

## Impact of Methanotrophic and N<sub>2</sub>O-reducing Bacterial Inoculation on CH<sub>4</sub> and N<sub>2</sub>O Emissions, Paddy Growth and Bacterial Community Structure in Paddy Field

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### ABSTRACT

Paddy field is one of the anthropogenic sources that produce greenhouse gases emission. This study aimed to investigate the impact of methanotrophic and N<sub>2</sub>O-reducing bacterial inoculation on CH<sub>4</sub> and N<sub>2</sub>O emissions, paddy growth and bacterial community structure in paddy field. Two treatments of 100% synthetic fertilizer (250 kg urea/ha) without biofertilizer and 50% synthetic fertilizer (125 kg urea/ha) with biofertilizer consisted of methanotrophic and N<sub>2</sub>O-reducing bacteria were applied in the paddy field. Inoculation of methanotrophic and N<sub>2</sub>O-reducing bacteria was able to reduce CH<sub>4</sub> and N<sub>2</sub>O emission up to 4.19 mg CH<sub>4</sub>/m<sup>2</sup>/day and 351.29 µg N<sub>2</sub>O/m<sup>2</sup>/day, respectively. Those bacterial applications were also able to increase paddy growth and yield productivity. According to DGGE profile, inoculation of the biofertilizer seemed to have a transient impact on bacterial communities in paddy soil at 36 days after transplanting (DAT) which showed the lowest similarity with all samples (a similarity index of 0.68). DGGE bands successfully excised have closest relative to uncultured bacteria which comprised 5 phyla, i.e. Proteobacteria (Alphaproteobacteria and Deltaproteobacteria), Nitrospirae, Actinobacteria, Firmicutes, and Acidobacteria. In this study, Alphaproteobacteria was the most dominant phylum. We provide basic information for developing the biofertilizer which supports sustainable agriculture.

## 1. Introduction

Paddy cultivation is one of the anthropogenic activities that produce greenhouse gases (GHG) emission, such as methane (CH<sub>4</sub>) and nitrous oxide (N<sub>2</sub>O) with a global warming potential (GWP) of 25 and 298 times higher than CO<sub>2</sub> (100-year horizon), respectively (IPCC 2007). Irrigation and fertilizer treatment intensely applied in paddy fields can promote GHG emissions. Indonesia is one of the top five countries contributing to atmospheric CH<sub>4</sub> and N<sub>2</sub>O emissions from paddy fields in 2010 (USEPA 2013). CH<sub>4</sub> is anaerobically produced by methanogens, then aerobically oxidized by methanotrophic bacteria in the oxidative layer of the soil. Conrad and Rothfus (1991) reported that 80% of CH<sub>4</sub> in paddy fields is oxidized by methanotroph. Meanwhile, N<sub>2</sub>O emission

is primarily generated through denitrification and nitrification process under flooding and drying period, respectively (Davidson *et al.* 1986). N<sub>2</sub>O can be reduced to N<sub>2</sub> by denitrifying bacteria possessing *nosZ* gene which encodes nitrous oxide reductase enzyme (Nos) (Henry *et al.* 2006). On the other hand, rice is the main staple food for 90% of the population in Asian countries, including Indonesia. Rice demand is estimated to increase as an increase in population growth. Consequently, it is necessary to develop environmentally friendly paddy cultivation which enhances paddy productivity without the increase of GHG emission or even be able to reduce CH<sub>4</sub> and N<sub>2</sub>O emission.

Most of the studies have focused to develop the management methods for mitigating GHG emissions (Yagi *et al.* 1996; Majumbar 2003; Malyan *et al.* 2016), while there are only few studies considering other methods. Manipulation of microbial communities in the soil might become one of the mitigation

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strategies in anthropogenic greenhouse gas emission (Singh *et al.* 2010). Therefore, inoculation of biofertilizer consisting of methanotrophic and N<sub>2</sub>O-reducing bacteria is considered to be an innovative approach to reduce CH<sub>4</sub> and N<sub>2</sub>O emission while at the same time it increases paddy productivity. In the previous studies, *Methylocystis rosea* BGM1 and *Methylobacter* sp. SKM14 was found to have *pmoA* gene, while *Methylococcus capsulatus* BGM9 has *mmoX* gene encoding enzymes involved in CH<sub>4</sub> oxidation (Rusmana and Akhdiya 2009). Additionally, *Methylocystis parvus* BGM3 and *M. capsulatus* BGM9 are known to have *nifH* and *nifD* gene (Bintarti *et al.* 2014), encoding dinitrogenase reductase enzyme and subunit of dinitrogenase enzyme which play an important role in N<sub>2</sub> fixation (Dedysh *et al.* 2004). N<sub>2</sub>O-reducing bacteria, *Ochrobactrum anthropi* BL2, proved to be able to reduce N<sub>2</sub>O by 5.41 µmol per ml of culture (Setyaningsih *et al.* 2010). In field experiments, methanotrophic and N<sub>2</sub>O-reducing bacteria were able to reduce CH<sub>4</sub> and N<sub>2</sub>O emission and enhance paddy growth and productivity in Sukabumi, Indonesia (Pingak *et al.* 2014; Sukmawati *et al.* 2015). Masrukhin *et al.* (2017) recently reported that inoculation of methanotrophic and N<sub>2</sub>O-reducing bacteria did not affect the total bacterial community in paddy fields in Sukabumi district, Indonesia. Microbial application in the fields often generates inconsistency in results. To evaluate the effectivity of methanotrophic and N<sub>2</sub>O-reducing bacteria, it is needed to conduct multi-location testing.

The objectives of this study were to evaluate the effectiveness of methanotrophic and N<sub>2</sub>O-reducing bacteria to reduce CH<sub>4</sub> and N<sub>2</sub>O emission and increase paddy growth and productivity in Tegal district, Indonesia. In addition, this study analyzed the impact of those bacteria on bacterial community structure in the paddy field using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE).

