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Full Length Research Paper

Antimicrobial activity of *Phoma* sp. URM 7221: An endophyte from *Schinus terebinthifolius* Raddi (Anacardiaceae)

Gustavo Bartolomeu Pedrosa Gomes da Silva[1], Karine Fagundes Silvino[1], Jadson Diogo Pereira Bezerra[2], Thaísa Gabriela Silva de Farias[3], Janete Magali de Araújo[1] and Tânia Lúcia Montenegro Stamford[4*]

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The discovery of new metabolites potentially bioactive against pathogenic microorganisms, mainly multidrug resistant, has aroused interest in endophytic fungi. The plant-associated microorganisms have been an important source for development of new compounds of biotechnological interest. This study aimed to investigate the antibacterial capacity of the endophytic fungus, *Phoma* sp. URM 7221 isolated from the medicinal plant *Schinus terebinthifolius* against human-pathogenic bacteria. An endophyte was isolated from *S. terebinthifolius* leaves. *Phoma herbarum* URM7221 was characterized morphologically and on the basis of ITS rDNA sequence. Primary antimicrobial activity was evaluated using the agar diffusion method and fermentation in liquid medium. Six different solvents were used to extract the active metabolites from fungal biomass and metabolic liquid. An antimicrobial activity test from the extract was carried out using a disk diffusion method with the endophytic extract containing the best antibacterial activity. Two tests were performed: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Partially purified secondary metabolite extracts were analysed by thin layer chromatography (TLC). Liquid metabolically bioactive compounds extracted with petroleum ether revealed a MIC of 25 and 500 µg.mL^{-1} against *S. aureus* and MRSA, respectively. Ether and methanol extracts were assessed by chemical analyses and contained phenolic compounds, triterpenes, steroids, reducing sugars, mono- and sesquiterpenes. The thin layer chromatography assay showed the activity of different antimicrobial compounds produced by *Phoma* sp. URM7221. This endophyte (URM7221) could be efficiently used for production of bioactive metabolites against pathogenic microorganisms, with significant biotechnological potential.

Key words: Bioactive compounds, endophytic fungi, pathogenic bacteria, multidrug-resistance, antibacterial agents.

INTRODUCTION

The overuse of antibiotics in medical practice has contributed to the increase in antibiotic-resistant microorganisms (Paes et al., 2014). Infections caused by Gram-positive bacteria with antibiotic resistance are one of the main reasons for the high mortality and morbidity rates reported for patients, resulting in costly treatment (Woodford and Livermore, 2009). *Staphylococcus aureus* is one of the most common pathogen in a diverse number of infectious diseases, including simple skin infections and invasive diseases that lead to bacteremia and sepsis (Kim et al., 2012; Rocha et al., 2015). This bacterium requires attention due to emergence of resistant strains to the primary antibiotics used in their treatment, such as methicillin. Methicillin-resistant *S. aureus* (MRSA) is a major cause of hospital morbidity and mortality (Siqueira et al., 2011). Similar to *S. aureus*, the genus *Enterococcus* includes some of the most important opportunistic pathogens. Their variable genome has influence on their adaptation to different environments (Arias and Murray, 2012).

The race to reduce the impact of infections caused by multidrug resistant bacteria, has reinforced the need to discover new antimicrobial substances. Studies have researched new compounds in plants and micro-organisms that live inside vegetal tissues, such as endophytes. Endophytes are microorganisms that live in association with plant tissue (Kusari et al., 2012) and many species are known to produce bioactive secondary metabolites with medicinal potential (Kusari et al., 2012). This suggests alternative paths to discovery of new bioactive substances, leading to development of new drugs derived from compounds produced by endophytes species (Kumar et al., 2011).

Several studies have demonstrated that endophytic fungi can produce numerous compounds with biological activities of interest, such as antitumor (Jin-long et al., 2011; Chandra, 2012), antimicrobial (Siqueira et al., 2011; Tayung et al., 2012; Bagchi and Banerjee, 2013; Pinheiro et al., 2013; Orlandelli et al., 2015), enzymes (Chandra, 2012), plant growth hormones (Hwang et al., 2011), leishmanicidal (Santiago et al., 2012), cytotoxic compounds (Li et al., 2011) and compounds with industrial and pharmaceutical potential (Meng et al., 2011; Wang and Dai, 2011), reinforcing the broad biotechnological potential of these microorganisms.

Schinus terebinthifolius Raddi belongs to Anacardiaceae family and is largely found in the coastal region of Brazil (Carvalho et al., 2013). This plant has used in traditional medicine as antipyretic, analgesic and in the treatment of urogenital diseases (Carvalho et al., 2013). Several studies have shown that *S. terebinthifolius* produce pharmacologically important substances, such as anticancer (Bendaoud et al., 2010; Matsuo et al., 2011), antimicrobial (Silva et al., 2010; Pereira et al., 2011), antioxidant (Bendaoud et al., 2010), healing purposes (Estevão et al., 2013) and antiproliferative (Queires et al., 2013).

Considering the impact on health human and the necessity of more studies verifying the biotechnological properties of endophytic fungi, the present study aims to 1) evaluate the antibacterial potential of endophytic fungi against Gram-positive pathogenic bacteria and 2) identify the class of bioactive metabolites produced by *Phoma* sp. (URM 7221) by thin layer chromatography (TLC).

MATERIALS AND METHODS

Endophytic fungi: Isolation and identification

S. terebinthifolius Raddi leaves were collected from different plants on the campus of Federal University of Pernambuco, Recife city, Pernambuco state, Brazil (08°03'07"S 34°56'59"O). For endophytic isolation, healthy leaves were selected as described by McInroy et al. (1995). The leaves were cut into 180 fragments of approximately 1 cm^2 and were cultured for up to 20 days at 30°C on Sabouraud agar and potato dextrose agar (PDA) media containing chloramphenicol (100 mg/L).

For morphological identification of endophytic fungi, the fungi were cultured on Synthetic Nutrient-poor Agar (SNA), malt extract agar (MEA) and potato dextrose agar (PDA) media for 10 days in the dark at 28°C and for 10 days under continuous UVA light. After this period of cultivation macro and microscopic characteristics of colonies were observed according to Boerema et al. (2004). For molecular analysis, 7-day old culture on PDA was used for DNA extraction with an UltraClean TM Microbial Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions.

Polymerase chain reaction for the part of ITS region (first and second internal transcribed spacer regions and intervening 5.8S nrDNA) amplification was performed with 50 µL volume, using Taq® DNA polymerase 1X buffer, 1.5 mM $MgCl_2$, 0.4 µM ITS1 and ITS4 primers (White et al., 1990), 0.2 mM dNTPs, 0.2 Taq® DNA polymerase and 25ng DNA. Amplification was done in a thermal cycler with the following conditions: 5 min at 95°C (1 cycle), 30 s at 95°C (30 cycles), 1 min at 62°C (annealing), 2 min at 72 °C (extension) and 5 min at 72°C (final extension). Amplification products were separated by electrophoresis on a 1% agarose gel, colored with GelRed® and visualized with UV light using molecular weight marker 1 kb plus (Fermentas®). PureLink PCR Purification Kit (Invitrogen®) was used according to manufacturer's recommendation. Amplification products were sequenced and electropherograms were edited by Staden Package software. The consensus sequence was blasted against sequences on GenBank

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using BLASTn. Sequences with the highest similarity percentage (99-100%) in comparison with the studied sequence were analysed.

Antimicrobial activity: Solid medium

Forty endophytic fungi isolated with distinct morphological characteristics were selected for antimicrobial activity. Four microorganisms were tested using an agar block test as previously described by Ichikawa et al. (1971). The four bacteria species used for the test were: *S. aureus* Rosenbach (UFPEDA02), MRSA (UFPEDA663), *Enterococcus faecalis* (Andrewes & Horder) Schleifer & Kilpper-Bälz (UFPEDA138), and *Bacillus subtilis* (Ehrenberg) Cohn (UFPEDA86). The inhibition zones obtained were compared with control information from the table of the Clinical and Laboratory Standards Institute (CLSI, 2013).

Fermentation in liquid medium

The endophytic fungus (URM 7221) that demonstrated the best antimicrobial activity using solid medium was selected to the test in liquid medium for extraction of active metabolites. After growth, fragments of 10 mm diameter of the endophytes were transferred to Erlenmeyer flasks (250 mL) with 50 mL of the liquid medium MPE (Hamada et al., 1974), Czapeck and M1 (20 g/L glucose, 200 g/L peptone) incubated for 2 days, at 30°C at 120 rpm. After this period, 10 mL of each pre-inoculum was transferred to flasks (500 mL) containing 90 mL of the same liquid medium and incubated in the same conditions. Every 24 h (during 120 h), 1 mL of fermentation broth was removed from the flask and centrifuged at 10.000 rpm for 3 min. From the supernatant fluid, 50 µL was used to perform the disk diffusion antimicrobial test (Kirby et al., 1966).

Endophytic extracts

After fermentation of endophytic fungus (URM 7221) for 48 h in MPE medium, the fungal biomass was separated from the metabolic liquid using Whatman filter paper 4 and centrifuged at 10.000 rpm for 3 min. Ethanol, methanol and acetone (1:10 g/mL) at pH 2.0, 7.0 and 9.0, respectively, were used to extract the active metabolites from the biomass. To extract the active substances present in the metabolic liquid petroleum ether, ethyl acetate and chloroform (2:1) were used. The pH of the metabolic liquid was adjusted to 2.0, 7.0 and 9.0, respectively. After 1 h of shaking, the samples were centrifuged at 10,000 rpm for 3 min and the solvent containing the extract was evaporated using a rotary evaporator at 55°C (Lyra et al., 1964).

Antimicrobial activity: Endophytic extract

The antimicrobial activity test of the endophytic extract was performed using a disk diffusion method, with sterile paper discs impregnated with 50 µL of each extract. The pH of the endophytic extract was adjusted to 7.0, to avoid interferences. The diameter of the inhibition zones around the discs was measured and was assessed using the same microorganisms tests as described in "Antimicrobial activity: solid medium" section. Chloramphenicol 1 mg/mL was used as a positive control, methanol was applied as solvent control, and the depleted liquid and water were used as negatives controls (Lyra et al., 1964).

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Two tests- MIC and MBC were used to assay the endophytic extract with the best antibacterial activity. A broth microdilution assay using 96-well microplate, performed according to the Clinical and Laboratory Standards Institute (CLSI, 2013) was used. All determinations were executed in triplicate.

Plant tissue extraction

For extraction of compounds with no antimicrobial activity, leaves of *S. terebenthifolius* (1 g) were immersed in 10 mL of hexane for two hours. The leaves were then immersed in 10 mL of ethyl acetate for two hours to extract bioactive compounds. Solvent was evaporated using a rotary evaporator at 55°C (Ceruks et al., 2007).

Secondary metabolic analyses

For partial purification of secondary metabolites, 10 µL aliquots of biomass extract (methanol) and metabolic fluid extract (ether) of *Phoma* sp. (URM 7221) were analysed by thin layer chromatography (TLC) on silica gel F254 (Merck®). Several mobile phases, such as ethyl acetate : acetic acid : formic acid : water (100:11:11:26, v/v), toluene : ethyl acetate (80:20; 97:3, v/v), acetone : n-butanol : phosphate buffer pH 5.0 (5:4:1, v/v), and specific visualization reagents were used (Robertson et al., 1956; Metz, 1961; Sharma and Darwra, 1991; Wagner and Bladt, 1996; Harbone, 1998). The chromatograms were run in saturated chambers.

The bioactive extracts obtained from *Phoma* sp. (URM 7221) were compared with compounds present in crude ethyl acetate extract of leaves by TLC using an agar overlay method (Rodrigues et al., 2009). Ethyl acetate: methanol (9:1) solution was used as mobile phase. An aqueous solution of 2,3,5-triphenyltetrazolium chloride (20 mg/mL) (Rodrigues et al., 2009) was used for visualization. Biological activity was determined by the formation of white and well defined inhibition zones against a red-purple background. Retention factor (Rf) values were measured and compared (Homans and Fuchs, 1970). All tests were performed in triplicate.

