Oxidation and Assimilation of Atmospheric Methane by Soil Methane Oxidizers

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The metabolism of atmospheric methane in a forest soil was studied by radiotracer techniques. Maximum $^{14}\text{CH}_4$ oxidation ($163.5 \text{ pmol of C cm}^{-3} \text{ h}^{-1}$) and ^{14}C assimilation ($50.3 \text{ pmol of C cm}^{-3} \text{ h}^{-1}$) occurred at the A_2 horizon located 15 to 18 cm below the soil surface. At this depth, 31 to 43% of the atmospheric methane oxidized was assimilated into microbial biomass; the remaining methane was recovered as $^{14}\text{CO}_2$. Methane-derived carbon was incorporated into all major cell macromolecules by the soil microorganisms (50% as proteins, 19% as nucleic acids and polysaccharides, and 5% as lipids). The percentage of methane assimilated (carbon conversion efficiency) remained constant at temperatures between 5 and 20°C , followed by a decrease at 30°C . The carbon conversion efficiency did not increase at methane concentrations between 1.7 and 1,000 ppm. In contrast, the overall methane oxidation activity increased at elevated methane concentrations, with an apparent K_m of 21 ppm (31 nM CH_4) and a $V_{\max}$ of $188 \text{ pmol of CH}_4 \text{ cm}^{-3} \text{ h}^{-1}$. Methane oxidizers from soil depths with maximum methanotrophic activity respired approximately 1 to 3% of the assimilated methane-derived carbon per day. This apparent endogenous respiration did not change significantly in the absence of methane. Similarly, the potential for oxidation of atmospheric methane was relatively insensitive to methane starvation. Soil samples from depths above and below the zone with maximum atmospheric methane oxidation activity showed a dramatic increase in the turnover of the methane assimilated (>20 times increase). Physical disturbance such as sieving or mixing of soil samples decreased methane oxidation and assimilation by 50 to 58% but did not alter the carbon conversion efficiency. Ammonia addition (0.1 or $1.0 \text{ } \mu\text{mol g [fresh weight]}^{-1}$) decreased both methane oxidation and carbon conversion efficiency. This resulted in a dramatic decrease in methane assimilation (85 to 99%). In addition, ammonia-treated soil showed up to 10 times greater turnover of the assimilated methane-derived carbon (relative to untreated soil). The results suggest a potential for microbial growth on atmospheric methane. However, growth was regulated strongly by soil parameters other than the methane concentration. The pattern observed for metabolism of atmospheric methane in soils was not consistent with the physiology of known methanotrophic bacteria.

One of the important global sinks for atmospheric methane is microbial oxidation in terrestrial environments (8, 11, 16, 26). Consumption of atmospheric methane has been reported for a range of oxic soils including forests, grasslands, heathlands, tundras, and deserts (9, 21, 23, 34, 36, 39). Atmospheric methane oxidation has also been reported for some agricultural soils, but the rates were generally lower than rates in nonagricultural systems (10). Approximately 20 to 60 Tg of methane from the atmosphere is consumed per year by microorganisms in soils (26). Interestingly, the soil organism(s) responsible for this methane consumption has so far evaded isolation and characterization.

Ecological studies have shown that atmospheric methane oxidizers must have unique physiological characteristics, including an ability to sustain a long-term oxidation of subatmospheric methane concentrations ($<3 \text{ nM CH}_4$). Methanotrophic bacteria known from culture collections may oxidize atmospheric methane temporarily but cannot maintain this ability over time (17, 29). Apparent K_m values for methane uptake by known methanotrophs are generally in the micromolar range (1 to $10 \text{ } \mu\text{M}$), suggesting an adaptation to elevated methane concentrations mainly. In contrast, Bender and Conrad (4) have shown that atmospheric methane oxidizers

have a high-affinity enzyme system and a rather low $V_{\max}$. The apparent K_m for methane uptake is 30 to 51 nM CH_4 and the $V_{\max}$ values fall often within a range of 0.7 to $3.6 \text{ nmol of CH}_4 \text{ h}^{-1} \text{ g (dry weight)}^{-1}$. The threshold concentration for methane uptake by soils is generally $<1 \text{ nM CH}_4$ (defined as the methane concentration below which no uptake occurs). Theoretically, these kinetic properties may be sufficient to permit maintenance and even cell growth with atmospheric methane as the substrate (7). However, several attempts to enrich for atmospheric methane oxidizers by using a range of methane concentrations have been unsuccessful (32).

