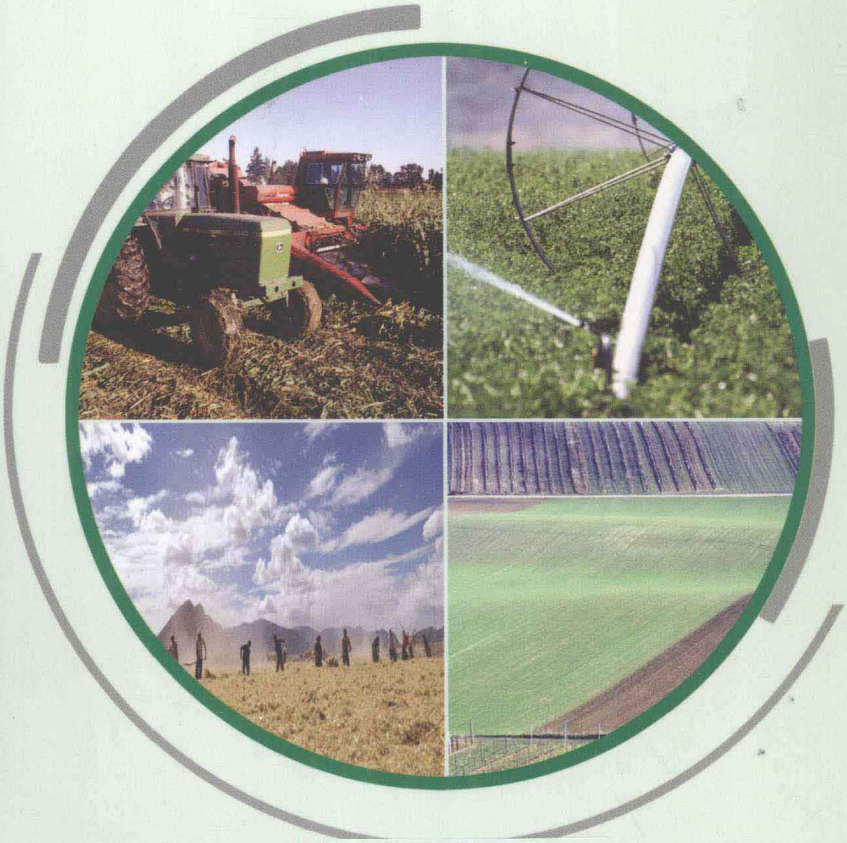
农业资源可持续利用与生态环境安全

——2009年农业资源利用学科全国博士生论坛论文集

主编 谢德体 倪九派 丁恩俊



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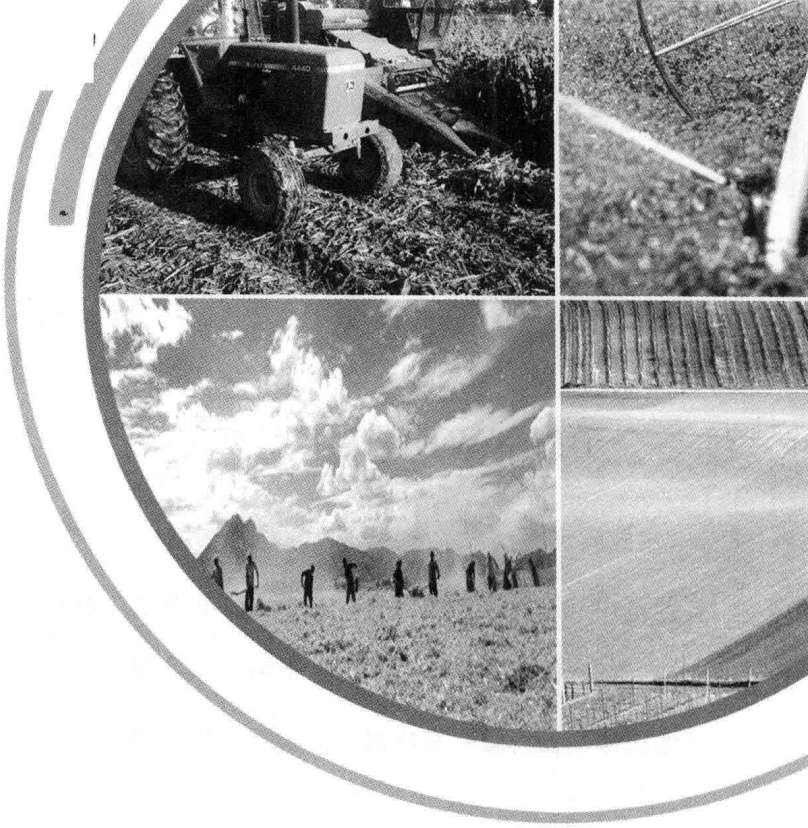