RESULTS

Endophyte isolation, identification and antimicrobial activity

Using culture media for isolation of endophytes, 220 endophytic fungi were isolated from leaves of *S. terebinthifolius*. Among these, 137 endophytes (64.62%) were obtained using Sabouraud agar medium and 75 (35.37%) using PDA medium.

The endophyte that showed the best antibacterial activity was identified. Morphological analysis was performed after 20 days culture on SNA, MEA and PDA media. The fungus grew on three different media with similar macroscopic characteristics (velvety-powdered texture, colony color ranging between light brown and dark pink, reverse light brown to reddish brown). Pycnidia

Table 1. Antibacterial activity of endophytic fungi isolated from leaves of *S. terebinthifolius* against human pathogenic bacteria.

Test microorganism	Inhibition zones (mm)						
	Endophytic fungi						
	F-10	F-61 (URM 7221)	F-100	F-105	F-169	F-178	F-188
Bacillus subtilis UFPEDA86	20 ± 0.0	35 ± 0.0	16,4 ± 0,94	16 ± 0.8	-	-	>10 ± 0.0
Enterococcus faecalis UFPEDA138	17 ± 0.0	23.7 ± 0.47	16.7 ± 0.47	>10 ± 0.0	-	>10 ± 0.0	-
Staphylococcus aureus UFPEDA02	-	35 ± 0.0	-	>10 ± 0.0	19 ± 0.0	>10 ± 0.0	>10 ± 0.0
Staphylococcus aureus (MRSA) UFPEDA663	16 ± 0.86	22,4 ± 0,47	-	-	-	-	>10 ± 0.0

F: Endophytic isolates; (MRSA): Methicillin-resistant *Staphylococcus aureus* resistant; (-): no activity.

Table 2. Susceptibility profile of test microorganisms to endophyte *Phoma* sp. (URM 7221) grown in MPE or M1 liquid fermentation.

Test Microrganisms	Inhibition zones (mm) over incubation time									
	24 h		48 h		72 h		96 h		120 h	
	MPE	M1	MPE	M1	MPE	M1	MPE	M1	MPE	M1
B. subtilis	14 ± 0.0	-	16.3 ± 0.94	17 ± 0.81	25 ± 0.94	12 ± 0.81	22.3 ± 0.47	15 ± 0.0	22.3 ± 0.47	15.6 ± 0.47
S. aureus	15 ± 0.0	-	31.3 ± 0.47	21 ± 0.47	29.3 ± 0.47	11 ± 0.81	15.3 ± 0.47	-	-	-
S. aureus MRSA	15 ± 0.0	-	26.4 ± 0.81	-	15 ± 0.81	-	-	-	-	-

-: No activity.

(91-111 × 58-203 μm) and conidia (4-5.5 × 2-2.5 μm) were formed only on SNA medium. Using ITS rDNA sequence to a megablast search of the NCBI GenBank nucleotide database, our sequence (KP966098) has high identity (98 to 100%) to sequences deposited as *Phoma* sp. and *P. herbarum* (HQ630963, Shrestha et al., 2011; KJ188712, Luo et al., 2014), among others. The morphological characters described above match the *P. herbarum* description by Boerema (2004). However, because the new publications (Chen et al., 2015) using multigene phylogenetic analyses on the taxonomy of this genus, the authors decided to identify the isolate only as *Phoma* sp. (URM 7221).

Forty endophytes were tested in the primary assay, and only seven (17.5%) expressed antimicrobial activity. The endophyte identified as *Phoma* sp. (URM 7221) showed the greatest activity against *B. subtilis* (inhibition zone of 35 mm), *E. faecalis* (23.7 mm), *S. aureus* (35 mm) and MRSA (22.4 mm) (Table 1).

Fermentation and endophytic extracts antimicrobial activity

Fermentation tests revealed that MPE medium was the most efficient and only Czapeck medium did not stimulate the production of active metabolites against the four bacteria tested. *E. faecalis* (UFPEDA138) was resistant to all endophytic extracts tested. The most efficientextract (using MPE medium) gave the best production of the bioactive principle and the largest inhibition zones against *S. aureus* (31 ± 0.47 3 mm after 48 h), MRSA (26.4 ± 0.81 mm, 48 h) and *B. subtilis* (25 ± 0.94 mm, 72 h). These results indicate that the production of bioactive metabolites with antimicrobial activity changes during incubation time (Table 2).

Antibacterial activity of biomass and metabolic liquid extracts obtained from *Phoma* sp. (URM 7221) and from leaves of *S. terebinthifolius* was evaluated (Table 3). The endophyte was subjected to liquid fermentation in MPE medium (pH 7.0) at 48 h. Petroleum ether (pH 7.0) was the most efficient solvent because it extracted bioactive metabolites active against *S. aureus* and MRSA, with inhibition zones reaching up to 23 mm. Ethyl acetate was able to partially extract bioactive compounds against the test microorganisms with inhibition zones between 10-18.6 mm. MIC and MBC values of petroleum ether extract were, respectively, 125 and 250 mg L^{-1} against *S. aureus*, and 500 and 1000 mg L^{-1} against MRSA.

Ethanol and methanol were used to extract active metabolites from fungal biomass and were effective against *S. aureus* and MRSA. Methanol (pH 2.0 and 7.0) showed inhibition zones ranging from 12 to 15 mm, with MIC and MBC of 250 and 500 mg L^{-1}, for *S. aureus* and 1000 mg L^{-1} and >1000 mg L^{-1} against MRSA. Acetone extracts were only active against *S. aureus*.

Table 3. Antimicrobial activity of crude extracts of *Phoma* sp. (URM 7221) obtained from liquid fermentation. MLE: Metabolic liquid exhausted; EXT: extract.

	Solvents		Inhibition zones (mm)					
			S. aureus (UFPEDA 02)			MRSA (UFPEDA 663)		
			pH2	pH7	pH9	pH2	pH7	pH9
Biomass	Acetone	EXT	21.6 ± 0.47	25 ± 0.0	22 ± 0.81	15 ± 0.0	17 ± 0.0	-
	Ethanol	EXT	23 ± 0.0	24 ± 0.0	12 ± 0.0	13.6 ± 0.47	11 ± 0.0	-
	Methanol	EXT	21.3 ± 0.94	21.6 ± 1.2	21 ± 0.0	17 ± 0.0	17 ± 0.0	17 ± 0.81
Metabolic liquid	Ethyl acetate	EXT	27.6 ± 0.94	28 ± 0.81	27 ± 0.81	21.6 ± 0.47	20 ± 0.0	20.6 ± 0.47
		MLE	-	-	-	-	-	-
	Chloroform	EXT	26.6 ± 0.47	25.6 ± 0.47	25.6 ± 0.47	19 ± 0.0	19 ± 0.0	19 ±0 .0
		MLE	-	-	-	11 ± 0.0	-	-
	Petroleum ether	EXT	-	11.6 ± 0.47	-	-	12 ± 0.0	-
		MLE	24 ± 0.0	24 ± 0.0	23.6 ± 0.47	19 ± 0.0	17.3 ± 0.47	17 ± 0.0

Chemical comparison of active compounds

Analysis of metabolic liquid by TLC suggested the presence of phenolic compounds, triterpenes, steroids, mono and sesquiterpenes. Analysis of fungal biomass showed reducing sugars and phenolic compounds. Using bioautographic assay, both extracts showed antimicrobial activity against *S. aureus* and MRSA. Petroleum ether extract showed Rf values of 0.65 and 0.6 for *S. aureus* and MRSA, respectively. Similar Rf values were observed for methanol extracts (0.125 and 0.15).

DISCUSSION

Studies of medicinal plants have received wide attention in recent years due to the large diversity of endophytic microorganisms that inhabit plant tissues (Siqueira et al., 2011). New species, useful for biotechnology applications, have been reported to produce extracellular active metabolites with pharmacological interest (Bagchi and Banerjee, 2013; Pinheiro et al., 2013). These microorganisms can also protect plants against phytopathogens, temperature, drought and world weather changes (Gundel et al., 2010). This research demonstrates the diversity of endophytic fungi living in leaves of *S. terebinthifolius* and the capacity of these endophytes to produce molecules with biotechnological importance. In this study, a unique endophytic species identified as *Phoma* sp. (URM 7221) produced more efficiently bioactive compounds against four Gram-bacteria species.

The genus *Phoma* comprises several species and varieties that are recognized as producers of antimicrobial compounds (Bezerra et al., 2015). Researchers have reported that *Phoma* species can be isolated from many plants, such as *Laguncularia racemosa* (Costa et al., 2012), *Amaranthus cruentus* (Pusz et al., 2015), *Taraxacum mongolicum* (Zhang et al., 2013a) and *Mitrajyna javanica* (Pharamat et al., 2013).An important taxonomic paper from CBS-KNAW (The Netherlands) highlights the importance of a polyphasic approach to characterise *Phoma* species and similar genera, and it presented an overview of the phytopathological importance of this genus (Aveskamp, 2010). This antimicrobial potential can be associated with production of phenolic secondary metabolites and steroids (Hwang et al., 2011). Similar to our results, these compounds have been identified by chemical prospecting, which might be related to antimicrobial activity presented by *Phoma* sp. (URM 7221).

According to Strobel et al. (2011), species of the genus *Phoma* can produce organic volatile compounds, of which sesquiterpenes are the most prominent. Hamayun et al. (2009) described that *P. herbarum* produced several metabolic compounds, because it is adaptable to many environmental conditions. These compounds can play a role in immunomodulation (Zhang et al., 2013b; Shen et al., 2014) and antitumor activity (Fang et al., 2011; Pharamat et al., 2013), which reinforces the importance of new surveys involving this fungal genus.

Studies in different countries have tested *Phoma* spp. antimicrobial activity against Gram-positive bacteria. Some authors studying this genus reported similar results. For example, Kumar et al. (2010) tested culture filtrate with the capacity to inhibit *S. aureus* (11 to 19 mm). In this study, primary assays proved antimicrobial activity of *Phoma* sp. (URM 7221), efficiently inhibiting *S. aureus* (11 to 35 mm) and MRSA (15 to 26.4 mm). In addition to the initial tests that showed activity against *B.*

subtilis (12 - 35 mm) and *E. faecalis* (23.7 ± 0.47 mm), other experiments demonstrated that *Phoma* sp. (URM 7221) extracts were not able to suppress the growth of these bacteria. Shukla et al. (2014) also demonstrated antimicrobial potential of this endophyte against *S. aureus* (18.3 ± 0.10 mm). Similar results were obtained by Zhang et al. (2013a) in a study of fungi associated with *Taraxacum mongolicum*, and the authors also found positive results against *S. aureus* (40 mm). Shen et al. (2012) has isolated endophytes from branches of *Phyllostachys edulis* and tested *P. herbarum* extracts against bacteria; it was not able to inhibit *S. aureus* and *B. subtilis*. The same fact occurred according to Pharamat et al. (2013) when evaluating fungal endophytes species obtained from *Mitrajyna javanica*.

Conclusions

Study of endophytic fungi from leaves of *S. terebinthifolius* revealed that different plants are capable of harboring endophytes, such as *Phoma* sp. (URM 7221) that demonstrated the most efficient antibacterial activity among other endophytes.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Full Length Research Paper

A very high frequency of hepatitis B and C virus infections during an active screening campaign in Abidjan

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Viral hepatitis is a serious public health problem affecting billions of people globally. Limited information is available on this issue in Cote d'Ivoire. The objectives of this study were to determine the prevalence and factors associated with hepatitis B virus (HBV) and hepatitis C virus (HCV) during an active screening campaign in Abidjan. A cross-sectional study was conducted at Pasteur Institute of Cote d'Ivoire from July 2015 to February 2016. The ethical clearance for this study was obtained from the National Ethical and Research Committee. An informed written consent was obtained from the participants of the study and administered a questionnaire related to the socio demographical information and risk factors of a possible route transmission HBV and hepatitis C virus (HCV). Blood samples were collected for the detection of HBS Ag, Ab-HBc IgG and Ab-HCV. Serological analyses were performed by Cobase 601 (Roche[R]). Data were analyzed by R software. A total of 1801 patients were recruited; among them 138 children (7.7%) aged from 0 to 15 years and 1663 adults (92.3%). The sex ratio was 1.2 (964/837). The overall prevalence of HBsAg was 30. 9% (557/1801) and 41.1% (702/1708) for Ab–HBc IgG. About HCV, the overall prevalence rate was 5.3% (95/1687), none of the children was HCV positive. The co-infection HBV/HCV rate was 0.95% (16/1687). HCV was associated with age and sexual risk behaviors. HBV was associated with gender, youth, sexual risk behaviors, and scarification. Our findings revealed a high prevalence of HBV. The measures to reduce the disease and its load transmission must be strengthened.