Predictions of future trends in atmospheric methane oxidation in relation to environmental changes require a basic understanding of the microbiology of the responsible methane-oxidizing microorganism(s). Despite this microorganism(s) being the only known biological consumer of atmospheric methane, several pertinent questions remain unanswered. For example, is atmospheric methane used as a true growth substrate? Is there more than one organism involved in the oxidation process, and why is the oxidation activity often suppressed for years following ammonia fertilization? In the present study, we have examined the oxidation and assimilation of atmospheric methane by microorganisms in a forest soil. Radiotracer techniques (^{14}C) were used to estimate methane assimilation and endogenous carbon turnover after changes in temperature, methane concentration, ammonia concentration, and soil structure.

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MATERIALS AND METHODS

Study site. Intact soil cores were collected in Klosterheden Forest Plantation in Western Jutland, Denmark. The forest was established on sandy, nutrient-poor, heathland soil in 1880 to 1888. The study site was vegetated by Norway spruce (*Picea abies*). The soil was a typical haploorthod (podzol) that had developed on the deposits from an outwash plain. The organic matter content varied from 86% g (dry weight)⁻¹ at the surface to 2% g⁻¹ at 16- to 20-cm depths. The pHs were 3.5 and 4.2 in the 0 and A horizons, respectively. Intact soil cores were brought back to the laboratory, and subsamples were incubated as outlined below. Unless stated otherwise, the experiments were carried out by using sandy soil from the zone with maximum potential atmospheric methane oxidation (12- to 18-cm depths).

Methane oxidation rates. Oxidation of atmospheric methane (1.7 ppm) was estimated from 1st order decreases in headspace methane concentration; oxidation at elevated methane concentrations (>100 ppm) was estimated from 0th order decreases. Methane (0.3 cm³) was sampled with a needle and syringe and analyzed on a Chrompack 438A gas chromatograph equipped with a flame ionization detector. The oven, injector, and detector temperatures were 100, 120, and 225°C, respectively. Gases were separated on a Hyasep Q column (2 m by 2 mm) with N₂ as the carrier gas (20 ml min⁻¹). The detection limit for methane was 0.1 ppm.

Metabolism of ¹⁴CH₄. The metabolism of atmospheric methane in soil samples was studied by using ¹⁴CH₄ as a tracer (0.25 MBq cm⁻³; 2.0 GBq mmol⁻¹; Amersham, Buckinghamshire, United Kingdom). The radiolabelled methane was purified as described previously (13). Unless stated otherwise, soil samples were incubated in serum bottles at 22°C with an initial methane concentration of <5 ppm (CH₄ plus ¹⁴CH₄). Incubation time varied between 6 and 24 h depending on the initial oxidation activity. The measured carbon conversion efficiency (percent assimilation) did not change during 6- to 24-h assays or at methane concentrations between 1.7 and 10 ppm. ¹⁴CH₄ was collected from the headspace and measured as ¹⁴CO₂ after combustion by flame ionization detector of the gas chromatograph (13).

After the incubation of soil samples with ¹⁴CH₄, ¹⁴CO₂ was stripped from the headspace of the serum bottles with air and recovered in CO₂ traps. The traps consisted of scintillation vials in series each with a 10-ml mixture of ethanolamine and ethylene glycol monomethyl ether (1:7, vol/vol). All radiolabelled samples were analyzed on a Packard 1600 TR liquid scintillation counter with 12 ml of Packard Insta-Gel Plus as a cocktail. The measured radioactivity was corrected for quenching by using external and internal standards.

