# Dynamics of methane oxidation and composition of methanotrophic community in planted rice microcosms

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# Pledge

I certify that the present thesis entitled:

**"Dynamics of methane oxidation and composition of methanotrophic community in planted rice microcosms"**

was carried out without any unlawful devices. I did not use any other than the described literature sources or technical devices. This work has never been submitted before in this or similar form to any other university and has not been used before any examination.

Marburg, 22.04.2008

Minita Shrestha

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## Dedication

I dedicate this work to:

- my husband,
- my grandparents,
- my parents
- my parents-in-law
- and those who will find it valuable.

The present work was carried out between April 2005 and March 2008 at the Max Planck Institute for Terrestrial Microbiology, Marburg, Germany under the supervision of Prof. Dr. Ralf Conrad.

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# Abbreviations

| Abbreviations | Full form |
|---|---|
| δ..... | delta |
| %…….. | percent |
| ‰…… | per mil |
| <……... | lesser than |
| >……... | greater than |
| $^{14}C$……. | radio carbon isotope |
| $^{13}C$ ……... | stable carbon isotope |
| AMO…… | ammonia monooxygenase enzyme |
| ANOVA……... | analysis of variance |
| ARB……. | Latin, "arbor" = tree |
| bp……. | base pair |
| C…….. | carbon |
| CCA……. | Canonical Correspondence Analysis |
| cDNA……... | copy DNA |
| $CH_4$…….. | methane |
| cm……..…… | centimeter |
| $CO_2$…….. | carbon dioxide |
| $CH_2F_2$…….. | difluoromethane |
| conc……. | concentration |
| dap…… | day after transplantation |
| DGGE……... | Denaturing Gradient Gel Electrophoresis |
| DM..... | demineralized water |
| DNA..... | deoxyribonucleic acid |
| dNTP..... | deoxyribonucleotide triphosphate |
| EB..... | elution buffer |
| Fig…… | figure |
| GC…… | gas chromatograph |
| GHGs…… | Greenhosue gases |

| | |
|---|---|
| ha…….. | hectar |
| h……..... | hour/hours |
| $H_2$……. | hydrogen |
| i.e…….. | that is |
| IPCC……. | Intergovernmental Panel for Climate Change |
| IRMS…… | Isotope ratio mass spectrometer |
| K…….. | potassium |
| kb……. | kilo base |
| M…….. | molar |
| min..... | minute/minutes |
| mg……..… | milligram |
| µl…….. | microliter |
| µM..... | micromolar |
| mm……... | millimeter |
| mM……... | millimolar |
| min..... | minute |
| MPN……. | most probable number |
| $N_2$..... | nitrogen gas |
| $NH_4^+$..... | ammonium |
| nM..... | nanomolar |
| $N_2O$..... | nitrous oxide |
| $NO_2^-$..... | nitrite |
| $NO_3^-$..... | nitrate |
| NCBI…… | National Center for Biotechnology Information |
| NMS..... | Nitrate mineral salts |
| rpm..... | revolutions per minute |
| RT-PCR ……... | reverse transcription-PCR |
| mRNA…….. | messenger RNA |
| OTU……. | operational taxonomic unit |
| P……... | phosphorous |
| PCR…….. | polymerase chain reaction |
| pM……. | picomolar |
| pMMO……. | particulate monooxygenase enzyme |

| | |
|---|---|
| *pmoA*…… | a subunit of particulate monooxygenase gene |
| PLFA…… | phospholipid fatty acids |
| ppb…… | parts per billion |
| ppmv…… | parts per million by volume |
| RNA…… | ribonucleic acid |
| rRNA…….. | ribosomal ribonucleic acid |
| RS……. | rhizospheric soil |
| RT…… | roots |
| s……... | second/seconds |
| SD…… | standard deviation |
| SIP…… | stable isotope probing |
| sMMO…….. | soluble methane monooxygenase enzyme |
| $SO_4^{2-}$……. | sulphate |
| T-RFLP…… | terminal restriction fragment length polymorphism |
| T-RF……. | terminal restriction fragment |
| U……..... | unit |
| v…….. | volume |

# Summary

Methanotrophs associated with the rhizosphere of rice plant oxidize $CH_4$ with molecular oxygen and use it as the sole source of carbon and energy. Thus, methanotrophs play an important role in global $CH_4$ budget by reducing $CH_4$ emissions from the rice ecosystems to the atmosphere. Rice plant ecosystems may exhibit different niches for methanotrophs as characterized by spatiotemporal variation of $CH_4$ and $O_2$ in the rice rhizosphere. Besides major limiting factors i.e., methane and oxygen, nitrogen (N) one of the limiting factors for rice yields, can also play an important role in methane oxidation. Therefore, in my Ph.D. research, I carried out a detailed study of the methanotrophic community structure and its activity in rice microcosms, which is important for the mechanistic understanding of $CH_4$ oxidation in soil.

The major two experiments were focused on methane oxidation process and methanotrophic community structure using planted rice microcosms that were incubated under controlled conditions in the greenhouse for 55 days (first experiment) and 88 days (second experiment). Methane oxidation process was determined by measuring the $CH_4$ flux in the presence and absence of difluoromethane as a specific inhibitor for methane oxidation. Composition of the methanotrophic community structure was investigated on the roots and in the rhizospheric soil by analyzing *pmoA* gene based on terminal restriction fragment length polymorphism (T-RFLP), and cloning and sequencing. *pmoA* gene encodes a subunit of the particulate methane monooxygenase, the key enzyme of methanotrophs. Abundant T-RFs were affiliated to cloned *pmoA* sequences. The metabolically active methanotrophs were analyzed by stable isotope probing of microbial phospholipids fatty acids (PLFA-SIP) and mRNA expression in the first and second experiment, respectively.

The first experiment was focused on the temporal variation of the methane oxidation process in rice microcosms. The active methanotrophs in the rice rhizosphere were identified by adding $^{13}C$-$CH_4$ directly to the rhizosphere of planted rice microcosms. Root and rhizospheric soil samples were collected after labelling to investigate changes in the total and active methanotrophic community. Both *pmoA* gene analyses based on T-RFLP, and cloning/ sequencing, and PLFA-SIP showed that type I and type II methanotrophic populations changed over time. However, PLFA-SIP showed that type I methanotrophs

were more active than type II methanotrophs indicating they were of particular importance on the roots as well as in the rhizospheric soil.

The second experiment was mainly focused on possible effects of different ammonium-N fertilizers on methane oxidation and composition of the methanotrophic community. For this purpose, planted rice microcosms were fertilized in three different ways; with only phosphorus and potassium (PK) as control, with urea (UPK) as N source, and ammonium sulphate (SPK) as N source. Methane fluxes were measured during the entire rice-cropping season and roots and rhizospheric soil samples were collected from six different growth stages of the rice plant. Methane oxidation rates in PK and UPK treatments were quite similar during most of the cropping season, revealing no nitrogen effect on methanotrophic activity. Ammonium sulphate largely suppressed methanogenesis providing an unfavorable environment for methanotrophs in SPK treatment. Canonical correspondence analysis ordination techniques were applied to investigate the correlation of treatments, environmental factors and process rates with *pmoA*-based T-RFLP profiles both on the roots and in the rhizospheric soil. On the roots, the methanotrophic community was significantly affected by different N-fertilizer treatments, whereas in the rhizospheric soil it was not affected. Overall, the community associated with rice roots was dominated by type I methanotrophs while community in rhizospheric soil was dominated by type II methanotrophs. In summary, I found that different nitrogen fertilizers affected the methanotrophic community structure without significantly affecting the rates of methane oxidation

Samples from the second experiment was in addition used to investigate the expression of *pmoA* mRNA and characterize the metabolically active methanotrophs that were responsible for *in situ* methane oxidation in the rhizospheric soil. No strong influence of N-fertilization was observed on active methanotrophs when comparing PK and UPK treatments. Instead, the temporal variation in substrate or nutrient concentrations seemed to play a relatively important role for the expression of *pmoA* genes in the treatments. Overall, I found that in rhizospheric soil type II methanotrophs were predominant in *pmoA* gene based study whereas type I methanotrophs were predominant in *pmoA* transcript analysis. Hence, type I methanotrophs apparently play an active role for methane oxidation, while type II methanotrophs constitute a background community that also persists under unfavorable conditions, such as in the SPK treatment.

Finally, I isolated methanotrophs from roots and rhizospheric soil samples of the microcosms. In total 11 isolates were obtained from roots (6 isolates) and rhizospheric soil (5 isolates). All the isolates were closely affiliated to type II methanotrophs, indicating that type II methanotrophs were the dominant culturable fraction of methanotrophs both on the roots and in the rhizospheric soil.

# Zusammenfassung

Methanotrophe in der Rhizosphäre der Reispflanze oxidieren $CH_4$ mit molekularem Sauerstoff und verwenden es als einzige Kohlenstoff- und Energiequelle. Im globalen $CH_4$-Haushalt spielen Methanotrophe durch die Reduzierung der $CH_4$-Emissionen aus Reis-Ökosystemen eine wichtige Rolle. Das Reis-Ökosystem stellt für Methanotrophe verschiedene Nischen zur Verfügung, die vor allem durch die räumliche und zeitliche Variation von $CH_4$ und $O_2$ charakterisiert sind. Neben den limitierenden Faktoren $CH_4$ und $O_2$ kann auch Stickstoff (N) – das ist auch ein limitierender Faktor für den Reis-Ertrag – für die Methanoxidation von Bedeutung sein. Im Rahmen meiner Doktorarbeit habe ich in Reis-Mikrokosmosmen die Struktur der methanotrophen Lebensgemeinschaften und deren Aktivität im Detail untersucht, da diese für unser Verständnis des Methanoxidationsprozesses im Boden sehr wichtig sind.

Die beiden wichtigsten Experimente konzentrierten sich auf den Prozess der Methanoxidation und die Struktur der methanotrophen Lebensgemeinschaft: Im Gewächshaus wurden unter kontrollierten Bedingungen - 55 Tage (1. Experiment), 88 Tage (2. Experiment) – Reispflanzen in Mikrokosmen gezüchtet. Methanoxidationsraten wurde durch Messung des $CH_4$-Flusses mit und ohne Zugabe von Difluormethan (spezifischer Hemmstoff für die $CH_4$-Oxidation) bestimmt. Die Zusammensetzung der methanotrophen Lebensgemeinschaft wurde an den Wurzeln und in der Rhizosphäre durch „terminal restriction fragment length polymorphism“ (T-RFLP) der *pmoA*-Gene analysiert, welche auch kloniert und sequenziert wurden. Das *pmoA*-Gen kodiert für eine Untereinheit der partikulären Methanmonooxygenase, dem Schlüsselenzym der Methanotrophen. Die häufigsten T-RFs konnten klonierten *pmoA*-Sequenzen zugeordnet werden. Die Analyse der metabolisch aktiven Methanotrophen erfolgte durch „stable isotope probing“ von mikrobiellen Phospholipidfettsäuren (PLFA-SIP) und durch Expression von mRNA der *pmoA* Gene im ersten bzw. zweiten Experiment.

Das erste Experiment konzentrierte sich auf die zeitliche Veränderung des Methanoxidationsprozesses iin Reismikrokosmosmen. Die Methanotrophen, welche in der Rhizosphäre des Reises aktiv waren, wurden durch Zugabe von $^{13}C$-$CH_4$ direkt in die Rhizosphäre der Reismikrokosmen identifiziert. Dabei wurden Wurzeln und

Rhizosphärenboden nach Markierung mit $^{13}C$ beprobt und mit Hilfe von T-RFLP und PLFA-SIP analysiert. Sowohl T-RFLP bzw. Klonieren/Sequenzieren der *pmoA*-Gene als PLFA-SIP zeigten, dass sich die Populationen von Typ I und Typ II Methanotrophen mit der Zeit änderten. PLFA-SIP zeigte weiterhin, dass Typ I Methanotrophe aktiver als Typ II Methanotrophe waren, sowohl an den Wurzeln als auch im Rhizosphärenboden.

Das zweite Experiment konzentrierte sich im Wesentlichen auf die möglichen Auswirkung von verschiedenen Ammonium-Düngern auf die Methanoxidation und die Zusammensetzung der methanotrophen Lebensgemeinschaft. Zu diesem Zweck wurden die Reismikrokosmen auf drei verschiedene Arten gedüngt: nur mit Phosphor und Kalium (PK) zur Kontrolle, mit Harnstoff (UPK) als Stickstoff-Quelle und mit Ammoniumsulfat (SPK) als Stickstoff-Quelle. Während der gesamten Reis-Wachstumsperiode wurde der Methanfluß gemessen. Die Wurzeln und der Rhizosphärenboden wurden zu sechs verschiedenen Zeitpunkten beprobt. Die Methanoxidationsraten unter Zugabe von PK und UPK waren über die Wachstumssaison hinweg sehr ähnlich und die methanotrophe Aktivität zeigte keine Veränderung aufgrund der N-Düngung. Ammoniumsulfat hingegen unterdrückte die Methanogenese weitgehend, so dass die SPK-Behandlung für die Methanotrophen ungünstig war. Die Korrelation zwischen Behandlung, Umweltfaktoren und Prozessraten einerseits und T-RFLP Profilen der *pmoA*-Gene andererseits wurde mit Hilfe der „Canonical Correspondence Analysis“ als statistischem Ordinationsverfahren sowohl an den Wurzeln als auch in der Rhizosphäre untersucht. Auf den Wurzeln wurde die methanotrophe Gemeinschaft signifikant von den verschiedenen Stickstoffdüngern beeinflusst. Dagegen wurde sie in der Rhizosphäre nicht beeinflusst. Die Lebensgemeinschaft auf den Reiswurzeln wurde von Typ I Methanotrophen, dagegen in der Rhizosphäre von Typ II Methanotrophen dominiert. Zusammenfassend kann man feststellen, dass die unterschiedliche Stickstoffdüngung zwar die methanotrophe Lebensgemeinschaft beeinflusste, aber nicht zu einer signifikanten Veränderung der Methanoxidationsaktivität führte.

Das zweite Experiment wurde auch benutzt, um die Expression von *pmoA*-mRNA zu untersuchen und so die metabolisch aktiven Methanotrophen zu charakterisieren, die verantwortlich sind für die in situ Methanoxidation in der Rhizosphäre. Beim Vergleich von PK- und UPK-Behandlung wurde kein starker Einfluß der Stickstoffdüngung auf die aktiven Methanotrophen festgestellt. Stattdessen war die zeitliche Variation der Substrat-

oder Nährstoffkonzentrationen anscheinend relativ wichtiger für die Expression der *pmoA*-Gene. Zusammenfassend kann man feststellen, dass im Rhizosphärenboden Typ II Methanotrophe dann vorherrschend waren, wenn die *pmoA*-Gene untersucht wurden, aber Typ I Methanotrophe vorherrschend waren, wenn die *pmoA*-Transkripte analysiert wurden. Daher scheinen Typ I Methanotrophe eine aktive Rolle bei der Methanoxidation zu spielen, während Typ II Methanotrophe mehr eine Grundgemeinschaft bilden, die auch unter ungünstigen Umständen (z.B. SPK-Behandlung) überdauert.

Schließlich habe ich Methanotrophe in Kultur genommen, die an der Wurzeln und im Rhizosphärenboden der Mikrokosmen leben. Insgesamt wurden 6 Isolate von der Wurzeln und 5 Isolate aus der Rhizosphäre erhalten. Alle Isolate waren eng verwandt mit Typ II Methanotrophen, was darauf hinweist, dass die kultivierbare Fraktion der Methanotrophen auf den Wurzeln und in der Rhizosphäre in erster Linie aus Typ II Methanotrophen besteht.

# Chapter 1

## General introduction

### 1.1 Greenhouses gases and global warming

The average global temperature is determined by the equilibrium between incoming energy from the sun and outgoing energy as heat from the earth. Greenhouse gases produce a warming effect by allowing incoming solar radiant energy to penetrate to the Earth's surface. Part of the outgoing infrared radiation is trapped by greenhouse gases in the lower atmosphere and then re-emitted. This process is referred to the "greenhouse effect", which adds to the net energy input of the lower atmosphere and thus leads to an increased global temperature ("global warming") (IPCC, 1990).

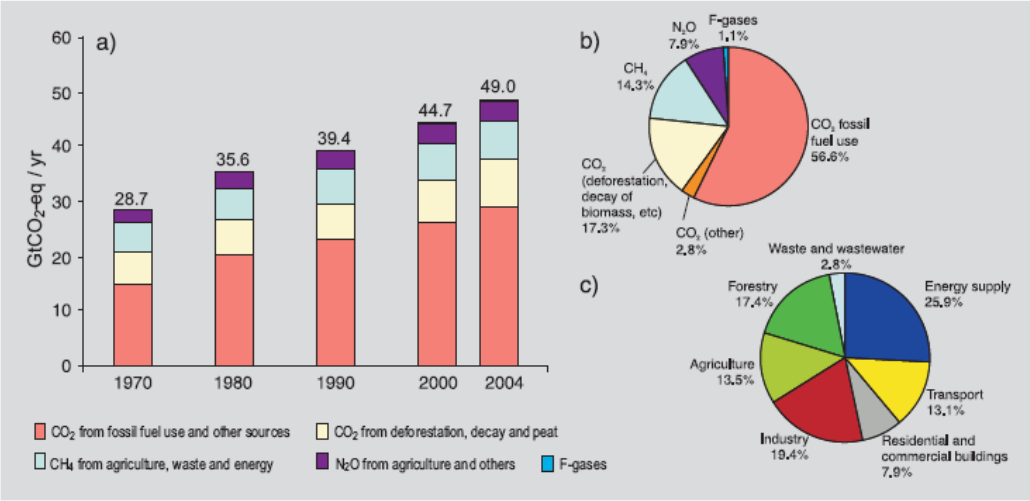



**Fig. 1.1:** (a) Global annual emissions of anthropogenic GHGs from 1970 to 2004 (b) Share of different anthropogenic GHGs in total emissions in 2004 in terms of $CO_2$-eq. (c) Share of different sectors in total anthropogenic GHG emissions in 2004 in terms of $CO_2$-eq. (Forestry includes deforestation) (IPCC, 2007).

Global greenhouse gas emissions due to human activities have grown since pre-industrial times, with an increase of 70% between 1970 and 2004 (Fig.1.1). The key greenhouse gases responsible for the enhanced greenhouse effect are carbon dioxide ($CO_2$), methane ($CH_4$), nitrous oxide ($N_2O$) and the man made chlorofluorocarbons (CFC's), which are

associated with the economic activities and food production (IPCC, 1996; Denier van der Gon, 1996).

Methane is an important greenhouse gas as it can absorb infrared radiation 25 times more effectively than carbon dioxide (Schlesinger, 1997). Due to this, methane is of great concern as a greenhouse gas. Although, the tropospheric $CH_4$ concentration is very low as compared to $CO_2$, methane accounts for 15 to 20% of global warming (IPCC, 2001). The global atmospheric concentration of methane has increased from a pre-industrial value of about 715 ppb to 1732 ppb in the early 1990s, and is 1774 ppb in 2005 (IPCC, 2007). Various anthropogenic methane sources are presented in Fig. 1.2.

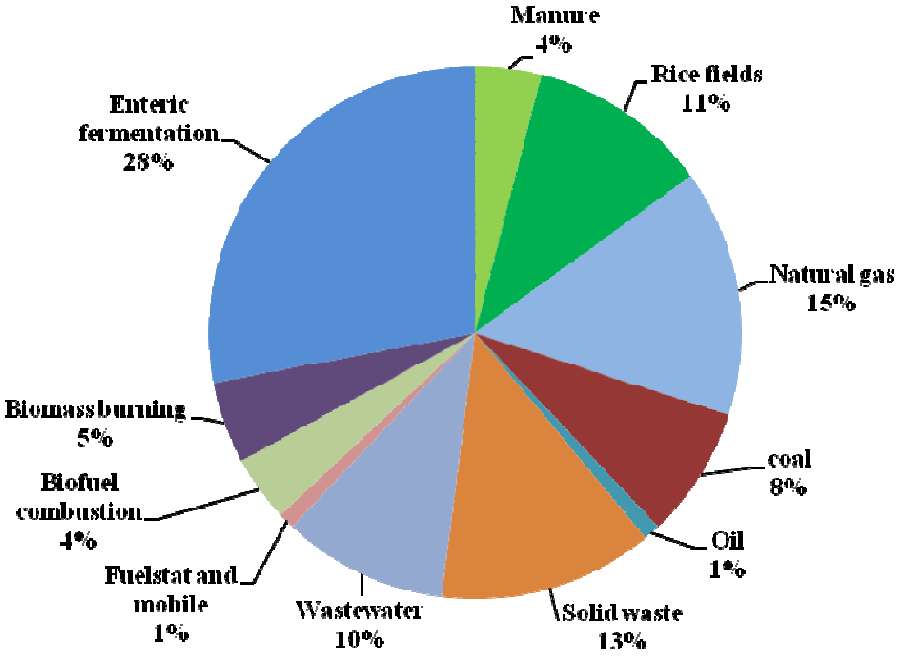



**Fig. 1.2:** Global anthropogenic methane budget. Total $CH_4$ emission due to anthropogenic activity in 2000 = 282.6 Tg $CH_4$ (U.S. EPA compilation, 2006).

### 1.2 Wetland rice fields as a source of methane

Rice fields are the most significant contributors of atmospheric $CH_4$ accounting for 11-13% of the World's total anthropogenic $CH_4$ emission (Lelieveld *et al.*, 1998; Wang *et al.*, 2004). According to the International Rice Research Institute statistics, World rice harvested area increased by approximately 33% from 115.5 Mha in 1961 to 153.3 Mha in 2004 (http://www.irri.org/science/ricestat/pdfs/WRS2005-Table02.pdf). According to an

estimate, rice production will need to expand by around 60% over the next 25 years to meet the demand of the World's growing human population (Cassman *et al*., 1998; Dubey, 2001; Neue, 1997), making rice cultivation a potential major cause of increasing atmospheric methane.

### 1.2.1 Methane formation in rice fields

In flooded rice fields, methane ($CH_4$) is produced by anaerobic bacteria (methanogens) as the terminal step of the anaerobic degradation of organic matter (Conrad, 1993; Denier van der Gon, 1996; Neue, 1993; Schütz *et al*., 1989). The anaerobic degradation of organic matter involves four main steps: a) hydrolysis of polymers by hydrolytic organisms, b) acid formation from simple organic compound by fermentative bacteria, c) acetate formation from metabolites of fermentations by homoacetogenic or syntrophic bacteria, and d) $CH_4$ formation from $H_2/CO_2$, acetate, simple methylated compounds or alcohols and $CO_2$ (Yao and Conrad, 2001). $CH_4$ is produced in rice fields after the sequential reduction of $O_2$, nitrate, manganese, iron and sulphate, which serve as electron acceptors for oxidation of organic matter to $CO_2$ (Yao *et al*., 1999). In paddy soil, acetate and $H_2$ are the two main intermediate precursors for $CH_4$ formation (Yao and Conrad, 1999). The rate of $CH_4$ produced in soil is controlled by several factors such as organic materials, temperature, pH and other soil factors.

### 1.2.2 Methane emission from rice fields

The net amount of $CH_4$ emitted from rice fields to the atmosphere is the balance of two opposite processes, production and oxidation. $CH_4$ emissions mainly consist of three closely coupled steps (Fig. 1.3) (Khalil and Shearer, 2006). First, the leftover soil carbon from previous years, straw, roots and organic compounds supplied by root exudation will supply the carbon needed for methane production. This carbon has to go through a number of decomposition steps involving various types of bacteria before it can be utilized by methanogens that produce methane in anaerobic environments. The second step is the transport of this deeper methane and its oxidation on the way to the atmosphere. The oxidation is found to take place mostly in the root zone, which has a supply of oxygen from the plant. In this root zone, or possibly just inside the root, are methanotrophic bacteria that utilize methane as a source of energy. The final step is the transport of methane out of the paddy system and into the atmosphere via several

pathways: bubbles, diffusion through the soil and water, and the transport of the methane through the root aerenchyma system of the plant. Of these the bubbles are important only in the early stages of rice growth (Denier *et al*., 1995; Li, 2000). Later, a very large fraction is the transport through the plant. This is in part because the oxidation creates strong gradients that move methane through the root zone while other pathways are less efficient. What gets out into the atmosphere through the plant is the rest of the methane that is not oxidized by the methanotrophs.

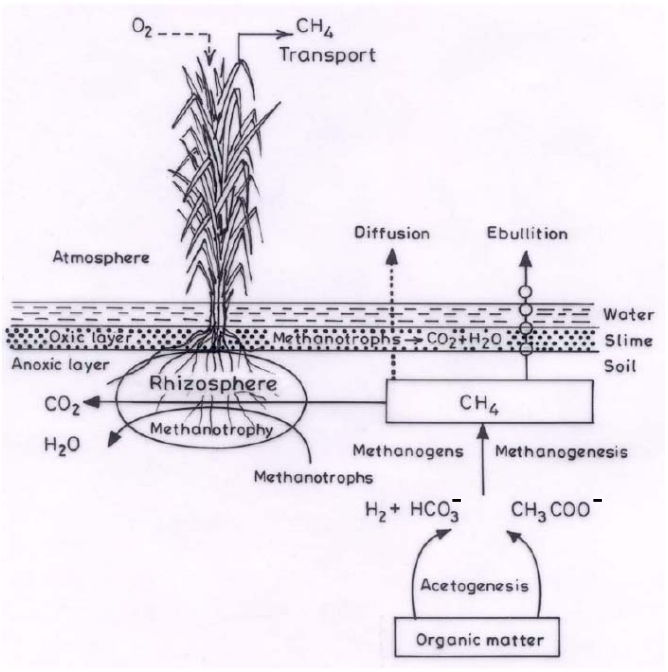



**Fig. 1.3:** Conceptual schematic diagram of methane production, oxidation and emission from paddy field (Dubey *et al*., 2005).

### 1.2.3 Methane oxidation in rice fields

In rice fields, methane oxidation greatly limits diffusion of methane to the atmosphere. Estimates of methane oxidation in the rice rhizosphere are reported as a range from 7 to 90% of the transported methane (EPP and Chanton, 1993; Denier van der Gon *et al*., 1996; Gilbert and Frenzel, 1998; Holzapfel-Pschorn *et al*., 1985) and a range from 7 to 52% if only data obtained from specific inhibitor studies are included.

Methanotrophic bacteria play an important role in global methane budget by consuming the potential amount of methane in rice fields. Methanotrophs associated with the

rhizosphere of rice plants oxidize $CH_4$ with molecular $O_2$ and use it as the sole source of carbon and energy. Methanotrophic bacteria are present in the aerobic soil layer, the roots, the soil surrounding the roots, so-called rhizosphere (Dubey and Singh, 2000; Gilbert and Frenzel, 1998; Joulian *et al*., 1997) and on the stem bases of flooded rice plants (Watanabe *et al*., 1997). The rice rhizosphere appears to be a very heterogeneous habitat for methanotrophs because both methane concentrations and oxygen released by roots are highly variable (Armstrong, 1970; Gilbert and Frenzel, 1995).

Overall methane dynamics in the paddy field is controlled by a complex set of parameters linking the biological and physical characteristics of soil environment like temperature, carbon source, nutrients, Eh, pH, soil microbes and properties of rice plants.

### 1.3 Anaerobic methane oxidation

Methane oxidation can occur in both aerobic and anaerobic environments, however, these are completely different processes involving different groups of prokaryotes. Aerobic methane oxidation is carried out by aerobic methanotrophs, and anaerobic methane oxidizers, discovered recently, thrive under anaerobic conditions and use sulfate or nitrate as electron donors for methane oxidation (Boetius, *et al*., 2000; Raghoebarsing *et al*., 2006).

Anaerobic methane oxidation associated with sulphate or iron reduction has been suggested to operate in rice fields (Murase and Kimura, 1994). However, there is no report of the isolation or molecular identification of bacteria, which affect anaerobic methane oxidation in rice fields.

### 1.4 Methane oxidising bacteria (Methanotrophs)

The methane oxidising bacteria are commonly known as methanotrophs. They are ubiquitous in nature and represent the important biogenic sink for the greenhouse gas methane. Since methanotrophs use methane and utilize methanol and other 1-carbon compounds as intermediates in their energy metabolism, they are members of larger group of bacteria called the methylotrophs. The methylotrophs are obligate 1-carbon utilizers. The first well-described methane oxidising bacterium was isolated from plant material in 1906 by Söhngen (Hanson *et al*., 1991). However, the habitats of

methanotrophs are numerous and include most freshwater, marine and terrestrial environments. The most methanotroph-rich communities are typically found in organic rich soils and sediments, sewage sludge and calcareous swamps (Hanson and Hanson, 1996).

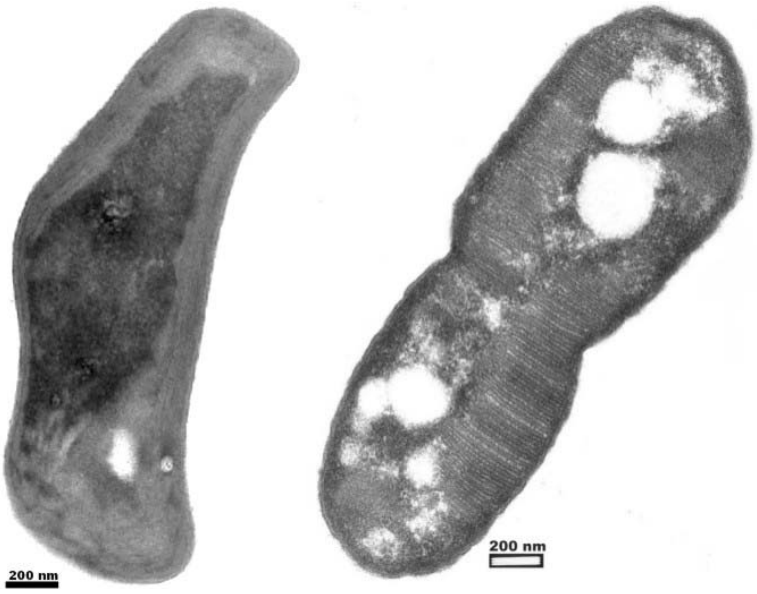



**Fig. 1.4:** Transmission electron micrographs of the type II methanotroph, *Methylocystis parvus* OBBP (left), and the type I methanotroph, *Methylomonas methanica* S1 (right). The micrographs illustrate the different intracytoplasmic membrane arrangements: paired membranes aligned to the periphery of the cell in type II methanotrophs, and bundles of vesicular discs in type I methanotrophs. The scale bars represent 200 nm (Binnerup *et al*., 2005).

Methanotrophs are strictly aerobic and gram-negative bacteria, and can be divided into two distinct physiological groups that are designated as type I and type II (Hanson and Hanson, 1996) on the basis of phylogeny, physiology, morphology and biochemistry, including characteristic phospholipid ester-linked fatty acids (PLFA) in their cell membranes (Fig. 1.4, Table 1.1). There are 13 recognized genera of methanotrophs (Bodrossy *et al*., 1997; Bowman *et al*., 1997; Dedysh *et al*., 2000, 2002; Hanson and Hanson, 1996; Heyer *et al*., 2005; Tsubota *et al*., 2005; Wise *et al*., 2001) consisting of both type I and type II methanotophs. Phylogenetic studies of 5S rRNA and 16S rRNA have confirmed the distinction between type I and type II methanotrophs and have placed

**Table 1.1:** List of characteristics for the differentiation of methanotrophs (Murell *et al*., 1998).

Characterisation of methanotrophs

| Genus | Phylogeny | Membrane type | Major PLFA | Formaldehyde assimilation pathway | Enzyme type | Mol% G+C content of DNA |
|---|---|---|---|---|---|---|
| *Methylomonas* | γ Proteobacteria | Type I | 16:1 | RuMP | pMMO | 51–59 |
| *Methylobacter* | γ Proteobacteria | Type I | 16:1 | RuMP | pMMO | 49–54 |
| *Methylomicrobium* | γ Proteobacteria | Type I | 16:1 | RuMP | pMMO | 50–60 |
| *Methylosphaera* | γ Proteobacteria | Type I | 16:1 | RuMP | pMMO | 43–46 |
| *Methylocaldum* | γ Proteobacteria | Type I | 16:1 | RuMP/serine | pMMO | 56–58 |
| *Methylococcus* | γ Proteobacteria | Type I | 16:1 | RuMP/serine | pMMO/sMMO | 59–66 |
| *Methylosinus* | α Proteobacteria | Type II | 18:1 | Serine | pMMO/sMMO | 62–63 |
| *Methylocystis* | α Proteobacteria | Type II | 18:1 | Serine | pMMO/sMMO | 62–63 |

RuMP, ribulose monophosphate pathway; pMMO, particulate methane monooxygenase; sMMO, soluble methane monooxygenase.

them, respectively, in the gamma and alpha subdivisions of the proteobacteria (Bowman, 2000; Bratina *et al*., 1992). Type I methanotrophs belong to the gamma subgroup of the proteobacteria, and include the species *Methylomonas*, *Methylosphaera*, *Methylomicrobium, Methylosarcina, Methylobacter, Methylocaldum, Methylococcus capsulatus, Methylohalobius*, and *Methylosoma*. They assimilate the formaldehyde produced from the oxidation of methane by using the ribulose monophosphate pathway (Fig 1.6). They contain predominantly 16-carbon fatty acids and possess bundles of intracytoplasmic membranes. The type II methanotrophs belong to the alpha subunit of the proteobacteria and include the species *Methylocystis, Methylocella, Methylocapsa* and *Methylosinus*. They assimilate formaldehyde by the serine pathway (Fig 1.6). They contain 18-carbon phospholipid fatty acids and possess paired intracellular membranes aligned to the periphery of the cell (Hanson and Hanson, 1996). Recently, two filamentous methane oxidizers, belonging to gammaproteobacteria, have been described, *Crenothrix polyspora* (Stoecker *et al*., 2006), which has a novel *pmoA*, and *Clonothrix fusca* (Vigliotta *et al*., 2007), which has a conventional *pmoA*. All of these methanotrophs that are belonging to *Alphaproteobacteria* and *Gammaproteobacteria* grow well at either neutral pH or slightly alkaline pH. However, there are two moderate acidophiles, *Methylocella* and *Methylocapsa*, which have pH optima of 5.0–5.5 and a lower limit of 4.0-4.5 (Dedysh *et al*., 1998, Dedysh, 2002). Quite recently, a new group of bacteria possessing methane monoxygenase gene has been cultured and fully sequenced. This new group of bacteria belonged to phylum *Verrucomicrobia* (Dunfield *et al*., 2007; Islam *et al*., 2008; Pol *et al*., 2007) and were extremely thermoacidophilic (optimum growth pH is below 2; optimum temperature is 55 °C).The phylogenetic positions of methanotrophs

belonging to α and γ-proteobacteria, β-proteobacteria (*amoA*), *Crenothrix* and *Verrucomicrobium*, and other cultured groups are shown in Fig. 1.5.

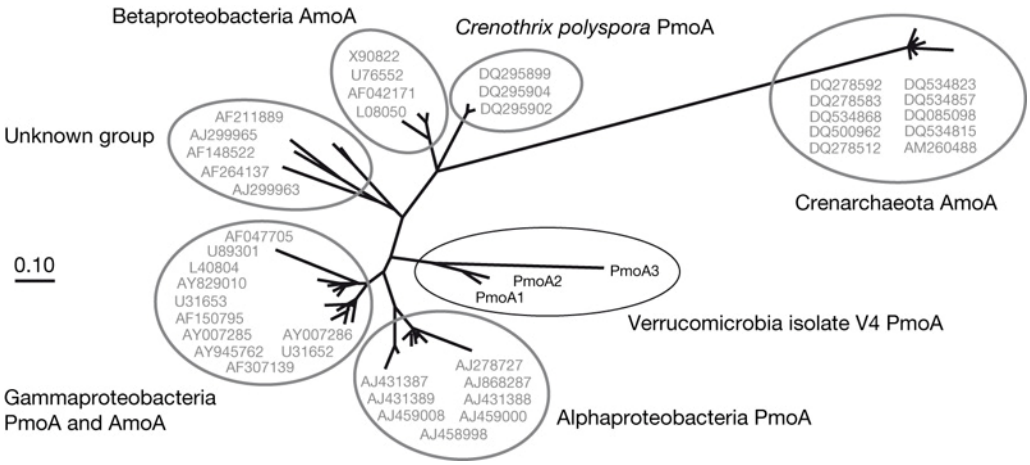



**Fig. 1.5:** Phylogenetic tree constructed from derived amino acid sequences encoded by *pmoA* and *amoA* genes (subunits of particulate methane monooxygenase or ammonia monooxygenase) and three sequences from Verrucomicrobia isolate V4 (Dunfield *et al*., 2007).

Methanotrophic communities occur mainly in two types of terrestrial ecosystems, where they have different functions (Conrad, 1996). The first ecosystem is well aerated upland soils where they occur and oxidize the $CH_4$ that is supplied from the atmosphere at low concentrations (<2.4 nM). The second ecosystem is wetland soils (e.g., rice fields) where they occur within oxic-anoxic interphase and oxidize $CH_4$ that is generated from the anoxic site of the soil at saturating concentrations (>1.2 mM).

Wetland soils, on one hand, are found to contain mostly cultivated taxa of methanotrophs including both type I and type II methanotrophs (Eller and Frenzel, 2001; Henckel *et al*., 2001; Shrestha *et al*., 2008). These genera generally have relatively high $K_m$ values (>1 μM). Upland soils, on the other hand, are often dominated by as yet-uncultured methanotrophs. These are represented by the *pmoA* sequence clusters USCα and USCγ (Ricke *et al*., 2005). Besides USCα and USCγ, the methanotrophs active in upland soil may also involve *Methylocystis* species (Knief *et al*., 2005) that express low $K_m$ values

after prolonged adaptation to low methane concentrations (Dunfield and Conrad, 2000; Dunfield *et al*., 1999).

### 1.4.1 Physiology, biochemistry and molecular biology of methanotrophs

The ability of methanotrophs to oxidize methane is due to the possession of the enzyme called methane monooxygenase (MMO). This enzyme oxidizes methane to methanol. The reaction uses reducing power to break the O-O bound in oxygen ($O_2$). One oxygen atom is incorporated into methanol ($CH_3OH$), while the other is converted to water ($H_2O$). The reducing power required for the oxidation of methane to methanol and for bacterial growth is derived from further oxidation of methanol, via formaldehyde (HCHO) and formate (HCOOH) to carbon dioxide ($CO_2$) (Fig. 1.6). Approximately 50% of the formaldehyde produced is assimilated into cell carbon and the remainder is oxidized to $CO_2$ and lost from the cell (Anthony, 1982).

The MMO enzyme has been the subject of extensive biochemical and molecular research. There are two distinct forms of this enzyme, the membrane-bound particulate methane monooxygenase (pMMO) and the cytoplasmic soluble methane monooxygenase (sMMO) (Hanson and Hanson 1996). The particulate methane monooxygenase (pMMO) is integrated into the inner membrane of the bacterial cell wall and is a copper protein (Zahn and DiSpirito 1996). The other methane monooxygenase is present within the cellular fluid (cytoplasm) and is therefore called the soluble methane monooxygenase (sMMO). This enzyme differs bio-chemically from the pMMO by having a di-ion center at the active site for methane oxidation (Rosenzweig *et al*., 1993). This enzyme is only expressed when copper deficiency prevents expression of pMMO. Methanotrophs expressing pMMO have higher growth yield, as pMMO is more specific and has a higher affinity for methane than sMMO (Hanson and Hanson, 1996). Only the pMMO is found universally in methanotrophs, except in the genus *Methylocella* (Dedysh *et al*., 2000; Theisen *et al*., 2005), and can therefore be used as a functional marker.

However, the pMMO enzyme complex shares many similarities with the ammonia monooxygenase (AMO) enzyme complex found in ammonia-oxidizing bacteria (Klotz and Norton, 1998). These similarities include a high degree of amino acid sequence identity, similar protein complex structures, and broadly similar substrate and inhibition profiles, while each play a crucial role in cell metabolism (Gilbert *et al*., 2000; Holmes *et*

*al*., 1995; Semrau *et al*., 1995). Methanotrophs and ammonia-oxidizing bacteria can oxidize both methane and ammonia; however, they can obtain energy only from the oxidation of methane and ammonium, respectively (Bedard and Knowles, 1989).

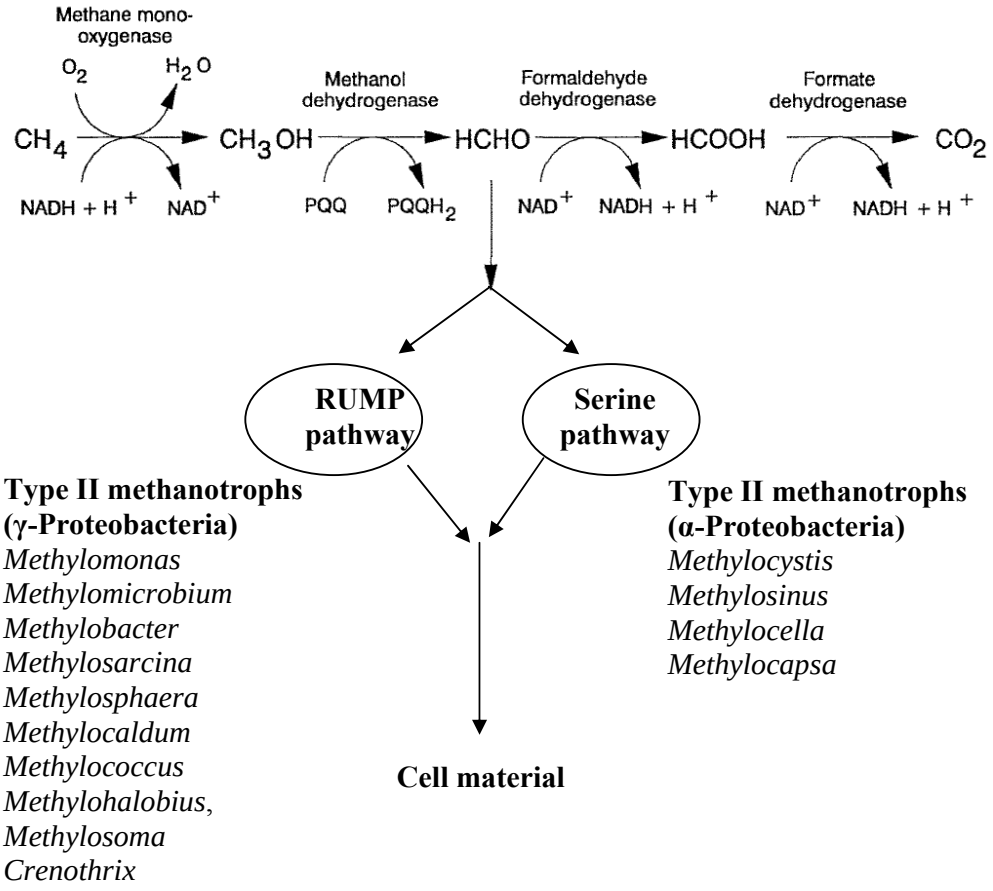



**Fig. 1.6:** Pathways for methane oxidation and formaldehyde assimilation in type I and type II methanotrophs (Murell, 1994, slightly modified).

### 1.4.2 16S rRNA phylogeny of methanotrophs

Yet uncultured methanotrophs can be detected with nucleic acid probes or by sequencing genes amplified by PCR directly from environmental samples (Giovannoni *et al*., 1988; Liesack and Stackebrandt, 1992; Olsen *et al*., 1986). These methods are useful for identification of taxa and for determination of the phylogenetic positions of microbes. Classification schemes (Hanson *et al*., 1991) have been strengthened because of the comparative sequence analysis of both the 5S and the 16S ribosomal RNA (rRNA) from a large number of methanotrophs and methylotrophs (Bowman, 1990; Bratina *et al*., 1992;

Tsuji *et al*., 1990). The position of different groups of methanotrophs based on 16S rRNA sequence and *pmoA* sequence are given in Fig. 1.7. Large numbers of 16S rRNA gene probes have been designed to amplify methanotrophs. To date, quite a few of these sets of methanotroph specific 16S rRNA probes have been used in environmental studies (Table1.2).

### 1.4.3 Functional gene phylogeny of methanotrophs

Study of functional genes is valuable because it leads to a better understanding of the activity of bacteria in different environments and their role in the cycling of biogeochemically important compounds, such as methane in the case of the methanotrophs. An advantage of using functional genes instead of 16S rRNA to study bacterial diversity is that they enable the potential functional diversity within an environment to be investigated. Although sMMO-based approaches are useful for studying methanotroph diversity in copper-depleted environments such as wetlands or contaminated aquifers, these genes, however, are not present in all known methanotrophs. A better approach is based on the pMMO, present in all known methanotrophs.

