Biogeosciences

Wetland restoration and methanogenesis: the activity of microbial populations and competition for substrates at different temperatures

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Abstract. Ljubljana marsh in Slovenia is a $16\,000$ ha area of partly drained fen, intended to be flooded to restore its ecological functions. The resultant water-logging may create anoxic conditions, eventually stimulating production and emission of methane, the most important greenhouse gas next to carbon dioxide. We examined the upper layer ($\sim$30 cm) of Ljubljana marsh soil for microbial processes that would predominate in water-saturated conditions, focusing on the potential for iron reduction, carbon mineralization (CO_2 and CH_4 production), and methane emission. Methane emission from water-saturated microcosms was near minimum detectable levels even after extended periods of flooding ($>$5 months). Methane production in anoxic soil slurries started only after a lag period of 84 d at 15°C and a minimum of 7 d at 37°C, the optimum temperature for methanogenesis. This lag was inversely related to iron reduction, which suggested that iron reduction out-competed methanogenesis for electron donors, such as H_2 and acetate. Methane production was observed only in samples incubated at 14–38°C. At the beginning of methanogenesis, acetoclastic methanogenesis dominated. In accordance with the preferred substrate, most (91%) *mcrA* (encoding the methyl coenzyme-M reductase, a key gene in methanogenesis) clone sequences could be affiliated to the acetoclastic genus *Methanosarcina*. No methanogens were detected in the original soil. However, a diverse community of iron-reducing *Geobacteraceae* was found. Our results suggest that methane emission can remain transient and low if water-table fluctuations allow re-oxidation of ferrous iron, sustaining iron reduction as the most important process in terminal carbon mineralization.

1 Introduction

For centuries, most European wetlands have been drained and used for agricultural and industrial needs. It is estimated that more than half of all peatlands in Europe were lost because of human activities (Nivet and Frazier 2004). However, the attitude toward wetlands changed as their functions were understood better, and today conservation efforts abound. One of the most important functions worth saving is the ability of wetlands to store and clean water. Major restoration concepts along these lines include ceasing agriculture and re-establishment of wetland hydrology (Rosenthal 2003). Since wetlands are by definition permanently or temporarily flooded areas, raising the water table to original levels is thus the first step for a wetland revival.

Peatlands are characterised by soils rich in organic matter. Peat accumulated since the last glaciations corresponds to 20–30% of the global soil carbon pool (Frolking et al., 2001; Gorham, 1991). Drained peat decomposes rapidly (Andriesse, 1988). When submerged again, oxygen is exhausted quickly. Fermentation products such as acetate and H_2 provide substrates for terminal oxidation processes. Reduction of terminal e$^-$ acceptors occurs sequentially according to thermodynamics: reduction of nitrate is followed by reduction of manganese, iron, sulphate, and finally by the reduction of CO_2 or methyl groups to CH_4 (Ponnamperuma, 1972). The water table, which affects oxygen availability and transport, is therefore an important determinant of gas emissions from peat soil (Aerts and Ludwig, 1997).

Iron is the fourth-most element in the earth's crust, and even in peatlands, iron reduction may shift the e$^-$ flow away from methanogenesis (Küsel et al., 2008). A wide variety of microbes can reduce iron oxides, but the *Geobacteracea*, members of the δ-Proteobacteria subdivision, have most regularly been found associated with bacterial iron reduction.

Methane is next to CO_2 the most important greenhouse gas (Intergovernmental Panel on Climate Change, 2007), and wetlands are the largest natural source of CH_4, contributing approximately 20% of the global emissions (Matthews, 2000). While methane production is a strictly anaerobic process confined to anoxic layers, methane oxidation in freshwater wetlands depends on the presence of oxygen. Methane oxidation may control methane fluxes reducing potential emissions substantially (Conrad and Frenzel, 2002).

Methanogenesis itself depends on a sequence of microbial processes starting with hydrolysis of polymers, followed by primary and secondary fermentation, and ending with the methanogenic conversion of acetate and H_2/CO_2 (Conrad and Frenzel, 2002). The effect of temperature on methanogenesis and methanogenic pathways has been studied in different sediments and soils showing a wide variety of reactions: methanogenesis may dominate at low temperatures, while electrons are diverted to iron reduction at the respective temperature optimum of mineralization (Metje and Frenzel, 2005). H_2/CO_2 may be used preferably by acetogens at low temperature, but by methanogens at higher temperature (Schulz and Conrad, 1996). Finally, a thermophilic methanogenic sub-population may co-exist with mesophiles, taking over at elevated temperatures (Fey and Conrad, 2000).

Ljubljana marsh in central Slovenia is a large fen area that was reclaimed for agriculture. A drainage system was established already in the 19th century, however, Ljubljana marsh has never achieved the expected productivity. Today, agriculture is declining with grazing and haying being the most important usage. The biggest threat for conservation is currently the expansion of the neighbouring city and its infrastructures.

Conservation and restoration efforts have been initiated by BirdLive Slovenija. Since becoming part of the Natura 2000 network, there have been attempts to re-establish the natural properties. Because seasonal flooding is important e.g. for certain bird habitats, management plans include raising the water table. This will result in anoxic conditions in the flooded soils potentially leading to increased CH_4 emissions. However, fluxes measured in the field showed only occasional minor CH_4 emissions (Danevčič and J. Hacin, personal communication, 2006). Microcosm studies indicated that CH_4 is produced only after a lag of two months, and only at elevated temperature (Stres et al., 2008).

