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Methanotrophy-driven accumulation of soil organic carbon in four paddy soils of Bangladesh

Running title: Methanotrophy in paddy soil

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ABSTRACT

Biological methane oxidation is a crucial process in the global carbon cycling that reduces methane emissions into the atmosphere from rice paddy fields and natural wetlands. However, soil organic carbon accumulation associated with microbial methane oxidation is poorly understood in wetland. The paddy soils with different origins of parent materials (Inceptisols, Entisols and Alfisols) were collected to investigate methane-derived carbon incorporation into soil organic matter from paddy fields of four different regions representing major rice production in Bangladesh. Following microcosm incubation with 5% (v/v) $^{13}CH_4$, soil ^{13}C-atom abundances significantly increased from background 1.08% to 1.88% - 2.78%, leading to a net accumulation of methane-derived soil organic carbon ranging from 120 to 307 mg kg^{-1} dry soil. Approximately 23.6 - 60.0% of the methane consumed was converted to soil organic carbon during microbial methane oxidation. The phylogeny of ^{13}C-labelled *pmoA* and 16S rRNA genes further revealed that canonical α (type II) and γ (type I) Proteobacteria were active methane oxidizers. Members within the *Methylobacter*- and *Methylosarcina*-affiliated type Ia lineages dominated active methane-oxidizing communities that were responsible for the majority of methane-derived carbon accumulation in three soils, while *Methylocystis*-affiliated type IIa lineage was the key contributor in one paddy soil of Inceptisols origin. These results suggest that methanotrophy-mediated synthesis of biomass played an important role on soil organic matter accumulation. This study thus provides a proof of concept that

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methanotrophs not only consume greenhouse gas methane but also serve as a key biotic factor in maintaining soil fertility.

Keywords: Soil organic carbon, methanotrophs, rice paddies, DNA-SIP, *pmoA* and 16S rRNA gene

INTRODUCTION

Soil organic carbon (SOC) plays a vital role on soil fertility by shaping soil physical structure and modulating soil biogeochemical processes in terrestrial ecosystems (Panakoulia *et al.*, 2017; Schjønning *et al.*, 2018). SOC is the key component of soil organic matter (SOM) which is originated from three major sources including shoot litter, root residues/exudates and microbial biomass (Nguyen, 2003). Microbe-derived carbon, rather than plant originated carbon, was arguably considered to be the primary source of the SOC (Barré *et al.*, 2018), especially in the deeper soil layers (Fontaine *et al.*, 2007; Schmidt *et al.*, 2011). The microbial residues and exudates occupied up to 80% of carbon in soil organic matter fractions (Gleixner *et al.*, 2002; Kiem and Kögel-Knabner, 2002; Liang and Balser, 2011), and microbial biomass accounts for 1–5% of SOC (Jenkinson, 1981) which is relatively stable and essential in the biochemical process of SOC and soil elemental cycling. The microbial biomass serves an engine for SOC turnover by balancing between SOM mineralization and stabilization processes (Sørensen, 1983; Simpson *et al.*, 2007; Liang and Balser, 2011).

Methane (CH_4) is a potent greenhouse gas emitted from both the natural wetlands and anthropogenic rice paddies, and its concentration in the atmosphere shows remarkable increases relative to pre-industrial era (Wahlen, 1993; Etminan *et al.*, 2016). Inundated rice paddy fields are one of the vital sources of atmospheric methane (Minami and Neue, 1994), broadly distributed in South-east Asia and other (sub)tropical areas in the world. Bangladesh is a sub-tropical low-lying deltaic country in South Asia and the third-largest rice-producing country with 36 million tons every year (BRRI, 2019). The tropical monsoon climate and low-lying land and irrigated rice farming system lead to enhanced methane emissions during the rice-growing seasons. It was estimated that the average methane release from the rice paddies in Bangladesh reaches 1,071 Gg yr^{-1} (Khan and Saleh, 2015). Therefore, understanding methane consumption and the fate of carbon metabolism associated to active methane oxidation is a key for sustainable rice production in Bangladesh, since aerobic methanotrophs arguably accounted for up to 80% of methane produced in paddy soils.

Methane in inundated rice fields is continuously produced by methanogenic degradation of organic matter under anoxic conditions (Reeburgh, 1996). Methanotrophs are the only biological sink of atmospheric methane in terrestrial ecosystems but can also consume methane at high concentrations (Conrad, 2009). For example, the concentration of methane up to 50,000 ppmv could be detected and consumed within the oxic zone of rice fields (Nouchi *et al.*, 1990; Eller and Frenzel, 2001). It is estimated that nearly 80% of CH_4 originated from submerged rice fields is reduced by methanotrophs (Frenzel *et al.*, 1992) and CH_4 emissions would likely double if the anaerobically produced methane is not consumed by the methanotrophs (Le Mer and Roger, 2001). In a complete process of methane oxidation, a proportion of methane is catalyzed to carbon-related intermediates, e.g. formaldehyde that is further assimilated into the methanotrophic cell biomass (Anthony, 1982). The synthesized microbial biomass either supports other organisms as carbon substrate (e.g. through predation and cross-feeding) (Bastviken *et al.*, 2003) or

becomes the organic component of the soil (Kindler *et al.*, 2009), contributing to the stable SOC as recently proposed (Miltner *et al.*, 2012; Kallenbach *et al.*, 2016).