The first oligonucleotide primers designed to amplify internal fragments of the genes encoding pMMO and AMO (ammonia monooxygenase) enzyme complexes were the A189f/A682r primer set (Holmes *et al*., 1999) (Table 1.3). The phylogeny of *pmoA*/*amoA* is reasonably congruent with the 16S rRNA gene phylogeny of the organisms from which the gene sequences were retrieved (Holmes *et al*., 1999; Kolb *et al*., 2003) (Fig. 1.7). Therefore, retrieval of *pmoA* and *amoA* sequences provides information on the phylogenetic position of these organisms. The A189f/A682r primers have been used extensively in environmental studies to provide a molecular profile and the diversity of the methanotrophs in various environments (Bourne *et al*., 2001; Holmes *et al*., 1999; Horz *et al*., 2002, 2001; Kolb *et al*., 2003) and have proved useful in detecting novel sequences (Holmes *et al*., 1999; Knief *et al*., 2003;). However, a new reverse *pmoA*-specific primer mb661r, used in conjunction with the A189f primer was designed and demonstrated specificity in amplifying *pmoA* sequences but not *amoA* sequences (Bourne *et al*., 2001; Costello and Lidstrom, 1999; Shrestha *et al*., 2008;). List of *pmoA* specific primer sets that were designed to study methanotrophic diversity is listed in Table 1.3.

Another potentially useful marker is the *mxaF* gene. PCR primers that specifically amplify a 550-bp fragment of *mxaF* sequences from methanotrophs have been used to extend the database of *mxaF* genes of methanotrophs and methylotrophs and to identify *mxaF* sequences in marine, soil and wetland samples (Holmes *et al.*, 1995; McDonald *et al.*, 1997; McDonald *et al.*, 1995). However, the *mxaF* gene is not specific for methanotrophs but also occurs in methylotrophs unable to use $CH_4$. A list of all the currently available functional gene based primers for methanotrophs are listed in table 1.3, and 1.4.

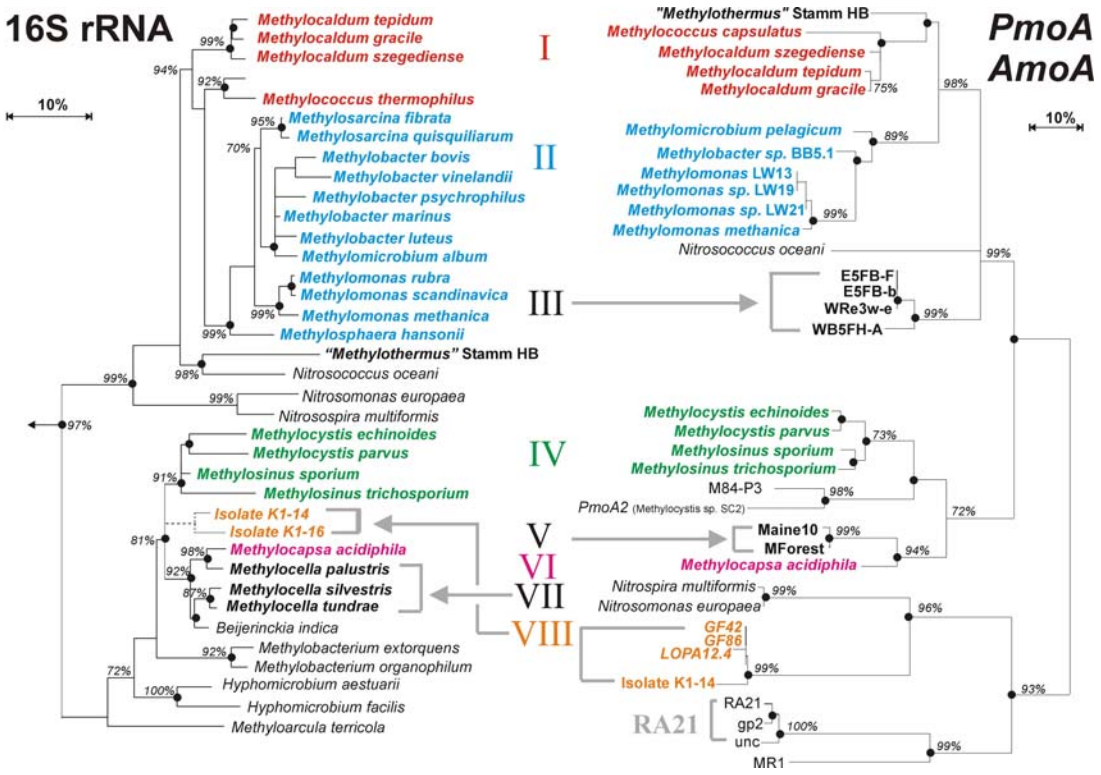



**Fig. 1.7:** Comparison of the phylogenies of the 16S rRNA gene and *pmoA*/*amoA* genes of methanotrophic bacteria. The dendogram of the 16S rRNA gene was calculated with Tree-Puzzle algorithm and confirmed with FstDNA-ML. The dashed line indicates a cluster that was inserted with the parsimony tool of the ARB software. The *pmoA*/*amoA* tree was calculated with Tree-Puzzle algorithm and confirmed with ProtML. The scales give 10% sequence distance (Kolb, Dissertation, Philipps University of Marburg, 2003).

**Table 1.2.** List of 16S rRNA gene probes targeting methanotrophs (McDonald *et al*., 2008).

| Type and probe | Sequence (5'-3') | Target |
|---|---|---|
| Type I methanotroph probes | | |
| 1071 | GGTCCGAAGATCCCCCGCTT | RuMP pathway methylotrophs |
| 1035-RuMP | GATTCTCTGGATGTCAAGGG | RuMP pathway methanotrophs |
| Mb1007[a] | CACTCTACGATCTCTCACAG | *Methylobacter* (*Methylomicrobium*)[a] |
| Mc1005 | CCGCATCTCTGCAGGAT | *Methylococcus* |
| Mm1007 | CACTCCGCTATCTCTAACAG | *Methylomonas* |
| MethT1dF | CCTTCGGGMGCYGACGAGT | Type I methanotrophs |
| MethT1bR | GATTCYMTGSATGTCAAGG | Type I methanotrophs |
| Type 1b | GTCAGCGCCCGAAGGCCT | Type I methanotrophs |
| Gm633 | AGTTACCCAGTATCAAATGC | *Methylobacter* and *Methylomicrobium* |
| Gm705[c] | CTGGTGTTCCTTCAGATC | Gamma methanotrophs except *Methylocaldum* |
| Mlb482 | GGTGCTTCTTCTAAAGGTAATGT | *Methylobacter* |
| Mlb662[d] | CCTGAAATTCCACTCTCCTCTA | *Methylobacter* |
| Mmb482 | GGTGCTTCTTCTATAGGTAATGT | *Methylomicrobium* |
| Mlm482 | GGTGCTTCTTGTATAGGTAATGT | *Methylomonas* |
| Mlm732a | GTTTTAGTCCAGGGAGCCG | *Methylomonas* group A |
| Mlm732b | GTTTGAGTCCAGGGAGCCG | *Methylomonas* group C |
| Mlc123 | CACAACAAGGCAGATTCCTACG | *Methylococcus* |
| Mlc1436 | CCCTCCTTGCGGTTAGACTACCTA | *Methylococcus* |
| Mcd77 | GCCACCCACCGGTTACCCGGC | *Methylocaldum* |
| M1184 | CCACTCGTCAGCGCCCGA | Type I methanotrophs |
| M1669[d] | GCTACACCTGAAATTCCACTC | *Methylobacter* and *Methylomonas* |
| M1983 | TGGATGTCAAGGGTAGGT | Type I methanotrophs |
| M1993 | ACAGATTCTCTGGATGTC | Type I methanotrophs |
| M11004[a] | TACGATCTCTCACAGATT | *Methylomicrobium* |
| Mh996r | CACTCTACTATCTCTAACGG | *Methylosphaera* |
| Type IF | ATGCTTAACACATGCAAGTCGAACG | Type I methanotrophs |
| Type IR | CCACTGGTGTTCCTTCMGAT | Type I methanotrophs |

| | | |
|---|---|---|
| Type II methanotroph probes | | |
| 9α | CCCTGAGTTATTCCGAAC | Serine pathway methylotrophs |
| 1034-Ser | CCATACCGGACATGTCAAAAGC | Serine pathway methanotrophs |
| Ms1020 | CCCTTGCGGAAGGAAGTC | *Methylosinus* |
| Type 2b | CATACCGGRCATGTCAAAAGC | Type II methanotrophs |
| MethT2R | CATCTCTGRCSAYCATACCGG | Type II methanotrophs |
| Am455[b] | CTTATCCAGGTACCGTCATTATCGTCCC | Alpha methanotrophs |
| Am976 | GTCAAAAGCTGGTAAGGTTC | Alpha methanotrophs |
| Ma464 | TTATCCAGGTACCGTCATTA | Type II methanotrophs |
| Mcell-1026 | GTTCTCGCCACCCGAAGT | *Methylocella palustris* |
| AcidM-181 | TCTTTCTCCTTGCGGACG | *Methylocella palustris* and *Methylocapsa acidiphila* |
| Mcaps-1032 | CACCTGTGTCCCTGGCTC | *Methylocapsa acidiphila* |
| Msint-1268 | TGGAGATTTGCTCCGGGT | *Methylosinus trichosporium* |
| Msins-647 | TCTCCCGGACTCTAGACC | *Methylosinus sporium* |
| Mcyst-1432 | CGGTTGGCGAAACGCCTT | All *Methylocystis* spp. |
| Type IIF | GGGAMGATAATGACGGTACCWGGA | Type II methanotrophs |
| Type IIR | GTCAARAGCTGGTAAGGTTC | Type II methanotrophs |

[a] Also called Mmb1007 (Gulledge *et al*., 2000). Primer Mg1004 (Eller *et al*., 2001) has an identical 15-bp overlap with Mb1007.
[b] Primer Ma450 is identical to part of Am455 (Eller *et al*., 2001).
[c] Primer Mg705 is identical to Gm705 (Eller *et al*., 2001).
[d] Primer Mg669 has an identical 15-bp overlap with Mlb662 (Eller *et al*., 2001).

**Table 1.3.** List of PCR primers used for amplification of *pmoA* genes from environmental samples (McDonald *et al*., 2008).

| Primer(s) | Sequence (5'-3') | Product size (bp)[b] |
|---|---|---|
| A189f[a]/A682r | GGNGACTGGGACTTCTGG/GAASGCNGAGAAGAASGC | 525 |
| mb661 | CCGGMGCAACGTCYTTACC | 510* |
| pmof1/pmor | GGGGGAACTTCTGGGGITGGAC/GGGGGRCIACGTCITTACCGAA | 330 |
| pmof2/pmor | TTCTAYCCDRRCAACTGGCC | 178 |
| *pmoA*206f/*pmoA*703b[d] | GGNGACTGGGACTTCTGGATCGACTTCAAGGATCG/GAASGCNGAGAAGAASGCGGCGACCGGAACGACGT | 530 |
| A650r | ACGTCCTTACCGAAGGT | 478* |
| mb661r_nd | CCGGCGCAACGTCCTTACC | 510* |
| *pmoA*for/*pmoA*rev | TTCTGGGGNTGGACNTAYTTYCC/CCNGARTAYATHMGNATGGTNGA | 281 |
| f326/r643 | TGGGGYTGGACCTAYTTCC/CCGGCRCRACGTCCTTACC | 358 |
| Mb601 R[c] | ACRTAGTGGTAACCTTGYAA | 432* |
| Mc468 R[c] | GCSGTGAACAGGTAGCTGCC | 299* |
| II 223 F[c]/II646 R[c] | CGTCGTATGTGGCCGAC/CGTGCCGCGCTCGACCATGYG | 444 |
| Mcap630[c] | CTCGACGATGCGGAGATATT | 461* |
| Forest675 R[c] | CCYACSACATCCTTACCGAA | 506* |

[a] Primer A189f is also known as A189gc.

[b] *, that is, when used in PCR with the primer A189f.

[c] Primers designed for real-time PCR quantification of subsets of methanotrophs.

[d] Primer set that enables the simultaneous detection of *pmoA1* and *pmoA2* at an annealing temperature of 60°C but only enables detection of *pmoA2* at 66°C (Tchawa *et al*., 2003).

**Table 1.4.** List of PCR primers used for amplification of *mmoX* genes from environmental samples (McDonald *et al*., 2008).

| Primer(s)[a] | Sequence (5'-3') | Product size (bp) |
|---|---|---|
| mmoXf882/mmoXr1403 | GGCTCCAAGTTCAAGGTCGAGC/TGGCACTCGTAGCGCTCCGGCTCG | 535 |
| mmoX1/mmoX2 | CGGTCCGCTGTGGAAGGGCATGAAGCGCGT/GGCTCGACCTTGAACTTGGAGCCATACTCG | 369 |
| 536f/877r | CGCTGTGGAAGGGCATGAAGCG/GCTCGACCTTGAACTTGGAGCC | 341 |
| mmoXr901[b] | TGGGTSAARACSTGGAACCGCTGGGT | 396[c] |
| A166f/B1401r | ACCAAGGARCARTTCAAG/TGGCACTCRTARCGCTC | 1,230 |
| 534f/1393r | CCGCTGTGGAAGGGCATGAA/CACTCGTAGCGCTCCGGCTC | 863 |
| met1/met4 | ACCAAGGAGCAGTTC/TCCAGAAGGGGTTGTT | |
| mmoX206f/mmoX886r | ATCGCBAARGAATAYGCSCG/ACCCANGGCTCGACYTTGAA | 719 |

[a] Primer mmoX1 was located at positions 2008 to 2037, and primer mmoX2 was located at positions 2347 to 2376. Primers A166f and B1401r are also known as mmoXA and mmoXD.

[b] mmoXr901_GC is also used for DGGE analysis with primer mmoX1(Iwamoto *et al*., 2000).

[c] When used in PCR with the primer mmoX1.

### 1.4.4 Methods to study methanotrophs

Several approaches have been adopted in molecular ecological studies for the detection and characterization of methanotrophs in the various ecosystems (McDonald *et al*., 2008). Culture-based techniques have successfully been used to isolate methanotrophs from environmental samples. However, culture-based methods are limited because either many methanotrophs do not grow on conventional media or it is very difficult to get them as a pure culture. Methanotrophs isolated from media or pure cultures constitute only a small fraction of the viable species diversity and the fraction of cells recovered from environmental samples is also believed to be a small fraction of those present (Bone *et al.*, 1986; Hanson and Wattenburg, 1991). In particular, the slow growth of methanotrophs, associated with non-methanotrophs that scavenge on nutrients on agar isolation plates, has hampered the studies. The physiological types of methanotrophs isolated from environmental samples may reflect the conditions used for enrichments and isolation attempts and thus may not be the dominant organisms in the original population (Amaral and Knowles, 1995; Bussman *et al*., 2006; Hanson and Wattenburg, 1991).

Another method for the identification of methanotrophs in natural environmental samples is phospholipids fatty acid analysis. Ester-linked phospholipid fatty acids (PLFAs) are a diverse group of cell membrane lipids and are known to degrade rapidly after cell death and, therefore, are representative of the living bacteria in soils or sediments (Vestal and White, 1989; White *et al*., 1979). They are polar lipids of which some are specific for subgroups of microorganisms, e.g. gram-negative or gram-positive bacteria, methanotrophic bacteria, fungi, mycorrhiza, and actinomycetes (Zelles, 1999). Individual PLFAs can thus be related to specific microbial populations. PLFAs are extracted from soil samples and subsequently analysed by gas chromatography (Frostegard *et al*. 1993; Zelles, 1999). Specific PLFAs are then identified and/or quantified and the result is evaluated by multivariate statistics. PLFA profiles of soil samples offer sensitive reproducible measurements for characterizing the numerically dominant portion of soil microbial communities without cultivating the organisms (Zelles, 1999). The technique gives estimates of both microbial community composition and biomass size, and the results represent the *in situ* conditions in soil. This approach has been particularly useful for detecting populations of type I and type II methanotrophs, allowing differentiation between type I and type II methanotrophs and also from all other organisms (Bowmann *et*

*al*., 1993). Type I methanotrophs produce $C_{16}$ fatty acids as their most abundant PLFAs, whereas type II methanotrophs produce a higher concentration of $C_{18}$ fatty acids (Bowman *et al*., 1993; Hanson and Hanson, 1996). However, a recent study showed that *Methylocystis heyeri* strains (type II methanotrophs) contained large amounts of 16:1ω8c, a PLFA that was previously thought to be associated with type I methanotrophs only. The major disadvantage of PLFA analysis is that it is not precise enough to identify bacteria to the species level. The specificity of PLFA profiling of bacterial populations can be significantly enhanced by applying isotopically labeled substrates to soils or sediments.

Stable isotope probing (SIP) of PLFA and nucleic acids is a novel technique to characterize structure and function of active microbial populations. Stable isotope probing (SIP) is a method, which attempts to link an organism's identity with its biological function under conditions approaching those *in situ*. The principle of SIP is based on the natural abundance of $^{13}C$ being approximately 1%. Consequently, addition of $^{13}C$ labelled substrate to an environmental sample will result in $^{13}C$ labelling of actively dividing bacteria when $^{13}C$ labelled substrate is used as a carbon source and incorporated into PLFA and nucleic acids (DNA/RNA) during their synthesis and replication. Phospholipid fatty acids stable isotope probing (PLFA-SIP) has become a popular approach for linking microbial community structure with its activity in the environment. In this approach, active soil microbial populations utilizing a $^{13}C$-labeled substrate will readily incorporate $^{13}C$ into membrane lipid components such as PLFAs. The resulting incorporation of label into specific PLFAs provides a "fingerprint" for the bacteria utilizing that substrate. There are many examples for the application of PLFA-SIP approach employed in an effort to characterize methanotrophs from different environment ( Bodelier *et al*., 2000; Bull *et al*., 2000; Boschker *et al*., 1998; Crossman *et al*., 2006, 2005; Knief *et al*., 2005, 2003). Similarly, Bodelier *et al*. (2000) carried out $^{14}C$-labeled PLFA slurry incubation study to characterize active methanotrophs from soil samples collected from rhizosphere compartment of rice microcosms with different fertilizers treatments.

Nucleic acids stable isotope probing (NA-SIP) is based on the incorporation of $^{13}C$-labelled substrates into nucleic acids, separation of labeled from unlabeled nucleic acids by density gradient centrifugation, and molecular identification of active populations carrying labeled nucleic acid. NA-SIP provides a very useful tool for studying active populations of methane oxidising bacteria in environmental samples, enabling active

members of a very diverse methanotroph community to be identified. The use of SIP has been potentially applied to study methanotrophic community by 16S rRNA gene analysis (Hutchens *et al*., 2004; Morris *et al*., 2002; Lin *et al*., 2004; Radajewski *et al*., 2002), *mmoX* (Hutchens *et al*., 2004; Lin *et al*., 2004), *mxaF* (Hutchens *et al*., 2004), and to a lesser extent *pmoA* (Radajewski *et al*., 2002) and *mxaF* (Morris *et al*., 2002). NA-SIP is limited by the necessity to assimilate a sufficiently large amount of $^{13}C$ into the nucleic acids so that density is significantly higher than that of unlabelled nucleic acids to allow differentiation by density gradient centrifugation. Detection and quantification of a specific mRNA molecule by reverse transcription PCR (RT-PCR) is a more promising tool to identify active microorganisms in the environment (see also the below section).

Other methods like *pmoA*-based microarray analysis (Bodressy *et al*., 2003, 2006) have also been increasingly being used these days. Microbial diagnostic microarrays (MDMs) consist of nucleic acid probe sets, with each probe being specific for a given strain, subspecies, species, genus, or higher taxon (Bodressy *et al*., 2003, 2006).

### 1.4.5 Functional diversity of methanotrophs

The diversity of functions within a microbial population is important for the multiple functions of a soil. The functional diversity of microbial communities has been found to be very sensitive to environmental changes (Kandeler *et al*., 1996; 1999; Zak *et al*., 1994). Functional diversity of microbial populations in soil may be determined by either expression of different enzymes (carbon utilization patterns, extra-cellular enzyme patterns) or diversity of nucleic acids (mRNA, rRNA) within cells.

mRNA molecules are gene copies used for synthesis of specific proteins by the cell and diversity of mRNA reflects the diversity and type of enzymes synthesized. Concentration of mRNA is correlated with the protein synthesis rate and as such with the activity of the microorganism. Therefore, the content and diversity of mRNA molecules will give a quite accurate picture of the *in situ* function and activity of the microbial community. A number of recent studies have focused on the analysis of expression of methane monooxygenase in the environment using *pmoA*-specific primer sets. This included the analysis of soil (Han and Semrau, 2004; Kolb *et al*., 2005), fresh water sediment (Cheng *et al*., 1999; Nercessian *et al*., 2005), landfill (Chen *et al*., 2007) and peatlands (Chen *et al*., 2008), providing direct *in situ* evidence of active methanotrophs.

### 1.4.6 Fingerprinting methods for the study of methanotrophic diversity

In the last decade molecular fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE; Muyzer *et al*., 1993), terminal restriction fragment length polymorphism (T-RFLP; Liu *et al*., 1997) or single strand conformation polymorphism (SSCP; Schwieger and Tebbe, 1998) became important and frequently used tools in microbial ecology in order to understand the spatial and temporal variability of microbial community structure and functions, e.g., in response to different treatments, agricultural practices, to pollution or to climate at a community level. These three techniques can be used to generate fingerprints not only of rRNA gene fragments but also of other functional genes PCR-amplified from total community DNA or cDNA (Prosser, 2002; Liesack and Dunfield, 2002). The general principle of most molecular fingerprinting techniques is based on the electrophoretic separation of marker gene fragments PCR-amplified from nucleic acids directly extracted from soil samples, due to differences in their nucleotide sequence.

Terminal restriction fragment length polymorphism (T-RFLP) is currently one of the most powerful methods in microbial ecology. T-RFLP analysis has been shown to be a consistent, high-resolution, and high-throughput cultivation-independent technique for rapidly comparing the diversity of bacterial DNA sequences amplified from PCR from environmental samples (Braker *et al*., 2001; Bremer *et al*., 2007; Buckley and Schmidt, 2001; Dunbar *et al*., 2001; Hartmann *et al*., 2007; Leuders and Friedrich, 2003; Marsh, 2005,1999; Noll *et al*., 2005; Osborn *et al*., 2000; Shrestha *et al*., 2008; Widmer *et al*., 2006). It is based on PCR amplification of 16S rRNA or functional gene with specific primers. The primers are labelled with a fluorescent tag at the terminus resulting in labelled PCR-products. The PCR products are digested with tetrameric restriction enzymes. The digested PCR products are subsequently loaded on an automated DNA sequencer where the labelled fragments are separated by high-resolution gel or capillary based electrophoresis. The output includes the fragments with different size and quantity and then these output data will be further normalized and analysed with multivariate analysis (Bremer *et al*., 2007; Noll *et al*., 2005).

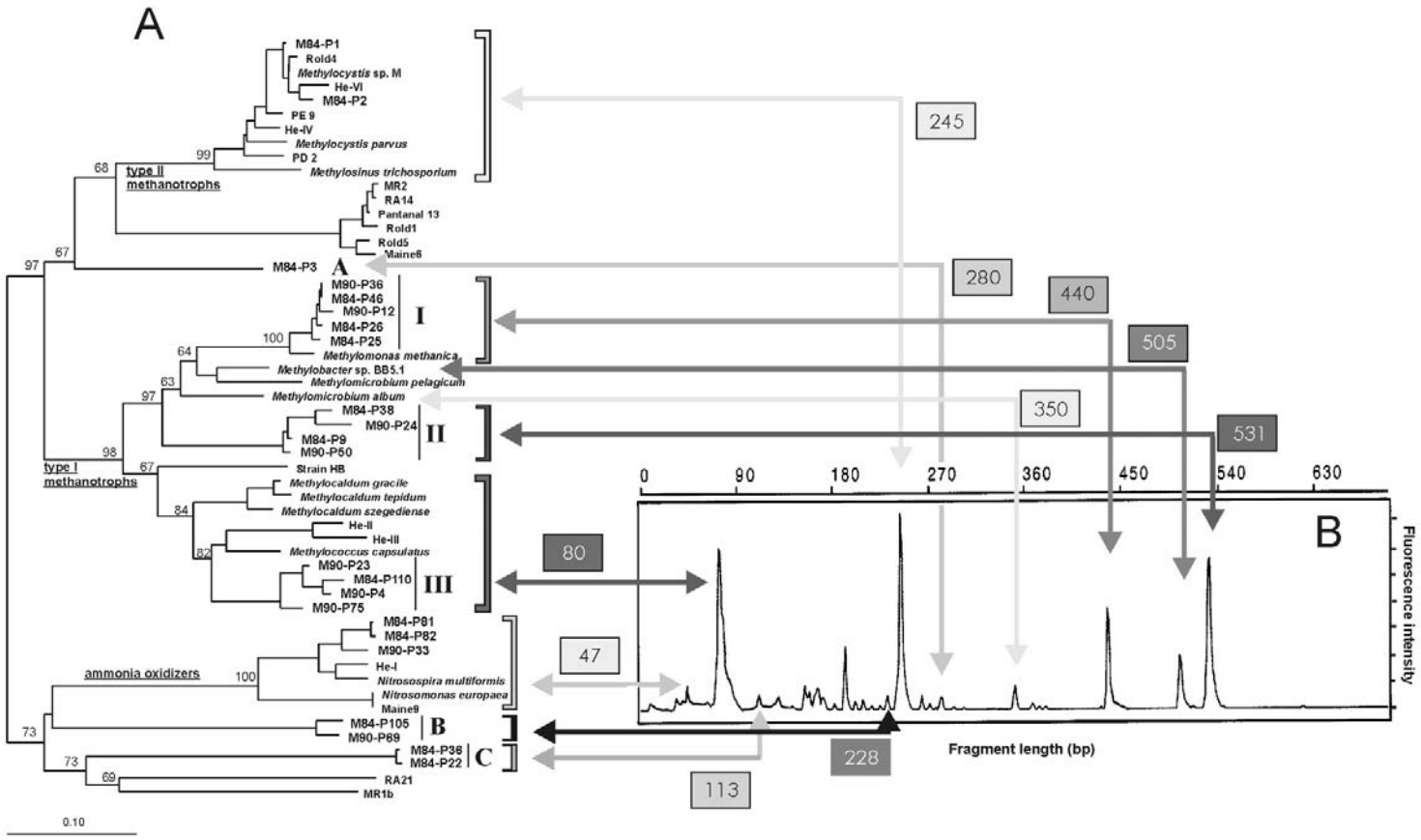



**Fig. 1.8:** A) Distance dendrogram constructed for partial *pmoA* and *amoA* gene sequences in relation to *pmoA*-based T-RFLP (B). Here different T-RFs can be affiliated to respective *pmoA* sequences as shown by arrow direction. (Horz *et al*., 2001).

### 1.4.7 Quantification of methanotrophs

The traditional method for enumerating methanotrophs in environmental samples has been most-probable-number (MPN) cultivation. Several studies have measured methanotroph numbers in the rice root rhizosphere and found $10^4$ - $10^6$ per g of soil (e.g., Bosse and Frenzel, 1997). Methanotroph numbers have also been determined by MPN in trichloroethylene-contaminated aquifers ($10^3$ - $10^5$ per g) (Takeuchi *et al*., 2001); swamp sediment ($10^6$ - $10^7$ per ml) (Miller *et al*., 2004); and wet meadow soil consuming atmospheric $CH_4$ ($10^5$ to $10^7$ per g) (Horz *et al*., 2002).

Fluorescence in situ hybridization (FISH) targeting the 16S rRNA gene has been used to identify (Eller and Frenzel, 2001) and enumerate (Dedysh *et al*., 2001, 2003) methanotrophs. The disadvantages of using FISH to enumerate methanotrophs are that it can only be used when the population is numerous enough to be counted under the microscope and when the 16S rRNA genes of the target organisms are known. Due to the many diversity studies of methanotrophs using *pmoA* phylogeny, many novel groups of methanotrophs can only be identified by *pmoA* sequence; hence, FISH cannot be used to enumerate these organisms. Therefore, other techniques have been developed that target the *pmoA* gene, including competitive reverse transcriptase PCR (Han and Semrau, 2004) and real-time PCR (Kolb *et al*., 2005, 2003) assays. The competitive reverse transcription-PCR approach was developed for both *pmoA* and *mmoX* using internal RNA standards and capillary electrophoresis and was successfully used to quantify the amount of mRNA transcript in both whole cells and a model soil slurry system (Han and Semrau, 2004). So far, this technique has not yet been applied to environmental samples. The quantitative real-time PCR assay for methanotrophs was developed from a method using SYBR green, previously used for detecting other bacteria, and *pmoA* specific primers designed to target five different groups of methanotophs in real-time PCR (Kolb *et al*., 2003). This assay was successfully used to quantify the methanotroph community in a number of environments, including a flooded rice field soil (Kolb *et al*., 2003) and forest soils (Kolb *et al*., 2005).

### 1.5 Effect of nitrogen fertilization on methane production and methane oxidation

Nitrogen fertilization is essential for achieving high rice yields and is widely practiced in rice cultivation. It has been assumed that an increase of rice production by 60% is the most appropriate way to sustain the estimated increase of the human population during the next three decades and for this reason, intensified global fertilizer application will be necessary. However, there is an ongoing discussion on the possible effects of nitrogen application on methane emission from the rice fields. Methane emission is a net consequence of methane production and oxidation, and both these processes are influenced by methane transport from the soil in which methane is produced and remained. Both methane production and oxidation are biological processes and affected by nitrogen fertilizer directly or indirectly (Schimel, 2000). Nitrogen application would also affect the methane transport through the effect of rice growth as rice plants are the main pathways for transporting methane from the anoxic soil to the atmosphere (Le Mer and Roger, 2001).

#### 1.5.1 Effects of nitrogen on methane production

Nitrogen fertilizers are supposed to stimulate methane production by enhancing rice plant growth, thus increasing the carbon supply for methanogens (Schimel, 2000; Fig. 1.9). Under flooded conditions, the growth of rice plant enhances methane emission by providing carbon sources and by favoring methane transport to the atmosphere (Dannenberg and Conrad, 1999). A direct stimulatory effect of nitrogen fertilization was also observed on methane production (Dan *et al*., 2001). Recently, Shan *et al*. (2008) observed that nitrogen application played an important role in the transformation of acetate to methane, indicating that N fertilizers might also affect methane production at the level of microbial community. Probably, nitrogen application might have stimulated the growth and activities of methanogens and conversion of acetate into methane by methanogens, thus leading to a decrease of acetate and an increase in methane emission.

#### 1.5.2 Effects of nitrogen on methane oxidation

There is an ongoing discussion on the possible effects of ammonium based nitrogen fertilizer on consumption of methane by methanotrophs depending on which environments, it has been applied (Bodelier and Laanbroek, 2004). Currently, there are

many contradictory results, reporting inhibition effects (Banik *et al*., 1996; Bosse *et al*., 1993; Cicerone and Shetter, 1981; Hütsch *et al*., 1994; Steudler 1989), stimulation effects (Bodelier *et al*., 2000; Dan *et al*., 2001; Krüger and Frenzel, 2003; Krüger *et al*., 2002 ; Mohanty *et al*., 2006) or no effects (Delgado and Mosier, 1996; Dunfield *et al*., 1995) of ammonium-based N-fertilization on methane oxidising bacteria. Schimel (2000) suggested these three different effects happen at different levels such as inhibitory effect at the biochemical level and stimulatory effect at the microbial level (Fig. 1.8).

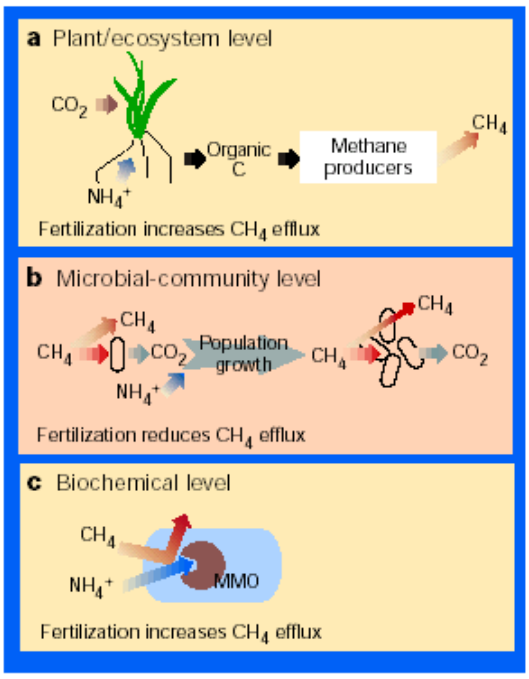



**Fig. 1.9:** Effects of ammonium fertilizer on methane dynamics in a rice ecosystem. **A.** At the plant/ecosystem level, nitrogen increases plant growth and carbon supply to the methane producers, stimulating methane flux from the soil. **B.** As Bodelier *et al* (2000) have shown, at the level of the microbial community, nitrogen stimulates the growth and activity of methane-oxidising bacteria (methanotroph), leading to reduced net efflux. **C.** At the biochemical level, ammonium inhibits methane consumption because of competition for methane monoxygenase, and so increases efflux. The balance between these processes may vary. But Bodelier *et al*. (2000) find that effects at the level of the microbial community dominated the rice ecosystem response to ammonium fertilization (Taken from Schimel, 2000).

Laboratory and field studies have demonstrated that $NH_4^+$ addition inhibits $CH_4$ oxidation in forest, grassland, arable and paddy soils (Cai and Yan, 1999; Hütsch *et al*., 1993; Mosier *et al*., 1991; Nesbit and Breitenbeck 1992; Steudler *et al*., 1989). Because of the similarity in shape and size of $CH_4$ and $NH_3$ molecules, and the relatively low specificity of the monooxygenase enzymes responsible, besides the oxidation of methane, the MMO convert ammonia to nitrite, and ammonia (Bedard and Knowles, 1989). Thus, it will reduce the amount of methane consumed by methanotrophic bacteria and it is hypothesized that the competition between $NH_4^+$ and $CH_4$ is the mechanism for the inhibitory effect of $NH_4^+$ on $CH_4$ oxidation (Schimel *et al*., 1993). The intermediates and end products of ammonia oxidation, i.e. hydroxylamine and nitrite, can be toxic to methanotrophic bacteria and will also lead to inhibition of methane consumption (Schnell, and King, 1995). Nitrite was found to inhibit $CH_4$ oxidation in the cultures of

*Methylomonas albus BG8* and *M. trichosperium OB3b* (King and Schnell, 1994). Furthermore, the addition of high amounts of ammonium salts to laboratory incubations may also affect methane oxidation due to osmotic stress (Whalen, 2000).

However, Bodelier *et al*. (2000) reported the stimulation of methanotrophic activity in paddy soil slurries after addition of N-fertilizers. Further, Eller and Frenzel (2000) showed that *in situ* rhizospheric methane oxidation, determined by the use of specific inhibitor $CH_2F_2$, decreased to zero upon depletion of ammonium in the soil. Identical results were found in natural rice paddies (Krüger, and Frenzel, 2003; Krüger *et al*., 2002). De Visscher and Cleemput (2003) published the first study that anticipated both inhibitory and stimulatory effects of ammonium on methane oxidation. They reported that $NH_4^+$ stimulates methane oxidation at high $CH_4$ concentrations and inhibit at low $CH_4$ concentrations. Nevertheless, experimental proof for a mechanism of nitrogen-based stimulation of methane oxidation in soil is still missing. Detailed knowledge on soil physicochemical parameters and on the type of methane oxidizers present in any particular environment is necessary to understand the modes of action of fertilizer application.

### 1.6 Objectives

- To study the spatial and temporal change on the activity and composition of methanotrophic community in the rice rhizosphere.
- To study the temporal variation in methane oxidation and methane concentration due to the application of different N-fertilizer treatments during different growth stages of the rice plants.
- To study the effect of different ammonium-N fertilizer treatments on the community structure of methanotrophs on the rice roots and in the rhizospheric soil during different growth stages of the rice plants.
- To study the effect of different ammonium-N fertilizer treatments on methane oxidation potential activity and metabolically active methanotrophs in the rhizospheric soil during different growth stages of the rice plants.
- To isolate the methanotrophs from rhizospheric soil and roots from rice microcosms treated with three different N-fertilizer treatments.

# Chapter 2

# Materials and methods

## 2.1 Materials

### 2.1.1 Microcosm Experiment

Rhizon soil sampler (Rhizosphere Research Products, The Netherlands)

Venoject blood collecting tubes (Terumo Europe N.V., Belgium)

Foil grab bags with stainless steel fitting (Analyt-MTC Messtechnik, Germany)

Perforated pipe (The Netherlands)

1.59 mm × 1.27 mm stainless steel tubing (Alltech GmbH, Germany)

3.00 mm ID isoversenic tubing (Gilson International, Germany)

Teflon tubing (Germany)

Rice field soil (used in experiment of chapter 3) from Italian Rice Research Institute in Vercelli, Italy, collected in 1998

Rice field soil (used in experiment of chapter 4) from Italian Rice Research Institute in Vercelli, Italy, collected in 2006

Rice seeds (*Oryza sativa* var. KORAL type japonica)

Urea, $(NH_2)_2CO$ (Sigma, Germany)

Ammonium sulphate, $[(NH_4)_2SO_4]$ (Sigma, Germany)

Potassium chloride, (KCL) (Fluka, Germany)

Potassium dihydrogen phosphate, $(KH_2PO_4)$ (Roth, Germany)

Demineralized water (MPI, Marburg, Germany)

$^{13}C$-labeled $CH_4$ (99 atom-% $^{13}C$, Isotec, USA)

pH meter (Microprocessor pH-Meter 539, WTW)

Peristaltic pump (Gilson International, Germany)

Gas Chromatography (Shimadzu GC-8A, Japan)

Ion Chromatography (Sykam, Germany)

SAFIRE microplate reader (TECAN, Germany)

### 2.1.2 PLFA extraction method

$CHCl_3$ (Merck, Germany)
Methanol (Merck, Germany)
KOH (Fluka, Germany)
Acetone (Merck, Germany)
Toluene (Merck, Germany)
Hexane (Merck, Germany)
Acetic acid (Merck, Germany)
Sep pak cartridges (Waters, Ireland)
Folded filters (Schleicher and Schuell, USA)
Pasteur pipettes (Germany)
Glass pipettes (Germany)
Round bottom flask (Ochs, Germany)
Furnace (Heraeus Instruments, Germany)
Centrifugation machine (Hettich EBA 8S, USA)
Rotary Evaporator (Laborta 4000, Heidolph, Germany)

### 2.1.3 Ammonium concentration analysis

*o*-Phthalaldehyde (Merck, Germany)
2-Mercaptoethanol (Merck, Germany)
Potassium dihydrogen phosphate (Roth, Germany)
Dipotassium hydrogen phosphate (Roth, Germany)
Ethyl acetate (Merck, Germany)
*t*-Butylmercaptan (2-Methyl-2-propanethiol) (Merck, Germany)
Pentane (Merck, Germany)
Fresh double distilled water (MPI, Marburg, Germany)
Separatory funnel (Ochs Germany)

### 2.1.4 Total nucleic acids extractions

Chloroform-isoamyl alcohol [24:1 (v/v)] (Sigma-Aldrich Chemie GmbH, Germany)
Phenol–chloroform–isoamyl alcohol [25:24:1 (v/v/v)] (Sigma-Aldrich Chemie GmbH, Germany)
Phenol (Water-saturated, Stabilized) (Applichem GmbH, Germany)
DEPC-pretreated water (Ambion, Europe)
Zirconium beads (0.1mm diameter) (Biospec Products Inc. Carl Roth, Germany)
Tris-HCl (Merck, Germany)
$Na_2EDTA$ (Merck, Germany)
Sodium dodecyl sulfate, 6% (v/v) (Merck, Germany)
TE buffer [10 mM Tris-HCl, 1 mM EDTA (pH 8.0)] (Promega, Germany)
Absolute Ethanol (Applichem GmbH, Germany)
RNA*later* ® (Ambion, Europe)
Phase Lock Gel Heavy 2 ml (Eppendorf vertrieb, Germany)

### 2.1.5 PCR and RT-PCR

*Methylocystis* strain H-17 (Culture collection from Prof. Dr. Peter Frenzel's Laboratory)
Primers (MWG-Biotech AG, Germany)
GoTaq ® Flexi DNA Polymerase (Promega, Germany)
Wizard ® SV Gel and PCR Cleanup System (Promega, Germany)
GenElute™ PCR Clean-up Kit (Sigma, Germany)
Nuclease-free water (Promega, Germany)
ImProm-II™ Reverse Transcription System (Promega, Germany)
RQ1 RNase-free DNase (Promega, Germany)
RNasin ® Ribonuclease Inhibition (Promega, Germany)
RNeasy MinElute Cleanup Kit (250) cat no. 74204(Qiagen, Germany)
Oligonucleotide primers (MWG Biotech, Germany)
Cycler Primus $96^{plus}$ (MWG Biotech, Germany)

### 2.1.6 Cloning and sequencing

pGEM-T Easy Vector System I (Promega, Germany)
Taq DNA polymerase, Recombinant (Invitrogen GmbH, Germany)
Micro Amp Fast Reaction Tube with cap (Applied Biosystems, Germany)

### 2.1.7 Terminal restriction fragment length polymorphism (T-RFLP)

SigmaSpin™ Post-Reaction Clean-Up Columns (Sigma, Germany)

HiDi-formamide (Applera, Germany)

Internal DNA fragment length standard (X-Rhodamine MapMarker® 30-1000 bp; BioVentures, USA)

ABI 310 automated sequencer (Applied Biosystems, USA)

GeneScan 3.71 software (Applied Biosystems, USA)

ABI 3130 automated sequencer (Applied Biosystems, USA)

GeneScan 5.1 software (Applied Biosystems, USA)

## 2.2 Methods

This Ph.D. research was based on two major experiments which were carried out as planted rice microcosm experiments in the greenhouse, MPI, Marburg, Germany. The soil used for these experiments was taken from drained paddy fields of the Italian Rice Research Institute, Vercelli, Italy in 1998 and 2006, stored in MPI, Marburg, Germany, under dry conditions and was used for the experiments of chapters 3 and 4, respectively.

The experimental set up and microcosm experiments have been explained in detail in the relevant chapters. Sequential preparation of microcosm set up and sample collection of roots and rhizospheric soil are illustrated in appendix. The other laboratory analyses methods have been described as below:

### 2.2.1 Measurement of $CH_4$ flux

Rates of $CH_4$ emission and oxidation were measured as described by Krüger and Frenzel (2003). Measurement of $CH_4$ emission and $CH_4$ oxidation rates were carried out once/twice a week. For the measurement of rates of $CH_4$ emission, triplicate microcosms were covered by static flux chambers made of plexi glass with 10 cm internal diameter and 40 or 82 cm height, depending on the height of the rice plants. The lower edge of the chambers was dipped into the floodwater to seal the chambers, while a built-in fan mixed the atmosphere inside the chambers and prevented the formation of gradients of gases present inside (see appendix). Gas samples were taken every 30 minutes for 3 hours. The rates of methane emission from rice soil microcosms were calculated by following the equation (Rolston, 1986):

$$f = (V/A)\ (\Delta C / \Delta t)$$

Where ‘f ’is the methane emission rate (mg $m^{-2}$ $h^{-1}$), ‘V’ is the volume of chamber above the surface water ($m^3$), ‘A’ is the cross-section area of pot ($m^2$), ‘ΔC’ is the concentration difference between zero and time t (mg $m^{-3}$), and ‘Δt’ is the time duration between the two sampling times (h).

Rates of $CH_4$ oxidation were measured in triplicate microcosms by adding 1% diflouromethane ($CH_2F_2$ 99%, ICI Chemicals, UK), a gaseous inhibitor specific for $CH_4$ oxidation (Miller *et al*., 1998), to the headspace of the flux chambers. This resulted in the

*in situ* inhibition of rhizospheric methane oxidation by introducing $CH_2F_2$ via the plant stem-rhizome-root aerenchyma system. Diflouromethane is a reversible inhibitor and promotes rapid recovery of methanotrophs after dissipation of the gas, thereby allowing repeated assays on the same microcosm. The gas samples were immediately analysed for $CH_4$, $CO_2$ and $CH_2F_2$ on a Shimadzu GC-8A gas chromatograph equipped with a flame ionization detector and a methanizer (Krüger *et al*., 2002). $CH_2F_2$ was analyzed to ensure the presence of sufficient inhibitor concentration. Production of methane is not affected by $CH_2F_2$ (Miller *et al*., 1998). The day after the measurement, the concentration of $CH_2F_2$ was again analyzed in order to confirm the complete removal of $CH_2F_2$ from the microcosms.

Methane oxidation rates were calculated from the differences in emission with and without inhibitor.