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前言

资源与环境问题是当今人类社会发展中所面临最严重的问题，备受社会关注。农业资源是人类生存和农业发展的基础，农业资源可持续利用重要性也逐渐凸现出来。2009年农业资源利用学科全国博士生论坛是国内首次针对农业利用学科而召开的全国性博士生论坛，本次论坛以“农业资源可持续利用”为主题，重点交流我国农业资源利用学科近年来的基础理论和应用技术研究成果以及博士生的研究成果，内容涉及农田物质循环与污染控制、土壤有机/无机/生物界面交互作用、养分资源管理与农产品安全、农业水土资源的管理与调控、土地可持续利用等研究领域的最新进展与成果。

本次论坛来自中国科学院南京土壤研究所、成都山地灾害与环境研究所、中国农业科学研究院、中国农业大学、南京农业大学、浙江大学、北京林业大学、华中农业大学、华南农业大学、广西大学、湖南农业大学、山西农业大学、扬州大学、福建农林科技大学、北京市农林科学研究院和西南大学16家单位的15位专家学者和36位博士研究生代表参加了会议，其中10余名专家学者作了精彩的特邀报告，20余位博士研究生也针对各自研究领域展现了最新研究成果。本论文集将是对这次论坛成果的一次集中展现，其不仅会有力地促进农业资源利用学科学术交流，同时对农业资源利用的教学和学科建设也具有重要的作用。

西南大学资源环境学院农业资源利用学科研究团队，近年来在“十一五”国家科技支撑计划重大项目“沿三峡库区坡耕地农业面源污染综合治理技术研究与示范”(2007BAD87B10)；“重庆地区喀斯特山地退化生态系统恢复与重建技术开发”(2006BAC01A16)及西南大学“211”生态学重点学科建设项目经费的资助下，召开了该次全国农业资源利用学科博士生论坛，本书即为这次论坛研究成果的结晶。

本书由谢德体教授主持编写，编写组成员有倪九派、丁恩俊等，全书由谢德体、丁恩俊统稿。

本书的编写得到了西南大学资源环境学院、研究生部的大力支持，西南师范大学出版社对本书的出版付出了大量的辛苦劳动，编写组对此表示深深感谢。

谢德体

2010年1月于西南大学

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Responses of methanogenic archaeal community to oxygen exposure in rice field soil①

Yanli Yuan[1,2], Ralf Conrad[2], Yahai Lu[1]

(*1. College of Resources and Environmental Sciences, China Agricultural University, Beijing 100193, China;*
2. Max-Planck Institute for Terrestrial Microbiology, Karl-von-Frisch-Str, 35043 Marburg, Germany)

Abstract: Methanogens are regarded as strict anaerobes and hence sensitive to O_2 exposure. It has been demonstrated that CH_4 production and emission from rice field soil are substantially reduced when soil is drained or aerated even shortly. However, the response of different methanogenic populations to O_2 stress remains unclear. Therefore, we determined CH_4 production and structure of the methanogenic community in a Chinese rice field soil after short-term (24 h) and long-term (72 h) exposure to O_2 under laboratory conditions. O_2 stress strongly inhibited CH_4 production, and the inhibitory effect increased with the duration of O_2 exposure. O_2 exposure also resulted in dramatic increase of ferric iron and sulfate concentrations. H_2 partial pressures were significantly reduced, most probably due to the competitive consumption by iron and sulfate-reducing bacteria. However, substrate competition couldn't explain the inhibition of acetoclastic methanogenesis, since acetate accumulated after O_2 exposure compared with the control. Quantitative (real time) PCR analyses of both archaeal 16S rRNA and *mcrA* genes (coding for a subunit of the methyl coenzyme M reductase) revealed that growth of the methanogenic populations was suppressed after O_2 exposure. However, terminal restriction fragment length polymorphism (T-RFLP) analyses of both 16S rDNA and 16S rRNA showed that the structure of the methanogenic archaeal community remained remarkably stable, and that acetoclastic *Methanosarcinaceae* were always dominant whether with or without O_2 exposure. Thus, O_2 stress apparently did not differentially affect the various methanogenic populations, but instead inhibited CH_4 production by enabling competition, generally suppressing growth, and differentially affecting existing enzyme activity.

