



**This is a postprint of an article published in
Shrestha, M., Abraham, W.-R., Shrestha, P.M., Noll, M., Conrad, R.
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
(2008) Environmental Microbiology, 10 (2), pp. 400-412.**

1 EMI-2007-0507, re-submitted revision
2 29 August 07

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

6
7

8 Minita Shrestha¹, Wolf-Rainer Abraham², Pravin Malla Shrestha¹, Matthias Noll^{1,3} and Ralf
9 Conrad^{1*}

10

11 ¹Max-Planck-Institut für terrestrische Mikrobiologie, Karl-von-Frisch-Straße, D-35043,
12 Marburg, Germany.

13 ²Helmholtz Centre for Infection Research, Research Group Chemical Microbiology,
14 Inhoffenstrasse 7, D-38124 Braunschweig, Germany.

15 ³Federal Institute for Materials Research and Testing, FG IV.I "Materials Resistance against
16 Microorganisms", Unter den Eichen 87, D-12205 Berlin, Germany

17

18 Running Title: Methanotrophs in rice microcosms

19

20 Keywords: Methane oxidation, Active methanotrophs, Rice microcosms, PLFA-stable isotope
21 probing, *pmoA* gene, rice roots, ¹³C incorporation.

22

23 * Corresponding author:

24 Ralf Conrad, Max-Planck-Institut für terrestrische Mikrobiologie, Karl-von-Frisch-Str., D-35043
25 Marburg, Germany. Phone: +49 (6421) 178 801. Fax: +49 (6421) 178 809. email:

26 conrad@staff.uni-marburg.de

1 **Summary**

2 Methanotrophs in the rhizosphere of rice field ecosystems attenuate the emissions of CH₄ into
3 the atmosphere and thus play an important role for the global cycle of this greenhouse gas.
4 Therefore, we measured the activity and composition of the methanotrophic community in the
5 rhizosphere of rice microcosms. Methane oxidation was determined by measuring the CH₄ flux
6 in the presence and absence of difluoromethane as a specific inhibitor for methane oxidation.
7 Methane oxidation started on day 24 and reached the maximum on day 32 after transplantation.
8 The total methanotrophic community was analysed by terminal restriction fragment length
9 polymorphism (T-RFLP) and cloning/sequencing of the *pmoA* gene, which encodes a subunit of
10 particulate methane monooxygenase. The metabolically active methanotrophic community was
11 analysed by stable isotope probing of microbial phospholipid fatty acids (PLFA-SIP) using ¹³C-
12 labeled CH₄ directly added to the rhizospheric region. Rhizospheric soil and root samples were
13 collected after exposure to ¹³CH₄ for 8 and 18 days. Both T-RFLP/cloning and PLFA-SIP
14 approaches showed that type I and type II methanotrophic populations changed over time with
15 respect to activity and population size in the rhizospheric soil and on the rice roots. However,
16 type I methanotrophs were more active than type II methanotrophs at both time points indicating
17 they were of particular importance in the rhizosphere. PLFA-SIP showed that the active
18 methanotrophic populations exhibit a pronounced spatial and temporal variation in rice
19 microcosms.

1 **Introduction**

2 Methane is a trace gas in the earth's atmosphere with important global warming implications.
3 Rice fields are an important source for atmospheric CH₄ contributing about 40 Tg y⁻¹ (Lelieveld
4 et al., 1998; Wang et al., 2004). Methane, which is produced in the soil, enters the roots of the
5 rice plants and is transported through the gas vascular system of the plants to the atmosphere and
6 oxygen is transported from the atmosphere into the roots. Hence, rice roots are partially oxic and
7 thus allow methanotrophic bacteria to be active in the rhizosphere (Conrad, 2004).

8 Methanotrophs associated with the rhizosphere of rice plants oxidize CH₄ with molecular O₂ and
9 use it as the main sole source of carbon and energy. Thus methanotrophs play an important role
10 in the global CH₄ budget by reducing CH₄ emissions from the rice ecosystems to the atmosphere
11 (Groot et al., 2003). Therefore, a better knowledge of the methanotrophic community structure
12 and its activity in paddy fields is important for the mechanistic understanding of CH₄ oxidation
13 in soil.

14 Methanotrophs are classified into two major physiological groups, type I (belonging to the
15 *Gammaproteobacteria*) and type II methanotrophs (belonging to the *Alphaproteobacteria*),
16 depending on the guanine and cytosine content of their DNA, intracellular membrane
17 arrangement, carbon assimilation pathway and phospholipid fatty acids (PLFA) composition
18 (Hanson and Hanson, 1996). Both, type I and type II methanotrophs have been detected in rice
19 field soil and on rice roots using cultivation techniques (Gilbert and Frenzel, 1998) as well as
20 cultivation-independent techniques that include PLFA analyses, cloning and sequencing of 16S
21 rRNA genes and functional genes (*pmoA*, *mmoX*, *mxoF*) (Bodelier et al., 2000; Bosse and
22 Frenzel, 1997; Eller and Frenzel, 2001; Henckel et al., 1999; Henckel et al., 2000; Horz et al.,
23 2001).

24 Methanotrophs are known to be sensitive to variation in CH₄ and O₂ concentrations (Bender
25 and Conrad, 1995; Henckel et al., 2000), and it has been suggested that the amount of available
26 CH₄ influences the competition between type I and type II methanotrophs. Type I methanotrophs
27 outcompete type II species under low CH₄ and high O₂ conditions, whereas type II species tend
28 to dominate under the opposite conditions (Amaral and Knowles, 1995; Graham et al., 1993).

1 Rice plant ecosystems may exhibit different niches for methanotrophs as characterized by
2 spatiotemporal variation of CH₄ and O₂. Moreover, CH₄ availability increases with distance from
3 the rice roots (Gilbert and Frenzel, 1998), while O₂ availability decreases with distance because
4 of lower root densities and consequently lower root O₂ release at larger distances (VanBodegom
5 et al., 2001). As a consequence, methanotrophic community structure in rice soil may shift with
6 changing conditions and over the season (Eller et al., 2005; Eller and Frenzel, 2001; Macalady et
7 al., 2002) quantified the temporal and spatial dynamics of methanotroph populations in a
8 California rice field using PLFA biomarker analyses, evaluating the relative importance of type I
9 and type II methanotrophs with depth and in relation to rice roots. However, the temporal change
10 of activity and active methanotrophic populations in the rice rhizosphere has not yet been
11 studied.

12 Currently, phospholipid fatty acids stable isotope probing (PLFA-SIP) has become a popular
13 approach for linking microbial community structure with its activity in the environment. In this
14 approach, active soil microbial populations utilizing a ¹³C-labeled substrate will readily
15 incorporate ¹³C into membrane lipid components such as PLFAs. The presence of particular
16 PLFAs is a distinct characteristic of methanotrophic bacteria allowing differentiation between
17 type I (16 carbon fatty acids: 16:0, 16:1) and type II methanotrophs (monounsaturated 18 carbon
18 fatty acids: 18:1 ω 9c, 18:1 ω 8) and also from all other organisms (Bowmann et al., 1993). PLFA-
19 SIP methodology has been successfully applied in several soils and sediments to identify active
20 CH₄-oxidizing bacteria (Boschker et al., 1998; Bull et al., 2000; Crossman et al., 2006; Knief et
21 al., 2003b). Similarly, Bodelier et al. (2000) carried out ¹⁴C-labeled PLFA slurry incubation
22 study to characterize active methanotrophs from soil samples collected from rhizosphere
23 compartment of rice microcosms with different fertilizers treatments. However, the temporal
24 change in active methanotrophic community in the rice rhizosphere has not yet been studied
25 using PLFA-SIP methodology. Furthermore, labeled CH₄ has not yet been applied to the rice
26 roots under close to in-situ conditions. Therefore, we conducted labeling experiments where
27 ¹³CH₄ was directly added to the rhizosphere of planted and fertilized rice microcosms mimicking
28 *in situ* conditions, and the total and active methanotrophic community was investigated with
29 respect to time by analyzing *pmoA* gene libraries and using PLFA-SIP, respectively.

1 **Results**

2 *Rates of CH₄ emission and oxidation*

3 The rhizosphere of rice microcosms was directly supplied with ¹³C-labeled CH₄ by circulating
4 ¹³C-CH₄-saturated water through permeable tubing buried in the soil (Fig.1). The rates of CH₄
5 emission and oxidation were similar in control and ¹³C-labeled microcosms during the
6 experimental period of 55 days after transplantation of rice seedlings (Fig. 2a, b). The CH₄
7 emission rates (in absence of inhibitor) gradually increased from the beginning and reached an
8 average value of 31 and 28 mg CH₄ m⁻² h⁻¹ on day 45 for the control (Fig. 2a) and the ¹³C-labeled
9 microcosms (Fig. 2b), respectively.

10 Methane oxidation rates were calculated as the difference between CH₄ emission rates in the
11 presence and absence of difluoromethane (CH₂F₂), a specific inhibitor of CH₄ oxidation (Miller
12 et al., 1998). Methane oxidation started on day 24 and reached the maximum on day 32 (Fig. 2a,
13 b), when nearly 60% of the anaerobically produced methane was oxidized prior to its emission to
14 the atmosphere (Fig. 3). However, this percentage value decreased rapidly afterwards. After the
15 addition of ¹³C-labeled CH₄, the δ¹³C values of the emitted CH₄ substantially increased in
16 comparison to the background δ¹³C-CH₄ values emitted from the control (unlabeled)
17 microcosms. The maximum δ¹³C was 1610‰ (2.85 atom-%) on day 38, decreased to 525‰
18 (1.68 atom %) on day 46 and increased slowly again and reached to 1588‰ (2.82 atom-%) on
19 day 54.

