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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

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Summary

Methanotrophs in the rhizosphere of rice field ecosystems attenuate the emissions of CH_4 into the atmosphere and thus play an important role for the global cycle of this greenhouse gas. Therefore, we measured the activity and composition of the methanotrophic community in the rhizosphere of rice microcosms. Methane oxidation was determined by measuring the CH_4 flux in the presence and absence of difluoromethane as a specific inhibitor for methane oxidation. Methane oxidation started on day 24 and reached the maximum on day 32 after transplantation. The total methanotrophic community was analysed by terminal restriction fragment length polymorphism (T-RFLP) and cloning/sequencing of the *pmoA* gene, which encodes a subunit of particulate methane monooxygenase**.** The metabolically active methanotrophic community was analysed by stable isotope probing of microbial phospholipid fatty acids (PLFA-SIP) using ^{13}C-labeled CH_4 directly added to the rhizospheric region. Rhizospheric soil and root samples were collected after exposure to $^{13}CH_4$ for 8 and 18 days. Both T-RFLP/cloning and PLFA-SIP approaches showed that type I and type II methanotrophic populations changed over time with respect to activity and population size in the rhizospheric soil and on the rice roots. However, type I methanotrophs were more active than type II methanotrophs at both time points indicating they were of particular importance in the rhizosphere. PLFA-SIP showed that the active methanotrophic populations exhibit a pronounced spatial and temporal variation in rice microcosms.

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} (Lelieveld et al., 1998; Wang et al., 2004). 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). Methanotrophs associated with the rhizosphere of rice plants oxidize CH_4 with molecular O_2 and use it as the main sole source of carbon and energy. Thus 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 into two major physiological groups, type I (belonging to the *Gammaproteobacteria*) and type II methanotrophs (belonging to the *Alphaproteobacteria*), depending on the guanine and cytosine content of their DNA, intracellular membrane arrangement, carbon assimilation pathway and phospholipid fatty acids (PLFA) composition (Hanson and Hanson, 1996). 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, cloning and sequencing of 16S rRNA genes and functional genes (*pmoA*, *mmoX*, *mxaF*) (Bodelier et al., 2000; Bosse and Frenzel, 1997; Eller and Frenzel, 2001; Henckel et al., 1999; Henckel et al., 2000; Horz et al., 2001).

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 outcompete type II species under low CH_4 and high O_2 conditions, whereas type II species tend to dominate under the opposite conditions (Amaral and Knowles, 1995; Graham et al., 1993).

Rice plant ecosystems may exhibit different niches for methanotrophs as characterized by spatiotemporal variation of CH_4 and O_2. Moreover, CH_4 availability increases with distance from the rice roots (Gilbert and Frenzel, 1998), while O_2 availability decreases with distance because of lower root densities and consequently lower root O_2 release at larger distances (VanBodegom et al., 2001). 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 et al., 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 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; Crossman et al., 2006; Knief et al., 2003b). 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 active methanotrophic community 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, we 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 libraries and using PLFA-SIP, respectively.

Results

Rates of CH_4 emission and oxidation

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.1). 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. 2a, b). 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. 2a) and the ^{13}C-labeled microcosms (Fig. 2b), respectively.

Methane oxidation rates were calculated as the difference between CH_4 emission rates in the presence and absence of difluoromethane (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. 2a, b), when nearly 60% of the anaerobically produced methane was oxidized prior to its emission to the atmosphere (Fig. 3). 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.

Soil pore water

Concentrations of CH_4 in pore water samples were similar in both the control and the ^{13}C-labeled microcosms. 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.

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.

Ammonium concentrations in the pore water of rhizospheric soil were similar in both control and ^{13}C-labeled treatments during the whole incubation period. The NH_4^+ concentrations started to decrease after 24 days and remained at about 10 μM after 38 days of transplantation. After the fertilisation on day 44, NH_4^+ concentration in pore water increased, but rapidly decreased again to 10-20 μM within 5 days of fertilization. The NO_2^- and NO_3^- concentrations in the pore water were below the detection limit (5 μM) during the entire experimental period. The pH of the pore water in the control and labeled microcosms varied between pH 6.8 and 7.6 at both rhizospheric and bulk regions (data not shown).

T-RFLP analysis of methanotrophic community

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 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 (see below). 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 (±1 bp) in all samples. All of these T-RFs were assigned by our own clone analysis (see below) 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).