Soil organic carbon also contain humic fractions that are heterogeneous high-molecular-weight organic materials, which can serve as electron acceptors for the anaerobic oxidation of organic compounds and hydrogen (Lovley *et al.*, 1996). It has raised the possibility that humic substances could catalyze the oxidation of organic carbon by mediating the reduction of the oxidized iron (Scott *et al.*, 1998), which indeed acts as electron acceptor for AOM (Beal *et al.*, 2009). Subsequent flux measurement showed significant lower methane emission upon the addition of humic acids in peat soils, implying the reduction of humic substance coupled to AOM (Blodau and Deppe, 2012). Stable-isotope probing showed that humic substances could as electron shuttle to fuel anaerobic methane oxidation coupled to iron (III) reduction (He *et al.*, 2019). Recent studies have shown that humic substances in a variety of environments facilitated anaerobic methane oxidation in denitrifying reactors (Bai *et al.*, 2019), wetland sediments (Valenzuela *et al.*, 2019) and paddy soils (Fan *et al.*, 2020). It is also estimated that humic substance-mediated AOM potentially contributed a global sink of methane up to 114 Tg of CH_4 per year in coastal wetlands (Valenzuela *et al.*, 2017), and played an important role on N_2O reduction as well (Valenzuela *et al.*, 2020). Intriguingly, it remains poorly understood about the accumulation of soil organic carbon derived from microbial methane oxidation in wetland environments.

Aerobic methanotrophs can be classified into α and γ Proteobacteria that are often termed as type II and type I methanotrophs, respectively. It thrives under high concentrations of methane conditions and regulates the aerobic methane oxidation rate in wetland and rice fields (Cai *et al.*, 2016; Zhao *et al.*, 2020). Although more recently discovered, Verrucomicrobia (designated as type III) (Op den Camp *et al.*, 2009; Knief, 2015) and candidate division NC10 (Ettwig *et al.*, 2009) also play important roles in reducing methane emission to the atmosphere from acidic and/or inorganic nitrogen-rich environments. Moreover, it appears that conventional methanotrophs dominated microbial methane oxidation in rice soil due to their extraordinarily faster growth rates relative to NC10 and Verrucomicrobia (Cai *et al.*, 2016). Despite the high diversity of methanotrophs in the paddy soils, the methane oxidation was usually controlled by distinct phylotypes of methanotrophs, the biogeography of which was explained best by soil pH (Zhao *et al.*, 2020). However, it is still unclear whether soils dominated by distinct active methanotrophic groups also have different methane consumption efficiency, since it is always challenging to estimate the ratio of methane carbon converted to SOC during methane oxidation, and to assess contribution of methanotrophy to soil organice matter in paddy fields with different soil parent materials origin. Methanotrophy is an active microbial process in rice paddy soils. We hypothesized that the methane-derived carbon could be an important source of new SOC input to the soils, and it is largely determined by active methanotrophic composition in paddy field soils. In this study, we employed a stable isotope tracing approach with DNA-based molecular tools to assess the conversion efficiency of methane-derived carbon into SOC, and to identify the active methanotrophs responsible for methane oxidation and assimilation based on the high throughput sequencing of the 16S rRNA and *pmoA* gene sequencing. The *pmoA* gene is a widely used functional molecular marker for the detection of active methanotrophs during methane oxidation and we applied (A189/mb661) primer sets to amplify the *pmoA* gene fragments (Costello and Lidstrom, 1999).

MATERIALS AND METHODS

Site description of paddy field

Soil samples were collected from the four different regions including Shirajgong (SG), Kushtia (KT), Mymensingh (MS) and Gazipur (GZ) districts, representing 90% of rice cultivated areas in Bangladesh (Supplementary Figure S1). The coordinates of four regions were a range between (23.91 °N to 24.74 °N) and (89.22 °E to 90.43 °E); mean annual temperature (25.2 °-28.9 ℃); average rainfall (1,593- 2,174 mm) and the elevation about (7-13 m), respectively (Table 1). The soils are classified as Entisol, Inceptisol and Alfisol under the Srikola, Ishurdi, Belabo and Salna soil series.

Table 1

The geographic and physicochemical properties of four rice paddy soils of Bangladesh

Soil characteristics	Shirajgong	Kushtia	Mymensingh	Gazipur
Abbreviation	SG	KT	MS	GZ
Coordinates	24.33 °N, 89.62 °E	24.00 °N, 90.43 °E	23.91 °N, 89.22 °E	24.74 °N, 90.42 °E
Soil series	Srikola	Ishurdi	Belabo	Salna
Soil order	Entisols	Inceptisols	Alfisols	Inceptisols
Mean annual rainfall (mm)	1610	1593	2174	2036
Mean annual temp (℃)	28.9	26.0	25.2	25.8
Elevation (m)	7.0	12.0	13.0	8.4
pH	5.37 ±0.12c	6.84 ±0.01a	5.68 ±0.02b	5.91 ±0.04b
SOC (g Kg^{-1})	11.1 ±0.01c	17.8 ±0.03a	15.4 ±0.03b	9.76 ±0.01c
TN (g Kg^{-1})	1.3 ± 0.00c	1.5 ± 0.00b	2 ±0.10a	1.3 ±0.10c
CEC(cmol Kg^{-1})	11.97 ±0.31b	13.87 ±0.23a	11.80 ±0.26b	7.78 ±0.06c
NO_3^- N (mg Kg^{-1})	3.21 ± 0.26c	7.31 ± 1.06a	4.78 ± 0.75b	4.67 ± 0.15b
NH_4^+ N (mg Kg^{-1})	12.67 ± 1.44a	12.73 ± 1.59a	4.97 ± 0.64c	8.50 ± 0.19b

Note: Abbreviation: SOC: Soil organic carbon; TN: Total nitrogen; CEC: Cation exchange capacity
The measurement was conducted in three replicates.