Methane oxidation rate = Methane emission rate with inhibitor **-** Methane emission rate without inhibitor

Stable isotope analysis of $^{13}C/^{12}C$ in gas samples was done using a gas chromatograph combustion-isotope ratio mass spectrometry (GCC-IRMS) system as described by Conrad *et al*. (2002) (refer to section 2.2.3.1)

### 2.2.2 Soil pore water

Pore water samples were collected weekly into Venoject blood collecting tubes (TERUMO EUROPE N.V., Belgium) from the rhizosphere (3 cm depth from the soil surface) and bulk (10 cm depth from the soil surface) regions of rice microcosms by using Rhizon pore water samplers (Rhizosphere Research Products, The Netherlands) (Fig 3.1 and Appendix Fig. C, D and E). Immediately after sampling, the tubes were heavily shaken by hand and an aliquot of gas sample from the headspace was collected with a pressure lock syringe and analyzed for $CH_4$ using a gas chromatograph equipped with FID detector as described in section 2.2.1. Then, the pore water was stored frozen (–20 °C) for determination of $^{13}C$-$CH_4$, pH, $NH_4^+$, $NO_2^-$, $NO_3^-$ and $SO_4^{2-}$. The $CH_4$ concentration ($C_{CH4}$) in the soil pore water was calculated as described (Krüger *et al*., 2001) below:

$$C_{CH4} = (m\ G_v)/(G_l\ M_v)\ [\mu M]$$

where m is the mixing ratio gas phase (ppmv), $M_v$ is the gas volume of an ideal gas (24.78 L $mol^{-1}$, at 25 °C), $G_v$ is the volume of gas headspace of the tube [L], and $G_l$ is the volume of liquid in the tube [L].

### 2.2.3 Analytical methods

#### 2.2.3.1 Measurement of $^{13}CH_4$ dissolved in the pore water

Stable isotope analysis of $^{13}C/^{12}C$ in gas samples (dissolved $CH_4$ in frozen pore water) was performed using a gas chromatograph combustion isotope ratio mass spectrometer (GC-C-IRMS) system (Conrad and Claus, 2005). The $CH_4$ and $CO_2$ in the gas samples (10–400 µl) were first separated in a Hewlett Packard 6890 gas chromatograph operating with a Pora Plot Q column (27.5 m length; 0.32 mm internal diameter; 10 µm film thickness; Chrompack, Frankfurt, Germany) at 25 °C and He (99.996% purity; 2.6 ml $min^{-1}$) as carrier gas. After conversion of $CH_4$ to $CO_2$ in the Finnigan Standard GC Combustion Interface III the gases were transferred into the IRMS. The working standards were $CO_2$ (99.998% purity; Messer-Griesheim, Germany) and methylstearate (Merck, Germany). The latter expressed in the delta notation versus PDB carbonate:

$$\delta^{13}C = 10^3(R_{sa}/R_{st} - 1)$$

with $R = {}^{13}C/{}^{12}C$ of sample (sa) and standard (st), respectively.

#### 2.2.3.2 Analysis of $NH_4^+$, $NO_2^-$, $NO_3^-$, and $SO_4^{2-}$ concentration, and pH in the pore water

Ammonium concentration was analysed fluorometrically by microscale analysis using a SAFIRE microplate reader (TECAN-Xfluor4, Germany) as described by Murase *et al.* (2006). The pore water sample was diluted to 100 times and 100 µl of diluted sample was mixed with 100 µl of 2 N KCl into 96-wells microtiter plates. Then 50 µl of reaction buffer [mixture of 15 mM *o*-phthalaldehyde (0.080g) and 50 mM 2-mercaptoethanol (137 µl) in 20 ml of 500 mM purified phosphate buffer (pH 6.8) and 20 ml fresh double distilled water (Corbin,1984)] was added to the 96-well microtiter plates and incubated at 63 °C for 10 min. After cooling to room temperature, the fluorescence intensity was

determined at an excitation wavelength of 410 nm and an emission wavelength of 470 nm with a SAFIRE microplate reader (TECAN, Germany). The resulting value was calculated in milli molar concentration after comparison with a standard curve prepared from known concentrations of ammonium sulphate.

Nitrate, nitrite and sulphate concentrations in the soil pore water samples were analysed by ion chromatography on an HPLC equipped with an LCA KSP column (Sykam, Germany) using $Na_2CO_3$ as the eluent using a refractive index detector and an UV-detector as described by Bak *et al*. (1991).

pH was measured using a pH meter.

### 2.2.4 Collection of soil and root samples

Rhizospheric soil and root samples were collected in triplicate from each of triplicate microcosms from all treatments and all sampling points. Before harvesting the plant, the upper 2-3 cm soil from all the rice microcosms was removed and discarded because this soil layer might have received oxygen from the overlaying water and not only from the rice roots. In the center of each rice microcosm (pot), a self-made nylon bag (25 μm mesh; 6 cm length and 9 cm radius) was placed through which water and nutrients could pass freely while roots were not able to penetrate, isolating the rhizospheric soil (soil from inside the bag and adhered to the roots) from the bulk soil (soil from outside the bag) (see Fig. 3.1 and appendix). Rhizospheric soil samples were homogenized in a sterile beaker with a sterile spatula and then put in aliquots of 500 mg into triplicate 2.0 ml Eppendorf cups. Subsequently, the roots were separated from the soil, were washed in deionized and sterile water and then collected in Falcon tubes. Immediately after sampling, roots and rhizospheric soil were stored at –80 $^{o}$C for PLFA and molecular analyses.

### 2.2.5 Moisture content determination

Approximately 1 g of soil was accurately weighed and then dried at 105 °C for 24 h (Janssen *et al*., 2002). The samples were reweighed after they were allowed to cool in a desiccator to room temperature. The drying and cooling procedure was repeated until constant mass (± 0.005 mg) was obtained.

The moisture content was calculated using following formula:

$$\% \text{ moisture content} = (I_w - F_w)/ F_w \times 100$$

where $I_w$= wet weight of the soil and $F_w$= dry weight of the soil

### 2.2.6 Methane oxidation potential assay

The methane oxidation potential activity was determined (chapter 5) for the rhizospheric soil and roots samples collected from plants harvested on 29, 40, 57, 62, 67, and 88 days after transplantation from all treatments. Soil slurries were prepared by mixing one part of soil and one part of demineralized and sterile water (grams per fresh weight gram). 2 ml of this slurry was placed into triplicate pressure tubes (25 ml), and tubes were closed with butyl stoppers. Similarly, triplicate tubes were prepared for washed and cut roots. The prepared tubes were flushed with synthetic air (21% $O_2$ in $N_2$) for 1 minute and subsequently, head space was supplemented with 50,000 ppmv $CH_4$. The tubes were then incubated horizontally on a roller at 30 °C in dark and depletion of $CH_4$ concentration was monitored over time for 72 hours. Headspace $CH_4$ was sampled with a pressure lock syringe and measured by GC. From the $CH_4$ depletion of the two parallel samples, linear regressions were calculated and $CH_4$ oxidation rates were calculated from the slope of these regression lines.

### 2.2.7 Molecular analyses of the methanotrophic community

#### 2.2.7.1 Total nucleic acids extraction

All solutions and glassware were made RNase-free by treatment with diethyl pyrocarbonate and the working area was cleaned with 2% Absolve$^{TM}$ (Perkin, USA). Total nucleic acids (chapter 3, 4, and 5) from the soil and root samples was extracted using a nucleic acids extraction protocol reported previously (Shrestha, Dissertation, 2007; Noll *et al*., 2005). The frozen roots were pulverized with a mortar and pestle after freezing in liquid $N_2$ and followed with extraction. Before the extraction, samples (rhizospheric soil and roots samples) were mixed with 700 µl of pre-cooled TPM buffer [50 mM Tris-HCl (pH 7.0), 1.7% (wt/vol) polyvinylpyrrolidon, 20 mM $MgCl_2$], and 0.5 g of zirconium beads (0.1 mm diameter). The mixture was shaken for 45 s at maximum speed in a bead beater (Fast Prep FP120, Bio 101, Thermo Savant, USA). Zirconium

beads, cell debris and soil particles were pelleted by centrifugation (5 min at 14,000 rpm at 4 °C), and the supernatant was transferred to a new reaction tube. The pellet was resuspended in 700 µl of a phenol-based lysis buffer [5 mM Tris-HCl (pH 7.0), 5 mM $Na_2EDTA$; 0.1% (wt/vol) sodium dodecyl sulfate, 6% (v/v) water-saturated phenol], followed by a second round of bead beating. After centrifugation at 14,000 rpm, the supernatants of the two bead-beating treatments were pooled and were extracted with 500 ml of water-saturated phenol, phenol–chloroform–isoamyl alcohol [25:24:1 (v/v/v)], and finally chloroform–isoamyl alcohol [24:1 (v/v)]. The total nucleic acids were precipitated from the aqueous phase with 1 volume of isopropanol and 3 M sodium acetate, pH 5.7 (1/10 volume of total sample solute). Finally, the total nucleic acid sample was washed twice with 70% ethanol, then air-dried, and resuspended in two tubes with 50 µl of TE buffer [10 mM Tris-HCl, 1 mM EDTA (pH 8.0)], one as a DNA extract and the other one for subsequent RNA isolation. Then, nucleic acids were checked for quality by electrophoresis in agarose gels after staining with ethidium bromide solution followed by gel documentation (photography using INTAS, Gel Jet Imager, Göttingen, Germany). Then, DNA purification was done by using the Wizard® DNA Clean-up System (Promega, Germany). Assessment of nucleic acid purity was done by determining the ratios of spectrophotometric absorbance of sample at 260/230 and 260/280.

#### 2.2.7.2 Total RNA isolation

Total RNA isolation was carried out for rhizospheric soil (chapter 5). For the removal of coextracted DNA, total nucleic acids after extraction was treated with 5 U DNase (Promega, Germany), 10 × DNAse buffer (Promega, Germany), in combination with 10 U RNasin (Promega, Germany), and incubated at 37 °C for 60 min. Finally, total RNA from the soil sample was recovered by using RNeasy kit (Qiagen, Germany). However, RNA from the roots sample could not be recovered. The integrity of the 16S and 23S rRNA was checked by electrophoresis on a 1% agarose gel. The gel was stained with ethidium bromide and photographed (INTAS, Gel Jet Imager, Germany).

#### 2.2.7.3 PCR amplification of *pmoA* gene

PCR amplification of the *pmoA* gene from rhizospheric soil and roots samples was done using primers sets A189f/A682r (Holmes *et al.*,1995), A189f/mb661r (Costello and Lidstrom, 1999), and A189f/nmb650r (see in section 5.3.7). The reaction was carried out

in 50 µl (total volume) mixtures containing 1 µl of template DNA, 10 µl of 5× reaction buffer (Promega, Germany), 1.5 mM $MgCl_2$, 200 µM each dNTP, 0.33 µM (each) primer (MWG-Biotech, Germany), and 2.5 U of Taq DNA polymerase (Promega, Germany). For the primer set A189f/mb661r, the thermal PCR profile was as follows: initial denaturation at 94 °C for 5 min; 15 cycles consisting of denaturation at 94 °C for 1 min, primer annealing at 62 °C for 45 s, and elongation at 72 °C for 60 s, followed by another twenty cycles consisting of denaturation at 94 °C for 1 min, primer annealing at 55 °C for 45 s and elongation at 72 °C for 60 s. The final elongation step was extended to 7 min at 72 °C. For the primers sets A189f/A682r and A189f/nmb650r, a touchdown thermal program was performed and consisted of an initial denaturation step of 3 min at 94 °C, followed by 11 touchdown cycles consisting of denaturation at 94 °C for 1 min, primer annealing at 64–55 °C (with -1 °C step) for 1 min and 24 further cycles at 55 °C for 1 min followed by 72 °C for 1 min extension. Final elongation step was extended to 7 min at 72 °C. A positive control was performed using genomic DNA of *Methylocystis* strain H-17, while a negative control was performed using $H_2O$ instead of DNA template. Amplification was performed in 0.2-ml reaction tubes using a DNA thermal cycler. Aliquots of the amplicons (5 µl) were checked by electrophoresis on a 1% agarose gel after staining with ethidium bromide solution for 30 minutes. PCR products were purified with GenElute™ PCR Clean-up Kit (Sigma, Germany) and used for further cloning and sequencing purpose.

#### 2.2.7.4 RT-PCR of *pmoA* transcripts

This analysis was carried out for the experiments described in chapter 5. RT-PCR was carried out using a two step RT-PCR kit according to the manufacturer's instructions (ImProm-II™ Reverse Transcription System, Promega, Germany). In the first step, cDNA synthesis was carried out in total volume of 20 µl containing 2 µl of template RNA and 20 pmol of A682r primer. Thus each RNA was reverse-transcribed to cDNA that was used as a template for PCR as the second step. In this step, primer set A189f/682r was used and a touchdown PCR program was performed as: initial denaturation step of 3 min at 94 °C, followed by 11 touchdown cycles consisting of denaturation at 94 °C for 1 min, primer annealing at 64–55 °C (with -1 °C step) for 1 min and 24 further cycles at 55°C for 1 min followed by 72 °C for 1 min extension. The final elongation step was extended to 7 min. An aliquot (1µl) of the PCR product from A189f/A682r primer set was used for

a second round of PCR with primer set A189f/mb661r. The PCR program was performed as: initial denaturation at 94 °C for 2 min; fifteen cycles consisting of denaturation at 94 °C for 1 min, primer annealing at 62 °C for 30 s, and elongation at 72 °C for 60 s followed by another twenty cycles consisting of denaturation at 94 °C for 1 min, primer annealing at 55 °C for 30 s and elongation at 72 °C for 60 s. The final elongation step was extended to 7 min. A negative control experiment was performed for all the samples as described above but after excluding reverse transcriptase enzyme. Also, a positive RT-PCR experiment was performed with control RNA provided in the kit (Promega, Germany) with all RT-PCR steps. Aliquots (5 µl) of the amplicons were checked by electrophoresis on a 1% agarose gel after staining with ethidium bromide solution for about 30 minutes and photographed.

#### 2.2.7.5 Cloning and sequencing

*pmoA* Clone libraries were constructed by using purified DNA or cDNA amplicons by using the pGEM-T Easy cloning kit (Promega, Germany). Several clones from rhizospheric soil samples and root samples were randomly selected for comparative sequence analysis. Cloned inserts were sequenced at the Max Planck Institute for Plant Breeding in Cologne, Germany, using the primers M13f and M13r targeting vector sequences. The partial *pmoA* gene sequences (from chapter 3) have been deposited in the EMBL, GenBank, and DDBJ nucleotide sequence databases under the following accession numbers: AM849616-AM849659 (RS-8); AM849660-AM849716 (RS-18); AM849717-AM849753 (RT-8); AM849754-AM849804 (RT-18). The partial *pmoA* gene sequences that were generated in fertilizer treatment study (chapter 4.3.6 and chapter 5.3.9) are still to be deposited in a nucleotide sequence database.

### 2.2.8 Phylogenetic analysis

The identities of the *pmoA* gene sequences were confirmed by searching the sequence databases using nucleotide blast (http://www.ncbi.nlm.nih.gov/BLAST/). Phylogenetic analyses of the *pmoA* gene and deduced amino acid sequences were carried out using the ARB program package (developed by O. Strunck and W. Ludwig; Technische Universität München [http://www.arb-home.de]). Sequences were manually aligned with the *pmoA* sequences obtained from the GenBank database. Regions of sequence ambiguity and incomplete data were excluded from the analyses. Results were depicted as a consensus

tree, combining the results of Tree-Puzzle, neighbor-joining approach, and maximum likelihood analyses of the data sets in chapter 3.

### 2.2.9 T-RFLP analysis

Analysis of terminal restriction fragment length polymorphism (T-RFLP) was performed for each DNA extract or cDNA samples in triplicate**.** The *pmoA* was amplified by PCR as described above (section 2.2.6.3) except a FAM (6- carboxyfluorescein)-labeled forward primer was used and the concentration of both forward and backward primers was increased to 0.66 µM for each reaction. For T-RFLP analysis, PCR products were run on the gel and the correct size was excised from the agarose gel and purified with Wizard ® SV Gel and PCR Cleanup System (Promega, Germany). After gel purification, approximately 100 ng of the amplicons were digested with 10 U of the restriction endonuclease MspI (Promega, Germany). The digestion was carried out in a total volume of 10 µl for 6 h at 37 °C followed with incubation at 65 °C for 20 min. The product was purified by using SigmaSpin™ Post-Reaction Clean-Up Columns (Sigma, Germany) following the manufacturer's instructions. Then aliquots (2.5 µl) of purified digested amplicons were mixed with 12-11 µl of deionized formamide (Applera, Germany) and 0.2-0.3 µl of an internal DNA fragment length standard (X-Rhodamine MapMarker® 30-1000 bp; BioVentures, USA). The mixtures were denatured at 94 °C for 3 minutes and then chilled on ice and finally loaded into an automated gene sequencer (ABI 310/ABI 3130, Applied Biosystems, Germany) fitted with ABI prism where the terminal restriction fragments (T-RFs) were separated by capillary electrophoresis. The length of fluorescently labeled T-RFs was determined by comparison with the internal standard using GeneScan 3.71/5.1 software (Applied Biosystems, Germany). Terminal restriction fragments between 50 and 550 bp with peak heights of >30 fluorescence units were used for T-RFLP analysis. Since T-RFs can vary slightly in size, T-RFLP patterns were inspected visually and peak size differences of up to 1-7 base pairs were confirmed by comparing the relative peaks of all patterns in all replicates and were considered identical and were clustered into a single TRF.

#### 2.2.9.1 Analysis of TRF profiles (Dunbar *et al.*, 2001)

i) Normalization of DNA quantity between replicate profiles.

The sum of all peak heights of >30 fluorescence units (i.e., the total fluorescence; 30 fluorescence units is the baseline noise threshold) in each replicate profile was calculated as an indication of the total DNA quantity represented by each profile. DNA quantity was normalized between replicate profiles to the smallest quantity by proportionally reducing the height of each peak in largest profiles. To accomplish this, the proportion of the smallest DNA quantity (i.e., total fluorescence) and a larger DNA quantity was calculated and used as a correction factor to adjust each peak height in the profile representing the larger DNA quantity. This procedure often eliminated peaks from larger profiles by reducing some peak heights below the baseline noise threshold (30 fluorescence units). Therefore, after adjustment of a profile, the new sum of peak heights of >30 fluorescence units was calculated, and the normalization procedure was repeated until, by iteration, the DNA quantity (i.e., total fluorescence) of the larger profile was equal to the quantity of the smaller profile.

ii) Creation of a derivative, reproducible sample profile.

For each sample, a derivative profile containing only the most conservative and reliable TRF information was created by identifying the subset of TRFs that appeared in all replicate profiles of a sample.

#### 2.2.9.2 Calculation of relative abundance of T-RFs (Dunbar *et al.*, 2001; Liesack and Dunfield, 2004)

After normalization of peak heights, the relative abundance (A*i)* of T-RFs in a sample, given in percent, were calculated as

$$Ai = ni/N$$

where *ni* represents the peak height of a T-RF (*i*) and *N* is the sum of all peak heights in a given T-RFLP pattern. To reduce data noise, only T-RFs with A*p* values ≥1% were considered for further analysis.

### 2.2.10 Phospholipid fatty acids-stable isotope probing (PLFA-SIP)

PLFA-SIP was carried out for the experiments described in chapter 3. Lipids were extracted from 3 g dry weight of rhizospheric soil and roots (n=3), taken from both control and $^{13}C$-labeled microcosms, using a modified Bligh and Dyer extraction method (Knief *et al*., 2003a). The frozen root samples were pulverized with a mortar and pestle after freezing in liquid $N_2$. The soil and roots samples were weighed in 30 ml glass bottle and 6 ml 50 mM phosphate buffer, 18 ml methanol and 7.5 ml chloroform were added to it and the bottles were shaken on a shaker at 300 rpm at room temperature under the hood for two hours. Then equal volumes (6 ml) of distilled water and of chloroform were added and the two phases were allowed to separate overnight at 4 °C. Next day the chloroform phase was dried by filtration through a sodium sulfate-containing phase separation filter and reduced in volume by rotary evaporation. The volume was reduced to about 1 ml and was fractionated on silicic acid column (Water, Ireland) into different polarity classes by sequential elution with chloroform, acetone and methanol (Zelles and Bai, 1993). The columns were pre-eluted with 2 ml of chloroform, and the sample was transferred to the column with three 2-ml washes of chloroform. Neutral lipids were eluted with 6 ml of chloroform, glycolipids were eluted with 6 ml of acetone, and polar lipids were eluted with 15 ml of methanol. The polar lipids were collected into a round-bottomed flask and the solvent was reduced with a rotary evaporator. The polar lipid extract was dissolved in 1 ml of methanol/toluene (1:1), 1 ml of methanolic KOH (0.2N) was added, and the mixture was incubated at 37 °C for 15 min. Finally 2 ml hexane, 0.3 ml 1 M acetic acid and 2 ml fresh double distilled water were added. The mixture was thoroughly mixed and centrifuged and fatty acid methyl esters were recovered from the organic phase of the sample. These resulting fatty acid methyl esters (FAME) were then sent to Braunschweig for further analysis by GC-C-IRMS in collaboration with Dr. Wolf Rainer Abraham, Braunschweig, Germany.

The fatty acid methyl esters were separated by gas chromatography (GC) and identified by their retention time compared to standards; peak identification was verified using mass spectrometry (MS). Fatty acids are designated by the total number of carbon atoms. The degree of unsaturation is indicated by a number separated from the chain length by a colon and is followed by ω*xc*, where *x* indicates the position of the double bond nearest to the aliphatic end (ω). The *c* indicates a *cis*-position of the double bond on the molecule.

Then the stable carbon-isotope compositions of the PLFA were determined using a Finnigan MAT Model 252 isotope ratio mass spectrometer coupled to HP 5890 GC with a Finnigan standard combustion interface (Abraham *et al*., 1998). To calculate isotope ratios ($\delta^{13}C$) for the PLFAs, $\delta^{13}C$ values of the FAMEs were corrected for the $^{13}C$-content of the carbon atom of the methyl group ($\delta^{13}C$ = -37.6‰) that was added during methanolysis

$$\delta^{13}C_{PLFA} = [(Cn + 1) \times \delta^{13}C_{Fame} - \delta^{13}C_{MeOH}]/Cn$$

where $\delta^{13}C_{PLFA}$ is the $\delta^{13}C$ of the fatty acid, C*n* is the number of carbons in the fatty acid, $\delta^{13}C_{Fame}$ is the $\delta^{13}C$ of the fatty acid methyl ester (FAME), and $\delta^{13}C_{MeOH}$ is the $\delta^{13}C$ of the methanol used for the methylating reaction (-37.6‰) to calculate the isotope ratios of the fatty acids (Abraham *et al*., 1998).

Total PLFAs were estimated based on peak areas in gas chromatograms with 16:0 as internal standard. From the difference in $\delta^{13}C$ values of the PLFA extracted from the $^{13}C$-labeled soils and those from the unlabeled soils (control), the amount of $^{13}C$ incorporation into each PLFA was calculated as ng $^{13}C$ incorporated per gram of soil or roots (Nold *et al*., 1999) as:

$$^{13}C \text{ incorporation} = (F_l - F_c)\,[PLFA]_l$$

where $F_l$ = fraction of $^{13}C$ in the $^{13}C$-labeled samples;

$F_c$ = fraction of $^{13}C$ in the unlabeled control sample; and

$[PLFA]_l$ = PLFA concentration in the $^{13}C$-labeled sample.

F was given by

$$F = {}^{13}C/({}^{13}C+{}^{12}C) = R/(R+1)$$

$$\text{where } R = (\delta^{13}C/1000+1)\,R_{VPDB}$$

The standard notation for expressing high-precision gas isotope ratio MS results in δ being defined as follows:

$$\delta\ (‰) = [(R_{FAME}/R_{VPDB}) - 1] \times 10^3$$

where $R_{FAME}$ and $R_{VPDB}$ are the $^{13}C/^{12}C$ isotope ratios corresponding respectively to the sample and to the international internal standard Pee Dee Belemnite, a South Carolinian carbonate rich in $^{13}C$ ($R_{VPDB} = 0.0112372 + 0.0000090$).

### 2.2.11 Statistical analyses

#### 2.2.11.1 Statistical analyses for chapter 3

Statistical analyses were performed using Microsoft Excel software. The two-sample t-test was used for comparing the means of relative abundances of (sum) T-RFs affiliated with type I and type II methanotrophs. Significant differences in $^{13}C$ incorporation into PLFAs obtained from rhizospheric soil and root samples and between groups of PLFAs representing type I and type II methanotrophs were also tested.

#### 2.2.11.2 Statistical analyses for chapter 4

All the statistical analyses were performed with R software package (Venables, Smith and the R Development Core Team, Version 2.6.2, 2008) in collaboration with Prof. Dr. Peter Frenzel, Max Planck Institute for Terrestrial Microbiology, Marburg, Germany. R is a free software environment and provides a wide variety of statistical and graphical techniques, and is highly extensible. In addition to R software, several other packages such as *locfit* (Clive Loader, 1999), *vegan* (Oksanen, 2005), *labdsv* (Roberts, 2007) are included which can be applied for different analyses as described below.

All the raw data from environmental ($CH_4$ and $NH_4^+$ concentration, pH and plant characteristic) and functional data ($CH_4$ oxidation rate) were analyzed by non-linear interpolation using R package *locfit* (Clive Loader, 1999). The interpolated data thus obtained were used for further statistic analyses.

T-RFs of different length were considered to be indicative of different *pmoA* operational taxonomic units (OTUs) present in a sample, and the relative peak heights were used as a

measure for the relative abundance of *pmoA*-OTUs. The effects of treatments, environmental factors and function on T-RFLP-profiles were explored by ordination techniques. An ordination based correspondence analysis (CA) was applied to analyze the treatment effects on community structure of methanotrophs from all six different growth periods. It uses the individual T-RFs obtained from each replicate microcosm and their relative abundance as input variables and calculates the position of all the T-RFs in a two-dimensional ordination. Samples with a similar community composition are placed closer together and samples with a dissimilar community composition are positioned further apart. Here in this study, CA ordination was used to plot the samples from different fertilizer treatments and fragments represented by different OTUs, using R package *vegan* (Oksanen, 2005).

To further identify OTUs (T-RFs) that differentiated methanotrophic communities by treatments, indicator species analysis was carried out as one of the visual techniques using R package *labdsv* (Roberts, 2007, http://ecology.msu.montana.edu/labdsv/R). The calculation of indicator value 'd' of a species was done as the product of the relative frequency and relative average abundance in clusters (Dufrene and Legendre, 1997). In this study, the indicator values were plotted as a range of different color codes in a heat-map for different nitrogen fertilizer treatments.

Canonical or constrained correspondence analysis (CCA) allows relating community variation to environmental variation. Environmental variables ($CH_4$ and $NH_4^+$ concentrations and pH along with plant characteristics i.e., tiller and leaves number) and functional data ($CH_4$ oxidation rates) were graphically correlated by CCA ordination using the above CA plots to evaluate relationships between overall community composition and environmental and functional variables. In this case, environmental variables were fitted with vectors while function data were fitted with contour lines. The fitted vectors are the arrows that tell about the direction (arrow points) of the most rapid change in the environmental variable. The length of the arrows indicates the correlation between ordination and environmental variable. Similarly, the contours are isolines, which are fitted as surfaces of environmental variables (functional factors) to ordinations. If the response is linear and vectors are appropriate, the fitted surface is a plane whose gradient is parallel to the arrow, and the fitted contours are equally spaced parallel lines perpendicular to the arrow (Oksanen, 2005).

Analysis of similarity (ANOSIM) was applied to test for significant differences in community composition among the treatments and also for significant effect of methane oxidation rates on community structure from three different treatments. This non-parametric procedure tests for significant differences between two or more groups, based on distance measures (Clarke, 1993). This method compares the ranks of distances between the groups and within the groups. The means of these two types of ranks are compared, and the resulting R test measures whether separation of community is found (R=1), or whether no separation occurs (R=0). The significance is based on permutation tests.

#### 2.2.11.3 Statistical analyses for chapter 5

One-way analysis of variance (ANOVA, $P<0.01$) was applied to test for the significant differences in methane oxidation potential rates for different treatments using SPSS software (version 11.5).

### 2.2.12 Media preparation for the isolation of methanotrophs

The protocol for preparation of nitrate mineral salts medium (NMS medium) (Bowmann, 2006) used is given below:

| Nitrate Mineral Salts Medium (NMS medium) | |
|---|---|
| $MgSO_4 \cdot 7H_2O$ | 1 g |
| $KNO_3$ | 1 g |
| $Na_2HPO_4 \cdot 12H_2O$ | 0.717 g |
| $KH_2PO_4$ | 0.272 g |
| $CaCl_2 \cdot 6H_2O$ | 0.2 g |
| Ferric ammonium EDTA | 5 mg |
| Trace element solution | 1 ml |

| Trace Element Solution | |
|---|---|
| Disodium EDTA | 0.5 g |
| $FeSO_4 \cdot 7H_2O$ | 0.2 g |
| $H_3BO_3$ | 0.03 g |
| $CoCl_2 \cdot 6H_2O$ | 0.02 g |
| $CuSO_4 \cdot 5H_2O$ | 0.03 g |
| $ZnSO_4 \cdot 7H_2O$ | 0.01 g |
| $MnCl_2 \cdot 4H_2O$ | 3 mg |
| $Na_2MoO_4 \cdot 2H_2O$ | 3 mg |
| $NiCl_2 \cdot 6H_2O$ | 2 mg |

In the first step, trace element solution was prepared by adding above components to distilled water and was brought to a volume of 1 litre and mixed thoroughly. In the second step, NMS medium was prepared by adding components to 1 litre of distilled water and thoroughly mixed. pH of the medium was adjusted to 6.8 using 0.1M KOH and then was distributed to culture tubes or serum bottles as specified. For solid media, agar (Difco, France) was added to a concentration of 1.5% (w/v) and boiled gently to dissolve the agar. Finally, the medium was autoclaved at 15 psi pressure (121 °C) for 15 min to sterilize.

# Chapter 3

## Activity and composition of methanotrophic bacterial communities in planted rice soil studied by flux measurements, analyses of *pmoA* gene and stable isotope probing of phospholipid fatty acids

## 3.1 Introduction

Methane is a trace gas in the earth's atmosphere with important global warming implications. Rice fields are an important source for atmospheric $CH_4$ contributing about 40 Tg $y^{-1}$ (Wang *et al*., 2004; Lelieveld *et al*., 1998). Methane, which is produced in the soil, enters the roots of the rice plants and is transported through the gas vascular system of the plants to the atmosphere and oxygen is transported from the atmosphere into the roots. Hence, rice roots are partially oxic and thus allow methanotrophic bacteria to be active in the rhizosphere (Conrad, 2004). Aerobic methanotrophs associated with the rhizosphere of rice plants oxidize $CH_4$ with molecular $O_2$ and use it as the sole source of carbon and energy. Thus aerobic methanotrophs play an important role in the global $CH_4$ budget by reducing $CH_4$ emissions from the rice ecosystems to the atmosphere (Groot *et al*., 2003). Therefore, a better knowledge of the methanotrophic community structure and its activity in paddy fields is important for the mechanistic understanding of $CH_4$ oxidation in soil.

Methanotrophs are classified as type I (belonging to the *Gammaproteobacteria*) and type II (belonging to the *Alphaproteobacteria*), based on several characteristics including phylogeny, guanine and cytosine content of their DNA, intracellular membrane arrangement, carbon assimilation pathways and phospholipid fatty acids (PLFAs) composition (Hanson and Hanson, 1996; Whittenbury *et al*., 1970). At present, there are 13 recognized genera of methanotrophs (Hanson and Hanson, 1996; Bowman *et al*., 1997; Bodrossy *et al*., 1997; Dedysh *et al*., 2000, 2002; Wise *et al*., 2001; Heyer *et al*., 2005; Tsubota *et al*., 2005) consisting of both type I and type II methanotophs. Both type I and type II methanotrophs have been detected in rice field soil and on rice roots using cultivation techniques (Gilbert and Frenzel, 1998) as well as cultivation-independent techniques that include PLFA analyses, fingerprinting techniques (T-RFLP, DGGE), and cloning and sequencing of 16S rRNA genes and functional genes (*pmoA*, *mmoX*, *mxaF*), (Bosse and Frenzel, 1997; Henckel *et al*., 1999, 2000; Bodelier *et al*., 2000; Eller and Frenzel, 2001; Horz *et al*., 2001; Macalady *et al*., 2002; Mohanty *et al*., 2006).

Methanotrophs are known to be sensitive to variation in $CH_4$ and $O_2$ concentrations (Bender and Conrad, 1995; Henckel *et al*., 2000), and it has been suggested that the amount of available $CH_4$ influences the competition between type I and type II methanotrophs. Type I methanotrophs seem to outcompete type II species under low $CH_4$

and high $O_2$ conditions, whereas type II species tend to dominate under the opposite conditions ( Graham *et al*., 1993; Amaral and Knowles, 1995). Rice plant ecosystems may exhibit different niches for methanotrophs as characterized by spatiotemporal variation of $CH_4$ and $O_2$ in the rice rhizosphere (Gilbert and Frenzel, 1995). Oxygen availability increases with plant age because of higher root-oxygen-releasing activity and higher root densities. Furthermore, methane availability increases with distance from the rice root, while oxygen availability decreases with distance (Gilbert and Frenzel., 1998). Thus, the rhizosphere appears to be a very heterogeneous habitat for methanotrophs. As a consequence, methanotrophic community structure in rice soil may shift with changing conditions and over the season (Eller *et al*., 2005; Eller and Frenzel, 2001). Macalady and colleagues (2002) quantified the temporal and spatial dynamics of methanotroph populations in a California rice field using PLFA biomarker analyses, evaluating the relative importance of type I and type II methanotrophs with depth and in relation to rice roots. However, the temporal change of activity and active methanotrophic populations in the rice rhizosphere has not yet been studied.

Currently, phospholipid fatty acids stable isotope probing (PLFA-SIP) has become a popular approach for linking microbial community structure with its activity in the environment. In this approach, active soil microbial populations utilizing a $^{13}C$-labeled substrate will readily incorporate $^{13}C$ into membrane lipid components such as phospholipid fatty acids (PLFAs). The presence of particular PLFAs is a distinct characteristic of methanotrophic bacteria allowing differentiation between type I (16 carbon fatty acids: 16:0, 16:1) and type II methanotrophs (monounsaturated 18 carbon fatty acids: 18:1ω9c, 18:1ω8) and also from all other organisms (Bowmann *et al*., 1993). PLFA-SIP methodology has been successfully applied in several soils and sediments to identify active $CH_4$-oxidizing bacteria (Boschker *et al*., 1998; Bull *et al*., 2000; Knief *et al*., 2003b; Crossman *et al*., 2006). Similarly, Bodelier *et al*. (2000) carried out $^{14}C$-labeled PLFA slurry incubation study to characterize active methanotrophs from soil samples collected from rhizosphere compartment of rice microcosms with different fertilizers treatments. However, the temporal change in the active methanotrophic community structure in the rice rhizosphere has not yet been studied using PLFA-SIP methodology. Furthermore, labeled $CH_4$ has not yet been applied to the rice roots under close to *in situ* conditions. Therefore, in this part of the study, I conducted labeling experiments where $^{13}CH_4$ was directly added to the rhizosphere of planted and fertilized

rice microcosms mimicking *in situ* conditions, and the total and active methanotrophic community was investigated with respect to time by analyzing *pmoA* gene analyses and using PLFA-SIP, respectively.

### 3.2 Objectives

- To study the spatial and temporal change on the composition of methanotrophic community in the rice rhizosphere.
- To study the temporal change of activity and active methanotrophic population in the rice rhizosphere.

## 3.3 Methods

### 3.3.1 Experimental setup

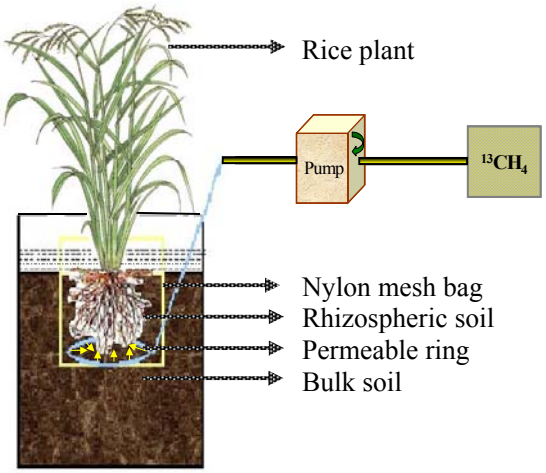



**Fig. 3.1:** Rice microcosm experimental set up.

### 3.3.2 Microcosm experiment

#### 3.3.2.1 Planted rice microcosms

Soil was taken from drained paddy fields of the Italian Rice Research Institute in Vercelli, Italy, in 1998 and was air-dried and stored at room temperature. The soil characteristics are described in Table 3.1. Immediately prior to its use, the soil was ground with a mechanical grinder and passed through a 2 mm sieve. Then the soil slurry was prepared with 1.8 kg soil, 940 ml demineralized water, and 60 ml of fertilizer solution [2.3 g nitrogen (N) as urea, 0.87 g phosphorous (P) as $KH_2PO_4$, and 1.85 g potassium (K) as KCl per liter], and finally filled into each microcosm pot with a volume of 2.5 L (height 16 cm, diameter 17 cm). The fertilizer composition corresponds per ha to 160 kg N as urea or ammonium sulphate, 140 kg $P_2O_5$ and 155 kg $K_2O$ as practically applied in rice fields. In the center of each pot, a self-made nylon bag (25 µm mesh; 6 cm length and 9 cm radius) was placed through which water and nutrients could pass freely while roots were not able to penetrate, isolating the soil inside the bag as rhizospheric soil from the bulk soil outside the bag (Fig. 3.1 and appendix). A ring of permeable tubing (7.5 cm diameter) was placed into the lower part of the nylon bag and was connected to a

reservoir foil grab bag (Analyt-MTC, Germany) containing demineralized water saturated with $^{13}C$-labeled $CH_4$ (99 atom-% $^{13}C$, Isotec, USA) and circulated directly to the microcosms with the help of peristaltic pumps (Fig. 3.1, appendix).

**Table 3.1:** Soil characteristics of rice field soil collected in year 1998.

| Variable | Portion |
|---|---|
| C (%) | 2.5 |
| N (%) | 0.15 |
| C/N | 17 |
| Texture (%) (clay/silt/sand) | 12/42/46 |

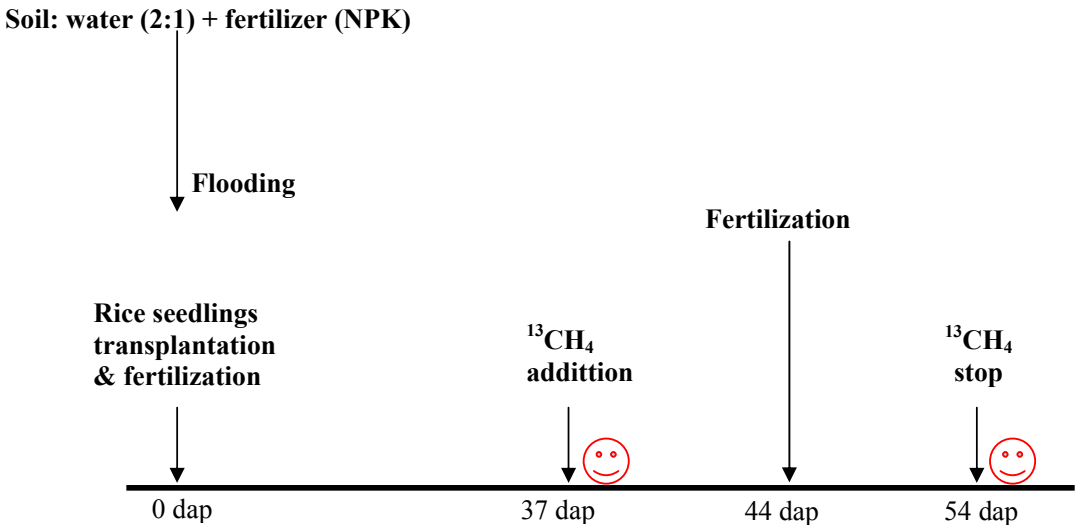



**Fig 3.2:** Schematic diagram of the experiment. dap indicates days after transplantation and ☺ indicates the sampling point for rhizospheric soil and roots samples.

In total, 18 microcosms were prepared among which 9 were for $^{13}C$-$CH_4$ labeling and another 9 were used as control without labeling ($^{12}C$-$CH_4$-labeling was not done), and were flooded with demineralized water giving a water depth of 5 cm above the soil surface and were incubated in the greenhouse with a relative humidity of 70%, 12 h photoperiod, and 30/22 ºC day/night temperature. After five days of flooding, one 14-day old rice seedling (*Oryza sativa* var. KORAL type japonica) germinated on moist filter paper at room temperature was transplanted into the center of the nylon bag in each pot, and 20 ml of fertilizer solution (same as above) was added. The day of transplantation was taken as day zero and the following incubation experiment was then conducted from May 29, 2005 till July 22, 2005 for a total of 55 days (Fig 3.2) under flooded conditions.

The $^{13}C$-labeled $CH_4$ solution was continuously circulated in the soil between day 37 and 54 after transplantation. On day 44, 20 ml of fertilizer solution was added a third time to the microcosms (Fig 3.2). In this study, fertilizer solution was added as a mixture of ammonium, phosphorous and potassium sources in order to fulfill the nutrient requirements for rice plant growth. Water lost due to evapotranspiration was daily replaced by addition of demineralized water to maintain a 5 cm water depth.

#### 3.3.2.2 Measurement of $CH_4$ flux

Rates of $CH_4$ emission and $CH_4$ oxidation were measured as described in chapter 2.2.1. Stable isotope analysis of $^{13}C/^{12}C$ in gas samples (methane flux) was performed using a gas chromatograph combustion isotope ratio mass spectrometer (GCC-IRMS) system as described in 2.2.3.1.

#### 3.3.2.3 Soil pore water

Pore water samples were collected weekly into Venoject blood collecting tubes from the rhizosphere (3 cm depth from the soil surface) and bulk (10 cm depth from the soil surface) regions of rice microcosms as described in 2.2.2.

#### 3.3.2.4 Analytical methods

The gas samples from the pore water were analyzed for $CH_4$ using gas chromatography equipped with FID detector as described in section 2.2.2. Stable isotope analysis of $^{13}C/^{12}C$ in gas samples (dissolved $CH_4$ in frozen pore water) was performed using a gas chromatograph combustion isotope ratio mass spectrometer (GC-C-IRMS) system as described in 2.2.3.1.

pH was measured using a pH meter.

### 3.3.3 Collection of soil and root samples

Rhizospheric soil and root samples were collected 44 (8 days after the beginning of $^{13}C$-labeling) and 55 (18 days after the beginning of $^{13}C$-labeling) days after transplantation from the $^{13}C$-labeled and control (unlabeled) microcosms in triplicate as described in chapter 2.2.4. The samples were designated as RS-8 and RS-18 for the rhizospheric soil,

and RT-8 and RT-18 for the roots, respectively where the numbers 8 and 18 stood for 8 and 18 days of incubation period after the beginning of $^{13}C$-labeling. The roots were washed in deionized sterile water. The soil and root samples were stored at –80 °C for molecular and PLFA-SIP analyses.

### 3.3.4 DNA extraction, PCR, cloning, and sequencing

Total DNA from the soil and root samples was extracted using total nucleic acids extraction protocol described in chapter 2.2.7.1. The frozen roots were pulverized with a mortar and pestle after freezing in liquid $N_2$. After extraction, DNA was checked for quality and quantity by electrophoresis in agarose gels stained with ethidium bromide, followed by gel documentation and then DNA purification was carried out using the Wizard® DNA Clean-up System (Promega, Germany).

PCR amplification of the *pmoA* gene was done using primers A189f and mb661 (Costello and Lidstrom, 1999) as described in chapter 2.2.7.3. Aliquots of the amplicons (5 µl) were checked by electrophoresis on 1% agarose gel including positive and negative controls.

Two clone libraries each of *pmoA* gene from DNA amplicons retrieved from rhizospheric soil and root samples were constructed using the pGEM-T Easy cloning kit (Promega, Germany). A total of 101 clones from rhizospheric soil samples (44 and 57 clones from RS-8 and RS-18 days samples, respectively) and of 88 clones from root samples (37 and 51 clones from RT-8 and RT-18 days samples, respectively) were randomly selected for comparative sequence analysis and proceeded as described in chapter 2.2.7.5.

### 3.3.5 Phylogenetic analysis

Refer to chapter 2.2.8.

### 3.3.6 T-RFLP analysis

Analysis of terminal restriction fragment length polymorphism (T-RFLP) was performed for each DNA extract in triplicates as described in chapter 2.2.9. The normalization procedure of each TRF profile was done as described in chapters 2.2.9.1 and followed

with calculation of relative abundance as described in chapters 2.2.9.2 and for statistical analysis.

### 3.3.7 Phospholipid fatty acids-stable isotope probing (PLFA-SIP)

Refer to chapter 2.2.10.

### 3.3.8 Statistical analysis

Refer to chapter 2.2.11.1.