In our study we focused on iron reduction and substrate availability as potential controls for CH_4 production. In addition, the community structures of Archaea and *Geobacteraceae* was analyzed, taking the latter as representatives of mesophilic iron reducers. The experiment covered a wide temperature range giving a full picture of the physiological capabilities and limitations of the microbial community. In a second experiment, we used water-saturated soil in microcosms to study the interplay between methanogenesis and CH_4 oxidation in a complex system. This work therefore provides fundamental information on the potential, extent, and distribution of different redox processes and associated microbial communities that could prevail after raising the water table level in the drained fen.

2 Materials and methods

2.1 Site description and field sampling

Ljubljana Marsh is a 16 000 ha fen region located in central Slovenia (45°58′ N, 14°28′ E). It is characterized by mineral-rich ground water and neutral pH. The average annual precipitation is 1400 mm, and the mean annual temperature is 10°C. A drainage-channel system was established in the 19th century and the landscape has been shaped ever since by peat extraction and agriculture. Today, 75% of the area is covered by grassland, 10% by forest, and 15% by cornfields. The experimental site is located on a grassland dominated by an *Arrhenatherion* (Seliškar, 1986). The water table is on average 50 cm below the surface, with pronounced seasonal fluctuations from flooded to 1 m below ground. The soil temperature in the upper 30 cm varies annually from 1°C to 20°C.

Samples were collected at the end of August in 2005 with ten individual soil cores (h=30 cm; d=15 cm) within a 10×5 m^2 area covering the upper 30 cm of soil. Soil samples were pooled, and plants and roots were removed to avoid litter input. The soil was homogenized by passing through a 3.15-mm sieve. Immediately after homogenization, aliquots (0.5 g) for molecular analysis were frozen and stored at -20°C. The remainder was kept at 4°C for maximally two weeks until the experiments were set up. The pooled soil contained 32% organic matter, 78% water, and had a pH (H_2O) of 7.6. For a more detailed description of this soil see Hacin et al. (2001).

2.2 Experimental design

Net methane fluxes were measured from 25 microcosms (d=7 cm; h=10 cm) filled with 250 g of homogenized soil and incubated for 65 days at 90% water holding capacity at 25°C in the dark. The water content was checked regularly by weighting the microcosms and adjusted with demineralised water, if necessary. CH_4 and CO_2 fluxes between soil and atmosphere were monitored monthly for 5 to 6 h after closing the headspace. No CH_4 emission was detectable after 65 days, and the microcosms were flooded and incubated for another 23 weeks at 25°C. Gas fluxes were measured as before. Methane oxidation was measured comparing fluxes with and without difluoromethane (CH_2F_2), a specific inhibitor of CH_4 oxidation (Miller and Oremland, 1998). Difluoromethane was added to a headspace concentration of 1% as described previously (Eller and Frenzel, 2001; Krüger et al., 2002).

Anaerobic processes were studied in anoxic soil slurries. Homogenized soil in 15 or 25 ml pressure tubes was mixed with sterilized anoxic distilled water at a ratio of 1:1.5

(vol/vol) to a total volume of 6.5 (effect of temperature, see below) and 10 ml (methanogenic pathways, see below). The tubes were closed with butyl rubber stoppers and flushed with N_2. In inhibition experiments, CH_3F, a specific inhibitor of acetoclastic methanogenesis, was added to the gas phase to a final concentration of 1% (Frenzel and Bosse 1996; Penning and Conrad 2006).

The effect of temperature was measured in 15-ml tubes incubated in a linear 0 to 80°C temperature gradient in the dark (Fey et al. 2001; Schulz et al. 1997). Tubes were incubated in duplicate at 30 temperatures for 115 days. CO_2 and CH_4 in the headspace were measured weekly, and H_2 at the end of the experiment. At the end of the experiment, 0.5 ml of each slurry was frozen at -20°C for molecular analysis; the remainder was used for pore water, Fe(II), and NH_4^+ analysis.

The methanogenic pathways were analyzed in 25-ml tubes at 25, 30, 37, 47, and 60°C for 68 days in the dark. Three replicates each with and without CH_3F were prepared. CO_2, CH_4, and H_2 concentrations were measured weekly. Additional replicates were incubated at 25°C. Every 1–2 weeks, three of these replicates were sacrificed for pore water, Fe(II) and NH_4^+ analyses (see below).

2.3 Analytical techniques

Gas phase analysis. Gases were measured as described previously (Bodelier et al., 2000). In short, CH_4 and CO_2 concentrations were measured on a SRI-8160A GC (SRI Instruments, Torrance, CA) with H_2 as carrier gas equipped with a methanizer and a flame ionization detector. H_2 concentrations were measured with a reducing gas detector (RGD2, Trace Analytical, Stanford, CA, USA). When H_2 concentrations were >200 ppm$_v$, a Shimadzu GC8A with N_2 as carrier gas and a thermal conductivity detector was used. Before the headspace was sampled, the tubes were shaken to equilibrate headspace and pore-water. The volume of gas removed from each tube was replaced with the same volume of N_2.

For *pore water analysis,* 2 ml slurry was centrifuged (5 min at 12 000$\times$ and 4°C), and the supernatant was filtered through a 0.2-μm filter. Organic acids and alcohols were measured by HPLC and GC, respectively, as described previously (Metje and Frenzel, 2005). Nitrate and sulphate were extracted from 0.15 g of wet soil or slurry by mixing with 600 μl of distilled water for 1 h. The extract was obtained as described above, and nitrate, nitrite and sulphate were determined by ion chromatography (Bak et al., 1991).

Reduced iron was extracted from 0.5 g of soil or 0.5 ml of soil slurry with hydrochloric acid (4.5 ml; 0.5 M) and measured spectrophotometrically (Lovley and Phillips, 1987b; Ratering and Schnell, 2000).

