Dynamics of Methane Production, Sulfate Reduction, and Denitrification in a Permanently Waterlogged Alder Swamp

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The dynamics of sulfate reduction, methanogenesis, and denitrification were investigated in a permanently waterlogged alder swamp. Molybdate, an inhibitor of sulfate reduction, stimulated methane production in soil slurries, thus suggesting competition for common substrates between sulfate-reducing and methanogenic bacteria. Acetate, hydrogen, and methanol were found to stimulate both sulfate reduction and methanogenesis in soil slurries, while trimethylamine mainly stimulated methanogenesis. Nitrate addition reduced both methane production and sulfate reduction, either as a consequence of competition or poisoning of the bacteria. Sulfate-reducing bacteria were only slightly limited by the availability of electron acceptors, while denitrifying bacteria were seriously limited by low nitrate concentrations. Arrhenius plots of the three processes revealed different responses to temperature changes in the slurry. Methane production was most sensitive to temperature changes, followed by denitrification and sulfate reduction. No significant differences between slope patterns were observed when comparing summer and winter measurements, indicating similar populations regarding temperature responses.

Sulfate reduction, methane production, and denitrification are important processes responsible for the terminal electron removal during decomposition of organic matter in anaerobic environments. Nitrate and sulfate serve as major electron acceptors for denitrifying and sulfate-reducing bacteria, respectively, while carbon dioxide and several simple organic compounds serve as electron acceptors or fermentable substrates for methanogenic bacteria. The competitive domination of sulfate reduction over methanogenesis in sulfate-rich environments has been well established as a consequence of thermodynamic and kinetic differences between the two processes (2, 20, 26).

Only a few investigations of competitive interactions between denitrifying, sulfate-reducing, and methane-producing bacteria have been carried out. Nitrogen oxides were shown to reduce methanogenesis in salt marsh sediments (4), lake sediments (18), and waterlogged soils (6). The effect was explained by substrate competition, redox changes, or enzyme poisoning. Sørensen (31) found that sulfate reduction in marine sediments stopped at the addition of sodium nitrate.

Most investigations of anaerobic metabolism in natural ecosystems have dealt with sulfate-rich marine sediments, where sulfate reduction is the dominating process (2, 27, 32), or eutrophic lake sediments, where most sulfate and nitrate is depleted in the hypolimnion and in the superficial sediment layers, leaving terminal carbon mineralization principally to methane-producing bacteria (20, 21). Investigations of anaerobic mineralization processes in terrestrial ecosystems have mainly focused on rice paddy soils (19, 28), acid peatlands (17, 38), or methane fluxes in relation to the global carbon cycles (3, 10).

In a previous study (37), we examined methane production, sulfate reduction, and denitrification in a permanently waterlogged alder swamp throughout the year. Methanogenesis was found to be the most important process calculated on an areal basis, followed by sulfate reduction, which was slightly lower but within the same order of magnitude. Denitrification constituted only a small percentage of the anaerobic respiration in the swamp. During the course of the year, sulfate and nitrate concentrations in the surface layers of the swamp vary from 0.1 to 1 mM and 10 to 15 μM, respectively. The temperature varies from approximately 0 to 15°C. As a consequence of the large seasonal variations in denitrification, sulfate reduction, and methanogenesis in the alder swamp, the questions of if the three processes are competitively related and how environmental factors influence the metabolic activity were raised.

Previously, dynamics of microbial responses to changes in physical and chemical environmental factors have only been described for single microbial processes or single environmental factors (1, 16, 23, 31). The aim of the present study was to examine whether denitrifying, sulfate-reducing, and methane-producing bacteria compete for common substrates and how changes in temperature and concentrations of potential electron donors and acceptors affect the three processes in the alder swamp.

MATERIALS AND METHODS

Sampling. The swamp investigated is a 1-ha permanently waterlogged stand of red alder (Alnus glutinosa) situated in the Dyrehaven forest north of Copenhagen, Denmark. The carbon input averages 335 g (dry weight) of litter m⁻² year⁻¹. Dry-matter contents range between 12 and 18%, while volatile solids determined by loss of ignition range between 78 and 80%. The pH in the soil is 6.1 to 6.7. Cores were collected with polyvinyl chloride tubes (inner diameter, 10.5 cm) and immediately brought back to the laboratory, where a slurry was prepared within 2 h. As the major activity of denitrification, sulfate reduction, and methane production occurs within the upper 10 cm of the soil (37), only these

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layers were used for the experiments. Slurries were made by blending the extruded cores anaerobically with anoxic interstitial water from the swamp. The slurries were anaerobically dispensed into 50-ml serum vials (20 ml in each), which were flushed with N2, stoppered with butyl rubber stoppers, and crimped with aluminum seals. All experiments on a specific date were made with one batch of slurry to minimize variations caused by differences in carbon content.

