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Methane production in aerated marshland and model soils: effects of microflora and soil texture

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Abstract

Under oxic conditions the importance of the indigenous microflora and the soil texture on methane production was investigated using marshland soils (clayey silt) and different textured model soils (clay, sand, gravel). Under an oxic partial pressure $>2.5\%$ O_2, soil slurries had a low methane production rate over a period of 50–70 h with acetate (0.35 ± 0.1 nmol g^{-1} h^{-1}) as well as with hydrogen (2.43 ± 0.51 nmol g^{-1} h^{-1}) as substrate. The rates amounted to 1 and 5%, respectively, compared to methane production rates under anoxic conditions. As soon as the oxygen concentration decreased to 2.5% (microaerophilic conditions) the methane production increased significantly (9.5 ± 3.3 nmol g^{-1} h^{-1} with acetate; 16.3 ± 3.3 nmol g^{-1} h^{-1} with hydrogen). In the absence of the indigenous microflora, the inoculated culture of *Methanosarcina mazei* (DSM 2053) did not produce any methane in soil slurries under oxic conditions. To inhibit methane oxidation, all samples (oxic and anoxic) were supplied with 60 nl acetylene ml^{-1} headspace. Furthermore, methane production in different textured model soils demonstrated that a high amount of negative surface charges increased methane production under oxic as well as under anoxic conditions. Consequently the methane production rates increased in the following sequence: sand < gravel < clayey silt (marshland soil) $\leq$ clay. Our results show that the indigenous microflora in combination with the sorptive quality of soil particles (clay, silt, organic matter) enables methanogenic activity in the presence of oxygen, promoting microscale anoxia within the slurries.

1. Introduction

Methanogenesis is one of the most prominent biological processes during the anaerobic decomposition of organic matter. Therefore, natural wetlands (marsh, fen and tundra) are a major global source of atmospheric methane (Aselmann and Crutzen, 1989; Crill et al., 1991; Pfeiffer, 1994; Mitch and Wu, 1995). Changing groundwater tables are typical for wetlands like marshes and thus these soils are characterized by changing oxygen conditions. Methanogenic bacteria are active in these soils if the conditions are anoxic and sufficiently reduced (Moore and Roulet, 1993).

Methanogenic bacteria are regarded as strictly anaerobic organisms without the ability to form spores or cysts. Growth and methane production were observed only under strictly anaerobic conditions (Whitman et al., 1992). However, Kiener and Leisinger (1983) showed that pure cultures of methanogenic bacteria can survive exposure to oxygen for at most 30 h. Strains with high oxygen tolerance were isolated from ecosystems with changing oxygen conditions. Longer periods of contact with oxygen led to a rapid decrease in viability. Fetzer et al. (1993) showed that the methane production potential under anoxic conditions of pure cultures significantly decreased after just 200 min incubation under air.

In contrast to pure cultures, the methanogenic microbial community in natural soils survives well under

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aerobic conditions. Wagner and Pfeiffer (1997) showed that the cell numbers of methanogens in oxic and anoxic soil layers of a typical marshland were similar. According to Mayer and Conrad (1990) the methanogenic population in paddy soils remained constant during dry fallow periods. Even in oxic desert soils, which are not commonly considered as habitats for anaerobic bacteria, low cell numbers of methanogenic bacteria were detected (Peters and Conrad, 1995). A rapid initiation of methane production in air-dried soil samples was reported, when they were incubated under submerged and anaerobic conditions (Mayer and Conrad, 1990).

As the oxygen tolerance of methanogenic bacteria in pure cultures and in natural ecosystems differs significantly their survival in soils may be a function of the biotic and abiotic environment. It is well known that metabolic activity of soil microorganisms is influenced by the nature and composition of soil particles (soil texture, surface charge, porosity) and the diversity of the natural microbial community (Stotzky and Rem, 1966; Marshall, 1975; van Loosdrecht et al., 1990; England et al., 1993; Costerton et al., 1995). The influence of these factors on methane production in the presence of oxygen and especially the contribution of aerobic and facultative anaerobic bacteria in this process so far have not been investigated. We studied methane production in thoroughly aerated slurries (marshland soils and different textured model soils) in the presence, and in the absence, of the indigenous microflora.