Key words: Methanogenic community; Rice field soil; Aeration; Substrate competition; Toxic effect

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Corresponding author: Yahai Lu, College of Resources and Environmental Sciences, China Agricultural University-Beijing 100193; Phone: +86-10-62733617, Fax: +86-10-62731016, E-mail: yhlu@cau.edu.cn

1 Introduction

Methanogens are regarded as strict anaerobes which do not form resting stages or spores for resisting to various environmental stress. Oxygen is a common stressor to anaerobes. In pure cultures, the exposure of anaerobic microorganisms to O_2 usually causes severe growth inhibition or even death (Imlay and Linn, 1986). However, it has been shown that methanogens possess different resistance capacities to O_2 stress. For instance, *Methanococcus vannielii* and *Methanococcus voltae* lose their viability after a brief exposure to O_2 (Kiener and Leisinger, 1983), while *Methanosarcina barkeri* strain Fusaro can survive in the presence of O_2 for up to 200 minutes without decrease of viability (Fetzer *et al.*, 1993) The methanogenic community in rice field soil consists mainly of acetoclastic *Methanosarcinaceae* and *Methanosaetaceae* and hydrogenotrophic *Methanomicrobiales*, *Methanobacteriales* and Rice cluster I (RC-I) lineages (Groβkopf *et al.*, 1998; Lueders and Friedrich, 2000; Wu *et al.*, 2006; Peng *et al.*, 2008). RC-I methanogens [now *Methanocellales* (Sakai *et al.*, 2008)] have been found to play a key role in CH_4 production from the plant-derived carbon in the rice rhizosphere (Lu and Conrad, 2005; Lu *et al.*, 2005). The analysis of the full genome sequence of an enriched RC-I culture reveals that these organisms possess an unique set of antioxidant enzymes, including a superoxide reductase (SOR) (Erkel *et al.*, 2006). Therefore, this group of methanogens may have a selective advantage over other methanogens for living in the rhizosphere where O_2 leakage from roots occurs (Erkel *et al.*, 2006).

In soil, however, resistance to aeration stress by methanogens may be more complex than in pure culture. It is likely that microscale anoxic sites are formed within soil aggregates which could provide a physical protection for the survival of methanogens under O_2 stress (Wagner *et al.*, 1999). The metabolic coupling of anaerobes and aerobes could provide another mechanism for the anaerobes to cope with O_2 stress (Wimpenny, 1981). It has been shown that methanogens can survive oxic periods in the soil environment (Mayer and Conrad 1990; Peters and Conrad 1995). Possibly some groups of methanogens can survive such oxic periods better than others, but this has not yet been studied. The only information available in anoxic rice soils is on the response of the activity of the methanogenic community to aeration stress (Ratering and Conrad, 1998). Effect on the community structure, on the other hand, has so far only been studied in microbial enrichment cultures (Wu and Conrad, 2001). Therefore, the objectives of the present study were to determine not only the response of the activity but also of the structure of the methanogenic community to O_2 exposure. Using a Chinese rice field soil it was found that although the activity of methanogens was substantially suppressed after O_2 exposure, the structure and size of the methanogenic populations remained remarkably stable.

2 Results and discussion

2.1 Biogeochemistry

The rice soil was collected from a paddy field in the southeastern China. The characteristics of field site and soil have been described previously (Peng *et al.*, 2008; Qiu *et al.*, 2008). The soil was pre-incubated anaerobically under laboratory condition for 3 weeks before O_2 exposure. During this pre-incubation, H_2 and acetate accumulated transiently (Fig. S1A). Fe(II) increased rapidly and reached a maximum at 8 days after pre-incubation. Sulfate was reduced to below the detection limit within 10 days (Fig. S1B). Nitrate was de-

tected only at a low concentration and depleted within 24 hours (data not shown). The production of small amount of CH_4 started already one day after onset of anoxic conditions, but increased substantially only after Fe(III) and sulfate were depleted (Fig. S1B).

The treatment with O_2 was started at 21 days after the pre-incubation. After repeated exposure to O_2 for 24 h (short-term treatment) and 72 h (long-term treatment), the soil slurries were flushed with N_2 for re-establishment of anoxic conditions. In the permanently anoxic control soil, CH_4 partial pressures in the headspace increased immediately and linearly (Fig. 1A). However, production of CH_4 was substantially suppressed in the O_2 treatments, and the inhibitory effect was larger in the long-term than the short-term treatment. In contrast to strong inhibition of CH_4 production, CO_2 production was slightly enhanced after O_2 exposure (Fig. 1B). In the control soil, H_2 partial pressures showed a burst after flushing with N_2 but then decreased to 0.4～2.2 Pa at day 8(Fig. 2C). Immediately after O_2 exposure H_2 partial pressures were very low but increased with time reaching similar values as in the control. Total concentration of Fe (II) in the control soil stayed constant at about 120 $\mu mol \cdot g^{-1}$ dw soil throughout the incubation (Fig. 1E). Sulfate concentration in the control soil stayed close to the detection limit (Fig. 1F). However, after the exposure to O_2, the concentrations of Fe(III) [decrease of Fe(II)] and sulfate were markedly increased. After return to anoxic condition, Fe(III) decreased again [increase of Fe(II)], while sulfate started to decrease when most of Fe(III) was consumed.

