Methyl Fluoride Affects Methanogenesis Rather than Community Composition of Methanogenic Archaea in a Rice Field Soil

Anne Daebeler1,2, Martina Gansen1*, Peter Frenzel1*

1 Department of Biogeochemistry, Max Planck Institute for Terrestrial Microbiology, Marburg, Germany, 2 Department of Microbial Ecology, Netherlands Institute of Ecology (NIOO-KNAW), Wageningen, The Netherlands

Abstract

The metabolic pathways of methane formation vary with environmental conditions, but whether this can also be linked to changes in the active archaeal community structure remains uncertain. Here, we show that the suppression of aceticlastic methanogenesis by methyl fluoride (CH₃F) caused surprisingly little differences in community composition of active methanogenic archaea from a rice field soil. By measuring the natural abundances of carbon isotopes we found that the effective dose for a 90% inhibition of aceticlastic methanogenesis in anoxic paddy soil incubations was <0.75% CH₃F (v/v). The construction of clone libraries as well as t-RFLP analysis revealed that the active community, as indicated by mcrA transcripts (encoding the a subunit of methyl-coenzyme M reductase, a key enzyme for methanogenesis), remained stable over a wide range of CH₃F concentrations and represented only a subset of the methanogenic community. More precisely, Methanocellaceae were of minor importance, but Methanosarcinaceae dominated the active population, even when CH₃F inhibition only allowed for aceticlastic methanogenesis. In addition, we detected mcrA gene fragments of a so far unrecognised phylogenetic cluster. Transcription of this phylotype at methyl fluoride concentrations suppressing aceticlastic methanogenesis suggests that the respective organisms perform hydrogenotrophic methanogenesis. Hence, the application of CH₃F combined with transcript analysis is not only a useful tool to measure and assign in situ acetate usage, but also to explore substrate usage by as yet uncultivated methanogens.

Introduction

Methanogenesis is the dominating terminal process in anoxic freshwater habitats like sediments and flooded soils. In rice fields, most labile organic carbon is derived from plant material, and carbohydrates are the primary source for anaerobes resulting eventually in acetate and H₂ + CO₂ as most important methanogenic precursors [1]. The theoretical ratio of acetate : H₂ + CO₂ usage equals 2 : 1 [2]. However, depending on the exact oxidation state of labile organic carbon, but also on competing microbial processes, this ratio may vary. Hence, the fraction of methane produced via acetate is an important variable in understanding what controls mineralization in anoxic environments.

The amount of acetate-derived methanogenesis can be assessed with CH₃F [methyl fluoride, fluoromethane], a specific inhibitor for aceticlastic methanogenesis. When applied for the first time in microbial ecology, CH₃F was assumed to be a specific inhibitor for methane oxidation and ammonium oxidation [3,4]. While providing direct access to processes, inhibitor experiments may be misleading, if specificity is confined to certain conditions [5]. Indeed, CH₃F turned out to be an efficient inhibitor of methane and ammonium monoxygenases. However, it soon became evident that it may also inhibit methanogenesis [6,7]. In anoxic incubations treated with CH₃F, approximately as much acetate accumulates as methane is lacking compared to untreated controls. Selectivity of CH₃F for suppression of aceticlastic methanogenesis was further validated in pure culture studies demonstrating that 1% v/v inhibited growth of and methanogenesis by pure cultures of aceticlastic Methanoseta and Methanosaeta. Other microbes, homoacetogenic, sulfate reducing and fermentative bacteria, and a methanogenic mixed culture based on hydrogen syntrophy, were not inhibited [7]. In Methanosarcina barkeri, which is able to use acetate and H₂ + CO₂ simultaneously, only acetate utilization was suppressed, when both acetate and hydrogen were supplied [7]. However, pure cultures are not necessarily representative for yet uncultured populations, and many operational taxonomic units (OTUs) have been designated to a phylogenetic clade and named from environmental sequence information alone. Hence, some populations may show a behavior different from that found in pure cultures.