2. Materials and methods

2.1. Investigation site

The investigation area 'Asseler Sand' (located at R:^{35}29,86/H:^{59}52,76 according to the Gauß–Krüger system of coordinates) is part of the Elbe river marshland near Hamburg, Northern Germany. The location represents a typical freshwater marshland soil (clayey silt, calcareous typic fluvaquent; Soil Survey Staff, 1992). The soil was characterized by an upper oxic horizon (Go, ranging down to 40 cm), a groundwater horizon (Gor) with varying oxygen conditions (between 40 and 60 cm) and a deeper anoxic horizon (Gr) with strong reduction features. The average groundwater table was about 40 cm. The profile investigated showed a high organic carbon content (2.5%), $CaCO_3$ amounted to 6.2% and pH of 7. Within the methane production zone (Gr horizon) the redox potential was lower than −180 mV and the number of methanogenic bacteria was 1×10^6 cells g^{-1} soil (wet weight ≈ twice dry weight).

2.2. Incubation experiments with marshland soils

Soil samples were taken from the anoxic layer (60–70 cm depth) of the profile. The fresh material was passed through a stainless steel sieve of 2 mm mesh size. Then 50 g of the homogenized soil were weighed into 250-ml Erlenmeyer flasks and mixed with 30 ml sterile and anoxic mineral solution. The flasks were closed with screw caps containing a septum. These samples were used to study:

(i) methane production of marshland soil samples under anoxic and oxic conditions. In the case of anoxic methane production the flasks were flushed with N_2/CO_2 (80:20 v/v). For the determination of methane production under oxic conditions the flasks used in the anoxic experiment were evacuated and flushed with synthetic air containing 20% oxygen. To inhibit methane oxidation, all samples (oxic and anoxic) were supplied with 60 nl acetylene ml^{-1} headspace (Watanabe et al., 1995). Acetate (20 mM) or hydrogen (H_2/CO_2, 80:20 v/v, pressurized 100 kPa) were used as substrate for methanogenic bacteria.

(ii) the role of the indigenous microflora for methane production in the presence of oxygen. Methane production rates in marshland soil samples were compared with sterilized samples inoculated with pure cultures of *Methanosarcina mazei*. The samples were sterilized for 2 h at 121°C. After sterilization each sample was inoculated with 5×10^6 cells ml^{-1} of a pure culture of *M. mazei* and incubated for 14 d anaerobically at 24°C with acetate as substrate. To start the experiment, the samples were flushed with synthetic air or with N_2–CO_2.

Three replicates were used for the anoxic experiments and four replicates for the experiments under oxic conditions. All slurries were shaken continuously at 24°C. Gas samples were taken from the headspace with a gastight syringe and analysed for the concentrations of methane and oxygen by gas chromatography. Methane production rates were calculated from the linear increase in methane concentration.

2.3. Characteristics of model soils

Model soils were prepared from clay (bentonite obtained from Serva, particle size <2 μm), sand (obtained from Merck, 0.3–0.5 mm) and gravel (2.0–6.0 mm). The control was natural marshland subsoil (60–70 cm depth) from the investigation area Asseler Sand with a clayey silt soil texture, treated in the same way as the other materials. The different soil materials had the following characteristics: bentonite clay consisting mainly of smectite, sand that was almost purely

quartz and gravel was composed of approx 40% mica and 40% feldspar (albite, orthoclase). The remaining material consisted mainly of kaolinite and serizite. The clayey silt marshland soil was 17.8% clay, 47.5% silt and 34.8% sand. The composition of the clay fraction was 19% smectite, 47% illite, 20% kaolinite and 14% chlorite. The mineral composition is an important factor, because it determines the cation exchange capacity and thus the ability of attachment of microorganisms. The minerals smectite, kaolinite, mica and serizite especially have an influence on the sorptive activity. The cation exchange capacity increased in the following sequence: sand (9.3 $mmol_c\ kg^{-1}$), gravel (36.7 $mmol_c\ kg^{-1}$), clayey silt marshland soil (238.4 $mmol_c\ kg^{-1}$) and bentonite (593.4 $mmol_c\ kg^{-1}$). The pH-value of the untreated bentonite was slightly alkaline (pH 8.8) whereas all the other materials had a pH-value of pH 6.6. After preparation of the model soils the pH of all materials was near neutral (pH 6.8–6.9). The highest number of chemoorganotrophic bacteria (plate counts) was found in the clayey silt marshland soil ($5.0 \pm 0.6 \times 10^6$ cells g^{-1}) whereas the model soils had cell numbers of 10^5 cells g^{-1} (wet weight).