The rice variety used in these areas was "BRRI dhan 29" and double-rice system was employed. Prior to soil sampling, the boro rice (BRRI dhan 29) was cultivated with 5-6 times irrigation using Shallow Tube Well (STW). The seed rate was about 30-35 kg ha^{-1} and the cultivated fields received urea fertilization about 250-300 kg N ha^{-1} yr^{-1}. From each field (around 300-400 m^2), three replicated soil samples were collected from 0-20 cm depth. Each composite sample was made by mixing three random cores after rice harvesting on May 2015, then homogenized by passing through a 2 mm meshed sieve and stored at 4 ℃ before incubation. About 200 g subsample of each soil was stored at -20 ℃ for microbial and molecular analyses. The remaining soils were air-dried to measure the physicochemical properties, including soil pH, soil total organic carbon, total nitrogen and the cation exchange capacity (CEC) as previously described (Wang *et al.*, 2015).

Stable isotope tracing of $^{13}CH_4$ metabolisms in soil

Triplicated samples of stable-isotope probing (SIP) experiments were constructed and incubated under three different atmospheric conditions, including "control" (incubated under ambient air), "High $^{12}CH_4$" (with 5% v/v $^{12}CH_4$) and "High $^{13}CH_4$" (with 5% v/v $^{13}CH_4$) in a similar manner to our previous studies (Sultana *et al.*, 2019). The fresh soil (equivalent to air-dried weight of 6.0 g soil) was placed into a 120-ml serum bottle sealed with a butyl stopper and incubated at 60% maximum water holding capacity at 28 ℃ in the dark. For "High $^{13}CH_4$" and "High $^{12}CH_4$" treatments, the ^{13}C-labeled (> 99% ^{13}C-atom purity) or unlabeled CH_4 gas was injected into the bottles at the first day of incubation to reach a methane gas mixing ratio at approximately 5% (equivalent to 50,000 ppmv). The headspace concentrations of methane were monitored twice a day by a gas chromatograph equipped with a flame-ionization detector (Shimadzu GC12-A), the standard curve was generated by a series of methane concentrations including 100, 1000, 5000, 10000, 50000, 80000 ppm. Destructive sampling was conducted when methane concentration decreased below 5,000 ppmv in microcosms, i.e., more than 90% consumption of the methane added to the microcosms. Soil samples were collected and frozen at -20 ^{0}C after incubation for 7, 15, 18 and 24 days for SG, KT, MS and GZ, respectively.

The potential of methane oxidation and production rates

The headspace concentration of methane was measured as described above. When the headspace concentration of methane showed no further decline and remained relatively unchanged, then soil samples were collected for the subsequent molecular analysis. The methane oxidation rate was then determined according to the following equation (eq. 1)

$$R_{ox} = (Ci - Ce) \times V \times p/(m \times t) \qquad \text{(eq. 1)}$$

where R_{ox} is the methane oxidation rate in μmol g^{-1} dry soil h^{-1}; Ci is the initial headspace concentration of CH_4 at day 0; Ce is the end point CH_4 concentration at the end of incubation; V = the net volume of the bottle (soil volume is excluded, L); p = methane density under standard condition (1.01×10^5 Pa, 273.15 K), i.e., 0.72 g L^{-1}; m = weight of dry soil (g) used in the incubation; t refers to incubation time (day) and more than 10 data points were used for each samples.

The methane production rates of all four paddy soils were measured as previously described with slight modification (Wassmann *et al.*, 1998). In brief, fresh soil samples (being equivalent to 10 g of air-

dried soil) were placed in 120-mL serum bottle sealed with a butyl stopper, and 40 mL distilled water was added to make soil slurries. The headspace gas of each bottle was replaced with N_2 by vacuum flushing before incubation at 28 ℃ in the dark. The headspace concentrations of methane were then measured after 3, 7, 11, 15 and 19-days of incubation by using a gas chromatograph as described above. The methane production rate was calculated as the slope of temporal increases in methane concentrations according to the following equation (eq. 3).

$$R_{prod} = (dc/dt) \times V \times Ws \times (Mw/Mv) \times T_0/(T_0+T) \quad \text{(eq. 2)}$$

Where R_{prod} is the methane production rate, ppmv day^{-1}; dc/dt = Headspace concentration changes of methane, ppmv day^{-1} ; V= the net volume of the bottle (L); Ws= soil weight after oven dry (g); Mw is the molecular weight of methane (12 g) ; Mv=Molecular volume (L); T_0 =Standard temperature (K); T= Temperature (K).

Determination of ^{13}C-abundance and ^{13}C-SOC conversion rates

Soil samples about 1.5 g from control and $^{13}CH_4$ trearments was stored at -80 ℃ freeze for about 6 hours and then frozen-dried for 48 hrs. The freeze-drying was conducted to obtain the fine particles of those vacuum freeze-dried soil and the relative abundance of soil ^{13}C-atom and soil organic carbon content were measured by an elemental analysis-isotope ratio mass spectrometry analyzer (Flash-2000 Delta V ADVADTAGE, Thermo Scientific, USA) as previously described (Li *et al.,* 2019). Soil ^{13}C-atom abundance and the ^{13}C-SOC conversion rates were calculated by following equations Eq. (3) and Eq. (4).

$$^{13}C_{soc} = SOC \times (^{13}C_e - {}^{13}C_b) \times 1000 /13 \quad \text{(eq. 3)}$$

Where $^{13}C_{soc}$ is the net increase of soil ^{13}C-SOC in soil samples with $^{13}CH_4$ amendment, μmol g^{-1} d.w.s; SOC = soil SOC content after incubation with $^{13}CH_4$, (g kg^{-1}); $^{13}C_e$= Soil ^{13}C-atom percent after incubation with $^{13}CH_4$ (%); $^{13}C_b$ = ^{13}C-atom percent in background soil (%); 13=Atomic number of carbon.

$$^{13}C_{\text{soc rate}} = {}^{13}C_{soc}/CH_4 \text{ consumed} \quad \text{(eq. 4)}$$

Where $^{13}C_{\text{soc rate}}$ refers to the conversion ratio of CH_4 to soil SOC after incubation with ^{13}C-CH_4 (%). $^{13}C_{soc}$ = net increase of soil ^{13}C-SOC, μmol g^{-1} dry soil; CH_4 consumed=$^{13}CH_4$ oxidized, μmol g^{-1} dry soil.