Another approach to determine methanogenic pathways uses isotopic signatures; for review see [8]. In short, methanogenesis from H₂ + CO₂ discriminates stronger against isotopically heavier carbon than does aceticlastic methanogenesis [8,9]. This difference can be used to calculate the contribution of these two
methanogenic pathways, provided the respective isotopic fractionation factors are known [8,10,11]. Indeed, combining the application of CH$_3$F with the analysis of isotopic signatures revealed the expected patterns [12].

The methanogenic community in rice fields mainly consists of versatile *Methanosarcinaceae* and strictly acetotrophic *Methanosaetaeae*, as well as of hydrogenotrophic *Methanomicrobiales*, *Methanobacteriales*, and *Methanocellales*; the latter were formerly known as rice cluster I [1,8,12–14]. Rice paddy soil is found to be compartmented into two habitats: rhizosphere and bulk soil. Methanogenic communities on rice roots are dominated by *Methanocellales*, with hydrogenotrophic methanogenesis contributing 60–80% to total methane production [15–17]. The influence of rice cultivars was found to be minor [18]. In bulk soil however, methane is mainly derived from acetate (50–83%), and *Methanosarcinaceae* are the prevailing methanogens [19,20]. The community structure of methanogens remains rather stable even under dry-wet cycles [21]. In summary, cell numbers fluctuate with management [21], but methanogenic communities in paddy fields of different geographical origin are highly related [22].

Here, we re-visit the inhibition of acetoclastic methanogenesis in a paddy soil asking not only how specifically CH$_3$F inhibits acetoclastic methanogenesis, but also for the response of different methanogenic archaea to this inhibitor. We studied the dose-response relationship of methanogenesis as a function of CH$_3$F concentration by combining process measurements with isotopic data and molecular analyses targeting the *mcrA* gene (encoding the subunit A of methyl coenzyme M reductase, a protein characteristic and essential for methanogenesis [23]). Since quite often only a minor fraction of a methanogenic community is metabolically active [16,25,26], we aimed at both the *mcrA* gene (community) and the respective mRNA (active community), as *mcrA* transcripts have been shown to be directly connected to energy metabolism and methanogenesis [24].

**Materials and Methods**

One kg bulk soil was sampled in spring 2008 from a rice field in the delta region of River Yangtze (Zhejiang Province, China) representing one of the major rice growing areas of the world. The particular field had been used for wetland rice production for about 2000 years [27–29]. Ten grams air-dried soil were mixed with ten milliliters oxygen-free distilled water in 26-ml pressure tubes. Tubes were capped with butyl rubber stoppers and flushed with N$_2$ for ten minutes. Different amounts of CH$_3$F corresponding to initial concentrations of 0.2, 0.3, 0.4, 0.6, 0.79, 0.99, 1.19, 1.57, 1.96, 2.72 and 3.85% were injected by syringe in two tubes each. Another three tubes did not receive CH$_3$F serving as control, and three were sampled immediately as primary soil material.

![Figure 1. Residuals, the difference between real and estimated size, of a FAM-labeled size standard used as ‘sample’ in t-RFLP analysis. Data from three replicate runs are shown. Fit: fifth order polynomial, red line; 95% prediction intervals: black lines. doi:10.1371/journal.pone.0053656.g001](image-url)
Water, tubes, and stoppers had been sterilized. The tubes were incubated for 14 days in the dark at 25°C. Methane, carbon dioxide and methyl fluoride in the headspace were measured repeatedly after sampling with a 0.25-ml pressure-lok syringe (Valco Instruments, USA) on a GC-FID (SRI-8610, SRI Instruments, USA). Only endpoint measurements are shown here. Quantification of lactate, formate, acetate, propionate, ethanol and butyrate were performed by analyzing filtered (ReZist, 0.2 μm PTFE, Schleicher and Schuell, Germany) pore water samples after 14 days of incubation by HPLC (SRI Instruments, USA).