The comparison of T-RFLP community profiles obtained from rhizospheric soil and root samples showed similar T-RF patterns but different relative abundances of the major T-RFs. Furthermore, two different sampling points were also conferred to the different relative abundances of the T-RFs in both rhizospheric soils as well as in root samples (Fig. 4a). 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 1. 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 1). The T-RFs affiliated to type II methanotrophs decreased with incubation time in the rhizospheric soil but increased on the roots.

Cloning and sequence analysis of pmoA gene

Sequences of *pmoA* genes retrieved from rhizospheric soil (Fig. 5) and root samples (Fig. 6) 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. 5) and on root samples, 65 among 88 clones (74%) were affiliated with type I methanotrophs (Fig. 6) 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 number of *pmoA*-sequences affiliated to *Methylomonas* sp. was 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 frequency over time (Fig. 5). 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. 5 and 6). Also sequences related to *Methylomicrobium album* were retrieved that exhibited a T-RF of 457 bp (Fig. 5 and 6) along with 350 bp as 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. 4b), were similar to the relative abundance of the same T-RFs detected in the DNA extracts (Fig. 4a).

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 we applied a ^{13}C-CH_4 labeling approach. Figure 7 shows the total abundance of phospholipid fatty acids (Fig. 7a) and the ^{13}C incorporation into phospholipid fatty acids (Fig. 7b) 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. 7a). Incorporation of ^{13}C into PLFA, on the other hand increased over time in both cases (Fig. 7b), 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. 7b). 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 after 18 days compared to 8 days of incubation. However, it decreased in some PLFAs (Fig. 7b), for example, in 18:1ω9 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

1. Furthermore, the ratio of ^{13}C incorporation between 8 and 18 days of ^{13}C-labeling (Fig. 7b) 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.

Discussion

In our study we have combined both physiological and bimolecular 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, we calculated the rates of CH_4 oxidation over the incubation period. As bimolecular tools, we 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). Ammonium concentrations also decreased during this time, which may be explained by efficient uptake of N by the rice plant (Arth et al., 1998). 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, we 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, we analyzed the methanotrophic community 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 and colleagues (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 cluster 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. Our study showed that this T-RF belongs to type I methanotrophs because, we used

pmoA specific primers (A189/Mb661) 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, we were able to calculate the relative abundance of the different methanotrophic populations by using T-RFLP analysis of the *pmoA* genes. Additionally, we were 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 1). 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 is 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 has 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. 7a). 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 we used DNA samples for the amplification of *pmoA*, we could not ascertain that the amplified *pmoA* product represented the metabolically active methanotrophs.

Therefore, we 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 our 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 indicates 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, we would not expect that any other organisms than methanotrophs assimilated ^{13}C-CH_4. Unexpectedly, we 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. We did not detect a peak for PLFA 18:1ω8, 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 our 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 agree 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 our 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. We speculate 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 our 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, we 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.

Experimental procedures

Planted rice microcosms

Soil was taken from drained paddy fields of the Italian Rice Research Institute in Vercelli, Italy and was air-dried and stored at room temperature. The soil characteristics have been described previously (Holzapfel-Pschorn and Seiler, 1986). Immediately prior to its use, the soil was passed through a 2-mm sieve, and soil slurry was prepared with 1.8 kg soil, 940 ml demineralized water, and 60 ml of fertilizer solution (9.89 g urea, 7.605 g KH_2PO_4, and 7.07 g KCl per liter), and finally filled into each microcosm with a volume of 2.5 L (height 16 cm, diameter 17 cm) pots. 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. 1). 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. 1).

In total, 18 microcosms were prepared among which 9 were for $^{13}CH_4$-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 28/22ºC 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. The incubation experiment was then

conducted for a total of 55 days under flooded conditions. The ^{13}C-labeled CH_4 solution was continuously circulated in the soil between day 37 and 54 after transplantation. After 44 days, 20 ml of fertilizer solution was added a third time to the microcosms. In our study, fertilization solution was added as a mixture of ammonium, phosphorous and potassium sources in split doses as applied in field practice 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.

Measurement of CH_4 flux

Rates of CH_4 emission and CH_4 oxidation were measured as described previously (Krüger and Frenzel, 2003). For the measurement of rates of CH_4 emission, triplicate microcosms were covered by static flux chambers, and gas samples were taken every 30 min for 3 hours. Rates of CH_4 oxidation were measured in parallel triplicate microcosms by adding 1% difluoromethane (CH_2F_2 99%, ICI Chemicals, UK), an inhibitor specific for CH_4 oxidation (Miller et al., 1998), to the headspace of the flux chambers. The gas samples were 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 as described previously (Krüger et al., 2002). After starting circulation of ^{13}C-labeled CH_4 through the soil, i.e. after day 37, gas samples were also taken into 10-ml glass vials, which had been previously flushed with N_2, for later analysis of the ^{13}C content of the CH_4. Stable isotope analysis of $^{13}C/^{12}C$ in gas samples was performed using a gas chromatograph combustion-isotope ratio mass spectrometry (GCC-IRMS) system as described previously (Conrad et al., 2002).