The substantial decrease of H_2 partial pressures after O_2 exposure is most likely due to the competitive consumption of H_2 by the iron reducers and sulfate reducers, which were activated due to the accumulation of Fe(III) and sulfate in the soil slurry. Iron- and sulfate-reducing bacteria can easily outcompete hydrogenotrophic methanogens as they have the lower K_m and K_s values for H_2 than methanogens (Robinson and Tiedje, 1984). Methane production resumed at days 10 and 23 after the short-term and long-term treatments with O_2, respectively (Fig. 1A). At this time both Fe(III) and sulfate had already been completely reduced.

In principle, the competitive inhibition of acetoclastic methanogenesis would also be possible if respiratory bacteria such as sulfate reducers, iron reducers and other aerobes could use the acetate more efficiently than the methanogens. However, acetate did not decrease but instead transiently accumulated, reaching a higher maximum in the long-term than in the short-term O_2 treatment (Fig. 1D). The calculated Gibbs free energy (G values) of CH_4 production from acetate in the O_2 treated soils was much more negative than in the permanently anoxic control (Fig. S2). Apparently, acetoclastic methanogenesis was thermodynamically under more favorable conditions in the O_2 treatments than the control. Therefore, something else than substrate competition must have suppressed the activity of the acetoclastic methanogens upon O_2 treatment. This result is consistent with previous observations in the Italian rice field soil (Ratering and Conrad, 1998). Killing of acetoclastic methanogens by O_2 exposure or inactivation of enzymes involved in CH_4 production may be possible explanations for the observed suppression.