## 2. Materials and Methods

### 2.1. Study Sites

The field experiment was conducted in Tegal, Central Java, Indonesia (7°02'44.8"S, 109°08'16.6"E). The weather parameters during the study period were 35.4°C maximum temperature, 22.6°C minimum temperature, 79% relative humidity and 2,211 mm annual rainfall. Paddy soil consisted of 23.31% of sand, 75.98% of silt, and 0.71% of clay, and classified

as silt loam soil based on United States Department of Agriculture. The soil is neutral soil (pH ~6.55) containing 3.30% of C and 0.12% of N categorized as high and low category, respectively. Ca<sup>2+</sup> and Mg<sup>2+</sup> concentration of the soil was very low (0.08 and 0.06 cmol/kg, respectively), K<sup>+</sup> concentration was low (0.29 cmol/kg), but Na<sup>+</sup> concentration was very high (1.99 cmol/kg).

### 2.2. Biofertilizer Preparation

Biofertilizer used in this experiment consisted of methanotrophic and N<sub>2</sub>O-reducing bacteria. Each methanotrophic bacterium (*Methylocystis rosea* BGM1, *Methylocystis parvus* BGM3, *Methylococcus capsulatus* BGM9, and *Methylobacter* sp. SKM14) was grown in nitrate mineral salt (NMS) liquid medium supplemented with 1% methanol (v/v). Meanwhile, N<sub>2</sub>O-reducing bacterium, *Ochrobactrum anthropi* BL2, was grown on denitrification liquid medium. Those bacteria were incubated at room temperature (±28°C) for 7-10 days until the total number of cells was 10<sup>8</sup> cells/ml. Bacterial cultures with the same volume were mixed before inoculated in the field.

### 2.3. Paddy Cultivation and Measurement of Paddy Growth and Yield

The land management and paddy cultivation system were conducted based on local farmer's practices. The experiment was carried out using a randomized block experiment with two treatments, i.e. without biofertilizer (B0) and with biofertilizer (B1). B0 was conducted with the application of 100% synthetic fertilizer (250 kg urea/ha) without biofertilizer, while B1 was performed by the application of 50% synthetic fertilizer (125 kg urea/ha) with biofertilizer. Both treatments were carried out at 3 blocks, where each block was divided into 2 plots designated for B0 and B1 in which the total area were 1,400 and 1,500 m<sup>2</sup>, respectively. The 25-day-old seedlings of Indonesian rice cultivar 'Memberamo' (*Oryza sativa*) were transplanted with the spacing of 20 x 20 cm (three plants per hill). The transplanting day was marked as 0 days after transplanting (DAT). Biofertilizer application was conducted by soaking the root of 25-day-old seedlings (root inoculation) in bacterial cultures for ±15 minutes before being transplanted in the fields, then given urea. Urea application on both treatments was carried out at 15 DAT. Paddy plants were grown under continuous flooding until two weeks before harvesting, then the plants were not irrigated until

the end of the experiment. Paddy plants growth and yield were randomly observed at 36, 69, and 106 DAT. Data analysis was performed by Independent t-test and Mann-Whitney U test using SPSS Statistics 22 for Windows (SPSS Inc., Chicago, IL, US).

#### 2.4. Measurement of CH<sub>4</sub> and N<sub>2</sub>O Emissions Rate

Gas sampling was randomly conducted using the lid closed chamber with a size of 55.5 x 55.5 x 89.9 cm<sup>3</sup> (L x W x H) that covers several paddy plants in each treatment. Gas samples were collected using a 10 ml syringe shortly after installation of the lid (t = 0) till 3 h after installation of the lid chamber (t = 3) at the rice vegetative and generative phase. CH<sub>4</sub> and N<sub>2</sub>O concentration were measured by gas chromatography equipped with flame ionization detector (FID) and electron capture detector (ECD), respectively. The increase of gas concentration for specific time intervals was used to calculate the CH<sub>4</sub> and N<sub>2</sub>O emissions rate as described previously by IAEA (1992).

#### 2.5. Soil Sampling and DNA Extraction

Soil sampling was carried out using the soil core sampler at 0 (before biofertilizer inoculation), 36 (rice vegetative phase), 69 (rice generative phase), and 106 DAT (harvesting day). Soil samples were randomly collected in triplicate from the site in the neighborhood of 10 cm of roots in each treatment. The same amounts of soil samples from the same treatment were well mixed to obtain a homogeneous composite sample representing the overall microbial communities from each treatment. DNA extraction from composite soil samples was conducted using DNeasy Powersoil Kit (Qiagen, Hilden, DE) based on the manufacturer's instruction. Quantity and purity of DNA were determined using NanoDrop 2000c (NanoDrop Technologies, Wilmington, DE, US), then DNA was stored at -20°C until use.

#### 2.6. PCR-DGGE

Amplification of 16S rRNA genes was performed by T1-Thermocycler (Biometra, Göttingen, DE). The V3 region of bacterial 16S rRNA genes was amplified using primer set, i.e. PRBA338f-GC (5'-CGCCCGCCGCGCGCGGGCGGGCGGGGGCACGGGGGGACTCCTACGGGAGGCAGCAG-3') and PRUN518r (5'-ATTACCGCGGC TGCTGG-3') (Øvreås *et al.* 1997). PCR reaction was performed in a total volume of 25 µl containing 12.5 µl GoTaq Green Master Mix 2x (Promega, Madison, WI, US), 1.25 µl primer forward (10 pmol), 1.25

µl primer reverse (10 pmol), 1 µl template DNA (~100 ng), and 9 µl nuclease-free water. The 16S rRNA genes amplification is started by an initial denaturation at 94°C for 5 min, followed by 25 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec, and extension at 72°C for 30 sec, then a final extension at 72°C for 5 min. PCR product of 16S rRNA gene amplified using the primer is approximately 200 bp. DGGE fingerprint was performed by DCode™ Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA, US). PCR product of 16S rRNA gene (22 µl) added with loading dye (5 µl) were separated in 8% (w/v) polyacrylamide gel (acrylamide-bisacrylamide [37.5:1]) with linear denaturant gradient ranging from 40-60%. 100% denaturant contains 7 M urea and 40% (v/v) formamide. The gel was electrophoresed at 100 V for 2 h, then 60 V at 10 h under 60°C in 1x TAE buffer (40 mM Tris base, 20 mM glacial acetic acid, 1 mM EDTA, pH 8.0). The gel was stained with ethidium bromide for 15 min, then visualized by using G:BOX Gel Documentation (Syngene, Frederick, MD, US). To phylogenetic analysis, each DGGE band was excised and eluted in 100 µl nuclease-free water under 4°C overnight. Eluted DNA was used as the template for the reamplification process using primer PRBA338f and PRUN518r without GC clamp under the same condition as above, then the reamplified PCR products were sequenced.