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). After heavy shaking by hand, an aliquot of

the headspace of the tubes was collected with a pressure lock syringe and analysed for CH_4. The CH_4 concentration in the soil pore water was calculated as described previously (Krüger et al., 2001). Then, the pore water was stored frozen (–20°C) for the later determination of ^{13}C-CH_4, pH, NH_4^+, NO_2^- and NO_3^-. Ammonium concentration was analysed by using a SAFIRE microplate reader (TECAN, Crailsheim, Germany) as described by (Murase et al., 2006), and nitrate and nitrite concentration by ion chromatography (Sykam, Gilphing, Germany) as described by Bak and colleagues (1991).

Collection of soil and root samples

Rhizospheric soil and root samples were collected after 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. The samples were designated as RS-8 and RS-18 for the rhizospheric soil, and RT-8 and RT-18 for the roots, respectively. The roots were washed in deionized sterile water. The soil and root samples were stored at –20°C for later molecular and PLFA-SIP analyses.

DNA extraction, PCR, cloning and sequencing

Total DNA from the soil and root samples was extracted using a DNA extraction protocol reported previously (Noll et al., 2005). 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 containing ethidium bromide, followed by DNA purification 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). 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). 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 5 min. Amplification was performed in 0.2-ml reaction tubes using a DNA thermal cycler (model 2400; PE Applied Biosystems). Aliquots of the amplicons (5 µl) were checked by electrophoresis on a 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 (49 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. 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 *pmoA* gene sequences 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); AM849759-AM849804 (RT-18).

Phylogenetic analysis

The identities of the *pmoA* gene sequences were confirmed by searching the sequence databases using BLAST (http://www.ncbi.nlm.nih.gov/BLASTn/). 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. Results were depicted as a consensus tree, combining the results of Tree-Puzzle, neighbor-joining approach, and maximum likelihood analyses of the data sets.

T-RFLP analysis

Analysis of terminal restriction fragment length polymorphism (T-RFLP) was performed for each total DNA extract in triplicate**.** The *pmoA* was amplified by PCR as described above except a FAM (6- carboxyfluorescein)-labeled forward primer was used. After purification with Qiaquick spin columns (Qiagen, Germany), 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. Aliquots (2.5 µl) of the digested amplicons were mixed with 12 µl of deionized formamide (Applera, Germany) and 0.2 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, Applied Biosystems, Germany) fitted with ABI prism where the terminal restriction fragments (T-RFs) were separated. The length of fluorescently labeled T-RFs was determined by comparison with the internal standard using GeneScan 3.71 software (Applied Biosystems, Germany). The accuracy of size calling between replicates was ±1 bp. The proportional abundance of individual T-RFs within a given T-RFLP pattern was determined as the peak height of the respective T-RF divided by the total peak height of all T-RFs detected within a fragment size range between 50 and 550 bp and was expressed as a fraction based on 1.0 (Lüdemann et al., 2000).

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

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 as described for DNA extraction. The weighed samples were extracted with the mixture of phosphate buffer, methanol and chloroform and further fractionated to neutral lipids, glyco- and phospholipids by column chromatography on a silicic acid column (Water, Ireland) into different polarity classes by sequential elution with chloroform, acetone and methanol (Zelles and Bai, 1993). The resulting fatty acid components were released and methylated by mild

alkaline methanolysis (White et al., 1979). The individual fatty acid methyl esters were identified using GC-mass spectrometry, and their stable carbon-isotope compositions 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. 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 incorporation = (F_e-F_c) $[PLFA]_e$, where F_e = fraction of ^{13}C in the ^{13}C-labeled samples; F_c = fraction of ^{13}C in the unlabeled control sample; and [PLFA] = PLFA concentration in the ^{13}C-labeled sample. F was given by $^{13}C/(^{13}C+^{12}C) = R/(R+1)$ where $R= (\delta^{13}C/1000+1)\ R_{VPDB}$.

Statistical analysis

Statistical analyses were performed using Microsoft Excel. The two-sample t-test was used for comparing the means of relative abundances of (sum or individual) 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 tpye I and type II methanotrophs were also tested.