Exchangeable ammonium was extracted from 0.15 ml soil slurry with 2 M KCl and determined fluorometrically using a microscale analysis (Goyal et al., 1988; Murase et al., 2006) on a SAFIRE micro-plate reader (TECAN, Crailsheim, Germany).

2.4 Calculations

Mineralization rates of soil organic matter were calculated from the measurements of CO_2 and CH_4 accumulation over time, using Origin Pro 6.1 (OriginLab Corp., USA). A single- or two- component exponential decay model was fitted using the equation:

$$Y_t = C_1 \cdot (1 - e^{(-k_1 \cdot (t-t_0))}) \tag{1}$$

for a single-component, and

$$Y_t = C_1 \cdot (1 - e^{(-k_1 \cdot (t-t_0))}) + C_2 \cdot (1 - e^{(-k_2 \cdot (t-t_0))}) \tag{2}$$

for a two-component exponential decay function (Murayama and Zahari, 1996a, b). Y_t is the amount of CO_2 or CH_4 in the gas phase after time t in days, t_0 is the length of the lag phase (for CO_2 accumulation $t_0=0$), C_1 and C_2 are the initial pools, and k_1 and k_2 are rate constants for the mineralization of labile and recalcitrant organic fractions, respectively. Microbially not degradable fractions are not considered here. The half-life of a fraction was calculated from

$$t_{1/2} = \ln(2) \cdot k^{-1} \tag{3}$$

2.5 Molecular methods

Microbial community DNA was extracted from 0.5 g of the original soil sample, or from 0.5 g of soil slurry incubated in a temperature gradient block. DNA was purified using the Fast DNA SPIN Kit for Soil (Q-Biogene, Carlsbad, CA, USA). Humic acids were removed by additional washes with guanidine thiocyanate solution (5.5 M; Metje and Frenzel, 2005). The concentration of extracted DNA was determined spectrophotometrically on a BioPhotometer (Eppendorf, Hamburg, Germany) at 260 nm. Enzymes and reagents for PCR reactions were obtained from Promega (Madison, WI, USA).

Archaeal 16S rRNA genes were amplified using primers A109f and A915r (Chin et al. 1999). For terminal-restriction fragment length polymorphism (T-RFLP) analysis, the reverse primer was labeled at the 5' terminus with 5-carboxyfluorescein, and PCR products were purified with the MinElute PCR Purification Kit (QiaGen, Düsseldorf, Germany). Approximately 50 ng of DNA was digested with *Taq*I restriction endonuclease (Promega Corp., Madison, WI, USA) at 65°C for 3 h. The procedures and equipment used for T-RFLP analysis were as described previously (Metje and Frenzel, 2005). The percentage abundance of each T-RF (Ap) was calculated as $Ap = ni \times 100/N$ in which ni represents the peak height of one distinct T-RF and N is the sum of all peak heights in a given T-RFLP profile.

The methanogenic communities were characterized by PCR amplification using three different *mcrA*-specific primer sets (Hales et al., 1996: ME1 and ME2; Luton et al., 2002: mcrAf and mcrAr; Springer et al., 1995: MCRf and MCRr). Only the primer set of Luton et al. (2002) yielded sufficient

PCR product. Partial 16S rRNA gene sequences of the iron-reducing *Geobacteraceae* were amplified with Geo564F and Geo840R primers (Cummings et al., 2003). For denaturing gradient gel electrophoresis (DGGE) analysis of *Geobacteraceae*, a GC clamp was added to the 5′ end of the Geo840R primer. All PCR reactions followed protocols described by the respective authors. PCR products were cleaned with the MinElute PCR Purification Kit (QiaGen, Düsseldorf, Germany) and cloned into *Escherichia coli* JM109 competent cells via the pGEM-T Easy Vector System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Selected clones were checked for correct insert size by vector-targeted PCR with vector-specific primers M13 and T7. Both strands of the correctly inserted DNA were sequenced using the ABI Prism Big Dye Terminator Cycle Ready Reaction Kit on an ABI 377 DNA sequencer (Applied Biosystems, Carlsbad, CA, USA) as specified by the manufacturer.

Geobacteraceae-targeting DGGE analysis was performed with a Bio-Rad system following the modified procedure of Cummings et al. (2003). The PCR product was loaded onto 1-mm thick 7.5% (wt/vol) polyacrylamide gels containing a 30–60% linear denaturing gradient; 100% of denaturant was defined as 7 M urea and 40% formamide. Electrophoresis was performed in 1 mM TAE buffer at 60°C and a constant voltage of 65 V for 15 h. After DGGE, gels were stained up to 1 h in 0.1% SYBR Green staining solution (Invitrogen, Carlsbad, CA, USA). Gels were scanned with a Typhoon Trio gel imager (GE Healthcare, UK). Gel images were linearized and analyzed with Photoshop CS2, version 9.02 (Adobe System Inc., San Jose, CA, USA). Bands were excised; each band was re-suspended in 20 μl of elution buffer (QiaGen, Düsseldorf, Germany). DNA was amplified as described above, and the amplification products were verified by checking their position after DGGE. Forty-five distinct bands were excised again, and DNA was eluted, amplified, cleaned and sequenced. Out of 45 excised bands, 30 different sequences were obtained; 14 of the remaining sequences were identical to other sequences, and one was a chimera. Two clone mixtures of 10 defined clones were made to obtain additional markers for DGGE community profiles.

