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**Wetland restoration
and methanogenesis**

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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 (~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 (CO₂ and CH₄ 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 and was inversely related to iron reduction, which suggested that iron reduction out-competed methanogenesis for electron donors, such as H₂ 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

Over the past 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

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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. Drained peat decomposes rapidly (Andriessse, 1988). When submerged again, oxygen is exhausted quickly. Fermentation products like 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).

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 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

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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 cause anoxic conditions in the flooded soils potentially leading to increased CH₄ emissions. However, fluxes measured in situ showed only occasional traces of CH₄ emissions (T. Danevčič and J. Hacin, personal communication, 2006). Microcosm studies indicated that CH₄ 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 CH₄ production. In addition, the structure of Archaea and iron reducing *Geobacteraceae* was analyzed. 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 CH₄ 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.

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2 Materials and methods

2.1 Site description and field sampling

Ljubljana marsh is a 16 000 ha large fen complex located in central Slovenia (45°58' N, 14°28' E). The average annual precipitation is 1400 mm, and the mean annual temperature is 10°C. The floodplain has developed in a glacial basin of tectonic origin which was gradually filled with river deposits (Šifrer, 1984). Groundwater is supplied mainly from the surrounding mountains by torrents and from the river Ljubljana, a karstic river, passing through the floodplain. Peat formation began 6000 years ago. Periodic changes of water regime resulted in the development of raised bog fragments on a lower fen plane with transitional fen areas of variable sizes. Natural development of the peatland ceased when fen-peat was reclaimed for agriculture while peat from raised bogs was cut as a fuel source. Drainage-channel systems were built throughout the marsh and the beds of river Ljubljana and its tributaries were redirected enabling water to flow off and soil to drain. Today, the water table is regulated by a control weir at river Ljubljana and by the drainage system. Peat excavation from the end of the 18th to the middle of 20th century left only small fragments of raised bog, with the remainder represented by fens with sedge peat a few cm to 3 m deep. Today, 75% of the area is covered by grasslands, 10% by forest, and 15% by cornfields.

Samples were collected in late August 2005. The experimental site is located on a grassland dominated by an *Arrhenatherion* (Seliškar, 1986), representing >50% of Ljubljana marsh grasslands. Groundwater is on average 50 cm below the surface, with pronounced seasonal fluctuations. The temperature at 30 cm depth ranges annually from 1°C to 20°C. Ten soil cores ($h=30$ cm; $d=15$ cm) were collected within a 10×5 m² 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 a maximum of 2 weeks until experiments were set up. The pooled soil contained 32% organic matter,

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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. 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 after adding difluoromethane (CH_2F_2), a specific inhibitor of CH_4 oxidation (Miller and Oremland, 1998), 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 and 10 ml, respectively. 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 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. After the tubes were opened, 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

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pore water, Fe(II) and NH_4^+ analyses (see below).

2.3 Analytical techniques

Gas phase analysis. CH_4 and CO_2 concentrations were measured on a GC with H_2 as carrier gas equipped with a methanizer and a flame ionization detector. Low H_2 concentrations were measured on a gas chromatograph with a reducing gas detector. When H_2 concentrations were $>200 \text{ ppm}_v$, a GC 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 $12\,000\times g$ and 4°C), and the supernatant was filtered through a $0.2\text{-}\mu\text{m}$ 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 $600 \mu\text{l}$ 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

Decomposition 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

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single- or two-component exponential decay model was fitted using the equation:

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

for a single-component, and

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

5 for a two-component exponential decay function (Murayama et al., 1996a; Murayama et al., 1996b). 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 decomposition of labile and recalcitrant organic fractions, respectively. The half-life of a fraction was calculated from

10 $t_{1/2} = \ln(2) \cdot k^{-1}$

2.5 Molecular methods

Microbial community DNA was extracted from 0.5 g of the original soil sample, or 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
15 additional washes with guanidine thiocyanate solution (5.5 M). The concentration of extracted DNA was determined spectrophotometrically on a BioPhotometer (Eppendorf, Hamburg, Germany) at 260 nm.