2.4. Incubation experiments with different textured model soils

Model soils prepared from clay, sand and gravel were saturated with tap water whereas the marshland soil was taken with its natural moisture. The material (160 ml each) was added to 500-ml flasks, closed with a screw cap with septum and sterilized for 2 h at 121°C. The sterile samples were inoculated with 4 g marshland soil and 20 ml medium was added. The carbonate-buffered medium had the following composition: 250 ml mineral soil solution (extracted from 200 g marshland soil), 250 ml dissolved organic carbon solution (extracted from 1000 g marshland soil, about 40 mg DOC l^{-1}), 5 ml trace element solution (Wolin et al., 1963), 10 ml vitamin solution (Bryant et al., 1971) and 490 ml distilled water. The model soils were incubated at 28°C for 12 months in darkness. At the end of the incubation the numbers of chemoorganotrophic bacteria were counted on DEV-gelatine agar (Merck No. 1.10685) and the pH-value of each material was measured (see above).

To start the experiment 50 g of each model soil (30 g of bentonite) were transferred into 250-ml Erlenmeyer flasks and supplied with 30 ml mineral solution. The mineral solution was carbonate-buffered (pH 6.7–6.9) and contained NH_4Cl (18.9 mM), $MgCl_2 \times 6H_2O$ (5 mM) and $CaCl_2 \times 2H_2O$ (2.7 mM). To each sample 1 ml (8×10^7 cells) of *Methanobacterium* strain AS-3 and *Methanosarcina* strain AS-80 was added. The cells were harvested by centrifugation during their exponentially growth phase and resuspended in mineral medium. The samples were incubated for 17 d anaerobically at 28°C on a shaker with acetate (20 mM) and H_2/CO_2 (80:20 v/v, pressurized 100 kPa) as energy and carbon sources.

To determine the methane production under aerobic conditions the model soils prepared as described were evacuated and flushed with synthetic air containing 20% oxygen. The methane oxidation was inhibited with 60 nl acetylene ml^{-1} headspace. As substrate, acetate (20 mM) was supplied again and in addition the headspace was pressurized with hydrogen (80:20 v/v, 100 kPa). For each sample four replicates were incubated on a shaker at 24°C. Gas samples were taken from the headspace with a gastight syringe and analysed for the concentration of methane and oxygen by gas chromatography. Methane production rates were calculated from the linear increase in methane concentration.

2.5. Bacterial cultures

Ms. mazei (Mah, 1980) was obtained from the Deutsche Sammlung von Mikroorganismen (DSM 2053). *Methanobacterium* strain AS-3 and *Methanosarcina* strain AS-80 were isolated from the marshland soil investigated. *Ms. mazei* was grown with acetate (20 mM) and *Ms.* strain AS-80 with methanol (20 mM) under a gas atmosphere of N_2/CO_2 (80:20 v/v). *Mb.* strain AS-3 used H_2/CO_2 (80:20 v/v, pressurized 150 kPa) as substrate. The composition of the mineral medium was described by Wagner and Pfeiffer (1997).

2.6. Gas analysis

Methane and oxygen concentrations were determined with a Carlo Erba (GC 6000 vega series 2) gas chromatograph. The instrument was equipped with a Heyesep D (100/120 mesh, 20 ft) and a Molesieve 5A (60/80 mesh, 7 ft) stainless steel column connected with a switching valve. Methane was analysed by a flame ionization detector (FID) and oxygen by a hot wire detector (HWD). All gas sample analyses were done after calibration with standards of known concentrations of the respective gases. The injector temperature was set at 100°C, the columns at 70°C and the FID at 200°C. The detector temperature of the HWD was 100°C and the filament cell temperature was 180°C. Helium was used as carrier gas.