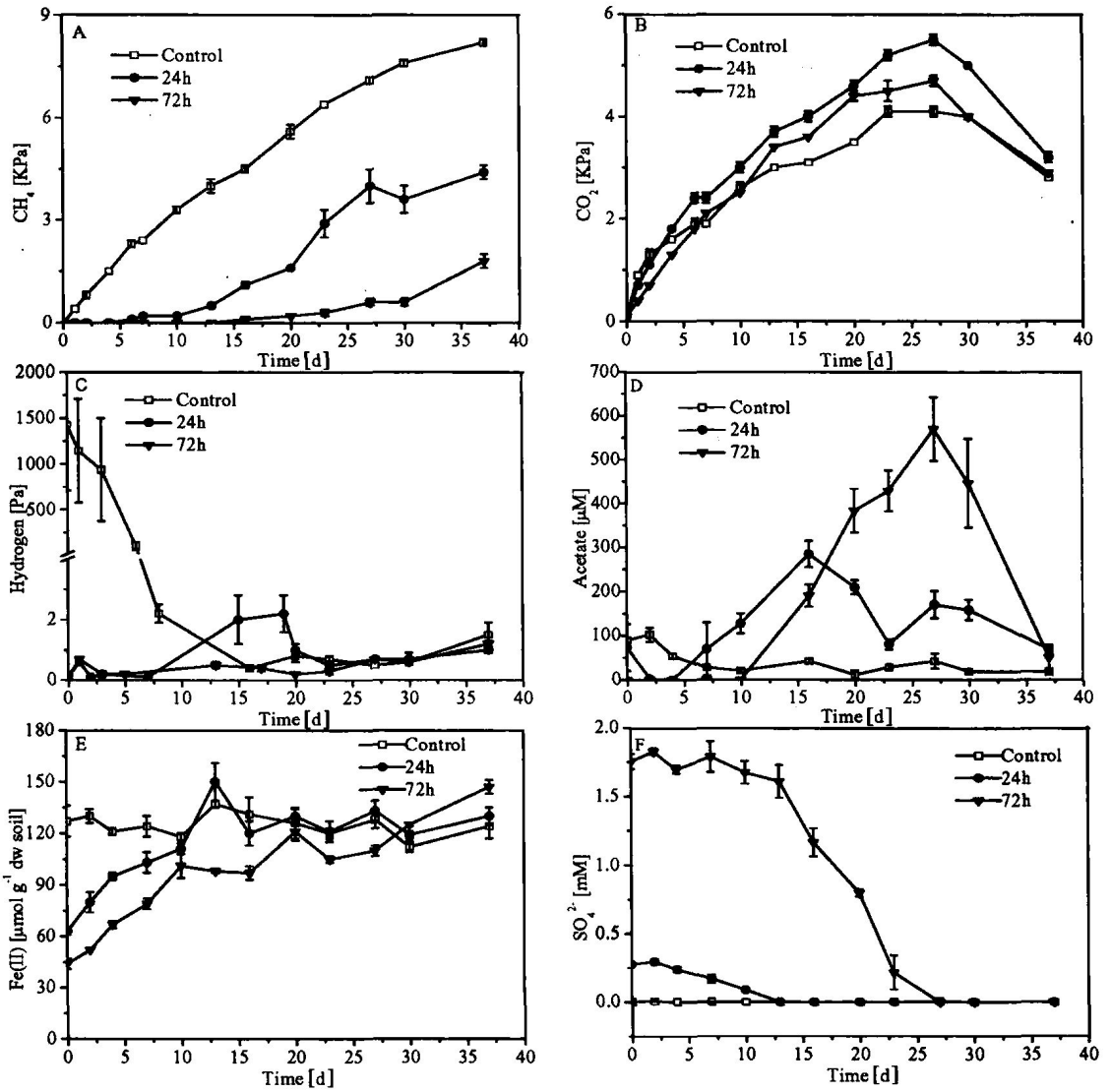

Figure 1. Biogeochemistry in the anoxic incubation of paddy soil. Shown is the time course of CH_4 (A), CO_2 (B) and H_2 (C) in the headspace, and acetate (D), ferrous iron (E) and sulfate (F) in the liquid phase (values are mean ± SE, n = 3). For the anoxic incubation, 8 g (d. w.) of soil was mixed with 12mL of distilled water in a 60-mL serum bottle. The soil was pre-incubated anaerobically for three weeks. Then, O_2 exposure was applied by bubbling with sterile pure O_2 for 5 min on a shaker. For the short-term stress (24 h), the 5-minute bubbling was repeated at 0 h and 12 h, while for the long-term stress (72 h), the treatment was repeated for six times at 0, 12, 24, 36, 48, and 60 h. In parallel, the control bottles were bubbled with sterile pure N_2 using the identical time schedule with 24 h treatment. After the O_2 exposure, all the bottles were bubbled for 5 minutes with pure N_2 to change conditions back to anoxic, and incubation was continued at 25 °C. At different time points, three bottles were sacrificed for gas measurements, liquid analysis and soil sampling. The partial pressures of CH_4, CO_2 and H_2 in the headspace were analyzed using gas chromatography (Roy *et al.*, 1997; Yao and Conrad, 2000). Ferrous ion in liquid samples was determined using the method modified from Lovely and Phillips (1987), sulfate was determined in an ion chromatographic system (Sykam) (Bak *et al.*, 1991), and acetate was analyzed using high-pressure liquid chromatography (Krumböck and Conrad, 1991)

2.2 Dynamics of the methanogenic archaeal community

A clone library of archaeal 16S rRNA genes was retrieved from soil sample during the recovery phase (day 21) after short-term treatment with O_2. The phylogenetic analysis of randomly selected clones showed that the archaeal community consisted mainly of *Methanosaetaceae*, *Methanosarci-*

naceae, *Methanomicrobiales*, RC-I, and RC-III (Table S1).

The dynamics of the archaeal community was determined using terminal restriction fragment length polymorphism (T-RFLP) analyses of both 16S rRNA genes (Fig. 2) and rRNA transcripts (Fig. 3). Six fragments (78 bp, 87 bp, 184 bp, 282 bp, 380 bp and 394 bp) were detected as major peaks in the T-RFLP profiles. Based on in-silico terminal restriction analysis of the archaeal sequences in this study and in the ARB database (Ricke *et al.*, 2005), these T-RFs could be assigned to the following lineages: *Methanomicrobiales* (78 bp), *Methanobacteriales* (87 bp), *Methanosarcinaceae* (184 bp), *Methanosaetaceae* (282 bp) and RC-III (380 bp). The 394-bp T-RF was associated with more than one lineage, which predominantly represented RC-I but occasionally also *Methanomicrobiales*.

In contrast to the strong effect on CH_4 production, both 16S rDNA and rRNA-based T-RFLP analyses indicated that O_2 exposure had little effect on the structure of archaeal community (Fig. 2 and 3). Only at the second day after O_2 exposure, a slight increase of RC-I methanogens was observed based on rDNA-based T-RFLP fingerprints. However, this was not confirmed by the RNA-level analyses. In addition, the 282-bp T-RF, indicative of acetoclastic *Methanosaetaceae*, increased after day 27 in the O_2-treated soils, but this was only seen in the RNA-based T-RFLP fingerprints, indicating possibly the increase of activity but no growth. This activity may be indicative for the end of suppression of acetoclastic methanogenesis by the previous O_2 exposure, as acetate no longer accumulated at this time but instead slowly decreased (Fig. 1D).

The total number of the methanogenic archaeal cells was estimated by determining the copy numbers of both archaeal 16S rRNA and *mcrA* genes using quantitative (real time) PCR. The copy number of archaeal 16S rRNA genes increased gradually with time in the control (Fig. 4). After the O_2 treatments, however, the copy numbers of archaeal 16S rRNA genes did not increase and thus, were in the end significantly lower than in the control. The copy numbers of the *mcrA* genes in both control and O_2 treatments followed a similar pattern as the 16S rRNA genes. Since the genome of methanogens contains 2～4 times more ribosomal RNA genes than *mcrA* genes (Erkel *et al.*, 2006, Table in supplemental material), copy numbers of the 16S rRNA gene were generally higher than those of the *mcrA* gene.