## 3.4 Results

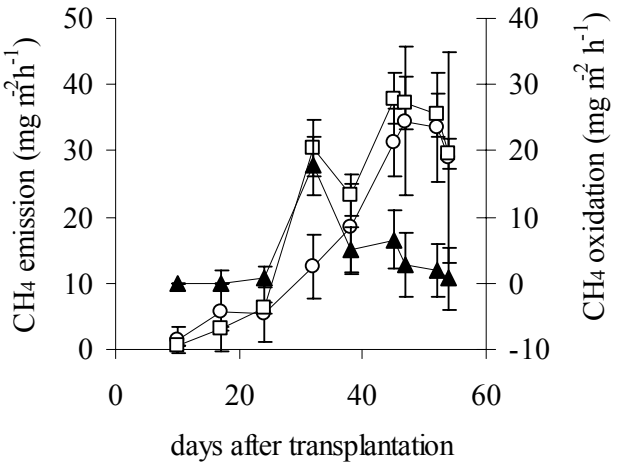
A microcosm experiment with rice plant was conducted for 55 days in the greenhouse. The rhizosphere of rice microcosms was directly supplied with $^{13}C$-labeled $CH_4$ by circulating $^{13}C$-$CH_4$-saturated water through permeable tubing buried in the soil (Fig. 3.1). The total community of methanotrophs and the incorporation of $^{13}C$ in PLFA of methanotrophs were analysed for two different incubation periods. The methane emission and methane oxidation rates were measured weekly throughout the period.

### 3.4.1 Rates of $CH_4$ emission and oxidation

The rates of $CH_4$ emission and oxidation were similar in control and $^{13}C$-labeled microcosms during the experimental period of 55 days after transplantation of rice seedlings (Fig. 3.3a and 3.3b). The $CH_4$ emission rates (in absence of inhibitor) gradually increased from the beginning and reached an average value of 31 and 28 mg $CH_4$ $m^{-2}$ $h^{-1}$ on day 45 for the control (Fig. 3.3a) and the $^{13}C$-labeled microcosms (Fig. 3.3b), respectively.

Methane oxidation rates were calculated as the difference between $CH_4$ emission rates in the presence and absence of diflouromethane ($CH_2F_2$), a specific inhibitor of $CH_4$ oxidation (Miller *et al*., 1998). Methane oxidation started on day 24 and reached the maximum on day 32 (Fig. 3.3a, 3b), when nearly 60% of the anaerobically produced methane was oxidized prior to its emission to the atmosphere (Fig. 3.4). However, this percentage value decreased rapidly afterwards. After the addition of $^{13}C$-labeled $CH_4$, the $\delta^{13}C$ values of the emitted $CH_4$ substantially increased in comparison to the background $\delta^{13}C$-$CH_4$ values emitted from the control (unlabeled) microcosms. The maximum $\delta^{13}C$ was 1610‰ (2.85 atom-%) on day 38, decreased to 525‰ (1.68 atom-%) on day 46 and increased slowly again and reached to 1588‰ (2.82 atom-%) on day 54 (Fig. 3.5).

a)




b)

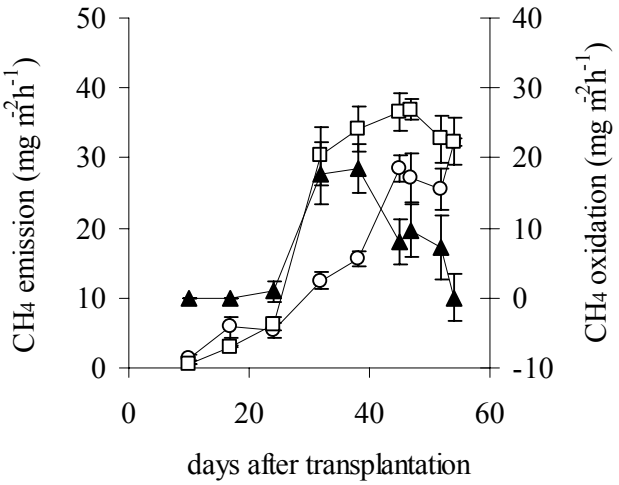



**Fig. 3.3:** Methane emission and methane oxidation rate in (a) control (unlabeled) and (b) labeled microcosms; $CH_4$ emission in the presence of inhibitor (□), in the absence of inhibitor (○), and $CH_4$ oxidation rate (▲); mean ± SD (n=3).

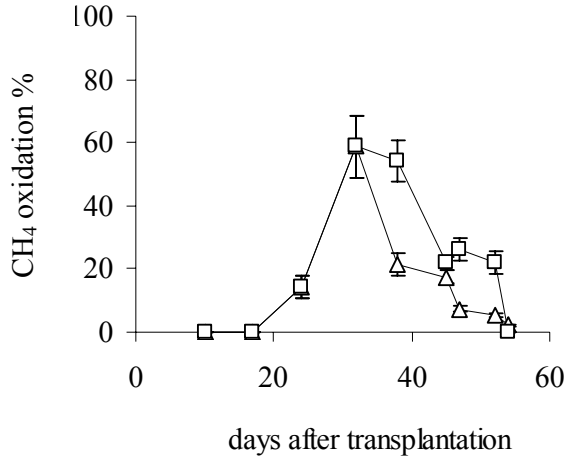



**Fig. 3.4:** Percent of $CH_4$ flux attenuated by $CH_4$ oxidation in the control (Δ) and the $^{13}C$-labeled microcosms (□); mean ± SD (n=3).

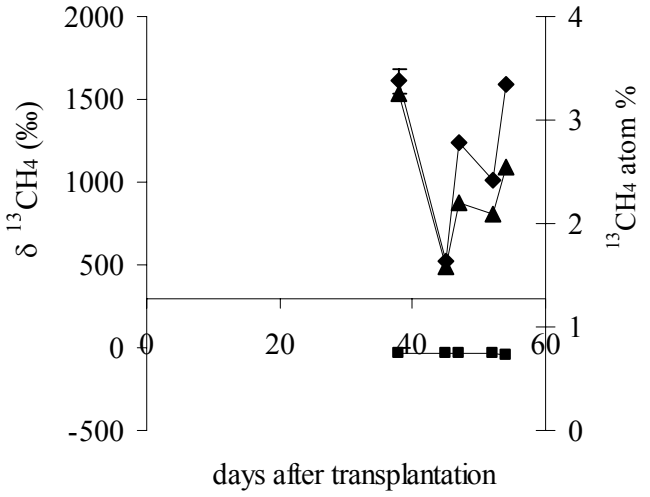


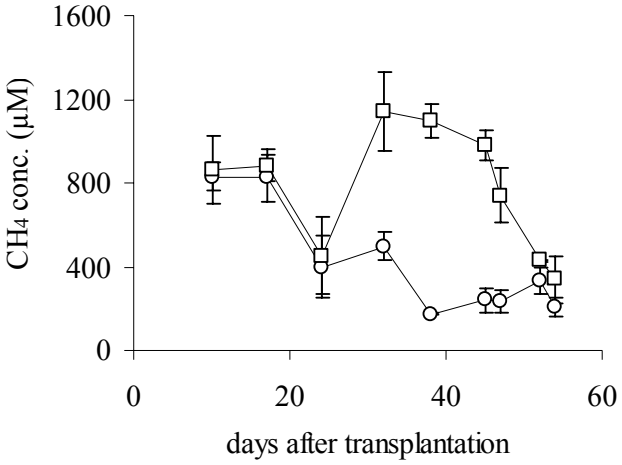

**Fig. 3.5:** $\delta^{13}C$ values of emitted $CH_4$ in the control (■) and $^{13}C$-labeled microcosms (◆); mean ± SD (n=3) and $^{13}C$ $CH_4$atom-% values in $^{13}C$-labeled microcosms (▲), mean ± SD (n=3).

### 3.4.2 $CH_4$ in the pore water

Concentrations of $CH_4$ in pore water samples were similar in both the control and the $^{13}C$-labeled microcosms (Fig. 3.6a, 6b). On average, $CH_4$ concentrations were lower in rhizospheric region (at 3 cm depth from the soil surface) than in bulk region (at 10 cm

depth from the soil surface). Until 17 days after transplantation, $CH_4$ concentrations were 800-900 μM at both regions in the control and the $^{13}C$-labeled microcosms. After 17 days, $CH_4$ in rhizospheric soil rapidly decreased to 400-500 μM and then gradually decreased to 200 μM until the end of the experiment. In the bulk soil, on the other hand, $CH_4$ concentrations increased up to 1100-1300 μM and later slowly decreased to about 300 μM after 52 days of transplantation.

a)




b)

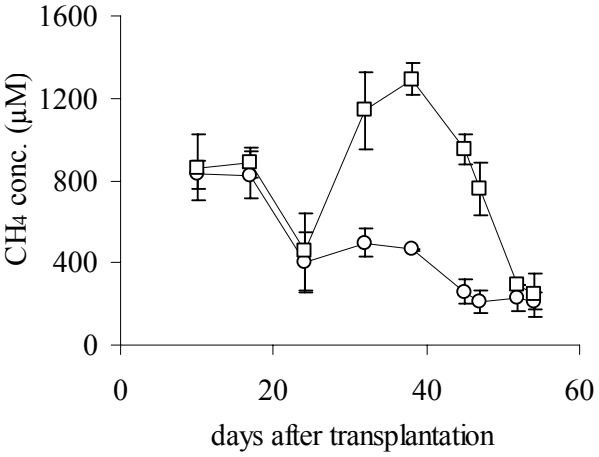



**Fig. 3.6:** Temporal variation in $CH_4$ concentrations obtained in a) control and b) labeled microcosms: in rhizospheric region (○) and in bulk region (□); mean ± SD (n=3).

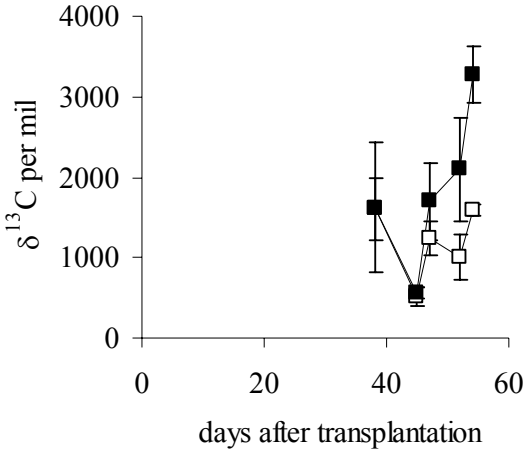



**Fig. 3.7:** $\delta^{13}C$ values of $CH_4$ in emitted flux (□) and in the pore water (■); mean ± SD (n=3).

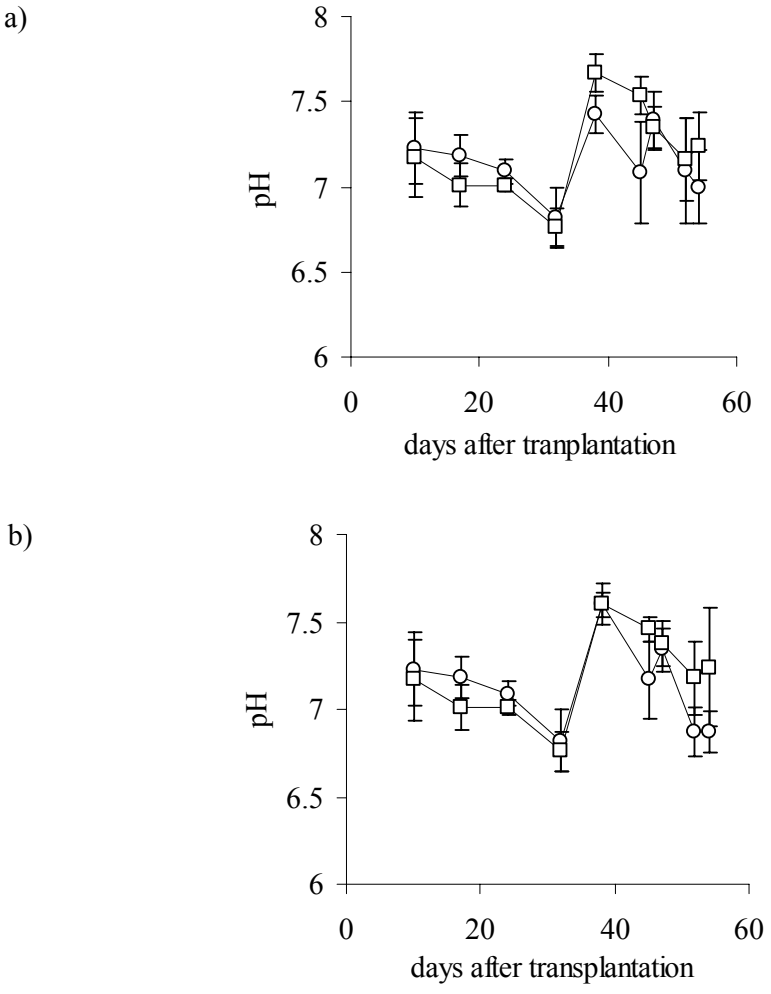



**Fig. 3.8:** Pore water pH in a) control and b) labeled microcosms: in rhizospheric region (○) and in bulk region (□); mean ± SD (n=3).

The $\delta^{13}C$ values of $CH_4$ in pore water were similar as those in the emitted $CH_4$. The initial $\delta^{13}C$ was 1624‰ (2.86 atom-%) on day 38, i.e., immediately after the beginning of the circulation of the $^{13}C$-labeled $CH_4$ solution in the soil, and decreased to 525‰ (1.68 atom-%) on day 46, and again gradually increased to 3272‰ (4.58 atom-%) on day 54 (Fig 3.7).

### 3.4.3 pH in the pore water

The pH of the pore water in the control and labeled microcosms varied between pH 6.8 and 7.6 at rhizospheric and bulk regions (Fig. 3.8).

### 3.4.4 T-RFLP analysis of methanotrophic community

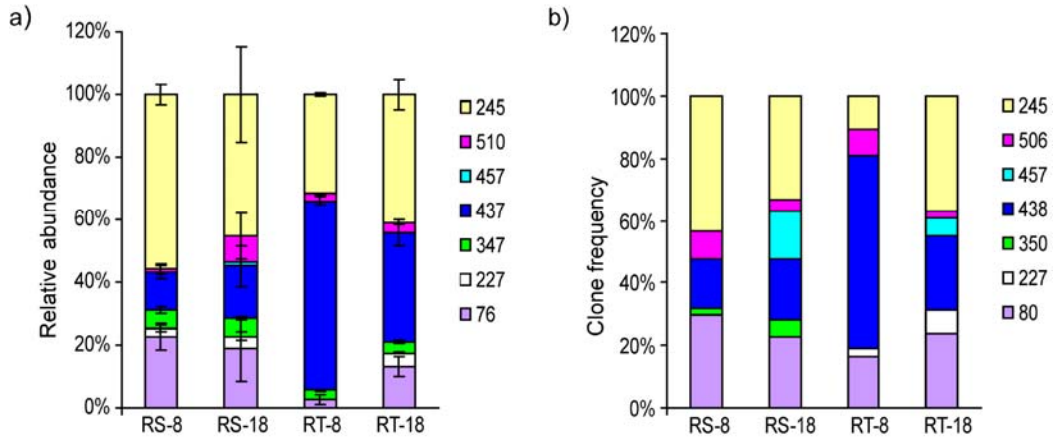



**Fig. 3.9:** Comparison of *pmoA* based (a) T-RFLP profiles (mean ± SD; n=3) and (b) clone frequencies obtained from rhizospheric soil and root samples. A total of 45, 56, 39, and 56 randomly selected clones were analysed from samples RS-8, RS-18, RT-8, and RT-18, respectively (RS = rhizospheric soil; RT = roots; numbers indicate time after beginning of $^{13}C$-labeling).

The methanotrophic community was investigated by T-RFLP analysis targeting the *pmoA* gene in DNA extracts from rhizospheric soil (RS) and root samples (RT) collected at 44 and 55 days after transplantation, i.e., 8 (RS-8, RT-8) and 18 (RS-18, RT-18) days after the beginning of $^{13}C$-labeling (fig 3.9a). Since the physiological data were similar in both control and labeled rice microcosms, molecular analyses were performed in labeled rice microcosms only. T-RFLP analysis produced highly reproducible patterns with T-RFs of 76, 227, 245, 347, 437, 457 and 510 bp lengths in all samples. All of these T-RFs were

assigned by my own clone analysis to the following methanotrophic genera, some of which, however, exhibited T-RFs that were slightly different from those determined by in-silico analysis: *Methylococcus/Methylocaldum* (80 vs. 76 bp); *Methylocystis/Methylosinus* (245 bp); *Methylomicrobium album* (350 vs. 347 bp and 457 bp); *Methylomonas* (438 vs. 437 bp), and *Methylobacter* (506 vs. 510 bp) (Fig 3.9a, 9b).

The comparison of T-RFLP community profiles obtained from the rhizospheric soil and root samples showed similar T-RF patterns but different relative abundances of the major T-RFs (Fig 3.9a). Furthermore, two different sampling points were also conferred to the different relative abundances of the T-RFs in both rhizospheric soil as well as in root samples (Fig. 3.9a). The relative abundance of T-RFs belonging to type I and type II methanotrophs in rhizospheric soil and root samples collected at different time points are summarized in Table 3.2. The T-RFs affiliated with type I methanotrophs were significantly ($P$<0.05) more abundant than those affiliated with type II methanotrophs in all samples with exception of RS-8, and in addition, they were more abundant on the roots than in the rhizospheric soil (Table 3.2). The T-RFs affiliated to type II methanotrophs decreased with incubation time in the rhizospheric soil but increased on the roots.

### 3.4.5 Cloning and sequence analysis of *pmoA* gene

Sequences of *pmoA* genes retrieved from rhizospheric soil (Fig. 3.10) and root samples (Fig. 3.11) were analysed by constructing phylogenetic trees. Phylogenetic analysis of *pmoA*-derived amino acid sequences revealed the presence of both type I methanotrophs (genera *Methylomonas*, *Methylobacter*, *Methylococcus*, *Methylocaldum* and *Methylomicrobium*) and type II methanotrophs (genera *Methylocystis* and *Methylosinus*) in rhizospheric soil as well as on root samples. In rhizospheric soil samples, 63 among 101 clones (63%) were affiliated with type I methanotrophs (Fig. 3.10) and on root samples, 65 among 88 clones (74%) were affiliated with type I methanotrophs (Fig. 3.11) while the remainder, i.e. 37% and 28%, respectively, were affiliated with type II methanotrophs being less abundant than those of type I methanotrophs. The *pmoA*-sequences affiliated to *Methylomonas* sp. were dominant among type I methanotrophs in all samples. The number of *pmoA*-sequences affiliated to *Methylocystis* sp. was 4-fold higher in RT-18 samples compared to those of RT-8, indicating an increase of clone

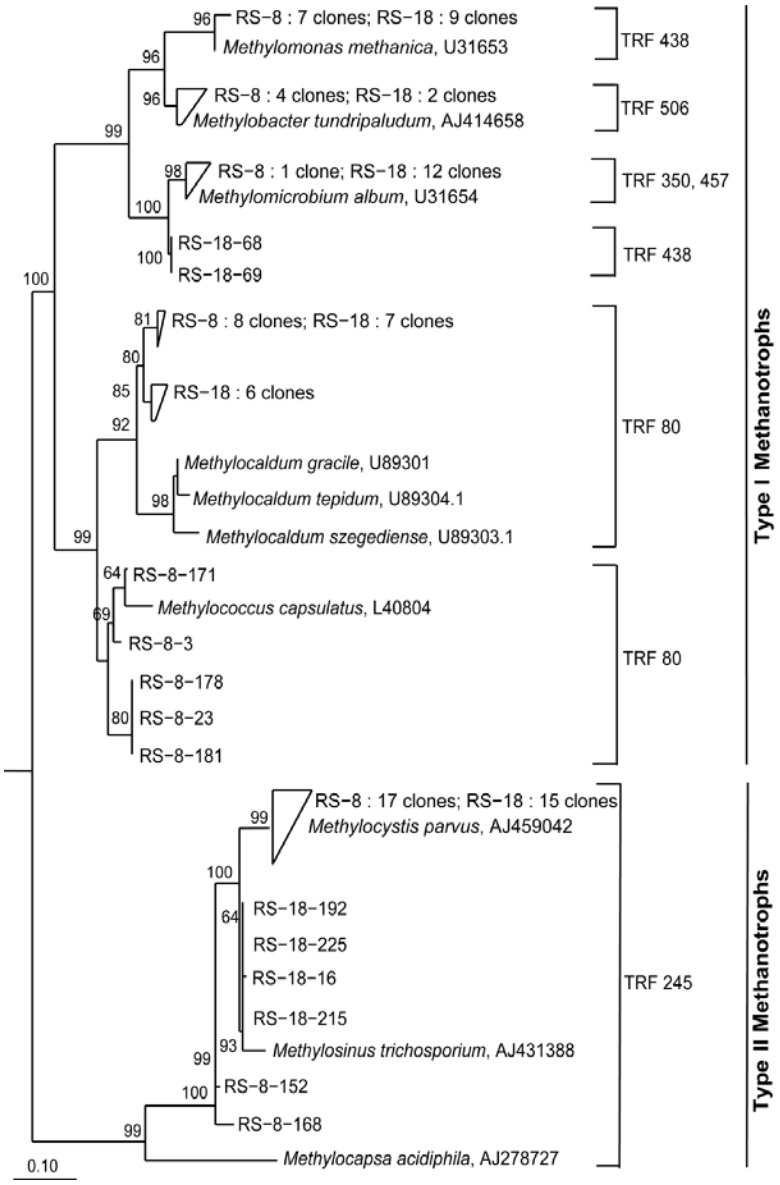



**Fig. 3.10:** Maximum likelihood tree showing the phylogenetic analysis of the derived amino acid sequences encoded by *pmoA* genes from rhizospheric soil samples. The numbers at the branch points are tree puzzle support values. Only values >60 are shown. Theoretical T-RF lengths using MspI are shown next to the sequences. The scale bar represents 10% sequence divergence.

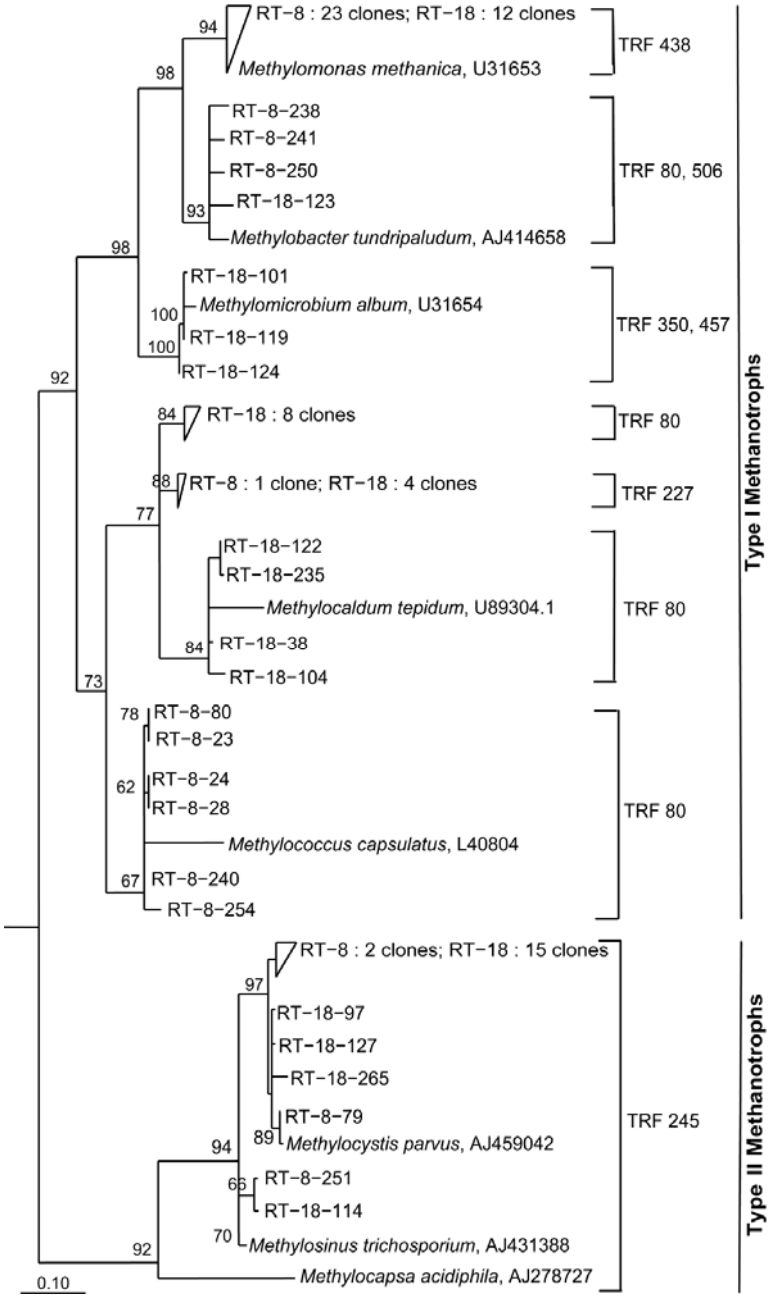



**Fig. 3.11:** Maximum likelihood tree showing the phylogenetic analysis of the derived amino acid sequences encoded by *pmoA* genes from root samples. The numbers at the branch points are tree puzzle support values. Only values >60 are shown. Theoretical T-RF lengths using MspI are shown next to the sequences. The scale bar represents 10% sequence divergence.

frequency over time (Fig. 3.11). Some clusters of type I methanotroph *pmoA* sequences were retrieved from rhizospheric soil (sequences corresponding to T-RF size of 80) and root (sequences corresponding to T-RF size of 80 and 227) samples, which could not be assigned to any sequence types that are deposited in public domain database (Fig. 3.10 and 3.11). Also sequences related to *Methylomicrobium album* were retrieved that exhibited a T-RF of 457 bp (Fig. 3.10 and 3.11) along with 350 bp that was reported previously (Horz *et al*., 2001).

T-RFLP analysis of individual clones mostly confirmed the assignment of the different T-RFs to the different genera of methanotrophs. The relative clone frequency of *pmoA*-sequences with the respective affiliated T-RFs, which were retrieved from rhizospheric soil and root samples (Fig. 3.9b), were similar to the relative abundance of the same T-RFs detected in the DNA extracts (Fig. 3.9a).

### 3.4.6 Incorporation of $^{13}C$ into PLFA of methanotrophs

To gain more information on the metabolically active methanotrophic community in rhizospheric soil and on root samples I applied a $^{13}C$-$CH_4$ labeling approach. Figure 3.12 shows the total abundance of phospholipid fatty acids (Fig. 3.12a) and the $^{13}C$ incorporation into phospholipid fatty acids (Fig. 3.12b) extracted from the rhizospheric soil and the roots incubated with $^{13}C$-$CH_4$ for 8 days (RS-8 and RT-8) and 18 days (RS-18 and RT-18), i.e., 44 and 54 days after transplantation. Total PLFA concentrations cannot be compared between rhizospheric soil and root samples, since they are expressed per gram dry soil versus gram dry root, respectively. However, total PLFA abundance increased over time in rhizospheric soil samples (RS-8 and RS-18), whereas it decreased on root samples (Fig. 3.12a). Incorporation of $^{13}C$ into PLFA, on the other hand increased over time in both cases (Fig. 3.12b), indicating the increased activity of methanotrophs.

On average, 16:1ω7, 16:1ω6, 16:0, 18:1ω7, 18:1ω9 and 18:0 were the dominant PLFAs labeled with $^{13}C$ in both rhizospheric soil and root samples (Fig. 3.12b). The PLFAs representing type I methanotrophs (16:1ω7, 16:1ω6 and 16:0) were significantly ($P<0.05$) more labeled with $^{13}C$ in all samples than those representing type II methanotrophs (18:1ω7, 18:1ω9 and 18:0). The incorporation of $^{13}C$ increased in most of the PLFAs

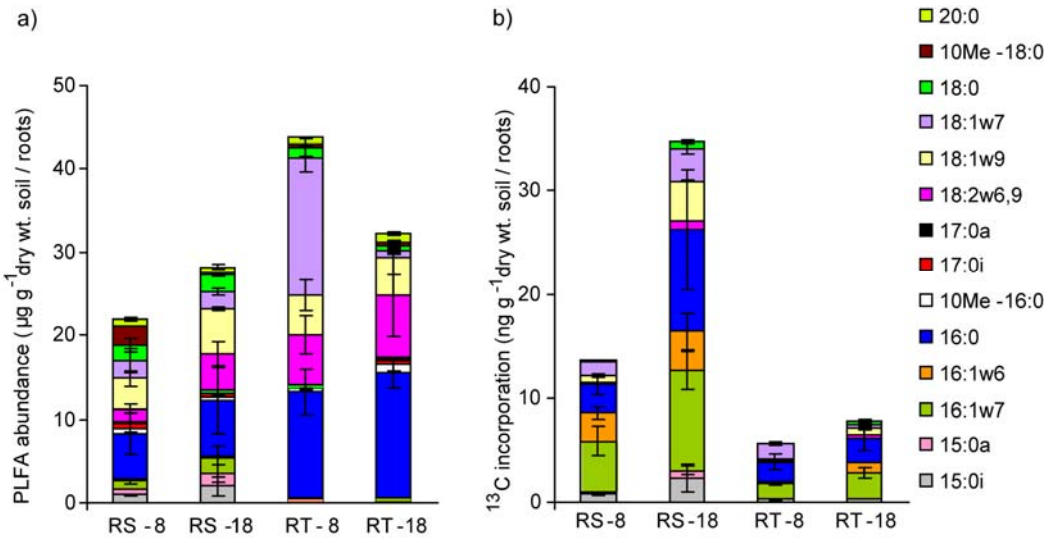



**Fig. 3.12:** PLFA abundance given as (a) total PLFA), and (b) $^{13}C$ incorporation into PLFA per gram of dry soil or root (RS = rhizospheric soil; RT = roots; numbers indicate time after beginning of $^{13}C$-labeling); mean ± SD (n=3).

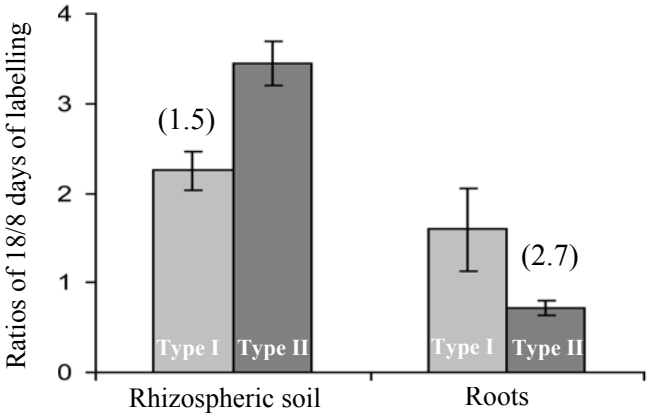



**Fig. 3.13:** Ratio of $^{13}C$ incorporation into PLFA (sum of type I and type II methanotrophs based on Fig 3.12; Table 3.2) between 8 and 18 days of labeling; mean ± SD (n=3); ($P$<0.05).

after 18 days compared to 8 days of incubation. However, it decreased in some PLFAs (Fig. 3.12b), for example, in 18:1ω7 PLFA on the root samples. The percent distribution of phospholipid fatty acids present in type I and type II methanotrophs in terms of $^{13}C$ incorporation per gram soil or gram root is summarized in Table 3.2. Furthermore, the ratio of $^{13}C$ incorporation between 8 and 18 days of $^{13}C$-labeling (Fig. 3.13) showed that after 18 days of incubation, type I methanotrophs exhibited a 2.7-fold higher $^{13}C$ incorporation than type II methanotrophs on the roots, whereas type II methanotrophs

exhibited a 1.5-fold higher $^{13}C$ incorporation than type I methanotrophs in the rhizospheric soil. The incubation experiments with $^{13}C$-$CH_4$ labeling furthermore showed that 15:0i and 18:2ω6,9 also became labeled with $^{13}C$. In samples retrieved from the RS-18, PLFA of 15:0i, a biomarker of Gram-positive bacteria (O´Leary and Wilkinson, 1988), represented 4% of total $^{13}C$ incorporation in PLFA. Similarly, PLFA of 18:2ω6,9, a biomarker of Eukaryotes (Frostegard and Baath, 1996), represented 2% and 6% of total $^{13}C$ incorporation in rhizospheric soil and root samples, respectively.

**Table 3.2.** Relative abundance of T-RFs and $^{13}C$ incorporation into phospholipid fatty acids (PLFA), characteristic for type I and II methanotrophs in rhizospheric soil (RS) and on rice roots (RT) sampled after 8 and 18 days of $^{13}C$-labeling, i.e., 44 and 54 days after transplantation.

| **Sample** | **RS-8** | **RS-18** | **RT-8** | **RT-18** |
|---|---|---|---|---|
| | | Relative abundance of T-RFs (%) | | |
| Type I † | 47[a] | 55[c] | 68[e] | 59[g] |
| Type II‡ | 53[b] | 45[d,m] | 32[f] | 41[h,m] |
| | | $^{13}C$ incorporation into PLFA (%) | | |
| Type I § | 73[a] | 62[c] | 58[e] | 71[g] |
| Type II ¶ | 16[b] | 20[d] | 28[f] | 15[h] |

†Sum of T-RFs 76, 227, 347, 437 and 457 bp, which were affiliated to type I methanotrophs in phylogenetic assignment.
‡Terminal restriction fragments (T-RFs) 245 bp, which were affiliated to type II methanotrophs in phylogenetic assignment.
§Sum of percent values of 16:0, 16:1w6 and 16:1w7 shown in Fig. 3.12b.
¶Sum of percent values of 18:0, 18:1w7 and 18:1w9 shown in Fig. 3.12b.

Numbers with different letters were statistically different ($P$<0.05).

## 3.5 Discussion

In this study, I combined both physiological and biomolecular analyses to elucidate the role of methanotrophs in the rhizospheric soil and on the roots of rice plants. To characterize the physiological state of methanotrophs, I calculated the rates of $CH_4$ oxidation over the incubation period. As biomolecular tools, I used T-RFLP and sequence analysis of the *pmoA* gene to assess the structure of the resident methanotroph community, and PLFA-SIP to determine the extent to which type I and type II methanotrophs actively assimilated $^{13}C$-$CH_4$.

Rates of $CH_4$ oxidation reached a maximum 32 days after transplantation. During this period, the overall $CH_4$ concentrations in the pore water had decreased. In addition, $CH_4$ concentrations were lower in the rhizospheric soil samples than in those of the bulk soil samples indicating increased methanotrophic activity in the rhizosphere and/or increased $CH_4$ loss by ventilation through the rice plant (Conrad and Klose, 2005; Gilbert and Frenzel, 1998). Rates of $CH_4$ oxidation decreased after reaching a maximum with 60% of the produced $CH_4$ being oxidized on day 32 following transplantation. Similar results have been obtained previously (Bodelier *et al*., 2000; Xu *et al*., 2004), demonstrating that $CH_4$ oxidation in rice fields is a dynamic process that seems to be regulated by various factors, including the age of the rice plant and nutrient availability for the microorganisms and/or plants. In particular, the decrease of $CH_4$ oxidation activity with the progress of the season has been observed previously (Dan *et al*., 2001; Eller and Frenzel, 2001; Krüger and Frenzel, 2003). Methanotrophic activity in the rice rhizosphere can be limited by available nitrogen (Bodelier *et al*., 2000; Dan *et al*., 2001; Krüger and Frenzel, 2003). Indeed, I found that a second fertilisation stimulated $CH_4$ oxidation albeit only briefly, similarly to the previous observations (Dan *et al*., 2001; Krüger and Frenzel, 2003). In order to better understand the dynamics of $CH_4$ oxidation in the context of microbial community, the methanotrophic community was analysed during this phase.

The resident populations of methanotrophs in the rhizospheric soil and on the roots were determined by targeting the *pmoA* gene, a functional gene marker for methanotrophs. Sequence analysis of several clone libraries showed the presence of both type I and type

II methanotrophs including the genera *Methylomonas*, *Methylobacter*, *Methylomicrobium*, *Methylococcus, Methylomicrobium, Methylocystis* and *Methylosinus* in both rhizospheric soil and on the roots. Such a diversity has been found previously in rice field ecosystems from Vercelli, Italy (Eller *et al*., 2005; Henckel *et al*., 2001; Horz *et al*., 2001) and elsewhere (Hoffmann *et al*., 2002; Jia *et al*., 2007). Most of the detected methanotrophic genera exhibited the characteristic size of the T-RFs reported by Horz *et al*. (2001). However, some of the T-RFs sizes observed in this study exhibited 2-4 base pairs difference to the theoretical T-RF size determined in-silico. A certain amount of variation between observed and predicted T-RF length remains that could be explained due to the application of different sequencing machines, dye labels, or fluctuations in laboratory temperature (Kaplan and Kitts, 2003), and even sometimes such variations appear to be sequence dependent (Kitts, 2001). Notable is the detection of a few sequences clustering with *Methylomicrobium* sp. and having a T-RF of 457 bp size, which has not been observed before. Furthermore, a few sequences were detected that clustered within the type I methanotrophs and have a T-RF of 227 bp size. Horz and colleagues (2001) had also detected sequences having a T-RF of 227 bp size from rice root samples but could not assign them to either *pmoA* or *amoA* sequences. This study showed that this T-RF belongs to type I methanotrophs because I used *pmoA* specific primers (A189f/mb661r) that do not amplify *amoA* sequences (Bourne *et al*., 2001; Costello and Lidstrom, 1999).

Due to relatively clear assignment of different T-RFs to the different methanotrophic genera, I was able to calculate the relative abundance of the different methanotrophic populations by using T-RFLP analysis of the *pmoA* genes. Additionally, I was able to quantify the number of *pmoA* sequences which could be unambiguously assigned to respective T-RFs. Despite both approaches showed that the general composition of the methanotrophic community was quite similar in the rhizospheric soil and on the roots, the relative abundance of individual methanotrophic genera was different and in addition, exhibited a shift between 44 and 54 days after transplantation. Thus, while the relative abundance of type II methanotrophs (T-RF of 245 bp) decreased in the rhizosphere soil, it increased on the roots. The reverse was observed for type I methanotrophs (Table 3.2). This result indicated that the different methanotrophic genera responded differently to spatiotemporal variations in the rice microcosms, which in turn gave a hint that different methanotrophic genera may have different $CH_4$, $O_2$ or nutrient requirements (see

Discussion below). Thus it was remarkable that type I methanotrophs were more abundant on the roots while type II methanotrophs were more abundant in the rhizospheric soil. Note, however, that the abundance of methanotrophic groups were only relative numbers within the total community of methanotrophs. The total community of methanotrophs might have most probably increased over time in the rhizospheric soil and decreased on the roots as indicated by the temporal change of the PLFA concentrations (Fig. 3.12a). PLFA biomarkers ideally provide information on microbial identity and biomass of living bacteria (Tunlid and White, 1992). However, this biomass also represents inactive bacteria and only a minor part of the PLFA detected belongs to methanotrophs, which occur on the order of $<10^7$ per gram dry rice soil, i.e., about 1% of total biomass (Eller *et al*., 2005; Joulian *et al*., 1997). Therefore, it was not possible to calculate the temporal change of the methanotrophic biomass from the T-RFLP and total PLFA analyses. Moreover, since I used DNA samples for the amplification of *pmoA*, I could not ascertain that the amplified *pmoA* product represented the metabolically active methanotrophs.

Therefore, I used PLFA-stable isotope probing method and supplied the rhizosphere of the rice microcosms with $^{13}C$-$CH_4$ between 37 and 54 days after transplantation. Although the labeled $CH_4$ consisted of 99 atom-% $^{13}C$, the $CH_4$ in the pore water and in the $CH_4$ flux contained only 3-5 atom-% $^{13}C$. This result showed that the $CH_4$ added through the permeable tubing into the rhizosphere became highly diluted by endogenously produced $CH_4$. However, the resulting $^{13}C$-content of the $CH_4$ allowed the detection of specifically $^{13}C$-labeled PLFA. PLFA-SIP had previously been used to characterize active methanotrophs in aquatic sediments (Boschker *et al*., 1998; Nold *et al*., 1999), but has so far not been applied in planted rice fields or planted rice microcosms. In this study of planted rice microcosms, mainly the PLFA (16:1ω7, 16:1ω6, 16:0, 18:1ω7, 18:1ω9, 18:0) became labeled with $^{13}C$ derived from $CH_4$. Although these PLFA are found in many different prokaryotes and eukaryotes (Spring *et al*., 2000) the labeling of them strongly indicated that type I and type II methanotrophs were active in rhizospheric soil and on root samples, and incorporated $^{13}C$ during the 8 and 18 days of incubation into 16:1ω7, 16:1ω6, 16:0 and 18:1ω7, 18:1ω9, 18:0 PLFA, respectively. However, I would not expect that any other organisms than methanotrophs assimilated $^{13}C$-$CH_4$. Unexpectedly, I detected small amounts (1-6%) of PLFA representing Gram-positive bacteria (15:0i) and eukaryotes (18:2ω6,9) that became labeled with $^{13}C$,

probably by cross-feeding. These PLFAs have been reported previously from rice paddy fields (Kimura and Asakawa, 2006). Recently, Raghoebarsing *et al*. (2005) showed that eukaryotic *Sphagnum* moss can be cross-fed by the $CO_2$ derived from methanotrophs living inside the moss. A peak for PLFA 18:1ω8 was not detected, which would be characteristic for *Methylocystis* sp. This might have been due to methodological limitations using a non-polar separation column as suggested by Knief *et al*. (2003b). Consistent with my study, the PLFA 18:1ω8 had also not been detected in rice fields by other researchers (Bai *et al*., 2000; Bossio and Scow, 1998; Reichardt *et al*., 1997).

During the 8 and 18 days of $^{13}C$-$CH_4$ labeling incubation, i.e., after 44 and 54 days of transplantation, the PLFA of methanotrophs became increasingly $^{13}C$-labeled, demonstrating their activity in the rhizospheric soil and on the roots. The incorporation of $^{13}C$ was significantly higher in the rhizospheric soil than on the roots ($P<0.05$), which is consistent with the relatively higher most probable number counts of methanotrophs (Eller and Frenzel, 2001). However, type I methanotrophs incorporated significantly more $^{13}C$ into their PLFAs than type II methanotrophs, in both soil and roots, indicating that type I methanotrophs were the more active population. In addition, $^{13}C$-incorporation into type I methanotrophs relatively increased with respect to time, while $^{13}C$-incorporation into type II methanotrophs decreased, albeit only on the roots. In the soil, on the other hand, it seemed to be opposite, i.e. the PLFA of type II methanotrophs became slightly more $^{13}C$-labeled with respect to time than PLFA of type I methanotrophs. These results indicated that type I and type II methanotrophs became increasingly more active on the roots and in the rhizospheric soil, respectively. These findings agreed with previous studies (Amaral and Knowles, 1995; Graham *et al*., 1993), which reported that competition between type I and type II methanotrophs depends upon the concentrations of $CH_4$ and $O_2$ and also the presence of nitrogen. Type I methanotrophs seem to prefer environments with plentiful $O_2$ and limited $CH_4$ concentrations, whereas type II methanotrophs dominate in environments with high concentrations of $CH_4$ and limited $O_2$. In my results, temporal changes in the activity of both type I and type II methanotrophs could be observed, with type I methanotrophs eventually exhibiting higher activity on the roots, while type II methanotrophs became more active in the rhizospheric soil. I speculated that type I methanotrophs were not be able to cope with the low $O_2$ concentrations in rhizospheric soil and thus became less active with time as $O_2$ availability decreases with distance from the roots. As a consequence, type II

methanotrophs became dominant instead. Consistent to my study, Macalady *et al*. (2002) suggested that both type I and type II methanotrophs coexist in rice paddies, but nevertheless occupy different niches with type I methanotrophs being more important in drained fields where $O_2$ reaches deeper soil layers and type II methanotrophs being more important in flooded fields where $CH_4$ availability is high.

In conclusion, I could effectively differentiate metabolically active methanotrophic community from the total methanotrophic community resident in the rhizospheric soil as well as on the roots from planted rice microcosms using the PLFA-SIP approach and community analysis approach. Both approaches demonstrated that type I and type II methanotrophic populations in the rhizospheric soil and on the rice roots changed differently over time with respect to activity and population size and that type I methanotrophs played a particularly important role in the rice field ecosystem. Furthermore, PLFA-SIP showed that the active methanotrophic populations exhibit a pronounced spatial and temporal variation in rice microcosms. This variation is probably due to different concentrations of methane, oxygen and probably nutrients, which provide different niches for the methanotrophs.