3. Results

3.1. CH_4 production of marshland soils

Marshland soil samples transferred from anaerobic to aerobic conditions showed two phases of methane

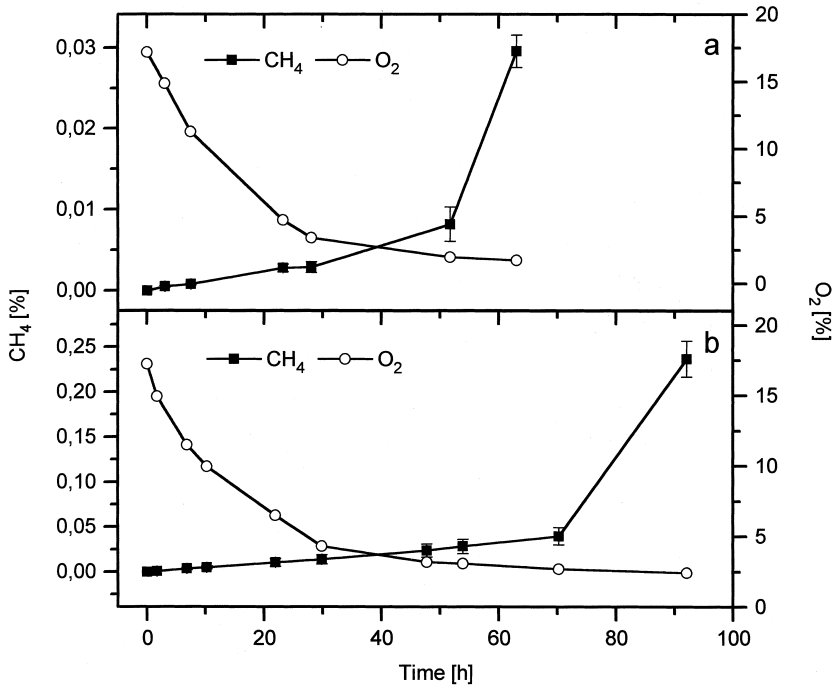

Fig. 1. Methane production of marshland soil after transfer to oxic conditions, (a) with acetate (20 mM) and (b) with H_2/CO_2 (80:20 v/v, pressurized 100 kPa) as substrate. The oxygen concentration was lowered from 18% (oxic conditions) to ≤2.5% (microaerophilic conditions) by the indigenous microflora. The samples were shaken at 24°C. Means ± standard error, $n = 4$.

production (Fig. 1). A constant rate was determined during the first 50 h with acetate as substrate (Fig. 1a). With hydrogen as substrate, the initial phase with linear increase in methane production lasted 70 h (Fig. 1b). During this first oxic phase with both substrates, the oxygen concentration decreased from about 18% to 2.5% (corresponded to 250–35 μM dissolved oxygen). In the second microaerophilic phase as the oxygen concentration decreased to <2.5%, the methane production increased.

The methane production rates in the presence of high oxygen concentrations (first phase) were considerably lower than the methane production of samples kept under strictly anaerobic conditions (Table 1). The rate under completely oxic conditions reached values of 0.35 nmol g^{-1} h^{-1} with acetate while seven times more methane was produced (2.43 nmol g^{-1} h^{-1}) with hydrogen. These rates were equivalent to 1% of the methane production under anoxic conditions with acetate and 5% with hydrogen as substrate.

The methane production rates during the second phase at microaerophilic conditions (<2.5%) with both substrates were higher than during the first phase of the experiment at oxic conditions (18–2.5%). The rates at microaerophilic conditions reached 20% (acetate) and 35% (hydrogen), respectively, compared to samples permanently kept under anaerobic conditions.

3.2. CH_4 production of *Ms. mazei* in sterilized soil samples

The importance of the indigenous microbial community for methane production in aerated samples could be demonstrated with sterilized soil slurries inoculated

Table 1
Methane production rates of marshland soils under anoxic, oxic (>2.5% oxygen) and microaerophilic conditions (≤2.5% oxygen). Means ± standard error

Substrate	CH_4 production rates (nmol g^{-1} dry weight h^{-1})								
	Anoxic conditions	n	r	Oxic conditions (18–2.5%)	n	r	Microaerophilic conditions[a] (≤2.5%)	n	r
Acetate	48.97 ± 6.10	3	0.997	0.35 ± 0.10	4	0.983	9.48 ± 3.28	4	0.959
H_2/CO_2	46.45 ± 3.52	3	0.985	2.43 ± 0.51	4	0.995	16.33 ± 3.29	4	0.998

[a] Methane production rates at low O_2 concentrations after consumption of oxygen by aerobic and facultative anaerobic bacteria. n is the number of replicates and r the correlation coefficient.