Methane produced from carbon dioxide (m CO2) was measured under inhibition of aceticlastic methanogenesis ($0.75% CH3F, see below), while methane produced from acetate (m acetate) was calculated from the balance to total methane produced in controls without inhibitor: m acetate = m total - m CO2.

Carbon isotopic signatures in methane and acetate were measured as described elsewhere [30]. $13C signatures are given in δ-notation referring to the respective standard material, Vienna Pee Dee Belemnite (VPDB) [8].

Total nucleic acids were extracted as described elsewhere [31]. For tRFLP analysis, mcrA gene fragments were obtained with primers ME1/ME2 [32], where the forward primer was labeled with FAM. PCR conditions were: initial denaturing at 94°C for 5 minutes, 35 cycles of 30 s at 94°C, 45 s at 55°C, 1.5 min at 72°C, and a final extension at 72°C for 5 min. Amplicons were digested with SAU96I and analyzed on a capillary sequencer (3130 Genetic Analyzer, Applied Biosystems). For reverse transcriptase PCR (RT-PCR), 5 μl sample were treated with DNA-free DNase (Qiagen) followed by exonuclease treatment (mRNA-ONLY Prokaryotic mRNA Isolation Kit, Epicentre Technologies) and cleaning (RNAeasy Mini Kit, Qiagen) according to manufacturers’ instructions. Reverse transcription and amplification was performed in one step combining reverse transcription (Reverse Transcription System, Promega, Germany) with 30 PCR cycles at

**Figure 2. Accumulation of acetate and methane (A), and the respective $13C signatures in % VPDB (B) depending on initial concentrations of methyl fluoride; $13C acetate is the combined signature for both C-atoms.** Data are endpoint measurements and not corrected for initial concentrations. The fitted dose-response curves follow a log-logistic model with the parameters ED50 (effective dose for 50% inhibition), upper limit, and slope, while the lower limit was fixed to the respective averages for 0% CH3F. ED50, ED90, and ED95 are marked by red lines. (C) Box-plot summarizing accumulation of methane and acetate in control (n = 3) and in samples with CH3F 0.75%, n = 6) after 14 days of anoxic incubation.

doi:10.1371/journal.pone.0053656.g002
conditions as described above, but without a FAM-label on primer ME1.

In tRFLP analysis measured fragment size may deviate from real (in silico) size. Different factors have been claimed to be responsible for size shifts [33,34], but a detailed residual analysis was lacking so far. Residuals, the difference between real and estimated size, were calculated by running a FAM-labeled size standard as ‘sample’ against a ROX-labeled size standard. Both standards were purchased from Eurogentec (Germany). The ‘fragment’ size of the FAM-labeled standard was calculated with the built-in software using a third order polynomial as calibration function. Even if the calibration curve gave nearly perfect fit, residuals showed a considerable non-linearity being best described by a fifth order polynomial (Figure 1; intercept = 16.67359, a = 20.3238648, b = 1.831838e-3, c = 23.81772e-06, d = 3.17735e-09, e = 28.61187e-13). This polynomial was used to correct measured TRF size making it comparable to in-silico fragment size.

Gene libraries for archaeal mcrA sequences were constructed using cDNA from the control samples and from samples incubated under 3.85% methyl fluoride, as well as DNA from the primary soil material. (RT)-PCR products were ligated into pGEM-T vector plasmids (Promega, Germany) and transformed into Escherichia coli competent cells JM109 (Promega, Germany) according to the manufactures’ instructions. The sequences were assembled with SeqManII (DNASTAR) and compared with sequences available in the GenBank database using the BLAST network service to determine the approximate phylogenetic affiliations. Alignment and phylogenetic analysis of the mcrA sequences from 69 DNA- and 91 mRNA-derived clones was done with ARB [35]. OTUs were defined by the average neighbor algorithm at 5% amino acid sequence divergence level; representative sequences for these OTUs were determined using mothur ver. 1.19.3 [36]. Sequence data have been submitted to GenBank under accession numbers JQ283291-JQ283438.