Selected substrates of potential importance to sulfate-reducing, methane-producing, and denitrifying bacteria were added to slurries to study their effects on the respective growth rates. All experiments were carried out in at least duplicate vials, and all additions were made from sterile solutions stored under N2. All compounds were added to give a final concentration of 1 mM in the vials. Hydrogen was added to the headspaces to give a partial pressure of 13.5 kPa.

In experiments in which temperature was the variable, the vials were incubated over a range of temperatures (0 to 25°C) covering the seasonal range in the swamp. In all other experiments, the incubation temperature was 15°C.

### Quantification of methane production, sulfate reduction, and denitrification.

**Methane production** was determined as the accumulation rate of methane in the headspaces of the vials, measured at intervals of 12 to 20 h. Before gas was sampled, the vials were shaken to equilibrate dissolved methane with the headspace. Rates of sulfate reduction were determined by injecting 100 μl of an anaerobic carrier-free Na235SO4 solution (Rissø, Denmark) containing approximately 10 μCi/ml into the vials. The vials were shaken and then incubated for 20 to 24 h. Sulfate reduction was terminated by injection of 2 ml of 10% (wt/vol) zinc acetate solution into each vial. Acid volatile sulfide was separated from the slurry after injection of 5 ml of anoxic 5 N HCl in a trapping train similar in principle to that described by Jørgensen and Fenchel (15). Trapped H235S and residual sulfate radioactivity were counted in a Packard Tri-Carb 3375 liquid scintillation spectrometer with Na235SO4 as the internal standard. One milliliter of 10% titanium chloride was added to vials containing molybdate immediately before acidification to avoid oxidation of sulfide during the formation of phosphomolybdate complexes (5). Sulfate reduction rates might be slightly underestimated because of isotopic exchange and sulfur formation from sulfide during the distillation procedure (11). Denitrification rates were measured by the acetylene blockage technique by injection of 4 ml of acetylene into the vials corresponding to a partial pressure of 12.16 kPa. This concentration has been found to be sufficient for complete noncompetitive blockage of N2O reduction in both soils and freshwater sediments (18, 36). Accumulation of N2O in the headspaces was measured at appropriate intervals, and corrections were made for the solubility of N2O in water according to Tiedje (36).

**Analytical procedures.** Methane concentrations were quantified on a Shimadzu 8A gas chromatograph equipped with flame ionization detectors. Gases were separated on a 2.5-m steel column packed with Porapak T 80/100 mesh (Waters Associates, Inc.) and with N2 as the carrier gas. The injector and detector temperature was 100°C, and the oven temperature was 80°C. N2O was quantified on a Packard 427 gas chromatograph with an electron capture detector. Separation was carried out on a 3-m steel column packed with Porapak Q 80/100 mesh and with N2 as the carrier gas. The injector temperature was 100°C, the detector temperature was 320°C, and the oven temperature was 60°C. All gas injections were carried out with 100- or 1,000-μl Pressurelok gastight syringes (Precision Sampling Corp.).

Interstitial water for sulfate and nitrate analyses was obtained by pressure filtration under N2 into a N2-flushed serum vial containing 0.2 g of zinc acetate. The interstitial water was then vacuum filtered through a 0.45-μm-pore-size membrane filter to remove precipitated ZnS. Sulfate was determined turbidimetrically as described by Tabatabai (34). Nitrate was determined after diazo formation with acidic sulfanilamide. The intensity of the formed azo dye was determined photometrically at 520 nm by flow injection analysis (as recommended in apparatus application note 6283, 1983; Tectator, Sweden).

### RESULTS

Preliminary experiments showed that sulfate reduction and methane production were linear for at least 48 h, while denitrification was linear for less than 1 h (data not shown). All experiments, therefore, were carried out within these limits. Sulfate concentrations in the slurries ranged between 200 and 400 μM, and nitrate concentrations were between 11 and 15 μM.

The effects of selected substrates and inhibitors on methane production, sulfate reduction, and denitrification are shown in Tables 1 and 2. The addition of sulfate had almost no stimulatory effect on sulfate reduction activity, indicating that the sulfate-reducing bacteria were not seriously limited by the lack of electron acceptors in the surface slurries. Hydrogen caused the most pronounced stimulation in all experiments, ranging from 30 to above 80%. Addition of acetate and ethanol also caused an enhancement of the sulfate reduction, while lactate only was found stimulating in one experiment.