20

21 *Soil pore water*

22 Concentrations of CH₄ in pore water samples were similar in both the control and the ¹³C-labeled
23 microcosms. On average, CH₄ concentrations were lower in rhizospheric region (at 3 cm depth
24 from the soil surface) than in bulk region (at 10 cm depth from the soil surface). Until 17 days
25 after transplantation, CH₄ concentrations were 800-900 μM at both regions in the control and the
26 ¹³C-labeled microcosms. After 17 days, CH₄ in rhizospheric soil rapidly decreased to 400-500
27 μM and then gradually decreased to 200 μM until the end of the experiment. In the bulk soil, on

1 the other hand, CH₄ concentrations increased up to 1100-1300 μM and later slowly decreased to
2 about 300 μM after 52 days of transplantation.

3 The δ¹³C values of CH₄ in pore water were similar as those in the emitted CH₄. The initial
4 δ¹³C was 1624‰ (2.86 atom-%) on day 38, i.e., immediately after the beginning of the
5 circulation of the ¹³C-labeled CH₄ solution in the soil, and decreased to 525‰ (1.68 atom-%) on
6 day 46, and again gradually increased to 3272‰ (4.58 atom-%) on day 54.

7 Ammonium concentrations in the pore water of rhizospheric soil were similar in both control
8 and ¹³C-labeled treatments during the whole incubation period. The NH₄⁺ concentrations started
9 to decrease after 24 days and remained at about 10 μM after 38 days of transplantation. After the
10 fertilisation on day 44, NH₄⁺ concentration in pore water increased, but rapidly decreased again
11 to 10-20 μM within 5 days of fertilization. The NO₂⁻ and NO₃⁻ concentrations in the pore water
12 were below the detection limit (5 μM) during the entire experimental period. The pH of the pore
13 water in the control and labeled microcosms varied between pH 6.8 and 7.6 at both rhizospheric
14 and bulk regions (data not shown).

15

16 *T-RFLP analysis of methanotrophic community*

17 The methanotrophic community was investigated by T-RFLP analysis targeting the *pmoA*
18 gene in DNA extracts from rhizospheric soil (RS) and root samples (RT) collected 44 and 55
19 days after transplantation, i.e., 8 (RS-8, RT-8) and 18 (RS-18, RT-18) days after the
20 beginning of ¹³C-labeling (see below). Since the physiological data were similar in both
21 control and labeled rice microcosms, molecular analyses were performed in labeled rice
22 microcosms only. T-RFLP analysis produced highly reproducible patterns with T-RFs of 76,
23 227, 245, 347, 437, 457 and 510 bp lengths (±1 bp) in all samples. All of these T-RFs were
24 assigned by our own clone analysis (see below) to the following methanotrophic genera, some
25 of which, however, exhibited T-RFs that were slightly different from those determined by in-
26 silico analysis: *Methylococcus/Methylocaldum* (80 vs. 76 bp); *Methylocystis/Methylosinus*
27 (245 bp); *Methylomicrobium album* (350 vs. 347 bp and 457 bp); *Methylomonas* (438 vs. 437
28 bp) and *Methylobacter* (506 vs. 510 bp).

1 The comparison of T-RFLP community profiles obtained from rhizospheric soil and root
2 samples showed similar T-RF patterns but different relative abundances of the major T-RFs.
3 Furthermore, two different sampling points were also conferred to the different relative
4 abundances of the T-RFs in both rhizospheric soils as well as in root samples (Fig. 4a). The
5 relative abundance of T-RFs belonging to type I and type II methanotrophs in rhizospheric soil
6 and root samples collected at different time points are summarized in Table 1. The T-RFs
7 affiliated with type I methanotrophs were significantly ($P < 0.05$) more abundant than those
8 affiliated with type II methanotrophs in all samples with exception of RS-8, and in addition, they
9 were more abundant on the roots than in the rhizospheric soil (Table 1). The T-RFs affiliated to
10 type II methanotrophs decreased with incubation time in the rhizospheric soil but increased on
11 the roots.

12

13 *Cloning and sequence analysis of pmoA gene*

14 Sequences of *pmoA* genes retrieved from rhizospheric soil (Fig. 5) and root samples (Fig. 6)
15 were analysed by constructing phylogenetic trees. Phylogenetic analysis of *pmoA*-derived amino
16 acid sequences revealed the presence of both type I methanotrophs (genera *Methylomonas*,
17 *Methylobacter*, *Methylococcus*, *Methylocaldum* and *Methylomicrobium*) and type II
18 methanotrophs (genera *Methylocystis* and *Methylosinus*) in rhizospheric soil as well as on root
19 samples. In rhizospheric soil samples, 63 among 101 clones (63%) were affiliated with type I
20 methanotrophs (Fig. 5) and on root samples, 65 among 88 clones (74%) were affiliated with type
21 I methanotrophs (Fig. 6) while the remainder, i.e. 37% and 28%, respectively, were affiliated
22 with type II methanotrophs being less abundant than those of type I methanotrophs. The number
23 of *pmoA*-sequences affiliated to *Methylomonas* sp. was dominant among type I methanotrophs in
24 all samples. The number of *pmoA*-sequences affiliated to *Methylocystis* sp. was 4-fold higher in
25 RT-18 samples compared to those of RT-8, indicating an increase of clone frequency over time
26 (Fig. 5). Some clusters of type I methanotroph *pmoA* sequences were retrieved from rhizospheric
27 soil (sequences corresponding to T-Rf size of 80) and root (sequences corresponding to T-RF
28 size of 80 and 227) samples, which could not be assigned to any sequence types that are

1 deposited in public domain database (Fig. 5 and 6). Also sequences related to *Methylomicrobium*
2 *album* were retrieved that exhibited a T-RF of 457 bp (Fig. 5 and 6) along with 350 bp as
3 reported previously (Horz et al., 2001).

4 T-RFLP analysis of individual clones mostly confirmed the assignment of the different T-
5 RFs to the different genera of methanotrophs. The relative clone frequency of *pmoA*-sequences
6 with the respective affiliated T-RFs, which were retrieved from rhizospheric soil and root
7 samples (Fig. 4b), were similar to the relative abundance of the same T-RFs detected in the DNA
8 extracts (Fig. 4a).

9

10 *Incorporation of ¹³C into PLFA of methanotrophs*

11 To gain more information on the metabolically active methanotrophic community in rhizospheric
12 soil and on root samples we applied a ¹³C-CH₄ labeling approach. Figure 7 shows the total
13 abundance of phospholipid fatty acids (Fig. 7a) and the ¹³C incorporation into phospholipid fatty
14 acids (Fig. 7b) extracted from the rhizospheric soil and the roots incubated with ¹³C-CH₄ for 8
15 days (RS-8 and RT-8) and 18 days (RS-18 and RT-18), i.e., 44 and 54 days after transplantation.
16 Total PLFA concentrations cannot be compared between rhizospheric soil and root samples,
17 since they are expressed per gram dry soil versus gram dry root, respectively. However, total
18 PLFA abundance increased over time in rhizospheric soil samples (RS-8 and RS-18), whereas it
19 decreased on root samples (Fig. 7a). Incorporation of ¹³C into PLFA, on the other hand increased
20 over time in both cases (Fig. 7b), indicating the increased activity of methanotrophs.

21 On average, 16:1 ω 7, 16:1 ω 6, 16:0, 18:1 ω 7, 18:1 ω 9 and 18:0 were the dominant PLFAs
22 labeled with ¹³C in both rhizospheric soil and root samples (Fig. 7b). The PLFAs representing
23 type I methanotrophs (16:1 ω 7, 16:1 ω 6 and 16:0) were significantly ($P < 0.05$) more labeled with
24 ¹³C in all samples than those representing type II methanotrophs (18:1 ω 7, 18:1 ω 9 and 18:0). The
25 incorporation of ¹³C increased in most of the PLFAs after 18 days compared to 8 days of
26 incubation. However, it decreased in some PLFAs (Fig. 7b), for example, in 18:1 ω 9 PLFA on
27 the root samples. The percent distribution of phospholipid fatty acids present in type I and type II
28 methanotrophs in terms of ¹³C incorporation per gram soil or gram root is summarized in Table

1 1. Furthermore, the ratio of ^{13}C incorporation between 8 and 18 days of ^{13}C -labeling (Fig. 7b)
2 showed that after 18 days of incubation, type I methanotrophs exhibited a 2.7-fold higher ^{13}C
3 incorporation than type II methanotrophs on the roots, whereas type II methanotrophs exhibited
4 a 1.5-fold higher ^{13}C incorporation than type I methanotrophs in the rhizospheric soil.

5 The incubation experiments with ^{13}C - CH_4 labeling furthermore showed that 15:0i and
6 18:2 ω 6,9 also became labeled with ^{13}C . In samples retrieved from the RS-18, PLFA of 15:0i, a
7 biomarker of Gram-positive bacteria (O'Leary and Wilkinson, 1988), represented 4% of total ^{13}C
8 incorporation in PLFA. Similarly, PLFA of 18:2 ω 6,9, a biomarker of Eukaryotes (Frostegard
9 and Baath, 1996), represented 2% and 6% of total ^{13}C incorporation in rhizospheric soil and root
10 samples, respectively.