Statistical analysis was done in R ver. 3.12.2 [37]. Dose-response models were fitted using package drc, ver. 2.2-1 [38]. Constrained correspondence analysis (CCA) and non-metric multidimensional scaling (NMDS) were done with package vegan ver. 2.1-0 [39], and a multivariate regression tree (MRT) was fitted with package mvpart ver. 1.4-0 [40]. Graphics were produced with package ggplot2 [41].

Results and Discussion

Metabolites and isotopic signatures

With increasing CH3F concentration, acetate accumulated while methane accumulation was reduced accordingly (Figure 2A) resulting in a highly significant negative correlation (r = 0.7, P = 0.0002). No other fermentation products, in particular
not formate, propionate, butyrate, or ethanol, did accumulate (data not shown). Along with the reduction of methanogenesis, both the δ^{13}C values of methane and acetate decreased (Figure 2B). The shift in δ^{13}C-CO₂ by about −20‰ VPDB between control (0% CH₃F) and incubations receiving ≥0.75% CH₃F is in accordance with a shift from mixed substrate usage to H₂ + CO₂ dependent methanogenesis [17,42]. Correspondingly, the relatively heavy carbon isotopic signature of −10‰ in acetate from control incubations implies that lighter acetate was preferentially consumed, thus enriching the remaining acetate in δ^{13}C. With increasing CH₃F concentration, δ^{13}C acetate continuously decreased until values stabilized around −23‰, as known for acetate derived from fermentation of organic matter in rice fields [12]. Thereby we can exclude that homoacetogenesis was an important process in the incubations, as otherwise the isotopic signature of acetate should have been substantially lower [43].

All fitted dose-response curves have ED₉₀ (effective dose for 90% inhibition) concentrations of <0.75% CH₃F. The dose-response curves for acetate and methane accumulation even showed ED₉₀ concentrations of <1%. The higher ED₉₀ for the isotopic signatures may be due to the rather gentle slope of the respective curves (Figure 2B).

If only aceticlastic methanogenesis was inhibited while acetogenesis proceeded, the sums of methane and acetate in control and fully inhibited samples (assumed at ≥0.75% CH₃F) should be equal. Indeed, no significant difference was found (Figure 2 C; two sample t-test, p = 0.87). On basis of the results of the different dose-response curves we conclude additionally that above 0.75% CH₃F virtually no acetate was consumed. Furthermore, our data does not indicate an effect on residual, hydrogenotrophic methanogenesis. In a previous experiment, hydrogenotrophic methanogenesis was found unaffected even at 4% CH₃F [6; in a hypersaline microbial mat from Solar Lake, Sinai]. However, in two incubations at elevated CH₃F concentrations (2.7 and 2.9%) not included in the dose-response fits, the amount of acetate produced was about 50% higher than the corresponding methane deficit. Methanogenesis and isotopic signatures, on the other hand, were not affected. Similar disproportionate acetate values have been reported before [44] and perhaps, these imbalances are caused by substrate heterogeneities, not by effects on methanogenesis.

Assuming that an initial CH₃F concentration of 0.75% inhibited aceticlastic methanogenesis, hydrogenotrophic methanogenesis contributed 18.3% to total methane production. The inhibitory concentration is within the range usually applied to rice field CH₃F [2,4,6,17,44–49] and other wetland soils [50–53]. A decade ago, CH₃F was thought to be a specific inhibitor for methane oxidation in general [3] and has been applied to chamber experiments quantifying methane oxidation from the difference between methane fluxes with and without CH₃F [Table 1]. Considering an ED₉₀ of <0.25% CH₃F for aceticlastic methanogenesis, these experiments may likely have underestimated the amount of methane oxidized due to co-inhibition of aceticlastic methanogenesis.