Methane production was markedly stimulated by addition of hydrogen to the slurries. Addition of methanol and trimethylamine also caused a stimulation of the methane
production, while acetate only caused a minor enhancement of the activity. Lactate, which cannot be utilized directly by any known methanogen, increased the methane production rate significantly.

The addition of sodium nitrate to the slurries stimulated denitrification 70 to 80 times, which clearly shows that denitrification activity was seriously limited by the availability of electron acceptors. Furthermore, the addition of glucose or acetate had no apparent effect upon the denitrification rates, unless nitrate was also added to the slurries, which confirmed that electron acceptor availability was the major growth-limiting factor for the denitrifying bacteria.

Competitive interactions between the three bacterial groups was studied by measuring metabolic activity after the addition of molybdate, a specific inhibitor of sulfate reduction (35), sulfate, or nitrate (Tables 1 and 2). When 1 mM sodium molybdate was added to soil slurries, sulfate reduction was almost completely inhibited, while methane production was stimulated 1.2 to 1.9 times. Sodium molybdate (5 mM) was found to inhibit methane production 12 to 28%, thereby reducing the stimulatory effect caused by inhibition of sulfate-reducing bacteria (data not shown). Nitrate (1 mM) was inhibitory to both sulfate reduction and methane production, reducing the activity to 1 to 4% and 15 to 30% of the normal activity, respectively.

As changes in anaerobic methane oxidation done by sulfate-reducing bacteria (13) would change the observed methane production rate in a manner similar to the alterations caused by the addition of molybdate or sulfate, oxidation of $^{14}$CH$_4$ was measured in the slurry by methods described by Zehnder and Brock (39) and Zehnder et al. (40). Virtually no methane oxidation was registered in the slurries, and the changes observed at the addition of sulfate were insignificant (data not shown).

The effects of different incubation temperatures on methane production, sulfate reduction, and denitrification are shown as Arrhenius plots in Fig. 1, and the related values of the calculations are shown in Table 3. As denitrification rapidly became electron acceptor limited, the nitrate concentration was raised to 1 mM in the slurries and the activity was measured during a period of 24 h.

The equality of the slopes of summer (6–24) and winter (1–26) activation energies for sulfate reduction and denitrification was confirmed ($P < 0.05$) by analysis of variance tests, followed by $t$ tests of the regression equations. The corresponding methanogenic activation energies were not equal when tested under the same conditions, but the slopes were found to be equal at $P < 0.05$ when June was compared with the other winter month (11–1).

Differences in activation energies between methane production, sulfate reduction, and denitrification were tested by analysis of variance and multiple $t$ tests of pooled data. Methane production was significantly different ($P < 0.05$) from the temperature characteristics of both sulfate reduction and denitrification, while differences between sulfate reduction and denitrification were significant only at $P > 0.30$.

When the relative importance of substrate concentrations and temperature were compared, temperature clearly had the most pronounced effect on methane production and sulfate reduction. The increase in temperature from winter to summer situations (0 to 15°C) resulted in methane production and sulfate reduction increases of 9.9 and 4.0 times, respectively. Increasing sulfate concentrations to 1 mM had almost no effect on sulfate reduction, whereas the addition of the most stimulatory electron donor (hydrogen) caused methane production and sulfate reduction increases of 4.3 and 1.8 times, respectively. Denitrification, on the other hand, was clearly more affected by electron acceptor availability than by temperature and addition of electron donors, as temperature increases from 0 to 15°C caused only a fivefold stimulation of the potential denitrification, compared with the 70- to 80-fold increase caused by addition of nitrate.

**DISCUSSION**

Sulfate reduction, methane production, and denitrification have previously been demonstrated to occur simultaneously within the same soil horizons of the Dyrehaven alder swamp (37) which makes this ecosystem suitable for studies of microbial competitive interactions. Despite the high content of volatile solids, the overall metabolic rates in the swamp are fairly slow compared with eutrophic lake sediments (12, 18), resulting in less steep sulfate and nitrate gradients.

The limitation of the denitrification process by electron acceptor availability and the fact that significant electron donor-determined stimulation occurred only upon the addition of nitrate could be explained by the low nitrate concentrations found in the slurry (11 to 15 mM). Assuming a $K_m$ value similar to values previously reported from marine sediments (50 to 344 mM nitrate) (24, 25), the denitrification system in the alder swamp was probably not saturated.