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Table 1. 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. (numbers with different letters were statistically different ($P<0.05$).

Sample	**RS-8**	**RS-18**	**RT-8**	**RT-18**
	Relative abundance of T-RFs (%)			
Type I[1]	47[a]	55[c]	68[e]	59[g]
Type II[2]	53[b]	45[d,m]	32[f]	41[h,m]
	^{13}C incorporation into PLFA (%)			
Type I [i]	73[a]	62[c]	58[e]	71[g]
Type II [ii]	16[b]	20[d]	28[f]	15[h]

[1] Sum of T-RFs 76, 227, 347, 437 and 457 bp, which were affiliated to type I methanotrophs in phylogenetic assignment.

[2] T-RFs 245bp, which were affiliated to type II methanotrophs in phylogenetic assignment.

[i] Sum of percent values of 16:0, 16:1ω6 and 16:1ω7 shown in Fig. 6b.

[ii] Sum of percent values of 18:0, 18:1ω7 and 18:1ω9 shown in Fig. 6b.

Figure legends

Figure 1: Rice microcosm experimental set up.

Figure 2: 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).

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

Figure 4: 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).

Figure 5: 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.

Figure 6: 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.

Figure 7: PLFA abundance given as (a) total PLFA), and (b) ^{13}C incorporation into PLFA per gram dry soil or root (RS = rhizospheric soil; RT = roots; numbers indicate time after beginning of ^{13}C-labeling); mean ± SD (n=3).

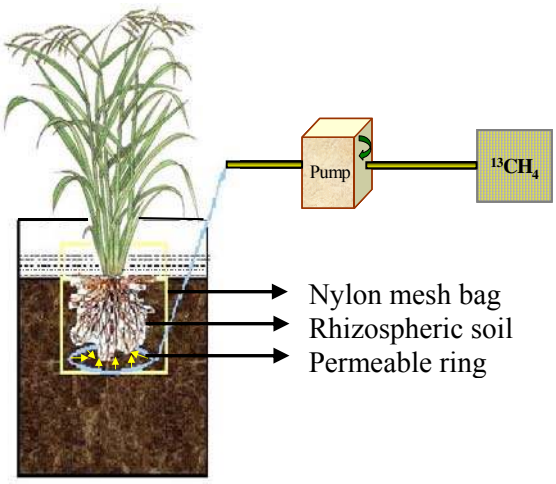
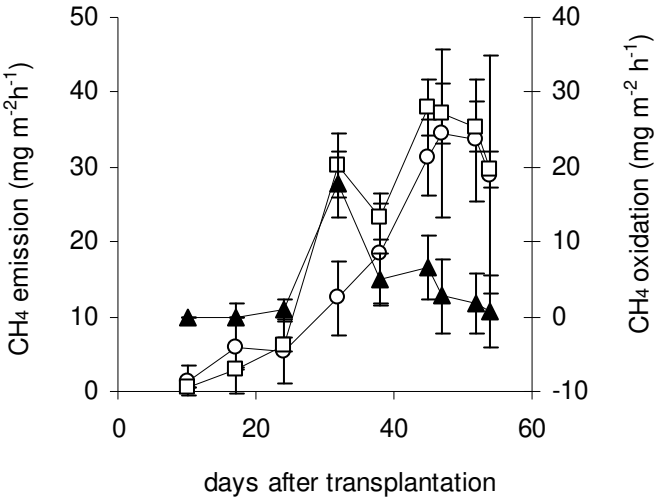

Pump
$^{13}CH_4$
Nylon mesh bag
Rhizospheric soil
Permeable ring

Figure 1

(a)


CH4 emission (mg m-2h-1)
CH4 oxidation (mg m-2 h-1)
days after transplantation
0
10
20
30
40
50
-10
0
10
20
30
40
60

(b)

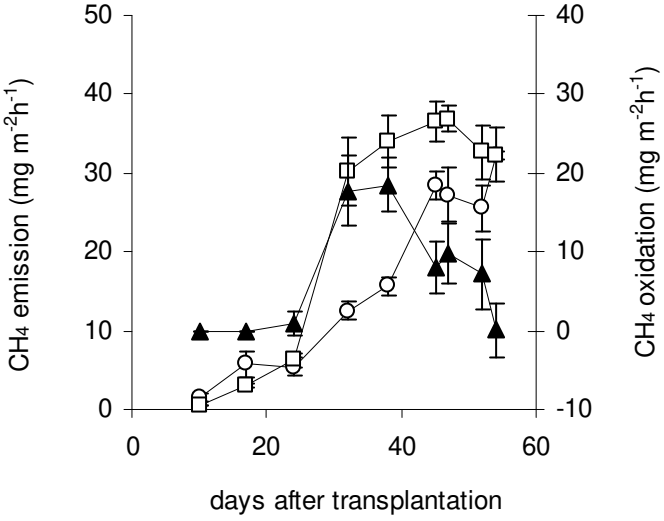

CH4 emission (mg m-2h-1)
CH4 oxidation (mg m-2h-1)
days after transplantation
0
10
20
30
40
50
-10
0
10
20
30
40
60