The methanogenic community

Community composition (DNA-based) and transcripts were analyzed by t-RFLP analysis as well as by cloning of the mcrA gene fragments and transcripts. Results of the t-RFLP analysis of the mcrA gene (Figure 3) indicated a high relative abundance of versatile Methanosarcinaeae (rRF 126, 133, 652, 683) and hydrogenotrophic Methanobacteriales (rRF 126, 663, 752). In addition, Methanocellales (rRF 133) were found in all incubations. Two rRFs could not be separated further: an in silico analysis of mcrA sequences from the clone library revealed that rRF 133 occurred in Methanocellales, the Fen cluster, and Methanosarcinaeae, while rRF 126 comprised both Methanobacteriales and Methanococcales. Despite this, t-RFLP patterns showed a distinct separation between total and active community in all analyses applied: CCA (Figure 3A) and MRT figure 3B) demonstrated consistently that a homogenous, active community was found across the whole CH₃F gradient applied. Furthermore, virtually the same separation was found with non-metric multidimensional scaling (NMDS; stress = 0.02, r² linear = 0.99; ordination not shown). As found recently for methanogens [21] and other microbial guilds [54], the active community consisted only of a subset of the total. Most remarkable was here the nearly complete absence of restriction fragments indicative for Methanobacteriales mcrA transcripts.

Cloning and sequencing allowed further differentiation. The DNA-based library constructed from soil sampled at the beginning of the experiment was dominated by sequences affiliated to Methanocellales, Methanosarcinaeae and Methanobacteriales, but also by a few members of the Fen cluster and a so far uncharacterized

| Table 2. Abundances of the 22 operational taxonomic units (OUTs) with a maximum intra-group distance of 5% (AA) in the clone library. |
|---------------------------------|--------|--------|--------|--------|
| OTU Affiliation | TRF | Start, mRNA | Control, mRNA | CH₃F, mRNA |
|-----------------|-----|-----------|--------------|-----------|
| 1 Msarc | 139 | 15 | 40 | 42 |
| 2 Mcell | 139 | 6 | 0 | 0 |
| 3 Mcell | 138 | 6 | 0 | 0 |
| 4 Mcell | 139 | 1 | 0 | 0 |
| 5 Mcell | 139 | 5 | 0 | 0 |
| 6 Mbac | 760 | 10 | 0 | 0 |
| 7 Mbac | 131 | 3 | 0 | 0 |
| 8 Mbac | 760 | 3 | 0 | 0 |
| 9 Mbac | 666 | 1 | 0 | 0 |
| 10 Mbac | 666 | 1 | 0 | 0 |
| 11 Mcell | 138 | 10 | 3 | 0 |
| 12 NN | 139 | 0 | 2 | 2 |
| 13 Msaeta | 131 | 2 | 0 | 0 |
| 14 Fen | 139 | 1 | 0 | 0 |
| 15 Msarc | 139 | 1 | 0 | 0 |
| 16 Mbac | 733 | 1 | 0 | 0 |
| 17 Mbac | 760 | 1 | 0 | 0 |
| 18 Mcell | 138 | 1 | 0 | 0 |
| 19 Mcell | 138 | 0 | 1 | 0 |
| 20 Msaeta-like | 139 | 0 | 2 | 0 |
| 21 Mcell | 139 | 0 | 0 | 1 |
| X² test, simulated p-values | Control, mRNA | CH₃F, mRNA |
| Start, mRNA | 0.0001 | 0.0001 |
| Control, mRNA | 0.05 |
cluster (Table 2). The latter (OTU 12; Table 2) were found in clones retrieved under CH3F suggesting a hydrogenotrophic mode of life. In accordance with our t-RFLP findings, only a minor fraction of this diversity could be retrieved from mRNA resulting in highly significant differences between DNA- and mRNA-based clone libraries (Table 2). Considering mRNA derived sequences as a proxy for group-specific activity, Methanobacteriales appeared to not produce methane at all. Similarly, Methanocellales seemed to have been much less important for methanogenesis than expected from their high dominance in the DNA-based clone library. With and without repression of aceticlastic methanogenesis, Methanosarcinaceae were the most active methanogens suggesting that they used acetate when possible, but shifted to H2 + CO2, if acetate usage was inhibited. This is in accordance with a previous experiment on Methanosarcina barkeri strain MS that was inhibited by CH3F when supplied with acetate, but not if grown on H2 + CO2 [7]. Methanosarcinaceae sequences detected here were affiliated to the type strain of Methanosarcina mazei (Figure 4) being able to use