DNA extraction and SIP gradient fractionation

Soil DNA was extracted from 0.5 g soil using the FastDNA spin kit (MP Biomedicals, Cleveland, OH, USA), following the manufacturer's instructions. DNA quantity and quality were measured by NanoDrop ND-1000 UV-visible light spectrophotometer (NanoDrop Technologies, USA). Soil DNA extracts from the treatment of "High $^{12}CH_4$" and "High $^{13}CH_4$" were subjected to CsCl isopycnic ultracentrifugation and fractionated to 14-15 density gradient fractions, to separate the heavy ^{13}C-labeled DNA from light ^{12}C DNA fractions as detailed in previous studies (Xia *et al.*, 2011; Lu and Jia, 2013). A digital refractometer (AR200, Reichert Tech., Ametek Inc., NY, USA) was used to determine the density of each gradient fraction. The polyethylene glycol 6000 (PEG 6000) was used to precipitate DNA in CsCl solutions before precipitation by 70% ethanol. The final DNA was dissolved in 30 μL of sterile water for downstream analysis.

Real-time quantitative PCR

DNA samples (including the total DNA extracts and DNA recovered from CsCl gradient fractions) were applied to real-time quantitative PCR (qPCR) using primer pair A189f-mb661r to quantify the copy number of *pmoA* genes on a CFX96 Optical Real-Time Detection System (Bio-Rad, Laboratories Inc., Hercules, CA, USA). The PCR reactions were carried out in a 20-μl mixture containing 10 μl of SYBR Premix Ex Taq (Takara Bio Inc, Japan), 0.5 μM of each primer and 1 μl of DNA template. The thermal conditions were described in (Supplementary Table S1). Standards containing 10^2-10^7 copies of *pmoA* gene-containing plasmids were prepared and used as described previously (Sultana *et al.*, 2019). The amplification efficiencies ranged from 92% to 105%, with R^2 values of 0.996 to 0.999.

MiSeq sequencing of 16S rRNA genes

MiSeq sequencing of the 16S rRNA genes was employed to assess the community structure of methanotrophs in soil total DNA and the active methanotrophs in the ^{13}C-DNA heavy fractions from the "High $^{13}CH_4$" soil treatments. The DNA of the same buoyant density fractions from the "High $^{12}CH_4$" treatments were also used for control. The universal primer pairs 515F and 907R were used to amplify the V4–V5 region of the 16S rRNA gene (Caporaso *et al.*, 2012). The thermal condition and primer detail of the PCR reaction were described in Supplementary Table S1. Paired-end sequencing (2 × 300 bp) was conducted using the MiSeq system (Illumina, USA). Raw sequences were subjected to the key quality control steps using Quantitative Insights Into Microbial Ecology software (QIIME) (Caporaso *et al.*, 2010), and low-quality sequences (quality score below 25, containing mismatched primers and ambiguous bases, and chimeras) were removed. A total of 1,131,823 high-quality reads were acquired. The high-quality sequences were applied to RDP MultiClassifier for the taxonomic assignment (Wang *et al.*, 2007). All sequences of known methanotrophs have been deposited in the RDP database so that the sequences in this study could be aligned and phylogenectically classified into the two methanotroph families (*Methylococcaceae* and *Methylocystaceae*). The taxonomic name of target sequences was converted to a txt file in order to make an accnos file used for Mothur software. The command "get.seq" of Mothur was then used to pick out the methanotrophic sequences of target from the fasta file. These sequences were then clustered into operational taxonomic units (OTU's) at 97% sequence similarity threshold by the UPARSE algorithm (Edgar, 2013). The representative sequences of the major MOB OTU's (containing ≥2% of total methanotrophic sequences in at least one of the samples) were used to construct a neighbor-joining phylogenetic tree by MEGA software. It should be noted that sequencing data is of compositional nature and ecological methods should be carefully evaluated by multiple statistic test (Gloor *et al*. 2017). The high-throughput raw sequencing reads of both the 16S rRNA and *pmoA* genes were deposited in NCBI Sequence Read Archive (SRA) under the project accession number PRJNA643911.

MiSeq sequencing of pmoA genes

The *pmoA* gene fragments in the ^{13}C-DNA heavy DNA fractions of "High $^{13}CH_4$" treatments were amplified using A189F/ mb661r primers and applied to paired-end sequencing (2 × 300 bp) on the MiSeq system (Illumina) as previously described (Cai *et al.*, 2016; Sultana *et al.*, 2019). The cycling profile for PCR was detailed in Supplementary Table S1. The raw paired-end reads were first assembled and quality filtered by Mothur (Schloss *et al.*, 2009). Putative frame-shifting reads were corrected or removed using the FrameBot program (Wang *et al.*, 2013). A total of 59,902 high-quality sequences were obtained. The FunGene Pipeline was also used to clustering the reads into operational taxonomic units (OTUs) at the genus-level with a distance cutoff of 0.07 (Degelmann *et al.*, 2010). The phylogenetic analysis of the representative sequences of major *pmoA* gene OTUs (containing ≥2% of sequences in at least one sample) was performed by MEGA using the neighbor-joining method.

Statistical analysis

One-way ANOVA followed by a Tukey's post-hoc test was conducted on SPSS version 10.0 (IBM Co., Armonk, NY, USA) to determine the significant difference of methane oxidation rates, soil ^{13}C-atom abundances, *pmoA* gene abundances between different treatments.