#### 2.7. Analysis of DGGE Profile

DGGE profile, band pattern in polyacrylamide gel, represents bacterial community structure. The band in DGGE profile corresponds to at least one unique nucleotide sequence of 16S rRNA fragments, representing a distinct dominant bacterial population in the community. The intensity of the band was frequently used to estimate the relative abundance of each population. The appearance and disappearance of bands in DGGE profiles indicate the shift of bacterial community structure (Muyzer *et al.* 1993). The position and intensity of bands in each sample were analyzed by LabImage Platform (Kapelan Bio-imaging, DE). One band that appeared in polyacrylamide gel was categorized as one operational taxonomy unit (OTU). Clustering analysis was carried out by using Paleontological Statistics (PAST3) software (Hammer *et al.* 2001). Clustering analysis based on the appearance and disappearance of bands at specific position was assessed by using Dice index and the dendrogram was constructed by using UPGMA method.

## 2.8. Phylogenetic Analysis

Nucleotide sequences were aligned with available database to identify the closest relatives using the Basic Local Alignment Sequence Tools for Nucleotide (BLAST-N) program at NCBI website (<http://ncbi.nlm.nih.gov/>).

## 3. Results

### 3.1. Paddy Growth and Yield

Paddy growth parameters observed in this experiment were plant height, root length, root wet weight, shoot wet weight, root dry weight, and shoot dry weight. Paddy plant height was significantly higher in biofertilizer application at 36, 69, and 106 DAT, compared to without biofertilizer ( $p \leq 0.05$ ) (Figure 1). Plant height with biofertilizer application was higher 3.29% than that of plant height without biofertilizer at 106 DAT. Root length, root wet weight, shoot wet weight, root dry weight, and shoot dry weight. In biofertilizer application were significantly greater over without biofertilizer, i.e. 11.32%, 29.12%, 16.19%, 43.50%, and 31.48%, respectively. Paddy plants yield showed significant differences between biofertilizer application and without biofertilizer ( $p \leq 0.05$ ) (Table 1; Figure 2). Yield parameters treated by methanotrophic and  $N_2O$ -reducing bacteria were significantly higher than without biofertilizer ( $p \leq 0.05$ ). The number of panicles observed at 69 and 106 DAT in biofertilizer application was significantly greater 10.51% than without biofertilizer ( $p \leq 0.05$ ) (Figure 2). Panicle length, number of grains per

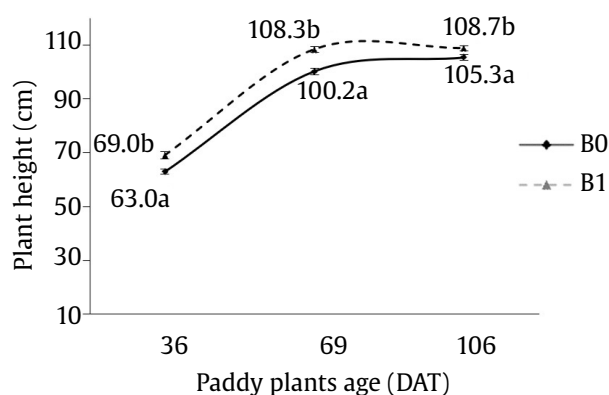


Figure 1. Paddy plants height without biofertilizer (B0) and with biofertilizer application (B1). The values followed by different letters in the same paddy plants age are significantly different according to Independent t-test and Mann-Whitney U test with  $p \leq 0.05$

panicle, and weight of 1,000 grains in biofertilizer application were significantly higher than that of the without biofertilizer ( $p \leq 0.05$ ), i.e. 9.09, 10.72, and 13.02%, respectively. In addition, the percentage of empty grains in biofertilizer application was significantly decreased by 13.03% compared to without biofertilizer ( $p \leq 0.05$ ). Grain yield of biofertilizer-inoculated plants was estimated at 49.99% higher than without biofertilizer ( $p \leq 0.05$ ).

Table 1. Paddy plants growth and yield without biofertilizer (B0) and with biofertilizer application (B1)

Parameters	Treatments	
	B0	B1
Growth parameters		
Root length (cm)	25.0a	27.9b
Root wet weight (g)	35.37a	45.67b
Shoot wet weight (g) <sup>a</sup>	161.43a	187.57b
Root dry weight (g)	8.20a	11.77b
Shoot dry weight (g) <sup>a</sup>	31.77a	41.77b
Yield parameters		
Panicle length (cm)	24.93a	27.20b
Number of grains per panicle	114a	126b
Percentage of empty grains (%)	22.77a	19.80b
Weight of 1000 grains (g)	27.01a	30.52b
Grain productivity estimation (ton/ha)	6.25a	9.38b

<sup>a</sup> represented shoot weight without grains

Different letters in the same parameter denote significant differences between without biofertilizer (B0) and with biofertilizer application (B1) at  $p \leq 0.05$  based on Independent t-test and Mann-Whitney U test

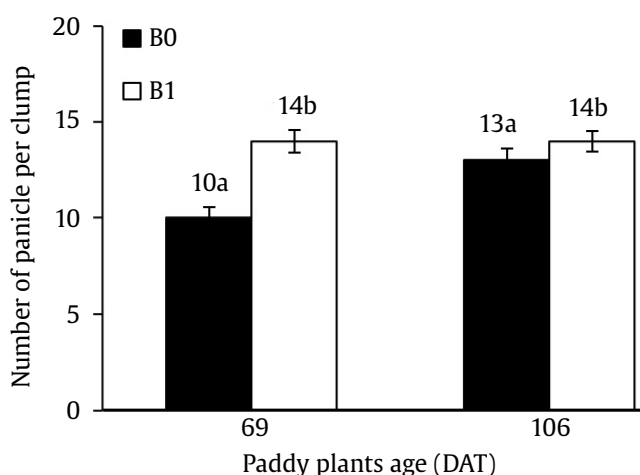


Figure 2. Number of panicles per clump without biofertilizer (B0) and with biofertilizer application (B1). The values followed by different letters in the same paddy plants age are significantly different according to Independent t-test and Mann-Whitney U test with  $p \leq 0.05$