The sequences obtained were compared to sequences in the GenBank database using the Basic Local Alignment Search Tool (BLAST) to find highly similar sequences (megablast). Chimeric 16S rRNA gene sequences were identified by Chimera Check of Ribosomal Database Project II (release 8.1) (Cole et al., 2005). Sequences were aligned and phylogenetically analyzed with the ARB software package (Ludwig et al., 2004) using neighbour-joining and Tree-Puzzle (Schmidt et al., 2002). Details are given in the respective figure captions. All sequences have been deposited in the GenBank and EMBL databases. *mcrA* sequences: EU753559-604; 16S rRNA-gene: EU753436-480 (Archaea), EU753481-510 (*Geobacteraceae*, excised bands) and EU753511-558 (*Geobacteraceae*, clones).
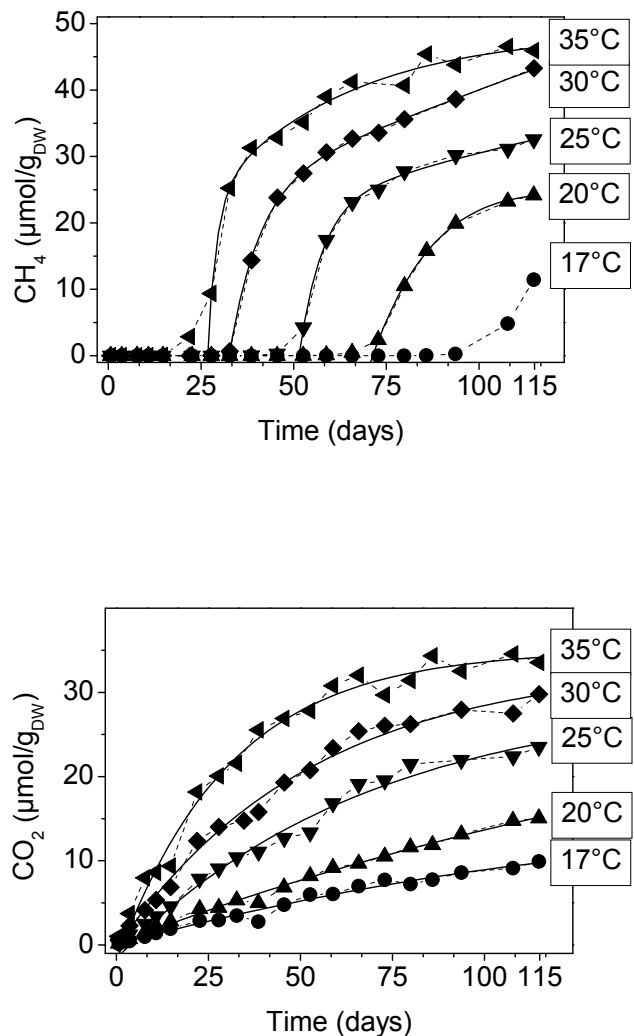

Fig. 1. Production of CH_4 **(A)** and CO_2 **(B)** over time in anoxic soil slurries at five temperatures. Solid lines were fitted by exponential decay models; for details see text.

3   Results

No CH_4 was emitted from the flooded microcosms before 162 days of incubation. Methane emission was observed first in the presence of the CH_4 oxidation inhibitor, CH_2F_2, suggesting CH_4 emissions were reduced by CH_4 oxidation once production had started. Similarly, the soil slurries started producing CH_4 only after a variable lag period, whereas CO_2 concentrations increased immediately (Fig. 1).

The amount of CO_2 in the gas phase of the soil slurries increased with time and with temperature (Figs. 1b, 2b). CO_2 accumulation with time followed a single-component exponential decay model below 30°C. Above 30°C a two-component exponential decay model gave a better fit. A two component model was also used to describe CH_4 production. The rate constants of mineralization of the labile organic
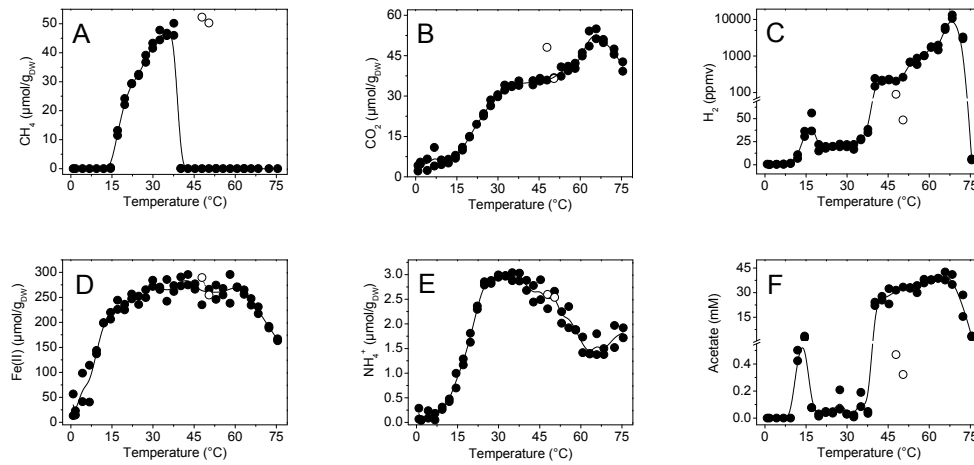
Fig. 2. Accumulation of CH_4 **(A)**, CO_2 **(B)**, and H_2 **(C)** in the gas phase and concentrations of Fe(II) **(D)**, NH_4^+ **(E)** and acetate **(F)** in pore water of anoxic soil slurries after incubation for 115 days. Open circles: see text for explanation. Solid lines connect the averages of the two replicates. Note the logarithmic scale in C.

matter, obtained from CO_2 accumulation values, increased with temperature from $0.01\,d^{-1}$ ($12°C$) to $0.21\,d^{-1}$ ($72°C$). However, rate constants of mineralization *via* methanogenesis varied from 0.04 to 0.17 independently of temperature. The half-lives of the labile organic matter calculated from CO_2 accumulation decreased with increasing temperatures from 120 to 3 days, whereas mineralization through methanogenesis required 5–13 days, regardless of the temperature (Fig. 3a). The lag phase of methanogenesis corresponded to the half life of labile organic matter calculated from CO_2 accumulation (Fig. 3b). We were unable to calculate precise mineralization rates of the refractory organic component at the given temperatures. Graphic analyses of refractory carbon mineralization suggested rate constants three orders of magnitude lower than for labile organic matter, with half-lives >20 y.