The increase of gene copies with time in the control indicated a net growth of methanogenic archaeal populations. Since the T-RFLP patterns did not much change despite net growth (Fig. 2), each group of the detected methanogens must have grown at the same rate. The relatively constant copy numbers of rRNA and *mcrA* genes in the O_2 treatments suggested that extensive death or inactivation of the different archaeal populations did not occur, though net growth apparently was suppressed. Furthermore, the duration of O_2 exposure did not show an effect on the copy numbers. This was obviously in contrast to CH_4 production, which showed a stronger inhibition after long-term compared to short-term O_2 exposure.

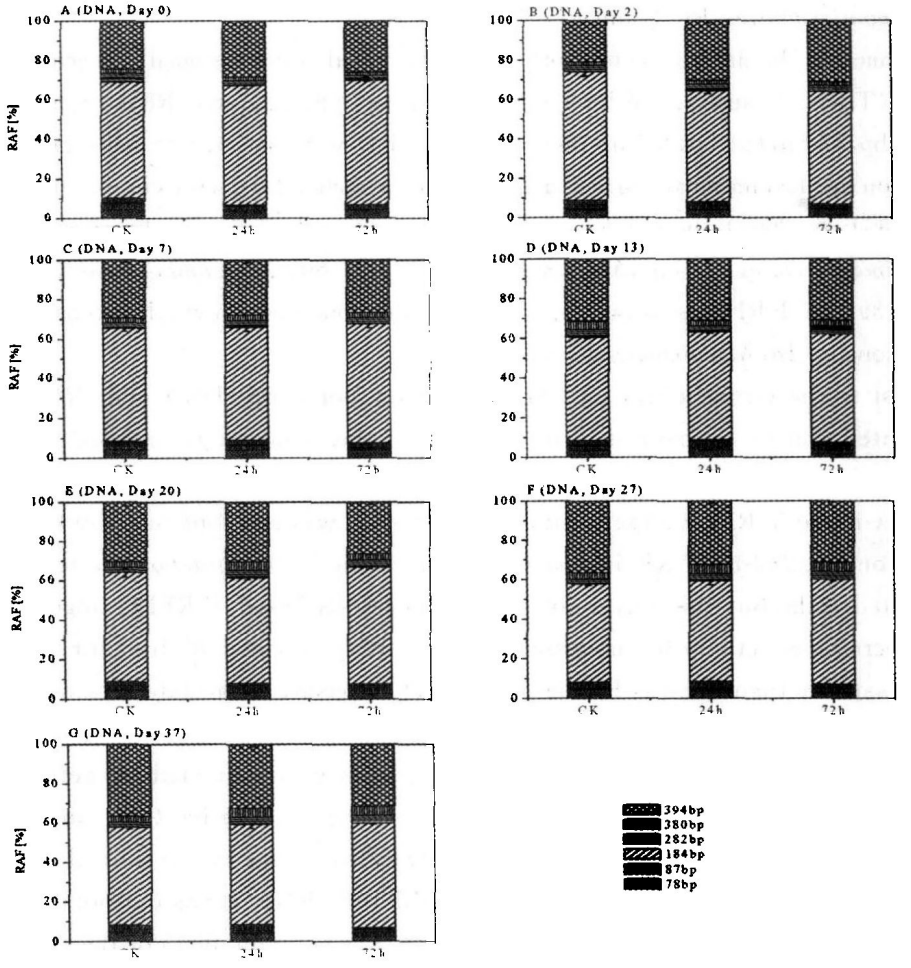

Figure 2. T-RFLP analyses targeting the archaeal 16S rRNA gene (DNA) at 7 different time points after short-term (24 h) and long-term (72 h) O_2 exposure and in the permanently anoxic control (CK). Shown is the percentage of individual T-RFs relative to the total integrated fluorescence (RAF = relative amplicon frequency; mean values ± SE; n= 3). Only T-RFs representing major methanogenic lineages are shown. DNA and RNA were co-extracted from soil samples using the phenol-chloroform extraction protocol modified from (Burgmann *et al.*, 2003). PCR reaction followed the protocol described in (Lueders and Friedrich, 2000). PCR products were purified and T-RFLP analysis was performed following the procedure as described previously (Chin *et al.*, 1999)

Interestingly, DNA-based T-RFLP profiling revealed that both *Methanosarcinaceae* and RC-I methanogens were dominant in the archaeal community, while RNA-level analysis showed that only *Methanosarcinaceae* were exclusively dominant. If we assume that DNA-level analysis represents total population, while RNA analysis more likely indicates the activity, this observation implies that the relative activity of *Methanosarcina* spp., i. e. the synthesis of proteins, was higher than of other methanogens and that this relation was true for all the different incubations. However, this interpretation may be deceptive. If the different methanogenic groups in the anoxic control were all growing at the same rate, as discussed above, but were not growing in the O_2-treatments, and since both DNA-based and RNA-based T-RFLP patterns were the same, independently whether the populations grew or not, the relative abundance of RNA-based T-RFs cannot represent a measure for the relative activity. Instead, RC-I methanogens must generally have smaller ribosome content per growing cell than *Methanosarcinaceae*. Hence, the relatively low abundance of the T-RF characteristic for RC-I in the RNA-based analysis does not necessarily indicate a low activity of this group.