11 12 **Discussion**

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

19 Rates of CH_4 oxidation reached a maximum 32 days after transplantation. During this
20 period, the overall CH_4 concentrations in the pore water had decreased. In addition, CH_4
21 concentrations were lower in the rhizospheric soil samples than in those of the bulk soil
22 samples indicating increased methanotrophic activity in the rhizosphere and/or increased CH_4
23 loss by ventilation through the rice plant (Conrad and Klose, 2005; Gilbert and Frenzel,
24 1998). Rates of CH_4 oxidation decreased after reaching a maximum with 60% of the produced
25 CH_4 being oxidized on day 32 following transplantation. Similar results have been obtained
26 previously (Bodelier et al., 2000; Xu et al., 2004), demonstrating that CH_4 oxidation in rice
27 fields is a dynamic process that seems to be regulated by various factors, including the age of
28 the rice plant and nutrient availability for the microorganisms and/or plants. In particular, the

1 decrease of CH₄ oxidation activity with the progress of the season has been observed
2 previously (Dan et al., 2001; Eller and Frenzel, 2001; Krüger and Frenzel, 2003). Ammonium
3 concentrations also decreased during this time, which may be explained by efficient uptake of
4 N by the rice plant (Arth et al., 1998). Methanotrophic activity in the rice rhizosphere can be
5 limited by available nitrogen (Bodelier et al., 2000; Dan et al., 2001; Krüger and Frenzel,
6 2003). Indeed, we found that a second fertilisation stimulated CH₄ oxidation albeit only
7 briefly, similarly to the previous observations (Dan et al., 2001; Krüger and Frenzel, 2003). In
8 order to better understand the dynamics of CH₄ oxidation in the context of microbial
9 community, we analyzed the methanotrophic community during this phase.

10 The resident populations of methanotrophs in the rhizospheric soil and on the roots were
11 determined by targeting the *pmoA* gene, a functional gene marker for methanotrophs. Sequence
12 analysis of several clone libraries showed the presence of both type I and type II methanotrophs
13 including the genera *Methylomonas*, *Methylobacter*, *Methylomicrobium*, *Methylococcus*,
14 *Methylomicrobium*, *Methylocystis* and *Methylosinus* in both rhizospheric soil and on the roots.
15 Such a diversity has been found previously in rice field ecosystems from Vercelli, Italy (Eller et
16 al., 2005; Henckel et al., 2001; Horz et al., 2001) and elsewhere (Hoffmann et al., 2002; Jia et
17 al., 2007). Most of the detected methanotrophic genera exhibited the characteristic size of the T-
18 RFs reported by Horz and colleagues (2001). However, some of the T-RFs sizes observed in this
19 study exhibited 2-4 base pairs difference to the theoretical T-RF size determined in-silico. A
20 certain amount of variation between observed and predicted T-RF length remains that could be
21 explained due to the application of different sequencing machines, dye labels, or fluctuations in
22 laboratory temperature (Kaplan and Kitts, 2003), and even sometimes such variations appear to
23 be sequence dependent (Kitts, 2001). Notable is the detection of a few sequences clustering with
24 *Methylomicrobium* sp. and having a T-RF of 457 bp size, which has not been observed before.
25 Furthermore, a few sequences were detected that cluster within the type I methanotrophs and
26 have a T-RF of 227 bp size. Horz and colleagues (2001) had also detected sequences having a T-
27 RF of 227 bp size from rice root samples but could not assign them to either *pmoA* or *amoA*
28 sequences. Our study showed that this T-RF belongs to type I methanotrophs because, we used

1 *pmoA* specific primers (A189/Mb661) that do not amplify *amoA* sequences (Bourne et al., 2001;
2 Costello and Lidstrom, 1999).

3 Due to relatively clear assignment of different T-RFs to the different methanotrophic genera,
4 we were able to calculate the relative abundance of the different methanotrophic populations by
5 using T-RFLP analysis of the *pmoA* genes. Additionally, we were able to quantify the number of
6 *pmoA* sequences which could be unambiguously assigned to respective T-RFs. Despite both
7 approaches showed that the general composition of the methanotrophic community was quite
8 similar in the rhizospheric soil and on the roots, the relative abundance of individual
9 methanotrophic genera was different and in addition, exhibited a shift between 44 and 54 days
10 after transplantation. Thus, while the relative abundance of type II methanotrophs (T-RF of 245
11 bp) decreased in the rhizosphere soil, it increased on the roots. The reverse was observed for
12 type I methanotrophs (Table 1). This result indicated that the different methanotrophic genera
13 responded differently to spatiotemporal variations in the rice microcosms, which in turn gave a
14 hint that different methanotrophic genera may have different CH₄, O₂ or nutrient requirements
15 (see Discussion below). Thus it is remarkable that type I methanotrophs were more abundant on
16 the roots while type II methanotrophs were more abundant in the rhizospheric soil. Note,
17 however, that the abundance of methanotrophic groups were only relative numbers within the
18 total community of methanotrophs. The total community of methanotrophs has most probably
19 increased over time in the rhizospheric soil and decreased on the roots as indicated by the
20 temporal change of the PLFA concentrations (Fig. 7a). PLFA biomarkers ideally provide
21 information on microbial identity and biomass of living bacteria (Tunlid and White, 1992).
22 However, this biomass also represents inactive bacteria and only a minor part of the PLFA
23 detected belongs to methanotrophs, which occur on the order of <10⁷ per gram dry rice soil, i.e.,
24 about 1% of total biomass (Eller et al., 2005; Joulain et al., 1997). Therefore, it was not possible
25 to calculate the temporal change of the methanotrophic biomass from the T-RFLP and total
26 PLFA analyses. Moreover, since we used DNA samples for the amplification of *pmoA*, we could
27 not ascertain that the amplified *pmoA* product represented the metabolically active
28 methanotrophs.

1 Therefore, we used PLFA-stable isotope probing method and supplied the rhizosphere of the
2 rice microcosms with ^{13}C - CH_4 between 37 and 54 days after transplantation. Although the
3 labeled CH_4 consisted of 99 atom-% ^{13}C , the CH_4 in the pore water and in the CH_4 flux
4 contained only 3-5 atom-% ^{13}C . This result showed that the CH_4 added through the permeable
5 tubing into the rhizosphere became highly diluted by endogenously produced CH_4 . However, the
6 resulting ^{13}C -content of the CH_4 allowed the detection of specifically ^{13}C -labeled PLFA. PLFA-
7 SIP had previously been used to characterize active methanotrophs in aquatic sediments
8 (Boschker et al., 1998; Nold et al., 1999), but has so far not been applied in planted rice fields or
9 planted rice microcosms. In our study of planted rice microcosms, mainly the PLFA (16:1 ω 7,
10 16:1 ω 6, 16:0, 18:1 ω 7, 18:1 ω 9, 18:0) became labeled with ^{13}C derived from CH_4 . Although these
11 PLFA are found in many different prokaryotes and eukaryotes (Spring et al., 2000) the labeling
12 of them strongly indicates that type I and type II methanotrophs were active in rhizospheric soil
13 and on root samples, and incorporated ^{13}C during the 8 and 18 days of incubation into 16:1 ω 7,
14 16:1 ω 6, 16:0 and 18:1 ω 7, 18:1 ω 9, 18:0 PLFA, respectively. However, we would not expect that
15 any other organisms than methanotrophs assimilated ^{13}C - CH_4 . Unexpectedly, we detected small
16 amounts (1-6%) of PLFA representing Gram-positive bacteria (15:0i) and eukaryotes (18:2 ω 6,9)
17 that became labeled with ^{13}C , probably by cross-feeding. These PLFAs have been reported
18 previously from rice paddy fields (Kimura and Asakawa, 2006). Recently, Raghoebarsing et al.
19 (2005) showed that eukaryotic *Sphagnum* moss can be cross-fed by the CO_2 derived from
20 methanotrophs living inside the moss. We did not detect a peak for PLFA 18:1 ω 8, which would
21 be characteristic for *Methylocystis* sp. This might have been due to methodological limitations
22 using a non-polar separation column as suggested by Knief et al. (2003b). Consistent with our
23 study, the PLFA 18:1 ω 8 had also not been detected in rice fields by other researchers (Bai et al.,
24 2000; Bossio and Scow, 1998; Reichardt et al., 1997).