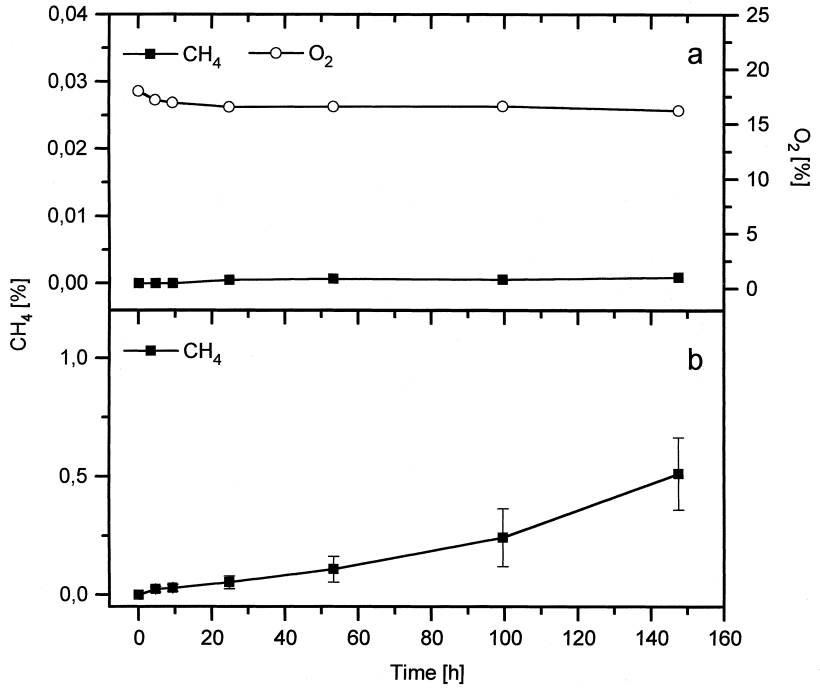

Fig. 2. Methane production by *Methanosarcina mazei* (DSM 2053) in sterilized soil, under (a) oxic and (b) anoxic conditions. Sterilized soil slurries containing acetate (20 mM) were inoculated with 5×10^6 cells ml^{-1} of a pure culture of *Ms. mazei* and shaken at 24°C. Means ± standard error, $n = 3$.

with *Ms. mazei*. Without the indigenous microflora *Ms. mazei* was not able to produce any methane in the presence of oxygen (Fig. 2a). Under anaerobic conditions the inoculated methanogenic culture produced methane with a rate of 14.3 ± 3.5 nmol g^{-1} h^{-1}(Fig. 2b).

3.3. CH_4 production of different textured model soils

The role of soil texture for the activity of methanogenic bacteria under oxic conditions could be demonstrated with model soils characterized by different soil textures. Under anoxic conditions the methane production rates of model soils showed a dependence on the different particle size classes (Fig. 3b). The methane production rates varied between 3.0 ± 1.3 nmol g^{-1} h^{-1} for the model soil made of sand and 15.7 ± 6.4 nmol g^{-1} h^{-1} for the clay material. The rate for the natural soil (clayey silt) was comparable with the model soil made with clay. In the presence of oxygen a relatively high methane production was determined in the marshland soil samples and in the model soils prepared with clay (Fig. 3a). The model soils made with gravel had a very small methane production rate. The experiment with sand showed no methane production. It should be noticed that even with sand oxygen had been consumed by the associated microflora (Fig. 4). Under oxic conditions the rates of clay and of the marshland soil material were in the same range. In the presence of oxygen the methane production rates with clay and marshland soil material reached about 30% of the rates under anoxic conditions. The model soils prepared with gravel reached about 3% of the rate under anoxic conditions.