Figure 4. Neighbor-joining tree based on 147 deduced amino acid positions from 949 mcrA sequences. Phylogenetic nodes verified by a maximum likelihood tree are marked with closed circles. The outer branches of distinct clusters are collapsed, and those containing OTUs defined in this study are marked in blue. Only representative sequences for the OTUs have been incorporated into the tree and are depicted as ‘OTU name (accession number, number of sequences representing the OTU). The corresponding tRFs were calculated in silico using the TRiFLe package [64] and are given to the right. Scale bar: 0.09 changes per amino acid position. The outgroup is Methanopyrus kandleri. doi:10.1371/journal.pone.0053656.g004
both these substrates, too [55]. It is intriguing that under CH$_3$F inhibition, no Methanocellales-related sequences could be retrieved anymore from mRNAs, resulting in a small yet still significant difference between the respective libraries (Table 2). While we cannot rule out a direct effect, shifting Methanosarcinaceae towards a hydrogenotrophic mode of life might also have changed competition for H$_2$ resulting in an indirect effect on Methanocellales.

Conclusion
While we found CH$_3$F to act specifically on acetlastic methanogenesis, the results obtained from the analysis of mcrA transcripts allow for relevant conclusions beyond this technical aspect. Community composition has often been regarded as a controlling factor for the flow of carbon and redox gradients through microbial communities. However, this experiment has shown how versatile Methanosarcinaceae are very well capable of delivering the same end-product under totally different conditions. This supports concepts developed to understand and predict the reaction of microbial communities to environmental changes [56,57]. Furthermore, this experiment demonstrates how the sensible applicability of selective inhibitors can help detect physiological traits of yet uncultivated microbes eventually supporting the design of cultivation strategies. Having found previously the same effect of CH$_3$F on methanogenesis in a soil from an Italian rice field [6] more than 10,000 km apart from that in China let us trust our findings are widely applicable.