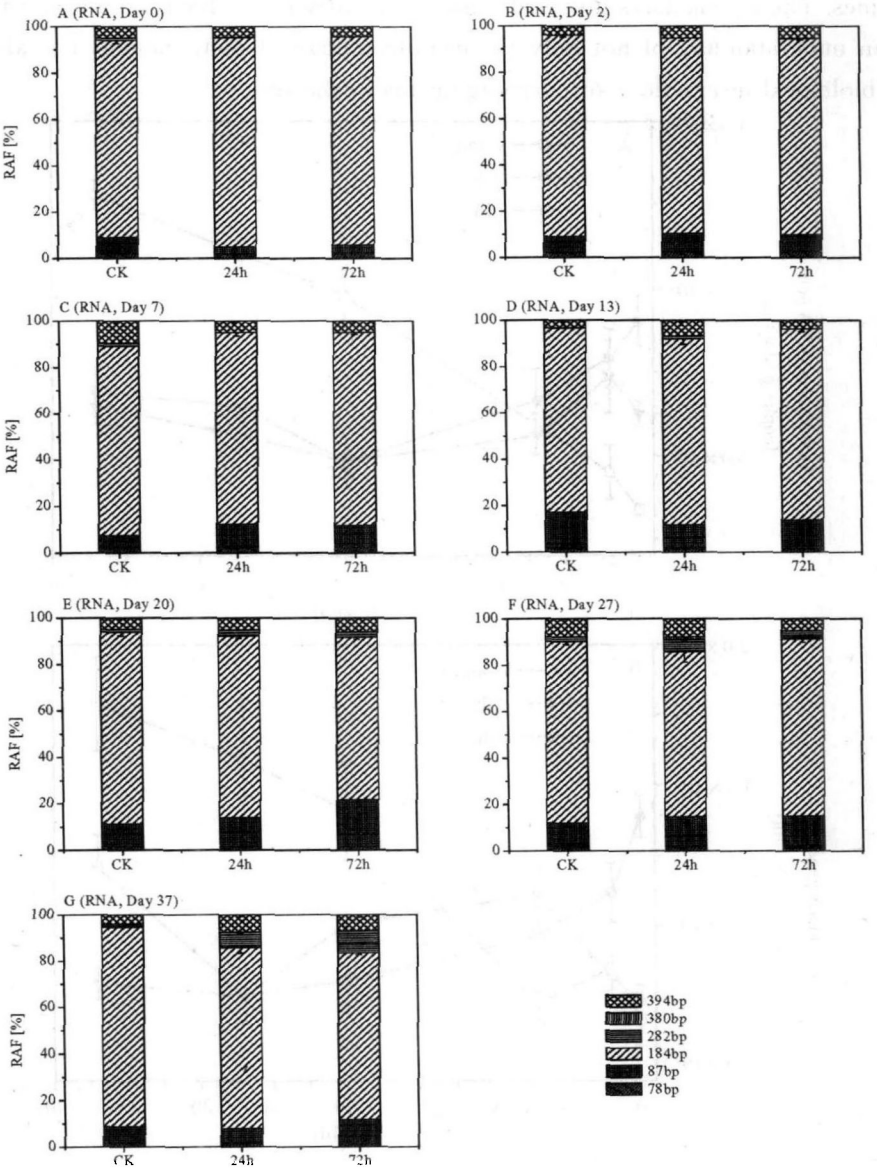

Figure 3. T-RFLP analyses targeting the archaeal 16S rRNA transcripts (RNA) at 7 different time points after short-term (24 h) and long-term (72 h) O_2 exposure and in the permanently anoxic control (CK). Shown is the percentage of individual T-RF relative to the total integrated fluorescence (RAF = relative amplicon frequency; mean values ± SE; n=3). Only T-RFs representing major methanogenic lineages are shown. For reverse transcription (RT), coextracted DNA was removed from the nucleic acid extracts by DNase digestion (Qiagen, Germany). RT-PCR was performed using the AccessQuick one tube RT-PCR system (Promega, Germany) following the protocol described previously (Lueders and Friedrich, 2002). Amplicons were purified and subjected to T-RFLP analysis as described in Figure 2

