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Two temperature optima of methane production in a typical soil of the Elbe river marshland

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Abstract

The soil of the Elbe river marshland was classified as calcareous freshwater marsh (USDA: typic fluvaquent). Methanogenic bacteria were found in oxic as well as in anoxic soil layers. Most probable number counts varied from 1.4×10^2 to 2.5×10^6 bacteria g^{-1} fresh weight. Methane production at different temperatures (5°C, 10°C, 15°C, 20°C) was studied with undisturbed soil cores. The highest methane production rates were measured at 10°C (0.31 nmol h^{-1} (g d.w.)$^{-1}$) and 20°C (0.58 nmol h^{-1} (g d.w.)$^{-1}$). Incubation experiments with disturbed soil material showed two optima of methane production at 10°C and in the mesophilic temperature range. Experiments with different substrates indicate that at 10°C acetate serves as substrate while at higher temperatures hydrogen is the main energy source for methanogens.

Keywords: Methane production; Most probable number counts; Temperature; Acetate; Hydrogen; Marsh soil; Soil core

1. Introduction

Methane is one of the most important climate relevant trace gases. Compared to carbon dioxide, methane is much more effective at absorbing infrared radiation from the earth's surface [1]. The concentration of methane in the troposphere has increased from 0.7 ppmv before industrial development to 1.7 ppmv at present [2]. Methane is increasing at an average global atmospheric concentration of about 1% per year [1,3]. Eighty to ninety percent of total methane emission originates from microbial processes [4].

In the past production and emission of methane from hydromorphic terrestrial ecosystems was investigated mainly in paddy fields [5–7]. In the northern hemisphere natural wetlands (marsh- and peatlands) are the most important sources of atmospheric methane. The world-wide wetland area has a size of 5.7×10^6 km^2, which is four times more than the global rice area under cultivation [8]. For marshlands great variations of methane emission between 0.0005 mg CH_4 m^{-2} day^{-1} to 500 mg CH_4 m^{-2} day^{-1} have been reported [9–11]. Little is known about how soil characteristics (soil texture, redox potential, temperature and others) influence the activity of methanogenic bacteria in terrestrial ecosystems [12–14].

One important factor for the microbial production of methane in soil is the temperature. Deep lake sediments have more or less constant temperature

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conditions compared with marshes and peats which are influenced more strongly by diurnal and seasonal temperature changes.

Within a certain range microbial activity increases with temperature [15]. Zeikus and Winfrey [16] showed that the optimal temperature range for methanogenesis in lake sediments was between 35°C and 42°C, which was considerably higher than the maximum temperature observed in situ. Dunfield et al. [17] found a temperature optimum for methane production at 25°C in samples of peat soils. They also showed that methane production was much more temperature-dependent (Q_{10} values 5.3–16) than the process of methane consumption (Q_{10} values 1.4–2.1). These results indicate that the microbial communities which are involved in the methane cycle are not well adapted to low temperatures. Westermann [18] studied the effect of incubation temperature on steady-state concentrations of different methanogenic substrates with slurries of two different swamps. His results, as well as the publications mentioned above, confirm that the methane production rates in a wide range increase linearly with increasing temperature.

Besides temperature methanogenic activity is influenced by the availability of substrate. The methanogenic bacteria utilize acetate, formate, methanol, ethanol, isopropanol, methylamines, and hydrogen as energy sources [19]. These substrates are produced during decomposition of organic matter by fermentative bacteria involved in the anaerobic foodchain. Lovley and Klug [20] hypothesized that fermentation and methanogenesis are the dominant processes in carbon flow under anaerobic conditions. Acetate and hydrogen are the most important methanogenic substrates in terrestrial ecosystems [21,22]. The availability of these substrates for methanogenic bacteria depends on the content of soil organic matter and on the activity of fermentative bacteria. Little is known about the adaptation of fermentative bacteria to low temperatures and the availability of methanogenic substrates at different temperatures [23,24]. Kelly and Chynoweth [25] found a correlation between methane production and the input of organic matter at constant temperatures for two small lakes in Michigan. At changing temperature conditions the effect of organic matter on methane production is overlaid with the influence of the temperature.