References
1. Conrad R, Frenzel P (2002) Flooded soils. In: Britton G, editor. New York: John Wiley & Sons, pp. 1316–1333.
2. Conrad R, Klose M (1999) How specific is the inhibition by methyl fluoride of acetoclastic methanogenesis in anoxic rice field soil? FEMS Microbiol Ecol 30: 47–56.
3. Orenland RS, Colburnton CW (1992) Evaluation of methyl fluoride and dimethyl ether as inhibitors for aerobic methane oxidation. Appl Environ Microbiol 58: 2893–2902.
4. Bodelier PLE, Frenzel P (1999) Contribution of methanothrophic and nitritifying bacteria to CH$_3$ and NH$_3$$^*$ oxidation in the rice rhizosphere using new methods for discrimination. Appl Environ Microbiol 65: 1826–1833.
5. Orenland RS, Copanie DG (1988) Use of "specific" inhibitors in biogeochemistry and microbial ecology. Adv Microb Ecol 10: 285–383.
6. Frenzel P, Bosse U (1996) Methyl fluoride, an inhibitor of methane oxidation and methane production. FEMS Microbiol Ecol 21: 25–36.
7. Janssen PH, Frenzel P (1997) Inhibition of methanogenesis by methyl fluoride: studies on pure and defined mixed cultures of anaerobic bacteria and archaea. Appl Environ Microbiol 63: 4532–4537.
8. Conrad R (2005) Quantification of methanogenic pathways using stable carbon isotopic signatures: a review and a proposal. Org Geochem 36: 739–752.
9. Whiticar MJ, Faber E, Schoell M (1986) Biogenic methane formation in marine and freshwater environments: CO$_2$ reduction vs. acetate fermentation - Isotope evidence. Geochem Cosmochim Acta 50: 693–709.
10. Kruger M, Eller G, Conrad R, Frenzel P (2002) Seasonal variation in pathways of CH$_4$ production and in CH$_4$ oxidation in rice fields determined by stable carbon isotopes and specific inhibitors. Global Change Biol 8: 265–280.
11. Conrad R, Chan OG, Claus P, Casper P (2007) Characterization of methanogenic Archaea and stable isotope fractionation during methane production in the profundal sediment of an oligotrophic lake (Lake Stechlin, Germany). Limnol Oceanogr 52: 1393–1406.
12. Penning H, Conrad R (2007) Quantification of carbon flow from stable isotope fractionation in rice field soils with different organic matter content. Org Geochem 38: 2053–2069.
13. Kruger M, Frenzel P, Kemnitz D, Conrad R (2005) Activity, structure and dynamics of the methanogenic archaeal community in a flooded Italian rice field. FEMS Microbiol Ecol 51: 323–331.
14. Sakai S, Conrad R, Liesack W, Imachi H (2010) Methanothermus arborae sp. nov., a hydrogentrophic methanogen isolated from rice field soil. ISMEJ 6: 2910–2923.
15. Chin KJ, Lueder T, Friedrich MW, Klose M, Conrad R (2004) Archaeal community structure and pathway of methane formation on rice roots. Microbiol Ecol 47: 59–67.
16. Lu Y, Conrad R (2005) In situ stable isotope probing of methanogenic archaea in the rice rhizosphere. Science 309: 1088–1090.
17. Penning H, Conrad R (2006) Effect of inhibition of acetoclastic methanogenesis on growth of archaeal populations in an anoxic model environment. Appl Environ Microbiol 72: 176–184.
18. Conrad R, Klose M, Noll M, Kemnitz D, Bodelier PLE (2006) Soil type links microbial colonization of rice roots to methane emission. Global Change Biol 14: 657–669.
19. Großkopf R, Janssen PH, Liesack W (1998) Diversity and structure of the methanogenic community in anoxic rice paddy soil microcosms as examined by cultivation and direct 16S rRNA gene sequence retrieval. Appl Environ Microbiol 64: 960–969.

Acknowledgments
The authors thank Zhi Hong Cao, Institute of Soil Science, CAS, Nanjing, (China), for providing the soil sample, and Peter Claus and Bellinda Schneider (MPI Marburg) for technical assistance. This is a contribution from the framework 'Biogeochemistry of paddy soil evolution'. This publication is publication no. 5385 of the Netherlands Institute of Ecology.