Because of the non-linear increase of CH_4 and CO_2 (Fig. 1), the accumulation during the experiment is given instead of a rate per day in Fig. 2. Two temperature optima for CO_2 accumulation were observed, one at $38°C$ and a second one at $65°C$ (Fig. 2b). Until the end of the experiment after 115 days, virtually no CH_4 production was observed below $14°C$. The lag phase for methanogenesis decreased with increasing temperature from a maximum of 80 days at $15°C$, to a minimum of 7 days at the temperature optimum. Around $15°C$, elevated levels of H_2 (50 ppmv; Fig. 2c) and acetate ($500\,\mu M$; Fig. 2f) coincided with a decreasing rate of Fe(II)-accumulation (Fig. 2d) and the onset of CH_4 accumulation, suggesting a transitional phase between Fe-reduction and methanogenesis. The average steady-state concentrations in methanogenic samples were 26 ppm$_v$ H_2 and 60 μM acetate (15–38°C; Fig. 2c, f). The respective concentrations were much lower when Fe-reduction was prevalent at temperatures <14°C, with 2 ppm$_v$ H_2 and acetate below the de-

tection limit. No other intermediate or substrate accumulated up to $40°C$. The temperature optimum for CH_4 production was $38°C$, where $>40\,\mu mol\,g\,dry\,wt^{-1}$ CH_4 accumulated after 115 days, with rates decreasing immediately when temperature increased (Fig. 2a). Ammonium concentrations were below the detection limit in the original soil. Above $10°C$, the concentration increased reaching an optimum at $32°C$ ($3.04\,\mu mol\,NH_4^+ \times g\,dry\,wt\text{-}1$).

Above $40°C$, very small amounts of CH_4 accumulated from the beginning, but reaching not more than 33 nmol $CH_4 \times g\,dry\,wt^{-1}$ at the end of the experiment. However, two samples, incubated at $48°C$ and $50°C$ (Fig. 2, open circles), showed a different pattern: CH_4 accumulated to high values, and alcohols, fatty acids, and H_2 accumulated less. H_2, fatty acids, and alcohols accumulated to high levels, with the highest concentrations at $66–76°C$.

A detailed analysis of the time course of iron reduction and methanogenesis was done at $25°C$. Fe(II) increased from the beginning till approximately 60 days (Fig. 4). The maximum concentrations of Fe(II) represented about 55–60% of the total iron content suggesting that roughly half of the chemically reducible iron was inaccessible for microbes. Methane production started while iron reduction began to decrease. At the onset of methanogenesis, hydrogenotrophic methanogensis contributed 30–75% (Fig. 4). After 68 days, however, acetoclastic methanogenesis was prevalent at 25, 30, and $37°C$ (95, 60, and 80%, respectively).

To analyze the microbial community and its changes, archaeal and *Geobacteraceae* – related 16S rRNA genes, and the *mcrA* gene were amplified, cloned, and analysed by T-RFLP or DGGE. The amount of DNA extracted was highest in the original soil sample and decreased in soil slurries as the temperature increased. Above $60°C$, no PCR products could be obtained. The archaeal 16S rRNA clone library of the
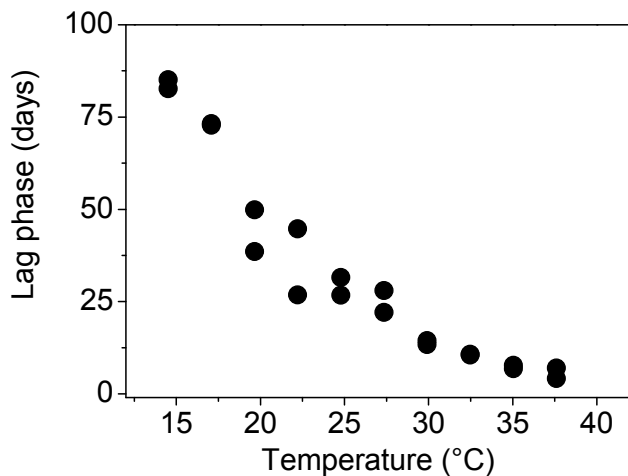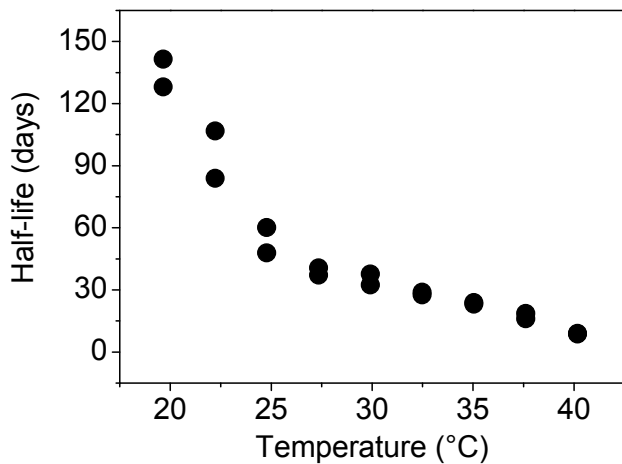
Fig. 4. Methanogenic pathways and iron reduction at 25°C. The values of CH_4 produced from either H_2/CO_2 or acetate were calculated from averages ($n=2$–3) of CH_4 concentrations measured in the presence (methanogenesis from H_2/CO_2 only) and in the absence of methyl fluoride. Fe(II): average ±SE, $n=6$.