All studies on methane production in terrestrial ecosystems mentioned above used disturbed sample material, although the complex natural soil structure should have an influence on the anoxic microbial community and their activity.

The purpose of this study was to investigate the temperature dependence on methane production of a natural freshwater marshland soil. Undisturbed soil cores and homogenized soil material were used.

2. Materials and methods

2.1. Investigation area

For our studies we used soil samples which were taken from the Elbe river marshland near Hamburg, Northern Germany. The location, which is called 'Asseler Sand', represents a typical freshwater marshland soil (USDA: typic fluvaquent). The soil was characterized by two different zones. The oxic layer reached to a depth of 40 cm. Beneath this zone the soil was permanently anoxic because of water saturation. Further details on the properties of the freshwater marsh soil at Asseler Sand will be given by Pfeiffer (in preparation).

2.2. Methane production in undisturbed soil cores

Undisturbed soil cores were taken with a steel corer. Sampling artefacts were minimized by using cylinders with a large diameter (11.4 cm), which were driven vertically into the anoxic soil layer (40–70 cm depth). Immediately after sampling and retrieval, the cylinders were closed with gastight seals at both ends. The top had two ports with ball taps (Fig. 1). One port was used to exchange the headspace atmosphere with argon to ensure anoxic conditions after sampling and to sample the produced gases in gastight bags (Linde, plastigas bag). At the end of the experiment the gas volume produced was determined. The other port had a screw cap with septum used as a syringe port. Gas samples were taken with a gastight syringe to analyse the headspace concentration of methane by gas chromatography.

Three cores were incubated under anaerobic conditions at 5°C, 10°C, 15°C and 20°C to determine

methane production rates. The headspace atmosphere of the 20°C cores after 112 days was exchanged with argon.

2.3. Methane production in homogenized soil material

The fresh soil material which originated from a soil depth of 60–70 cm was successively sieved (8, 4 and 2 mm mesh sieve). Samples of soil (30 g fresh weight) were put into serum bottles (120 ml) and each was supplied with 5 ml sterile and anoxic tap water and with 0.1 g organic matter (air-dried and ground roots of *Phragmites australis*). The serum bottles were closed with black rubber stoppers and flushed with N_2/CO_2 (80:20 v/v).

Methane production was determined at 5°C, 10°C, 15°C, 22°C, 28°C and 32°C. Before gas samples were taken the bottles were shaken vigorously for 30 s to equilibrate the methane concentration between the liquid and gas phases. Fourteen replicates were incubated for each temperature. Methane production rates were calculated from the linear increase of the methane concentration during the first 6 days.

2.4. Methane production potentials with different substrates

Methane production was determined with H_2/CO_2, acetate and glucose as substrates. The material used originated from a soil depth of 40–45 cm. Acetate and glucose were supplied as solutions (10 mM) and H_2/CO_2 as gas (80:20 v/v, pressurized 150 kPa).

Samples of soil (15 g fresh weight) were supplied with 6 ml of the nutrient solution or with sterile and anoxic tap water (control). The serum bottles (25 ml) were closed with black rubber stoppers, flushed with N_2 or H_2/CO_2, and shaken at 10°C or 20°C. Three replicates were incubated for each substrate and temperature.

2.5. MPN counts

The number of methanogenic bacteria was determined in a three-tube most probable number (MPN) analysis using a 10-fold serial dilution of soil in growth medium. The mineral medium had the following composition: NH_4Cl (18.9 mM), $MgCl_2 \cdot 6H_2O$ (5 mM), $CaCl_2 \cdot 2H_2O$ (2.7 mM) and $K_2HPO_4 \cdot 3H_2O$ (1.76 mM). The medium was carbonate-buffered (pH 6.8–7.0) and also supplemented with yeast extract (0.2%), resazurin (1 mg l^{-1}) as redox indicator, cysteine hydrochloride (0.5 g l^{-1}) and sulfide (1 mM) as reducing agent, trace element solution [26] and vitamin solution [27].