The reduction in methane production and sulfate reduction upon addition of nitrate is in accordance with previous studies (4, 6, 18, 32), but whether this is a competitive or inhibitory phenomenon needs further investigation. It may, however, be concluded that the competitive role of denitrifying bacteria in the alder swamp is minimal in the situ nitrate concentrations and denitrification rates.

The $K_m$ values of 50 to 100 mM sulfate for sulfate-reducing bacteria in freshwater sediments reported by Ingversen et al. (12) and Smith and Klug (29) may explain why sulfate-reducing bacteria, as opposed to denitrifying bacteria, were not limited by electron acceptor availability, as the sulfate concentration in the slurries of our study generally exceeded 200 mM sulfate.

The stimulation of both sulfate reduction and methane production upon the addition of hydrogen and acetate indicates that these substrates may be subject to competition in

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**TABLE 2. Effect of various compounds on denitrification rates**

<table>
<thead>
<tr>
<th>Date</th>
<th>Control</th>
<th>Acetate</th>
<th>Glucose</th>
<th>Nitrate</th>
<th>Acetate + nitrate</th>
<th>Glucose + nitrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>6–24</td>
<td>0.02 (0.00)</td>
<td>0.02 (0.00)</td>
<td>0.03 (0.00)</td>
<td>1.67 (0.16)</td>
<td>1.66 (0.27)</td>
<td>2.43 (0.45)</td>
</tr>
<tr>
<td>11–1</td>
<td>0.02 (0.00)</td>
<td>0.02 (0.00)</td>
<td>0.02 (0.00)</td>
<td>1.40 (0.11)</td>
<td>1.45 (0.07)</td>
<td>2.08 (0.19)</td>
</tr>
</tbody>
</table>

* All compounds were added to give a final concentration of 1 mM. The nitrate concentrations in the slurries were 11 μM for 6–24 and 15 μM for 11–1. Values shown are from triplicate vials.
FIG. 1. Arrhenius plots of log sulfate reduction, denitrification, and methane production rates in the swamp. The different lines show the calculated regression equations of the three processes.

The addition of molybdate to soil slurries confirmed competition for common substrates between the two bacterial groups. The molybdate-stimulated enhancement of methane production, however, was much smaller than the basic sulfate reduction activity, implying that only a part of the electron and carbon flow proceeding through sulfate reduction can be utilized by methanogenic bacteria. Sulfate-reducing bacteria are able to metabolize an array of substrates other than hydrogen and acetate. Smith and Klug (30) suggested that sulfate-reducing bacteria metabolized
lactate, propionate, and free amino acids in eutrophic lake sediments. Cappenberg (8) showed that lactate was the major electron donor of sulfate reduction in Lake Vechten sediments. Methanol, which had earlier been considered not to be utilizable by sulfate-reducing bacteria, and ethanol were found to stimulate sulfate reduction in our study. Recently, several authors have described sulfate-reducing bacteria able to grow on methanol as an energy source although the growth rates are slow (7, 22). Lactate failed to stimulate sulfate reduction in one of our experiments. Similar observations have been described by Ingvorsen et al. (12) in experiments with eutrophic lake sediments. This may be due either to a saturation of the lactate-metabolizing sulfate-reducing bacteria or to competition performed by other bacteria able to utilize lactate, or lactate might be an ecological unimportant intermediate for sulfate reduction in the swamp. The presence of lactate-utilizing sulfate-reducing bacteria in the swamp was confirmed by lactate enrichment studies (data not shown) and the stimulation of sulfate reduction on 6–24.

Different responses to temperature changes of different microbial populations can play an important role in ecosystems, where the competitive balances are sensitive to minor environmental changes. Harder and Veldkamp (9) studied the competition between facultatively and obligately psychrophilic marine bacteria in chemostats and found that the outcome of competition between the two bacterial species was dependent upon the incubation temperature. In contrast to these well-defined mixed culture studies, denitrification, methane production, and sulfate reduction measured in slurries from the alder swamp are terminal processes dependent upon the preceding mineralization processes in the anaerobic food chain. Temperature plots of each anaerobic process, therefore, must be composed of activation energies of that specific food chain.