Figure 2

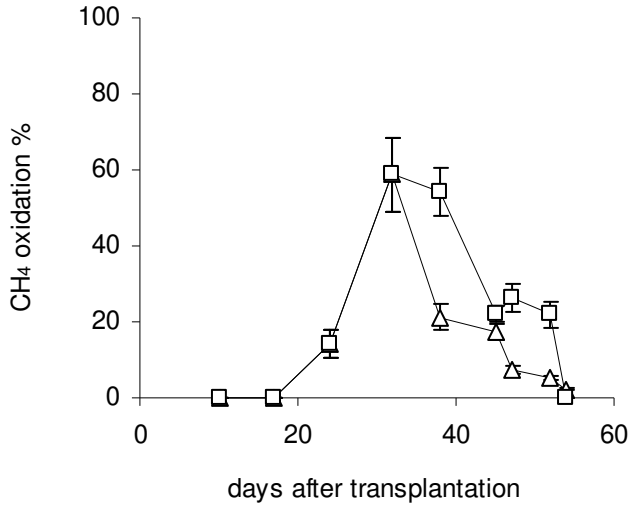
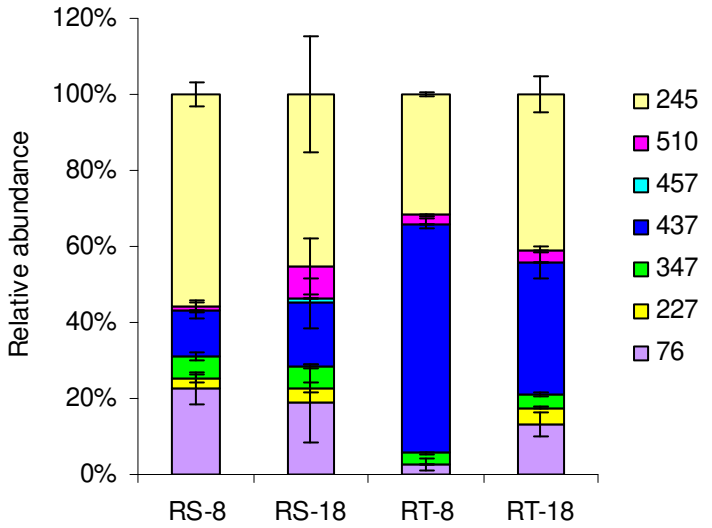

100
80
60
40
20
0
CH4 oxidation %
0
20
40
60
days after transplantation

Figure 3

a)


120%
100%
80%
60%
40%
20%
0%
Relative abundance
RS-8
RS-18
RT-8
RT-18
245
510
457
437
347
227
76

b)

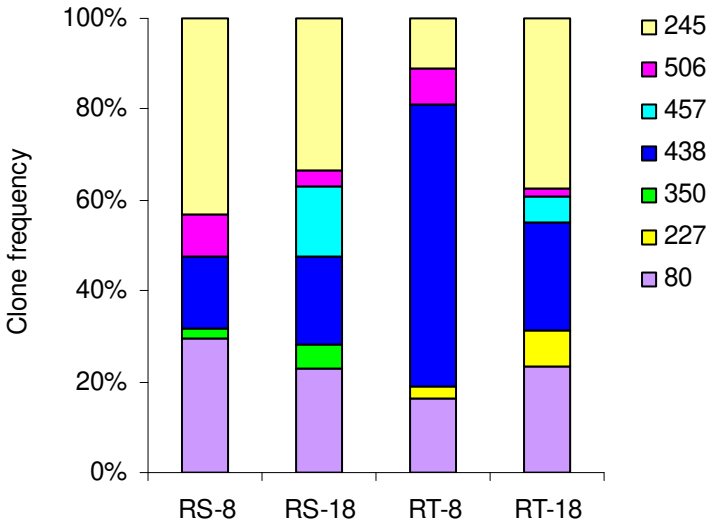

100%
80%
60%
40%
20%
0%
Clone frequency
RS-8
RS-18
RT-8
RT-18
245
506
457
438
350
227
80