The MPN method was applied to count the cell numbers in two different experiments.

(i) At first we determined the cell numbers in the vertical profile to a depth of 150 cm. As energy and carbon sources acetate (20 mM) and H_2/CO_2 (80:20 v/v, pressurized 150 kPa) were used. The bottles were incubated at 28°C for 30 days in darkness.

(ii) Secondly, the cell numbers were determined in the presence of different substrates at 10°C and 28°C. Either acetate, formate (20 mM) or H_2/CO_2 (80:20 v/v, pressurized 150 kPa) was supplied. The material used originated from the anoxic soil layer (60–70 cm deep). The numbers of methanogens were determined four times for each substrate and each temperature. The bottles were incubated at 10°C for 90 days and at 28°C for 30 days.

All preparation steps were done under strictly anaerobic conditions. Each bottle was analysed for CH_4 production by gas chromatography. Bottles were counted as positive if the headspace concentration of methane was higher than 30 ppmv. The MPN index was calculated by means of MPN tables [28].

2.6. Methane analysis

Methane concentrations were determined with a gas chromatograph (United Technologies Packard 437A) equipped with a Porapak-Q stainless steel column (80/100 mesh) connected to a flame ionization detector. The injector temperature was set at 40°C, the column at 35°C and the detector at 140°C. Nitrogen was used as carrier gas.

2.7. Statistical analysis

Significant differences between all data populations being the results of the experiments described in Section 2.3Section 2.5 were analysed by the Kruskal-Wallis one-way analysis of variance. In the case of comparing two data populations the nonparametric *U*-test according to Mann-Whitney was used [29].

3. Results

3.1. Vertical profile of cell numbers

The vertical profile of cell numbers showed the distribution of methanogens within the investigated marshland soil.

Methanogenic bacteria were found in oxic (Go horizon) as well as in anoxic (Gr horizon) soil layers (Fig. 2). The cell numbers ranged from 1.4×10^2 to 2.5×10^6 cells g^{-1} soil. Low numbers in the range of 10^2–10^3 were found in soil depths between 25 and 35 cm and 50 and 55 cm, mostly in sandy sedimentation layers. The average number of methanogens in the upper 70 cm of soil amounted to 3.4×10^4 cells (g soil)$^{-1}$. The average cell number from a soil depth of 70–150 cm was about 15 times higher (9.8×10^5 cell (g soil)$^{-1}$).

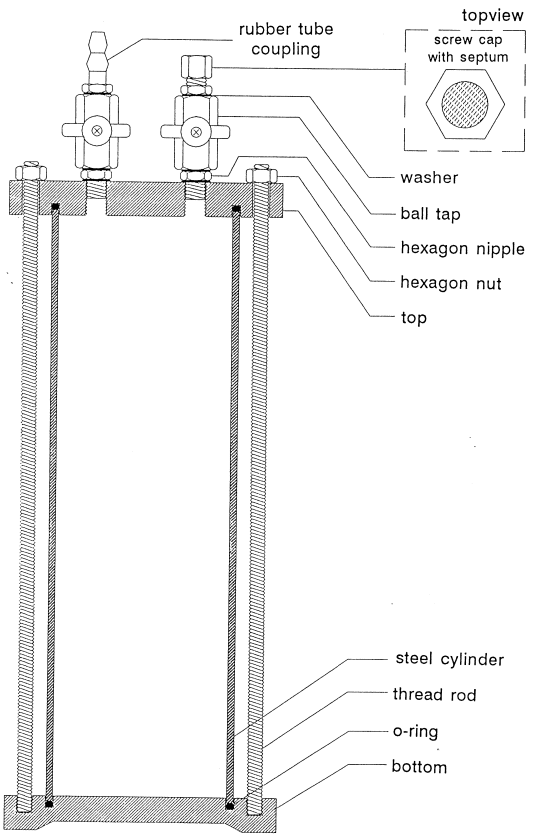

Fig. 1. Construction of the steel corer. The system was used for sampling of undisturbed soil cores and for the measurement of methane production under anaerobic conditions at different temperatures. The original height was 35 cm.