¹⁴CH₄ assimilated into microbial biomass was determined after converting total soil organic matter to carbon dioxide (CO₂ plus ¹⁴CO₂) by CrO₃ oxidation (37). Prior to the CrO₃ oxidation, soil samples (1 to 5 g) were homogenized and dried for 24 h at 70°C. Samples were then incubated for 2 h at 130°C in 180-ml reaction vessels in the presence of 50 ml of CrO₃ reagent (2 M CrO₃ dissolved in a 2:1 mixture of concentrated sulfuric and phosphoric acids). Labelled and nonlabelled CO₂ produced during the chemical oxidation was stripped from the vessels with N₂ and recovered in CO₂ traps as described above. Alternatively, smaller soil samples (0.25 to 1 g [dry weight]) were incubated for 2 h at 130°C with 10 ml of CrO₃ reagent in 160-ml serum bottles sealed with Teflon stoppers and aluminum crimps.

It was assumed that the ¹⁴C recovered from the soil samples was associated with the organic rather than the inorganic carbon fraction. Significant amounts of labelled inorganic carbon (e.g., carbonates) did not accumulate due to a low soil pH of 3.5 to 4.2. This was confirmed by comparing ¹⁴C recovery from soil samples before and after acidification with 1 M H₂SO₄. Recovery of labelled bacterial biomass from the soil matrix by CrO₃ oxidation was 93 to 95%. This was estimated by quantitative recovery of known amounts of ¹⁴C-labelled cells of *Methylosinus trichosporium* OB3b added to unlabelled soil.

Fractionation of labelled microbial biomass. ¹⁴C-labelled organic matter extracted from soil samples was separated into proteins, lipids, nucleic acids and polysaccharides, and low-molecular-weight compounds by a solvent fractionation scheme (29). Quadruplicate soil samples (5 g [dry weight]) were extracted sequentially in 50-ml Pyrex glass centrifuge tubes with methanol-dichloromethane, trichloroacetic acid, and KOH. After each extraction step, the remaining soil and cell residues were precipitated by centrifugation (3,000 × g, 20 min, 5°C). Labelled proteins were quantified as ¹⁴CO₂ after CrO₃ oxidation of the KOH extract.

Stratification of atmospheric methane oxidizers. The stratification of microorganisms oxidizing atmospheric methane was determined by measuring the depth-specific assimilation of ¹⁴CH₄ in an intact soil core (26 by 3.4 cm). The core was stoppered, and 0.2 ml of ¹⁴CH₄ (0.25 MBq cm⁻³; 3,000 ppm) was added repeatedly to the core headspace until a total of 0.85 cm³ of ¹⁴CH₄ had been consumed. Additional ¹⁴CH₄ was added when the headspace methane concentration had decreased to <1 ppm (the initial concentration was 8 ppm). After incubation for 65 h at 20°C, the core was sectioned into 3-cm slices and sieved. Soil samples were dried (24 h, 70°C), and three 1-g samples of soil from each depth were assayed for radiolabelled biomass by CrO₃ oxidation as described above. A second set of subsamples from the sectioned core (three 8-g samples of fresh soil from each depth) was transferred to 38-ml serum bottles, and the depth distribution of potential methane oxidation was measured at the ambient methane concentration (1.7 ppm). The incubation with ambient air continued for 21

FIG. 1. Distribution of ¹⁴C in cell material extracted from soil samples incubated with atmospheric concentrations of ¹⁴CH₄. LMW, low-molecular-weight compounds; NAP, nucleic acids and polysaccharides.

days to obtain a measure of the turnover of the labelled microbial biomass. The headspace was flushed with air at days 1, 2, 5, and 21, and ¹⁴CO₂ was collected and analyzed as described above.

Effect of temperature. The effect of temperature on oxidation and assimilation of atmospheric methane was studied for sieved soil samples incubated with ¹⁴CH₄. Triplicate samples (10 g [fresh weight] [gfw]) were transferred to 55-ml vials and incubated at 5, 10, 20, or 30°C with 0.1 ml of ¹⁴CH₄ (25 kBq cm⁻³). The incubation time varied between 8 and 24 h depending on the oxidation activity at each temperature. The initial headspace methane concentration was 2.5 ppm. Methane oxidation and assimilation were determined by gas chromatography and CrO₃ oxidation as described above.