RESULTS

Accumulation of soil organic carbon by methanotrophy

Assuming linear kinetics, following 7-27 days of soil incubation for the same amount of CH_4 consumption, the highest CH_4 oxidation rate was observed in SG soil, and the other three soils showed similar but significantly lower rates of CH_4 oxidation (Figure 1a). The soil ^{13}C-atom abundances were significantly increased from background 1.08% to 2.36% (SG), 1.88% (KT), 2.78% (MS) and 2.04% (GZ), with a mean value of 2.27% (Figure 1b). The net production of soil ^{13}C-SOC calculated was 167, 171, 307 and 120 mg kg^{-1} from the "High $^{13}CH_4$" sample treatments of SG, KT, MS and GZ soils, respectively (Table 2). The ratios of methane-derived carbon converted to newly accumulated soil organic carbon were 32.54%, 33.55%, 60.02% and 23.60% in SG, KT, MS and GZ soils, respectively (Table 2).

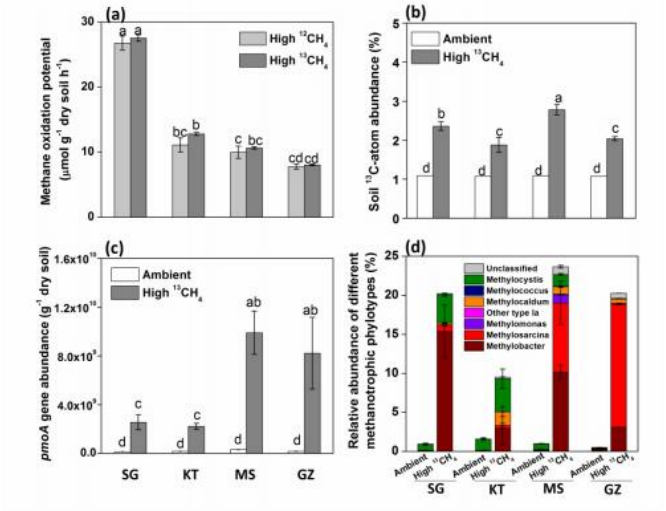

Fig. 1 Methane oxidation potentials, ^{13}C-atom abundances, abundances and compositions of methanotrophs in four paddy soils of Bangladesh following microcosm incubation at ambient air condition or after amendment of ^{13}C-labelled or unlabeled methane. (a) Methane oxidation potential was estimated as the linear rate of methane consumption by soils; (b) The ^{13}C-atom percent in soil organic carbon were measured to assess the assimilation of methane-derived carbon by soil methanotrophs after incubation; (c) The abundance of methanotrophs were estimated by quantification of *pmoA* genes using the real-time quantitative PCR assays; (d) The compositions of methanotrophic taxon from the 16S rRNA gene sequences of soil total DNA. "Ambient" represents incubation under natural atmospheric air condition, and "$^{12}CH_4$" and "$^{13}CH_4$" refer to the soil microcosm incubation amended with 5% v/v $^{12}CH_4$ and $^{13}CH_4$, respectively. The error bars represented the standard errors of triplicate microcosms.

Table 2

Methane oxidation and soil organic carbon accumulation in ^{13}C-CH4 amended soils.

Soil	$^{13}CH_4$ oxidized (μmol g^{-1} *d.s*)	Soil Organic Carbon (g kg^{-1})	^{13}C-SOC increase (mg kg^{-1})	Conversion ratio of CH_4 to soil SOC (%)	Methane production rate (nmol g^{-1} d.s. day^{-1})
SG	39.54±1.40a	13.1±0.01c	167±14.9b	32.54%	64±4.65a
KT	39.23±1.41a	21.3±0.02a	171±39.4b	33.55%	10±0.98d
MS	39.34±1.52a	18.2±0.02b	307±23.8a	60.02%	33±3.24b
GZ	39.12±0.65a	12.5±0.01c	120±6.61c	23.60%	22±0.54c

Note: The abbreviation of SG, KT, MS and GZ is the same as Table 1. SOC: soil organic carbon.

Dynamics of methanotrophic communities

Being consistent with the incorporation of methane-derived carbon into soil organic matter, the abundances of methanotrophs, estimated by qPCR of *pmoA* genes, exhibited a significant increase in soils following incubation under high concentration of methane (Figure 1c). The addition of methane led to an about 1.2-4.5-fold increase in *pmoA* gene abundance in soils (Figure 1c). Similarly, high-throughput sequencing of 16S rRNA genes from the total DNA extracts showed a 5.9-39.2 fold increase in the relative abundance of the methanotrophs following the methane-amended incubation compared to ambient air control (Figure 1d). Particularly, *Methylobacter* and *Methylosarcina*-affiliated type Ia methanotrophs consisted of the most abundant lineages of methanotrophs in SG, MS and GZ soils, while a similar abundance of *Methylocystis*-affiliated type IIa and *Methylobacter*-affiliated Ia methanotrophs were observed in KT soil (Figure 1d).

Stable-isotope probing of ^{13}C-methanotrophs

Following the isopycnic centrifugation of the extracted DNA, quantification of *pmoA* genes in each fractionated DNA extract demonstrated active cell propagation of methanotrophs (Figure 2a), being consistent with ^{13}C increase of SOC (Figure 1b) in all four soils fed with high concentrations of $^{13}CH_4$. The *pmoA* gene was mostly detected in the "light" fractions (with CsCl buoyant density around 1.715 g ml^{-1}) of "High $^{12}CH_4$" control treatment, but the highest abundance of *pmoA* genes moved toward "heavy" DNA fractions (buoyant density around 1.735 g ml^{-1}) in the "High $^{13}CH_4$" treatments (Figure 2a).