Key words: Hepatitis B virus, hepatitis C virus, seroprevalence, risk factors, Cote d'Ivoire.

INTRODUCTION

According to the World Health Organization, more than 240 million people are infected with the hepatitis B virus (HBV) worldwide, and the majority lives in developing countries (WHO, 2013), such as Côte d'Ivoire. Every year, there are more than 600000 deaths due to the complications related to the infection. HBV's association with liver diseases, such as the primary liver carcinoma and cirrhosis, is clearly established (Hammad and Zaghloul, 2009). HBV and HCV are easily transmissible through sexual, parenteral, and vertical routes (Pozzetto

and O. Garraud, 2011). Several behavioral, environmental, and cultural factors may also be responsible for these infections (Kramvis and Kew, 2007). In Africa, after the vertical and sexual transmissions, HBV and HCV infections are due to cultural practices (levirate, sorority, sexual rituals, scarification, piercing, and tattoos) or medical surgeries (Simpore et al., 2007). Several authors have reported different prevalence of HBV and HCV among sub-populations in Cote d'Ivoire with estimates which vary according to the studied population and methods that are used. In fact, the authors reported that 12.1% HIV co-infected children (Rouet et al., 2008), 3.2% children without HIV; 7.53% blood donors; and 17.6% hemodialysis patients were seropositive for HBs Ag (Kouassi-M'Bengue et al., 2010, 2012, 2013). For HCV infection, the authors reported that 2.5% among blood donors (Kouassi-M'Bengue et al., 2012) were seropositive. However, there is no reliable national survey of HBV and HCV exposure in the population is most likely to benefit from the early detection, surveillance and treatment. Given this gap in our understanding of HBV and HCV in Cote d'Ivoire, we launched an active screening campaign in Abidjan in collaboration with the National Hepatitis Infection Control Programs and the support of a private partner.

The aim of this study was to determine the prevalence and factors associated with HBV and HCV in Abidjan (Cote d'Ivoire).

MATERIALS AND METHODS

A partnership of five years (2015-2020) was signed between Cote d'Ivoire government and an international pharmaceutical laboratory. As a result of this contract, Pasteur Institute of Côte d'Ivoire (IPCI), hosting the National Reference Center (CNR) of viral hepatitis, has been designated as a technical implementation partner. A national awareness campaign was launched through the media to get a larger sensitization of the whole population.

A cross-sectional study was conducted at IPCI from July 2015 to February 2016. Voluntary patients or subjects addressed by healthcare staff to IPCI were enrolled in the study. The subjects responded to a range of questions such as socio demographic information, such us age, marital status, profession, educational background (literacy, schooling), knowledge about hepatitis B and C virus, site of living; other data on the risk factors of the possible route of HBV and HCV transmission like the sharing of toothbrush and cutting objects, sexual risk behaviors, history of blood transfusion, tattooing or piercing body, scarification, and the history of HBV vaccination was obtained. Blood samples were collected for the detection of HBS Ag, Ab-HBc IgG and Ab-HCV. Serological analyses were performed by Cobas e 601 (Roche [R]).

Ethical issues

The ethical clearance for this study was obtained from the National Ethical and Research committee. We obtained an informed written consent from all participants of the study. Biological parents or relatives have given their consent for infants.

Data analysis

Data were entered into Epi Info 3.5 and analyzed by R sofware. Qualitative variables were compared using the Fisher exact test and quantitative variables by analysis of variance. A percentage with 95% confidence interval (CI) was used to describe the prevalence. OR with 95% CI was calculated for each association. A p values less than 5% was considered as significant.

RESULTS

A total of 1801 patients were screened for HBsAg and 1687 for anti-HCV Ab. In all, the sex-ratio (M/F) = 1.15 and 1.16, respectively for HBs Ag detection and anti-HCV Ab. The overall mean age was 36 (± 15) years, ranging from 2 months to 85 years.

The overall prevalence of HBsAg was 30.9% (557 positive) in 1801 subjects. We noted that 41.1% (702/1708) subjects had positive Ab-HBc IgG without any detection of HBS Ag.

HBsAg prevalence in <20, 20-29, 30-39, 40-49 and >50 age groups was 13.4, 39.8, 37.4, 31.1 and 18.1%, respectively, as well as it showed an increase with age from 13.4% in <20 age group and 39.8% in 20-29 group, respectively, then a decrease from 37.4% in 30-39 age group to 18.1% in the older group >50 years old (Figure 1A). When the overall prevalence of HBsAg marker was analyzed according to gender, the prevalence was 36.7 and 24.3% in male and female subjects, respectively. The difference was found to be statistically significant (OR = 1.8, P < 0.01) (Figure 1B).

The seroprevalence of anti-HCV Ab was found to be 5.6%, corresponding to 95 seropositive subjects out of 1687 participants. The prevalence of anti-HCV in <20, 20-29, 30-39, 40-49 and >50 age groups was1.2, 3.3, 2.9, 3.3 and 18%, respectively. For these subjects, the anti-HCV prevalence increased with age (Figure 2A).

The overall prevalence of anti-HCV in 781 female subjects was 5.4% (42 positive), which was lower than that in the 906 male subjects, 5.8% (53 positive). The difference was found not to be statistically significant (P=0.67) (Figure 2B).

Regarding the seropositivity of HBV and HCV dual carriage, the presence of both HBsAg and anti-HCV antibodies was noted in 16 cases out of 1687 tested subjects (0.9%). These 16 patients were male in 87.5% and female in 12.5%. The proportion of co-infection was the same among the 20-29 and 30-39 years (31.3%), for 40-49 and >50 age group it was respectively 12.5 and 25%.

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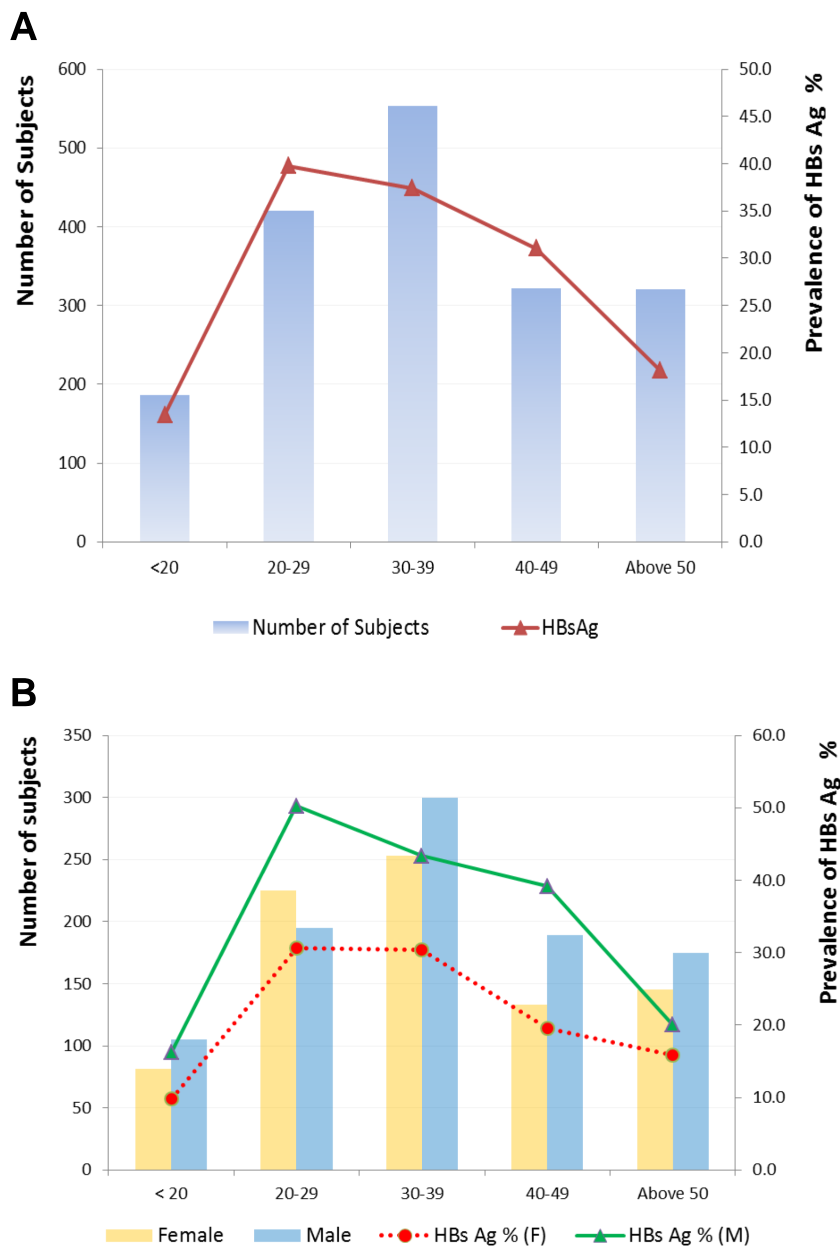

Figure 1. Serological features of HBsAg prevalence in Abidjan. Bars represent the number of subjects in each group indicated by left vertical axis, and the points represent the prevalence in each group indicated by right vertical axis. The horizontal axis represents the age ranges. **A**, Overall characterization of HBsAg by age group. **B**, Overall characterization of HBsAg by gender, M, male, F, female.

Table 1 lists the frequency of potential risk factors reported by groups (positive and negative) HBs Ag. On the subject of HBV carriage, HBsAg positivity was associated with male gender (OR= 1.8; p < 0.01), age (OR =2.9; p < 0.01), history of sexual risk behaviors (OR: 2.9; p < 0.01), family position (OR = 1.5; p = 0.0005) living in Abidjan (OR =1.5, p=0.002) and low socio economical level. It was defined by not salaried (OR=1.3; p = 0.016) and promiscuity (OR =1.3; p=0.014), scarification was also a risk factor (OR=1.6; p<0.01). Conversely, there were no significant differences between HBV prevalence in people who benefited from a blood transfusion and those who have not (OR = 0.7; p= 0.048). That is the same thing for the people who were vaccinated against hepatitis B or not (OR=0.7; p=0.013). There was no significant difference between sharing toothbrush (OR = 1.2; p =0.69), sharing cutting objects (OR=0.96; p=0.77), body piercing (OR =1.3; p=0.22), tattooing (OR =1.2; p=0.5) a consumption of alcohol or drug (OR = 1.1 and 1.5; p=0.39 and 0.46). Literacy,

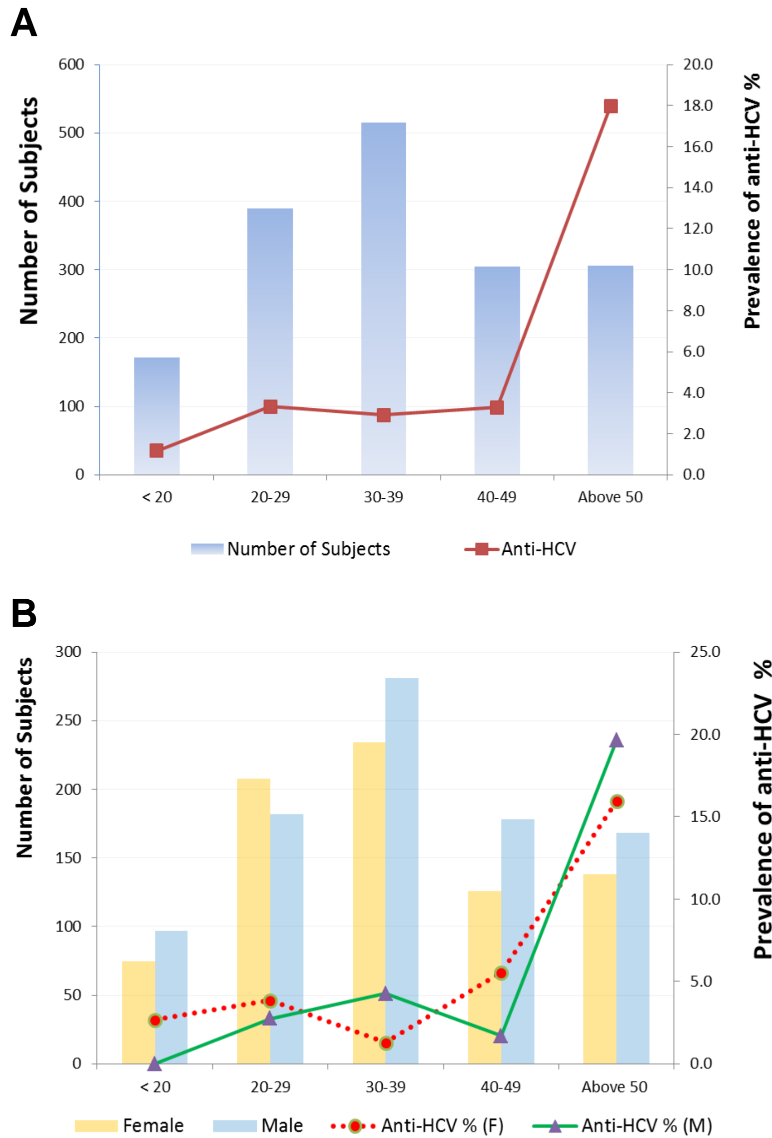

Figure 2. Serological features of anti-HCV prevalence in Abidjan. Bars represent the number of subjects in each group. Indicated by left vertical axis, and the points represent the prevalence in each group indicated by right vertical axis. The horizontal axis represents the age ranges. A, Overall characterization of anti-HCV by age group. B, Overall characterization of anti-HCV by gender, M, male, F, female.

schooling and knowledge about HBV are not associated with HBs Ag reactive with OR and p respectively 1.3, 0.12; 1.2, 0.008; and 0.96, 0.76.