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
20 products were purified with the Mini Elute PCR Purification Kit (QiaGen). Approximately 50 ng of DNA was digested with Taq I restriction endonuclease (Promega) 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 (A_p)

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was calculated as $A_p = n_i \times 100/N$ in which n_i 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 amplification using three different *mcrA*-specific primer sets (Hales et al., 1996; Luton et al., 2002; Springer et al., 1995). 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 Mini Elute 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-specific 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.). Bands from two DGGE gels were excised; each band was resuspended in 20 μ l of elution buffer (QiaGen). 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

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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.

5 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.,
10 2004) using neighbour-joining. 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).

3 Results

15 No CH₄ was emitted from the flooded microcosms before 162 days of incubation. Methane emission was observed first in the presence of the CH₄ oxidation inhibitor, CH₂F₂, suggesting CH₄ emissions were reduced by CH₄ oxidation once production has started. Similarly, the soil slurries started producing CH₄ only after a variable lag period, whereas CO₂ concentrations increased immediately (Fig. 1).

20 The amount of CO₂ in the gas phase of the soil slurries increased with time and with temperature (Figs. 1b and 2b). CO₂ 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 CH₄ production. The rate constants of decomposition of the labile organic matter, obtained from CO₂ accumulation values, increased with temperature from 0.01 d⁻¹
25 (12°C) to 0.21 d⁻¹ (72°C). However, rate constants of decomposition via methanogenesis varied from 0.04 to 0.17 independently of temperature. The half-lives of the la-

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labile organic matter calculated from CO₂ accumulation decreased with increasing temperatures from 120 to 3 days, whereas half-life was 5-13 days when calculated from methanogenesis regardless of the temperature (Fig. 3a). The lag phase of methanogenesis corresponded to the half life of labile organic matter calculated from CO₂ accumulation (Fig. 3b). We were unable to calculate precise decomposition 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 years.

Because of the non-linear increase of CH₄ and CO₂ (Fig. 1), the accumulation during the experiment is given instead of a rate per day in Fig. 2. Two temperature optima for CO₂ 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 CH₄ 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 H₂ (50 ppm_v; Fig. 2c) and acetate (500 μM; Fig. 2f) coincided with a decreasing rate of Fe(II)-accumulation (Fig. 2d) and the onset of CH₄ accumulation, suggesting a transitional phase between Fe-reduction and methanogenesis. The average steady-state concentrations in methanogenic samples were 26 ppm_v H₂ and 60 μM acetate (15–38°C; Fig. 2c and f). The respective concentrations were much lower when Fe-reduction was prevalent at temperatures <14°C, with 2 ppm_v H₂ and acetate below the detection limit. No other intermediate or substrate accumulated up to 40°C. The temperature optimum for CH₄ production was 38°C, where >40 μMol g dry wt⁻¹ CH₄ 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 μMol NH₄⁺ g dry wt⁻¹).

Above 40°C, very small amounts of CH₄ accumulated from the beginning, but reaching not more than 33 nmol CH₄ 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:

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CH₄ accumulated to high values, and alcohols, fatty acids, and H₂ accumulated less. H₂, 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 methanogenesis 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* – 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 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. 6) (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. No PCR-products were retrieved above 55°C. The dominating fragment (185 bp; A_p 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 A_p up to 6 and 30%, respectively. It is suggested that the T-RFs of 185 bp represents members of the *Methanosarcinaceae*, 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;

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Penning and Conrad, 2006). The T-RF of 185 bp may represent a member of RC-VI (Kemnitz et al., 2004). In accordance with acetoclastic methanogenesis prevailing at optimal temperature, 46 *mcrA* clone sequences from a methanogenic slurry were represented by acetoclastic *Methanosarcina* sp. and hydrogenotrophic RC-I at a ratio of 91:9 (46 clones total).