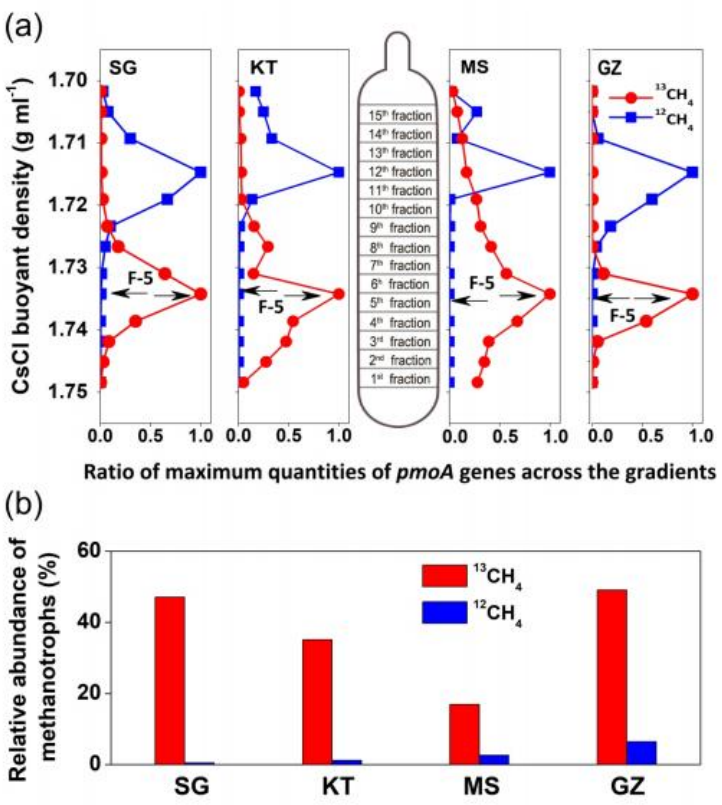

Fig.2 The distributions of ^{13}C-labelled methanotrophs based on the qPCR of *pmoA* genes and the amplicon sequencing of 16S rRNA and *pmoA* genes following DNA-based stable isotope probing of high $^{13}CH_4$ amendments. (a) The quantitative distribution of *pmoA* genes across the entire buoyant density gradient of the DNA fractions from soil microcosms incubated with either ^{13}C-labelled or unlabeled CH_4 as control. The normalized percentages represent the ratios of the *pmoA* gene number for each DNA gradient to the maximum number for each treatment. (b) The percent of methanotroph-affiliated reads in the total 16S rRNA genes sequenced from the heavy DNA fraction (fraction-5) of both the $^{13}CH_4$- and $^{12}CH_4$-treatments, respectively.

The labeling of methanotrophs were further supported by high-throughput 16S rRNA gene sequence analsysis of the whole microbial communities in the heavy DNA fractions (fraction No. 5) from both the high $^{13}CH_4$ and $^{12}CH_4$ amendments. The relative abundances of methanotrophs-affiliated 16S rRNA genes in the ^{13}C-DNA were 49.1% (SG), 47.1% (GZ), 35.1% (MS) and 16.9% (KT), and low abundance of 6.4% (SG), 0.5% (GZ), 1.2% (MS) and 2.6% (KT) in the heavy DNA from control treatment. It thus suggested that methanotrophs were ^{13}C-labeled and spun down to the lower fraction of ultracentrifugation tube with heavy buoyant density, leading to significantly enriched abundance of methanotrophs in the ^{13}C-DNA from the "High $^{12}CH_4$" treatment (Figure 2b).

Active methanotrophs in soils

To identify the taxonomic identity of active methanotrophs, phylogenetic analyses were performed on both ^{13}C-labeled 16S rRNA and *pmoA* genes amplified from the heavy DNA fraction of "High $^{13}CH_4$" treatment. The taxonomic classification of ^{13}C-labeled methanotrophic 16S rRNA genes revealed that type Ia methanotrophs dominated active methane oxidizers in SG, MS and GZ soils, but the compositions of active type Ia-related methanotrophs in these three soils were different. Particularly, *Methylobacter*-like phylotypes were the most abundant phylotypes accounting for about 63.4% of all methanotrophs in soil SG, while *Methylosarcina*-like phylotypes contributed 66.6% of active methanotrophs in soil GZ (Figure 3 and Supplementary Figure S2). In MS soil, however, *Methylobacter* and *Methylosarcina*-like phylotypes showed similar relative abundances. The analysis of ^{13}C-labeled *pmoA* gene revealed a generally higher relative abundance of type Ia in these soils compared to 16S rRNA gene analysis, i.e., the predominance of either *Methylobacter* or *Methylosarcina* (Figure 3 and Supplementary Figure S3). Notably, in KT soil, *Methylocystis* was the most abundant active methanotrophs (77.5%) based on the 16S rRNA gene phylogenetic classification; It should also be noted that the phylogeny of ^{13}C-labeled *pmoA* genes revealed most abundant was the *Methylobacter* related type Ia methanotrophs in this soil (Figure 3).

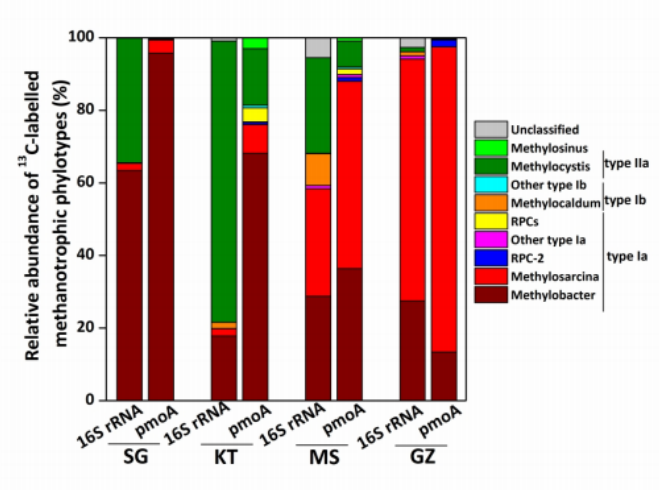

Fig. 3 The compositions of ^{13}C-labelled methanotrophs based on MiSeq amplicon sequencing of the 16S rRNA and *pmoA* genes following DNA-based stable isotope probing of high $^{13}CH_4$ treatment.