Figure 4

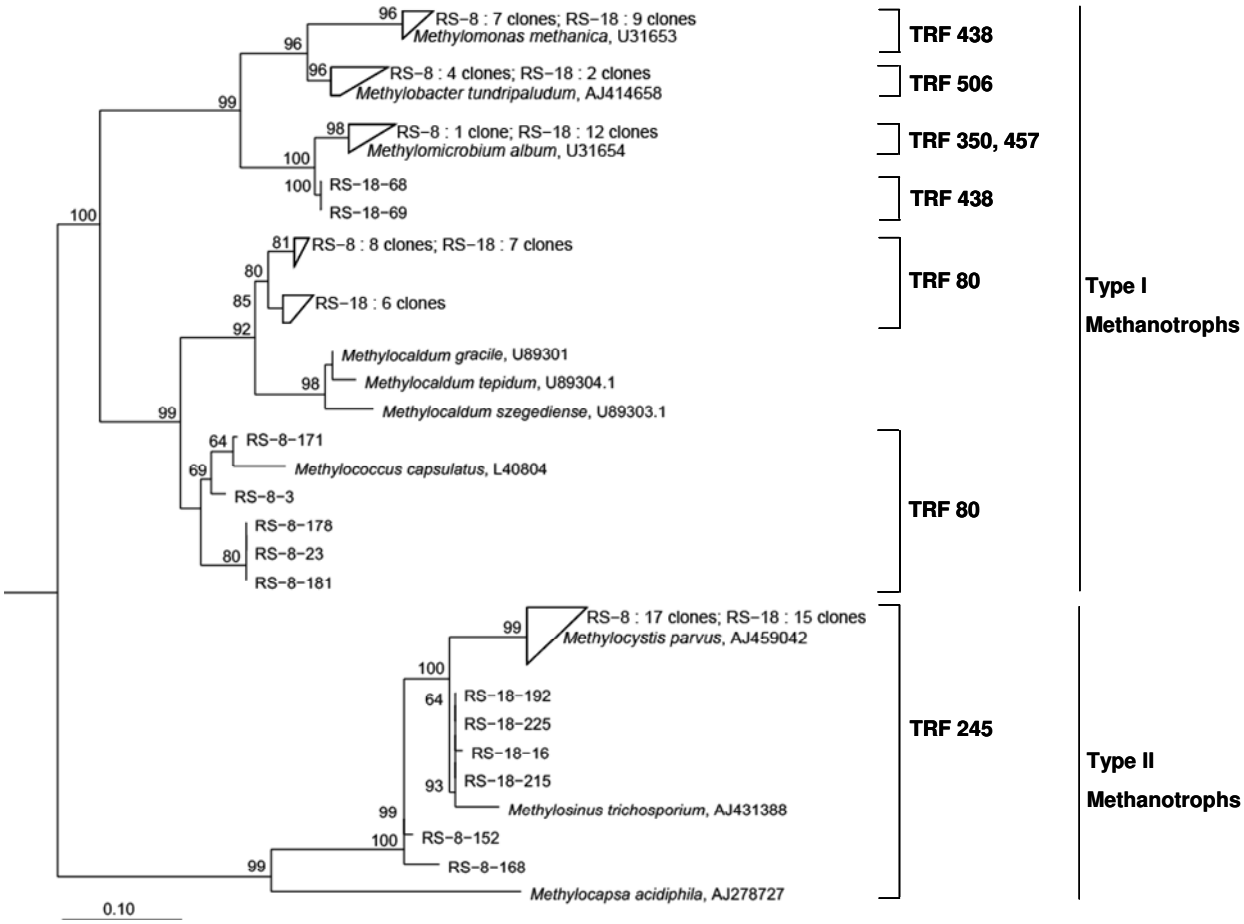

RS−8 : 7 clones; RS−18 : 9 clones
Methylomonas methanica, U31653
RS−8 : 4 clones; RS−18 : 2 clones
Methylobacter tundripaludum, AJ414658
RS−8 : 1 clone; RS−18 : 12 clones
Methylomicrobium album, U31654
RS−18−68
RS−18−69
RS−8 : 8 clones; RS−18 : 7 clones
RS−18 : 6 clones
Methylocaldum gracile, U89301
Methylocaldum tepidum, U89304.1
Methylocaldum szegediense, U89303.1
RS−8−171
Methylococcus capsulatus, L40804
RS−8−3
RS−8−178
RS−8−23
RS−8−181
RS−8 : 17 clones; RS−18 : 15 clones
Methylocystis parvus, AJ459042
RS−18−192
RS−18−225
RS−18−16
RS−18−215
Methylosinus trichosporium, AJ431388
RS−8−152
RS−8−168
Methylocapsa acidiphila, AJ278727
0.10
TRF 438
TRF 506
TRF 350, 457
TRF 438
TRF 80
TRF 80
TRF 245
Type I
Methanotrophs
Type II
Methanotrophs