# Chapter 4

## Effect of different ammonium-N fertilizers on methane oxidation and methanotrophic community structure in rice rhizosphere at different growth stages of rice plant

## 4.1 Introduction

Nitrogen (N) fertilization is one of the essential factors for achieving high rice yields. Rice production has to be increased by 60% in next decades to meet the demands of the growing human population (Cassman *et al*., 1998). It has been thought that increasing amounts of N-fertilizer will have to be applied to maximize rice yields as per the estimation of rice production. As a consequence, the expected intensification of rice production will most likely lead to increased atmospheric methane emissions in the near future, if the proper mitigation strategies are not applied.

Methanotrophic bacteria (methane oxidising bacteria, MOB), the only biological sink of methane, play an important role by reducing the potential amount of emitted methane from rice fields into the atmosphere. Approximately 10-30% of the methane produced by methanogens in rice paddies is consumed by aerobic methane-oxidising bacteria associated with the roots of rice. Factors that limit or even inhibit the activities of methane oxidising bacteria have major effects on the global methane budget. Besides major limiting factors i.e., methane and oxygen, nitrogen (N) one of the limiting factor for rice yields, can also play an important role in methane oxidation and may become an inhibiting or stimulating factor for growth of methanotrophs. There is ongoing discussion on the possible effects of ammonium based nitrogen fertilizer on consumption of methane by methanotrophs depending on which environment it has been applied (Bodelier and Laanbroek, 2004). Currently, there are many contradictory results, reporting inhibition effects (Banik *et al*., 1996; Bosse *et al*., 1993; Cicerone and Shetter, 1981; Hütsch *et al*., 1994; Steudler 1989), stimulation effects (Bodelier *et al*., 2000; Dan *et al*., 2001; Krüger *et al*., 2002; Krüger and Frenzel, 2003) or no effects (Delgado and Mosier, 1996; Dunfield *et al*., 1995) of ammonium-based N-fertilization on methane oxidising bacteria. Interestingly, De Visscher and Cleemput (2003) have anticipated for the first time that $NH_4^+$-N inhibits $CH_4$ oxidation at low $CH_4$ concentrations and stimulates $CH_4$ oxidation at high $CH_4$ concentrations. So far many mechanisms have been proposed for stimulation and inhibition effects on methane oxidation and methanotrophs but none of them have yet been experimentally verified.

Schimel (2000) proposed that at the level of the microbial community, nitrogen stimulates the growth and activity of methane-oxidising bacteria and at the biochemical level, ammonium inhibits methane consumption because of competition for methane monoxygenase. Because of similarity in size and structure of $CH_4$ and $NH_3$ molecules, and the relatively low specificity of the monoxygenase enzymes responsible, both methanotrophs and ammonia oxidizers can oxidize $CH_4$ and $NH_4^+$ (Bedard and Knowles, 1989). Furthermore, the intermediates and end products of ammonia oxidation, i.e., hydroxylamine and nitrite can be toxic to methanotrophic bacteria and may also lead to inhibition of methane consumption (Schnell and King, 1995). However, in rice fields methanotrophs have to compete with other different microorganisms and also with the rice plant itself for nitrogen. Therefore, methanotrophs may undergo severe nitrogen limitation as the plants grow, and application of nitrogen fertilization may relieve nitrogen limitation and stimulate methanotrophic growth and activity (Bodelier *et al*., 2000; Dan *et al*., 2001).

Different fertilizer types were reported to affect methane production and methane emission differently (Yao and Chen, 1994; Schutz *et al*., 1989). Urea is the most common N-fertilizer and widely used in rice cultivation. Ammonium sulphate is also used in many places and this fertilizer is generally used as a possible mitigation strategy for methane emission. Ammonium sulphate has been found to reduce methane emission in rice fields due to competitive inhibition of methanogens by sulphate reducers for hydrogen and acetate substrates (Minamikawa *et al*., 2005; Cai *et al*., 1997; Denier van der Gon and Neue, 1994; Lindau, 1994).

Although, there are an increasing number of studies related to the effects of N-fertilization on the methane oxidation process, the effects on the process-governing actors, i.e. the methanotrophic community, have not been studied in detail. The question arises whether the methanotrophic community structure plays any role in the response of the methanotrophs to different nitrogen availability.

Bodelier *et al.* (2000) and Mohanty *et al.* (2006) showed that type I methanotrophs are stimulated while type II methanotrophs are inhibited by ammonium-based nitrogen fertilizers. However, these results were obtained in rice field soil slurry incubation. So far, no detailed study has yet been carried out to investigate the effect of available ammonium-N fertilizer on methane oxidation and methanotrophic community structure

under *in situ* condition during the entire rice-growing season. Furthermore, in rice agriculture the common practice is application of fertilizers in split doses before flooding, at tillering stage and before panicle initiation, thus creating different ammonium-N environments at different growth stages of the rice plant. It is possible that the nitrogen availability will shape the methanotrophic community structure differently at different growth stages of the rice plant. Therefore, this research was mainly focused on the study of the effect of different ammonium based-nitrogen fertilizers (urea and ammonium sulphate) on methane oxidation and methanotrophic community structure at different growth stages of rice plant using rice microcosms under controlled conditions in the greenhouse.

## 4.2 Objectives

- To study the temporal variation in methane oxidation and methane concentration due to the application of different N-fertilizer treatments during different growth stages of the rice plants.
- To study the effect of different ammonium-N fertilizer treatments on the community structure of methanotrophs on the rice roots and in the rhizospheric soil during different growth stages of the rice plants.

## 4.3 Methods

### 4.3.1 Experimental setup

The experimental set up is as shown in Fig. 4.1.

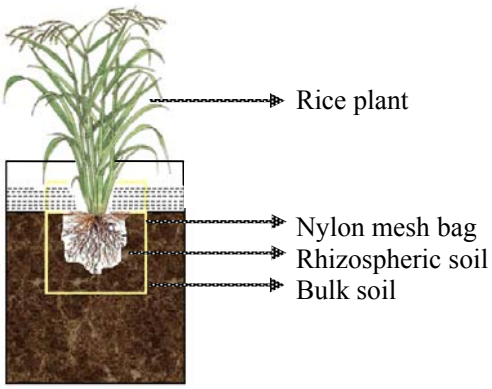



**Fig. 4.1:** Rice microcosm experimental set up.

### 4.3.2 Microcosm experiment

#### 4.3.2.1 Planted rice microcosms

Soil was taken from drained paddy fields of the Italian Rice Research Institute in Vercelli, Italy, in 2006 and was air-dried and stored at room temperature. The soil characteristics are described in table 4.1. Immediately prior to its use for preparing the microcosms, the soil was ground with a mechanical grinder and passed through a 2 mm sieve. Then the soil slurry was prepared with 1.6 kg soil, 900 ml demineralized water, and 50 ml of fertilizer solution [2.3 g nitrogen (N) as urea/ ammonium sulphate, 0.87 g phosphorous (P) as $KH_2PO_4$, and 1.85 g potassium (K) as KCl per liter], and filled into each microcosm pot with a volume of 1.6 L (height 11 cm, diameter 16 cm). This fertilizer composition corresponds per ha to 160 kg N as urea or ammonium sulphate, 140 kg $P_2O_5$ and 155 kg $K_2O$ as practically applied in rice fields. In the center of each pot, a self-made nylon bag (25 μm mesh; 6 cm length and 9 cm radius) was placed through which water and nutrients could pass freely while roots were not able to penetrate, isolating the soil inside the bag as rhizospheric soil from the bulk soil outside the bag (Fig.4.1 and appendix).

**Table 4.1:** Soil characteristics of rice field soil collected in year 2006.

| Variable | Portion |
|---|---|
| C (%) | 1.43 |
| N (%) | 0.13 |
| C/N | 11 |
| Texture (%) (clay/silt/sand/stones >2mm) | 14/61/25/2 |

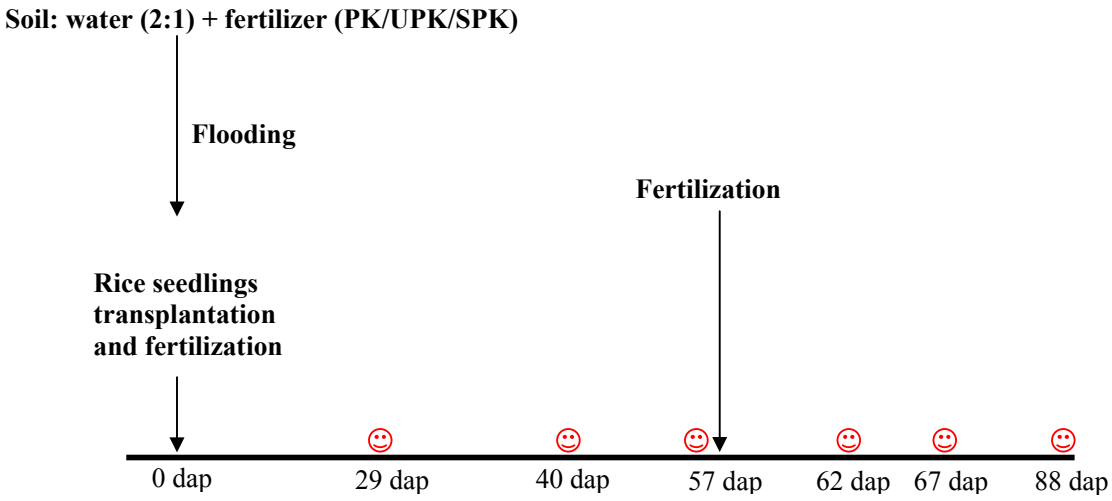



**Fig. 4.2:** Schematic diagram of the experiment. dap indicates days after transplantation and ☺ indicates the soil sampling point.

In this study, three different ammonium-N fertilizer treatments were applied: i) PK as control treatment without N source, ii) UPK with urea as N source, and iii) SPK with ammonium sulphate as N source. All three N-fertilizer treatments were accompanied with phosphorous (P) and potassium (K) and thus a mixture of fertilizer solution was added in split doses as applied in rice fields in order to fulfill the nutrient requirements for rice plant growth. In total, 75 microcosms were prepared with 25 for each treatment. The prepared microcosms were flooded with demineralized water giving a water depth of 5 cm above the soil surface and were incubated in the greenhouse with a relative humidity of 70%, 12 h photoperiod, and 28/22 ºC day/night temperature. After five days of flooding, one 12-day old rice seedling (*Oryza sativa* var. KORAL type japonica) germinated on moist filter paper at room temperature was transplanted into the center of the nylon bag in each pot, and 20 ml of fertilizer solution (same as above) was added. The day of transplantation was taken as day zero and the following incubation experiment was

then conducted from September 20, 2006 till December 12, 2006 for a total of 88 days under flooded conditions (Fig. 4.2). On dap 57, 20 ml of fertilizer solution was added a third time to the microcosms. Water lost due to evapotranspiration was daily replaced by addition of demineralized water to maintain a 5 cm water depth.

#### 4.3.2.2 Plant parameters

Once every week total plant height, tiller number and leave number were determined. Plant height was defined as the distance from the soil surface to the uppermost part of the canopy.

#### 4.3.2.3 Measurement of $CH_4$ flux

Rates of $CH_4$ emission and $CH_4$ oxidation were measured as described in chapter 2.2.1. From the $19^{th}$ day of transplantation, measurements of methane emission rates and methane oxidation rates were carried out once/twice a week. For the measurement of $CH_4$ emission rates, triplicate microcosms were covered by static flux chambers made of plexi glass with 10 cm internal diameter and 40 or 82 cm height, depending on the height of the rice plants as described in chapter 2.2.1. Gas samples were taken from all three treatments (PK, UPK and SPK) in every 30 minutes for 3 hours. $CH_4$ oxidation rates were measured as the flux in the presence and absence of inhibitor by adding 1% diflouromethane to the headspace of the flux chambers in triplicate microcosms. The gas samples were immediately analysed for $CH_4$ and $CH_2F_2$ on a Shimadzu GC-8A gas chromatograph as described previously in chapter 2.2.1.

#### 4.3.2.4 Soil pore water

Pore water sampling was carried out once/twice a week from the very first day after transplantation. Pore water samples were collected into Venoject blood collecting tubes from the rhizosphere and bulk regions of rice microcosms by using Rhizon pore water samplers and further treated as described in chapter 2.2.2.

### 4.3.3 Analytical Methods

The gas samples from the pore water were analyzed for $CH_4$ using gas chromatography equipped with FID detector as described in section 2.2.1.

Ammonium, nitrate, nitrite and sulphate concentrations were analyzed as described in chapter 2.2.3.2. pH was measured using a pH meter.

### 4.3.4 Collection of soil and roots samples

Rhizospheric soil and root samples were collected 29, 40, 57, 62, 67, and 88 days after transplantation in triplicate samples from each of the triplicate microcosms as described in 2.2.4. In this study, a total of 9 replicates was sampled at each sampling date for each treatment. The rhizospheric soil samples were assigned as PK-RS-X, UPK-RS-X, and SPK-RS-X, and roots samples were assigned as PK-RT-X, UPK-RT-X, and SPK-RT-X for PK, UPK and SPK treatments, respectively. In these notations, X varies according to different sampling times (29, 40, 57, 62, 67, and 88), e.g. PK-RS-29, UPK-RS-29, and SPK-RS-29. The roots were washed in deionized sterile water and were stored at –80 $^{o}$C for molecular analyses. In addition to chapter 2.2.4, rhizospheric soil samples were mixed with 1 ml of RNAlater® immediately after sampling. After 24 h of incubation at 4 $^{o}$C, soil samples were stored at –80 $^{o}$C for molecular analyses.

### 4.3.5 Moisture content determination

Refer to chapter 2.2.5.

### 4.3.6 Total nucleic acids extraction, PCR, cloning and sequencing

Before extraction of total nucleic acids from roots and rhizospheric soil, soil samples were thawed and RNAlater® was removed by centrifuging at 5000 × g for 5 minutes. The samples were washed twice with one-quarter-strength Ringer solution in order to remove the remaining RNAlater® (Shrestha, Dissertation, 2007) and immediately used for extraction of nucleic acids as described in chapter 2.2.7.1. Finally, the *pmoA* gene was amplified using primers set A189f/mb661r (Costello and Lidstrom, 1999) and A189f/nmb650r (detail in chapter 5.3.7) as described in chapter 2.2.7.3.

For cloning, a pool of 9 replicate samples was done representing one sample for each treatment from 57 dap. Six clone libraries of *pmoA* genes were constructed using DNA amplicons retrieved from root samples (57 dap) and rhizospheric soil (57 dap) from each of three fertilizer treatments. The clone libraries were constructed using the pGEM-T Easy cloning kit (Promega, Germany). A total of 97 clones from root samples (47, 37 and

13 clones from PK-57, UPK-57 and SPK-57 samples, respectively) and 83 clones from rhizospheric soil samples (33, 24 and 26 clones from PK-57, UPK-57 and SPK-57 samples, respectively) were randomly selected for comparative sequence analysis and proceeded as described in chapter 2.2.7.5.

### 4.3.7 Phylogenetic analysis

Refer to chapter 2.2.8.

### 4.3.8 T-RFLP analysis

Analysis of terminal restriction fragment length polymorphism (T-RFLP) was performed for each DNA extract in replicates as described in chapter 2.2.9. The *pmoA* was amplified using FAM (6-carboxyfluorescein)-labeled forward primer as described in chapter 2.2.9. However, using reverse primer mb661 in conjunction with labeled forward primer (A189), the amplified products from SPK-57 and SPK-88 dap of roots samples could not be obtained. Therefore, these samples were amplified using the newly designed reverse primer nmb650 (explained in chapter 5.3.8) in conjunction with labeled forward primer (A189). All PCR amplified products were gel purified with Wizard ® SV Gel and PCR Cleanup System (Promega, Germany) and then proceeded for T-RFLP analysis as described in chapter 2.2.9. The normalization procedure of each TRF profile was done as described in chapters 2.2.9.1 and followed with calculation of relative abundance as described in chapters 2.2.9.2 and for statistical analysis.

### 4.3.9 Statistical analyses

Refer to chapter 2.2.11.2.

## 4.4 Results

A microcosm experiment with rice plant was conducted for 88 days under controlled conditions in the greenhouse. Three different treatments were applied to investigate the effect of different nitrogen fertilizers on methane oxidation and total methanotrophic community structure on the roots and in the rhizospheric soil. The methane oxidation process was studied during the whole incubation period along with the analysis of pore water profile. The total community of methanotrophs was analyzed using terminal restriction fragment length polymorphism (T-RFLP) for six different time points during the rice-growing season. Further, the effect of treatments, environmental factors and functions were investigated using statistical ordination techniques.

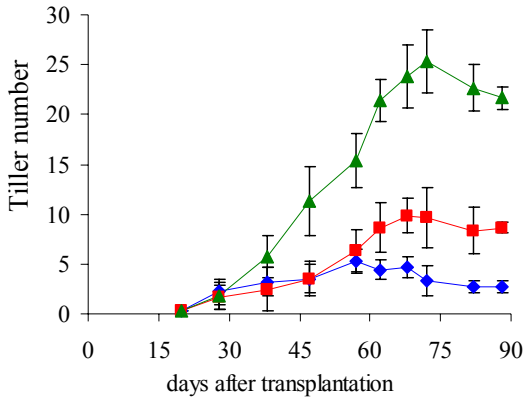
### 4.4.1 Plant parameters

The vegetative growth of rice plants (plant height, number of tiller, and the number of leaves) was more pronounced in SPK treatment than in UPK and PK treatments (Fig. 4.3). The plant growth was similar in PK and UPK till 57 dap, but after 57 dap, i.e., after fertilizer addition, number of tillers and leaves increased in UPK treatment as compared to PK treatment.

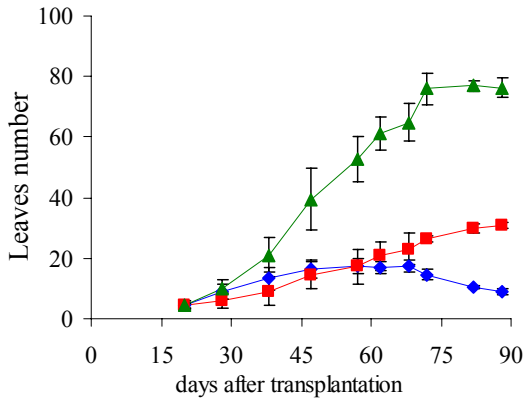
### 4.4.2 Rates of $CH_4$ emission and oxidation

The $CH_4$ emission rates (in absence of inhibitor) gradually increased from the beginning and reached to a maximum value of 19 mg $CH_4$ $m^{-2}$ $h^{-1}$ on day 48 and 21 mg $CH_4$ $m^{-2}$ $h^{-1}$ on day 65 for PK (control) and UPK treatments, respectively (Fig. 4.4a). In SPK treatment, the emission rate remained always lower than 1 mg $CH_4$ $m^{-2}$ $h^{-1}$ for most of the rice-growing season. The $CH_4$ emission rates were higher in PK treatment till day 57 after transplantation and after that values decreased and remained lower for the rest of the cropping period, while in UPK treatment $CH_4$ emission rates significantly increased after 57 dap as compared to earlier days and fluctuated for rest of the late period. The same trend of increase in methane emission rates after 57 dap was observed in SPK treatment but at much lower rates. However, no differences were observed in methane emission rates between PK and UPK treatments except on 43 dap and 65 dap.

a)




b)




c)

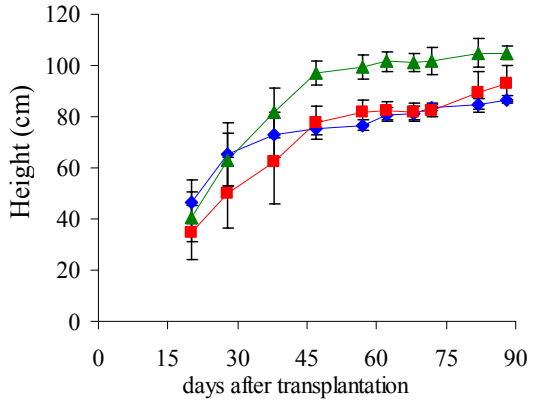
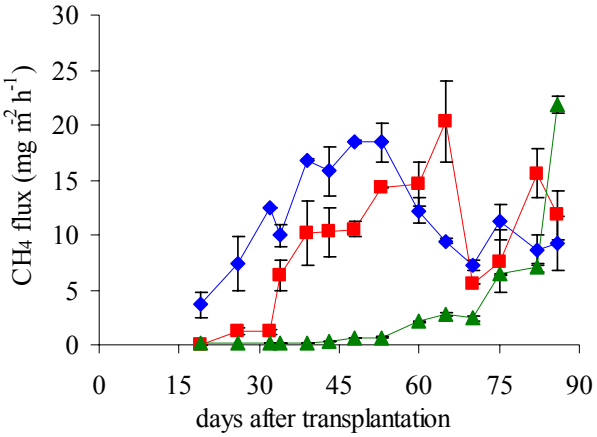
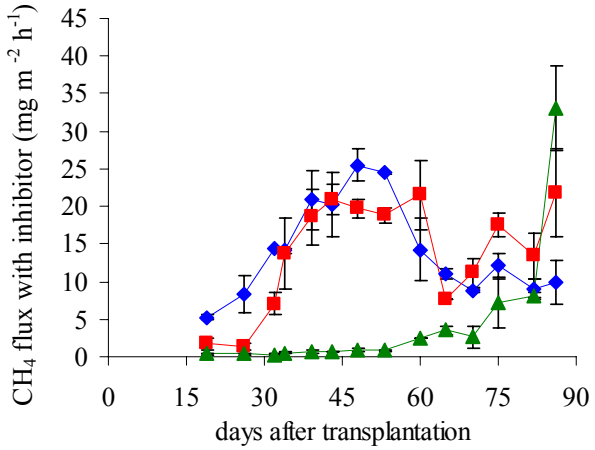



**Fig. 4.3:** Rice plant growth parameters a) number of tiller, b) number of leaves, and c) plant height (cm) in three treatments: PK, UPK, SPK ; mean ± SD (n=3-20).

a)




b)




c)

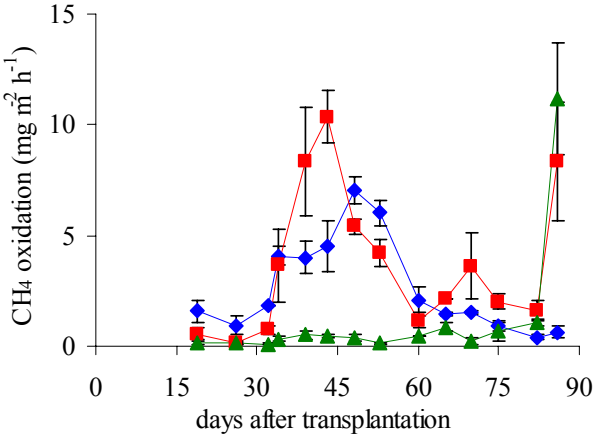



**Fig. 4.4:** a) Methane flux ($CH_4$ emission in the absence of inhibitor) and b) methane flux $CH_4$ emission in the presence of inhibitor, and c) methane oxidation rates in three treatments: PK, UPK, SPK ; mean ± SD (n=3-9).

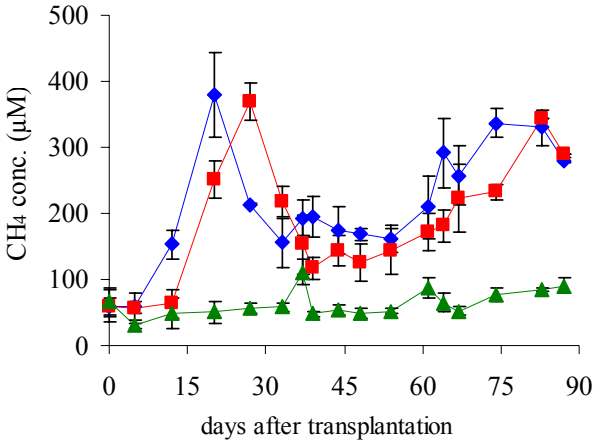
Methane oxidation started to increase after day 30 and reached the maximum value of 7 mg $CH_4$ $m^{-2}$ $h^{-1}$ on day 48 and of 11 mg $CH_4$ $m^{-2}$ $h^{-1}$ on day 43 in PK and UPK treatments, respectively (Fig. 4.4c). These values contributed to nearly 28% and 50% of the anaerobically produced methane that were oxidized prior to its emission to the atmosphere. In SPK treatment, methane oxidation rates were less than 1 mg $CH_4$ $m^{-2}$ $h^{-1}$ during the whole incubation period. However, high methane emission and methane oxidation rates were observed on day 88, the end of incubation, in SPK treatment (Fig. 4.4a, 4.4b, and 4.4c).

### 4.4.3 $CH_4$ in the pore water

The $CH_4$ concentration dissolved in the pore water rapidly increased in both rhizospheric and bulk regions 15 days after transplantation in PK and UPK treatments, while in SPK treatment the values did not change much (Fig. 4.5a and 4.5b). On average, $CH_4$ concentration was lower in the rhizospheric region (at 3 cm depth from the soil surface) than in the bulk region (at 10 cm depth from the soil surface). The maximum value of $CH_4$ concentration in the rhizospheric region (Fig. 4.5a) was around 400 μM on 20 and 30 days after transplantation in PK and UPK, respectively. These values decreased and remained at around 200 μM between 30 and 60 days and then again increased in the late rice-growing season. In the bulk soil (Fig. 4.5b), $CH_4$ concentration increased up to 800 μM on around 40 dap and later slowly decreased to about 500 μM on 48 dap and remained between 300-400 μM for the rest of the time. There were no major differences in $CH_4$ concentrations between PK and UPK treatments in the rhizospheric as well as in the bulk region during most of the incubation period.

In SPK treatment, the maximum $CH_4$ concentration in the pore water of the rhizospheric region was 112 μM on 37 dap, otherwise it remained lower to an average of 50-60 μM during most of the time (Fig. 4.5a). Similarly in the bulk region (Fig. 4.5b), the maximum $CH_4$ concentration in pore water was 170 μM on 37 dap. However, 54 days after transplantation, $CH_4$ concentration in pore water started to increase slowly in both rhizospheric and bulk regions.

a)




b)

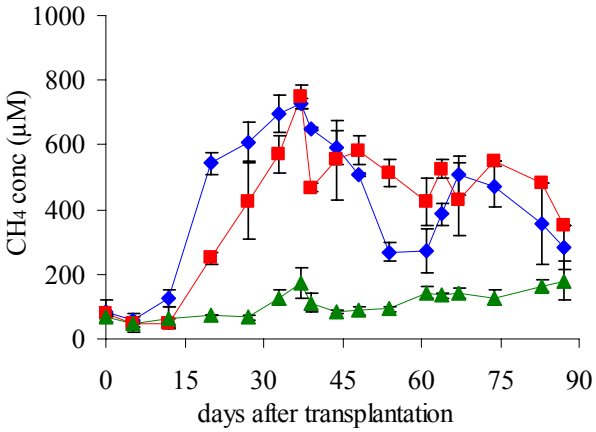
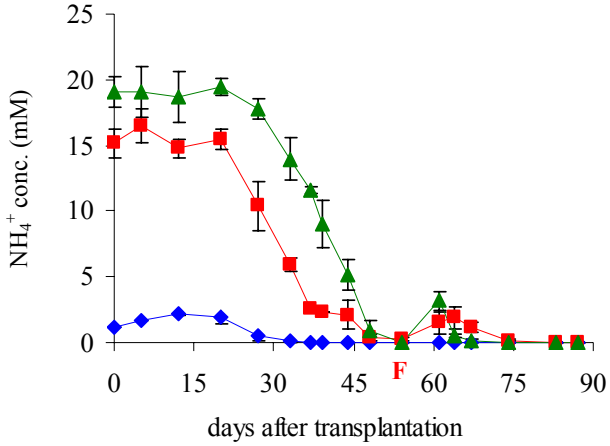



**Fig. 4.5:** Porewater $CH_4$ concentrations in a) rhizospheric region and b) bulk region in three treatments; PK, UPK, SPK; mean ± SD (n=3-9).

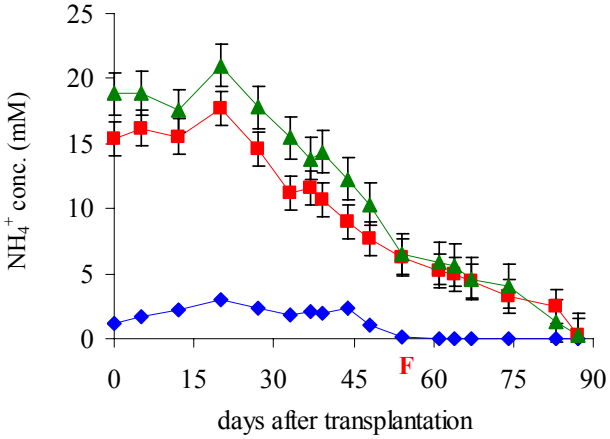
### 4.4.4 $NH_4^+$, $NO_2^-$, $NO_3^-$, $SO_4^{2-}$ and pH in the pore water

Ammonium concentrations dissolved in the pore water showed a similar pattern of ammonium consumption in the N-fertilizer treatments (Fig. 4.6a and 4.6b). Initially, $NH_4^+$ concentration remained constant but 20 days after transplantation it started to rapidly decrease both in rhizospheric region and bulk regions in all three treatments, indicating rapid consumption by rice plant. In PK treatment, where no nitrogen source was added, $NH_4^+$ concentration was around 2 mM, probably due to the residual nitrogen fertilizer or organic material source from rice field, and was undetectable after 40 days of transplantation. In the N-fertilizer treatments, i.e., in UPK and SPK treatments,

a)




b)




c)

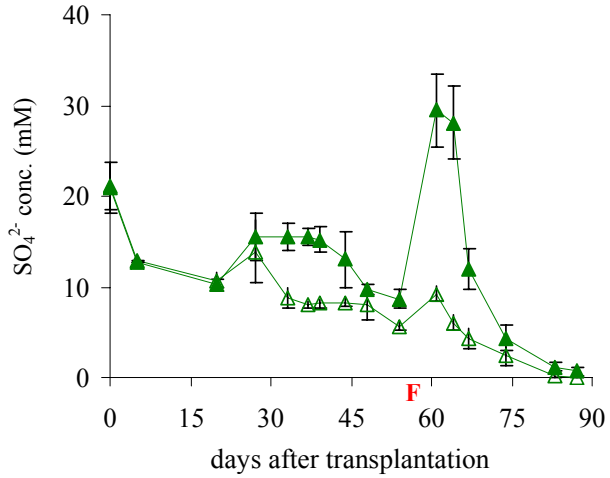
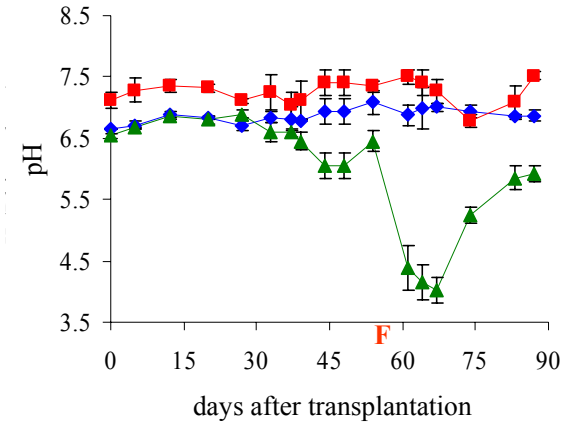



**Fig. 4.6:** Porewater $NH_4^+$ concentrations in a) rhizospheric region and b) bulk region in three treatments; PK UPK, SPK, and c) porewater $SO_4^{2-}$ concentrations in rhizospheric region ( ) and in bulk region ( ); mean ± SD (n=3-9). **F** indicates addition of fertilizer on the following day.

where ammonium as nitrogen was added, $NH_4^+$ concentration depleted on 57 dap. After fertilizer addition on 57 dap (Fig. 4.6a), a small peak was observed in UPK and SPK treatments at about 2-3 mM concentration, which after a short time, completely disappeared from the rhizospheric region indicating high consumption by plants. In bulk region, $NH_4^+$ concentration slowly decreased over time and reduced to less than 0.1 mM on 88 dap (Fig. 4.6b). UPK and SPK treatments provided similar trend of $NH_4^+$ consumption pattern during whole incubation period.

The $NO_2^-$ and $NO_3^-$ concentrations in the pore water were below the detection limit (5 µM) during the entire experimental period.

a)




b)

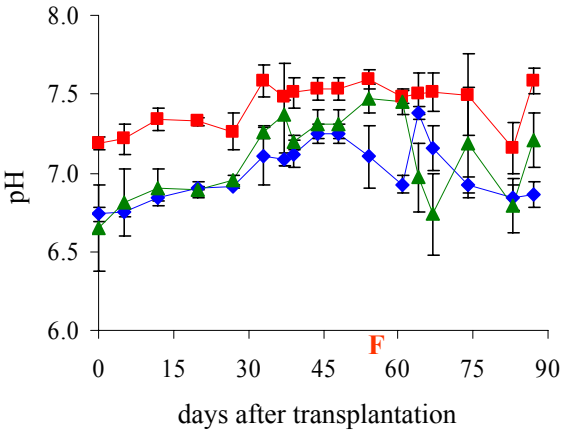



**Figure 4.7:** Pore water pH in a) rhizospheric region, and b) bulk region in three treatments. PK, UPK, SPK, mean ± SD (n=3-9).

In SPK treatment (Fig. 4.6c), sulphate concentration was found at an average of 13 mM in rhizospheric region and 10 mM in bulk region till 57 days after transplantation. The values increased to 29 mM after fertilizer addition on 57 dap in the rhizospheric region and started to decrease again after 64 days. Finally, sulphate concentration decreased to less than 1 mM and 0.1 mM in the rhizospheric and bulk regions, respectively.

The pH of the pore water in PK treatment varied between pH 6.5 - 7.1 and 6.5 - 7.5 in rhizospheric and bulk regions, respectively (Fig. 4.7a, b). Similarly, the pH of pore water in UPK treatment varied between pH 6.8 - 7.5 and 7.2 - 7.6 in rhizospheric and bulk regions, respectively (Fig. 4.7a, b). In SPK treatment, pH varied between 6.7 - 7.5 in bulk region. However, in the rhizospheric region, where it was initially around pH 6.5, it decreased after 57 dap to around pH 4 and subsequently slowly recovered back to pH 5.9 on 88 dap (Fig. 4.7a, b).

### 4.4.5 Moisture content determination

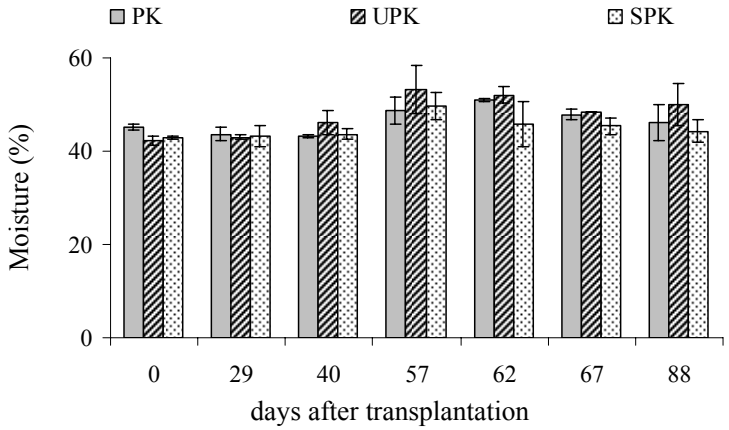



**Fig. 4.8:** Moisture content determined from rhizospheric soil samples collected from different sampling dates; mean ± SD (n=3).

Moisture content was determined for the rhizospheric soil after sampling at different time points (Fig. 4.8). The moisture content was in the range of 42-56% in all treatments and on all sampling days and there was no significant change among the treatments during the entire rice-growing season

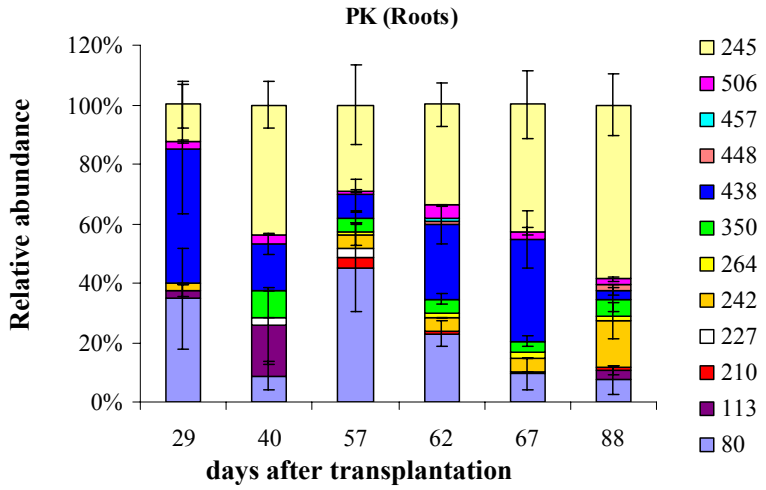
### 4.4.6 Methanotrophic community structure based on *pmoA* gene on roots and in rhizospheric soil samples

Methanotrophic community structure was investigated on roots (RT) and in rhizospheric soil (RS) samples from six different time points of rice-growing season, i.e., 29, 40, 57, 62, 67 and 88 days after transplantation. The *pmoA* gene was successfully amplified from three replicates of three rice microcosms from three different fertilizer treatments for each sampling day (3 replicates × 3 microcosms × 3 treatments = 27 samples per sampling day) using *pmoA* gene specific primers. *pmoA* gene-based methanotrophic community was subsequently resolved by T-RFLP analysis. T-RFLP analysis produced highly reproducible T-RFLP patterns with T-RFs of 80, 113, 210, 227, 242, 245, 264, 350, 438, 448, 457 and 507 base pairs (bp) lengths in almost all replicate samples (Fig. 4.9 and 4.12).

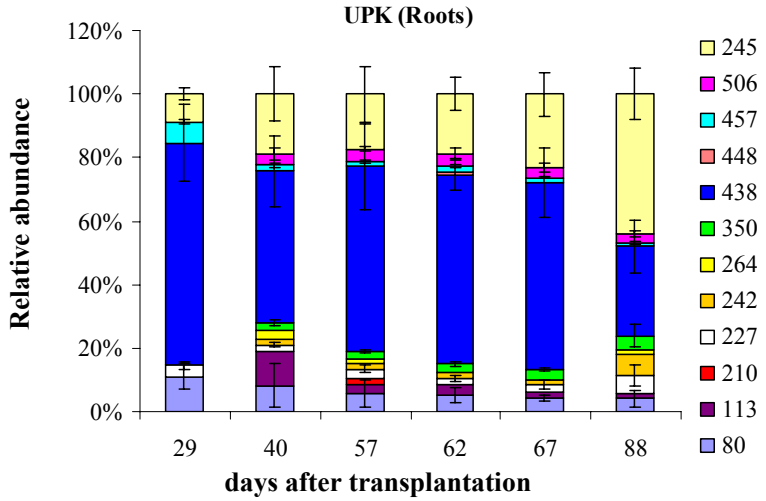
#### 4.4.6.1 Methanotrophic community structure on roots

T-RFLP profiles obtained from the roots-associated methanotrophic community revealed a diverse pattern among the three treatments as well as at the different growth stages of rice plants (Fig. 4.9a, b, c). T-RFLP pattern in UPK treatment seemed to be constant throughout the season with high relative abundances of T-RF of 438 bp except on 88 dap, while T-RFLP pattern in PK and SPK treatments were different at different sampling points. For example, in PK treatment, relative abundance of T-RFs of 80, 245 and 438 bp changed at almost all sampling time points. Similarly, in SPK treatment, relative abundance of T-RF of 438 bp was initially high on 29 dap, decreased with time and then completely disappeared on 62 dap (Fig. 4.9c). However, this T-RF appeared again on 67 dap and the relative abundance increased on 88 dap. Similarly, relative abundance of T-RF of 245 bp size was low on 29 dap, increased afterwards and remained almost constant during 4 consecutive sampling points and again decreased on 88 dap (Fig. 4.9c). Overall, T-RFs of 80, 245, and 438 bp were quite common in all treatments as well as throughout the rice growing season (Fig. 4.9a, b, and c). On 88 dap, T-RFLP pattern generally lead to a higher number of T-RFs as compared to early growth stages in all treatments indicating highest diversity.

a)




b)




c)

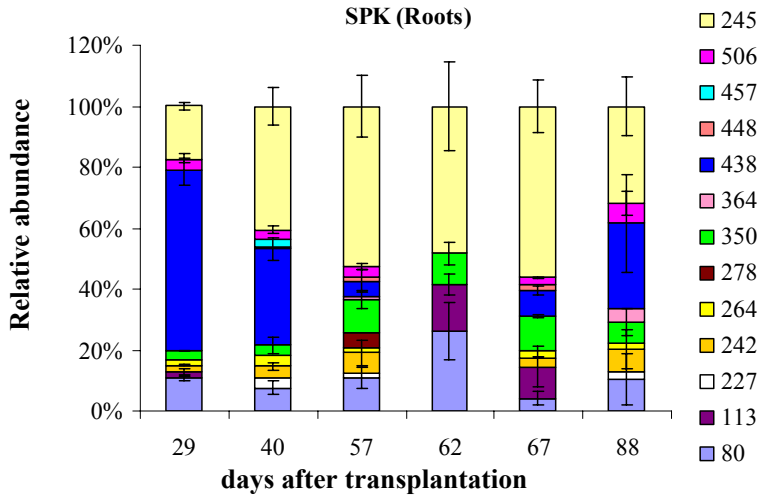



**Fig. 4.9:** *pmoA* gene based T-RFLP profiles from root samples from a) PK treatment, b) UPK treatment, and c) SPK treatment from different sampling points; mean ± SD (n =5-9).

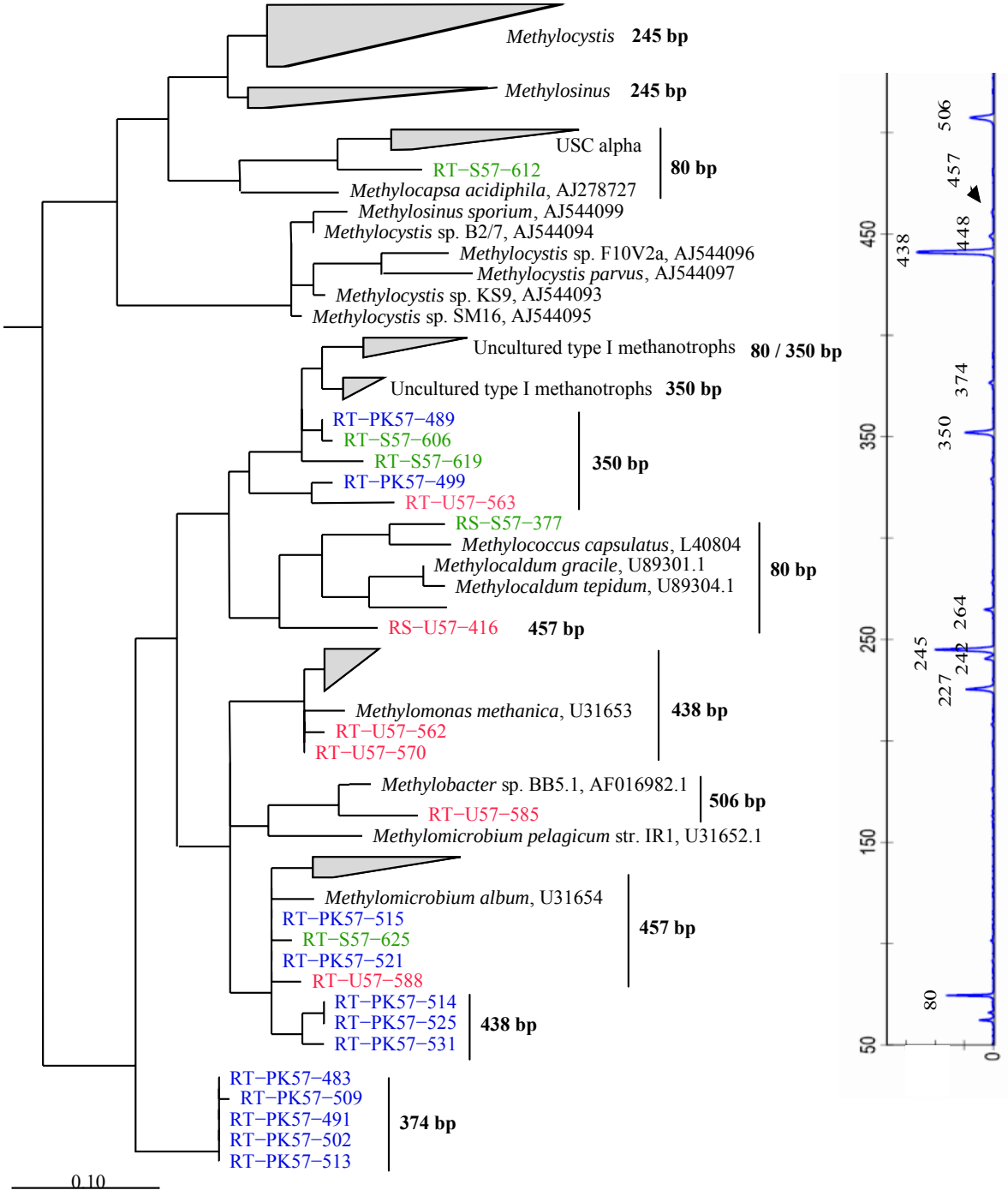



**Fig. 4.10:** Neighbor-joining tree showing the phylogenetic analysis of the derived amino acid sequences encoded by *pmoA* genes from rhizospheric soil and roots samples. Blue, red and green color indicates representative clones from PK, UPK and SPK treatments. Similarly, name of clones starting from RS belongs to rhizospheric soil samples and name of clones starting from RT belongs to roots samples. Theoretical T-RF lengths using MspI are shown next to the sequences. The scale bar represents 10% sequence divergence. Figure at the right hand side indicates a T-RFLP profile from RS-UPK29, *pmoA* transcript (refer to chapter 5) with different peak positions.