### 3.2. CH<sub>4</sub> and N<sub>2</sub>O Emissions

Measurement of CH<sub>4</sub> and N<sub>2</sub>O emission rates was performed to determine the accumulation and absorption rate of CH<sub>4</sub> and N<sub>2</sub>O in both treatments during the rice vegetative and generative phase. CH<sub>4</sub> emission rate from paddy fields without biofertilizer was 6.11 and 1.32 mg CH<sub>4</sub>/m<sup>2</sup>/day at the rice vegetative and generative, respectively. On the other hand, paddy fields treated with biofertilizer was able to absorb CH<sub>4</sub> up to 4.11 mg CH<sub>4</sub>/m<sup>2</sup>/day at the rice vegetative phase and 4.19 mg CH<sub>4</sub>/m<sup>2</sup>/day at the rice generative phase (Figure 3). Meanwhile, N<sub>2</sub>O was emitted up to 248.11 µg N<sub>2</sub>O/m<sup>2</sup>/day from paddy fields without biofertilizer at the rice vegetative phase, then absorbed in the rice generative phase that reached 80.04 µg N<sub>2</sub>O/m<sup>2</sup>/day. A different trend was showed by paddy fields with biofertilizer, where N<sub>2</sub>O was absorbed up to 319.63 and 351.29 µg N<sub>2</sub>O/m<sup>2</sup>/day at the rice vegetative and generative phase, respectively (Figure 4).

### 3.3. Soil Bacterial Community Structure

According to the DGGE profile of each treatment, there were 38 different OTUs that appeared from the paddy soil samples in both the rice vegetative and generative phases. The number of OTUs in paddy soil sample without biofertilizer (23-29 OTUs) were generally greater than biofertilizer application (15-28 OTUs). We found the bacterial community shift, i.e. the decrease in the number of detected OTUs, but an increase in the intensity of some OTUs (Figure 5a;

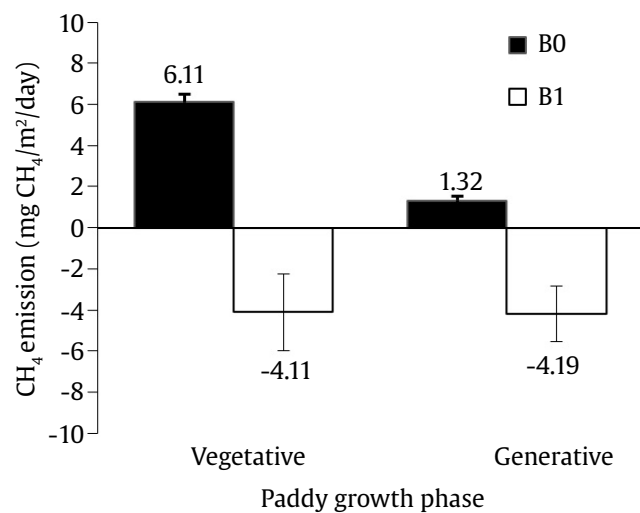


Figure 3. CH<sub>4</sub> emissions from paddy fields without biofertilizer (B0) and with biofertilizer application (B1) at the rice vegetative and generative phase

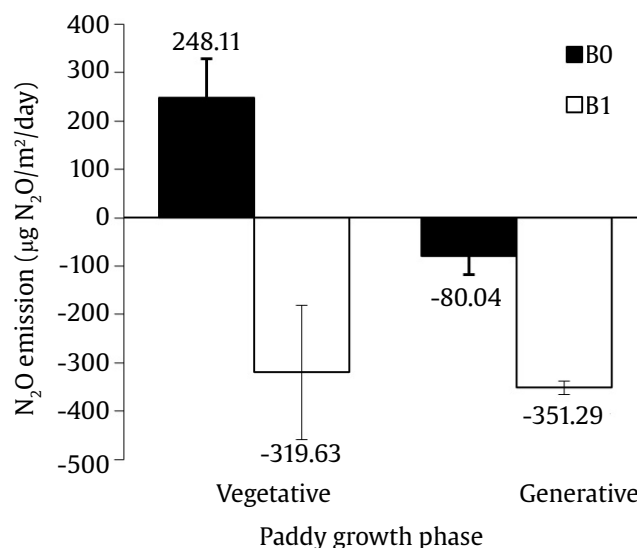


Figure 4. N<sub>2</sub>O emissions from paddy fields without biofertilizer (B0) and with biofertilizer application (B1) at the rice vegetative and generative phase

Table 2). The number of OTUs in paddy soil without biofertilizer application increased after transplanting to the highest level of 29 OTUs at 36 DAT (36B0), then it gradually decreased at 69 and 106 DAT (28 and 24 OTUs, respectively). On the other hand, the number of OTUs in biofertilizer application declined after transplanting to the lowest level of 15 OTUs at 36 DAT (36B1). Thereafter, these OTUs increased to 25 OTUs at 69 DAT (69B1) and then decreased to 24 OTUs at 106 DAT (106B1). In addition, there was one specific band in 0B1, 69B0, and 106 B0, respectively, and two specific bands that only appeared in 36B0 (Figure 5a).