Geobacteraceae-specific 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. 5). 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 ($n=78$) clustered within the gammaproteobacteria: 75% with the *Geobacteraceae*, 23% with *Desulfuromonas*- and *Pelobacter*-Group of iron reducers, and one sequence was affiliated to *Anaeromyxobacter*.

The clones and DGGE bands were grouped into nine groups (Table 1). Most of *Geobacteraceae*-sequences were closely related to *Geobacter chapelleii* (Group II, 24%) and *Pelobacter propionicus* (groups III and VI, 20%). The vast majority of bands were positioned on the gel between 40 and 55% denaturant in the gradient. The three bands positioned at 53–55% denaturant (Group VIII) were affiliated with *Pelobacter actylenicus*. The bands positioned at even higher denaturant concentrations (not shown) belonged to *Aneromyxobacter*. Few bands were present exclusively at high or low temperatures, and most bands were present throughout the temperature range, differing only in relative intensity. At lower temperatures, the most pronounced bands belonged to groups II, III, and IX; bands 01–2 and 12–1 were detected only at low temperatures. Bands belonging to groups V, VI, and VIII were more pronounced between 30 and 40°C, but visible at higher temperatures, too. Some bands belonging to groups I, IV, and V and band 15–1 were clearly apparent only at 30–40°C.

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If methanogenesis had occurred regularly in this soil, it would have recovered quickly after flooding had caused anoxic conditions. However, CH₄ 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 CH₄ production. Iron reduction started immediately after the start of incubation and production of CH₄ was completely suppressed until ~2/3 of iron was reduced.

Iron reducers have a higher affinity for H₂ and acetate than methanogenic archaea, and they can grow at lower H₂ and acetate concentrations (Lovley et al., 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. 5, Table 1). In samples incubated at lower temperatures, the predominating groups were more closely affiliated with acetate oxidizers (*Desulfuromonas acetoxidans* and *Geobacter chapelleii*). Competition for acetate is suggested by the very low acetate concentration in the Fe-reducing samples (Fig. 2d and f). *Methanosaeta* is reported to have an acetate threshold of 20 μM, with 200–500 μM 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 evi-

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dent. H₂ concentration in these samples was ≤ 1 nM, but may have allowed minor CH₄ formation (Figs. 2c and 4) (Karadagli et al., 2007). This agrees with observations in a rice field and in rice soil microcosms, in which only minor residual CH₄ production from H₂/CO₂ occurred during the Fe-reducing phase (Frenzel et al., 1999; Krüger et al., 2002; Lueders et al., 2002). The higher levels of H₂ and acetate in 15°C-samples coincided with the onset of iron reduction and 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 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 fresh-water 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 et al., 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 CH₄ production are able to use acetate corresponding well to the high fraction of acetoclastic methanogenesis. The acetate concentrations of 60 μ M 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 (Leyboet al., 2006). A minor fraction of methanogenic archaea in our samples were affiliated with RC-I. Members of RC-I are hydrogenotrophic and more active at low H₂ concentrations (Lu et al., 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 (Conrad 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, CO₂ and CH₄, 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, 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 CH₄ 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 the production of CH₄ 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. Hence, intermittent drainage events, preferably during the warm season, may be the most promising strategy mitigating CH₄ emissions. In addition, the microcosm-experiment has shown that CH₄ may be oxidized by methanotrophs be-

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fore it can escape into the atmosphere. Overall, our results suggest that restoration strategies proposed for Ljubljana marsh will not necessarily increase CH₄ emission.

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Table 1. Affiliation of sequences from clones and DGGE bands (Fig. 5) with cultivated *Geobacteraceae*, and with *Desulfuromonas* and *Pelobacter* species.