Differences in temperature responses may either be due to differences in temperature ranges of the linear part of the Arrhenius plot or a consequence of different slopes (activation energies) of the plots. Harder and Veldkamp (9) reported little difference in activation energies of the facultatively and obligately psychrophilic populations and concluded that the differences in competitive outcome rather was a consequence of differences in temperature ranges for optimal growth than in activation energies. Our studies show significant differences in Arrhenius slopes between the three investigated processes, while no differences in optimal growth temperatures were observed within the normal temperature ranges in the swamp. The two organisms studied by Harder and Veldkamp were both aerobic heterotrophic pseudomonads utilizing oxygen as the terminal electron acceptor. In anaerobic ecosystems, however, the bacteria responsible for terminal electron removal might belong to different metabolic groups, yielding different energy outcomes, and involving both eubacterial and archaebacterial species. The various enzymatic pathways involved in these metabolic types might result in different responses to physical and chemical alterations of the environment, as observed in our Arrhenius plots.

Abdollahi and Nedwell (1) argued that variations in the Arrhenius constant of a microbial population throughout the year could be regarded as variations in variables other than temperature (bacterial numbers, and electron donor and acceptor concentrations). In studies of temperature influences on sulfate reduction in salt marsh sediments, they found no significant variations in Arrhenius constants during the course of the year, implying that temperature was the dominant variable in these environments. In our studies, the Arrhenius constants of methane production, denitrification, and sulfate reduction vary between 8 and 20%, also indicating that variables other than temperature are influencing these processes.

Compared with other measurements of temperature coefficients of methanogenesis and denitrification, our values are slightly higher. Kelly and Chynoweth (16) observed methanogenic $Q_{10}$ values of 1.2 to 4.7 in freshwater sediments compared with our averages of 4.58 ± 0.65. Stanford et al. (33) described an average $Q_{10}$ value of 2 for denitrification in different soils compared with the average of 2.9 ± 0.07 found in our investigation. On the other hand, our sulfate reduction temperature coefficients of 2.48 ± 0.17 are slightly lower than values from marine sediments (3.4 to 3.9 [1, 14]) and limnic sediments (2.9 [12]).

Most dynamic studies of microbial competitive interactions have focused on the significance of electron donor and acceptor relations, while little is known about the role of physical parameters, such as temperature. In natural ecosystems, a variety of variables may determine the outcome of competition for growth-limiting substrates. Our experiments indicate, that in environments exposed to large temperature fluctuations, the balance between terminal anaerobic bacte-

### TABLE 3. Characteristics of the Arrhenius plots of the three processes studied

<table>
<thead>
<tr>
<th>Process</th>
<th>Date</th>
<th>Slope</th>
<th>$E^\theta$</th>
<th>$Q_{10}'$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanogenesis</td>
<td>6–24–85</td>
<td>-5.76</td>
<td>23.67</td>
<td>-0.996</td>
</tr>
<tr>
<td></td>
<td>10–1–85</td>
<td>-5.02</td>
<td>19.45</td>
<td>-1.000</td>
</tr>
<tr>
<td></td>
<td>11–1–85</td>
<td>-6.06</td>
<td>22.29</td>
<td>-0.990</td>
</tr>
<tr>
<td></td>
<td>1–26–86</td>
<td>-4.82</td>
<td>18.81</td>
<td>-0.998</td>
</tr>
<tr>
<td>Sulfate reduction</td>
<td>6–24–85</td>
<td>-3.37</td>
<td>12.85</td>
<td>-0.854</td>
</tr>
<tr>
<td></td>
<td>10–1–85</td>
<td>-3.26</td>
<td>12.49</td>
<td>-0.953</td>
</tr>
<tr>
<td></td>
<td>11–1–85</td>
<td>-3.52</td>
<td>13.89</td>
<td>-0.954</td>
</tr>
<tr>
<td></td>
<td>1–26–86</td>
<td>-2.83</td>
<td>11.73</td>
<td>-0.936</td>
</tr>
<tr>
<td>Denitrification</td>
<td>6–24–85</td>
<td>-3.75</td>
<td>14.90</td>
<td>-0.972</td>
</tr>
<tr>
<td></td>
<td>10–1–85</td>
<td>-3.67</td>
<td>13.94</td>
<td>-0.946</td>
</tr>
<tr>
<td></td>
<td>11–1–85</td>
<td>-3.82</td>
<td>14.55</td>
<td>-0.987</td>
</tr>
<tr>
<td></td>
<td>1–26–86</td>
<td>-3.96</td>
<td>15.83</td>
<td>-0.970</td>
</tr>
</tbody>
</table>

*a* Slope of the Arrhenius plot (log metabolic rate: 1/T - 10^{-1} \cdot K^{-1}).

* Activation energy or temperature characteristics (kcal \cdot mol^{-1}).

* Temperature coefficient caused by a 10-degree increase in temperature.
ria might be altered during the course of the year as a consequence of changes in temperature.

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LITERATURE CITED