There were 23 DGGE bands successfully excised from the polyacrylamide gel. Sequencing analysis showed that those bands have closest relative to uncultured bacteria including 5 phyla, i.e. Proteobacteria (Alphaproteobacteria and Deltaproteobacteria), Nitrospirae, Actinobacteria, Firmicutes, and Acidobacteria, during the entire paddy cultivation period (Table 3). Most of the bands belong to Alphaproteobacteria. Shift in bacterial composition occurred during the rice cultivation period (Table 2). In the paddy field without biofertilizer application, OTU 15 (deltaproteobacteria) and OTU 17 (type II methanotroph bacterium) were the predominant OTUs whose relative abundance ranged from 8 to 18% during the rice cultivation period (0, 36, 69, and 106 DAT). A different trend was observed in biofertilizer application where OTU 17 (type II methanotroph

Table 2. Relative abundance of bacterial OTUs in each sample

Treatments	Number of OTUs	Dominant OTUs	Percentage of dominant OTUs (%)	OTUs that appeared in all samples	Percentage of OTUs frequently appeared (%)
OB0	23	17 (15%), 15 (14%)	29	17 (15%), 15 (14%), 38 (10%), 18 (5%), 19 (5%), 23 (4%), 22 (3%), 29 (3%), 9 (2%), 10 (2%), 13 (2%), 34 (2%), 7 (1%)	68
OB1	28	17 (14%), 38 (9%)	23	17 (14%), 38 (9%), 19 (7%), 15 (7%), 18 (5%), 23 (5%), 13 (3%), 22 (3%), 34 (3%), 10 (2%), 29 (2%), 7 (1%), 9 (1%)	62
36B0	29	17 (18%), 15 (8%)	26	17 (18%), 15 (8%), 38 (7%), 18 (6%), 19 (5%), 23 (4%), 29 (3%), 10 (3%), 7 (2%), 13 (2%), 22 (2%), 34 (2%), 9 (1%)	63
36B1	15	17 (20%), 18 (12%)	32	17 (20%), 18 (12%), 23 (10%), 15 (9%), 19 (9%), 38 (8%), 13 (4%), 7 (3%), 10 (3%), 34 (3%), 9 (2%), 22 (2%), 29 (2%)	87
69B0	28	17 (17%), 15 (12%)	29	17 (17%), 15 (12%), 38 (7%), 18 (5%), 19 (5%), 23 (5%), 22 (2%), 29 (2%), 34 (2%), 7 (2%), 9 (2%), 10 (2%), 13 (2%)	65
69B1	25	17 (20%), 15 (13%)	33	17 (20%), 15 (13%), 38 (9%), 23 (7%), 18 (5%), 19 (4%), 10 (2%), 13 (2%), 22 (2%), 29 (2%), 34 (2%), 7 (1%), 9 (1%)	70
106B0	25	17 (18%), 15 (11%)	29	17 (18%), 15 (11%), 38 (7%), 18 (6%), 19 (4%), 22 (4%), 23 (4%), 13 (3%), 29 (3%), 7 (2%), 9 (2%), 10 (2%), 34 (2%)	68
106B1	24	38 (12%), 17 (11%)	23	38 (12%), 17 (11%), 15 (9%), 19 (6%), 23 (6%), 9 (4%), 18 (4%), 7 (3%), 10 (3%), 13 (3%), 22 (3%), 29 (3%), 34 (2%)	69

bacterium, 14%) and OTU 38 (Firmicutes bacterium, 9%) became predominant at 0 DAT. At 36 DAT, OTU 17 (type II methanotroph bacterium, 20%) and OTU 18 (*Methylocystis* sp., 12%) were predominant, then OTU 15 (deltaproteobacteria, 13%) and OTU 17 (type II methanotroph bacterium, 20%) were commonly found at 69 DAT. Dominant OTUs at 106 DAT in biofertilizer application were similar to 0 DAT, i.e. OTU 17 (type II methanotroph bacterium, 11%) and OTU 38 (Firmicutes bacterium, 12%). There were

some dominant OTUs in 36B0 that not detected in 36B1 (Figure 5a). The DGGE profile also showed 13 dominant OTUs appeared in both treatments along sampling time (Table 2). Those OTUs were affiliated to Acidobacteria, *Methylobacterium* sp., *Nitrospira* sp., deltaproteobacteria, Coriobacteriaceae bacterium, *Pseudolabrys* sp., Firmicutes bacterium, type II methanotroph bacteria (2 OTUs), and *Methylocystis* sp. (4 OTUs).