DISCUSSIONS

The present study employed a stable isotope tracing technique to investigate the contribution of methane oxidizers to the soil organic carbon accumulation. Following an incubation of four rice paddy soils with methane concentration that was in the range found in this environment (50000 ppmv $^{13}CH_4$) under field conditions (Shrestha *et al.*, 2008), soil ^{13}C contents significantly increased in all paddy soils (Figure 1b). The newly accumulated ^{13}C in these soils might represent viable soil microbiota and/or dead biotic biomass, but it should derive exclusively from methanotrophy metabolisms (either directly or *via* the methane-driven food web) as methanotrophs are capable of using methane as sole carbon and energy source for their growth. It is estimated that on average, 37.4% of the methane-carbon was assimilated by the MOB to synthesize cell organic molecules in soils studied (Table 2), which was comparable to atmospheric methane assimilation rate of 31 to 43% found in the forest soils (Roslev *et al.*, 1997). Flooded paddy fields are an important source of atmospheric methane, releasing up to 36 Tg methane gas globally per year (Saunois *et al.*, 2016). However, this might account for a minor part of the methane produced from the paddy fields, and the majority of methane (up to 80%) has already been consumed by methanotrophs before being released to the atmosphere (Frenzel *et al.*, 1992). Therefore, up to 1.07 Tg CH_4 yr^{-1} were microbially consumed (Khan and Saleh, 2015), which could contribute to 0.25 to 0.64 Tg methane-derived SOC, assuming that the conversion rates of methane-derived carbon to SOC varied from 23.6% to 60.0% in this study (Table 2). It should be noted that from an ecosystem level perspective, plant-driven methanogenesis may also play more important roles on soil organic matter accumulation by delivering large amounts of substrates that can be utilized by specific trophic guilds of Bacteria and/or Archaea. Meanwhile, at different level inorganic species (particularly ammonium) could have profound impacts on methanotrophic communities and methanognesis direcely andor indirectly (Bodelier and Laanbroek, 2004). Nontheless, not only can methanotrophs relieve the emission of the greenhouse gas methane from paddy soils, they can also mitigate the loss of SOC due to soil heterotrophic respiration and to stabilize the storage of soil organic matter in waterlogged paddies (Stein, 2020; Valenzuela *et al.*, 2020). Multi-omics approaches and single-cell techniques in future may provide important tools to deciphere these complex interactions at the level of whole microbial community in soil.

Methanotrophs rely on methane-derived carbon assimilation for growth while oxidizing methane to carbon dioxide for energy acquisition. The methane carbon assimilated by methanotrophs was used for the synthesis of the carbon backbone of the organic molecule (Hanson and Hanson, 1996), including nucleic acids. Therefore, ^{13}C-DNA-based stable isotope probing can be used as a powerful tool for tracking the carbon assimilation by a diverse guild of microorganisms that perform methane oxidation and evaluating their relative contribution to methane uptake and soil food web (Radajewski *et al.*, 2003). In the present study, *pmoA* genes were greatly enriched in the heavy DNA fractions from soils fed with $^{13}CH_4$ (Figure 2a), suggesting the active growth of methanotrophs supported by methane-carbon assimilation and successful ^{13}C-labeling of these organisms. The proportion of methane oxidizers could account for up to >45% of the total ^{13}C-labeled microbes based on the 16S rRNA gene sequence analysis (Figure 2b), and was much lower (<1%) in the heavy DNA fractions from samples without supplemented high concentrations of methane as control treatment. This suggested MOB can store a considerable amount of methane-derived carbon through biomass synthesis and cell propagation under the high methane concentration, such as in inundated wetland ecosystems. In addition, it is likely that the ^{13}C-labelling of other heterotrophic microorganisms could be associated with the methane oxidation (Murase and Frenzel, 2007).

Type Ia methanotrophs showed high growth rate in all soils following methane amendment (Figure 1d), and became the dominant community of active methanotrophs in three of the all soils (Figure 3), being consistent with previous observations that high-concentration of methane stimulated the growth of type I rather than type II methanotrophs (Krause *et al.*, 2012). However, it should be noted that the concentration of methane in this study was exceptionally high up to ~5%, being significantly higher than those commonly observed in the dissolved water of wetland under field conditions (Zhang *et al.*, 2010). Intriguingly, type II methanotrophs were also detected with potentially high methane oxidizing activity based on ^{13}C-labelled 16S rRNA gene analysis in KT soil (Figure 3, Supplementary Figure S2), which was not frequently observed in rice paddy soils (Sultana *et al.*, 2019). It was proposed that soil pH might have played an important role in the distribution of active methanotrophs in paddy soils, and dominant type II MOB methanotrophic activity would most likely occur in acidic environments (Zhao *et al.*, 2020). However, the KT soil with potential high methanotrophic activity in the present study had the highest pH 6.84 among all four soils tested (Table 1), indicating that the other environmental factor(s) might also be important in modulating the activity and growth of type II methanotrophs in this soil. It is noteworthy that phylogenetic analysis of ^{13}C-*pmoA* genes from KT soil revealed more abundant type Ia MOB participating in methane oxidation. This discrepancy by analyzing different marker genes could be due to primer bias, and it appeared that the *pmoA* primers used here resulted in more frequent detection of type I methanotrophic communities (Figure 3). Nevertheless, both the *pmoA* and 16S rRNA gene phylogenies confirmed the relatively higher activity of type II methanotroph in methane consumption in KT soil compared to type II methanotrophs activities in the other three soils.