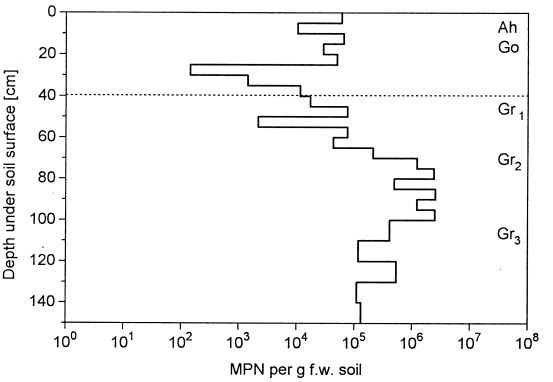

Fig. 2. Vertical profile of cell numbers of methanogenic bacteria. MPN counts were determined in fresh marshland soil (f.w. = fresh weight). The incubation was done at 37°C with acetate (20 mM) under an atmosphere of H_2/CO_2 (80:20 v/v, pressurized 150 kPa). The 95% confidence limits amounted to log 0.670 in a three-tube MPN analysis. Ah = top soil accumulated with organic matter, Go = oxidized groundwater horizon, Gr = groundwater horizon with strong reduction features.

For the Gr horizons there were two different domains to be distinguished. The first was a former surface mineral horizon at a soil depth of 70–100 cm with well mixed humified organic matter (fAhGr). In this zone the highest numbers of methanogens were found (1.7×10^6 cells (g soil)$^{-1}$). In the second zone, at 100–150 cm, cell numbers were about 10 times lower than at 70–100 cm depth.

3.2. Cell numbers recovered on different substrates

The investigation of cell numbers recovered on hydrogen, acetate, and formate showed the differences of methanogenic bacteria due to the substrate for the two determined temperature optima.

At 10°C the cell numbers varied between 3.1×10^2 cells g^{-1} soil with hydrogen and 1.9×10^5 cells g^{-1} soil with formate as substrate (Fig. 3). Within this temperature level the Kruskal-Wallis test showed highly significant differences ($P = 0.0096$) for the MPN counts determined with different substrates. According to the Mann-Whitney test the numbers of methanogens grown with formate showed significant differences relative to the cell numbers of methanogens grown with hydrogen ($P = 0.014$) and acetate ($P = 0.029$).

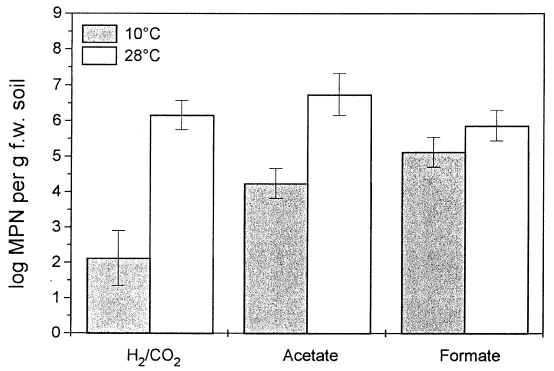

Fig. 3. Cell numbers of methanogenic bacteria. MPN counts were determined in fresh marshland soil (f.w. = fresh weight) with H_2/CO_2 (80:20 v/v, pressurized 150 kPa), acetate or formate (20 mM) at 10°C and 28°C (means ± standard errors, $n = 4$). The samples used originated from a soil depth of 60–70 cm. MPN counts at 10°C and 28°C showed highly significant differences for each substrate (P values for hydrogen = 0.014, acetate = 0.014 and formate = 0.043).

At 28°C the cell numbers varied between 1.0×10^6 and 9.7×10^6 cells g^{-1} soil. In contrast to 10°C there were no significant differences ($P = 0.1148$) within the 28°C temperature group.

The comparison of cell numbers at 10°C with cell numbers at 28°C carried out with the Mann-Whitney test furthermore showed highly significant differences for each substrate (P values for hydrogen = 0.014, acetate = 0.014 and formate = 0.043).