25 During the 8 and 18 days of ^{13}C - CH_4 labeling incubation, i.e., after 44 and 54 days of
26 transplantation, the PLFA of methanotrophs became increasingly ^{13}C -labeled, demonstrating
27 their activity in the rhizospheric soil and on the roots. The incorporation of ^{13}C was significantly
28 higher in the rhizospheric soil than on the roots ($P < 0.05$), which is consistent with the relatively
29 higher most probable number counts of methanotrophs (Eller and Frenzel, 2001). However, type

1 I methanotrophs incorporated significantly more ^{13}C into their PLFAs than type II
2 methanotrophs, in both soil and roots, indicating that type I methanotrophs were the more active
3 population. In addition, ^{13}C -incorporation into type I methanotrophs relatively increased with
4 respect to time, while ^{13}C -incorporation into type II methanotrophs decreased, albeit only on the
5 roots. In the soil, on the other hand, it seemed to be opposite, i.e. the PLFA of type II
6 methanotrophs became slightly more ^{13}C -labeled with respect to time than PLFA of type I
7 methanotrophs. These results indicated that type I and type II methanotrophs became
8 increasingly more active on the roots and in the rhizospheric soil, respectively. These findings
9 agree with previous studies (Amaral and Knowles, 1995; Graham et al., 1993), which reported
10 that competition between type I and type II methanotrophs depends upon the concentrations of
11 CH_4 and O_2 and also the presence of nitrogen. Type I methanotrophs seem to prefer
12 environments with plentiful O_2 and limited CH_4 concentrations, whereas type II methanotrophs
13 dominate in environments with high concentrations of CH_4 and limited O_2 . In our results,
14 temporal changes in the activity of both type I and type II methanotrophs could be observed,
15 with type I methanotrophs eventually exhibiting higher activity on the roots, while type II
16 methanotrophs became more active in the rhizospheric soil. We speculate that type I
17 methanotrophs were not be able to cope with the low O_2 concentrations in rhizospheric soil and
18 thus became less active with time as O_2 availability decreases with distance from the roots. As a
19 consequence, type II methanotrophs became dominant instead. Consistent to our study,
20 Macalady et al. (2002) suggested that both type I and type II methanotrophs coexist in rice
21 paddies, but nevertheless occupy different niches with type I methanotrophs being more
22 important in drained fields where O_2 reaches deeper soil layers and type II methanotrophs being
23 more important in flooded fields where CH_4 availability is high.

24 In conclusion, we could effectively differentiate metabolically active methanotrophic
25 community from the total methanotrophic community resident in the rhizospheric soil as well as
26 on the roots from planted rice microcosms using the PLFA-SIP approach and community
27 analysis approach. Both approaches demonstrated that type I and type II methanotrophic
28 populations in the rhizospheric soil and on the rice roots changed differently over time with
29 respect to activity and population size and that type I methanotrophs played a particularly

1 important role in the rice field ecosystem. Furthermore, PLFA-SIP showed that the active
2 methanotrophic populations exhibit a pronounced spatial and temporal variation in rice
3 microcosms. This variation is probably due to different concentrations of methane, oxygen and
4 probably nutrients, which provide different niches for the methanotrophs.

6 **Experimental procedures**

7 *Planted rice microcosms*

8 Soil was taken from drained paddy fields of the Italian Rice Research Institute in Vercelli, Italy
9 and was air-dried and stored at room temperature. The soil characteristics have been described
10 previously (Holzapfel-Pschorn and Seiler, 1986). Immediately prior to its use, the soil was
11 passed through a 2-mm sieve, and soil slurry was prepared with 1.8 kg soil, 940 ml
12 demineralized water, and 60 ml of fertilizer solution (9.89 g urea, 7.605 g KH_2PO_4 , and 7.07 g
13 KCl per liter), and finally filled into each microcosm with a volume of 2.5 L (height 16 cm,
14 diameter 17 cm) pots. In the center of each pot, a self-made nylon bag (25 μm mesh; 6 cm length
15 and 9 cm radius) was placed through which water and nutrients could pass freely while roots
16 were not able to penetrate, isolating the soil inside the bag as rhizospheric soil from the bulk soil
17 outside the bag (Fig. 1). A ring of permeable tubing (7.5 cm diameter) was placed into the lower
18 part of the nylon bag and was connected to a reservoir foil grab bag (Analyt-MTC, Germany)
19 containing demineralized water saturated with ^{13}C -labeled CH_4 (99 atom-% ^{13}C , Isotec, USA)
20 and circulated directly to the microcosms with the help of peristaltic pumps (Fig. 1).

21 In total, 18 microcosms were prepared among which 9 were for $^{13}\text{CH}_4$ -labeling and another 9
22 were used as control without labeling (^{12}C - CH_4 -labeling was not done), and were flooded with
23 demineralized water giving a water depth of 5 cm above the soil surface and were incubated in
24 the greenhouse with a relative humidity of 70%, 12 h photoperiod, and 28/22°C day/night
25 temperature. After five days of flooding, one 14-day old rice seedling (*Oryza sativa* var.
26 KORAL type japonica) germinated on moist filter paper at room temperature was transplanted
27 into the center of the nylon bag in each pot, and 20 ml of fertilizer solution (same as above) was
28 added. The day of transplantation was taken as day zero. The incubation experiment was then

1 conducted for a total of 55 days under flooded conditions. The ^{13}C -labeled CH_4 solution was
2 continuously circulated in the soil between day 37 and 54 after transplantation. After 44 days, 20
3 ml of fertilizer solution was added a third time to the microcosms. In our study, fertilization
4 solution was added as a mixture of ammonium, phosphorous and potassium sources in split
5 doses as applied in field practice in order to fulfill the nutrient requirements for rice plant
6 growth. Water lost due to evapotranspiration was daily replaced by addition of demineralized
7 water to maintain a 5 cm water depth.

8

9 *Measurement of CH_4 flux*

10 Rates of CH_4 emission and CH_4 oxidation were measured as described previously (Krüger and
11 Frenzel, 2003). For the measurement of rates of CH_4 emission, triplicate microcosms were
12 covered by static flux chambers, and gas samples were taken every 30 min for 3 hours. Rates of
13 CH_4 oxidation were measured in parallel triplicate microcosms by adding 1% difluoromethane
14 (CH_2F_2 99%, ICI Chemicals, UK), an inhibitor specific for CH_4 oxidation (Miller et al., 1998), to
15 the headspace of the flux chambers. The gas samples were analysed for CH_4 , CO_2 and CH_2F_2 on
16 a Shimadzu GC-8A gas chromatograph equipped with a flame ionization detector and a
17 methanizer as described previously (Krüger et al., 2002). After starting circulation of ^{13}C -labeled
18 CH_4 through the soil, i.e. after day 37, gas samples were also taken into 10-ml glass vials, which
19 had been previously flushed with N_2 , for later analysis of the ^{13}C content of the CH_4 . Stable
20 isotope analysis of $^{13}\text{C}/^{12}\text{C}$ in gas samples was performed using a gas chromatograph
21 combustion-isotope ratio mass spectrometry (GCC-IRMS) system as described previously
22 (Conrad et al., 2002).

23

24 *Soil pore water*

25 Pore water samples were collected weekly into Venoject blood collecting tubes (TERUMO
26 EUROPE N.V., Belgium) from the rhizosphere (3 cm depth from the soil surface) and bulk (10
27 cm depth from the soil surface) regions of rice microcosms by using Rhizon pore water samplers
28 (Rhizosphere Research Products, The Netherlands). After heavy shaking by hand, an aliquot of

1 the headspace of the tubes was collected with a pressure lock syringe and analysed for CH₄. The
2 CH₄ concentration in the soil pore water was calculated as described previously (Krüger et al.,
3 2001). Then, the pore water was stored frozen (−20°C) for the later determination of ¹³C-CH₄,
4 pH, NH₄⁺, NO₂[−] and NO₃[−]. Ammonium concentration was analysed by using a SAFIRE
5 microplate reader (TECAN, Crailsheim, Germany) as described by (Murase et al., 2006), and
6 nitrate and nitrite concentration by ion chromatography (Sykam, Gilphing, Germany) as
7 described by Bak and colleagues (1991).

8

9 *Collection of soil and root samples*

10 Rhizospheric soil and root samples were collected after 44 (8 days after the beginning of ¹³C-
11 labeling) and 55 (18 days after the beginning of ¹³C-labeling) days after transplantation from the
12 ¹³C-labeled and control (unlabeled) microcosms in triplicate. The samples were designated as
13 RS-8 and RS-18 for the rhizospheric soil, and RT-8 and RT-18 for the roots, respectively. The
14 roots were washed in deionized sterile water. The soil and root samples were stored at −20°C for
15 later molecular and PLFA-SIP analyses.

16

17 *DNA extraction, PCR, cloning and sequencing*

18 Total DNA from the soil and root samples was extracted using a DNA extraction protocol
19 reported previously (Noll et al., 2005). The frozen roots were pulverized with a mortar and pestle
20 after freezing in liquid N₂. After extraction, DNA was checked for quality and quantity by
21 electrophoresis in agarose gels containing ethidium bromide, followed by DNA purification
22 using the Wizard[®] DNA Clean-up System (Promega, Germany).

23 PCR amplification of the *pmoA* gene was done using primers A189f and mb661 (Costello
24 and Lidstrom, 1999). The reaction was carried out in 50 µl (total volume) mixtures containing 1
25 µl of template DNA, 10 µl of 5× reaction buffer (Promega, Germany), 1.5 mM MgCl₂, 200 µM
26 each dNTP, 0.33 µM (each) primer (MWG-Biotech, Germany), and 2.5 U of Taq DNA
27 polymerase (Promega, Germany). The thermal PCR profile was as follows: initial denaturation at
28 94°C for 5 min; 15 cycles consisting of denaturation at 94°C for 1 min, primer annealing at 62°C

1 for 45 s, and elongation at 72°C for 60 s, followed by another twenty cycles consisting of
2 denaturation at 94°C for 1 min, primer annealing at 55°C for 45 s and elongation at 72°C for 60
3 s. The final elongation step was extended to 5 min. Amplification was performed in 0.2-ml
4 reaction tubes using a DNA thermal cycler (model 2400; PE Applied Biosystems). Aliquots of
5 the amplicons (5 µl) were checked by electrophoresis on a 1% agarose gel including positive and
6 negative controls.