Author Contributions
Conceived and designed the experiments: PF. Performed the experiments: AD. Analyzed the data: AD MG PF. Wrote the paper: AD PF.
39. Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, et al. (2011) vegan: Community Ecology Package. 2.2-0 ed.
40. De'ath G (2002) Multivariate regression trees: a new technique for modeling species-environment relationships. Ecology 83: 1105–1117.
41. Wickham H (2009) ggplot2. Elegant graphics for data analysis; Gentleman R, Hornik K, Parmigiani G, editors. Dordrecht: Springer. 1–212 p.
42. Whiticar MJ (1999) Carbon and hydrogen isotope systematics of bacterial formation and oxidation of methane. Chem Geol 161: 291–314.
43. Gehebck JT, Risatti JB, Hayes JM (1989) Carbon isotope effects associated with autotrophic acetogenesis. Org Geochem 14: 441–446.
44. Conrad R, Klose M (2000) Selective inhibition of reactions involved in methanogenesis and fatty acid production on rice roots. FEMS Microbiol Ecol 34: 27–34.
45. Banker BC, Khateke HK, Aitfodd DP, DeLaune RD, Lindau CW (1995) Methane sources and sinks in paddy rice soil: relationship to emissions. Agric Ecosys Environ 53: 243–251.
46. Bodleye PLE, Hahn AP, Arth I, Frenzel P (2000) Effects of ammonium-based fertilisation on microbial processes involved in methane emission from soils planted with rice. Biogeochemistry 51: 225–257.
47. Denier van der Gon HAC, Neue HU (1996) Oxidation of methane in the rhizosphere of rice plants. Biol Fert Soils 22: 359–366.
48. Jia ZJ, Cai ZC, Xu H, Li XP (2001) Effect of rice plants on CH4 production, transport, oxidation and emission in rice paddy soil. Plant Soil 230: 211–221.
49. Roy R, Kluiver HD, Conrad R (1997) Early initiation of methane production in anoxic rice soil despite the presence of oxidants. FEMS Microbiol Ecol 24: 311–320.
50. Keane M, Torburt G, Kuesl R (2000) Competition of Fe(III) reduction and methanogenesis in an acidic fen. FEMS Microbiol Ecol 65: 88–101.
51. Jerman V, Metje M, Mandic-Mulec I, Frenzel P (2009) Wetland restoration and methanogenesis: the activity of microbial populations and competition for substrates at different temperatures. Biogeosciences 6: 1127–1138.
52. Metje M, Frenzel P (2005) Effect of temperature on anaerobic ethanol oxidation and methanogenesis in an acidic peat from a northern wetland. Appl Environ Microbiol 71: 8191–8200.
53. Krause S, Luke C, Frenzel P (2010) Succession of methanotrophs in oxygen-methane counter-gradients of flooded rice paddies. ISME J 4: 1603–1617.
54. Liu Y (2010) Methanosarcinales. In: Timmis KN, editor. Handbook of Hydrocarbon and Lipid Microbiology. Berlin Heidelberg: Springer. pp. 595–604.
55. Conte J, Del Giorgio PA (2011) Composition influences the pathway but not the outcome of the metabolic response of bacteria to resource shifts. PLoS One 6: e25266.
56. Allison SD, Martin JB (2006) Resistance, resilience, and redundancy in microbial communities. Proc Natl Acad Sci U S A 103: 11512–11519.
57. Boeckx P, van Cleemput O (1997) Methane emission from a freshwater wetland in Belgium. Soil Sci Soc America J 61: 1250–1256.
58. Epp MA, Chanton JP (1993) Rhizospheric methane oxidation determined via the methyl fluoride inhibition technique. J Geophys Res 98: 18415–18418.
59. Holman MA, Carlson RG (2001) Methane oxidation associated with submerged vascular macrophytes and its impact on plant diffusive methane flux. Biogeochemistry 52: 207–224.
60. King GM (1996) In situ analyses of methane oxidation associated with the roots and rhizomes of a bur reed, Sparganium eurycarpum, in a Maine wetland. Appl Environ Microbiol 62: 4548–4555.
61. Moosavi SC, Crill PM (1998) CH4 oxidation by tundra wetlands as measured by a selective inhibitor technique. J Geophys Res-Atmos 103: 29093–29106.
62. Popp TJ, Chanton JP, Whiting GJ, Grant N (2000) Evaluation of methane oxidation in the rhizosphere of a Carex dominated fen in north central Alberta, Canada. Biogeochemistry 51: 259–281.
63. Junier P, Junier T, Witzel KP (2006) TRIFlE, a program for in silico terminal restriction fragment length polymorphism analysis with user-defined sequence sets. Appl Environ Microbiol 72: 6452–6456.