It has been explicitly summarized about the diversity and habitat preferences of aerobic methanotrophs in physichemically distinct environment (Knief, 2015). The long-term adaption of type I and type II methanotrophs to abiotic and/or biotic factors have evolved distinct phenotypes such as cell sizes, morphology and specific growth rate. Microbes are considered the engine that drives the global elemental cycling (Falkowski et al., 2008), but the contribution of microbial biomass to soil organic matter from the specialized functional guilds is rarely investigated (Miltner *et al.*, 2012). Methane oxidation is an ecologically and agriculturally important process with significance consequence (Knief, 2015), and it appears that the conversion rates of CH_4 to SOC are affected by the size of methanotrophic communities as expressed by the relative abundance of methanotrophic phylotypes, although the rates were less affected by the presence of Type I methanotrophs (Figure 1). For instance, the influence of Type I *Methylobacter* was not apparent in the conversion of CH_4 to soil C in SG soil (Table 2). Intriguingly, the fixed carbon derived from methane (Table 2) accounted only for a small fraction of total soil organic carbon pool (Supplementary Table S2), representing 2.19%, 3.64%, 5.59% and 1.59% in $^{13}CH_4$-amended paddy soils of SG, KT, MS and GZ, respectively. Meanwhile, our results also showed significant labeling of the obligate methanol oxidizers Methylobacterium in SG, MS and GZ soils. Crossing-feeding of methylotrophs on methanol released during methane oxidation has been demonstrated before (Zheng et al., 2014), but what compound actually was exchanged have not been fully deciphered yet. Future study is warranted towards a more accurate budget of methane-driven accumulation and turnover of SOM in a wide variety of wetland ecosystems.

Due to the limited number of soil samples used in this study, confident correlations cannot be made between multiple environmental factors tested and the variations in methanotrophic compositions (supplementary Table S3). However, there are some discernible patterns which might be of importance in explaining the distribution of different methanotrophic phylotypes in the rice paddy fields. For instance, the methane production rate varied in four different soils (Table 2 and Supplementary Figure S4),

according to our flooded sample incubation under anaerobic conditions. It is also interesting to note that the highest relative abundance of *Methylobacter*-affiliated type Ia methanotrophs in SG soil with the highest methane production rate, while KT soil with the lowest methane production contained the most abundant *Methylocystis*-affiliated type IIa methanotrophs (Figure 3). Since our incubation was conducted after a flooding procedure and replacing all the headspace gas with N_2 in the bottles, the emitted methane gas should mostly originate from the methanogenic archaea in the soils without being partially oxidized by methanotrophs. Therefore, the methane production rate observed in the microcosms might well reflect the potential activities of methanogens. Methanogens and methanotrophs are the most important microbial groups in methane cycles and closely associated with each other in inundated rice paddy fields (Conrad, 2007), and different combination of dominant methanogenic and methanotrophic phylotypes were indeed observed in previous studies on rice paddy fields (Lee *et al.*, 2014; Liu *et al.*, 2016; Vaksmaa *et al.*, 2017). It is thus reasonable to speculate that the selection of different types of methanotrophs might be related to the methane-producing organisms and dynamics in soils. Meanwhile, the KT soils with more abundant active type II methanotrophs also had the highest pH and cation exchange capacity. Nonetheless, the emission of CO2 and likely N2O was not measured in this study, and the production of these greenhouse gases is closely associated with microbial oxidation of CH4 by aerobic methanotrophs (Stein, 2020). The total global warming potential considering all greenhouse gases would provide a more comprehensive view on the sequestration and respiration of soil organic carbon in paddy field. Given the abiotic properties greatly influence the assembly of active methanotrophs (Kaupper *et al.*, 2020), future studies with adequate samplings and/or maniupulation of abiotic factors warrants attention to decipher the biochemical processes responsible for microbial turnovers of CH_4, CO_2 and N_2O in soils

It should be noted that soil organic carbon is relatively stabilized in soils (Table 1), but apparent increase was observed in soils after microcosms incubation (Table 2). The discrepancy may likely result from the different details of soil pretreatments as SOC in background soil samples was determined immediately upon the arrival of soil samples at relatively coarse texture. However, all soil samples were subjected to the same degree of homogenization, and the significant enrichment of ^{13}C atom percent in SOC provide strong evidence for the net increase of SOC in soils. Meanwhile, methanotrophy-induced SOC may be closely associated with diazotrophy in paddy soil as previously shown in peatland (Larmola *et al.*, 2014). Accumulation of SOC can lead to high C/N ratio, thus trigging biological nitrogen fixation. $^{15}N_2$ tracing indicated that up to 45 kg N ha^{-1} could be fixed by biological nitrogen fixation per year in the paddy soil that received no fertilizers (Bei *et al.*, 2013). Recent studies provide solid evidence on taxonomic identities of active diazaotrophs linked to nitrogen fixation activity in paddy soil by DNA-SIP (Li *et al.*, 2019). Understanding the retention time of fresh microbial biomass and its coupling with nitrogen fixation would be the key for better understandings of microbially-mediated process on soil health.

CONLUSIONS

In this study, we revealed a significant incorporation of methane-derived carbon into soil organic matter through microbial biomass buildup associated with microbial methane oxidation under high methane enriched gas in four different rice paddy soils of Bangladesh. The oxidation and assimilation of labeled ^{13}C-CH_4 occurred in association with methanotrophy, providing strong evidences for accumulation of soil organic carbon (^{13}C-SOC) via methane-driven trophic web. The conversion efficiency of methane-derived carbon into newly input of soil organic carbon varied greatly among soil samples studied, and it could be explained by the labeling of phylogenetically distinct methanotrophs. Type Ia lineages dominated methanotrophy-mediated accumulation of soil organic carbon in three soils while type IIa-methanotrophs was more actively engaged in methane-carbon turnover in one soil sample. Our study highlight the important roles of methanotrophs not only on reducing greenhouse gas emission but also on sustaining soil organice matter.

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