3.3. Methane production

The influence of different temperatures on methane production was investigated using undisturbed soil cores as well as homogenized soil material. The results of both experiments indicated the existence of a low temperature optimum of methanogenesis for the investigated marshland soil.

The undisturbed soil cores showed high variances of methane production rates at each temperature. Thus it was not possible to carry out the necessary statistical calculations. But the natural soil structure and the physico-chemical conditions were represented much better in these experiments, where methane production corresponded better to the in situ conditions of the investigation site. On the other hand, the homogenized soil material resulted in a smaller variance of methane measurements and more replicates could be obtained. Therefore it was possible to confirm the result of the 10°C temperature optimum by statistical analysis.

The soil cores, incubated at 10°C, 15°C and 20°C, showed nearly the same progress of methane production (Fig. 4). Two different phases could be distinguished. The first phase (initial phase) showed a lower methane production rate than the second phase (final phase). The latter was characterized by an abrupt increase in methane production. At the low temperature the initial phase was extended. At 20°C it lasted 8 days, at 10°C 19 days. At the end of the experiment the headspace of all cores was almost saturated with concentrations of about 50% CH_4.

As the cores of 5°C showed a low methane production during the first 97 days the experiment was extended to 240 days. After this time the progress of methane production was similar to that at the three other temperatures (results are not shown). A greater increase in methane concentration (beginning of the final phase) could be observed after 118 days. At the

Table 1
Production rates of methane in undisturbed soil cores during the initial and final phases of incubation at different temperatures as well as produced gas volumes and concentrations of methane at the end of the experiment

	5°C	10°C	15°C	20°C	20°C after exchanging headspace atmosphere
CH_4 production rate 'initial phase' (nmol h^{-1} (g d.w.)$^{-1}$)	0.08 ± 0.01	0.31 ± 0.03	0.19 ± 0.02	0.58 ± 0.06	no initial phase
CH_4 production rate 'final phase' (nmol h^{-1} (g d.w.)$^{-1}$)	2.55 ± 0.06	19.89 ± 0.92	14.70 ± 0.35	23.54 ± 1.85	7.01 ± 0.63
CH_4 (%)	47	53	55	56	29
Gas volume (ml)	360	1250	928	1573	560

Means ± standard error, $n = 3$.

end of the experiment the headspace methane concentration reached a value of about 50%.

Production rates of methane during the initial and final phases as well as gas volumes and concentrations of gas produced in the soil cores are shown in Table 1. At 5°C the smallest amount of methane was produced. At 10°C more methane was produced than at 15°C and the most methane was found at 20°C.

The highest methane production rate with 0.58 nmol h^{-1} (g d.w.)$^{-1}$ was determined at 20°C. This value was about two times higher than at 10°C. The methane production rates during the final phases at all temperatures were several times higher than during the initial phase. This result indicates that the conditions in the soil cores compared to in situ conditions changed during the experiment.

After exchanging the headspace atmosphere of the 20°C cores (after 112 days) with argon, methane production continued without an initial phase (Fig. 5). But the methane production rate with 7.01 nmol h^{-1} (g d.w.)$^{-1}$ was 3.5 times lower than before the exchange of headspace gas.

The results of the experiment with homogenized soil material were similar to the results of the investigation with undisturbed soil cores (Table 2). There was one temperature optimum at 10°C and another in the mesophilic temperature range. At 10°C the methane production rate was 3.4 times higher than at 5°C and 1.6 times higher than at 15°C.

As it was possible to handle 14 parallels for each temperature, we were able to confirm the low temperature optimum by statistical analysis. The Kruskal-Wallis test showed significant differences between 5°C, 10°C and 15°C at the $P < 0.01$ level. The Mann-Whitney test showed highly significant differences in the comparison between 5°C and 10°C ($P = 7.45 \times 10^{-6}$) as well as between 10°C and 15°C ($P = 7.48 \times 10^{-6}$). In addition the parametric t-test according to Kolmogorov-Smirnov was permissible for the comparison between 10°C and 15°C. This test also showed highly significant differences for the data populations at the $P < 0.01$ level.