Table 2 lists the frequency of potential risk factors reported by groups reactive or not with HCV-ab. Factors significantly associated with HCV infection were increasing age (OR =10.6; p<0.001) literacy (OR=3.5; p<0.001), schooling (OR= 2.5, p<0.001), living in Abidjan and family position. Conversely, no association was observed between HCV seropositivity and gender, tattooing, and sexual risk behaviors.

DISCUSSION

A cross-sectional study to assess the epidemiology of HCV and HBV prevalence among the general population living in Abidjan and other cities in Cote d'Ivoire was reported. To the best of our knowledge, this is the largest community-based epidemiologic study of HCV and HBV infections from Cote d'Ivoire during a national continual screening campaign. Most of the previous studies in this field were limited due to the short duration of the survey and scarce sample of populations (Assi et al., 2011). The

Table 1. Distribution of hepatitis B virus serological marker and principal risk factors in the study population; July 2015 to February 2016.

Risk Factor		HBV-Positive subjects		HBV-Negative subjects		OR (CI 95%)	p-value
		N	%	N	%		
Age	>=56	29	14.4	173	85.6	2.9 (1.9 – 4.6)	<0.001
	0-55	528	33	1071	70		
Sex	Female	203	24.3	634	75.7	1.8 (1.5 – 2.2)	<0.001
	Male	354	36.7	610	63.3		
Residence	Abidjan	440	29.4	1057	70.6	1.5 (1.2- 1.9)	0.002
	Out	117	38.5	187	61.5		
Family position	Couple	117	26	332	74	1.5 (1.2- 1.9)	0.0005
	Alone	412	35.2	759	64.8		
Salaried	Yes	327	30.4	749	69.6	1.3 (1.1- 1.6)	0.016
	No	213	36.3	374	63.7		
Promiscuity	Yes	145	36.3	254	63.7	1.3 (1.1- 1.7)	0.014
	No	389	29.9	914	70.1		
Sexual risk behaviors	Yes	436	34	846	66	1.7 (1.3- 2.2)	<0.001
	No	94	23.1	313	76.9		
Scarification	Yes	100	41	144	59	1.7 (1.3- 2.2)	<0.001
	No	419	29.4	1007	70.6		
Vaccination	Yes	91	26.5	252	73.5	0.7 (0.5-0.9)	0.013
	No	375	33.7	739	66.3		
Blood Transfusion	Yes	52	25.2	154	74.8	0.7 (0.5-0.99)	0.048
	No	474	32	1005	68		
Literacy	Yes	493	31.9	1052	68.1	1.3 (0.9-1.8)	0.12
	No	49	26.3	137	73.7		
Schooling	Basic	115	27.8	298	72.2	1.2 (0.9- 1.6)	0.08
	High	427	32.4	891	67.6		
Knowledge	Yes	369	32.3	775	67.7	0.96 (0.8- 1.2)	0.76
	No	133	33.1	269	66.9		
Drug	Yes	5	33.3	10	66.7	1.5 (0.5- 4.2)	0.46
	No	524	31.2	1158	68.8		
Tattoo	Yes	29	34.5	55	65.5	1.2 (0.7- 1.9)	0.5
	No	501	31	1114	69		
Body Piercing	Yes	33	37.1	56	62.9	1.3 (0.8- 2.1)	0.22
	No	496	30.9	1109	69.1		
Share toothbrush	Yes	11	34.4	21	65.6	1.2 (0.6- 2.4)	0.69
	No	516	31.1	1143	68.9		
Sharing of cutting objects	Yes	170	30.9	381	69.1	0.96 (0.8- 1.2)	0.77
	No	365	31.5	792	68.5		
Alcohol	Yes	159	32.8	326	67.2	1.1 (0.8- 1.4)	0.39
	No	360	30.6	815	69.4		

OR: Odds ratios, CI: confidence interval. Univariate analysis

Table 2. Distribution of hepatitis C virus serological marker and principal risk factors in the study population; July 2015 to February 2016

Risk factor		**HCV-Positive subjects**		**HCV-Negative subjects**		**OR (CI 95%)**	**p-value**
		N	**%**	**N**	**%**		
Age	>=56	49	25.3	145	74.7	10.8 (6.9 – 16.5)	<0.001
	0-55	46	3.1	1447	96.9		
Sex	Female	42	5.4	739	94.6	1.09 (0.7 – 1.7)	0.67
	Male	53	5.8	853	94.2		
Residence	Abidjan	69	4.9	1333	95.1	1.9 (1.2- 3.1)	0.005
	Out	26	9.1	259	90.9		
Family position	Couple	33	7.8	391	92.2	1.7 (1.0- 2.7)	0.03
	Alone	53	4.8	1042	95.2		
Salaried	Yes	63	6.3	936	93.7	1.1 (0.7- 1.7)	0.63
	No	32	5.7	530	94.3		
Promiscuity	Yes	24	6.3	358	93.7	1.3 (0.8- 2.0)	0.31
	No	60	5.0	1149	95.0		
Sexual risk behaviors	Yes	50	4.2	1148	95.8	0.5 (0.3- 0.8)	0.004
	No	30	7.9	350	92.1		
Scarification	Yes	12	5.2	221	94.8	1.00 (0.5- 1.9)	0.99
	No	68	5.1	1259	94.9		
Blood Transfusion	Yes	13	6.7	181	93.3	1.4 (0.7- 2.5)	0.32
	No	69	5.0	1313	95.0		
Literacy	Yes	61	4.2	138	95.8	3.5 (2.0- 5.9)	<0.001
	No	24	13.5	154	86.5		
Schooling	Basic	37	9.4	357	90.6	2.5 (1.6- 4.0)	<0.001
	High	48	3.9	1178	96.1		
Knowledge	Yes	47	4.5	1009	95.5	2.04 (1.29- 3.23)	0.002
	No	34	8.7	357	91.3		
Drug	Yes	1	6.7	14	93.3	1.3 (0.03- 8.7)	0.5
	No	82	5.2	1489	94.8		
Tattoo	Yes	1	1.3	77	98.7	0.2 (0.005- 1.3)	0.11
	No	82	5.4	1428	94.6		
Body Piercing	Yes	6	7.2	77	92.8	1.4 (0.5- 3.4)	0.26
	No	77	5.1	1423	94.9		
Share toothbrush	Yes	1	3.1	31	96.9	0.6 (0.01- 3.5)	0.48
	No	83	5.4	1465	94.6		
Alcohol	Yes	18	3.5	501	96.5	0.6 (0.3- 0.9)	0.03
	No	65	6.0	1013	94.0		

OR: Odds ratios, CI: confidence interval. Univariate analysis

descriptive epidemiologic data presented in this study can provide new insight into the contribution of HCV and HBV in the etiology of the liver disease in Cote d'Ivoire.

Data analysis revealed a very high prevalence (30.9%) of hepatitis B and confirmed a relative mean prevalence (5.3%) of hepatitis C. These results show a higher HBV and HCV prevalence than in the target groups as reported by other studies. So, Adoba et al. (2015) reported a prevalence of 14.5 and 0.5% for 200 barbers in Ghana; Coppola in a retrospective review study notified 0 to 15% HBV for immigrant coming from sub-Saharan Africa (Coppola et al., 2015); even in a retrospective study concerning 578 patients with chronic hepatitis conducted in Ethiopia, 22.3 and 3.6%, respectively for HBV and HCV were reported (Taye et al., 2015). However, regarding the rate of HCV, our results are lower than hemodialyzed and thalassemia patient with 33.5 and 37.1%, respectively (Malhotra et al., 2016; Ramadan et al., 2016).

Nevertheless, our rates are lower than the ones reported by Robert et al. (2016); in a population of HIV seropositive men which make sex with men with 49.4% for HBV and 10.4% for HCV. This high prevalence can be explained by the fact that it was the first year of national screening campaign and many people are interested. However, many patients are involved by medical prescription. A study realized in Kenya had recruited 389 patients with jaundice; HBs Ag seropositive was 50.6%, higher than our work (Missiani et al., 2016). Another study in Cameroon, concerning 97 hemodialyzed patients reported 6.2 and 20.6% seroprevalence for HBV and HCV (Halle et al., 2016).

This study also revealed a high rate of seropositivity to Ab-HBc-Ig G without any detection of HBs Ag in 41.1% cases. In this work, we did not research anti HBs, so our interpretation of these cases is a prior contact with HBV. The detection of the anti HBs Ab would have allowed us to decide between a passive immunization or the hypothesis of occult hepatitis. Concerning HBV and C co-infection, our prevalence was similar (0.9 vs. 0.2%) to that reported by Ojide in Nigeria (Ojide et al., 2015). The virological and molecular aspects of HBV/HCV coinfection are poorly comprehended. Although, liver disease activity and progression are generally more severe in the presence of double infection, an inverse relationship in the replicative. Levels of the two viruses exists, suggesting direct or indirect (that is, mediated by host immune responses) viral interference (Raimondo et al., 2008).

The analysis by gender has revealed that the seroprevalence of hepatitis B among males is significantly higher than that found in females. Our study is consistent with the results obtained by Deng et al. (2013) in 2013 in China (6.54% compared 3.87%) and Makuwa et al. (2009) in Gabon in 2009 (16.2% compared 9.9%); and no plausible explanation has been given for the higher rate in males in the general population but probably due to the higher exposure to occupational HBV risk factors in men, or else females clear the HBV more efficiently as compared to males.

Age group of 16 to 55 years is the most affected (35%), compared to the older (14.4%). These results show that young people are the most affected by HBV, $p<0.001$. These results are similar to those of Makuwa et al. (2009) who reported a prevalence of 22.22% among young men in the same age group in urban areas of Gabon. The low prevalence of individuals in the age group above 50 years of age could indicate that several people in this group might have died from cirrhosis or liver cancer due to the lack of medical care.

Regarding HCV, the higher prevalence was observed in advanced age groups; indeed, no anti-HCV seropositive case was found in 138 children younger than 16 years, nevertheless, the seropositivity increased progressively from adults (3.8%) to older persons (18%). The higher prevalence of HCV in older people could be attributed to a longer exposure to risk factors for HCV transmission; for instance iatrogenic transmission resulting from inadequately sterilized equipments, inappropriate use of supplies, etc. This risk factor is considered as the main risk associated with HCV infection in majority of the participants from the general population included in this study. This same risk is the primary cause for HCV transmission in many outbreaks documented in the United States and the European Union Heath care (Centers for Disease Control and Prevention, 2011; Rantala, 2008).