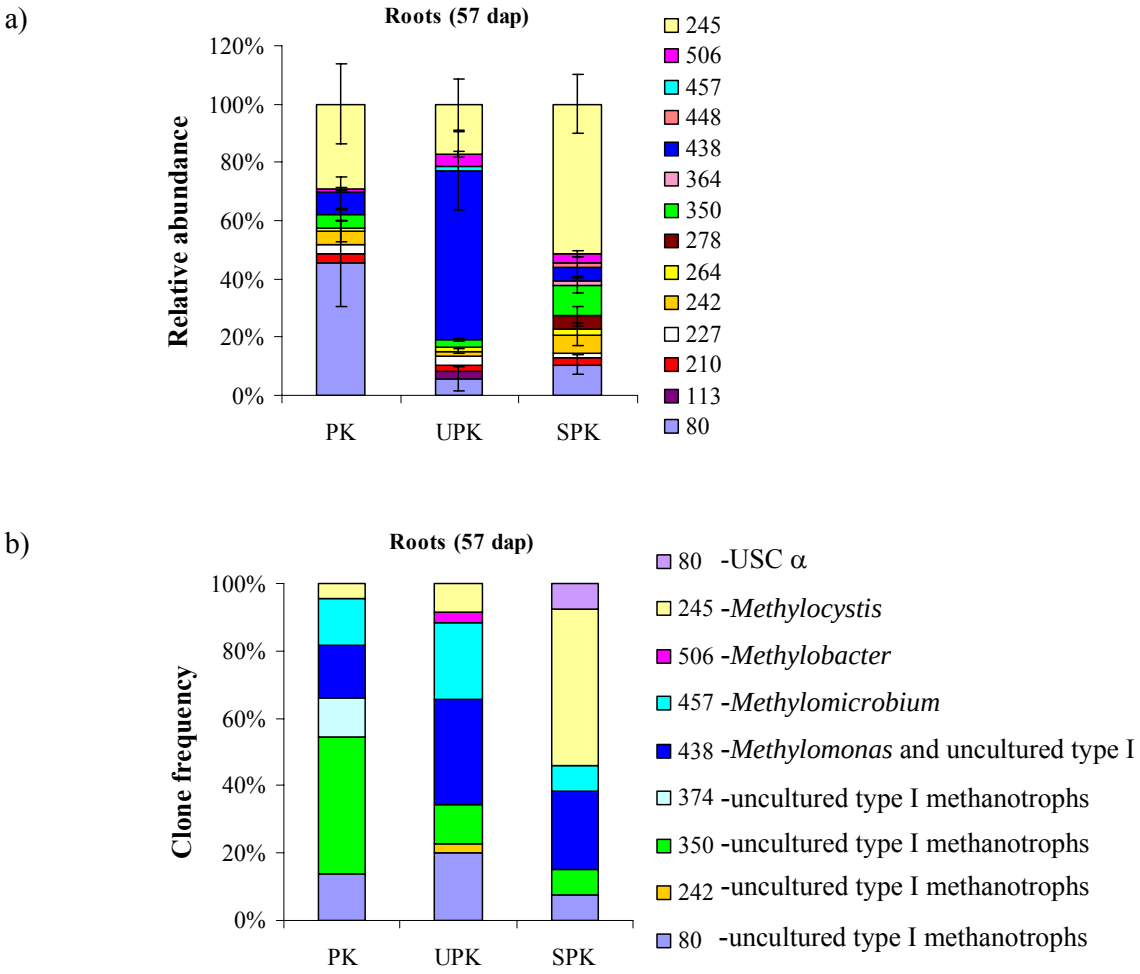



**Fig. 4.11:** Comparison of *pmoA* based (a) T-RFLP profiles (mean ± SD; n=5-9) and (b) clone frequencies obtained from root samples. A total of 47, 37, and 13 randomly selected clones were analysed from root samples of PK, UPK and SPK treatments, respectively.

Three clone libraries were generated from roots samples on 57 dap as PK-57, UPK-57, and SPK-57 (Fig. 4.10a and 4.11b). The T-RFs were assigned to respective genera of methanotrophs by using the clone libraries obtained from PCR products from roots samples as 80 bp – *Methylococcus* and *Methylocaldum*, uncultured type I methanotrophs, and USC α, 245 bp – *Methylocystis* and *Methylosinus* (type II methanotrophs), 438 bp – *Methylomonas*, 457 bp – *Methylomicrobium*, 506 bp – *Methylobacter*, and 227, 243, 264 and 350 bp – uncultured type I methanotrophs (Fig. 4.10). However, T-RFs of 113, 210, 278, and 448 could not be assigned to any of the sequences obtained from the clone libraries. Instead, 11% of clones could be assigned to T-RF of 374 bp that was not represented in the T-RFLP profiles (Fig. 4.11a and 4.11b), as it was only present as a

minor peak that was removed due to the normalization procedure (chapter 2.2.9). The relative clone frequency of *pmoA*-sequences with the respective affiliated T-RFs that were retrieved from root samples (Fig. 4.11b), were similar to the relative abundance of the same T-RFs detected in the DNA extracts (Fig. 4.11a). After affiliation of T-RFs to the respective sequences, root samples revealed a diverse community pattern, representing both type I and type II methanotrophs, on the basis of relative abundance and composition, in all three treatments as well as in all growth stages. Overall, type I methanotrophs were predominant in most of the roots samples.

#### 4.4.6.2 Methanotrophic community structure in rhizospheric soil

T-RFLP profiles obtained from rhizospheric soil samples comprised a total of 10 T-RFs after normalization procedure as described in chapter 2.2.9. All treatments exhibited T-RFLP patterns with similar composition with few changes in relative abundance during different growth stages. T-RF of 245 bp comprised up to 60% of the total relative abundance in each T-RFLP profile in all treatments and sampling points, except for PK treatment on 29 dap (Fig. 4.12a, b, c) indicating the importance of this T-RF in rhizospheric soil. Further details indicated that methanotrophs affiliated to other T-RFs behaved differently in different treatments. For example, T- RF of 438 bp was present in all samples of PK (Fig. 4.12a) and UPK (Fig. 4.12b) treatments, while in SPK treatment (Fig. 4.12c) this T-RF was present only on 29 and 40 dap and absent in the rest of the sampling time points indicating a shift in composition of type I methanotrophic community. Similarly, T-RF of 506 bp on 57 dap and T-RF of 350 bp on 29, 62, 67 and 88 dap were more abundant in SPK treatment as compared to PK and UPK treatments. Among all T-RFs, T-RFs of 80, 350, 438, 242 and 245 bp seemed to be stable in PK and UPK treatments whereas T-RFs of 350, 242 and 245 bp seemed to be stable in SPK treatment. Overall, rhizospheric soil on 29 dap represented the highest number of T-RFs in all treatments indicating highest diversity. T-RFs of 350, 242 and 245 bp were found to be stable and common T-RF among all treatments during the entire rice growing season (Fig. 4.12a, b, c).

In total, three clone libraries were generated from rhizospheric soil on 57 dap as PK-57, UPK-57, and SPK-57. These clone libraries and other clone libraries from another experiment (chapter 3) were used to further assign the T-RFs, obtained from T-RFLP analysis, to respective genera of methanotrophs as 80 bp – *Methylococcus* and

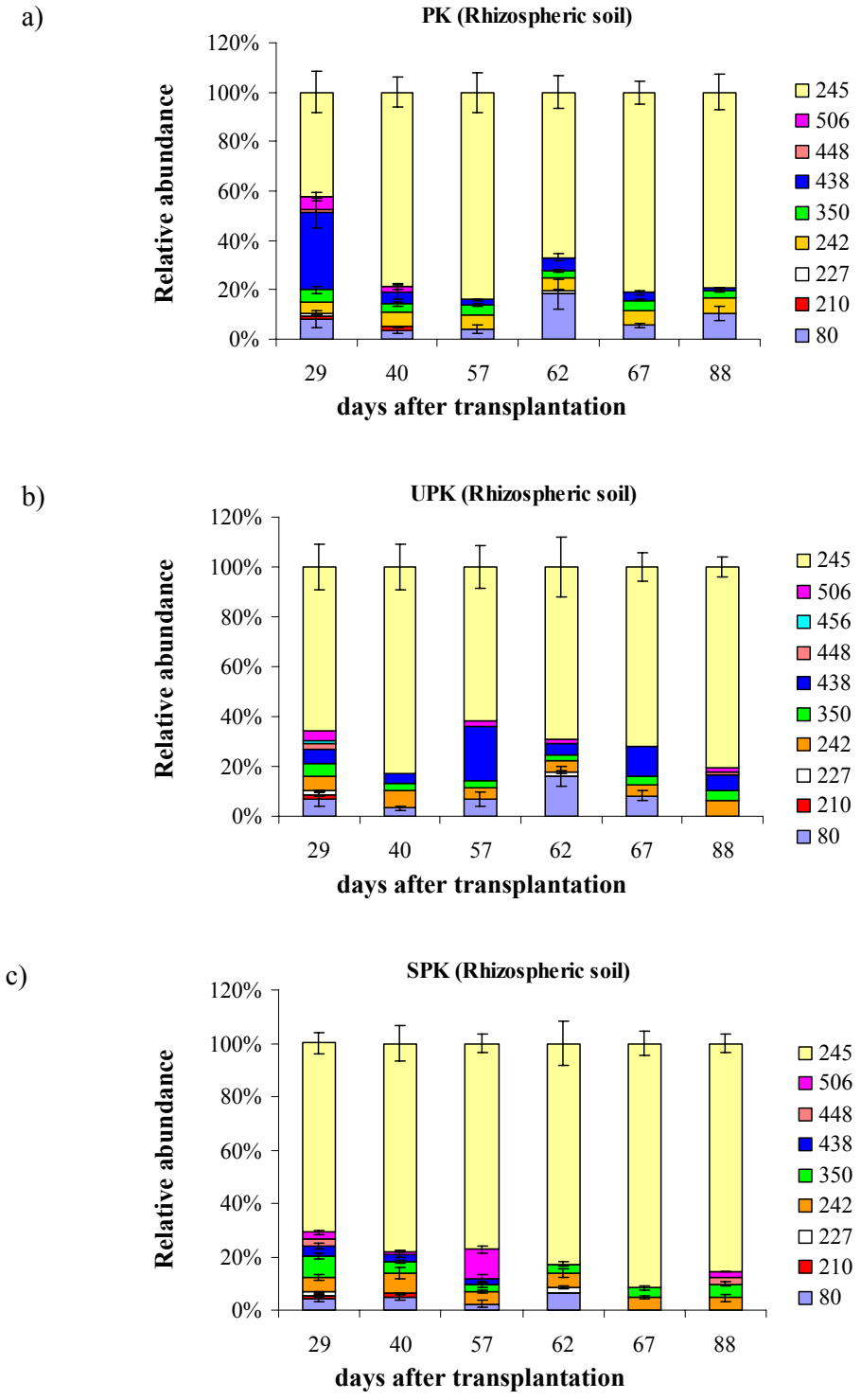



**Fig. 4.12:** *pmoA* gene based T-RFLP profiles from rhizospheric soil samples from a) PK treatment, b) UPK treatment, and c) SPK treatment from different sampling points; mean ± SD (n =5-9).

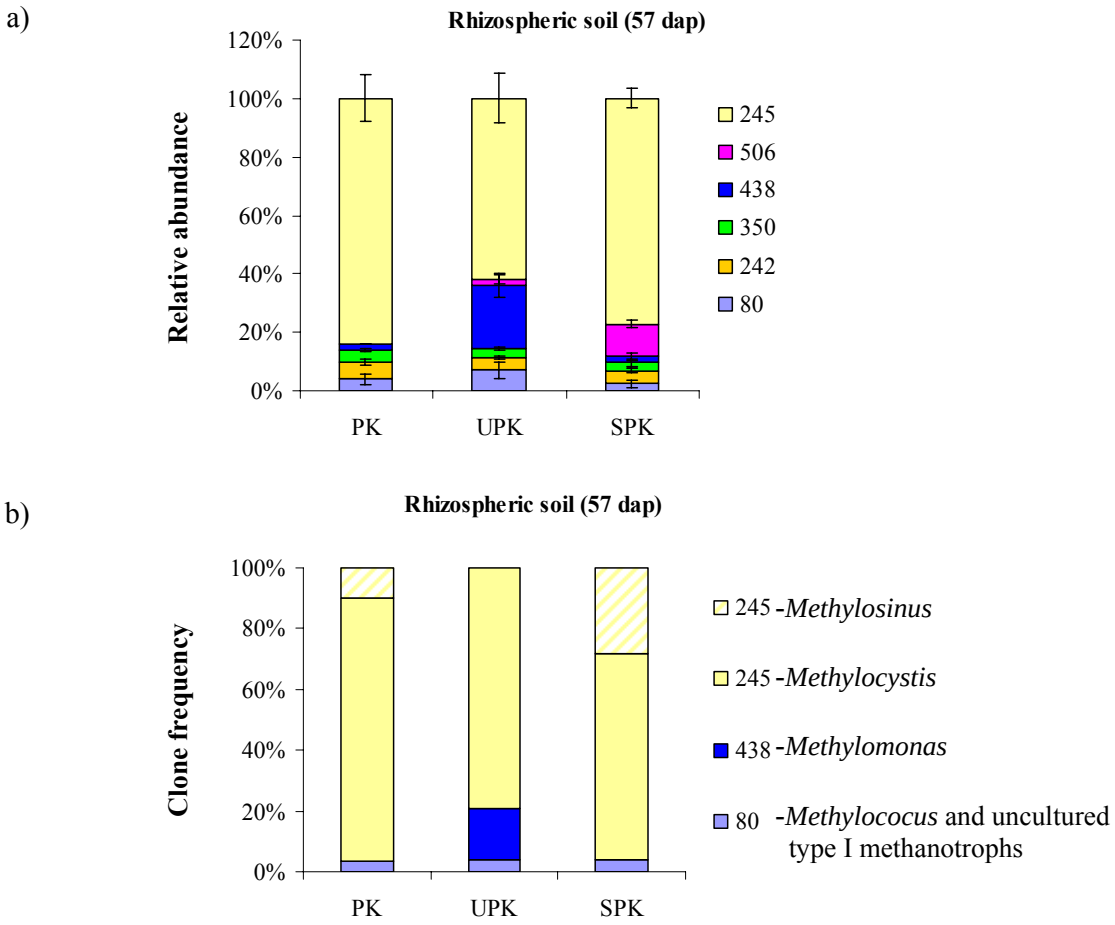



**Fig. 4.13:** Comparison of *pmoA* based (a) T-RFLP profiles (mean ± SD; n=5-9) and (b) clone frequencies obtained from rhizospheric soil samples. A total of 33, 24, and 26 randomly selected clones were analyzed from rhizospheric soil samples of PK, UPK and SPK treatments, respectively.

*Methylocaldum* and uncultured type I methanotrophs, 245 bp – *Methylocystis* and *Methylosinus* (type II methanotrophs), 438 bp – *Methylomonas*, 457 bp – *Methylomicrobium*, 507 bp – *Methylobacter*, and 227, 242, and 350 bp – uncultured type I methanotrophs as shown in Fig. 4.10. However, no representative clones could be assigned to T-RFs of 210 and 448 bp sizes. The relative clone frequency of *pmoA*-sequences with the respective affiliated T-RFs (Fig. 4.13b), were similar to the relative abundance of the same T-RFs detected in the DNA extracts (Fig. 4.13a). Cloning and sequencing analyses of samples from 57 dap revealed that among type II methanotrophs, *Methylosinus* could be distinguished as 6% and 27% from all type II methanotrophs, which made up 87% and 96% of all methanotrophs in PK and SPK treatment,

respectively (Fig. 4.13b). However, no clones from *Methylosinus* were obtained from UPK treatment.

The affiliation of T- RFs to the respective sequences indicated that the rhizospheric soil consisted of both type I and type II methanotrophs in all three treatments as well as in all growth stages. However, type II methanotrophs were predominant in all samples from PK, UPK, and SPK treatment, consisting of 81%, 79%, and 69% respectively.

### 4.4.7 Effect of nitrogen fertilizer treatments on methanotrophic community structure

The raw data (environmental and functional data) were first introduced to local regression method using R package *locfit* and interpolated data thus obtained from smoothing curves were used for further statistical analyses. Examples of the smoothed curves of $NH_4^+$ concentrations (at 3 cm depth) and $CH_4$ concentrations (at 10 cm depth) are shown in Fig. 4.14a and 4.14b, respectively.

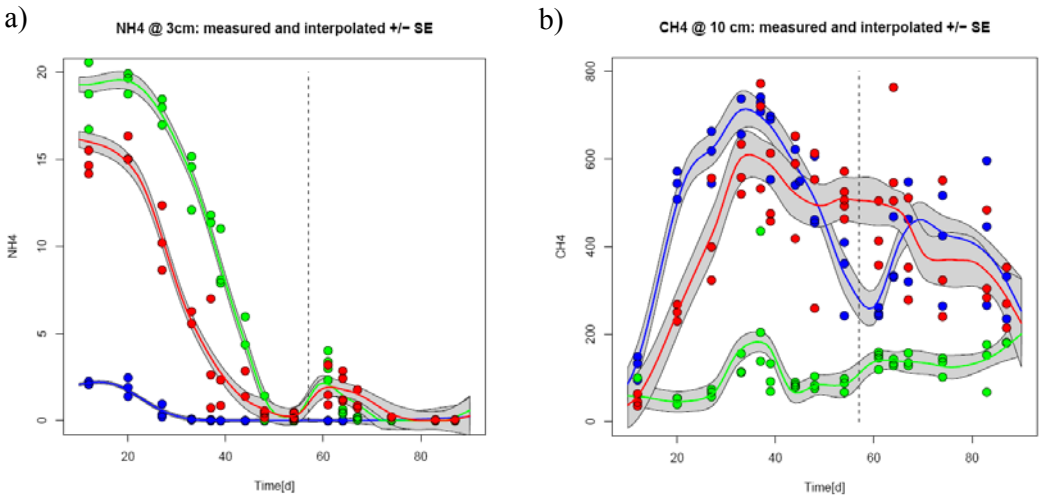



**Fig. 4.14:** Smoothing curves for a**)** $NH_4^+$ concentration in rhizospheric region (at 3 cm depth) and b) $CH_4$ concentration in bulk region (at 10 cm depth) obtained by local regression fit using *locfit*. Blue, red and green color codes for PK, UPK, and SPK treatments. The solid circles in different colors indicate raw values and color lines (blue, red and green) indicate smooth fit from interpolated data. The grey lines above and below the smooth curve are 95% confidence bands. The dashed line indicates the additions of fertilizers on 57 dap.

The T-RFLP data was further analyzed by ordination-based correspondence analysis (CA) to obtain the treatment effects on the composition of the methanotrophic community structure on the roots and in the rhizospheric soil. The results of CA can be visualized in an ordination diagram for roots and rhizospheric soil samples (Fig. 4.15a and 4.15b). On the roots (Fig. 4.15a), methanotrophic community composition based on T-RFLP profiles were significantly affected by different nitrogen fertilizer treatments (ANOSIM statistic R: 0.3696; $P$<0.001; based on 1000 permutations). This can also be seen from the CA ordination plot (Fig. 4.15a) that methanotrophic communities from three treatments were separately clustered except in few of the samples.

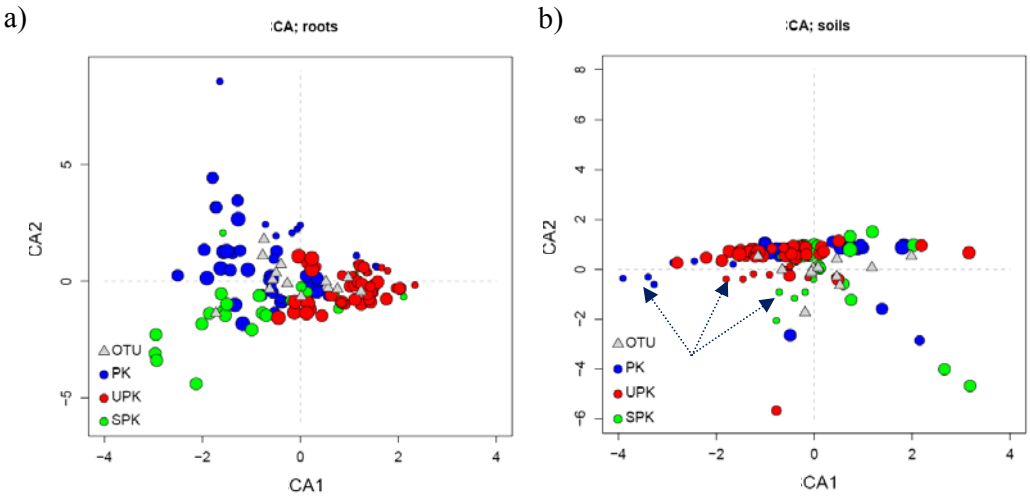
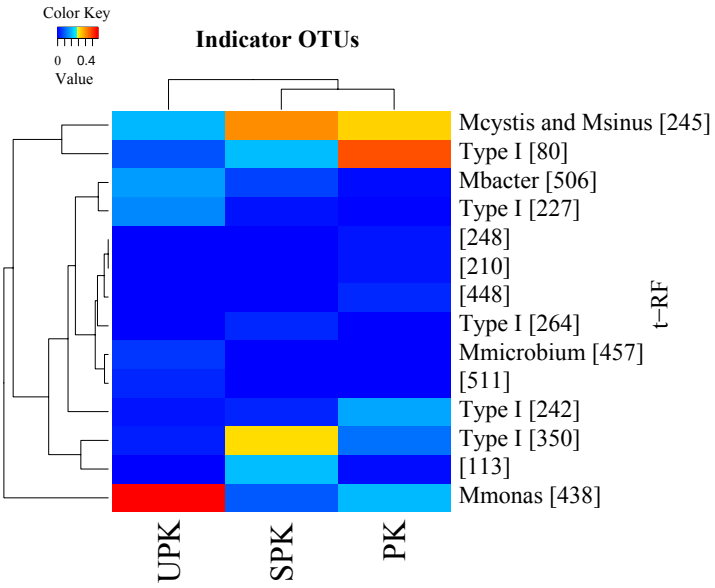



**Fig. 4.15:** CA ordination plot, based on Chi-squared distance, for the samples and T-RFs based on *pmoA* T-RFLP data from a) roots and b) rhizospheric soil samples. Triangles indicate different OTUs, i.e., T-RFs with different lengths. Colored circles code for samples from different treatments: blue, red and green circles for PK, UPK, and SPK treatments. The size of circles is proportional to the plant age. Three dashed arrows directing towards to the circles of early plant age (29 dap) indicates a separate cluster from other samples. The eigenvalues of the 1$^{st}$ and 2$^{nd}$ axes in the ordination diagram are as follows: $\lambda_1$ = 0.22, $\lambda_2$ = 0.06 for diagram 'a' and $\lambda_1$ = 0.07, $\lambda_2$ = 0.05 for diagram 'b'.

In the rhizospheric soil, methanotrophic community composition based on T-RFLP profiles were little affected by different nitrogen fertilizer treatments (ANOSIM statistic R: 0.06128; $P$<0.001; based on 1000 permutations). CA ordination plot (Fig. 4.15b) showed that methanotrophic communities from three treatments were closely clustered in the centroid. Only few samples from early plant age (29 dap), as represented

a)




b)

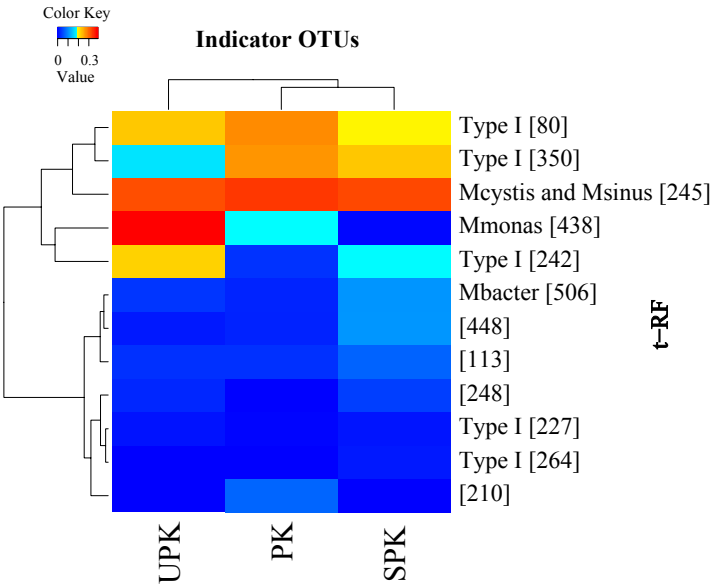



**Fig. 4.16:** Heat map showing species indicator values obtained for OTUs (T-RFs) for different treatments from a) roots, and b) rhizospheric soil. The color key bar on the top indicates the range of indicator values as blue to red where red color indicates the maximum value for each OTU. Different *pmoA*-OTUs are on the right hand side and dendogram on the left hand side shows the grouping of OTUs.

by smallest size of the circles, seemed to cluster separately for different treatments (indicated by dashed arrows in Figure 4.15b).

Indicator species analysis was further used to identify the T-RFs that were more prevalent and specific to different treatments in both roots and rhizospheric soil (Fig. 4.16a and 4.16b). This procedure examines the relative abundances and presence or absence of individual T-RFs in a predefined group compared to those expected to occur by chance (Dufrene and Legendre, 1997). On the roots samples (Fig. 4.16a), OTU affiliated to *Methylococcus*, *Methylocaldum,* and uncultured type I methanotrophs (type I methanotrophs, T-RF of 80 bp) had high indicator value for PK (control) treatment, OTU affiliated to *Methylomonas* (type I methanotrophs-TRF of 438 bp) had high indicator value for UPK treatment, and OTU affiliated to *Methylocystis and Methylosinus* (type II methanotrophs T-RF of 245 bp) had high indicator value for SPK treatment. In rhizospheric soil samples (Fig. 4.16b), OTU affiliated to *Methylocystis* and *Methylosinus* (type II methanotrophs, T-RF of 245 bp) had indicator value for PK, UPK, and SPK treatments while OTU affiliated to *Methylomonas* (type I methanotrophs T-RF of 438 bp) *had* additionally high indicator value for UPK treatment.

In Fig. 4.17, the two major indicator species were overlaid on the CA ordination plot shown in Figure 4.15a. The new plot (Fig. 4.17) shows the OTUs affiliated to *Methylomonas* (T-RF of 438 bp) and to *Methylococcus*, *Methylocaldum* and uncultured type I methanotrophs (T-RF of 80bp) as isolines with their relative abundance, demonstrating that UPK and PK treatments clustered separately because of the respective dominance of the two different indicator species.

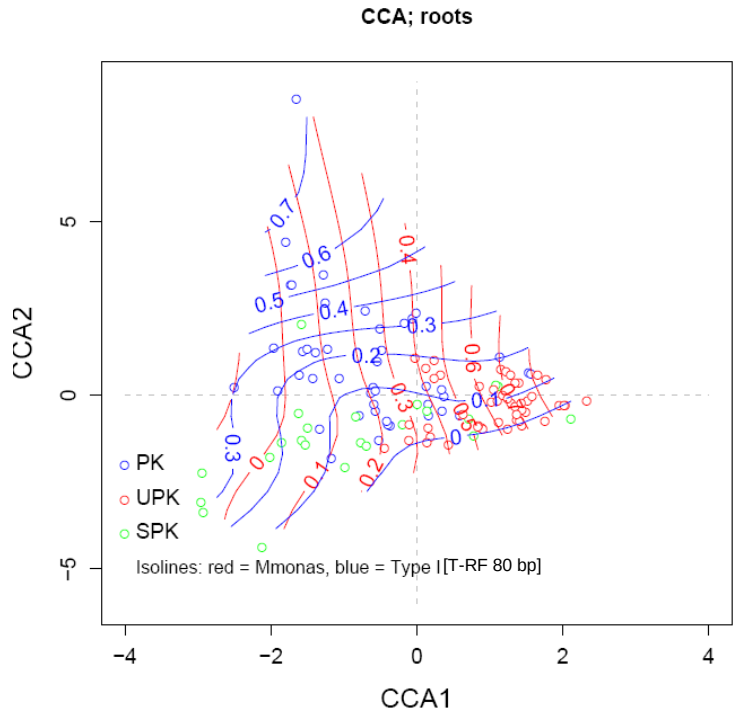
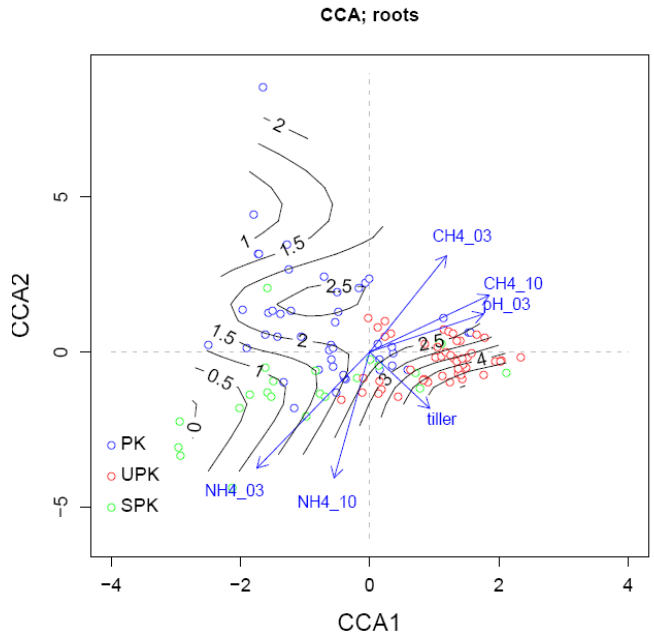



**Fig. 4.17:** CCA ordination plot from Fig. 4.15a with overlay of two indicator species *Methylomonas* and uncultured type I methanotrophs (T-RF 80 bp) as depicted by red and blue isolines with relative abundance values (shown in numbers) indicating separate clusters with different treatments.

### 4.4.8 Effect of environmental and functional factors on methanotrophic community structure

The T-RFLP data were further analyzed by ordination-based CCA to investigate the influence of environmental factors i.e., ammonium and methane concentrations, pH and tiller number, and functional factor i.e., methane oxidation rates, on the composition of the methanotrophic community structure. Environmental and functional variables were overlaid as constraints on CA ordination plots shown in Figures 4.15a and 4.15b. Some variables were removed from the ordination plot by the software itself because they were collinear (redundant) (e.g., pH at 10 cm in Fig. 4.18a). The correlation of treatment and most of the environmental factors were significant ($P$<0.001) with CCA ordination as shown in Table 4.2.

a)




b)

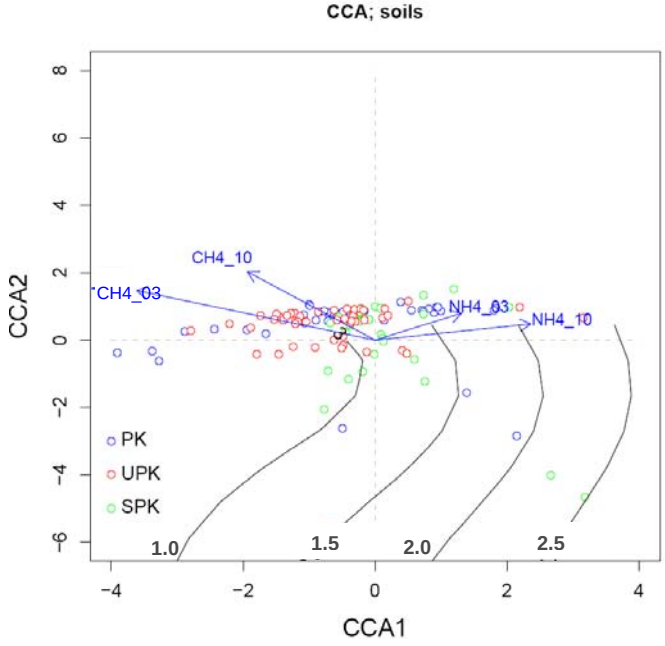



**Fig. 4.18:** CCA ordination plots based on Chi-squared distance for the effect of environmental variables (as shown by vectors) on the composition of methanotrophic community on a) roots and b) in rhizospheric soil, based on the samples from replicate microcosms of different treatments. The angles between the species vectors indicate correlation among species. Colored open circles indicate the samples from different treatments as blue, red and green circles code for PK, UPK, and SPK treatments. CH4_03 and CH4_10 indicate $CH_4$ concentrations at rhizospheric and bulk regions, respectively. NH4_03 and NH4_10 indicate $NH_4^+$ concentrations at rhizospheric and bulk regions, respectively. Isolines with numbers indicate different $CH_4$ oxidation rates. The

eigenvalues of the 1st and 2nd axes in the ordination diagram are as follows: $\lambda_1 = 0.22$, $\lambda_2 = 0.06$ for diagram 'a' and $\lambda_1 = 0.07$, $\lambda_2 = 0.05$ for diagram 'b'.

CCA ordination plot created from roots samples (Fig. 4.18a) showed that $CH_4$ concentration at bulk region (10 cm depth) and high $CH_4$ oxidation rates plotted as isolines separated UPK from PK and SPK treatments and $NH_4^+$samples at rhizospheric region (3 cm depth) seemed to separate SPK treatment from PK and UPK treatments. This is also seen from the correlation of the vectors with the ordination (Table 4.2). However, $CH_4$ oxidation rates did not play significant role on the community level (Table 4.3). CCA ordination plot from soil samples (Fig. 4.18b) showed that almost all environmental variables lay far apart from all samples since all the samples from different treatments were clustered together in the centroid.

**Table 4.2:** Effect of vectors on CCA ordination plot for roots.

| Vectors | CCA1 | CCA2 | $r^2$ | Pr (>r) | *P*-value |
|---|---|---|---|---|---|
| $NH_4$_03 | -0.58761 | -0.80915 | 0.2270 | <0.001 | *** |
| NH4_10 | -0.25613 | -0.96664 | 0.2021 | <0.001 | *** |
| CH4_03 | 0.52359 | 0.85197 | 0.1431 | <0.001 | *** |
| CH4_10 | 0.82229 | 0.56906 | 0.1033 | 0.002 | ** |
| pH_03 | 0.89206 | 0.45192 | 0.0745 | 0.015 | * |
| pH_10 | 0.92540 | -0.37899 | 0.3369 | <0.001 | *** |
| tiller | 0.57393 | -0.81890 | 0.0508 | 0.057 | . |
| leave | 0.25993 | -0.96563 | 0.0411 | 0.093 | . |

Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

The named axes give direction cosines of the vectors and $r^2$ gives the squared correlation coefficient. For plotting, the axes should be scaled by the square root of $r^2$ .The significances Pr (>r), or *P*-values are based on 1000 permutations.

**Table 4.3:** Analysis of similarities (ANOSIM) tested for different methane oxidation rates on roots associated methanotrophic community.

| $CH_4$ oxidation rate | ANOSIM statistic R | Significance value |
|---|---|---|
| 1 | 0.106 | 0.001 |
| 2 | 0.0104 | 0.3 |
| 3 | 0.00393 | 0.5 |
| 4 | -0.044 | 0.8 |

Significance values are based on 1000 permutations.

## 4.5 Discussion

The previous part of my study (chapter 3) indicated that the activity and population size of methanotrophs differed between the rice roots and rhizospheric soil as well as between two different sampling points. Such variation was assumed to be due to different concentrations of methane, oxygen and probably nutrients, which provide different niches for the methanotrophs. Therefore, this part of my research was mainly focused on the study of the effect of different nitrogen fertilizer treatments (PK, UPK, and SPK) on methane oxidation and methanotrophic community structure on the rice roots and in the rhizospheric soil during six different growth stages of rice plant. For this purpose, methane oxidation rates were compared among the different nitrogen fertilizer treatments and the methanotrophic communities in these treatments were studied based on the *pmoA* gene analysis using T-RFLP fingerprinting method. T-RFLP data were analyzed using CCA ordination techniques in order to investigate the effect of treatments, and environmental and functional factors.

Fertilization of rice plants with different ammonium-N fertilizers along with phosphorous and potassium, resulted in more healthy and dense plants (highest number of tiller and leave). The root development was better in SPK treatment followed by UPK treatment as compared to PK treatment (a control without nitrogen addition). These findings were supported by the rapid depletion of ammonium in both UPK and SPK treatments during most of the rice-growing season indicating the rapid consumption of ammonium by rice plant. Therefore, it could be assumed that increased plant growth might have affected plant ventilation (via better roots development) and roots exudation, which might have affected methane turnover. This assumption was supported here by the increased methane flux in UPK and SPK treatment after fertilization on 57 dap, while such response was absent in PK treatment indicating the influence of nitrogen fertilizer compared to other fertilizers.

$CH_4$ flux and $CH_4$ oxidation rates were similar in PK and UPK treatments for most of the time during the cropping period indicating no N-fertilizer treatment effects on methane oxidation in this study. Similar effect was observed in previous studies (Bykova *et al*., 2007; Cai *et al*., 1997; Delgado and Mosier, 1996; Dunfield *et al*., 1995). This might be

explained by the proposed mechanism given by Schimel (2000). According to Schimel (2000), at the plant/ecosystem level, nitrogen fertilizers stimulate $CH_4$ production by increasing rice plant growth and increasing the carbon supply for methanogens thus stimulating methane flux from the soil (Dan *et al*., 2001; Bodelier *et al*., 2000). Similarly, at the level of the microbial community, nitrogen stimulates the growth and activity of methanotroph, leading to reduced net efflux (Bodelier *et al*., 2000). Therefore, in my study, where high amount of nitrogen fertilizer was applied in UPK treatment, it could be assumed that increased methane production by methanogens at the same time, might have been counter balanced by methane oxidation by methanotrophs. As a consequence, no effect could be observed in either methane production or methane oxidation process. However, a transient positive influence was observed for methane oxidation in UPK treatment after fertilizer addition on 57 dap. Similar result was also reported by Krüger and Frenzel (2003).

Consistent to other studies (Minamikawa *et al*., 2005; Cai *et al*., 1997; Kesheng and Zhen, 1997; Denier van der Gon and Neue, 1994; Lindau, 1994), my study also demonstrated that in the SPK treatment, low methane concentrations and low methane flux were observed. This was because of suppression of methanogenesis by high sulphate concentrations. Therefore, SPK treatment was not comparable with PK and UPK treatments. There was no decrease in sulphate concentration as observed in rhizospheric region till 57 dap, which indicated regeneration of sulphate by the oxidation of reduced sulfur. The dense roots in SPK treatment might have released sufficient amounts of $O_2$ required for this process. After fertilization on 57 dap (Fig. 4.6c), sulphate concentration increased by a factor of three and resulted in a decrease of pH in the pore water of the rhizospheric region (Fig. 4.7a). pH value slowly recovered back consistent with a decrease of the sulphate concentration. The decreased sulphate concentration probably improved the conditions for $CH_4$ production, and might be the explanation for the increased pore water methane concentrations, methane flux, and methane oxidation rates in the late period. Similar increase of methane flux in the late period was reported by Minamikawa *et al*. (2005).

The determination of *pmoA*-based T-RFLP profiles and subsequent affiliation to clone sequences and CA ordination indicated that the methanotrophic community structure was significantly affected by different treatments; however, the effect was stronger on the

roots and weaker in the rhizospheric soil (Fig. 4.12 and 4.15b). This indicated that the roots and rhizospheric soil provided two completely different environments for methanotrophs (Henckel *et al*., 2000).

The methanotrophic community associated with the roots and the rhizospheric soil contained both type I and type II methanotrophs. This finding was consistent to the experiment described in chapter 3 and to the reports by Eller and Frenzel (2001) and Henckel *et al* (2000), all using the soil from the same rice-field. However, type I methanotrophs were dominant on the root samples (except few samples of SPK treatment) (Fig. 4.9). The methanotrophic community composition as well as relative abundance consistently changed in PK and SPK treatments at all sampling time points, but in UPK treatment a relatively stable methanotrophic community existed throughout the period except on 88 dap (Fig. 4.9). In further detail, the relative abundance of type I and type II methanotrophs changed a lot in PK and SPK treatments, while in UPK treatment type I methanotrophs were predominant consisting of more than 75% (especially *Methylomonas* with more than 50%) of the total community at all sampling time points except on 88 dap. These findings indicated a strong temporal variation in PK and SPK treatment while not in UPK treatment. My result showed that PK and SPK treatments suffered from $NH_4^+$ (Fig. 4.6) and $CH_4$ (Fig. 4.5) limitation, respectively, during most of the times of rice-growing season. Furthermore, $NH_4^+$ concentration at rhizospheric region (at 3 cm depth) seemed to be responsible for the change in methanotrophic community in SPK treatment in comparison to PK and UPK treatments as revealed by CCA ordination (Fig 4.18a and Table 4.2). This indicated a response of low $CH_4$: $NH_4^+$ ratio on methanotrophic community in SPK treatment.

In UPK treatment $CH_4$, $O_2$ and $NH_4^+$ concentrations were probably sufficient allowing a relatively consistent and stable methanotrophic community throughout the entire rice-growing season except on 88 daps. Moreover, $CH_4$ concentration at bulk region (at 10 cm depth) and high methane oxidation rates seemed to be responsible for separating methanotrophic community from that of other treatments as revealed by CCA ordination (Fig. 4.18a and Table 4.2). However, there was no significant effect of methane oxidation rates on methanotrophic community in UPK treatment (Table 4.3). Another explanation of a consistent methanotrophic community with dominant type I methanotrophs (especially genus *Methylomonas*) in UPK treatment might also be due to their adaptability

to urea-N fertilizer. The rice fields in Vercelli, Italy (the source of soil that was used in my experiments) have been continuously fertilized with urea as N source for many years. Therefore, urea-N on the roots might lead to a selection of adapted methanotrophic community from the soil around the roots since rice field soil may serve as a seed bank for methanotrophs (Eller and Frenzel, 2001).

Interestingly, indicator species for each treatment (Fig. 4.16a and 4.17) revealed that on the roots, OTUs (T-RF of 80 bp) affiliated to uncultured type I methanotrophs (separate cluster deeply branched from *Methylocaldum* and *Methylococcus), Methylomonas* and *Methylocystis* were the important species for PK, UPK and SPK treatments, respectively. Besides these indicator species, there were few other genera that seemed also to be important for respective treatments. For instance, five sequences of uncultured type I methanotrophs (T-RF of 374 bp) was obtained only in PK treatment on 57 dap and none in other treatments. Another example was the presence of a single clone that was closely related to high-affinity methanotrophs belonging to upland soil cluster alpha (USCα) on the roots samples of SPK treatment on 57 dap (Fig. 4.10). The appearance of USCα which harbors in low methane concentrations (Henckel *et al*., 2000; Holmes *et al*., 1999; Knief *et al*., 2003; Kolb *et al*., 2005) could be attributed to low $CH_4$ concentration in SPK treatment. Thus, all of these findings suggested that on the roots, methanotrophic genera responded differently to different N-fertilizer treatments as evidenced by statistical analysis revealing significant effect of treatments on methanotrophic community. Nevertheless, the inhibition effect of $SO_4^{2-}$ fertilizer resulting in low $CH_4$ concentration, might have selected a different methanotrophic community in SPK treatment.

In case of rhizospheric soil, all treatments (PK, UPK and SPK) were dominated by a relatively stable type II methanotrophic community at almost all sampling time. This indicated that type II methanotrophic community structure appeared to be stable and did not change with time. Similar findings were reported by Henckel *et al*. (1999, 2000) and Bodelier *et al*. (2000). Indicator species for each treatment (Fig. 4.16b) showed that in the rhizospheric soil, OTUs affiliated to *Methylocystis* (type II methanotrophs) were the important species for PK, UPK, and SPK treatments while *Methylomonas* was an additional important species for UPK treatment. Analysis of the genus level of type I methanotrophs revealed further differences among the treatments, based on *pmoA* T-RFLP profiles and *pmoA* sequences. For instance, T-RF of 438 bp, affiliated to

*Methylomonas* was dominant on 29 dap in PK treatment while it was dominant on 57 dap in UPK treatment. As an another examples, in SPK treatment, T-RF of 506 bp, affiliated to *Methylobacter,* was increasingly dominant on 57 dap which was quite rare in PK and UPK treatments. Thus, these findings suggested that methanotrophs, especially type I methanotrophs, in rhizospheric soil responded differently to different N-fertilizer treatments. Nevertheless, weak but significant effect of nitrogen fertilizer was observed in overall methanotrophic community in the rhizospheric soil, probably due to high relative abundance of type II methanotrophs.