Band and clone group designation	No. of clones and/or bands	Closest cultured relative, GenBank accession no.
Group I	6	<i>Geobacter psychophilus</i> , AY653549
Group II	19	<i>Geobacter chapelleii</i> , U41561
Group III	6	<i>Geobacter propionicus</i> , CP000482
Group IV	4	<i>Geobacter humireducens</i> , AY187306
Group V	4	<i>Geobacter pelophilus</i> , U96918
Group VI	9	<i>Pelobacter propionicus</i> , CP000482
Group VII	5	<i>Geobacter pickeringii</i> , DQ145535
Group VIII	7	<i>Pelobacter acetylenicus</i> , X70955
Group IX	11	<i>Desulfuromonas acetoxidans</i> , M26634
Band 01–2	1	<i>Geobacter humireducens</i> , AY187306
Band 12–1	1	<i>Geobacter argillaceus</i> , DQ145534
Band 15–1	1	<i>Geobacter hephaestius</i> , AY737507

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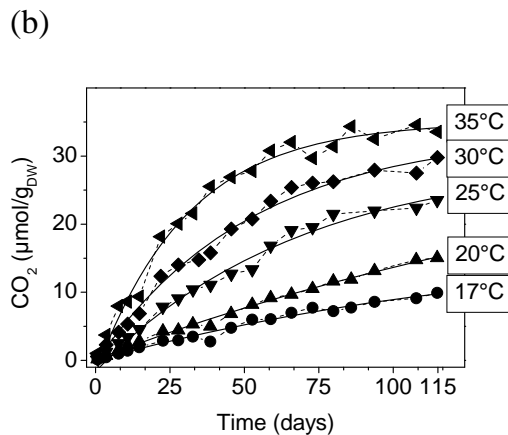
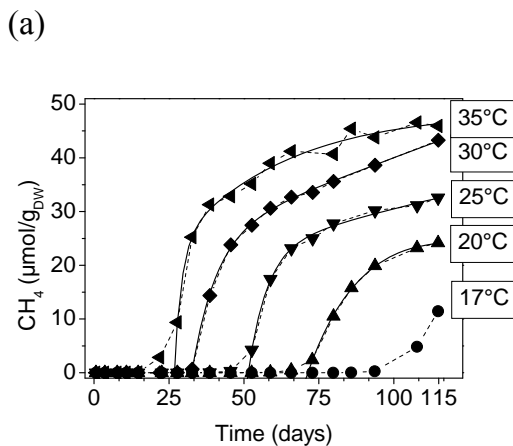


Fig. 1. Production of CH_4 (a) and CO_2 (b) over time in anoxic soil slurries at five temperatures. Solid lines were fitted by exponential decay models; for details see text.

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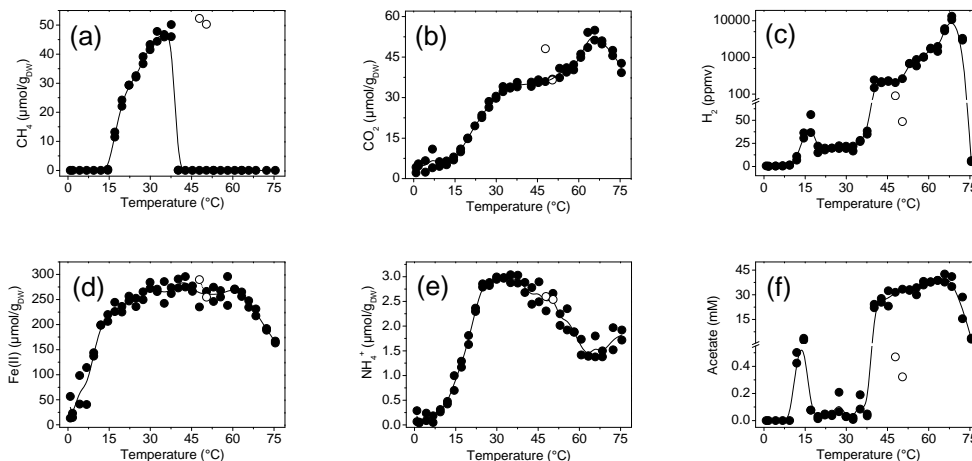


Fig. 2. Accumulation of CH₄ (a), CO₂ (b), and H₂ (c) in the gas phase and concentrations of Fe(II) (d), NH₄⁺ (e) and acetate (f) in pore water of anoxic soil slurries after incubation for 115 days. Open circles: see text for explanation. Solid lines connect the averages of the two replicates. Note the logarithmic scale in C.