This study also reports an HBV carriage in 12.3% of children under 16 years; a significant difference was observed between younger (0-5 years) and older children (6-15 years), (7.3% vs. 14.4%) $p=0.25$. At this stage, the study cannot demonstrate the evidence of a vertical transmission or a horizontal infection. In fact, some traditional practices could explain the high prevalence of HBV in children, particularly, mothers using saliva to heal baby's wound. However, vertical transmission probably plays an important role, as in Cote d'Ivoire, no efficient action (HBV free screening during pregnancy, vaccination at birth and not at six weeks) is taken to fight against it.

The lack of significant difference in the prevalence of HBV among people when taking into account their history of blood transfusion can be explained by the improvement of blood safety in Cote d'Ivoire. In fact, HIV, hepatitis B and hepatitis C, and the bacterium *Treponema pallidum* subspecies *pallidum* are routinely detected in blood donations. However, the prevalence of 25.2% of HBV among transfused persons to 32% in non-transfused shows that the contamination by a residual risk of blood transfusion remains (Ouattara et al., 2006).

Conclusion

This study reports a high prevalence of HBV and HCV

carriage in Cote d'Ivoire. The results of our study showed that youth, gender, sexual risk behaviors, scarification, living out of Abidjan in one hand and in the other hand old age, sexual risk behaviors, literacy, schooling are the common risk factors for transmission, respectively of HBV and HCV infection in the community. A better organization and increased awareness campaigns on HBV and HCV will reduce their prevalence. The measures to reduce the disease and its load transmission must be strengthened.

Conflict of interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Diversity and composition of methanotrophs in paddy soil as affected by different long-term fertilizer management from double-cropping paddy fields in Southern China

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Methane (CH_4) is the most important greenhouse gas, which was produced from paddy fields. The CH_4 production and emission were affected by methane–oxidizing bacteria (methanotrophs). Therefore, it is significant to investigate the effects of fertilizer applications on the change of soil methanotrophs, which affected CH_4 emission. The objective of this paper was to describe changes of CH_4 and diversity and composition of methanotrophs in paddy soil in relation to the application of crop residues, mineral fertilizer, and manure based on a long-term field experiment. In this study, static chamber-gas chromatography technique, real-time polymerase chain reaction (PCR) and Illumina high-throughput sequencing of the 16S rRNA gene, respectively, were used to analyze the CH_4 emissions from paddy fields, soil methanotrophs abundance and community diversity from May to October 2014 under five fertilization treatments: mineral fertilizer (MF), rice residue and mineral fertilizer (RF), low manure rate and mineral fertilizer (LOM), and high manure rate and mineral fertilizer (HOM), as compared to without fertilizer input (CK). The results indicated that CH_4 from fertilization treatments displayed different emission patterns during early and late rice growth period. HOM treatment had the highest CH_4 emissions during early and late rice growth period with 5.074 and 6.099 g m^{-2}, respectively. Some methanotrophs genera (*Methylosinus, Crenothrix, Methylocaldum, Methylomicrobium* and *Methylomonas*) were identified at the early and late rice main growth stages. The abundance and composition of soil methanotrophs were affected by long-term fertilization managements. The methanotrophs abundance was inhibited under MF treatment, while they were stimulated under RF, LOM and HOM treatments. The abundance and community composition of methanotrophs in paddy soil were affected by fertilizers of mineral, crop residues, and manure. It was concluded that application with organic and crop residues enhance the abundance and community composition of methanotrophs in double-cropping paddy fields in Southern China through a long-term fertilizer experiment.

Keywords: CH_4, long-term fertilization, methanotrophs diversity, methanotrophs composition, paddy field.

INTRODUCTION

Methane (CH_4) is the most greenhouse gas in the atmosphere and contributes approximately 18% to global

warming (IPCC, 2007). The atmospheric CH_4 concentration was affected by CH_4 production and oxidation. Paddy field is one of the major sources of CH_4, which annually emits 60 Tg CH_4 into the atmosphere (Lowe, 2006). Rice is the major food crops to feed people, especially in Asia (Krüger and Frenzel, 2003; Conrad et al., 2006). Therefore, some agronomic practices need to be strengthened and improved to obtain higher grain yield, such as fertilizer applications.

The CH_4 emission is affected by methanotrophs in the surface soil layer and rhizosphere, then it releases into the atmosphere. That is, CH_4 emission from paddy field was influenced by methanotrophs, which were gram-negative bacteria that utilize CH_4 as their sole source of carbon and energy (Lowe, 2006). According to the physiology, phylogeny, morphology and biochemistry, methanotrophs were classified into three main groups (type I, type II, and type X) (Hanson and Hanson, 1996). The growth and activity of methanotrophs was influenced by many factors, such as soil conditions, fertilizer application and vegetation cover (Hanson and Hanson, 1996; Zheng et al., 2008). And the fertilizer applications and rice plant are the important factors that affect growth and activity of methanotrophs.

At present, molecular approaches is widely used to assess the diversity and activity of methanotrophs, which used phylogenetic and functional gene probes to detect and analyze methanotrophs from samples (Murrell et al., 1998). In addition to the 16S rRNA gene, the presence and abundance of CH_4 oxidizers were also shown by functional genes of methanotrophs (Fjellbirkeland et al., 2001; Horz et al., 2001). For discriminating the methanotrophs, in earlier studies, different polymerase chain reaction (PCR) primers were designed to amplify 16S rRNA gene fragments of different groups CH_4 oxidizers (Henckel et al., 1999). High-throughput sequencing of the 16S rRNA gene was used to determine that the composition and diversity of microbial in response to soil tillage, different crop rotation and fertilizer applications. Soil methanotrophs community structure was changed after long-term fertilizer applications (Zheng et al., 2008). The relative data was used to measure abundance of operational taxonomic units (OTUs) and to calculate indices of richness and shannon of soil samples.

Our studies indicated that long-term fertilization managements could lead to significant changes in diversity of some soil microbe, enzyme activities, such as aerobic bacterial, actinomycete, fungus and β-glucosidase (Tang et al., 2014). Soil methanotrophs play an important role in carbon cycling in terrestrial ecosystems. The fertilizer application is the important factor that affects growth and activity of methanotrophs. A viable option was applied with manure and crop residue to changing CH_4 emissions, methanotrophs abundance and community composition in paddy field? Therefore, the objectives of this research were to: (1) CH_4 emissions from paddy fields respond to the long-term fertilization managements, and (2) long-term fertilizer managements lead to changes in methanotrophs abundance and community composition in paddy field. Therefore, the gas and soil samples were collected from a long-term fertilization experimental field and the CH_4 emissions from paddy fields, the methanotrophs abundance and community composition were studied using static chamber-gas chromatography technique, real-time PCR and Illumina high-throughput sequencing based on both 16S rRNA gene, respectively.

MATERIALS AND METHODS

Sites and cropping system

The experiment was established in 1986. It was located in Ning Xiang County (28°07' N, 112°18' E, and altitude 36 m) of Hunan Province, China. Under a continent monsoon climate, the annual mean precipitation was 1553 mm and potential evapotranspiration of 1354 mm. The monthly mean temperature was 17.2°C. Soil texture of the plough layer (0 to 20 cm) was silt clay loam with 13.71% sand and 57.73% silt. At the beginning of the study, the surface soil characteristics (0 to 20 cm) were as follows: soil organic carbon (SOC) 29.4 g kg^{-1}, total nitrogen 2.0 g kg^{-1}, available N 144.1 mg kg^{-1}, total phosphorous 0.59 g kg^{-1}, available P 12.87 mg kg^{-1}, total potassium 20.6 g kg^{-1}, and available potassium 33.0 mg kg^{-1}. There were three crops in a year, barley (*Hordeum vulgare* L.), early rice, and late rice (*Oryza sativa* L.). Barley was sown in the middle of November and harvested in early May of the following year. Early rice was then transplanted and harvested in the middle of July. The growth period of late rice lasted from late July to the end of October.

Experiment design

The experiment had five treatments: control (without fertilizer input, CK), mineral fertilizer (MF), rice residue and mineral fertilizer (RF), low manure rate and mineral fertilizer (LOM), and high manure rate and mineral fertilizer (HOM). The design ensured all fertilized treatments received equal N amount (the amount of N in mineral fertilizer plus that from rice residue or manure). The mineral fertilizers included urea, ordinary superphosphate, and potassium chloride. Details about the fertilizer management are listed in Table 1. Before rice transplanting seedling, manure and air-dried rice residue were incorporated into soil surface. The cultivation depth was about 20 cm. For early rice, and late rice, 70 and 60% of N was applied at seeding, and the remaining N was applied at top dressing stages. All the P_2O_5 and K_2O were applied at seeding stages. There were three replications and each plot size was 66.7 m^2. We referred to the data for the individual cropping periods from May to October, 2014.

1) For the RF treatment, rice straw return rate (air dry) was 2780 and 3600 kg ha^{-1} for early and late rice. (2) For the LOM treatment, manure application rate (decomposed) was 2625.0 and 2670.0 kg

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Table 1. Nutrient supply from rice straw, chicken manure and mineral fertilizer under different fertilizer treatments (kg ha^{-1}).

Treatments	Early rice			Late rice			Total		
	N	P_2O_5	K_2O	N	P_2O_5	K_2O	N	P_2O_5	K_2O
CK	0+0*	0+0	0+0	0+0	0+0	0+0	0	0	0
MF	142.5+0	54.0+0	63.0+0	157.5+0	43.2+0	81.0+0	300.0	97.2	144.0
RF	124.4+18.1	50.4+3.6	38.3+24.7	133.0+24.5	37.8+5.4	48.2+32.8	300.0	97.2	144.0
LOM	96.0+46.5	33.0+21.0	33.6+29.4	110.2+47.3	21.8+21.4	51.1+29.9	300.0	97.2	144.0
HOM	49.6+92.9	12.0+42.0	4.2+58.8	63.0+94.5	0.5+42.7	21.2+59.8	300.0	97.2	144.0

*Input from mineral fertilizer + input from organic fertilizer. The treatments are without fertilizer (CK), mineral fertilizer (MF), crop residue and mineral fertilizer (RF), low manure rate and mineral fertilizer (LOM), and high manure rate and mineral fertilizer (HOM).

ha^{-1} for early and late rice. (3) For the HOM treatment, manure application rate (decomposed) was 5250.0 and 5340.0 kg ha^{-1} for early and late rice. (4) The N, P_2O_5, and K_2O content of air-dry early rice straw was 0.65, 0.13, and 0.89%; N, P_2O_5, and K_2O content of air-dry late rice straw was 0.68, 0.15, and 0.91%, respectively; and N, P_2O_5, and K_2O content of decomposed chicken manure was 1.77, 0.80, and 1.12%, respectively.

Sample collection

Samples were collected in 2014. In each plot, soil samples in the rhizosphere soil were collected from the rice plants root at different rice growth stages. Three samples were collected from each plot. The samples were immediately frozen until further analyses could be performed.

CH_4 emissions from paddy fields were investigated using the static chamber-GC technique at 9:00 to 11:00 in the morning during rice growth period. From the second day after transplanting of early or late rice, gases sample were collected weekly.

Measurement of CH_4

The quantities of CH_4 emission were survey with a gas chromatograph (Agilent 7890A) equipped with flame ionization detector (FID). CH_4 was separated using 2 m stainless-steel column with an inner diameter of 2 mm 13×MS column (60/80 mesh), with FID at 200°C.

CH_4 fluxes were calculated with the following equation (Liebig et al., 2010):

$$F = \rho h \, [273 / (273+T)] \, dC / dt$$

where F is the CH_4 emission (mg m^{-2} h^{-1}); T is the air temperature (°C) inside the chamber; ρ is the CH_4 density at standard state (0.714 kg m^{-3} for CH_4); h is the headspace height of the chamber (m); and dC/dt is the slope of the curve of gas concentration variation with time.

The total CH_4 emissions (g $CH_4 \cdot m^{-2}$) were sequentially computed from the emissions between every 2 adjacent intervals of the measurements, based on a non-linear, least-squares method of analysis (Singh et al., 1996).