Figure 5

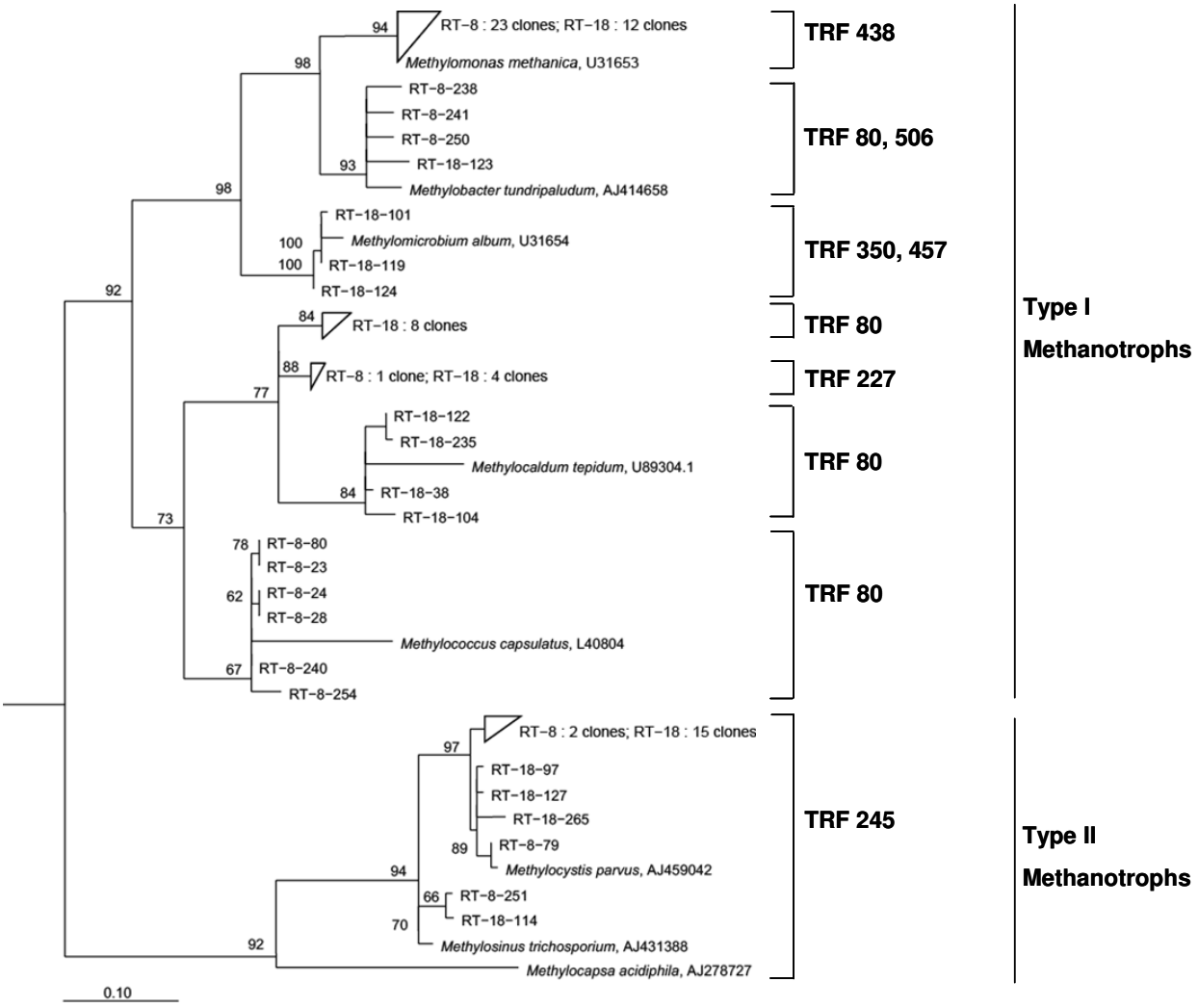
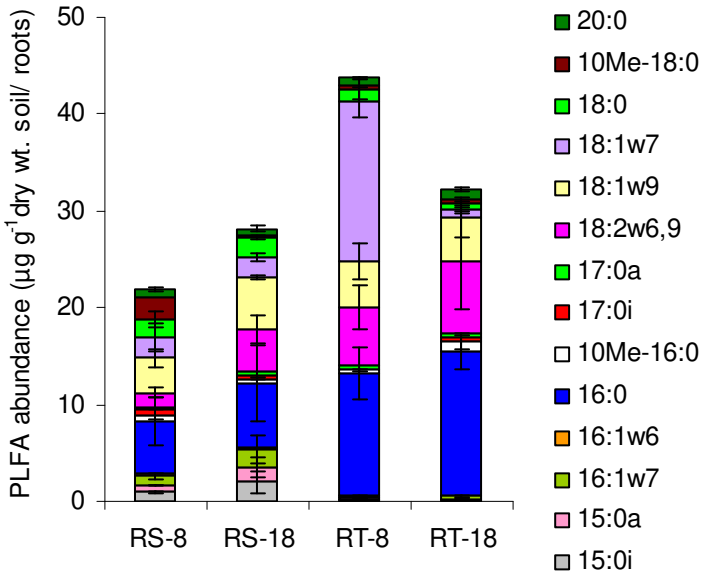