Fig. 3. (A) Half-life of labile soil organic matter calculated from CO_2 production in anoxic soil slurries incubated at different temperatures. **(B)** Lag phase for CH_4 production in anoxic soil slurries incubated at different temperatures.

original soil consisted exclusively of crenarchaeal sequences with no methanogenic representatives (45 of 46 clone sequences; one was a chimera). All sequences belonged to the yet uncultured rice cluster VI (RC-VI) of Crenarchaeota (Fig. 5) (Chin et al., 1999; Kemnitz et al., 2004).

T-RFLP analysis of archaeal 16S rRNA genes from samples incubated at various temperatures yielded four distinct T-RFs of 185, 382, 394, and 735. The original soil yielded only two T-RFs of 140 and 185 bp. The dominating fragment (185 bp; Ap 46–100%) was present in all samples. T-RFs of 382, 394, and 735 bp were found in samples incubated at 20–38°C. The 382- and 394-bp T-RFs increased at higher temperatures (30–38°C), with an Ap up to 6 and 30%,
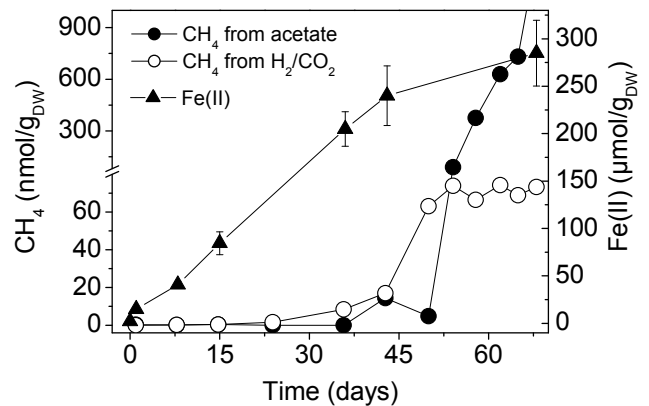
respectively. It is suggested that the T-RFs of 185 bp represents members of *Methanosarcinaceae* or RC-VI (Kemnitz et al. 2004), 382 bp members of the euryarchaeal RC-III, and the T-RF of 394 bp members of the methanogenic RC-I, (Kemnitz et al., 2004; Lu et al., 2005; Penning and Conrad, 2006). This affiliation is consistent with the clone library: archaeal 16S rRNA gene clone sequences from the original soil belonged exclusively to RC-VI (Fig. 5), and *mcrA* clone sequences from a methanogenic slurry incubated at 35°C were represented by acetoclastic *Methanosarcina* and hydrogenotrophic RC-I at a ratio of 91:9 (Fig. 6).

Geobacteraceae-targeting PCR yielded a strong product for all samples incubated below 40°C. There was no obvious difference in DGGE bands between samples incubated at 1–27°C (Fig. 7). In samples incubated at 30–35°C, the community shifted and stabilized again at 38°C. Banding patterns remained constant up to 60°C, at which the bands became fainter, suggesting lower target numbers. The *Geobacteraceae* 16S rRNA gene clone library from the original soil contained 48 different clones. DGGE-bands were excised, re-amplified and sequenced.