DNA Extraction and PCR

For each sample, DNA was isolated from 0.5 g of soil using the MoBio PowerSoil™ DNA Isolation Kit (Carlsbad, CA, USA). Extractions were executed according to the manufacturer's protocol. All genomic DNA concentration and purity were determined by NanoDrop spectrophotometry (Thermo Scientific, Wilmington, DE, USA). PCR was performed at an initial denaturation temperature of 94°C for 3 min, followed by 20 cycles of 94°C for 45 s, 53°C for 30 s, and 65°C for 90 s. A final elongation step at 65°C was run for 10 min. PCR products were purified using the Qiagen™ PCR purification kit following the manufacturer's protocol with the exception of eluting in sterile water (Qiagen, Valencia, CA, USA) and quantified in Qubit 2.0 Fluorometer (Invitrogen, NY, USA).

Illumina high-throughput sequencing of 16S rRNA genes

Primers 515F and 806R (Caporaso et al., 2010) were used to target the V3–V4 region of the 16S rRNA gene with the addition of a barcoded sequence and the required Illumina adapters. Sequencing was performed on an Illumina (Illumina, Miseq–OE Biotech Company; Shanghai, China) with two paired-end read cycles of 300 bases each. Sequence analysis and OTUs identification was according to the methods of Giongo et al. (2010) and Fagen et al. (2012). Reads were trimmed to remove low quality bases and to remove the first 11 bases corresponding to the primer region by a script based on Trim2 (Huang et al., 2003), and then the reads were separated by barcode. Paired reads were assembled using FLASH to the reference greengenes (Reyon et al., 2012) 16S SSU rRNA database.

IFA analysis of methanotrophs bacteria

The methanotrophs genera were selected using the indirect immunofluorescence (IFA) method (Svetlana et al., 2007). Eight polyclonal antibodies specific for 8 methanotrophs genera, namely, *Methylosinus*, *Crenothrix*, *Methylobacter*, *Methylocaldum*, *Methylococcus*, *Methylomicrobium*, *Methylomonas*, and *Methylosarcina* were applied. The cross-reactivity and specificity of the antibodies were tested previously and found to be species-specific (Svetlana et al., 2007). The total number of methanotrophs was calculated as the sum of 8 pre-selected methanotrophs genera.

Diversity indices

To estimate bacterial diversity of each sample, the number of OTUs, richness and shannon index were calculated using Mothur (Shannon, 1963). The phylogenetic distribution of OTUs was constructed by QIIME software.

Data analysis

The data were analyzed as a randomized complete block, using the

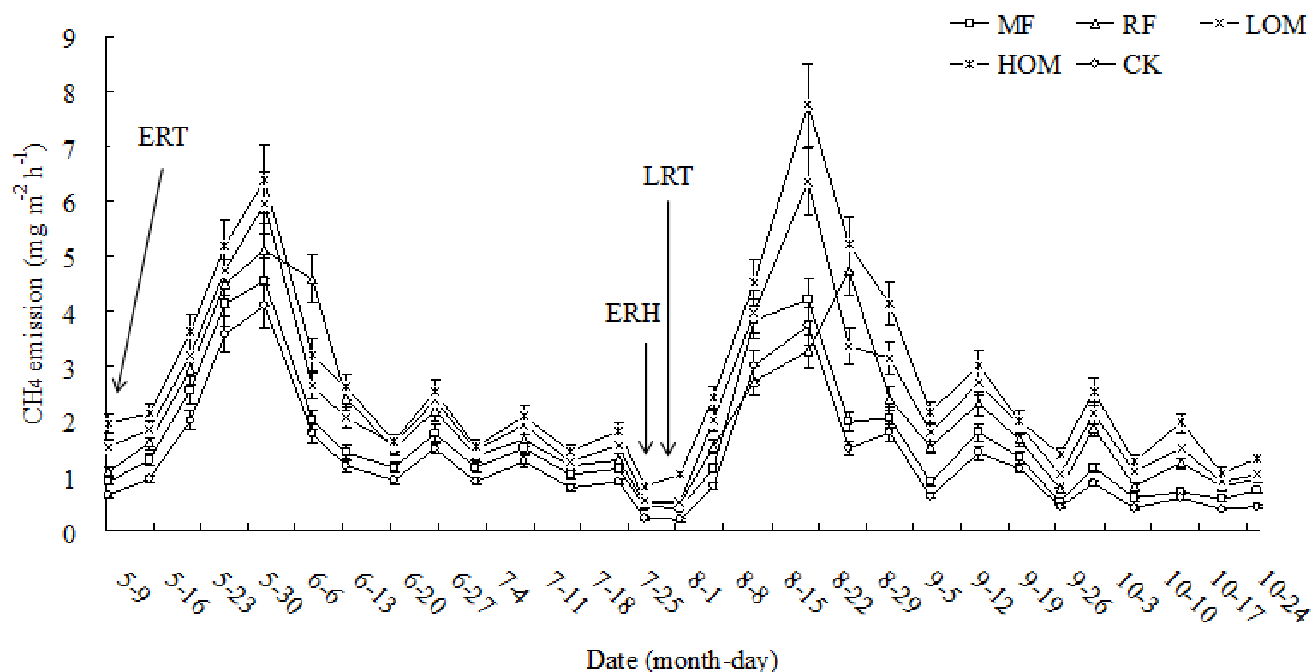

Figure 1. CH_4 emission from paddy fields affected by long–term fertilizer managements during early and late rice growth period. MF: Mineral fertilizer; RF: crop residue and mineral fertilizer; LOM: low manure rate and mineral fertilizer; HOM: high manure rate and mineral fertilizer; CK: without fertilizer. ERT: early rice transplanting; ERH: early rice harvesting; LRT: late rice transplanting. CH_4 emission rate is the mean of values measured within each treatment (n=3). Bars indicate standard deviation.

PROC ANOVA procedure of SAS (SAS Institute, 2003). Mean values were compared using the least significant difference (LSD) test and a probability value of 0.05 was considered to indicate statistical significance.

RESULTS

CH_4 emission

During early rice growing season, the CH_4 emission was low after early rice transplanting, but increased quickly until the peak appeared at 23 days after transplanting, and then declined to a low and stable level (Figure 1). The CH_4 emission were significantly different among treatments with the sequence of HOM>LOM>RF>MF>CK (P<0.05) during early rice growing season (Figure 1).

During late rice growing season, CH_4 emission mainly focused at tillering stage and the peak value of CH_4 emission was observed at 24 days after transplanting. Then, the emission rate decreased to a low level with relative stability. The sequence of treatments in CH_4 emission was HOM>LOM>RF>MF>CK (Figure 1).

During early rice growing season, the accumulate CH_4 emission of CK was significantly lower than MF, RF, LOM, and HOM (P<0.05) and the sequence with different treatments was HOM>LOM>RF>MF>CK (Table 2). The total CH_4 emissions from paddy fields during late rice growing season were 3.212 g m^{-2} in MF, 3.961 g m^{-2} in RF, 4.881 g m^{-2} in LOM, 6.099 g m^{-2} in HOM, and 2.548 g m^{-2} in CK. The order of treatments in total CH_4 emission was HOM>LOM>RF>MF>CK (Table 2). Meanwhile, HOM had larger total CH_4 emissions than other treatments during early and late rice growing season.

Operational taxonomic units of methanotrophs bacteria

At the early rice main growth stages, the root methanotrophs bacteria of rice plants from the LOM treatment had the highest number of OTUs (Figure 2). And the highest values of the OTUs in LOM with 76 at seedling stage (SS), the highest values of the OTUs in MF, RF, CK with 23, 41, and 47 at tillering stage (TS) among the different treatments. At the early rice main growth stages, the OTUs values were significantly different among treatments with the sequence of LOW>CK>RF>HOM>MF (Figure 2). At the late rice main growth stages, the highest values of the OTUs at SS in LOM and HOM with 53 and 40, and then dramatically declined to a low level. And the highest values of the OTUs were observed at TS in MF and RF with 39 and 62 after transplanting. Then, the OTUs values dramatically decreased to a low level.

Genetic diversity indices of methanotrophs

At early and late rice main growth stages, the root

Table 2. Effects of long–term fertilizer managements on CH_4 emission from paddy fields during whole growth period of early and late rice (g $CH_4 \cdot m^{-2}$).

Treatments	Early rice	Late rice	Total
MF	3.470±0.147^{c}	3.212±0.176^{d}	6.682±0.323^{d}
RF	4.418±0.131^{b}	3.961±0.141^{c}	8.379±0.271^{c}
LOM	4.521±0.128^{b}	4.881±0.114^{b}	9.402±0.242^{b}
HOM	5.074±0.100^{a}	6.099±0.093^{a}	11.173±0.193^{a}
CK	2.886±0.083^{d}	2.548±0.074^{e}	5.434±0.157^{e}

MF: Mineral fertilizer; RF: crop residue and mineral fertilizer; LOM: low manure rate and mineral fertilizer; HOM: high manure rate and mineral fertilizer; CK: without fertilizer. Values are presented as mean ± SE (n = 3). Means in each column with different letters are significantly different at the $P < 0.05$ level.

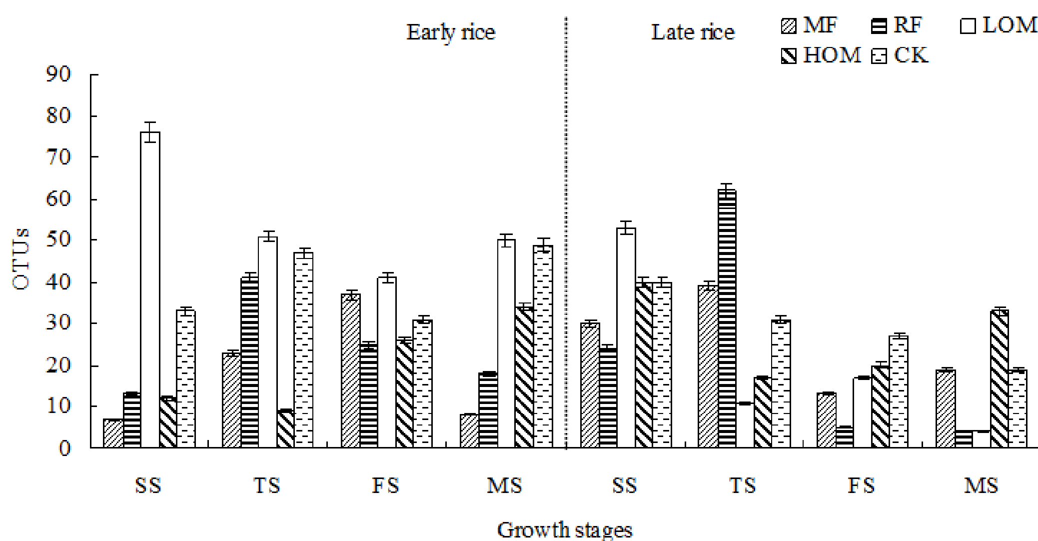

Figure 2. Operational taxonomic units of methanotrophs bacteria with different fertilizer treatments at rice main growth stages. SS: Seedling stage; TS: tillering stage; FS: full heading stage; MS: maturity stage. MF: mineral fertilizer; RF: crop residue and mineral fertilizer; LOM: low manure rate and mineral fertilizer; HOM: high manure rate and mineral fertilizer; CK: without fertilizer. Values are presented as mean ± SE (n = 3).

methanotrophs of rice plants from the paddy fields had the highest values of the richness indexes at TS and SS, respectively, and had the lowest values of the richness indexes at maturity stage (MS). Meanwhile, at early and late rice main growth stages, the values of the richness indexes were higher in HOM than in MF, RF, LOM and CK. The order of treatments in the values of the richness indexes was HOM>LOM>RF>CK>MF (Table 3).

At early and late rice main growth stages, the root methanotrophs of rice plants from the paddy fields had the highest values of the shannon indexes at TS and SS, respectively and had the lowest values of the shannon indexes at MS. Meanwhile, at the early and late rice main growth stages, the values of the shannon indexes were higher in HOM and LOM than in MF, RF and CK. The order of treatments in the values of the shannon indexes was HOM>LOM>RF>CK>MF (Table 3).