In conclusion, different nitrogen fertilizers affected methanotrophic community structure without significantly affecting the rates of methane oxidation.

# Chapter 5

## Study of metabolically active methanotrophs in the rice rhizosphere using *pmoA* transcripts at different growth stages of rice plant

## 5.1 Introduction

In the last few decades, cultivation-independent molecular techniques have been intensively applied to investigate the microbial diversity and quantify predominant organisms in natural microbial communities (Neufeld and Mohn, 2006). Some of the widely used techniques for studying the methanotrophic community diversity include 16S rRNA gene and functional gene (*pmoA, mmoX, mxaF*) targeted polymerase chain reaction (PCR) based cloning and sequencing (Miller *et al*., 2004; Uz *et al*., 2003), microarray analysis (Bodrossy *et al*., 2006), degenerating gradient gel electrophoresis (DGGE) (Bodelier *et al*., 2000; Bourne *et al*., 2001; Eller *et al*., 2005; Jia *et al*., 2007: Wise *et al*., 1999) and terminal restriction fragment length polymorphism (T-RFLP) (Horz *et al*., 2005; 2001; Pester *et al*., 2004; Shrestha *et al*., 2008). Although these DNA-based approaches provide the genetic potential of methanotrophic community present in different environments, they do not give information about metabolically active methanotrophs. One of the possibilities to overcome this limitation is to study the mRNA transcripts, for instance, *pmoA* transcripts in case of methanotrophs.

In addition to PCR-based gene detection, analysis of mRNAs of indicator genes should significantly enhance our understanding of active functional groups in the environment. Detection of mRNAs, which have a short half-life (Robinson *et al*., 1998), is the best indicator of metabolically active cells or specific activity in a complex microbial community. Although detection of mRNAs has been hindered by difficulties in the extraction of intact RNA from environmental samples (Saleh-Lakha *et al*., 2005), recent advances in extraction methods for mRNA (Bürgmann *et al*., 2001; Griffiths *et al*., 2000; Shrestha, Dissertation, 2007) may facilitate the direct analysis of functional gene expression of active methanotrophs in the environment. A number of recent studies have focused on the analysis of expression of methane monoxygenase in different environment using *pmoA* specific primer sets. These include the analysis of soil (Han and Semrau, 2004; Kolb *et al*., 2005), fresh water sediment (Cheng *et al*., 1999; Nercessian *et al*., 2005), landfill (Chen *et al*., 2007) and peatlands (Chen *et al*., 2008), providing direct *in situ* evidence of active methanotrophs. However, expression of *pmoA* in rice rhizosphere under *in situ* conditions has not yet been investigated.

Therefore, in this part of my research, I have compared the methanotrophic community composition and diversity, related to different fertilizer treatment and different growth stages, based on *pmoA* gene and *pmoA* transcripts. This approach shall distinguish total diversity of methanotrophs from that of metabolically active methanotrophs in the rhizospheric soil at the time of sampling, and the latter might then be correlated with the physiological conditions.

## 5.2 Objectives

- To study the effect of different ammonium-N fertilizer treatments on methane oxidation potential activity and metabolically active methanotrophs in the rhizospheric soil during different growth stages of the rice plants.
- To compare the effect of different N-fertilizer treatments on total and metabolically active methanotrophs in rhizospheric soil.

## 5.3 Methods

This part of study was based on the experimental setup and microcosm experiment that was used in chapter 4.

### 5.3.1 Experimental setup

Refer to chapter 4.3.1.

### 5.3.2 Microcosm experiment

Refer to chapter 4.3.2.

### 5.3.3 Collection of soil and root samples

Rhizospheric soil and root samples used were collected from the microcosm experiment described in chapter 4.3.2.

### 5.3.4 Methane oxidation potential assay

Refer to chapter 2.2.6.

### 5.3.5 Total nucleic acids extraction

Total nucleic acids from the soil and root samples was extracted using total nucleic acids extraction protocol as described in chapter 2.2.7.1.

### 5.3.6 Total RNA isolation

Total RNA isolation was carried out as described in chapter 2.2.7.2.

### 5.3.7 RT-PCR of *pmoA* transcripts

Refer to chapter 2.2.7.4.

### 5.3.8 Terminal restriction fragment length polymorphism (T-RFLP)

As described in chapter 2.2.7.4, a semi-nested approach was used for analysis of terminal restriction fragment length polymorphism (T-RFLP) of *pmoA* gene transcripts. T-RFLP

of *pmoA* gene transcripts was performed by using cDNA products from the rhizospheric soil samples in triplicate as described in chapter 2.2.9. In the first PCR step, cDNA products were amplified using the primer set A189f/A682r. In the second PCR step, an aliquot (1 µl) of the amplified product from the first step was used for PCR amplification using the primer set A189f/mb661r where a FAM (6-carboxyfluorescein)-labeled forward primer was used. However, for a few samples, RT-PCR with the primer set A189f/mb661r did not work. Therefore, a degenerate reverse primer, nmb650r, was designed based on the 650 reverse primer (Bourne *et al*, 1999) and was used in the second round of PCR (mentioned in chapter 4.3.8). Before performing these amplifications, this new reverse primer was tested against mb661 reverse primer on few of the samples which were successfully amplified using A189f/mb661r primer set for T-RFLP analysis to check the primer performance. The result showed that both A189f/mb661r and A189f/nmb650r primer sets gave the similar T-RFLP patterns (Fig.5.1). Therefore, the nmb650 was used as reverse primer in conjunction with the forward primer A189 for the rest of the samples to amplify the PCR products for T-RFLP analysis.

All PCR amplified products were purified using gel purification with Wizard ® SV Gel and PCR Cleanup System (Promega, Germany) and then used for T-RFLP analysis as described in chapter 2.2.9. The normalization procedure of each TRF profile was done as described in chapter 2.2.9.1 and followed with calculation of relative abundance as described in chapter 2.2.9.2.

### 5.3.9 Cloning and sequencing

To affiliate the different TRFs and to analyze the clone frequency, three clone libraries were generated from mRNA extracts of rhizospheric soil samples (PK-40, UPK-40, and SPK-40 dap).

### 5.3.10 Phylogenetic analysis

Refer to chapter 2.2.8.

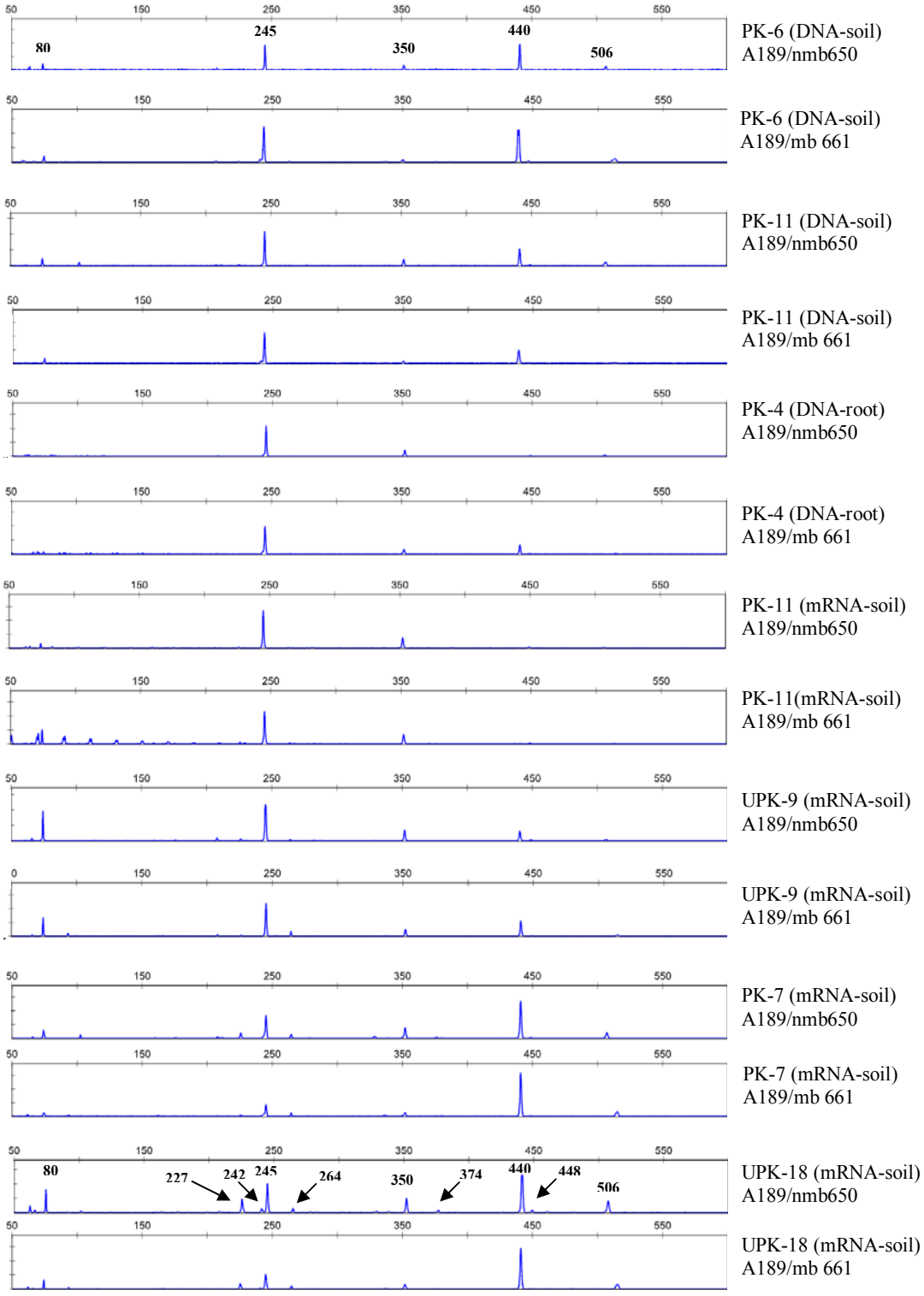



**Fig. 5.1:** Comparison of T-RFLP patterns based on *pmoA* gene as revealed by primer sets A189f/nmb650r and A89f/mb661r on few samples. T-RFLP analysis was carried out to check

reproducibility in different samples. Name of samples at right hand side indicate different DNA samples from rhizospheric soil (DNA-soil), roots (DNA-root) and mRNA sample from rhizospheric soil (mRNA-soil) with two different primer sets. The numbers in T-RFLP profiles indicate the length (bp) of the different T-RFs.

## 5.4 Results

### 5.4.1 Methane oxidation potential

In this study, methane oxidation potential was determined on the roots (Fig. 5.2) and in the rhizospheric soil (Fig. 5.3) using different treatments from six different growth stages i.e., 29, 40, 57, 62, 67, and 88 days after transplantation except for root samples from 29 dap.

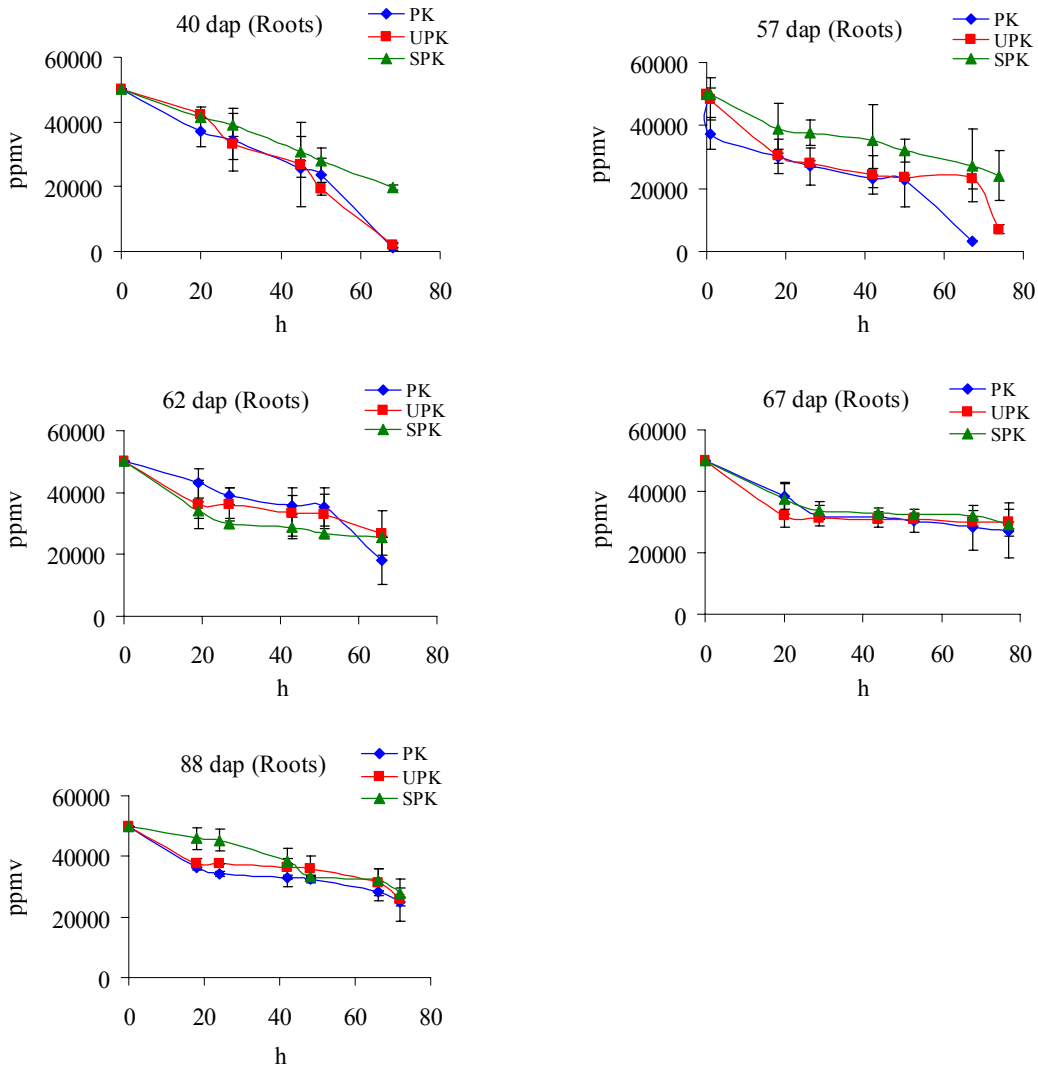



**Fig. 5.2:** Oxidation of $CH_4$ at a mixing ratio of 50,000 ppmv in tubes containing roots from six different sampling time points (40, 57, 62, 67 and 88 dap as shown at top of each plot) were observed at different incubation period (hour). Blue, red and green colors code for PK, UPK, and SPK treatments; mean ± SD (n=3).

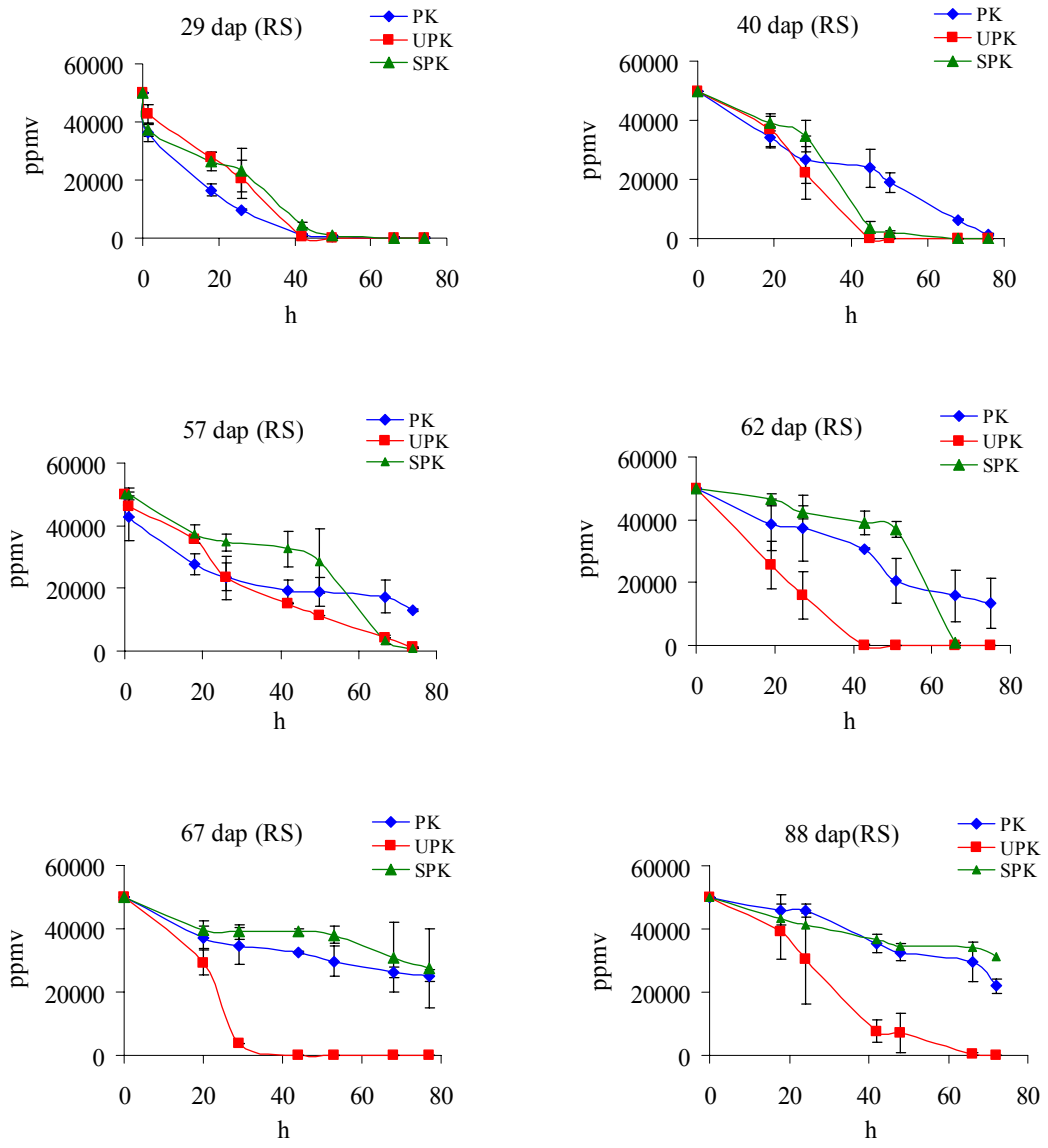



**Fig. 5.3:** Oxidation of $CH_4$ at a mixing ratio of 50,000 ppmv in tubes containing rhizospheric soil from six different sampling time points (29, 40, 57, 62, 67, and 88 dap as shown at top of each plot) were observed at different incubation period (hour). Blue, red and green colors code for PK, UPK, and SPK treatments; mean ± SD (n=3).

On the roots, methanotrophs exhibited immediate and fast potential activities on 40 dap. After 40 dap, roots samples exhibited much less activity with a long lag phase (Fig. 5.2). Methane oxidation potential rates were not calculated for the roots samples. Methane oxidation started immediately in all rhizospheric soil samples (PK, UPK, and SPK treatments) without any lag phase (Fig. 5.3). Methane oxidation potential rates were

significantly ($P$<0.01) different among the treatments on 62 and 88 dap. The methane oxidation potential (MOP) rates were significantly lower ($P$<0.01) in control than in UPK treatment during entire rice growing season. There was no significant differences ($P$>0.01) in MOP rates of two nitrogen fertilizer treatments until 57$^{th}$ days of transplantation. However, MOP rates dropped significantly ($P$<0.01) in SPK treatment after 62 dap and remained significantly lower than in control and UPK treatments.

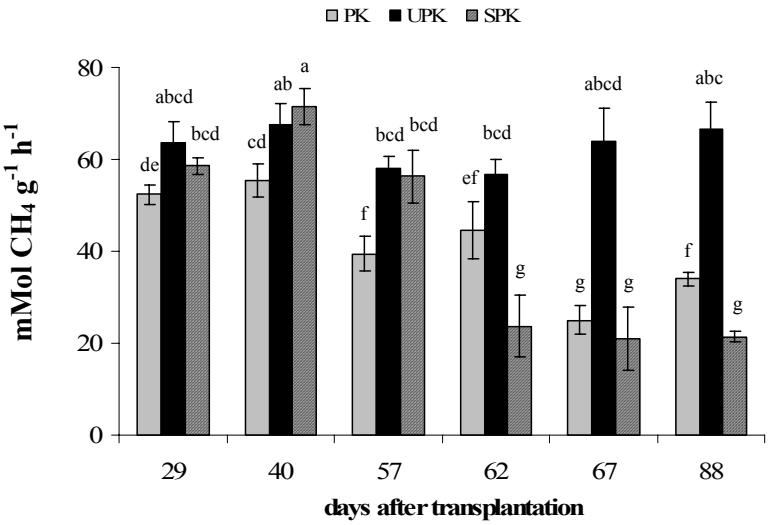
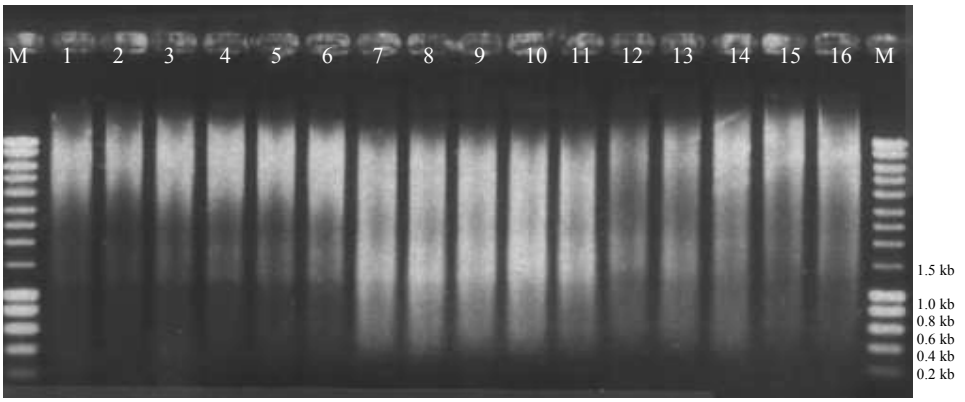



**Fig. 5.4:** Methane oxidation potential measured in rhizospheric soil samples (calculated from result shown in Fig. 5.3) from different sampling periods in PK, UPK, and SPK treatments; mean ± SD (n=3). Different letters indicate significant difference ($P$<0.01) between the means of the different treatments.

### 5.4.2 Amplification of *pmoA* transcripts in rhizospheric soil

Total RNA was isolated using the same nucleic acids extract obtained from chapter 4.3.6. High quality of total nucleic acids (absorbance ratio of 280/260 = 1.83) and total RNA was obtained (Fig. 5.5a and Fig. 5.5b). cDNA synthesized from total RNA extract from rhizospheric soil samples was successfully amplified for *pmoA* transcripts of 509 bp length by using a semi-nested approach using primer sets A189f/A682br (Fig. 5.6, Lanes 1-3) in the first step and then A189f/mb661r or A189f/nmb650r (Fig. 5.6, Lanes 4-6) in the second step. To ensure complete removal of genomic DNA, a negative control of same RNA template was also simultaneously run with all RT-steps excluding the reverse transcriptase enzyme (Fig. 5.6, Lanes 7-9).

a)




b)

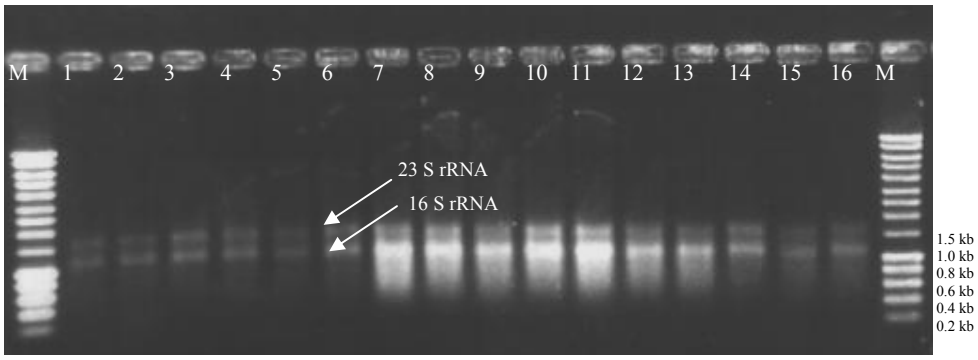



**Figure 5.5:** Gel electrophoresis of a) total nucleic acids obtained from rhizospheric soil (Lanes 1-16; M is size marker and b) total RNA after DNA digestion (Lanes 1-16; M is size marker).

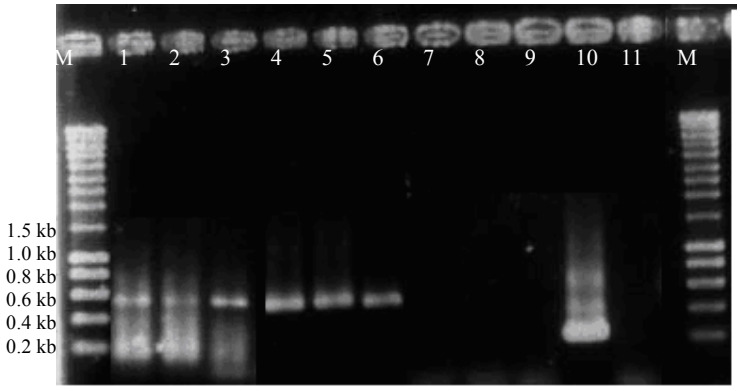



**Figure 5.6:** Gel electrophoresis of RT-PCR products of mRNA transcripts obtained using the primer set A189f /A682r (Lanes 1-3), primer set A189f /mb661r (Lanes 4-6), negative control as RNA templates without RT-step (Lanes 7-9), positive control (RNA control provided in RT-PCR kit) for RT-PCR (Lane 10), negative control (without template) (Lane 11), and M is the size marker.

### 5.4.3 T-RFLP and cloning/sequencing of *pmoA* transcripts in rhizospheric soil

*pmoA* transcripts that were presumably responsible for *in situ* methane oxidation were successfully retrieved from rhizospheric soil samples from six different growth stages of the rice plant (Fig 5.7). Here, T-RFLP patterns of transcripts from three replicates from the same microcosm as well as from three replicate microcosms of each treatment were analysed for tube to tube and pot to pot variations. Variation among the replicates was checked for major T-RF peaks, which were highly reproducible. Therefore, the relative abundances of T-RFs of all the replicates (5-9 replicates) were averaged for each treatment and time point.

T-RFLP analysis produced highly reproducible patterns with T-RFs of 80, 113, 210, 227, 242, 245, 264, 278, 350, 364, 440, 448, 457 and 506 base pairs (bp) lengths in most of the samples. T-RFLP profiles obtained from rhizospheric soil, revealed a highly diverse pattern of the active methanotrophic community at the different sampling times (Fig. 5.7 a, b, c). Furthermore, PK and UPK treatment revealed similar T-RFLP patterns, while SPK treatment showed a different one.

In PK treatment (Fig. 5.7a), composition and relative abundances of major T-RFs, (T-RFs of 80, 245, 350, and 438 bp) changed with sampling times. For example, T-RF of 438 and 245 bp were dominant with relative abundance of 52% and 16% on 29 dap and adversely changed to 12% and 74% on 40 dap (Fig. 5.7a). Similarly in UPK treatment (Fig. 5.7b), composition and relative abundances of major T-RFs, (T-RFs of 80, 245, 350, and 438 bp) changed with sampling times. T-RF of 264 bp appeared at all sampling time points except on 67 dap. T-RF of 506 bp was obtained in almost all samples except on 40 dap. Additionally, small changes in composition and abundance in T-RFs of 113, 210, 227, 242, 375 bp sizes could be observed in UPK treatment at different sampling time points.

In SPK treatment (Fig. 5.7c), two major T-RFs i.e. 245 and 350 bp size were abundant at all sampling time points and the relative abundance of these two T-RFs consecutively changed during the four initial sampling time points. The T-RF of 245 bp was predominant, while the T-RF 350 bp was less dominant. The other T-RFs, however, appeared and disappeared sporadically.

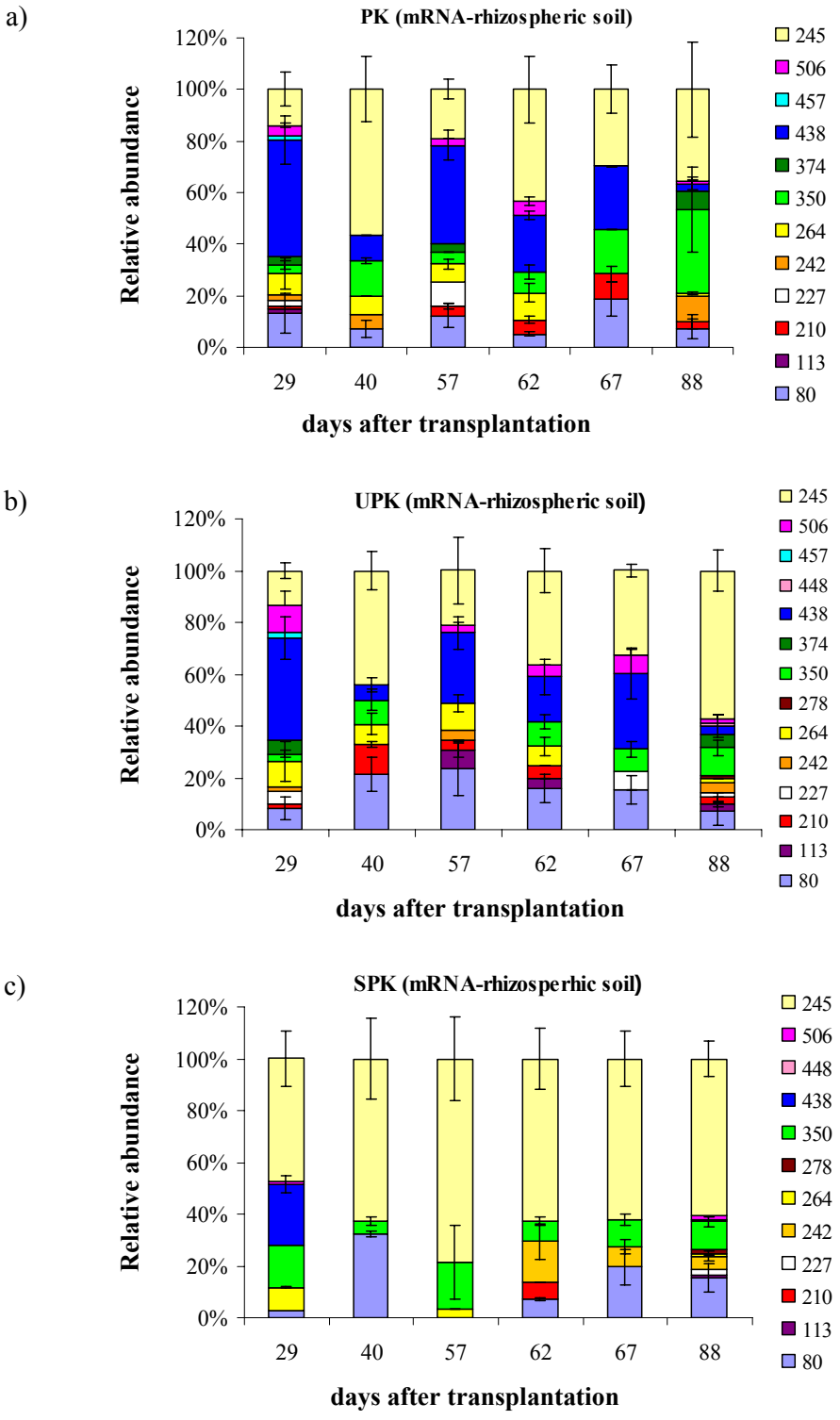



**Fig. 5.7:** Relative abundance calculated for *pmoA* transcripts from rhizospheric soil samples from a) PK treatment, b) UPK treatment, and c) SPK treatment from different sampling points; mean ± SD (n = 5-9).

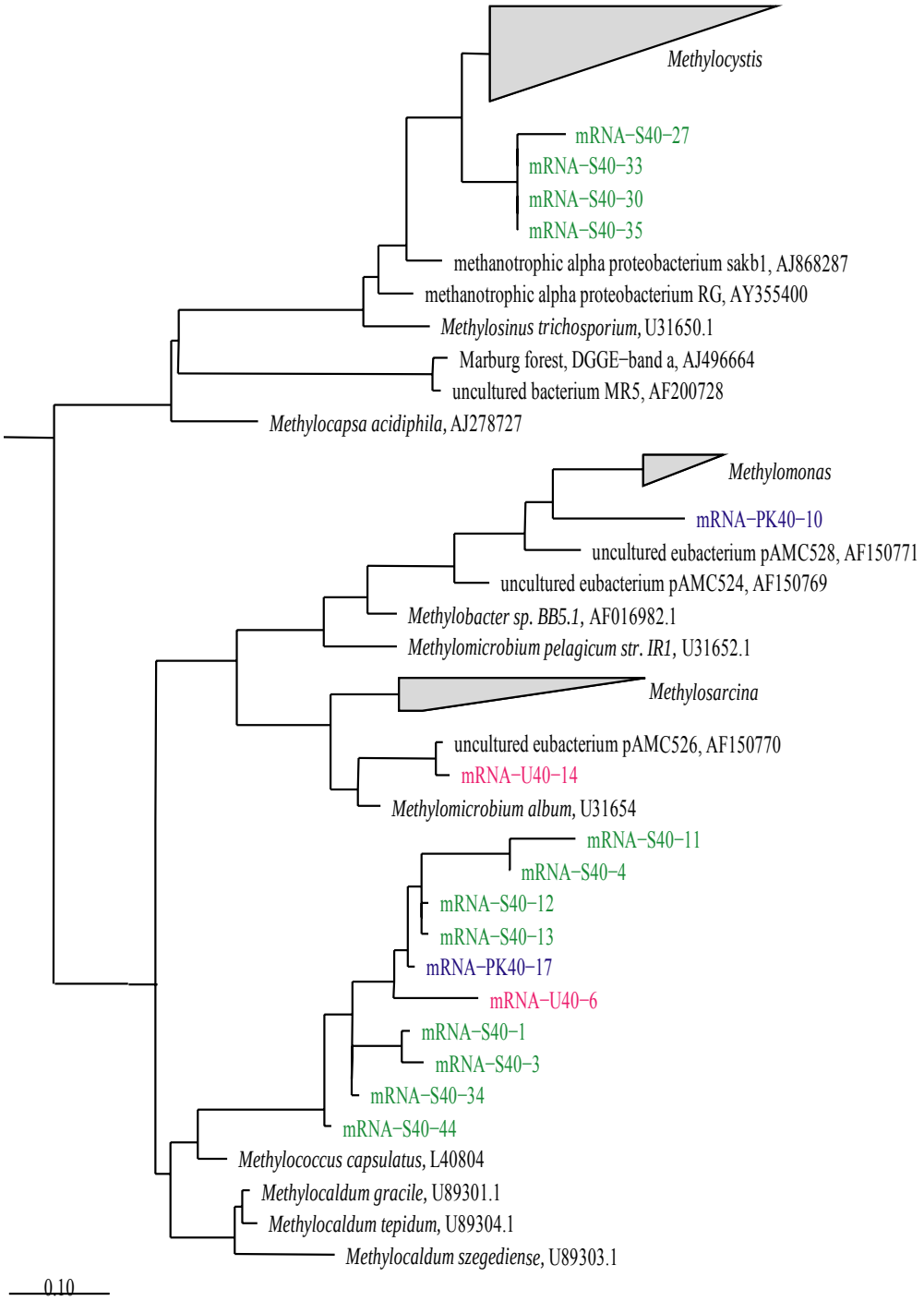



**Fig. 5.8:** Neighbor-joining tree showing the phylogenetic analysis of the derived amino acid sequences encoded by *pmoA* genes from rhizospheric soil. Blue, red and green color indicates representative clones from PK, UPK and SPK treatments, respectively. The scale bar represents 10% sequence divergence.

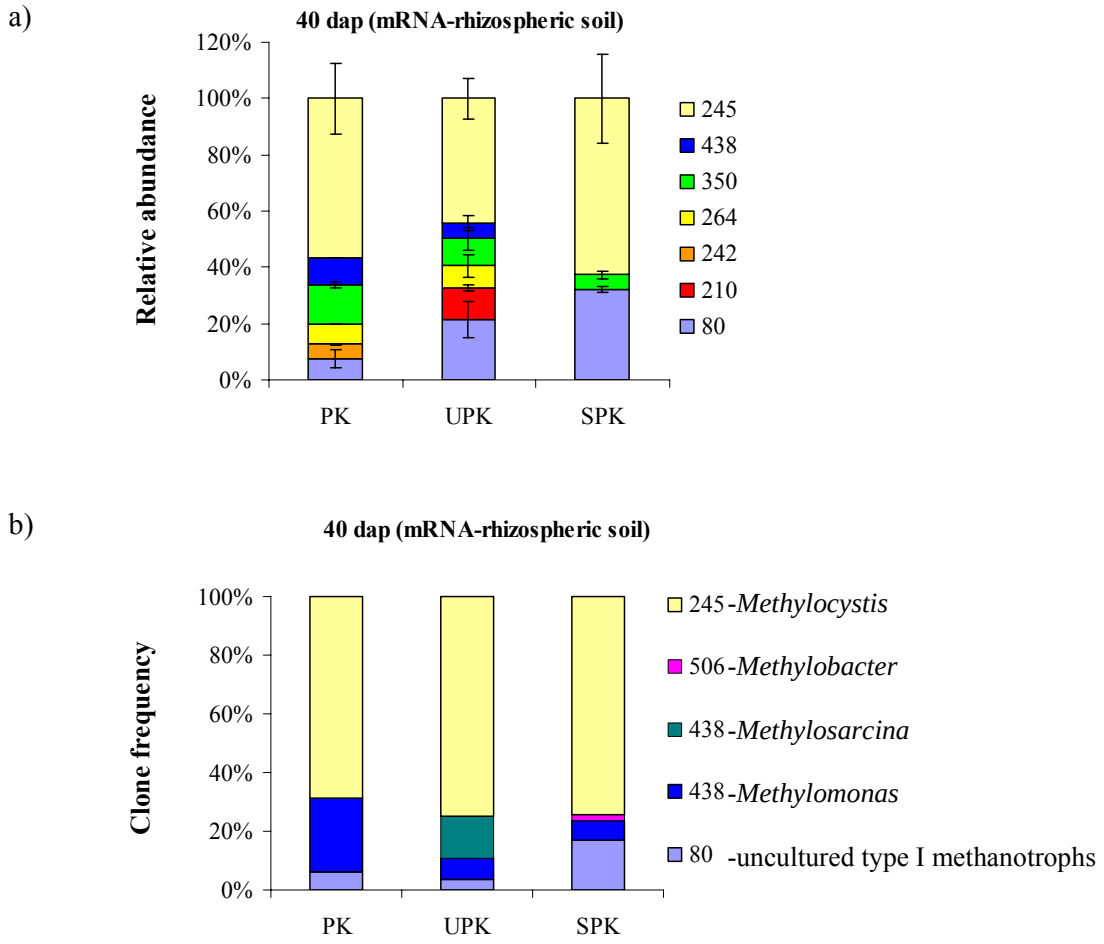



**Fig. 5.9:** Comparative analysis between a) Relative abundance, and b) clone frequency for *pmoA* transcripts retrieved from rhizospheric soil samples from PK, UPK, and SPK treatment from 40 dap; mean ± SD (n = 5-9) for T-RFLP analysis.

Cloning and sequencing of *pmoA* transcripts was carried out for rhizospheric soil samples from 40 dap (Fig. 5.8 and 5.9b). The T-RFs were assigned to the respective genera of methanotrophs by using the clone libraries obtained from PCR products from rhizospheric soil samples as 80 bp – uncultured type I methanotrophs, 245 bp – *Methylocystis* (type II methanotrophs), 438 bp – *Methylomonas*, and *Methylosarcina,* and 506 bp – *Methylobacter*. However, using these clone libraries none of the obtained sequences could be affiliated to the unknown T-RFs (113, 210, 278, and 448). Clone frequency revealed a similar pattern as the relative abundance of T-RFs, however, clone frequency provided a less diverse transcription pattern than that obtained by T-RFLP analysis (Fig. 5.9a and 5.9b).

After affiliation of T-RFs to the respective sequences, rhizospheric soil samples revealed a diverse transcription pattern, representing both type I and type II methanotrophs, on the basis of relative abundance and composition, in all treatments as well as at all growth stages. Overall, relative abundance of type I methanotrophs transcript was high in most of the PK and UPK treatment samples whereas, relative abundance of type II methanotrophs transcript was high in most of the SPK treatment samples.

## 5.5 Discussion

In the previous part of my study (chapter 4), *pmoA* gene-based T-RFLP profiles did not show any strong effect of different nitrogen fertilizer treatments on the methanotrophic community structure in the rhizospheric soil. Instead, a relatively stable community pattern was observed. In the present part, my research mainly focused on *pmoA* expression and the metabolically active methanotrophs that were responsible for *in situ* methane oxidation in rhizospheric soil under different fertilizer treatments and at different growth stages of the rice plant. Furthermore, the metabolically active diversity of methanotrophs was compared with the total methanotrophic diversity.

### 5.5.1 Methane oxidation potential

Methane oxidation potential tests were performed to assess the effect of different fertilizer treatments (PK, UPK, and SPK) on methanotrophic activity associated with roots and rhizospheric soil. Lower methane oxidation rates with long lag phase were obtained on the roots as compared to rhizospheric soil, indicating a quite different physiological state of methanotrophs between roots and rhizospheric soil. However, a direct comparison of the rates in soil and roots is not possible, because of the reference to either root mass or soil mass, respectively (Eller and Frenzel, 2001). Quick response in the methane oxidation potential measurement throughout all the growth stages indicated the presence of active methanotrophs in rhizospheric soil for all treatments. This result was further confirmed by the detection of *pmoA* expression in all samples. Methane oxidation potential rates were significantly lower in control treatment than in UPK treatment during entire rice growing season and than in SPK treatment at least initial three growth stages thus indicating the stimulating effect of nitrogen fertilizer on methanotrophic community. This result was in consistent to the findings reported by Bodelier et al (2000) where they have also found the higher potential activity in nitrogen fertilizer (urea or $(NH_4)_2HPO_4$) added soil slurry as compared to unfertilized soil slurry. However, the pattern of methane oxidation potential activity was not consistent to *in situ* methane flux measurements result (chapter 4) where methane oxidation rate or methane flux were almost similar between control and UPK treatments for most of the rice growing season. Probably, the presence of rice plant or roots exudates might play a role in the process that occurred in the rice microcosms, which could not be retrieved in soil slurry rather than the direct effect of any

factor like nitrogen fertilizer on 0.25 g of dry soil per ml (Bodelier et al, 2000).

### 5.5.2 Gene expression of methanotrophs in rhizospheric soil

The T-RFLP analysis of *pmoA* transcripts showed a diverse pattern of expression that corresponded to the potentially active genera observed in the community fingerprints of methanotrophs in the different treatments. The transcription pattern also indicated rapid changes of transcriptional activity over time, indicating a strong temporal variation in all treatments. This suggested that metabolically active methanotrophs responded fastly and actively to changing environments (Henckel *et al*., 2000) responding to temporal variation in $CH_4$ and $NH_4^+$ concentrations (chapter 4.4.3 and chapter 4.4.4) and probably to variation in $O_2$ concentrations released by roots, during rice-growing season (Frenzel *et al*., 1992).

Interestingly, the number of T-RFS of *pmoA* transcripts retrieved from PK and UPK treatments was higher than those retrieved from SPK treatment (Fig. 5.7). This indicated that PK and UPK treatment revealed a higher diversity of active methanotrophs than SPK. A complete overview of T-RFLP pattern revealed that *pmoA* transcripts retrieved from PK and UPK treatments were similar, whereas *pmoA* transcripts retrieved from SPK treatment were quite different. Nevertheless, some differences among the transcripts in PK and UPK treatments were evident at different sampling times (Fig. 5.7a and 5.7b).

Type I methanotrophs were dominant in all rhizospheric soil samples of PK and UPK treatments except at some time points (40 dap in PK, and 40 and 88 dap in UPK treatment) (Fig. 5.7a and 5.7b). This result revealed that in PK and UPK treatments, type I methanotrophs were mainly responsible for *in situ* methane oxidation in rhizospheric soil during most of the time of rice-growing season. Recently, Noll *et al*. (2008) and Qiu *et al*. (2008) also reported that type I methanotrophs were metabolically active in urea-treated soil slurry and in rice field experiments, respectively, using RNA based-stable isotope probing of 16S rRNA.