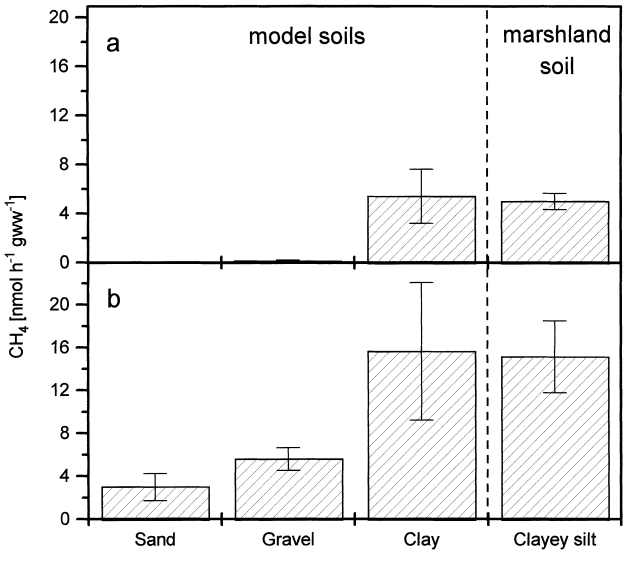

Fig. 3. Methane production rates of different textured model soils inoculated with methanogenic bacteria, under (a) oxic and (b) anoxic conditions (ww means wet weight). Sterilized material had been inoculated with 2% marshland soil to establish the indigenous microflora. To start the experiment, the samples were inoculated with 8×10^6 cells of *Methanobacterium* strain AS-3 and *Methanosarcina* strain AS-80. Acetate (20 mM) and H_2/CO_2 (80:20 v/v, pressurized 100 kPa) were supplied as substrate. Means ± standard error, $n = 3$.

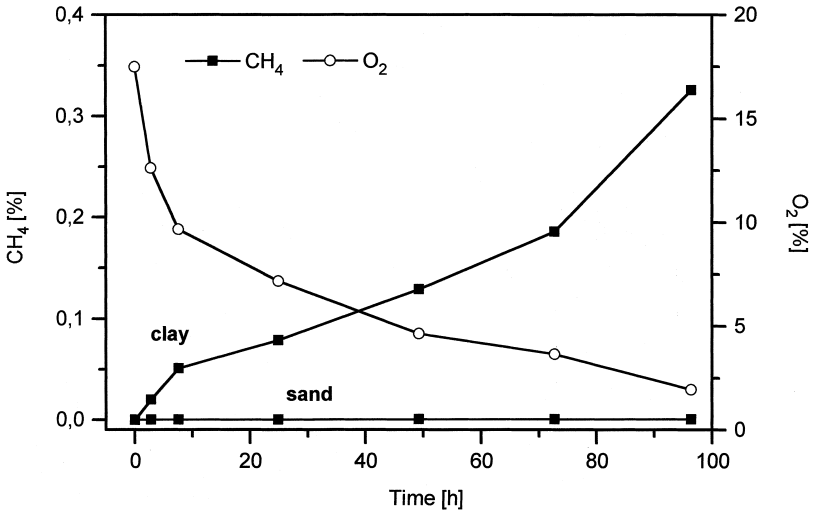

Fig. 4. Oxygen consumption by the indigenous microflora and methane production of inoculated methanogenic bacteria in comparison of the model soils made of sand and clay.

The methane production rates of the model soil made with clay, gravel and sand under oxic and anoxic conditions all showed significant differences ($P < 0.05$) according to the Mann–Whitney test. When the methane production rate of the model soil made with gravel and sand were compared, the significance of the differences were similar.

4. Discussion

Growth and activity of methanogenic bacteria were observed only under strictly anaerobic conditions (Hungate, 1967; Zehnder and Wuhrmann, 1977; Zinder, 1993).

The data presented here demonstrate methane production in the presence of oxygen. Experiments with marshland soil and different textured model soils show that the aerobic and facultative anaerobic microflora in association with soil particles such as clay, enable methane production within aerated soil slurries. Because the soil material was homogenized and shaken vigorously during the experiments, limited diffusion of oxygen into soil aggregates is unlikely.