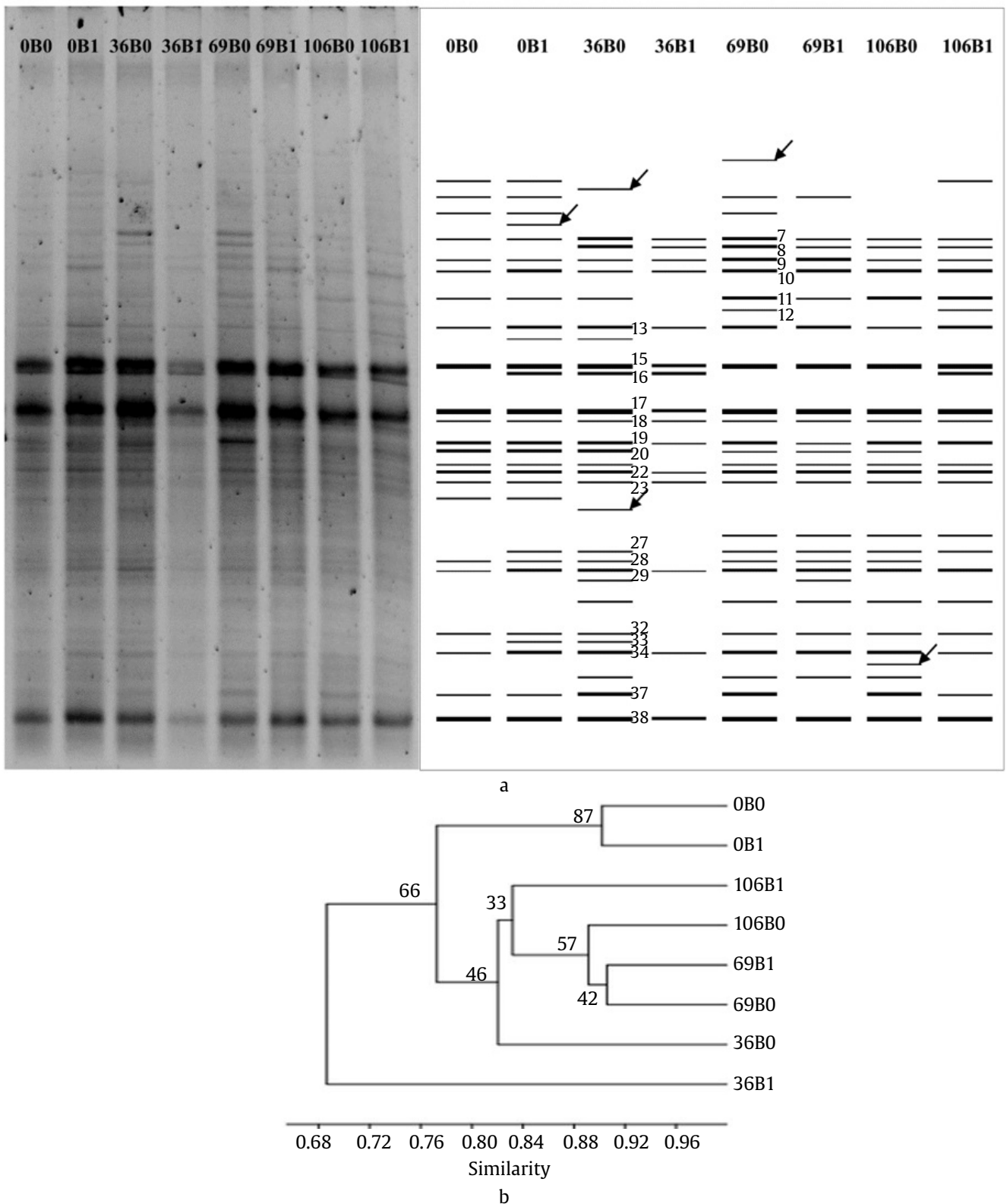


Figure 5. (a) DGGE profile of 16S rRNA gene from paddy soil (left), DGGE illustration by using LabImage Platform (right). A total of 23 bands marked by numbers in the right side were excised for sequencing analysis. Black arrows show the unique bands. (b) Clustering analysis of total bacteria in paddy fields. 0B0: without biofertilizer at 0 DAT; 0B1:with biofertilizer at 0 DAT; 36B0:without biofertilizer at 36 DAT; 36B1:with biofertilizer at 36 DAT; 69B0:without biofertilizer at 69 DAT; 69B1:with biofertilizer at 69 DAT; 106B0: without biofertilizer at 106 DAT; 106B1: with biofertilizer at 106 DAT

Table 3. Closest relative, identity, and accession number of 16S rRNA fragments excised from DGGE gel

OTU <sup>a</sup>	Closest relative	Phyla/subphyla	Identity (%)	NCBI accession number
7	Uncultured Acidobacteria bacterium clone AlertB07	Acidobacteria	91	JF508288.1
8	Uncultured <i>Methylocystis</i> sp. isolate DGGE gel band b1-1	Alphaproteobacteria	92	GU227561.1
9	Uncultured <i>Methylobacterium</i> sp. clone: SB-P-1-A11	Alphaproteobacteria	95	LC038999.1
10	Uncultured <i>Nitrospira</i> sp. isolate DGGE gel band PS-22	Nitrospira	92	JX163885.1
11	Uncultured <i>Methylocystis</i> sp. isolate DGGE gel band b1-1	Alphaproteobacteria	87	GU227561.1
12	Uncultured <i>Methylocystis</i> sp. isolate DGGE gel band b1-1	Alphaproteobacteria	90	GU227561.1
13	Uncultured <i>Methylocystis</i> sp. isolate DGGE gel band b1-1	Alphaproteobacteria	95	GU227561.1
15	Uncultured delta proteobacterium clone 1806	Deltaproteobacteria	89	KX367710.1
16	Uncultured <i>Methylocystis</i> sp. isolate DGGE gel band b1-1	Alphaproteobacteria	83	GU227561.1
17	Uncultured type II methanotroph isolate DGGE gel band LFWIII c44	Alphaproteobacteria	96	HM755803.1
18	Uncultured <i>Methylocystis</i> sp. isolate DGGE gel band b1-1	Alphaproteobacteria	92	GU227561.1
19	Uncultured <i>Methylocystis</i> sp. isolate DGGE gel band b1-1	Alphaproteobacteria	96	GU227561.1
20	Uncultured type II methanotroph isolate DGGE gel band LFWIII c44	Alphaproteobacteria	95	HM755803.1
22	Uncultured <i>Methylocystis</i> sp. isolate DGGE gel band b1-1	Alphaproteobacteria	95	GU227561.1
23	Uncultured type II methanotroph isolate DGGE gel band LFWIII c44	Alphaproteobacteria	95	LC065356.1
27	Uncultured <i>Methylocystis</i> sp. isolate DGGE gel band b1-1	Alphaproteobacteria	92	GU227561.1
28	Uncultured alpha proteobacterium clone NL10BD-01-G02	Alphaproteobacteria	89	FM252884.1
29	Uncultured Coriobacteriaceae bacterium clone Z3AcetMagB56	Actinobacteria	94	KX350886.1
32	Uncultured Acidimicrobiales bacterium clone 2363	Actinobacteria	89	KX368267.1
33	Uncultured Acidimicrobineae bacterium clone OTU1801	Actinobacteria	96	KM060560.1
34	Uncultured <i>Pseudolabrys</i> sp. clone B49	Alphaproteobacteria	96	MF449388.1
37	Uncultured <i>Anaeromyxobacter</i> sp. clone: UH-33	Deltaproteobacteria	89	AB265927.2
38	Uncultured Firmicutes bacterium clone B1	Firmicutes	85	EU620703.1

<sup>a</sup>The OTU number are indicated in Figure 5a

Clustering analysis was performed to evaluate the impact of biofertilizer inoculation on soil bacterial community structure. According to the dendrogram, bacterial community structure before biofertilizer application in both treatments (OB0 and OB1) was clustered in one group with a high similarity index (0.90). The result showed that inoculation of methanotrophic and N<sub>2</sub>O-reducing bacteria seemed

to have a transient impact on bacterial communities in paddy soil (Figure 5b). It was observed at 36 DAT (36B1) that had the lowest similarity index compared to other samples (0.68). Then, bacterial community structure in biofertilizer application at 69 and 106 DAT were relatively similar to bacterial communities from paddy fields without biofertilizer application with a similarity index of 0.91 and 0.83, respectively



(data not shown). Soil samples of 36B0, 69B0, 69B1, 106B0, and 106B1 also formed one cluster, where 69B0 and 69B1 were clustered with the highest similarity (0.91). It could denote the change of bacterial communities because of the biofertilizer inoculation which only occurred at 36 DAT, but it did not seem at 0, 69, and 106 DAT.