All sequences ($n=74$) clustered within the order *Desulfuromonadales,* including the the Fe(III) reducing genera *Geobacter*, *Desulfuromonas* and *Pelobacter.* One sequence was affiliated to the genus *Anaeromyxobacter.* The clones and the respective DGGE bands were assigned to groups I-XI (Fig. 8). The vast majority of bands were positioned on the gradient gel between 40 and 55% denaturant. The bands positioned at denaturant concentrations >55% (not shown) belonged to the iron reducer *Anaeromyxobacter.* Most bands were present throughout the whole temperature range but differing in relative intensity.
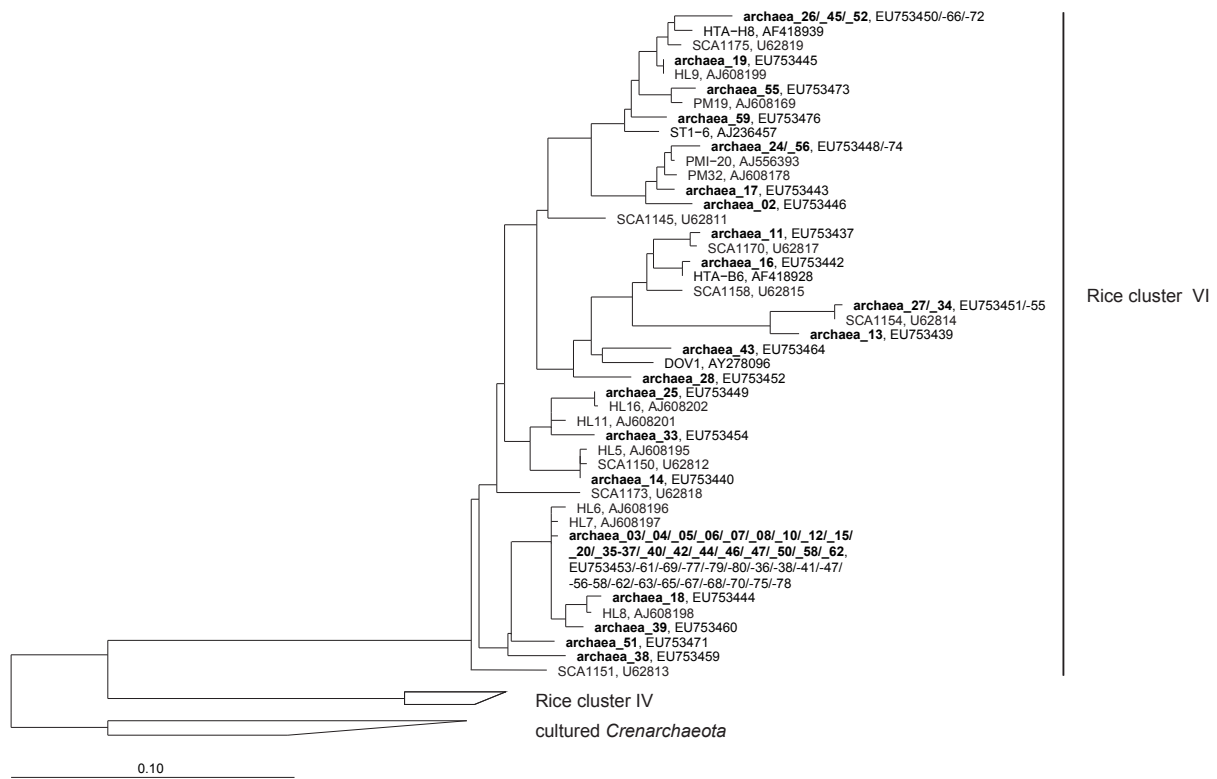
Fig. 5. Crenarchaeota, rice cluster VI: neighbour-joining tree of 16S rRNA gene sequences. Clones retrieved from the original soil (bold), from upland (Bintrim et al., 1997) and flooded soils (Kemnitz et al., 2004). GenBank accession numbers of the sequences are indicated. Scale bar: estimated number of base changes per nucleotide position.

4 Discussion

If methanogenesis had occurred regularly in this soil, it would have recovered quickly after flooding had caused anoxic conditions. However, CH_4 production began only after a long lag phase. This implies that either methanogenesis was inhibited by another process, and/or methanogen abundance was low. Denitrification and sulphate reduction could be ruled out as competing processes, because the respective electron acceptors were virtually absent. Fe(III), in contrast, proved to be a major electron sink. The length and course of iron reduction coincided well with the lag phase for CH_4 production. Iron reduction started immediately after the start of incubation and production of CH_4 was completely suppressed until ~2/3 of iron was reduced.

Iron reducers have a higher affinity for H_2 and acetate than methanogenic archaea, and they can grow at lower H_2 and acetate concentrations (Lovley and Phillips, 1986; Lovley and Phillips, 1987a). Fe-reducing bacteria form a polyphyletic functional group. While a PCR-assay directed against a functional gene common to all Fe-reducing bacteria is not available, the PCR-assay targeting the 16S rRNA gene covers a significant fraction of these organisms (Cummings et al., 2003). *Geobacteraceae* can use short-chain fatty acids,

monoaromatic compounds, and hydrogen as electron donors (Lovley, 2000). Most of the DGGE-bands in samples incubated above 30°C could be affiliated with iron reducers that can use very diverse electron donors (*Geobacter pelophilus, Pelobacter acetylenicus,* and *Pelobacter propionicus*; Fig. 7). At lower temperatures, one of the pronounced bands belonged to group XI that could be affiliated to the acetate oxidizing *Desulfuromonas acetoxidans*. Competition for acetate is suggested by the very low acetate concentration in the Fe-reducing samples (Fig. 2d, f). *Methanosaeta* is reported to have an acetate threshold of 20 μM, with 200–500 μM being claimed to be necessary for *Methanosarcina* (Jetten et al., 1990). Since *Methanosaeta* was not detected, the acetate concentration measured at temperatures <14°C is well in accordance with competition with iron reducers for acetate, while a direct temperature control of methanogenic populations was not evident. H_2 concentration in these samples was ≤1 nM, but may have allowed minor CH_4 formation (Fig. 2c, 4) (Karadagli and Rittmann, 2007). This agrees with observations in a rice field and in rice soil microcosms, in which only minor residual CH_4 production from H_2/CO_2 occurred during the Fe-reducing phase (Frenzel et al., 1999; Krüger et al., 2002; Lueders and Friedrich, 2002). The higher levels of H_2 and acetate in 15°C-samples coincided with the onset

Fig. 6. Methanogenic *Archaea*: Tree-Puzzle tree of *mcrA*-sequences. The tree was calculated with 10 000 puzzling steps, the Whelan-Goldman substitution model, parameter estimation using neighbor-joining, a filter 20–100%, and 160 valid columns. Sequences retrieved after incubation at 35°C for 115 days are printed in bold. Scale bar: estimated number of changes per amino acid position. Root: *Methanopyrus kandleri* (AF414042). Clone sequences were aligned against an ARB-database with ~2500 *mcrA* sequences. Nearest cultivated and environmental neighbors were identified after adding the clone sequences with the quick add tool (parsimony) to an existing working tree with ~2500 sequences. Since clone sequences were forming coherent clusters, only 17 representatives out of 45 sequences were subsequently used together with the nearest cultivated and environmental sequences to generate the initial maximum-likelihood tree.
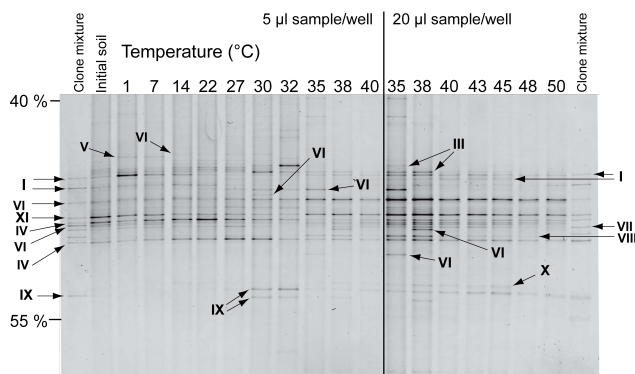