Structure of the methanotrophs bacteria community

It was indicated that some methanotrophs genera (*Methylosinus, Crenothrix, Methylobacter, Methylocaldum, Methylococcus, Methylomicrobium, Methylomonas* and *Methylosarcina*) were identified at main growth stages of early rice and late rice. It was shown that some methanotrophs genera were detected at some growth stages of early rice (Table 4). *Methylosinus, Crenothrix, Methylocaldum, Methylomicrobium* and *Methylomonas* were detected more often in all treatments at early rice main growth stages. *Methylobacter* were detected more often in LOM at the SS and MS. *Methylococcus* were detected more often in CK at main growth stages of early rice. *Methylosarcina* were detected more often in HOM and CK at early rice main growth stages.

Methylosinus, Crenothrix, Methylomicrobium,

Table 3. Genetic diversity indices of methanotrophs with different fertilizer treatments during rice main growth stages.

Rice	Treatment	Items							
		Richness index				Shannon index			
		SS	TS	FS	MS	SS	TS	FS	MS
Early rice	MF	22.4±0.65^{b}	25.4±0.73^{b}	19.7±0.57^{bc}	16.8±0.49^{b}	2.75±0.08^{c}	3.17±0.09^{b}	2.68±0.08^{b}	2.24±0.06^{b}
	RF	23.8±0.69^{ab}	26.8±0.77^{ab}	21.5±0.62^{ab}	17.8±0.51^{ab}	2.97±0.09^{ab}	3.45±0.10^{ab}	2.84±0.08^{ab}	2.42±0.07^{ab}
	LOM	24.3±0.70^{ab}	27.2±0.79^{ab}	21.9±0.63^{ab}	18.2±0.53^{ab}	3.06±0.09^{a}	3.57±0.10^{a}	2.92±0.08^{ab}	2.51±0.07^{ab}
	HOM	24.7±0.71^{a}	28.3±0.82^{a}	22.3±0.64^{a}	18.5±0.53^{a}	3.18±0.09^{a}	3.63±0.10^{a}	3.07±0.09^{a}	2.63±0.08^{a}
	CK	23.0±0.66^{ab}	26.0±0.75^{ab}	20.3±0.59^{c}	17.4±0.50^{ab}	2.85±0.08^{bc}	3.34±0.10^{ab}	2.75±0.08^{b}	2.38±0.07^{b}
Late rice	MF	23.4±0.68^{a}	20.8±0.60^{b}	18.7±0.54^{b}	15.1±0.44^{b}	2.82±0.08^{c}	2.73±0.08^{c}	2.67±0.08^{c}	2.21±0.06^{c}
	RF	24.7±0.71^{a}	22.3±0.64^{ab}	19.7±0.57^{ab}	15.8±0.46^{ab}	3.02±0.09^{ab}	2.97±0.09^{ab}	2.95±0.09^{ab}	2.47±0.07^{ab}
	LOM	25.2±0.73^{a}	22.8±0.66^{ab}	20.4±0.59^{ab}	16.2±0.47^{ab}	3.14±0.09^{a}	3.04±0.09^{a}	3.02±0.09^{ab}	2.58±0.07^{a}
	HOM	25.6±0.74^{a}	23.4±0.68^{a}	20.8±0.60^{a}	16.6±0.48^{a}	3.26±0.09^{a}	3.18±0.09^{a}	3.14±0.09^{a}	2.66±0.08^{a}
	CK	24.2±0.70^{a}	21.5±0.62^{ab}	19.4±0.56^{ab}	15.6±0.45^{ab}	2.95±0.09^{bc}	2.86±0.08^{bc}	2.84±0.08^{bc}	2.35±0.07^{bc}

SS: Seedling stage; TS: tillering stage; FS: full heading stage; MS: maturity stage. MF: mineral fertilizer; RF: crop residue and mineral fertilizer; LOM: low manure rate and mineral fertilizer; HOM: high manure rate and mineral fertilizer; CK: without fertilizer. Values are presented as mean ± SE (n = 3). Means in each column with different letters are significantly different at the $P < 0.05$ level.

Methylomonas and *Methylosarcina* were detected more often in all treatments at late rice main growth stages. *Methylocaldum* were detected more often in MF, RF, HOM and CK at main growth stages of late rice. *Methylococcus* were not detected in LOM and HOM at late rice main growth stages (Table 5).

Heatmap in the different fertilizer treatments during rice growth period

The 8 most abundant genera for five treatments at early rice main growth stages were analyzed and the differences were shown in the heatmap (Figure 3). The abundances of *Methylomonas* and *Crenothrix* were significantly higher in different treatments at main growth stages of early rice. *Methylosinus* were relatively more abundant in different treatments at SS. *Methylocaldum* were relatively more abundant in different treatments at SS and MS. On the other hand, *Methylobacter* and *Methylococcus* were not detected in different treatments at main growth stages of early rice. *Methylomicrobium* and *Methylosarcina* were not detected in different treatments at full heading stage (FS).

At the late rice main growth stages, the relative abundances of *Methylomonas* were significantly higher in different treatments. *Methylosinus* were relatively more abundant in different treatments at SS. *Methylomicrobium* were relatively more abundant in different treatments at FS and MS. *Methylosarcina* were relatively more abundant in different treatments at MS. On the other hand, *Methylococcus* and *Methylocaldum* were not detected in different treatments at main growth stages of late rice. *Methylomicrobium* were less abundant in different treatments at SS and TS. *Crenothrix* were less abundant in different treatments at MS (Figure 4).

Phylogenetic tree analysis of methanotrophs gene clones among different fertilizer treatments

At the early rice main growth stages, clustering analysis allowed the identifcation of OTUs responsible for the community shifts in different fertilization at genera level. The results showed that OTUs *Methylomonas* and *Crenothrix* were especially abundant in the soil samples. When phylogenetic trees were constructed using abundance belonging to each OTUs, *Methylocaldum* and *Methylosarcina* were grouped into a tight and distinct cluster, *Methylomonas* and *Crenothrix* were also grouped into a tight and distinct cluster. One-third of the clones in OTUs were grouped into a cluster including *Methylocaldum* (Figure 5).

At the late rice main growth stages, the results showed that OTUs *Methylomonas* and *Methylocaldum* were especially abundant in the soil samples. When phylogenetic trees were constructed using abundance belonging to each OTUs, *Crenothrix* and *Methylocaldum* were grouped into a tight and distinct cluster, *Methylomonas* and *Methylosarcina* were also grouped into a tight and distinct cluster. Quarter of the clones in OTUs was grouped into a cluster including *Methylocaldum* (Figure 5).

DISCUSSION

Effects of fertilizer applications on CH_4 emission

CH_4 emission is complex processes including production

Table 4. Genera of selected methanotrophs in different treatments at main growth stages of early rice.

Stage	Treatment	Methanotrophs							
		Methylosinus	*Crenothrix*	*Methylobacter*	*Methylocaldum*	*Methylococcus*	*Methylomicrobium*	*Methylomonas*	*Methylosarcina*
SS	MF	+	+	-	+	-	-	+	+
	RF	+	+	-	+	-	+	+	-
	LOM	+	+	+	+	-	+	+	-
	HOM	+	-	-	+	-	-	-	+
	CK	+	+	-	+	+	+	+	+
TS	MF	+	+	-	+	+	-	+	+
	RF	+	+	-	+	-	+	+	+
	LOM	+	+	-	+	-	+	+	+
	HOM	-	-	-	+	-	+	+	+
	CK	+	+	-	+	+	+	+	+
FS	MF	+	+	-	+	+	+	+	-
	RF	+	+	-	+	+	+	+	-
	LOM	+	+	-	-	-	+	+	-
	HOM	+	+	-	+	+	+	+	+
	CK	+	+	-	+	+	+	+	+
MS	MF	+	-	+	+	-	+	-	+
	RF	+	+	-	+	-	+	+	-
	LOM	+	+	+	+	+	+	+	+
	HOM	+	+	-	-	-	+	+	+
	CK	+	+	-	+	+	+	+	+

+ is present, it exist; - is absent, did not exist.

Table 5. Genera of selected methanotrophs in different treatments at main growth stages of late rice.

Stage	Treatment	Methanotrophs						
		Methylosinus	*Crenothrix*	*Methylocaldum*	*Methylococcus*	*Methylomicrobium*	*Methylomonas*	*Methylosarcina*
SS	MF	+	-	+	+	+	+	+
	RF	+	+	+	+	+	+	+
	LOM	+	+	-	-	+	+	+
	HOM	+	+	+	-	+	+	+
	CK	+	+	+	+	+	+	+

Table 5. Contd.

Stage	Treatment	Methanotrophs						
		Methylosinus	*Crenothrix*	*Methylocaldum*	*Methylococcus*	*Methylomicrobium*	*Methylomonas*	*Methylosarcina*
TS	RF	+	+	+	-	+	+	+
	LOM	+	+	-	-	+	+	-
	HOM	+	+	+	-	+	-	+
	CK	+	+	+	-	+	+	+
FS	MF	+	+	-	-	+	+	-
	RF	-	+	-	+	+	-	+
	LOM	+	+	+	-	+	+	+
	HOM	+	+	+	-	+	+	+
	CK	+	+	+	+	+	+	+
MS	MF	-	+	+	-	+	+	+
	RF	+	-	-	-	+	-	+
	LOM	+	-	-	-	-	+	+
	HOM	+	+	+	-	+	+	+
	CK	-	+	+	+	+	+	+

+ is present, it exist; - is absent, did not exist.

and oxidation. CH_4 production was regulated by vegetation type, fertilizer managements, soil temperature, soil moisture, root activity and many other factors (Wassmann et al., 2004; Kallenbach et al., 2010; Ma et al., 2008). In this study, the total CH_4 emission from paddy fields during rice growth period were much higher in HOM, LOM and RF as compared to CK (Figure 1 and Table 2), which was similar to the result by Wang et al. (2013). The reasons was (1) microbial activities were improved after returning rice residues, manure into the soil for that supplements of carbon source and energy for microbial activities to accelerate consumption of soil oxygen and decrease of soil redox potential (Eh); (2) methanogens became active for the large quantities of carbon source, which provided reactive substrate for CH_4 emission from paddy fields. Meanwhile, compared with the RF, LOM and HOM, we also observed that MF decreased the CH_4 emission and resulted in a positive CH_4 emission from paddy fields. CH_4 emission was probably reduced because of excessive soil inorganic N level. Reduced root growth due to lower soil inorganic N level as a result of absence of N fertilization also probably reduced the methanotrophs activities, thereby resulting in lower CH_4 emission in MF. Several researchers (Bronson and Mosier, 1994; Powlson et al., 1997) have reported that N fertilization reduced CH_4 emission compared to no N fertilization. However, CH_4 emissions at early and late rice growth periods were decreased to a large extent after field drying, the reason may be (1) the methanogens activities were limited when the soil aeration was increased after field drying; and (2) the ability for transportation and emission of CH_4 was limiting, which the rice plant physiological activities was decreased.

Effects of fertilizer applications on soil methanotrophs diversity

Organic and crop residues fertilization resulted in increasing methanotrophs bacteria diversity (Figure 2), showing that application of organic, crop residues significantly influenced methanotrophs bacteria composition, and mineral fertilization influenced methanotrophs bacteria composition to a lesser degree.