#### 4. Discussion

Several studies have demonstrated that methanotrophic bacteria (*M. rosea* BGM1, *M. parvus* BGM3, *M. capculatus* BGM9, and *Methylobacter* sp. SKM14) was capable of oxidizing CH<sub>4</sub> and fixing N<sub>2</sub> under laboratory condition (Rusmana and Akhdiya 2010). BGM1, BGM3, and BGM9 isolates had nitrogenase activity up to 10.9, 7.1, and 15.3 nM/h/ml culture, respectively (Maisaroh 2009). *Ochrobactrum anthropi* BL2 could reduce N<sub>2</sub>O *in vitro* (Setyaningsih *et al.* 2010). Methanotrophic and N<sub>2</sub>O-reducing bacteria were able to enhance plant growth and reduce CH<sub>4</sub> and N<sub>2</sub>O emission in the paddy fields in Sukabumi, Indonesia (Pingak *et al.* 2014; Sukmawati *et al.* 2015). The similar results were obtained in this study where inoculation of methanotrophic and N<sub>2</sub>O-reducing bacteria positively induced plant growth and yield (Figure 1; Figure 2; Table 1) and reduced CH<sub>4</sub> and N<sub>2</sub>O emissions from paddy fields (Figure 3; Figure 4). In this study, the yield of biofertilizer-inoculated plants was greater than that of without biofertilizer, which might be caused by the ability of methanotrophic bacteria in N<sub>2</sub> fixation. Nitrogen is a chemical compound used by plants in large quantities during their growing period, e.g. tillering and grain filling stage. In addition, the high growth of paddy plants might be resulted from the changes in root morphology, including root length, due to the interaction of inoculant bacteria, plant roots, and indigenous microbes. The benefits of this change is the more efficient acquisition of soil nutrients. This result is consistent with the previous studies which showed the plant growth enhancement, i.e. plant height, root length, shoot dry weight, and root dry weight in the biofertilizer application were higher than that of without biofertilizer up to 12.50%, 37.06%, 67.60%, and 143.32%, respectively. Number of panicles, grains per panicles and weight of 1,000 grains were 16.49%, 48.28%, and 42.07% higher in the biofertilizer application, and empty grains was lower in the biofertilizer application (Sukmawati *et*

*al.* 2015). Pingak *et al.* (2014) also demonstrated that plant height, shoot wet weight, shoot dry weight, and root dry weight 100% synthetic fertilizer treatment. Grain productivity improvement was also shown by the biofertilizer and 25% synthetic fertilizer treatment (62.5 kg/ha) and the biofertilizer and 20% synthetic fertilizer (50 kg/ha), whose grain productivities were greater up to 67.53 and 42.07% compared to those in the application of 100% synthetic fertilizer (250 kg/ha), respectively (Pingak *et al.* 2014; Sukmawati *et al.* 2015).

CH<sub>4</sub> emission was greater at the vegetative than the generative phase of paddy plants, as shown in the paddy field without biofertilizer application (Figure 3). In contrast, CH<sub>4</sub> emission in the paddy field inoculated with the biofertilizer was reduced in both paddy growth phases (Figure 3). It was due to the ability of methanotrophic bacteria in CH<sub>4</sub> oxidation. During the vegetative phase, the paddy plants were flooded, then dried in the late generative phase. The flooded conditions will decrease redox potential (Eh) of the soil which provides a favorable condition for methanogens to produce CH<sub>4</sub> (Malyan *et al.* 2016). Moreover, there are a higher amount of root exudate in the rice vegetative phase as substrates for methanogens (Aulakh *et al.* 2001). High concentration of fertilizer is commonly applied in the early and late vegetative phases. Nitrogenous fertilizer can enhance CH<sub>4</sub> production caused by the increase of plant growth along with an increase in root exudate (Bodelier *et al.* 2000). High concentration of ammonium-based fertilizer was able to inhibit the activity of methane monooxygenase involved in CH<sub>4</sub> oxidation (Mishra *et al.* 2018). On the contrary, low concentration of ammonium-based fertilizer may stimulate methane oxidation activity by bacteria which impact CH<sub>4</sub> emission reduction (Cai *et al.* 1997; Seo *et al.* 2014; Mishra *et al.* 2018). Nitrate addition could inhibit methanogenesis in paddy soils that cause a decrease in CH<sub>4</sub> emission (Wang *et al.* 2018). However, there was no obvious effect of nitrogenous fertilizer application on CH<sub>4</sub> emission (Adviento-Borbe *et al.* 2015; Fan *et al.* 2016). Meanwhile, N<sub>2</sub>O emission in the paddy field without biofertilizer was greater at the rice vegetative phase compared to the rice generative phase (Figure 4). We found lower N<sub>2</sub>O emission in biofertilizer application plots caused by the N<sub>2</sub>O-reducing activity of *O. anthropi* BL2. Biofertilizer application was able to reduce N<sub>2</sub>O emission with almost the same amount in both vegetative and generative phase of

paddy plants (Figure 4). Higher  $N_2O$  emission in the vegetative phase might be due to the high activity of indigenous denitrifying bacteria and a higher concentration of ammonium resulted from fertilizer N application (Adviento-Borbe *et al.* 2015; Fan *et al.* 2016). Carbon substrates also affect the abundance of denitrifiers, and the denitrification rate tends to high in rice-growing season (Henrich and Haselwandter 1997). In the rice generative phase,  $N_2O$  was absorbed in the 100% urea treatment without biofertilizer ( $-80.04 \mu\text{g } N_2O/\text{m}^2/\text{day}$ ) (Figure 4). It was presumably due to a decrease in the concentration of ammonium in the soil during this phase, so that indigenous  $N_2O$ -reducing bacteria use  $N_2O$  as a substrate instead of ammonium.