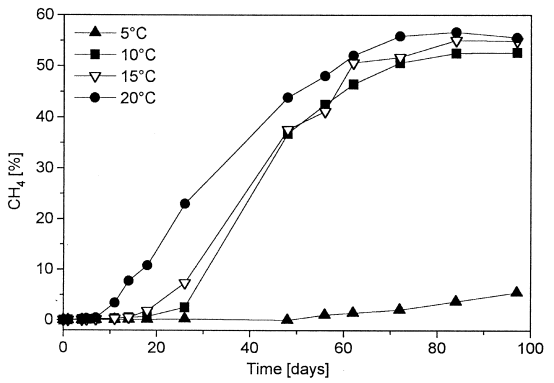

Fig. 4. Progress of methane production in undisturbed soil cores during anaerobic incubation at different temperatures for 97 days. The methane concentration was analysed in the headspace atmosphere of the soil cores.

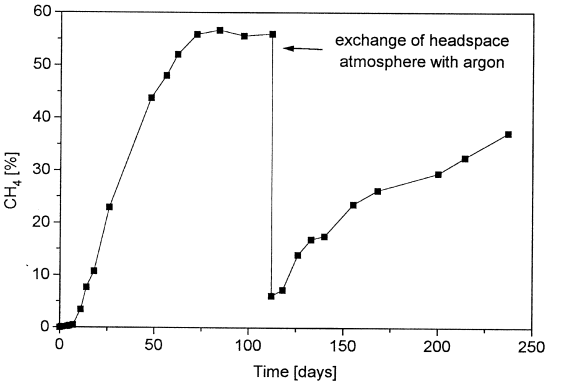

Fig. 5. Progress of methane production in undisturbed soil cores during anaerobic incubation at 20°C. The headspace atmosphere was exchanged with argon after 112 days.

3.4. *Influence of different substrates on methane production*

The influence of different substrates on methane production was determined with selected soil samples

Table 2
Methane production rates of homogenized soil samples at different temperatures

Temperature (°C)	CH_4 production rate (nmol h^{-1} g^{-1} f.w.)
5	3.08 ± 0.25
10	10.51 ± 0.96
15	6.48 ± 0.77
22	27.20 ± 3.14
28	30.84 ± 4.56
32	42.71 ± 4.84

The samples were supplied with 0.1 g organic matter (air-dried and ground roots of *Phragmites australis*). Means ± standard error, $n = 14$.

at 10°C and 20°C. Acetate and H_2/CO_2 can be used directly by the methanogens, while glucose has to be converted to methanogenic substrates by fermentative bacteria. The results showed the significance of various substrates at different temperatures.

With acetate as additional substrate the methane production rates at 10°C amounted to 0.166 nmol h^{-1} (g d.w.)$^{-1}$ This was two times higher than with H_2/CO_2 or glucose (Table 3). At 20°C the highest methane production rate was found with H_2/CO_2 (44.15 nmol h^{-1} (g d.w.)$^{-1}$), while the lowest rate was found with acetate (15.89 nmol h^{-1} (g d.w.)$^{-1}$) as substrate. Controls without any additional substrate showed a very low methane production rate compared to the samples supplied with substrate. Their rates were in the range of the natural methanogenic activity.

4. Discussion

Methanogenic bacteria were found within the whole profile of the freshwater marsh soil. Even in the upper oxic layer (Go horizon) about 10^4 cells (g soil)$^{-1}$ of acetotrophic and hydrogenotrophic methanogens were found. These values were only 10 times lower than in the anoxic soil layers (Gr horizon).

This is surprising because the methanogenic bacteria are strictly anaerobic organisms without the ability to form cysts or spores [30]. In pure culture they die within a few hours after aeration [31]. On the other hand Mayer and Conrad [14] showed that the cell numbers of methanogenic bacteria (10^4–10^6 (g d.w.)$^{-1}$) in paddy soils were nearly the same during oxic or anoxic conditions.