Fig. 7. *Geobacteraceae* and related *Desulfuromonadales*: DGGE-profiles of 16S rRNA gene fragments from the original soil and after incubation at different temperatures. Individual bands that yielded sequence data are labelled with group numbers (Roman numerals). The denaturant gradient is indicated to the left in %.

methanogenesis (Fig. 2). Considering that prior to incubation no methanogens could be detected, these data indicate that fermentation and methanogenesis had not yet reached a steady state.

The archaeal 16S rDNA gene sequences retrieved from the original soil fell all into rice cluster VI, a group of non-methanogenic Crenarchaeota. Rice cluster VI has been found in various freshwater and terrestrial habitats, but its physiology is still unknown (Kemnitz et al., 2004; Ochsenreiter et al., 2003). These data support the assumption that methanogenesis in the upper 30 cm of Ljubljana marsh soil is not a process that occurs naturally, even after longer periods of flooding. Our results indicate that rare representatives of a methanogenic community were present and became enriched during incubation, at least at temperatures above 15°C. Indeed, methanogens have been shown to survive prolonged oxic conditions (Mayer and Conrad, 1990; Peters and Conrad, 1995). Hence, re-establishing a methanogenic community is to be expected after flooding, but it will have to cope with the competition for electron donors by Fe-reducing bacteria.

The *Methanosarcinaceae* detected in samples with high CH_4 production are able to use acetate corresponding well to the high fraction of acetoclastic methanogenesis. The acetate concentrations of 60 μM in the methanogenic samples, however, were still lower than thresholds reported for pure cultures (Jetten et al., 1990), but as high as observed in a methanogenic rice field soil (Leybo et al., 2006). A minor

Fig. 8. *Geobacteraceae* and related *Desulfuromonadales*: neighbour-joining tree of 16S rRNA gene sequences retrieved from the original soil, or recovered from DGGE bands (Fig. 7). First a stable bootstrap tree with sequences from cultured and uncultured species with >1300 bases was constructed. Afterwards clone sequences and sequences from DGGE (in bold) were added by quick add (parsimony) as implemented in ARB, considering a total of 313 base positions. Closed symbols: ≥90%; open symbols: ≥80%; scale bar: estimated number of base changes per nucleotide position. As root *Escherichia coli* (AJ567617) was used.

fraction of methanogenic archaea in our samples were affiliated with RC-I. Members of RC-I are hydrogenotrophic and more active at low H_2 concentrations (Lu and Conrad, 2005), which allows them to grow in syntrophy with secondary fermenters (Lueders et al., 2004). RC-I is often found in periodically oxic environments, suggesting that they are well adapted to oxic stress during periods of drought (Con-

rad et al., 2006). Recently, this was supported by a complete genome analysis of RC-I (Erkel et al., 2006), predicting the presence of a complex set of enzymes and mechanisms protecting this organism against oxidative stress.

Soil organic matter is a complex mixture of different components. In short-term experiments, CO_2 and CH_4, the end-products of carbon mineralization, often increase

linearly with time. For this long-term experiment, lasting 115 days, two-component decay models describing the different degradability of a labile and a refractive fraction gave a better fit (Murayama and Zahari, 1996b). The labile fraction had a half-life of about 130 days at 20°C, the in situ summer temperature in the soil layer studied. The shorter half-life calculated for methanogenesis was lacking an obvious temperature response suggesting that production of methanogenic precursors and overall mineralization were only loosely coupled. The temperature optimum for methanogens was relatively high compared to northern wetlands (Metje and Frenzel, 2005; Metje and Frenzel, 2007), but comparable to that found for methanogenesis in an Italian rice field soil (Fey et al., 2001). This suggests a common adaptation to the climate in the Mediterranean, and a latitudinal differentiation of microbial populations in flooded soils. Furthermore, the outliers in CH_4 accumulation at 48–50°C (Fig. 2a) indicate the presence of a cryptic moderately thermophilic population like that found in an Italian rice field soil (Fey et al., 2001). However, the adaptive value of such a trait is entirely unclear.

We have shown that re-establishment of a methanogenic population and, hence, production of CH_4 was limited by competition for substrates, mainly for acetate. In the presence of Fe(III), methanogenesis will be largely delayed over a wide temperature range. The long-term effects after restoring a high water-table are more difficult to predict: timing of methanogenesis does not only depend on competing terminal processes, but also on labile organic carbon. Its availability will depend on plant exudates and litter input that were not part of our study. However, if the water-table is allowed to fluctuate, the iron oxidation-reduction cycle is expected to continue, as corroborated by other studies (Küsel et al., 2008). Hence, intermittent drainage events, preferably during the warm season, may be the most promising strategy mitigating CH_4 emissions. In addition, the microcosm-experiment has shown that CH_4 may be oxidized by methanotrophs before it can escape into the atmosphere. Overall, our results suggest that restoration strategies proposed for Ljubljana marsh will not necessarily increase CH_4 emission.

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