7 Two clone libraries each of *pmoA* gene from DNA amplicons retrieved from rhizospheric
8 soil and root samples were constructed using the pGEM-T Easy cloning kit (Promega,
9 Germany). A total of 101 clones from rhizospheric soil samples (49 and 57 clones from RS-8
10 and RS-18 days samples, respectively) and of 88 clones from root samples (37 and 51 clones
11 from RT-8 and RT-18 days samples, respectively) were randomly selected for comparative
12 sequence analysis. Cloned inserts were sequenced at the Max Planck Institute for Plant Breeding
13 in Cologne, Germany, using the primers M13f and M13r targeting vector sequences.

14 The *pmoA* gene sequences have been deposited in the EMBL, GenBank, and DDBJ
15 nucleotide sequence databases under the following accession numbers: AM849616-AM849659
16 (RS-8); AM849660-AM849716 (RS-18); AM849717-AM849753 (RT-8); AM849759-
17 AM849804 (RT-18).

18

19 *Phylogenetic analysis*

20 The identities of the *pmoA* gene sequences were confirmed by searching the sequence databases
21 using BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>). Phylogenetic analyses of the DNA and
22 deduced amino acid sequences were carried out using the ARB program package (developed by
23 O. Strunck and W. Ludwig; Technische Universität München [<http://www.arb-home.de>]).

24 Sequences were manually aligned with the *pmoA* sequences obtained from the GenBank
25 database. Regions of sequence ambiguity and incomplete data were excluded from the analyses.

26 Results were depicted as a consensus tree, combining the results of Tree-Puzzle, neighbor-
27 joining approach, and maximum likelihood analyses of the data sets.

28

1 *T-RFLP analysis*

2 Analysis of terminal restriction fragment length polymorphism (T-RFLP) was performed for
3 each total DNA extract in triplicate. The *pmoA* was amplified by PCR as described above except
4 a FAM (6- carboxyfluorescein)-labeled forward primer was used. After purification with
5 Qiaquick spin columns (Qiagen, Germany), approximately 100 ng of the amplicons were
6 digested with 10 U of the restriction endonuclease MspI (Promega, Germany). The digestion was
7 carried out in a total volume of 10 μ l for 6 h at 37°C. Aliquots (2.5 μ l) of the digested amplicons
8 were mixed with 12 μ l of deionized formamide (Applera, Germany) and 0.2 μ l of an internal
9 DNA fragment length standard (X-Rhodamine MapMarker[®] 30-1000 bp; BioVentures, USA).
10 The mixtures were denatured at 94°C for 3 minutes and then chilled on ice and finally loaded
11 into an automated gene sequencer (ABI 310, Applied Biosystems, Germany) fitted with ABI
12 prism where the terminal restriction fragments (T-RFs) were separated. The length of
13 fluorescently labeled T-RFs was determined by comparison with the internal standard using
14 GeneScan 3.71 software (Applied Biosystems, Germany). The accuracy of size calling between
15 replicates was ± 1 bp. The proportional abundance of individual T-RFs within a given T-RFLP
16 pattern was determined as the peak height of the respective T-RF divided by the total peak
17 height of all T-RFs detected within a fragment size range between 50 and 550 bp and was
18 expressed as a fraction based on 1.0 (Lüdemann et al., 2000).

19

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

21 Lipids were extracted from 3 g dry weight of rhizospheric soil and roots (n=3), taken from both
22 control and ¹³C-labeled microcosms, using a modified Bligh and Dyer extraction method (Knief
23 et al., 2003a). The frozen root samples were pulverized with a mortar and pestle after freezing in
24 liquid N₂ as described for DNA extraction. The weighed samples were extracted with the mixture
25 of phosphate buffer, methanol and chloroform and further fractionated to neutral lipids, glyco-
26 and phospholipids by column chromatography on a silicic acid column (Water, Ireland) into
27 different polarity classes by sequential elution with chloroform, acetone and methanol (Zelles
28 and Bai, 1993). The resulting fatty acid components were released and methylated by mild

1 alkaline methanolysis (White et al., 1979). The individual fatty acid methyl esters were identified
2 using GC-mass spectrometry, and their stable carbon-isotope compositions were determined
3 using a Finnigan MAT Model 252 isotope ratio mass spectrometer coupled to HP 5890 GC with
4 a Finnigan standard combustion interface (Abraham et al., 1998). To calculate isotope ratios
5 ($\delta^{13}\text{C}$) for the PLFAs, $\delta^{13}\text{C}$ values of the FAMES were corrected for the ^{13}C -content of the
6 carbon atom of the methyl group ($\delta^{13}\text{C} -37.6\text{‰}$) that was added during methanolysis. Total
7 PLFAs were estimated based on peak areas in gas chromatograms with 16:0 as internal standard.
8 From the difference in $\delta^{13}\text{C}$ values of the PLFA extracted from the ^{13}C -labeled soils and those
9 from the unlabeled soils (control), the amount of ^{13}C incorporation into each PLFA was
10 calculated as $\text{ng } ^{13}\text{C}$ incorporated per gram of soil or roots (Nold et al., 1999) as: ^{13}C
11 incorporation = $(F_e - F_c) [\text{PLFA}]_e$, where F_e = fraction of ^{13}C in the ^{13}C -labeled samples; F_c =
12 fraction of ^{13}C in the unlabeled control sample; and $[\text{PLFA}] = \text{PLFA concentration in the } ^{13}\text{C}$ -
13 labeled sample. F was given by $^{13}\text{C}/(^{13}\text{C}+^{12}\text{C}) = R/(R+1)$ where $R = (\delta^{13}\text{C}/1000+1) R_{\text{VPDB}}$.

14

15 *Statistical analysis*

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

21

22 **Acknowledgements**

23 We thank Peter Claus and Melanie Klose for excellent technical assistance and Esther Surges for
24 isotope ratio measurements. This study was financially supported by the Fonds der Chemischen
25 Industrie. Minita and Pravin Shrestha received a doctoral fellowship from the Max-Planck
26 Society.

References

- Abraham, W. R., Hesse, C., and Pelz, O. (1998) Ratios of carbon isotopes in microbial lipids as an indicator of substrate usage. *Appl. Environ. Microbiol.* **64**: 4202-4209.
- Amaral, J. A. and Knowles, R. (1995) Growth of methanotrophs in methane and oxygen counter gradients. *FEMS Microbiol. Lett.* **126**: 215-220.
- Arth, I., Frenzel, P., and Conrad, R. (1998) Denitrification coupled to nitrification in the rhizosphere of rice. *Soil Biol. Biochem.* **30**: 509-515.
- Bai, Q., Gattinger, A., and Zelles, L. (2000) Characterization of microbial consortia in paddy rice soil by phospholipid analysis. *Microb. Ecol.* **39**: 273-281.
- Bak, F., Scheff, G., and Jansen, K. H. (1991) A rapid and sensitive ion chromatographic technique for the determination of sulfate and sulfate reduction rates in freshwater lake sediments. *FEMS Microbiol. Ecol.* **85**: 23-30.
- Bender, M. and Conrad, R. (1995) Effect of CH₄ concentrations and soil conditions on the induction of CH₄ oxidation activity. *Soil Biol. Biochem.* **27**: 1517-1527.
- Bodelier, P. L. E., Roslev, P., Henckel, T., and Frenzel, P. (2000) Stimulation by ammonium-based fertilizers of methane oxidation in soil around rice roots. *Nature* **403**: 421-424.
- Boschker, H. T. S., Nold, S. C., Wellsbury, P., Bos, D., DeGraaf, W., Pel, R. et al. (1998) Direct linking of microbial populations to specific biogeochemical processes by ¹³C-labelling of biomarkers. *Nature* **392**: 801-805.
- Bosse, U. and Frenzel, P. (1997) Activity and distribution of methane-oxidizing bacteria in flooded rice soil microcosms and in rice plants (*Oryza sativa*). *Appl. Environ. Microbiol.* **63**: 1199-1207.
- Bossio, D. A. and Scow, K. M. (1998) Impacts of carbon and flooding on soil microbial communities - phospholipid fatty acid profiles and substrate utilization patterns. *Microb. Ecol.* **35**: 265-278.

- Bourne, D. G., McDonald, I. R., and Murrell, J. C. (2001) Comparison of pmoA PCR primer sets as tools for investigating methanotroph diversity in three Danish soils. *Appl. Environ. Microbiol.* **67**: 3802-3809.
- Bowmann, J. P., Sly, L. I., Nichols, P. D., and Hayward, A. C. (1993) Revised taxonomy of the methanotrophs: description of *Methylobacter* gen. nov., emendation of *Methylococcus*, validation of *Methylosinus* and *Methylocystis* species, and a proposal that the family *Methylococcaceae* includes only the group I methanotrophs. *Int. J. Syst. Bacteriol.* **43**: 735-753.
- Bull, I. D., Parekh, N. R., Hall, G. H., Ineson, P., and Evershed, R. P. (2000) Detection and classification of atmospheric methane oxidizing bacteria in soil. *Nature* **405**: 175-178.
- Conrad, R. (2004) Methanogenic microbial communities associated with aquatic plants. In *Plant Surface Microbiology*. Varma, A., Abbott, L., Werner, D., and Hampp, R. (eds). Berlin: Springer, 35-50.
- Conrad, R. and Klose, M. (2005) Effect of potassium phosphate fertilization on production and emission of methane and its ¹³C-stable isotope composition. *Soil Biol. Biochem.* **37**: 2099-2108.
- Conrad, R., Klose, M., and Claus, P. (2002) Pathway of CH₄ formation in anoxic rice field soil and rice roots determined by ¹³C-stable isotope fractionation. *Chemosphere* **47**: 797-806.
- Costello, A. M. and Lidstrom, M. E. (1999) Molecular characterization of functional and phylogenetic genes from natural populations of methanotrophs in lake sediments. *Appl. Environ. Microbiol.* **65**: 5066-5074.
- Crossman, Z. M., Wang, Z. P., Ineson, P., and Evershed, R. P. (2006) Investigation of the effect of ammonium sulfate on populations of ambient methane oxidising bacteria by ¹³C-