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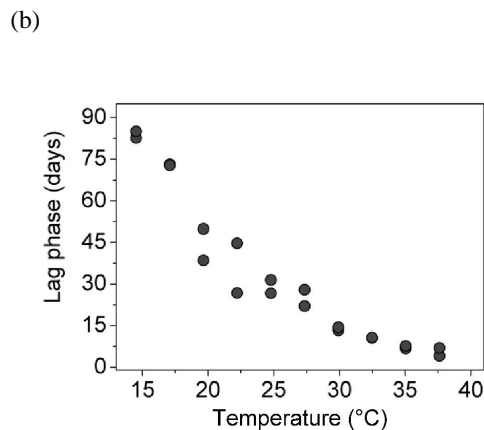
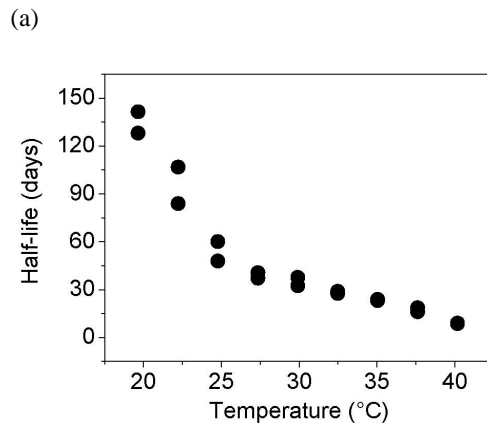


Fig. 3. (a) Half-life of labile soil organic matter calculated from CO₂ production in anoxic soil slurries incubated at different temperatures. (b) Lag phase for CH₄ production in anoxic soil slurries incubated at different temperatures.

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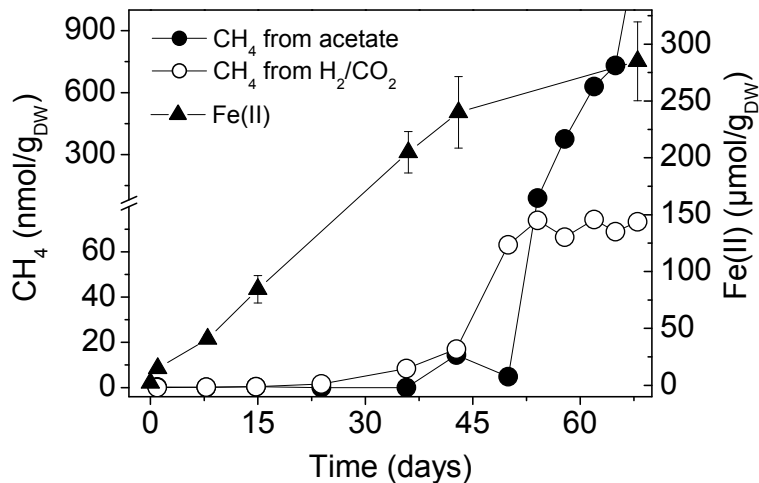


Fig. 4. Methanogenic pathways and iron reduction at 25°C. The values of CH₄ produced from either H₂/CO₂ or acetate were calculated from averages ($n=2-3$) of CH₄ concentrations measured in the presence (methanogenesis from H₂/CO₂ only) and in the absence of methyl fluoride. Fe(II): average \pm SE, $n=6$.