RT−8 : 23 clones; RT−18 : 12 clones
Methylomonas methanica, U31653
RT−8−238
RT−8−241
RT−8−250
RT−18−123
Methylobacter tundripaludum, AJ414658
RT−18−101
Methylomicrobium album, U31654
RT−18−119
RT−18−124
RT−18 : 8 clones
RT−8 : 1 clone; RT−18 : 4 clones
RT−18−122
RT−18−235
Methylocaldum tepidum, U89304.1
RT−18−38
RT−18−104
RT−8−80
RT−8−23
RT−8−24
RT−8−28
Methylococcus capsulatus, L40804
RT−8−240
RT−8−254
RT−8 : 2 clones; RT−18 : 15 clones
RT−18−97
RT−18−127
RT−18−265
RT−8−79
Methylocystis parvus, AJ459042
RT−8−251
RT−18−114
Methylosinus trichosporium, AJ431388
Methylocapsa acidiphila, AJ278727
0.10
TRF 438
TRF 80, 506
TRF 350, 457
TRF 80
TRF 227
TRF 80
TRF 80
TRF 245
Type I Methanotrophs
Type II Methanotrophs

Figure 6

a)


PLFA abundance (µg g-1dry wt. soil/ roots)
50
40
30
20
10
0
RS-8
RS-18
RT-8
RT-18
20:0
10Me-18:0
18:0
18:1w7
18:1w9
18:2w6,9
17:0a
17:0i
10Me-16:0
16:0
16:1w6
16:1w7
15:0a
15:0i

b)

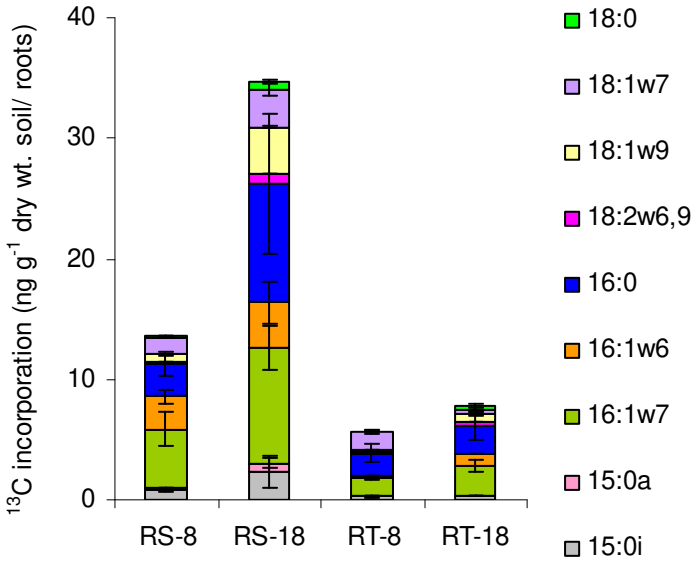

13C incorporation (ng g-1 dry wt. soil/ roots)
40
30
20
10
0
RS-8
RS-18
RT-8
RT-18
18:0
18:1w7
18:1w9
18:2w6,9
16:0
16:1w6
16:1w7
15:0a
15:0i

Figure 7