Metabolic activity of the facultative anaerobes may reduce the oxygen partial pressure and produce substances which might be used by methanogenic bacteria as substrates. Gottschal and Szewzyk (1985) found that 80–90% of all bacteria investigated in an estuarine tidal mud-flat belong to the group of facultatively anaerobic species. Most of these bacteria were able to live under oxygen-limiting conditions by respiration and fermentation at the same time (Wimpenny and Necklen, 1971; Linton et al., 1975). As part of this syntrophic relationship hydrogen, formate and acetate are consumed by the methanogens which benefit the metabolism of the facultative anaerobic bacteria (Thiele and Zeikus, 1988; Stams, 1994). We assume that the close association of methanogenic and facultatively-anaerobic bacteria has a strong effect on the survival and metabolic activity of methanogenic bacteria in oxic–anoxic interfaces. This is of ecological significance because the marshland soil as well as other anaerobic habitats (Jørgensen, 1977; Revsbech et al., 1979; Revsbech and Ward, 1984) are periodically in contact with oxygen.

Previous investigations reported various cocultures of aerobic and anaerobic bacteria, e.g. sulfate-reducing and methanogenic bacteria, grown under oxygen-limiting conditions (Gerritse et al., 1990; Wimpenny and Abdollahi, 1991; Gerritse et al., 1992; Gerritse and Gottschal, 1993). Brune et al. (1995) showed that the hindgut of termites was not as strictly anaerobic as for example the rumen of cattle. The oxic and anoxic processes in termites were combined in cramped surroundings inside the hindgut with a wide spectrum of redox potentials.

The second factor which enabled methane production to occur under oxic conditions was the presence of soil particles. The surface area and the amount of negative charges determined the sorptive activity for microorganisms and nutrients (Stotzky, 1966; Heijnen et al., 1992).

As the cell numbers and oxygen consumption activity of the associated microflora were similar for all materials the different methane production found may be interpreted as a function of the various sorptive quality of the materials. The cation exchange capacity increased in the sequence: sand < gravel < clayey silt (marshland soil) < clay. Similarly, the surface area of the main soil texture increased: sand (particle size < 0.25 mm) = 0.09 $m^2\ g^{-1}$, silt = 0.1–1.0 $m^2\ g^{-1}$

and clay = 5– > 400 m^2 g^{-1} (Koensler, 1989; Heim, 1990). Clay and the marshland soil showed the highest methane production rates in the presence and absence of oxygen. van Loosdrecht et al. (1987a,b) showed that the hydrophobicity and electrophoretic mobility of bacteria could be taken as an indicator for their adhesion properties. Methanogenic bacteria like *Methanosarcina barkeri* as well as other bacteria have a hydrophobic cell surface and a low electrophoretic mobility which supported the attachment of the organisms to the surface of soil particles (Grotenhuis et al., 1992).

The results show that the anaerobic methane producing bacteria survived in the presence of oxygen. Kiener and Leisinger (1983) showed that the arrangement of cells in packets like *Methanosarcina* protected this colony-forming unit during extended periods of oxygen stress. Other investigations have indicated that methanogenic bacteria are potentially viable under oxic conditions at the level of individual cells (Kirby et al., 1981; Kiener et al., 1988; Kengen et al., 1991; Meile et al., 1995). However, in contrast to microaerophilic strains of sulfate-reducing bacteria (Dilling and Cypionka, 1990; Marschall et al., 1993) no investigations which have reported the same physiological potential of methanogenic bacteria.

Our results suggest that a spatial coupling of aerobic respiration and anaerobic methane formation represent a well established part of the methane production process in natural ecosystems influenced by changing oxygen conditions. Only in combination with the sorptive capacity of natural soil particles like clay, silt or soil organic matter can the indigenous microflora develop a protective effect on the methanogenic bacteria against oxygen.

Furthermore, our findings are of great importance for modelling of methane emissions from natural wetlands. Current methane models use the groundwater level as an important forcing parameter for the calculation of methane release (Walter et al., 1996). Our results show significant methane production rates under microaerophilic conditions (CH_4 production rates with acetate = 9.5 nmol g^{-1} h^{-1} and with hydrogen = 16.3 nmol g^{-1} h^{-1}, see Table 1). They indicate that these rates must be included in the calculations of methane emission rates, because the zone of the investigated marshland with microaerophilic conditions (Gor horizon) beneath the groundwater table for most of the year. As shown before, methanogens survive oxic phases (Wagner and Pfeiffer, 1997) and the results presented show that they will also contribute to methane production during oxic periods, even if a significant amount of the methane formed under aerobic conditions is consumed directly by methanotrophic bacteria.

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