- labelling and GC/C/IRMS analysis of phospholipid fatty acids. *Soil Biol. Biochem.* **38**: 983-990.
- Dan, J. G., Krüger, M., Frenzel, P., and Conrad, R. (2001) Effect of a late season urea fertilization on methane emission from a rice field in Italy. *Agric. Ecosyst. Environ.* **83**: 191-199.
- Eller, G. and Frenzel, P. (2001) Changes in activity and community structure of methane-oxidizing bacteria over the growth period of rice. *Appl. Environ. Microbiol.* **67**: 2395-2403.
- Eller, G., Krüger, M., and Frenzel, P. (2005) Comparing field and microcosm experiments: a case study on methano- and methylo-trophic bacteria in paddy soil. *FEMS Microbiol. Ecol.* **51**: 279-291.
- Frostegard, A. and Baath, E. (1996) The use of phospholipid fatty acid analysis to estimate bacterial and fungal biomass in soil. *Biol. Fertil. Soils* **22**: 59-65.
- Gilbert, B. and Frenzel, P. (1998) Rice roots and CH₄ oxidation - the activity of bacteria, their distribution and the microenvironment. *Soil Biol. Biochem.* **30**: 1903-1916.
- Graham, D. W., Chaudhary, J. A., Hanson, R. S., and Arnold, R. G. (1993) Factors affecting competition between type-I and type-II methanotrophs in 2-organism, continuous-flow reactors. *Microb. Ecol.* **25**: 1-17.
- Groot, T. T., VanBodegom, P. M., Harren, F. J. M., and Meijer, H. A. J. (2003) Quantification of methane oxidation in the rice rhizosphere using ¹³C-labelled methane. *Biogeochem.* **64**: 355-372.
- Hanson, R. S. and Hanson, T. E. (1996) Methanotrophic bacteria. *Microbiol. Rev.* **60**: 439-471.
- Henckel, T., Friedrich, M., and Conrad, R. (1999) Molecular analyses of the methane-oxidizing microbial community in rice field soil by targeting the genes of the 16S rRNA,

- particulate methane monooxygenase, and methanol dehydrogenase. *Appl. Environ. Microbiol.* **65**: 1980-1990.
- Henckel, T., Jäckel, U., and Conrad, R. (2001) Vertical distribution of the methanotrophic community after drainage of rice field soil. *FEMS Microbiol. Ecol.* **34**: 279-291.
- Henckel, T., Roslev, P., and Conrad, R. (2000) Effects of O₂ and CH₄ on presence and activity of the indigenous methanotrophic community in rice field soil. *Environ. Microbiol.* **2**: 666-679.
- Hoffmann, T., Horz, H. P., Kemnitz, D., and Conrad, R. (2002) Diversity of the particulate methane monooxygenase gene in methanotrophic samples from different rice field soils in China and the Philippines. *Syst. Appl. Microbiol.* **25**: 267-274.
- Holzappel-Pschorn, A. and Seiler, W. (1986) Methane emission during a cultivation period from an Italian rice paddy. *J. Geophys. Res.* **91**: 11803-11814.
- Horz, H. P., Yimga, M. T., and Liesack, W. (2001) Detection of methanotroph diversity on roots of submerged rice plants by molecular retrieval of *pmoA*, *mmoX*, *mxoF*, and 16S rRNA and ribosomal DNA, including *pmoA*-based terminal restriction fragment length polymorphism profiling. *Appl. Environ. Microbiol.* **67**: 4177-4185.
- Jia, Z., Kikuchi, H., Watanabe, T., Asakawa, S., and Kimura, M. (2007) Molecular identification of methane oxidizing bacteria in a Japanese rice field soil. *Biol. Fertil. Soils* **in press**: doi:10.1007/s00374-007-0186-x.
- Joulian, C., Escoffier, S., LeMer, J., Neue, H. U., and Roger, P. A. (1997) Populations and potential activities of methanogens and methanotrophs in rice fields - relations with soil properties. *Eur. J. Soil Biol.* **33**: 105-116.
- Kaplan, C. W. and Kitts, C. L. (2003) Variation between observed and true terminal restriction fragment length is dependent on true TRF length and purine content. *J. Microbiol. Methods* **54**: 121-125.

- Kimura, M. and Asakawa, S. (2006) Comparison of community structures of microbiota at main habitats in rice field ecosystems based on phospholipid fatty acid analysis. *Biol. Fertil. Soils* **43**: 20-29.
- Kitts, C. L. (2001) Terminal restriction fragment patterns: a tool for comparing microbial communities and assessing community dynamics. *Curr. Iss. Intest. Microbiol.* **2**: 17-25.
- Knief, C., Altendorf, K., and Lipski, A. (2003a) Linking autotrophic activity in environmental samples with specific bacterial taxa by detection of ¹³C-labelled fatty acids. *Environ. Microbiol.* **5**: 1155-1167.
- Knief, C., Lipski, A., and Dunfield, P. F. (2003b) Diversity and activity of methanotrophic bacteria in different upland soils. *Appl. Environ. Microbiol.* **69**: 6703-6714.
- Krüger, M., Eller, G., Conrad, R., and Frenzel, P. (2002) Seasonal variation in pathways of CH₄ production and in CH₄ oxidation in rice fields determined by stable carbon isotopes and specific inhibitors. *Global Change Biol.* **8**: 265-280.
- Krüger, M. and Frenzel, P. (2003) Effects of N-fertilisation on CH₄ oxidation and production, and consequences for CH₄ emissions from microcosms and rice fields. *Global Change Biol.* **9**: 773-784.
- Krüger, M., Frenzel, P., and Conrad, R. (2001) Microbial processes influencing methane emission from rice fields. *Global Change Biol.* **7**: 49-63.
- Lelieveld, J., Crutzen, P. J., and Dentener, F. J. (1998) Changing concentrations, lifetime and climate forcing of atmospheric methane. *Tellus* **50B**: 128-150.
- Lüdemann, H., Arth, I., and Liesack, W. (2000) Spatial changes in the bacterial community structure along a vertical oxygen gradient in flooded paddy soil cores. *Appl. Environ. Microbiol.* **66**: 754-762.

- Macalady, J. L., McMillan, A. M. S., Dickens, A. F., Tyler, S. C., and Scow, K. M. (2002) Population dynamics of type I and II methanotrophic bacteria in rice soils. *Environ. Microbiol.* **4**: 148-157.
- Miller, L. G., Sasson, C., and Oremland, R. S. (1998) Difluoromethane, a new and improved inhibitor of methanotrophy. *Appl. Environ. Microbiol.* **64**: 4357-4362.
- Murase, J., Noll, M., and Frenzel, P. (2006) Impact of protists on the activity and structure of the bacterial community in a rice field soil. *Appl. Environ. Microbiol.* **72**: 5436-5444.
- Nold, S. C., Boschker, H. T. S., Pel, R., and Laanbroek, H. J. (1999) Ammonium addition inhibits ¹³C-methane incorporation into methanotroph membrane lipids in a freshwater sediment. *FEMS Microbiol. Ecol.* **29**: 81-89.
- Noll, M., Matthies, D., Frenzel, P., Derakshani, M., and Liesack, W. (2005) Succession of bacterial community structure and diversity in a paddy soil oxygen gradient. *Environ. Microbiol.* **7**: 382-395.
- O'Leary, W.M. and Wilkinson, S.G. (1988) Gram-positive bacteria. In *Microbial Lipids, vol.1*. Ratledge, C. and Wilkinson, S. G. (eds). London: Academic Press, 117-185.
- Raghoebarsing, A. A., Smolders, A. J. P., Schmid, M. C., Rijpstra, W. I. C., Wolters-Arts, M., Derksen, J. et al. (2005) Methanotrophic symbionts provide carbon for photosynthesis in peat bogs. *Nature* **436**: 1153-1156.
- Reichardt, W., Mascarina, G., Padre, B., and Doll, J. (1997) Microbial communities of continuously cropped, irrigated rice fields. *Appl. Environ. Microbiol.* **63**: 233-238.
- Spring, S., Schulze, R., Overmann, J., and Schleifer, K. H. (2000) Identification and characterization of ecologically significant prokaryotes in the sediment of freshwater lakes: molecular and cultivation studies. *FEMS Microbiol. Rev.* **24**: 573-590.
- Tunlid, A. and White, D.C. (1992) Biochemical analysis of biomass, community structure, nutritional status, and metabolic activity of microbial communities in soil. In *Soil*

- Biochemistry*, vol.7. Stotzky, G. and Bollag, J. M. (eds). New York: Marcel Dekker, 229-262.
- VanBodegom, P., Goudriaan, J., and Leffelaar, P. (2001) A mechanistic model on methane oxidation in a rice rhizosphere. *Biogeochem.* **55**: 145-177.
- Wang, J. S., Logan, J. A., McElroy, M. B., Duncan, B. N., Megretskaia, I. A., and Yantosca, R. M. (2004) A 3-D model analysis of the slowdown and interannual variability in the methane growth rate from 1988 to 1997 [Review]. *Global Biogeochem. Cycles* **18**: B3011-doi:10.1029/2003GB002180.
- White, D. C., Davis, W. M., Nickels, J. S., King, J. D., and Bobbie, R. J. (1979) Determination of sedimentary microbial biomass by extractible lipid phosphate. *Oecologia* **40**: 51-62.
- Xu, Z. J., Zheng, X. H., Wang, Y. S., Han, S. H., Huang, Y., Zhu, J. G., and Butterbach-Bahl, K. (2004) Effects of elevated CO₂ and N fertilization on CH₄ emissions from paddy rice fields. *Global Biogeochem. Cycles* **18**: B3009.
- Zelles, L. and Bai, Q. Y. (1993) Fractionation of fatty acids derived from soil lipids by solid phase extraction and their qualitative analysis by GC-MS. *Soil Biol. Biochem.* **25**: 495-507.