The response of methanotrophs bacteria community structures to agricultural managements was also represented by Nicol et al. (2003). In this study, it was shown that higher number of OTUs

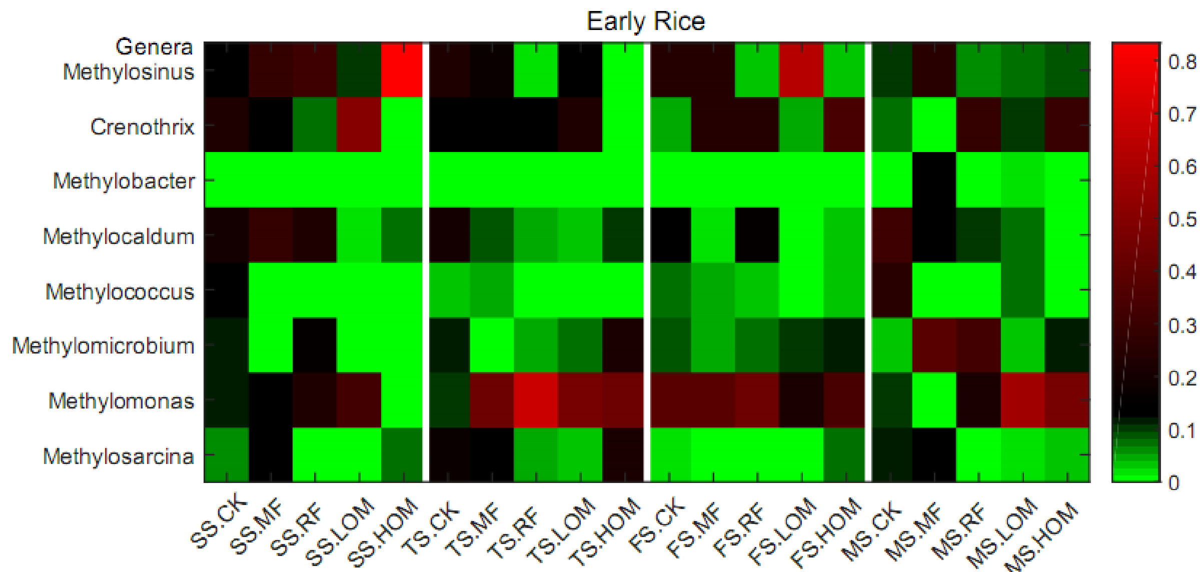

Figure 3. Heatmap illustrating the 8 most abundant methanotrophs genera in the different fertilizer treatments at early rice main growth stages. SS: seedling stage; TS: tillering stage; FS: full heading stage; MS: maturity stage. MF: mineral fertilizer; RF: crop residue and mineral fertilizer; LOM: low manure rate and mineral fertilizer; HOM: high manure rate and mineral fertilizer; CK: without fertilizer.

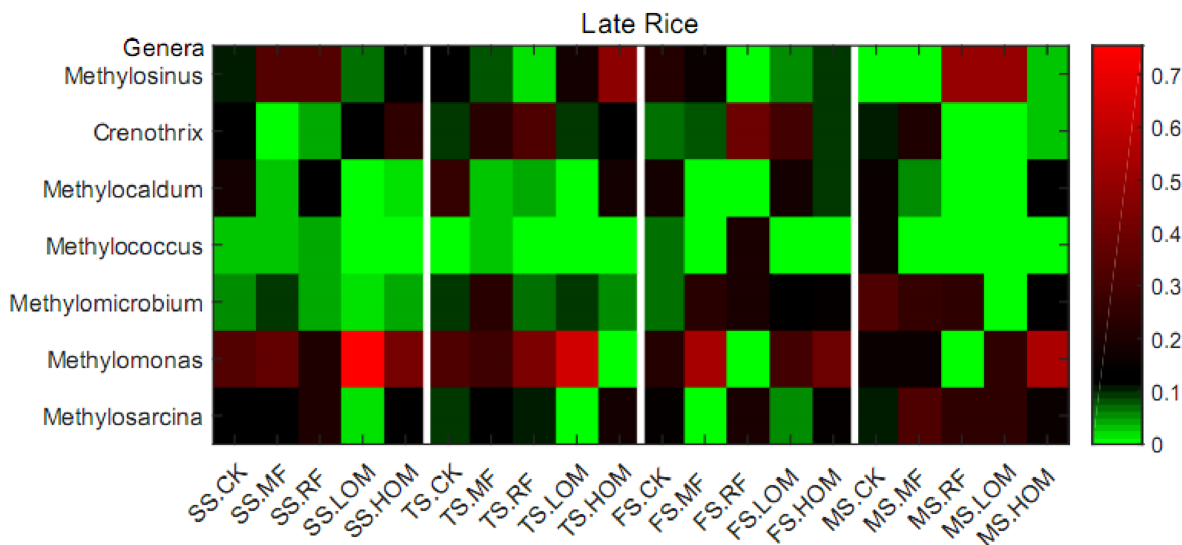

Figure 4. Heatmap illustrating the 7 most abundant methanotrophs genera in the different fertilizer treatments at late rice main growth stages. SS: seedling stage; TS: tillering stage; FS: full heading stage; MS: maturity stage. MF: mineral fertilizer; RF: crop residue and mineral fertilizer; LOM: low manure rate and mineral fertilizer; HOM: high manure rate and mineral fertilizer; CK: without fertilizer.

with organic, crop residues, than that of the mineral fertilizer. The relationship of methanotrophs bacteria and organic, crop residues, and mineral fertilizer in the experiment soil, proposes that organic crop residues is an important nutrient for the found taxa, which is relative to methanotrophs bacteria growth processes.

The methanotrophs microbial diversity was shown by the richness index and shannon index which was larger in HOM, LOM, and RF treatments, which agree with Zheng et al. (2008), who found that soils under NPK and recycled crop residues had higher levels of methanotrophs diversity compared with the conventional fertilization. Meanwhile, shannon index and richness index was significantly higher when application with organic and crop

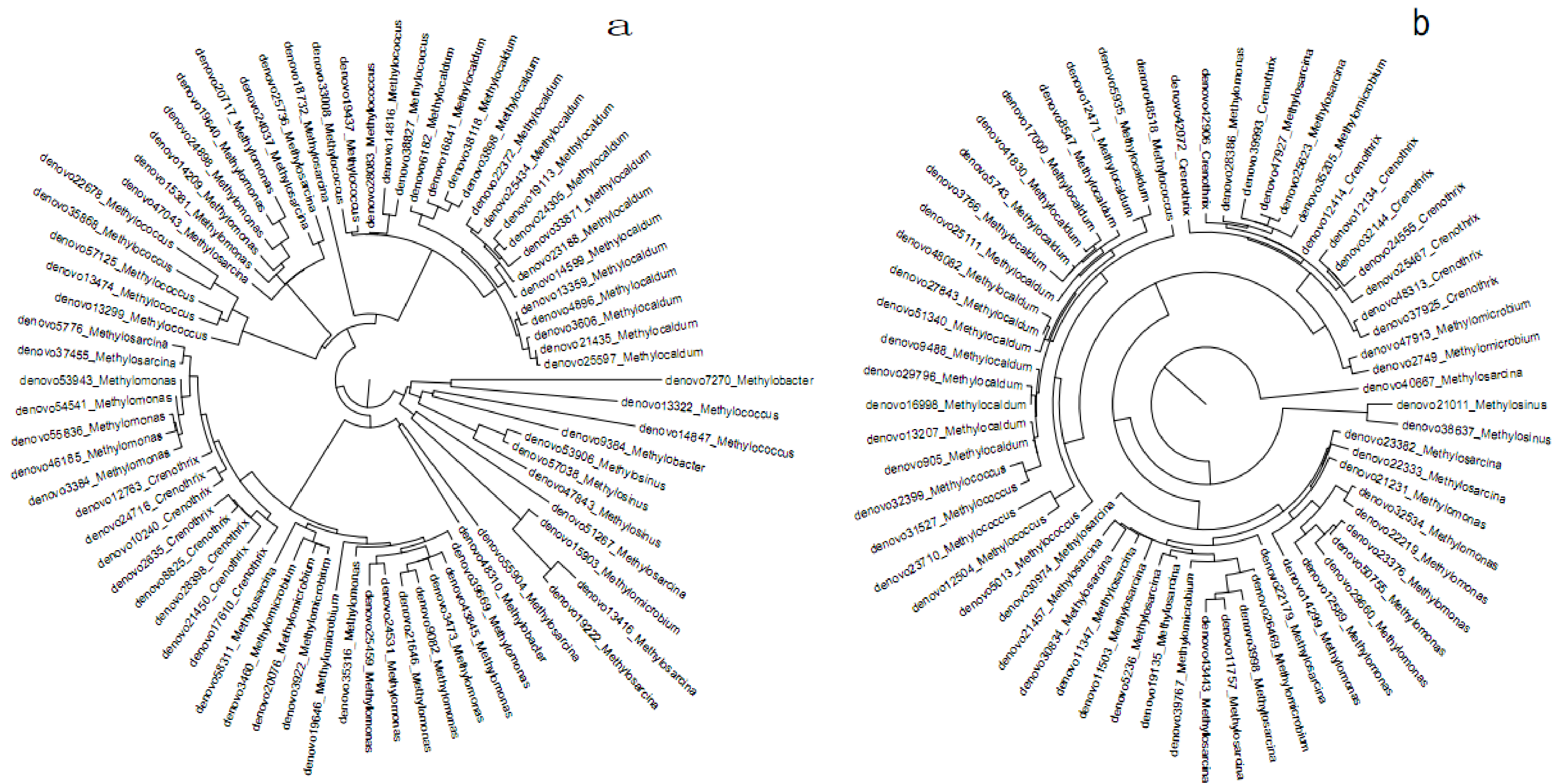

Figure 5. Phylogenetic distribution of OTUs of the clone libraries of 16S ribosomal RNA genes for soil samples under different treatments at early rice (a) and late rice (b) main growth stages.

residues, indicating that the application of organic and crop residues based on application of NPK, with higher rate of C:N, stimulates methanotrophs diversity on the soil. That is, rate of C:N in rice and organic straw substrates is higher than that of other mineral fertilizer (Garcia and Rice, 1994), which provides more substrate to microbial explaining higher shannon index and richness index found in this work on plots where rice was the growing season. Meanwhile, it was key factors that root exudates influenced methanotrophs communities, for that they provide the carbon source to soil microbial (Badri and Vivanco, 2009). Some studies indicated that roots may regulate the soil methanotrophs community in their rhizosphere, change the soil physical and chemical properties, and control the growth of competing plant species (Nardi et al., 2000), which may also affect the

methanotrophs diversity in rhizosphere.

Effects of fertilizer applications on soil methanotrophs community

According to the effects of different fertilizer treatments on methanotrophs abundance, five treatments (MF, RF, LOM, HOM and CK) were chosen to analyze the methanotrophs community structure. Some distinct differences in the diversity indices were found between the mineral fertilizer and application of organic; crop residues fertilizer treatments indicated that the soil methanotrophs community was changed with different fertilization managements. And the diversity pattern was shown in HOM and LOM treatments. In addition to the chemical NPK, the recycled application of crop residues and organic proposal were important to maintain methanogenic bacteria in paddy soils (Conrad and Klose, 2006). It is well-known that the soil CH_4 oxidation rate can be changed by different CH_4 concentrations, thus the growth, activities and community structure of methanotrophs were changed (Bender and Conrad, 1995). In conclusion, according to sequences and phylogenetic analysis, it was shown that soil methanotrophs community structure was changed by different fertilizer treatments.

Some study indicated that the genera of methanotrophs were different between paddy soils and forest soils (Mohanty et al., 2006). In this study, the 8 most abundant genera for five treatments at early rice main growth stages were shown in the heatmap (Figure 3), and the 7 most abundant genera for five treatments at late rice main growth stages were showed in the heatmap (Figure 4), indicated that composition significantly varied among the five estimated treatments (MF, RF, LOM, HOM and CK). Our results showed that methanotrophs were related to the genera of *Methylosinus, Crenothrix, Methylocaldum, Methylomicrobium* and *Methylomonas* with the most usual methane oxidizers in paddy soil with different fertilizer treatments, which were in agreement with an earlier study (Fjellbirkeland et al., 2001).

Conclusions

The results indicated that with the same nitrogen application rate, different organic-inorganic mixed fertilizer application, such as RF, LOM, and HOM, caused substantial CH_4 emissions during early and late rice growth period compared with those from the conventional MF treatment. Meanwhile, the abundance and composition of methanotrophs was affected by long-term fertilization managements. In the MF treatment, methanotrophs abundance was inhibited, compared to the RF, LOM and HOM treatments. The methanotrophs diversity of the HOM and LOM treatments was distinguished from RF, MF and CK. Furthermore, methanotrophs community composition was changed with different treatments based on the sequences and phylogenetic analysis. And the higher ratio genera of *Methylosinus, Crenothrix, Methylomicrobium, Methylocaldum* and *Methylomonas* were found in the five treatments. In summary, the application of mineral fertilizer was an important factor that affected the abundance of methanotrophs and application of organic; crop residues enhance the abundance of methanotrophs in double-cropping paddy fields in Southern China. It is important to understand that the main effective factors for abundance and composition of methanotrophs, was linked to soil ecosystem processes and sustainable developing management of rice cultivation.

Conflict of Interests

The authors have not declared any conflict of interest.

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