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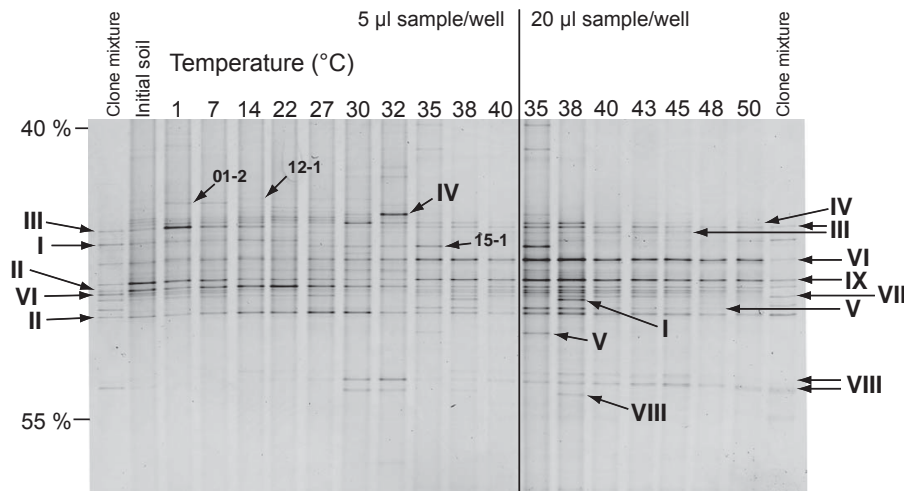


Fig. 5. *Geobacteraceae*: DGGE-profiles of 16S rRNA gene fragments from the original soil and after incubation at different temperatures. Individual bands that yielded sequence data are labelled with Group numbers (Roman numerals) or band designations (see Table 1). The denaturant gradient is indicated to the left in %.

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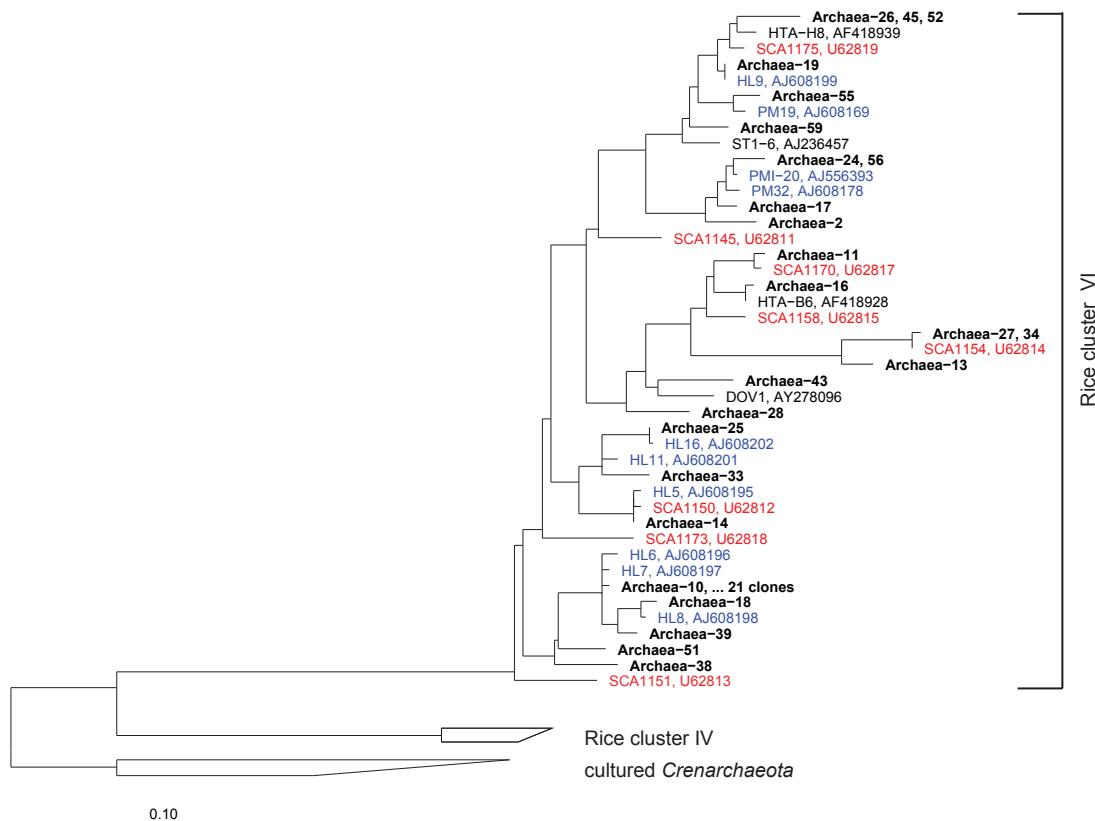