Microbial inoculation would cause long-term, transient, or even no prominent impact in the equilibrium of soil microbes communities. The inoculation may directly has positive effect on plant growth and protection, and may also have positive and negative impacts on the indigenous microbial population (Trabelsi and Mhamdi 2013). Inoculation of methanotrophic and  $N_2O$ -reducing bacteria seemed to have a transient impact on bacterial communities in paddy soil at 36 DAT, then the bacterial community structure at 69 and 106 DAT was almost similar to 0 DAT (Table 2). Several studies showed the transient impact on soil microbial communities occurred after the inoculation of biocontrol bacteria, such as *Streptomyces melanosporofaciens* (Prevost *et al.* 2006) and *Pseudomonas fluorescens* 2P24 (Gao *et al.* 2012). Masrukhin *et al.* (2017) showed that inoculation of methanotrophic and  $N_2O$ -reducing bacteria had no significant effect on soil bacterial community in lowland paddy fields in Sukabumi, Indonesia based on the observations at 0, 60 and 120 DAT. In our study, there were some predominant OTUs in 36B0 which were not detected in 36B1. It might be due to the decrease in the relative abundance of those OTUs in 36B1. We suggest that this shift can be interpreted as addition of dominant general bacteria by methanotrophic bacteria, including from the inoculant. The loss of certain bacterial species may not change the function of the system. It is due to the same function may be carried out by different bacterial species (Kennedy 1999; Nannipieri *et al.* 2003). At 36 DAT, *Methylocystis* sp. was predominant in the biofertilizer application, where the  $CH_4$  emission was lower than without biofertilizer treatment (Figure 5a; Table 3). However, Lee *et al.* (2014) reported that the

relative abundance of *Methylocystis* was highest at transplantation time, showing very low  $CH_4$  emission. Predictably, methanotrophic bacteria used in this study well survived and exhibited high level of  $CH_4$  oxidation activity, even though the paddy growth in the biofertilizer application was greater than without biofertilizer. DGGE fingerprint analysis supports the idea that there was an interaction between these bacteria, plants and indigenous bacteria. Information about the changes in indigenous bacterial communities resulted from microbial inoculation will be important to develop the microbial inoculants which favor the plants and other beneficial microbes (Gadhav *et al.* 2018).

In this present study, bacterial diversity in the paddy fields without biofertilizer treatment at the rice vegetative phase might be governed by a higher urea application supplying available N in the soil. Higher concentrations of nutrients caused by fertilizer may cause higher bacterial diversity in the rice vegetative phase (Pittol *et al.* 2017). The activity and growth of such bacteria in soil around rice roots are stimulated after fertilizer application (Bodelier *et al.* 2000). In the rice generative and ripening phase (69 and 106 DAT), the number and intensity of OTUs were relatively similar in both treatments (Table 2), which probably due to the nutrients that were still abundant in the two treatments treatments. The decrease in bacterial diversity from 69 to 106 DAT might be caused by the decrease of root exudates. Root exudates contain amino acid, sugar, and organic acid providing nutrients for microbial growth and colonization process, and may indirectly affect microbial diversity. High levels of root exudates may support high numbers of microbial diversity. Paddy roots released exudates that contain higher amounts of those compounds at an early stage until panicle initiation, yet it gradually decreased from flowering towards later stages or maturity (Aulakh *et al.* 2001; Jackson and Ilamurugu 2014).

Phylogenetic analysis showed that the detected bacteria belong to 5 phyla, i.e. Proteobacteria (Alphaproteobacteria and Deltaproteobacteria), Nitrospirae, Actinobacteria, Firmicutes, and Acidobacteria. Alphaproteobacteria was the most dominant group in the paddy soil of Tegal district. It has been reported that the most common phylum of paddy rhizosphere and soil was Proteobacteria (Lu *et al.* 2006; Breidenbach and Conrad 2015; Masrukhin *et al.* 2017; Pittol *et al.* 2017). Proteobacteria in paddy

fields may particularly contribute to N<sub>2</sub> fixation, plant growth promotion, organic substances degradation, iron and sulfate reduction, and methane oxidation. Bao *et al.* (2014) also reported that microbes in paddy roots are responsible for CH<sub>4</sub> oxidation and N<sub>2</sub> fixation which the majority derived from type II methanotrophic bacteria of the family *Methylocystaceae*, such as *Methylosinus* spp. Symbiotic between paddy plant and diazotrophic methanotroph may permit paddy plants to acquire nitrogen via N<sub>2</sub> fixation, mainly under low-N fertilizer (Minamisawa *et al.* 2016). Type II methanotroph bacterium and *Methylocystis* sp. belonging to Alphaproteobacteria, which predominate in this study, probably contribute to CH<sub>4</sub> oxidation and N<sub>2</sub> fixation. Those bacteria, in particular represented by OTU 17 and OTU 18, were mainly found as the most dominant OTUs in the biofertilizer application at 36 DAT (Table 2; Table 3).

In conclusion, inoculation of methanotrophic bacteria *Methylocystis rosea* BGM1, *Methylocystis parvus* BGM3, *Methylococcus capulatus* BGM9, *Methylobacter* sp. SKM14, and N<sub>2</sub>O-reducing bacteria *Ochrobactrum anthropi* BL2 was able to reduce CH<sub>4</sub> and N<sub>2</sub>O emissions, promote paddy plants growth and productivity, and increase the efficiency of nitrogen fertilizer utilization. The inoculation of methanotrophic and N<sub>2</sub>O-reducing bacteria seemed to have a transient impact on bacterial communities in paddy fields. Alphaproteobacteria was the predominant phylum in the paddy fields without and with biofertilizer application during the rice vegetative and generative phase. This results can be a basic information for developing biofertilizer that supports sustainable agriculture.

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