Table 1. Relative abundance of T-RFs and ¹³C 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 ¹³C-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, 16:1 ω 6 and 16:1 ω 7 shown in Fig. 6b.

ⁱⁱ Sum of percent values of 18:0, 18:1 ω 7 and 18:1 ω 9 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; CH₄ emission in the presence of inhibitor (□), in the absence of inhibitor (○), and CH₄ oxidation rate (); mean ± SD (n=3).

Figure 3: Percent of CH₄ flux attenuated by CH₄ oxidation in the control (Δ) and the ¹³C-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 ¹³C-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) ¹³C incorporation into PLFA per gram dry soil or root (RS = rhizospheric soil; RT = roots; numbers indicate time after beginning of ¹³C-labeling); mean ± SD (n=3).

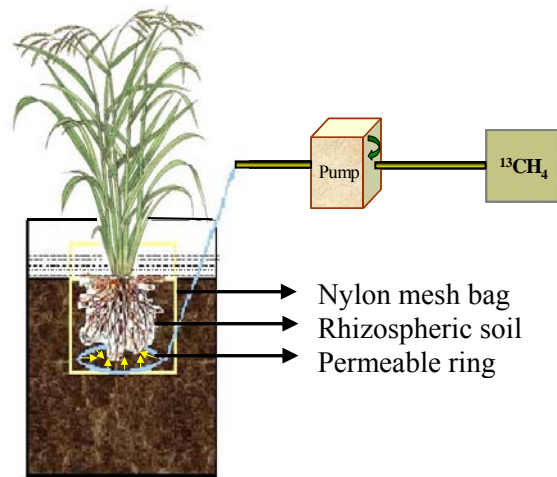
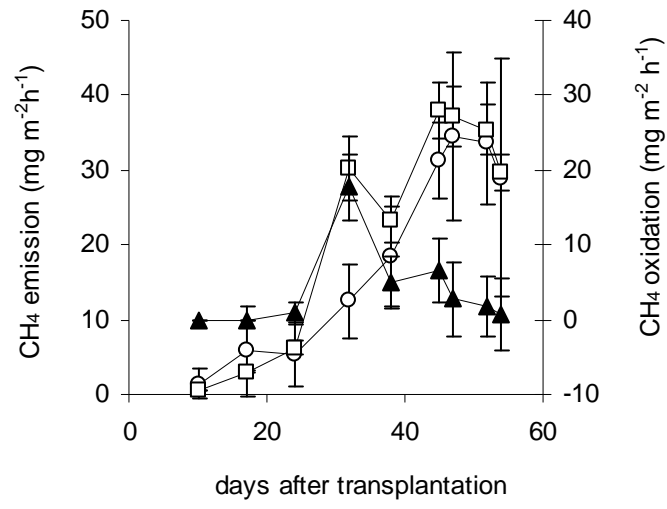


Figure 1

(a)



(b)

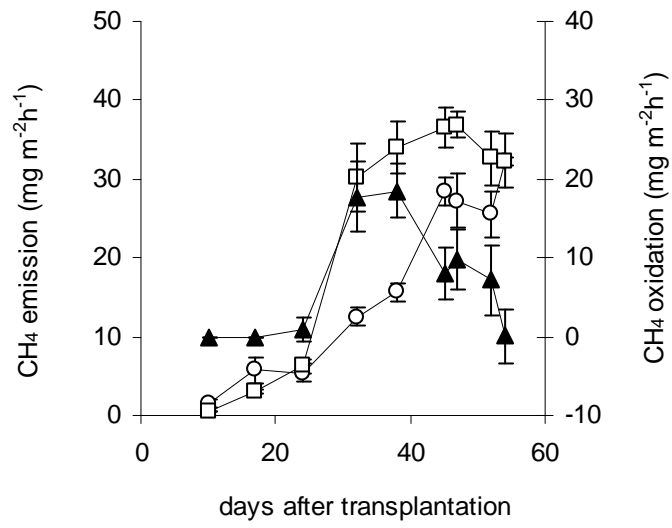


Figure 2

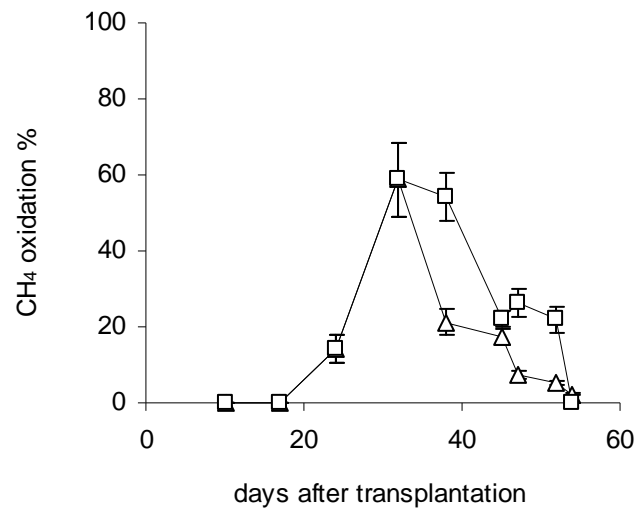
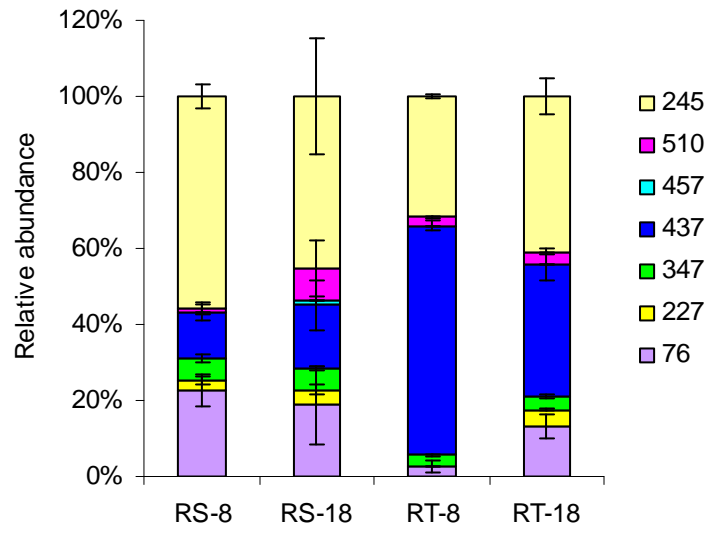


Figure 3

a)



b)