3 Conclusion

In conclusion, our study revealed a remarkable discrepancy between the effect of O_2 exposure on the activity and the structure dynamics of the methanogenic community in rice field soil. The observed effects of O_2 exposure on the activity of methanogens were neither completely explained by competition with iron reducers and sulfate reducers, nor by differential suppression of growth or enzyme synthesis in acetoclastic versus hydrogenotrophic methanogens. Therefore, we hypothesize that CH_4 production, acetoclastic CH_4 production in particular, is in addition affected on the level of the

existing enzymes. The mechanisms involved remain currently unresolved. To uncover the mechanisms will require an understanding of not only the eco-physiology of methanogens but also the complex physical and biological interactions of microorganisms in the soil.

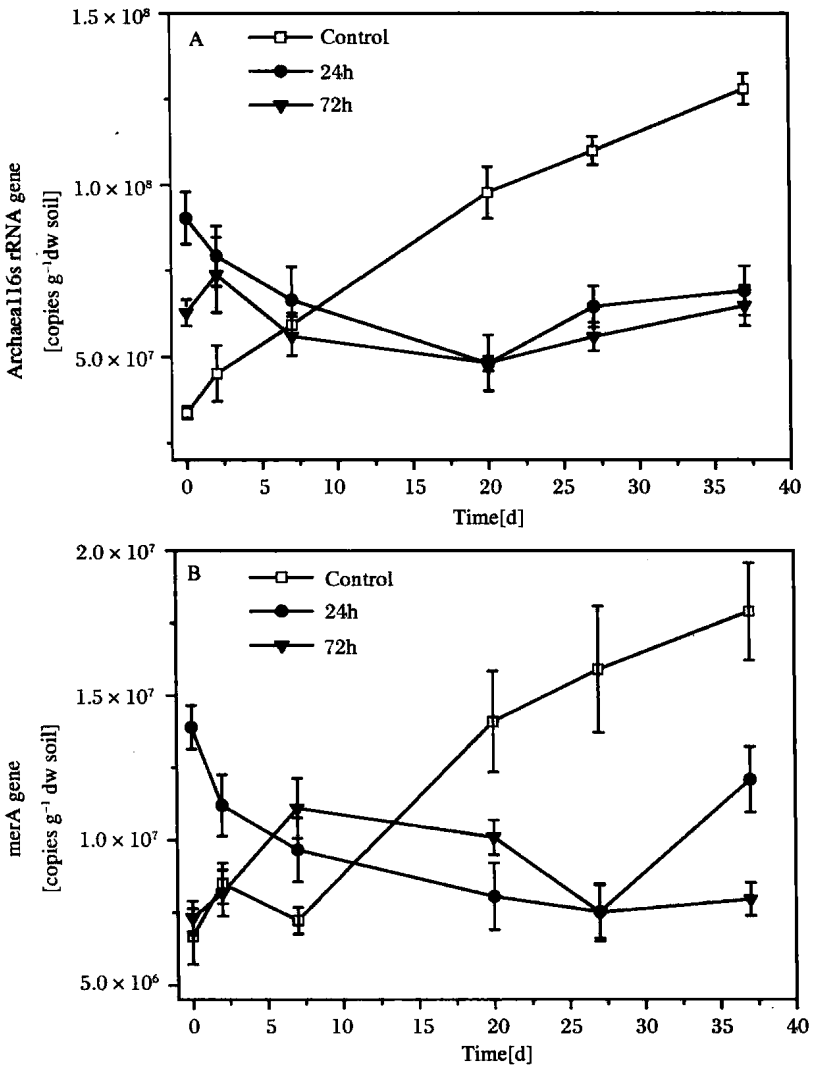

Figure 4. Temporal changes of 16S rRNA gene copies of total archaea (A) and *mcrA* gene copies (B) of methanogens (values are mean ± SE, n=3). The copy numbers of both archaeal 16S rRNA and *mcrA* genes were determined using quantitative PCR. The DNA standard for the quantification of the *mcrA* gene copies was prepared using the genomic DNA extracted from the liquid culture of *Methanosarcina barkeri*™ (DSM804) according to the extraction protocol (Lueders and Friedrich, 2002). The DNA standard for total archaeal 16S rRNA genes was prepared using partial 16S rDNA fragments (sequence length was about 800 bp) amplified from genomic DNA of *M. barkeri* using the protocol as described previously (Groβkopf *et al.*, 1998). Quantification of the archaeal 16S rRNA gene was carried out using the primers 364f (Burggarf *et al.*, 1997) and 934r with the same conditions as described in (Kemnitz *et al.*, 2004) and quantification of the *mcrA* genes was done using the primers ME1f/ME3r (Hales *et al.*, 1996) with the same conditions as described in (Wilms *et al.*, 2007)

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