Fig. 6. *Crenarchaeota*, rice cluster VI: neighbour-joining tree of 16S rRNA gene sequences. Clones retrieved from the original soil (black), from terrestrial (red, Bintrim et al., 1997) (red) and freshwater sites (blue) (Kemnitz et al., 2004). GenBank accession numbers of the sequences are indicated. Scale bar: estimated number of base changes per nucleotide position.

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Fig. 7. Methanogenic *Archaea*: neighbour-joining tree of *mcrA*-sequences. Sequences retrieved after incubation at 35°C for 115 days are printed in bold. Scale bar: estimated number of changes per amino acid position.

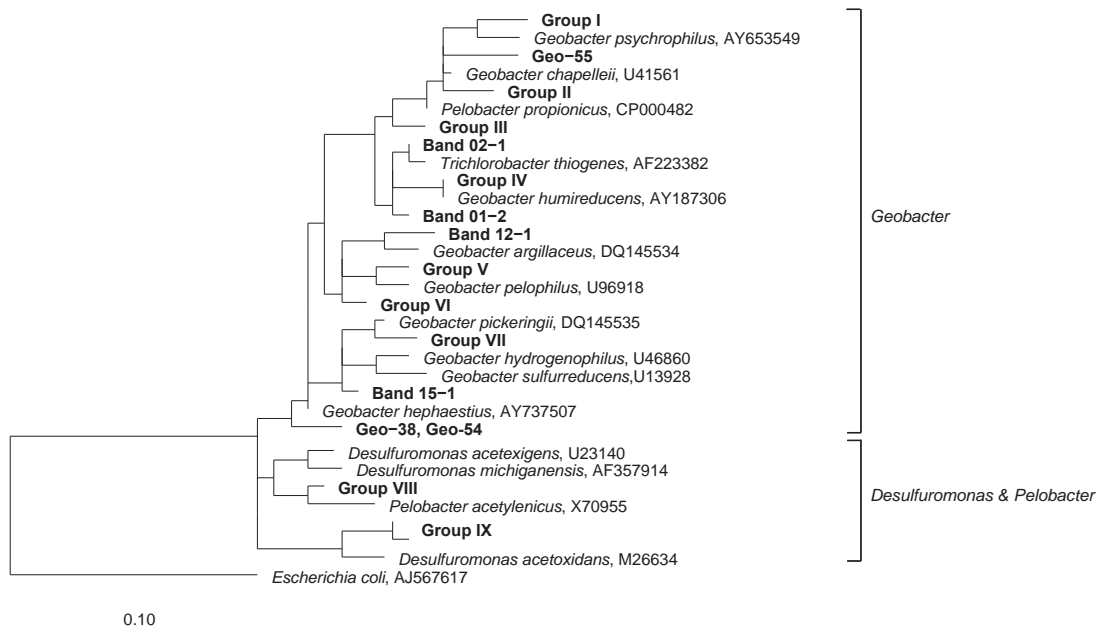


Fig. 8. *Geobacteraceae*: neighbour-joining tree of 16S rRNA gene sequences retrieved from the original soil, or recovered from DGGE bands (Fig. 5). A total of 313 base positions was considered. Scale bar: estimated number of base changes per nucleotide position.

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