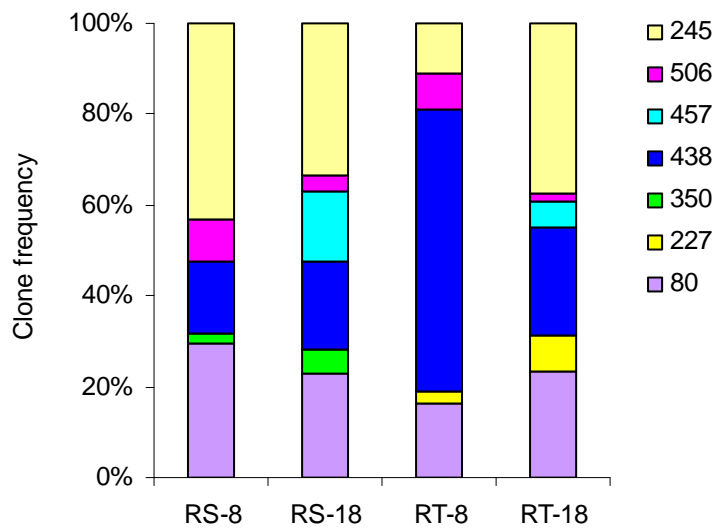


Figure 4

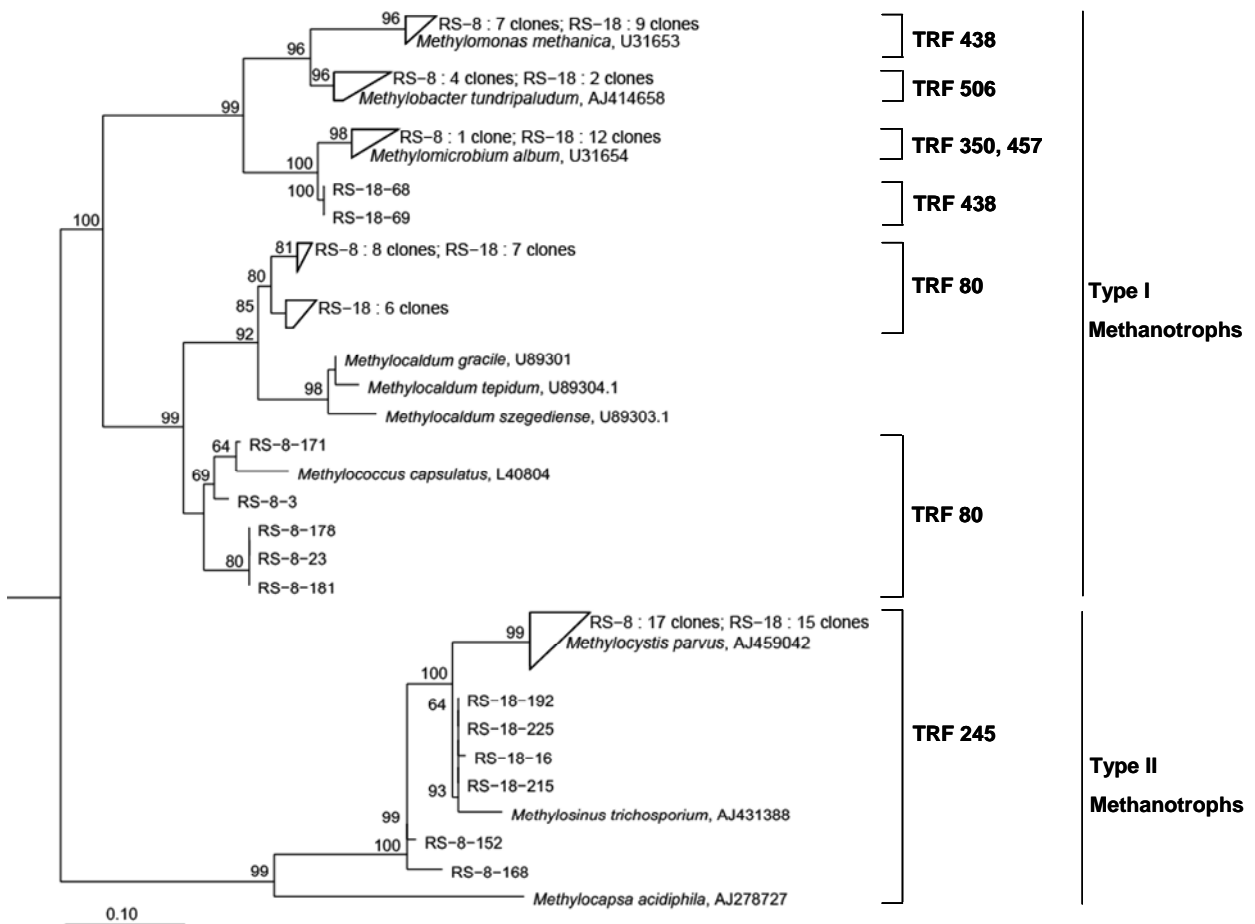


Figure 5

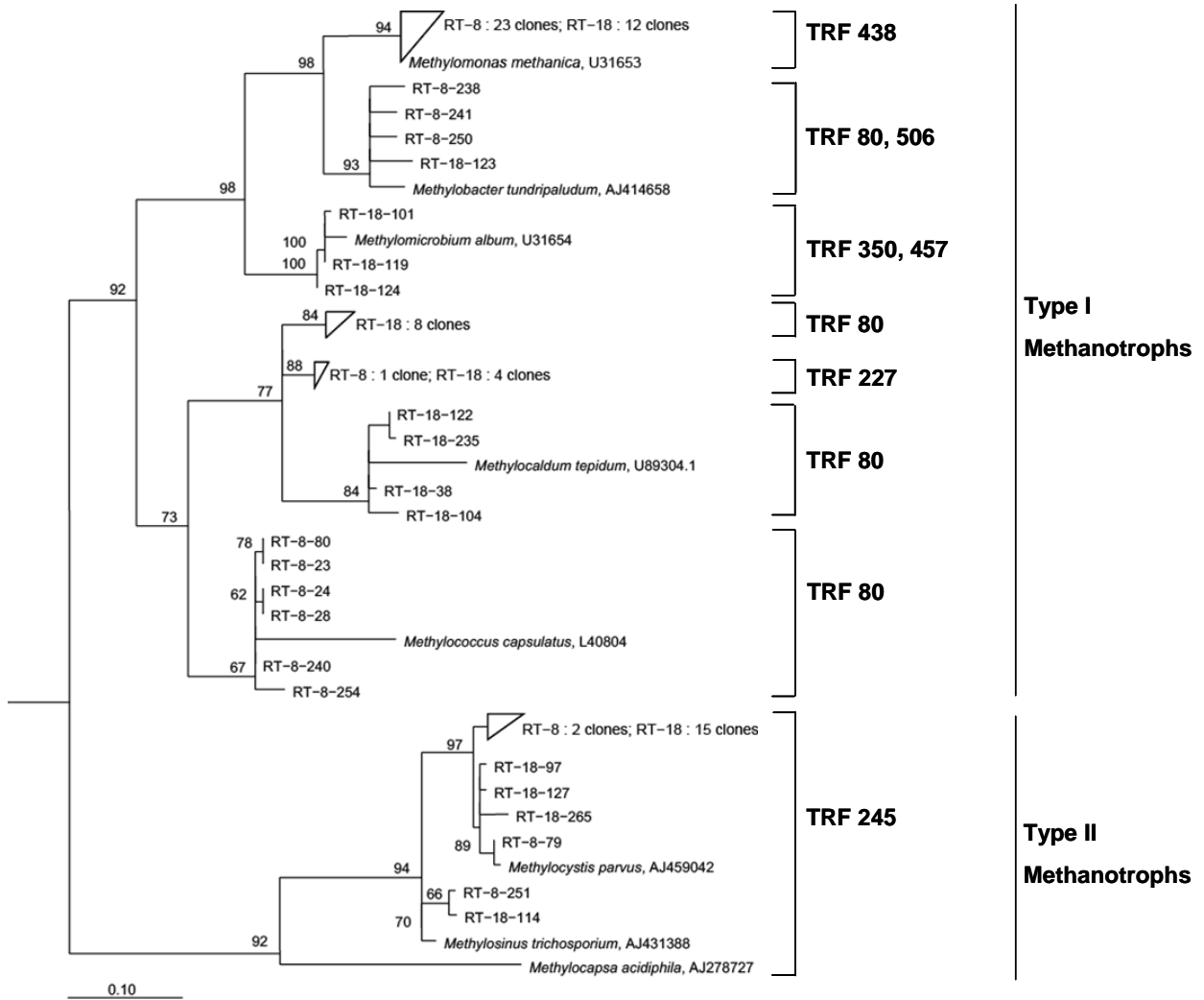
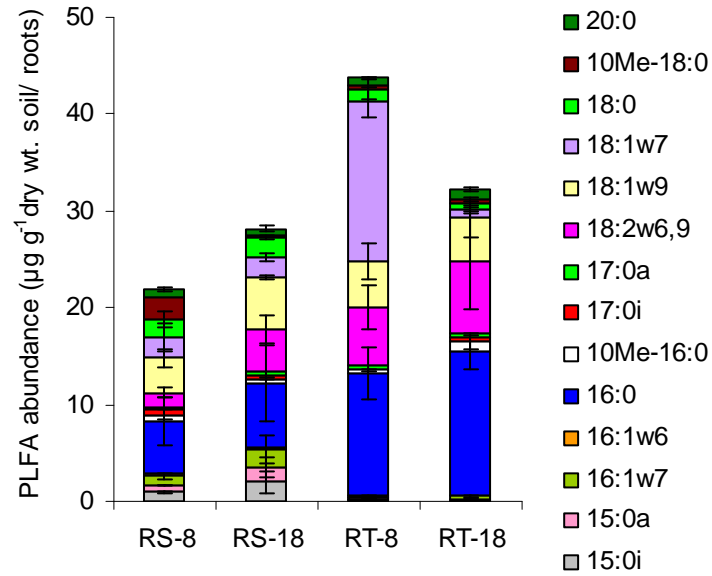


Figure 6

a)



b)

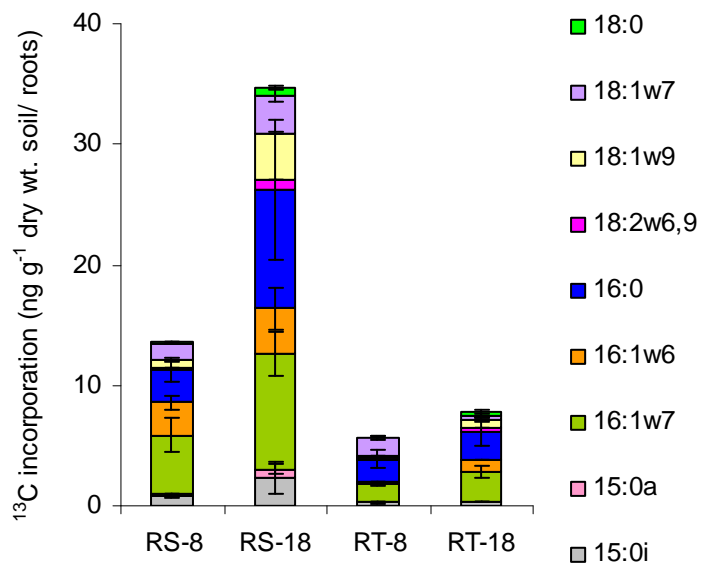


Figure 7