OTUs are shown by the topology, with bootstrap values >60% indicated at branch
702 nodes. Each OTU representative sequence is taxonomically annotated to a known
703 strain from GenBank with the closest phylogenetic relation.

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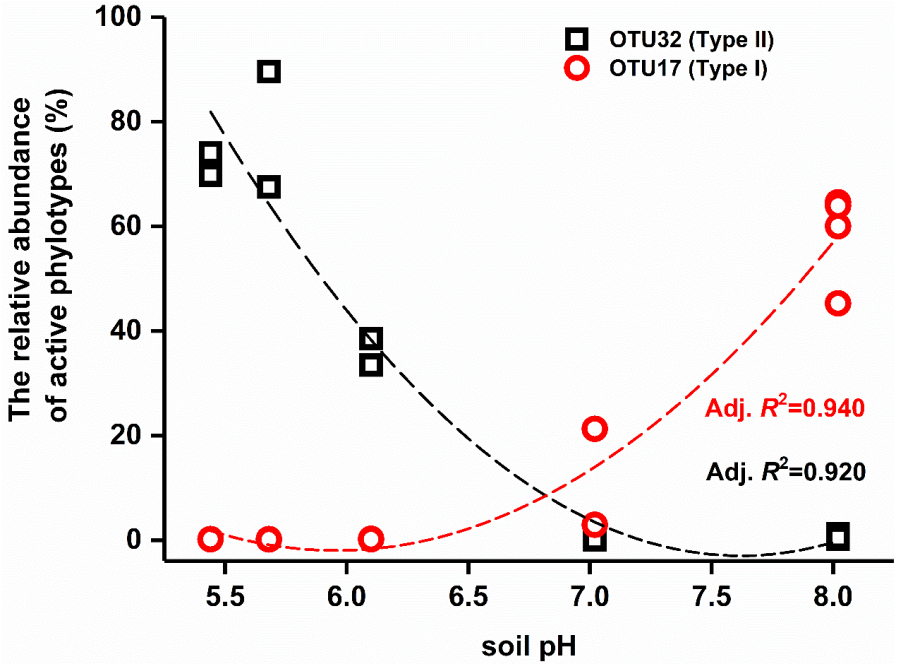

Figure 4 The relationship between soil pH and the relative abundance of major ^{13}C-labelled methanotrophic phylotypes (OTUs). OTU17 and OTU32 are the same OTUs displayed in Fig. 3.