Type II methanotrophs were dominant in rhizospheric soil samples of SPK treatment (Fig. 5.7c) indicating that type II methanotrophs were mainly responsible for *in situ* methane oxidation under those conditions. The predominance of type II methanotrophs in

SPK treatment was supported by the previous findings (Henckel *et al*., 2000, Hanson and Hanson, 1996) which suggested that type II methanotrophs survive better than type I methanotrophs under adverse conditions. Conditions in SPK treated soil were probably adverse since $CH_4$ production was inhibited by high sulphate concentrations and $CH_4$ concentrations were low (see chapter 4).

### 5.5.3 Comparative diversity of total and active methanotrophs in rhizospheric soil

Total methanotrophic community structure revealed a similar pattern of T-RFs at all growth stages of plant development in all treatments whereas active methanotrophic structure revealed a more diverse and changeable pattern of T-RFs. This result is reasonable since the total community consists of both live (active and inactive) and dead cells, whereas the active community only consists of metabolically active cells, which respond to changing environmental conditions.

Total methanotrophic diversity in rhizospheric soil samples consisted of mainly type II methanotrophs with more than 60% relative abundance. This result is quite consistent with previous studies (Eller and Frenzel, 2001; Henckel *et al*, 2000; Gilbert and Frenzel., 1995) which repeatedly reported the predominance of type II methanotrophs in rice field soils. In contrast, the active methanotrophic diversity consisted mainly of type I methanotrophs in most of the samples of PK and UPK treatments, while mainly of type II methanotrophs in most of the samples of SPK treatment. However, higher relative abundance of type I methanotrophs revealed from *pmoA* transcripts compared to that obtained from *pmoA* gene could be due to higher background of type II methanotrophs in *pmoA* gene-based T-RFLP profile.

On the genus level, T-RFLP profiles and subsequent phylogenetic analysis indicated that not all of the methanotrophs detected as *pmoA* gene were also detected as *pmoA* mRNA and vice versa. For example, the T-RF of 242 bp was present in almost all *pmoA* gene-based T-RFLP patterns whereas, it was not found in transcription patterns. By contrast, the T-RF of 264 bp was found as transcript in almost all samples but was not detectable in the *pmoA* gene-based T-RFLP patterns. Another example was the presence of four sequences affiliated to *Methylosarcina* (type I methanotrophs) in the rhizospheric soil samples of UPK treatment on 40 dap (Fig. 5.11), which could not be obtained from the

mRNA-based clone library of PK and SPK treatments, and *pmoA* gene-based clone libraries.

### 5.5.4 Conclusions

Methane oxidation potential assays indicated the methanotrophic activity in all treatments, which was also indicated by the successful mRNA extraction. Expression of *pmoA* transcripts did not reveal any significant influence of N-fertilization on metabolically active methanotrophs when comparing control and UPK treatments. Instead, the composition of active methanotrophic communities did change over time and probably a temporal variation in substrate or nutrient concentrations played a more important role for expression of the *pmoA* genes of methanotrophs in all treatments. In total, the addition of different fertilizers affected significantly both the activity and the composition of the metabolically active methanotrophs in rhizospheric soil during entire rice growing season. However, the treatments in which the active methanotrophic community composition differed most did not correspond to treatments with the most different activities, showing that potential activity was uncoupled to composition of active methanotrophs. Probably, going one step further by quantifying *pmoA* transcript numbers may provide positive (great) relevance with methane oxidation potential rates, which unfortunately was not performed for this study. Overall, type I methanotrophs were predominant in *pmoA* transcript analysis thus apparently playing an active role for methane oxidation in rhizospheric soil, while type II methanotrophs persists under unfavorable conditions, such as in the SPK treatment. Hence, type I methanotrophs apparently play an active role for methane oxidation in rhizospheric soil, while type II methanotrophs constitute a background community that also persists under unfavorable conditions, such as in the SPK treatment.

# Chapter 6

## Isolation of methanotrophs from rhizospheric soil and roots of planted rice microcosms

## 6.1 Introduction

It is clear from the studies described so far (chapter 3, 4 and 5) that the molecular analyses tools are very useful for studying methanotrophic community structure and diversity. However, to understand the physiology and function of the methanotrophs in detail, one has to do isolation, purification and multiplication of cells to provide sufficient biomass. However, cultivation on the basis of the presently described media generally resulted in the isolation of less than 1% of the viable species.

Methanotrophs have been isolated from a variety of environments including rice fields (Dianou and Adachi, 1999; Gillbert and Frenzel, 1998; Heyer *et al*., 2002), freshwater lakes (Heyer *et al*., 2002), wetlands (Dedysh *et al*., 1998, 2000, 2002), hot spring (Tsubota *et al*., 2005). However, there is a relatively small number of methanotrophic species that were isolated from rice fields in comparison to the high diversity of methanotrophs retrieved by using molecular tools.

In this part of my PhD research, I have isolated methanotrophs from the roots and rhizospheric soil samples collected on 57 dap.

## 6.2 Objectives

Isolation of methanotrophs from rhizospheric soil and roots from rice microcosms treated with three different N-fertilizer treatments.

## 6.3. Methods

### 6.3.1 Isolation of methanotrophs

For the isolation of methanotrophs, rhizospheric soil slurry and roots samples were used after measurement of methane oxidation potentials in samples from 57 dap. The soil slurry in the tube was provided with 5% $CH_4$ and 20% $O_2$ and was incubated horizontally on a roller at 30 °C as described in chapter 2.2.6 in dark. In case of root samples, 1 g of roots was transferred to a 250-ml serum bottle containing nitrate mineral salts (NMS) medium (see chapter 2.2.12 for composition). The head space contained 5% $CH_4$ and 20% $O_2$. The bottles were incubated on a shaker at 30 °C in dark. After four weeks of incubation, the medium containing soils or root samples was serially diluted up to a dilution of $10^{-6}$. From the terminal dilution step ($10^{-6}$), a 100 µl aliquot was streaked on NMS agar plates (see chapter 2.2.6 for composition). The plates were placed into an anaerobic jar filled with 5% $CH_4$ and 20% $O_2$ and incubated at 30 °C in dark. When the colonies became visible, they were continuously transferred to fresh agar plates until a pure culture was obtained indicated by uniform colony morphology. Purity was confirmed by sequencing the 16S rRNA and *pmoA* genes using cells of a single colony.

### 6.3.2 Genomic DNA extraction

Individual colonies were scrapped out using sterile toothpicks and were mixed with 200 µl of a $1/4^{th}$ ringer solution and 100 µl of a 10% (wt/vol) solution of sodium dodecyl sulfate. About 0.5 g of sterile zirconium beads (0.1mm diameter) was added, and the suspension was shaken for 1 min at 5.5m/s in a bead beater (Fast Prep FP120, Bio 101, Thermo Savant, USA). Then two freeze thaw cycles were conducted by rapidly cooling in liquid nitrogen for 20 s followed by heating in a 100 °C water bath for 10 min. Cell debris was pelleted at 14,000 rpm for 10 min at 4 °C, and the supernatant was mixed with 0.5 vol of ammonium acetate buffer pH 7.2. Centrifugation was carried out at 14,000 rpm for 5 min at 4 °C. The supernatant was mixed with 2.5 volumes of absolute ethanol and then incubated at –80 °C for 60 min. DNA was then precipitated by centrifuging at 14,000 rpm for 30 min at 4 °C. DNA thus obtained was washed once with 70% ethanol and dried in a vacuum dryer. Finally, the DNA was resuspended in 50 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) and stored at –20 °C.

### 6.3.3 Polymerase chain reaction (PCR) of bacterial 16S rRNA genes

PCR was carried out using primers 27f and 1492r (Lane, 1991), which amplify 16S rRNA genes of a wide range of members of the domain *Bacteria* from positions 28 through 1491 (*E. coli* numbering [Brosius *et al*., 1978]). The reaction mixture contained 1 µl of template DNA, 10 µl of 5× reaction buffer (Promega, Germany), 3 µl of 25 mM $MgCl_2$ (Promega, Germany), 5 µl of 10 mM dNTP mix (Promega, Germany) 0.5 µl of 33 pmol (each) primer (MWG-Biotech, Ebensburg, Germany), and 2.5 U of *Taq* DNA polymerase (Promega, Germany). Finally, the total volume was made up to 50 µl with sterile water. Amplification was performed in 0.2 ml reaction tubes using a DNA thermal cycler (ABI 9600; PE Applied Biosystems). The thermal PCR profile was as follows: initial denaturation for 2 min at 94 °C; 30 cycles, consisting of denaturation at 94 °C for 45 s, primer annealing at 48 °C for 60 s, and elongation at 72 °C for 120 s. The final elongation step was extended to 12 min. Aliquots of the 16S rRNA gene amplicons (5 µl) were checked by electrophoresis on a 1% agarose gel.

### 6.3.4 Polymerase chain reaction (PCR) of *pmoA* gene

PCR was carried out using the *pmoA* gene specific primers A189f and mb661r as described in chapter 2.2.7.1.

### 6.3.5 PCR purification

The PCR products thus amplified from 16S rRNA gene and *pmoA* gene were purified by using GebElute™ PCR clean-up kit (Sigma, Germany) following the instructions of the manufacturer.

### 6.3.6 Cycle sequencing

For cycle sequencing, the BigDye Terminator kit v3.1 (Applied Biosystem, Germany) was used. The PCR reagent mix was prepared by combining the following reagents (on ice) in a 0.5-ml microcentrifuge tube: 2 µl Ready Reaction Premix, 1 µl BigDye Sequencing Buffer, 3.2 pmol (forward or reverse) primer, 60 - 80 ng (2 µl) of purified PCR product and distilled water to make the volume 10 µl. Tubes were placed in a thermal cycler (Applied Biosystems - 9600) preheated to 104 $^{o}$C. The program used was

as follows: initial denaturation at 96 °C for 30s; 25 cycles, consisting of denaturation for 10 s at 94 °C, primer annealing at 50 °C for 5 s, and final elongation at 60 °C for 4 min. The product was then kept at 4 °C until and purified by using SigmaSpin™ post-reaction clean-up columns (Sigma, Germany) as described in the manufacturer's protocol. Sequences were generated with an ABI-3130 sequencer (Applied Biosystems) and analyzed with the sequence analysis software version 5.1 (MPI, Marburg).

### 6.3.7 Phylogenetic analysis

Identities of the 16S rRNA gene sequences were confirmed for methanotrophs by searching the sequence databases using nucleotide blast (http://www.ncbi.nlm.nih.gov/BLAST/). The partial 16S rRNA gene sequences (~600 base pairs) were added to a database consisting of about 50,000 complete or partial bacterial 16S rRNA sequences. This database was part of the ARB program package (Ludwig *et al*., 2004). The 16S rRNA gene sequences were integrated into the database with the automated alignment tool of the ARB program package. The resulting alignments were manually checked and corrected, if necessary. In addition, if needed for comparison, 16S rRNA gene sequences were obtained from the NCBI database (www.ncbi.nlm.nih.gov) and inserted into the ARB database. Phylogenetic dendrograms were constructed by using neighbor-joining and the ARB program package.

Similarly, identities of the *pmoA* gene sequences were confirmed by searching the sequence databases using nucleotide blast (http://www.ncbi.nlm.nih.gov/BLAST/). Phylogenetic analyses of the DNA and deduced amino acid sequences were carried out using the ARB program package (developed by O. Strunck and W. Ludwig; Technische Universität München [http://www.arb-home.de]). Sequences were manually aligned with the *pmoA* sequences obtained from the GenBank database. Regions of sequence ambiguity and incomplete data were excluded from the analyses.

## 6.4. Results and discussion

The 57 dap samples were chosen for isolation of methanotophs as ammonium-N concentration was very low in these samples (Fig. 4.6a). For the isolation of predominant methanotrophic bacteria, highest positive dilution step was used for the isolation process. Pure isolates were obtained after repeated sub-culturing of a colony. Some of the enriched microbes were lost during the colony transfer process and some could not be obtained in the pure form, especially those belonging to type I methanotrophs (purification is still in progress). This is normal, as isolation of methanotrophs is problematic due to slow growth rates and also due to the growth of other non-methane utilizing bacteria during cultivation (Escoffier *et al*., 1997; Hanson *et al*., 1992; Bowman, 2006).

Until now, I have obtained twelve pure methanotrophic isolates. 16S rRNA partial gene and *pmoA* gene sequences showed that all 12 isolates belonged to type II methanotrophs (Fig. 6.1 and 6.2). Therefore, members of type II methanotrophs represented the culturable fraction of dominant methanotrophs in both rhizospheric soil and roots. Type I methanotrophs have so far not been isolated from the rice field ecosystem, though their abundance has been shown by molecular approaches in previous (Eller and Frenzel, 2001; Horz *et al*., 2001) and my own studies (chapter 3, 4, and 5).

Out of eleven isolates analysed, in total six isolates (I-RT-PK2, I-RT-PK3, I-RT-PK10, I-RT-UPK1, I-RT-PK2, and I-RS-SPK19) exhibited 16S rRNA gene sequence similarities of >98% with the taxonomically described methanotrophic species (Table 6.1). These isolates were thus identified to the species level (Stackebrandt and Goebel, 1994). Two Out of eleven isolates analysed, in total six isolates (I-RT-PK2, I-RT-PK3, I-RT-PK10, I-RT-UPK1, I-RT-PK2, and I-RS-SPK19) exhibited 16S rRNA gene sequence similarities of >98% with the taxonomically described methanotrophic species (Table 6.1). These isolates were thus identified to the species level (Stackebrandt and Goebel, 1994). Two isolates (I-RS-SPK16 and I-RT-PK1) exhibited 16S rRNA gene sequence similarities of>96% and <98% with taxonomically described methanotrophic species (Table 6.1) and thus were assigned to the genus level, which is either *Methylocystis* or *Methylosinus*. Three isolates (I-RS-PK19, I-RS-PK21, and I-RS-SPK17) showed 16S rRNA gene sequence similarities of <95% with the taxonomically described methanotrophic species (Figure 6.1 and Table 6.1) and thus are probably novel at the genus level.

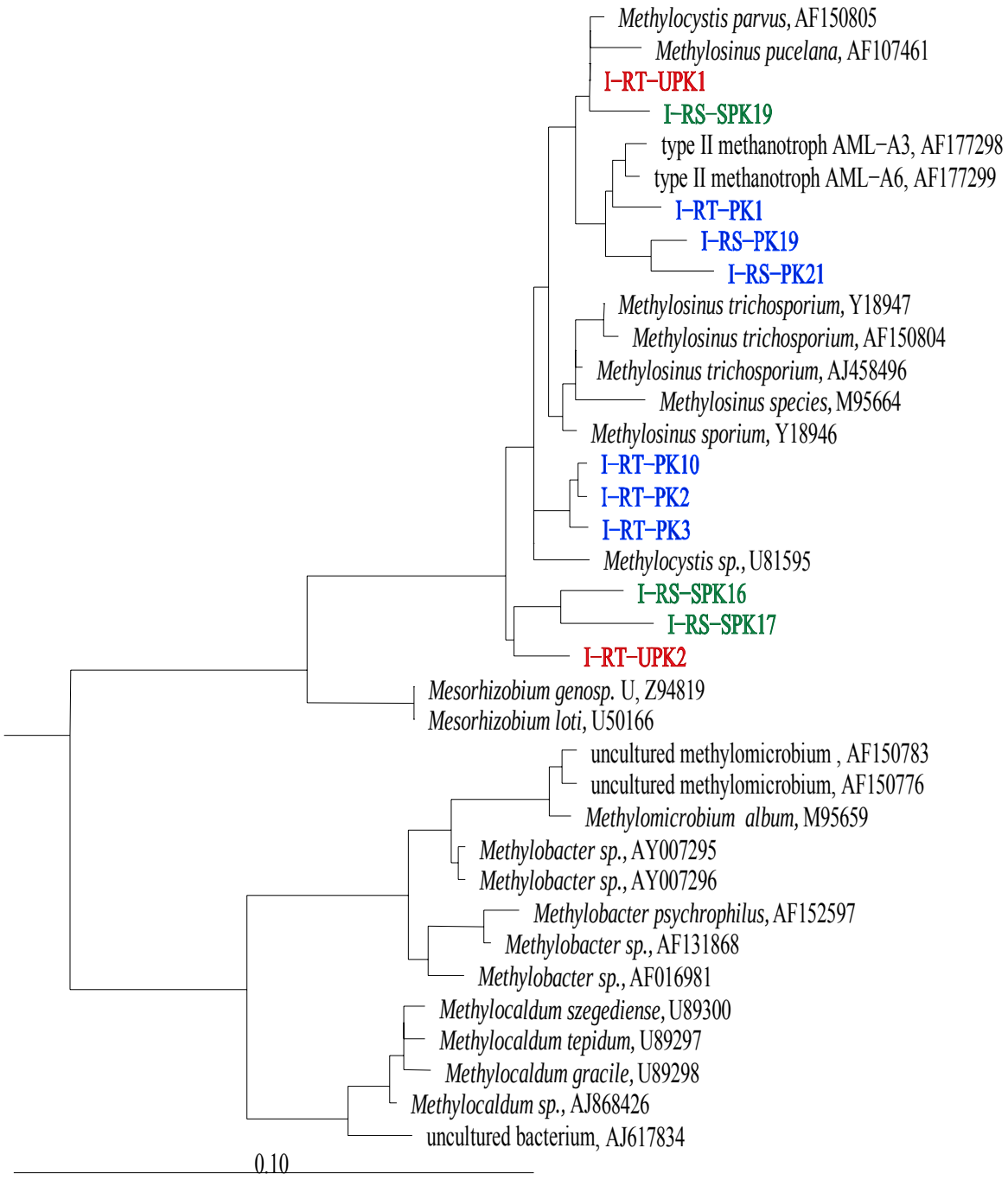



**Fig. 6.1:** Phylogenetic relationship between 16S rRNA gene sequences of isolates from roots (I-RT) and rhizospheric soil samples (I-RS) and representatives of the methanotrophs. Blue, red and green color indicates representative isolates from PK, UPK and SPK treatments, respectively. The scale bar represents 10% sequence divergence.

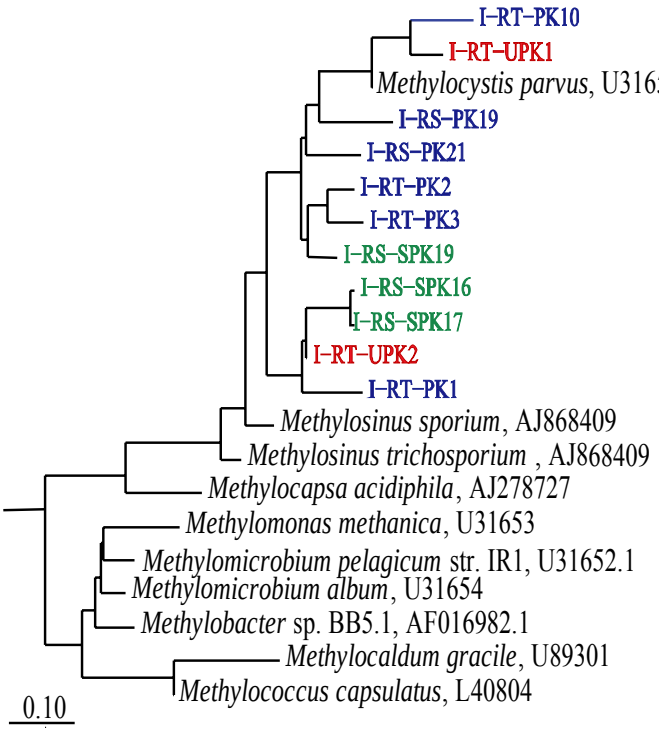



**Fig. 6.2:** Phylogenetic relationship between the *pmoA* gene sequences of isolates from roots (I-RT) and rhizospheric soil samples (I-RS) and representatives of the methanotrophs. Blue, red and green color indicates representative isolates from PK, UPK and SPK treatments, respectively. The scale bar represents 10% sequence divergence.

**Table 6.1:** Analysis of isolates based on partial 16S rRNA gene sequences, including the closest taxonomically described relatives as identified by using ARB program package.

| Isolates | Seq. length (bp) | Close relative | Accession no. | % identity |
|---|---|---|---|---|
| **I-RT-PK1** | 679 | *Methylocystis parbus* | AF150805 | 96.5 |
| **I-RT-PK2** | 679 | *Methylosinus trichosporium* | MTR458496 | 99.8 |
| **I-RT-PK3** | 638 | *Methylosinus trichosporium* | MTR458496 | 99.5 |
| **I-RT-PK10** | 639 | *Methylosinus trichosporium* | MTR458496 | 99.8 |
| **I-RT-UPK1** | 619 | *Methylosinus trichosporium* | MTR458496 | 99 |
| **I-RT-UPK2** | 740 | *Methylosinus trichosporium* | MTR458496 | 99.3 |
| **I-RS-PK19** | 809 | *Methylosinus pucelana* | AF107461 | 91.5 |
| **I-RS-PK21** | 825 | *Methylosinus pucelana* | AF107461 | 93.7 |
| **I-RS-SPK16** | 801 | *Methylosinus sporium* | Y18946 | 96 |
| **I-RS-SPK17** | 829 | *Methylosinus sporium* | Y18946 | 94 |
| **I-RS-SPK19** | 500 | *Methylosinus pucelana* | AF107461 | 99 |

# Chapter 7

## General discussion

Methanotrophs, inhabitants of the rice rhizosphere, play an important role in the reduction of methane emissions from rice fields. In rice fields, they are subjected to many environmental and field management parameters, which may have a significant impact on their function, activity and community composition. To study this in greater detail, I have investigated methane oxidation and the community structure of methanotrophs in planted rice microcosms under controlled conditions in the greenhouse. However, in such greenhouse studies, not all parameters can be simulated. Eller *et al* (2005) have compared the field and microcosm experiments and shown that activity patterns of methanotrophs agreed well between both experiments excluding the quantity data.

I have carried out two major experiments in consecutive years. The first experiment (labelling experiment, in short - 'LE') was conducted from May 29 till July 22, 2005 for total 55 days. The second experiment (fertilizer experiment, in short - 'FE') was conducted from September 20 till December 12, 2006 for total 88 days. However, the controlled temperature was different for these two experiments as the day/night temperature was maintained at 30/22ºC for LE, while at 28/22ºC for FE. The major difference between these two experiments was that in LE, I applied two treatments, i.e., with (labelled rice microcosms) and without addition of $^{13}C$-$CH_4$ (control rice microcosms), and both types of rice microcosms were given similar treatments (e.g. fertilization). In FE, on the other hand, I applied three different nitrogen fertilizer treatments i.e., PK (without nitrogen source), UPK (with urea as nitrogen source) and SPK (with ammonium sulphate as nitrogen source). Fertilization was carried out in both experiments in the similar manner but on different dates (on 43 dap for LE and on 57 dap for FE). In both LE and FE-UPK, urea was added as ammonium nitrogen fertilizer, so that these two experiments are comparable in this aspect to each other.

In both experiments, I had carried out physiological analyses to investigate the dynamics of methane oxidation process over time during the rice growing season. For this purpose, I had measured the methane emission rates and methane oxidation rates and analyzed the pore water chemistry at different growth stages of rice plant. Pore water samples were

collected from rhizospheric region (3 cm) and bulk region (10 cm depth) to analyze $CH_4$ concentration, $NH_4^+$ concentration, and pH. However, analysis of $NH_4^+$ concentrations and plant characteristics were not done for LE.

Both experiments showed a temporal variation in $CH_4$ emission rates, $CH_4$ oxidation rates, and $CH_4$ concentrations dissolved in the pore water, which correlated well with other field and rice microcosm studies (Eller and Frenzel, 2005; Krüger and Frenzel, 2001; Schütz *et al*., 1989). $CH_4$ oxidation rates started after 20 dap, reached a maximum between 30 and 40 dap, and decreased during the rest of the period (till 55 dap) in both experiments (Fig. 3.3 and Fig. 4.4a). In particular, the decrease of $CH_4$ oxidation activity with the progress of the season has been observed previously (Dan *et al*., 2001; Eller and Frenzel, 2001; Krüger and Frenzel, 2003). However, $CH_4$ emission rates (Fig. 3.3 and 4.4a), $CH_4$ oxidation rates (Fig. 3.3 and 4.4b) and $CH_4$ concentrations (Fig. 3.6 and 4.5), were higher in LE than in FE (UPK treatment). This could be due to longer day light exposure to plants in LE. After a third fertilization on 43 and 57 dap in LE and FE, respectively, a transient positive influence of ammonium was observed for methane oxidation in both experiments, similar to previous observations (Dan *et al*., 2001; Krüger and Frenzel, 2003). In summary, a temporal variation of $CH_4$ oxidation occurred as a general trend which indicated that methane oxidation is a dynamic process that seems to be regulated by various factors, including the age of the rice plant, substrate and nutrient availability for the microorganisms and/or plants.

The role and composition of methanotrophs in rice rhizosphere was investigated after collecting the roots and the rhizospheric soil from different growth stages of rice plants. In LE, roots and rhizospheric soil samples were collected from two time points i.e., 43 and 55 dap whereas, in FE, roots and rhizospheric soil samples were collected from six time points i.e., 29, 40, 57, 62, 67 and 88 dap. I used *pmoA* gene analyses based on T-RFLP fingerprinting method and cloning and sequencing to assess the structure of the methanotroph community in both experiments.

T-RFLP analysis produced highly reproducible T-RFLP patterns with major T-RFs of 80, 226, 245, 350, 438, 457 and 506 base pairs (bp) lengths in almost all replicate samples (Fig. 3.9a, 4.9, and 4.11). Exceptionally, T-RF of 113, 210, 242, 264, 278, 448 bp length which represented minor T-RFs (with relatively low relative abundance) were not,

detected in LE. The T-RFs were assigned to respective genera of methanotrophs by sequence analysis of several clone libraries obtained from PCR products of respective roots and rhizospheric soil samples. T-RFs were assigned to *Methylococcus* and *Methylocaldum* (80 bp), *Methylocystis* and *Methylosinus* (245 bp), *Methylomicrobium* (350 and 457 bp), *Methylomonas* (438 bp), *Methylobacter* (506 bp), and five different clusters of uncultured I methanotrophs (80 bp, 227 bp, 243 bp, 264 bp, 350 bp) Fig. 3.10, 3.11, and 4.10). However, T-RFs of 113, 210, 278, and 448 bp could not be assigned to any of the sequences obtained from the clone libraries.

Thus, phylogenetic analysis of *pmoA*-derived amino acid sequences revealed the presence of both type I methanotrophs (genera *Methylomonas*, *Methylobacter*, *Methylococcus*, *Methylocaldum*, *Methylomicrobium* and uncultured type I methanotrophs with T-RF of 80 bp size) and type II methanotrophs (genera *Methylocystis* and *Methylosinus*) on root samples as well as in rhizospheric soil in all samples. Such a diversity has been found previously in rice field ecosystems from Vercelli, Italy (Eller *et al*., 2005; Henckel *et al*., 2001; Horz *et al*., 2001) and elsewhere (Hoffmann *et al*., 2002; Jia *et al*., 2007). Notable is the detection of sequences having T-RF of 350 and 457 bp size clustering with *Methylomicrobium* sp. in LE (Fig. 3.10 and 3.11) and sequences having T-RF of 350 bp size clustering together with uncultured type I methanotrophs having T-RF of 80 bp in FE (Fig. 4.10). Similarly, sequences having T-RF of 374 bp were detected in FE only (Fig. 4.10) clustering separately in type I methanotrophs. Interestingly, sequences having T-RF of 80 bp size representing a separate cluster deeply branched from *Methylococcus* and *Methylocaldum* were repeatedly observed in both experiments.

The general composition of the methanotrophic community was quite similar on the roots and in the rhizospheric soil referring to both LE and FE-UPK experiments. Furthermore, type I methanotrophs were dominant on the roots, while type II methanotrophs were dominant in the rhizospheric soil. Interestingly, *Methylomonas* (type I methanotrophs) was the most dominant genus found on the roots samples from both UPK experiments (LE and FE) suggesting that *Methylomonas* might play an important role in urea treated roots. This was further supported by the indicator species test using R software with *labdsv* package that was carried out for FE. However, the relative abundance of individual methanotrophic genera (especially type I methanotrophs) was different and in addition, exhibited a shift between/among the different sampling time points in both the

roots and the rhizospheric soil from both experiments. Nevertheless, in case of FE-UPK, the observed shift in the relative abundance of individual methanotrophic genera was not pronounced. The root samples in FE exhibited a relatively stable community structure with exception on 29 and 88 dap as compared to other two treatments (PK and SPK have not yet been discussed here). The shift observed in the rhizospheric soil could be related to type I methanotrophs which were not easily visible due to high relative abundance of type II methanotrophs at all sampling points. This result indicated that the different methanotrophic genera responded differently to spatiotemporal variations in the rice microcosms, which in turn gave a hint that different methanotrophic genera may have different $CH_4$, $O_2$ or nutrient requirements.

Note, however, that the abundance of methanotrophic groups were only relative numbers within the total community of methanotrophs as they were based on T-RFLP analyses only. The quantitative determination of methanotrophic biomass using real-time PCR quantification method would be interesting, but has not yet been done. Moreover, since I used DNA samples for the amplification of *pmoA*, I could not ascertain that the amplified *pmoA* product represented the metabolically active methanotrophs. For this purpose, PLFA-SIP was used in LE and mRNA approach was used in FE to determine the extent to which type I and type II methanotrophs perform actively by assimilating $^{13}C$-$CH_4$ (added directly to the rice microcosms) and by *pmoA* gene expression. Notably, the analysis of PLFA-SIP was obtained for roots and rhizospheric soil, while mRNA analysis could only be achieved for the rhizospheric soil.

For PLFA-stable isotope probing method, $^{13}C$-$CH_4$ was supplied to the rhizosphere of the rice microcosms between 37 and 54 days after transplantation. Although the labeled $CH_4$ consisted of 99 atom-% $^{13}C$, the $CH_4$ in the pore water and in the $CH_4$ flux contained only 3-5 atom-% $^{13}C$. This result showed that the $^{13}C$-$CH_4$ added through the permeable tubing into the rhizosphere became highly diluted by endogenously produced $CH_4$. However, the resulting $^{13}C$-content of the $CH_4$ allowed the detection of specifically $^{13}C$-labeled PLFA. This indicated that the idea of adding labelled substrate directly to the rhizosphere, while mimicking *in situ* condition was successful and such kind of labelling experiment can be performed in the future.

In this study, mainly the PLFA 16:1ω7, 16:1ω6, 16:0, 18:1ω7, 18:1ω9, and 18:0 became labeled with $^{13}C$ derived from $CH_4$. The PLFA of methanotrophs became increasingly $^{13}C$-labeled at two subsequent sampling times, demonstrating the activity of the methanotrophs in the rhizospheric soil and on the roots. However, type I methanotrophs incorporated significantly more $^{13}C$ into their PLFAs than type II methanotrophs, in both soil and roots, indicating that type I methanotrophs were the more active population. In addition, type I and type II methanotrophs became increasingly more active on the roots and in the rhizospheric soil with respect to time (Fig 3.12 b and 3.13). Thus, the temporal changes in the activity of both type I and type II methanotrophs was observed. Although the above PLFAs are found in many different prokaryotes and eukaryotes (Spring *et al*., 2000), labelling of them strongly indicated that type I and type II methanotrophs were active in rhizospheric soil and on root samples. Thus, such specificity of addition of labelled substrate $^{13}C$-$CH_4$ for methanotrophs is the main key idea behind using the PLFA-SIP method to investigate the metabolically active methanotrophs. Every technique has its own pros and cons. One of the limitation of such kind of labelling technique is that the labelling amount, generally needed to be assimilated by the microorganism, is not known and there is always a possibility of cross-feeding in case of labelling for long incubation period. Moreover, PLFA-SIP method does not give any information on species level of microorganisms.

To overcome such limitation, T-RFLP and cloning/sequencing was carried out by targeting *pmoA* mRNA of rhizospheric soil in FE. This approach was proven a powerful tool for investigation of the metabolically active methanotrophs that were responsible for methane oxidation in rhizospheric soil. Although detection of mRNAs has been hindered by difficulties in the extraction of intact RNA from environmental samples (Saleh-Lakha *et al*., 2005), recent advances in extraction methods for mRNA may facilitate the direct analysis of functional gene expression of active methanotrophs in the environment (Shrestha, Dissertation, 2007; Bürgmann *et al*., 2001; Griffiths *et al*., 2000). A number of recent studies have focused on the analysis of expression of methane monoxygenase in different environment using *pmoA* primer sets (Han and Semrau, 2004; Kolb *et al*., 2005; Cheng *et al*., 1999; Nercessian *et al*., 2005; Chen *et al*., 2007; Chen *et al*., 2008), providing direct evidence of active methanotrophs. In this study, for the first time, I had successfully used *pmoA* mRNA to study the metabolically active methanotrophic communities that were present in the rhizospheric soil sample under *in situ* conditions.

The T-RFLP analysis of *pmoA* mRNA showed a diverse pattern of transcripts that corresponded to the potentially active genera observed in the community fingerprints for methanotrophs. The transcription pattern also indicated rapid changes of transcriptional activity over time, indicating a strong temporal variation in all treatments. mRNA approach based on T-RFLP pattern after affiliation to sequence analysis, revealed that active methanotrophic diversity consisted mainly of type I methanotrophs in rhizospheric soil samples (UPK treatment) at most of the sampling time points of the rice-growing season.

In conclusion, I could effectively detect the metabolically active methanotrophic community in the rhizospheric soil as well as on the roots from planted rice microcosms using the PLFA-SIP and *pmoA* mRNA approaches (not applied on the roots). In addition, I could further differentiate these community from the total methanotrophic community using *pmoA* gene based community analysis. All three approaches demonstrated the presence of both type I and type II methanotrophic populations in the rhizospheric soil and on the rice roots. Furthermore, *pmoA* gene based community analysis revealed that type II methanotrophs were dominant in the rhizospheric soil whereas type I methanotrophs were dominant on the roots thus indicating that rhizospheric soil and the roots constitute two different environments probably providing different ecological niches for methanotrophs (Henckel *et al*., 2000). However, for the first time, both PLFA-SIP and *pmoA* mRNA analyses strongly revealed that despite the dominance of type II methanotrophs in rice rhizospheric soil, type I methanotrophs were the metabolically active ones and responsible for *in situ* methane oxidation in the rice rhizosphere. Importantly, these two approaches corresponded quite well with each other. In addition, dominance of type I methanotrophs on the roots was revealed from two independent experiments (LE and FE-UPK), and PLFA-SIP analysis showed that the metabolically active methanotrophs were type I. *pmoA* mRNA analysis, which unfortunately could not yet be achieved with root material, will further clarify this result. The isolation of methanotrophs (11 isolates) from the roots and rhizospheric soil samples indicated that the culturable fraction of dominant methanotrophs does not necessarily be metabolically active or dominant one.

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# Appendix

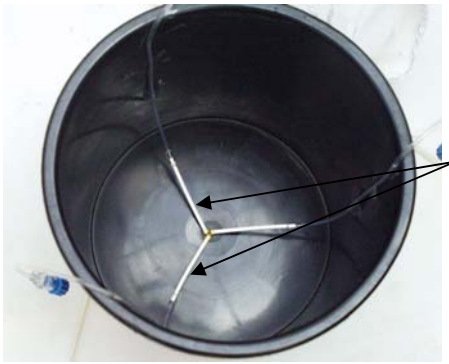


**Fig. A:** Set up for experiment 1 and 2.

**Fig. B:** Set up for experiment 1.

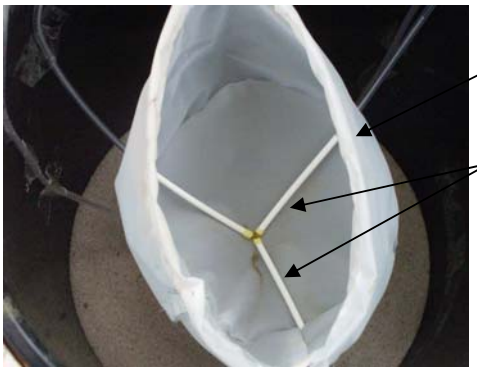


**Fig. C:** Set up for experiment 1 and 2.

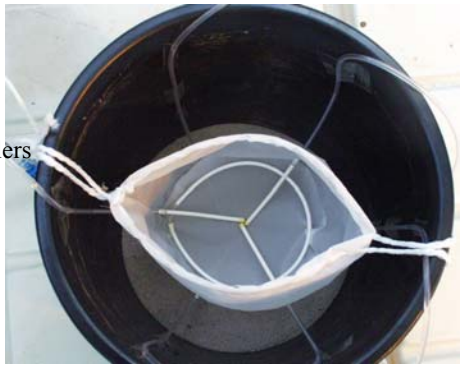

**Fig. D:** Set up for experiment 1.


**Fig. E:** Set up for experiment 1 and 2.

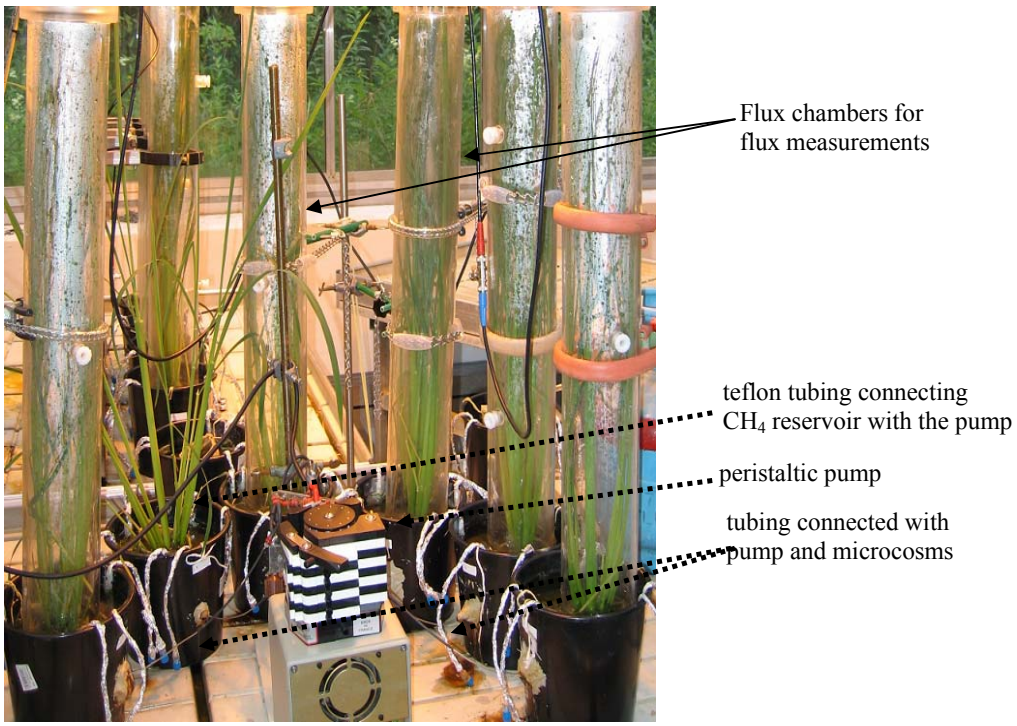


**Fig. F:** Set up for experiment 1.

**Fig: G:** Set up with flux chambers

**Fig. H:** Set up for experiment 1 and 2.

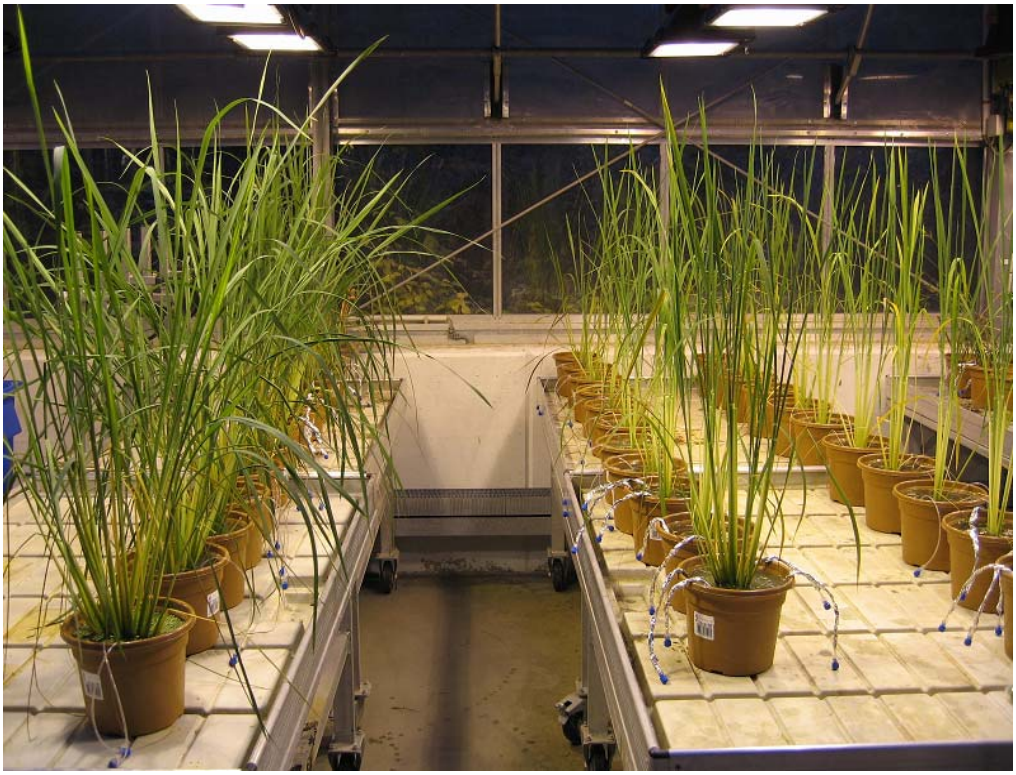

**Fig. I:** Set up for experiment 2.

**Fig. J:** Set up for experiment 2

**Sampling time:**

**Fig. K:** Rice plant with nylon bag was separated out from the rest of bulk soil**.**

**Fig. L:** UPK treated rhizosphere (Expt. 2)

**Fig. M:** SPK treated rhizosphere (Expt. 2)

**Fig. N:** Rhizospheric soil isolated from bulk soil

**Fig. O:** Rhizospheric soil collected from SPK

**Fig. P:** Washed roots from PK

**Fig. Q:** Washed roots from UPK

**Fig. R:** Washed roots from SPK

**Fig. S:** Plant growth at the end of the experiment 2.

**Fig. T:** Grain filling stage.

**Fig. U:** Grain filling stage.

# Curriculum vitae

**Minita Shrestha**
**Citizenship: Nepalese**

## Academic achievements

**Apr. 2005 – Mar. 2008:** Ph.D. work at the Max Planck Institute for Terrestrial Microbiology, Marburg under the supervision of Prof. Dr. Ralf Conrad.
**Jan. 2002 – Aug. 2003:** M.Sc. in Water and Wastewater Engineering with thesis entitled "Kinetics of the Anaerobic Sulphate Removal Process" from Asian Institute of Technology (AIT), Bangkok, Thailand.
**1998 - 2000:** M.Sc. in Chemistry with major subject as Organic Chemistry from the Central Department of Chemistry, Tribhuvan University, Kathmandu, Nepal.

## Working experiences

**Sep. 2000 – Dec. 2001:** Worked as a Research and Development Executive/ Senior Analyst in Nepal Pharmaceuticals Laboratory Private Nepal, Birganj, Nepal.
**July 1995 - Oct. 1998:** Worked as a Quality Control Executive/Analyst in Nepal Pharmaceuticals Laboratory Private Nepal, Birganj, Nepal.

## Rewards and scholarships

**Apr. 2005 – Mar. 2008:** Stipendium from Max Planck Institute Society to pursue Ph.D. in Max Planck Institute for Terrestrial Microbiology, Marburg, Germany.
**Jan. 2002 – Aug. 2003:** DANIDA scholarship to study M.Sc in Water and Wastewater Engineering at Asian Institute of Technology, Thailand.

## Conferences attended

**April 12-14, 2007:** Poster entitled "Spatial and temporal variation of active methanotrophs in planted rice microcosms" presented in "Microbial Adaptation to Stress and Environment" Symposium in Marburg, Germany.

**April 01-04, 2007:** Poster entitled "Spatial and temporal variation of active methanotrophs in planted rice microcosms" presented in VAAM Annual Conference, Osnabrück, Germany.

**August 20-25, 2006:** Poster entitled "Methane oxidation and incorporation of $^{13}C$ into PLFA of methanotrophs in planted rice microcosms" presented in ISME Conference ($11^{th}$ International Symposium on Microbial Ecology), Vienna, Austria.

**October 23-25, 2003:** Oral Presentation entitled "Kinetics of the anaerobic sulphate removal process" in First International Symposium on South Asian Water Environment, Asian Institute of Technology, Bangkok, Thailand.