This fact is in accordance with our result, namely that in situ methanogenic bacteria survive quite well under aerobic conditions. The reasons are not yet understood. However, the ability of methanogens from the investigated marsh soil to survive in the presence of oxygen is of ecological significance for the methane emission rate of marsh soils. Moore and Roulet [32] showed that methane production in peatland soils decreased in relation to a lowered water table. Their results indicated that changing groundwater levels determined the proportion of methane production and consumption in hydromorphic soils. Besides methane production the oxidation of methane in the oxic layer has a considerable effect on the total amount of methane emitted [33]. A higher groundwater table caused less methane oxidation presumably due to associated changes in oxygen gradients [34]. It seems to be possible that the activity of methanogenic bacteria in the soil layer with changing oxygen conditions leads to additional methane production in the investigated marsh soil and thus to a higher methane emission if the groundwater table is higher.

Table 3
Methane production at 10°C and 20°C with different substrates compared to a control without any substrate

Substrate	CH_4 production rate (nmol h^{-1} (g d.w.)$^{-1}$)	
	10°C	20°C
Acetate	0.166 ± 0.077	15.89 ± 6.51
H_2/CO_2	0.085 ± 0.015	44.15 ± 0.64
Glucose	0.088 ± 0.035	31.72 ± 12.50
Control without substrate	0.014 ± 0.001	0.099 ± 0.04

Means ± standard error, $n = 3$.

Most methanogenic bacteria are metabolically active between 4°C and 45°C. Koyama [35] showed that methane production increases with temperatures up to 40°C.

The results presented show that the increase of methane production with temperature is not linear. Two temperature optima in methane production at 10°C and in the mesophilic temperature range could be demonstrated. The 10°C temperature optimum showed highly significant differences with 5°C and 15°C.

We conclude that the two temperature optima indicate the existence of two different methanogenic communities in the investigated freshwater marsh soil. This suggestion is supported by the fact that the methane production rate at 10°C was much higher with acetate than with H_2/CO_2 or glucose. Glucose, lactate and ethanol [36] stimulate the methanogens which are connected with the H_2-producing bacteria. One population is adapted to the average annual temperature at a soil depth of 50 cm, which is about 9°C, and uses acetate as energy source. The other methanogenic population uses hydrogen which is produced to a greater extent at higher temperatures.

This interpretation is in accordance with the re-

sults of Svensson [37], who reported two different communities of methanogenic bacteria in peat. The first group used acetate as substrate with a temperature optimum of 20°C and the other oxidized hydrogen at a temperature optimum of 28°C.

Our results emphasize that using undisturbed soil cores leads to better results in comparison to experiments with disturbed soil material when the influence of ecological parameters on methane production is investigated. Especially gas flux and the availability of dissolved nutrients are influenced by the natural pore system and thus have certain effects on the composition and activity of the microbial population. However, for the procedure of statistical analysis of the results it is necessary to use homogenized soil material, because this kind of soil sample showed little variance.

As the investigation of cell numbers with various substrates showed significant differences between 10°C and 28°C, we propose that the availability of different substrates at lower and higher temperatures is the reason for the existence of two different methanogenic populations. At 28°C the cell numbers with hydrogen, formate and acetate were nearly the same. Surprisingly, the cell numbers at 10°C were significantly lower with hydrogen in comparison to formate or acetate, although most of the hydrogenotrophic methanogens are able to use formate.

Our results showed that at 10°C a community of methanogenic bacteria existed which was obviously well adapted to low temperatures. These methanogens grew very well with formate or acetate but showed a reduced ability to use hydrogen as substrate. Conrad et al. [38] showed that in paddy soils the H_2-producing bacteria were more inhibited at low temperatures than the methanogenic bacteria. Furthermore homoacetogenesis at low temperatures is a dominant process in anoxic ecosystems such as lake sediments and cold climate soils [39,40]. Above this, in sediments and paddy soils acetate is the main substrate for methanogenesis with a proportion of about 80% [41,42].

We conclude that there is an adaptation of methanogens from the marshland area governed by the availability of the substrates, which are low molecular organic acids like acetate and formate at low temperatures. At higher temperatures hydrogen serves as the main substrate for methanogens. To confirm the results experiments are in progress to isolate methanogens at different temperatures and with different substrates.

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