Effect of methane concentration. The effect of methane concentration on oxidation and assimilation of methane was studied for sieved soil samples incubated with ¹⁴CH₄. Triplicate samples (10 gfw) were transferred to 55-ml vials and incubated at 20°C with 5, 100, 1,000, or 10,000 ppm CH₄ and 0.2 ml of ¹⁴CH₄ (0.25 MBq cm⁻³). The incubation time varied between 12 and 90 h depending on the oxidation activity at each methane concentration. Methane oxidation and assimilation rates were determined as described above. The kinetics of methane oxidation (V_{max} and apparent K_m for uptake) was estimated from Eadie-Hofstee plots and nonlinear regression analysis of total ¹⁴CH₄ turnover at the different methane concentrations (measured as the recovery of ¹⁴CO₂ plus ¹⁴C biomass).

Effect of disturbances. The effect of soil mixing and ammonia addition on oxidation and assimilation of atmospheric methane was studied for soil subsamples incubated with ¹⁴CH₄. Methane metabolism was examined after the following manipulations: mechanical mixing of the soil (mixed with a spoon), sieving of the soil (using a 2-mm mesh), or ammonia addition to soil (0.1 or 1.0 μmol of NH₄Cl gfw⁻¹). Undisturbed ("intact") soil subsamples collected with cutoff syringes served as controls. The two ammonia solutions (NH₄Cl) were gently sprayed onto the soil crumbs; the control and the homogenized soil samples received an equal volume of autoclaved deionized water (0.25 ml). Triplicate 20-g soil samples were transferred to 55-ml vials and incubated at 20°C with 0.2 ml of ¹⁴CH₄ (0.25 MBq cm⁻³). The incubation with ¹⁴CH₄ was initiated 2 h after the soil manipulations. Control, mixed, and sieved samples were incubated for 4 h, and samples with 0.1 or 1.0 μmol of NH₄⁺ gfw⁻¹ added were incubated for 5 and 22 h, respectively (to partly offset differences in initial oxidation activity). Atmospheric methane oxidation and assimilation were determined as described above. After the incubations with ¹⁴CH₄, subsamples from each set of treatment (three 15-g samples of soil) were incubated for 96 h with ambient air in stoppered 55-ml vials to monitor the release of ¹⁴CO₂ (carbon turnover).

RESULTS

Atmospheric methane assimilation. Atmospheric methane was used as a source of cell carbon by microorganisms in Klosterheden Forest soil (Fig. 1). The assimilated ¹⁴CH₄ was incorporated into all major cell macromolecules, e.g., 50% as protein, 19% as nucleic acids and polysaccharides, and 5% as lipids. Low-molecular-weight compounds such as cell metabolites accounted for 26% of the label recovered. Further fractionation of the lipids showed that atmospheric methane was assimilated into membrane lipids including phospholipid fatty acids (data not shown). In the zone with maximum methanotrophic activity (12 to 18 cm), 38 to 43% of the ¹⁴CH₄ oxidized was converted into microbial biomass; the remaining

TABLE 1. Effect of temperature on oxidation and assimilation of atmospheric methane by soil methane oxidizers

Temp (°C)	Methane oxidation (pmol of C cm ⁻³ h ⁻¹)	Methane assimilation (pmol of C cm ⁻³ h ⁻¹)	Carbon conversion efficiency (%)
5	14.9	6.3	42
10	25.6	10.0	39
20	40.0	16.7	42
30	44.5	9.0	20

label was recovered as ¹⁴CO₂. The relative methane assimilation decreased to 34% at the soil surface and below the zone with maximum methanotrophic activity (at 0- to 3- and 18- to 21-cm depths, respectively).

Effect of temperature. Atmospheric methane oxidation and assimilation rates in soil samples increased with increasing temperatures between 5 and 20°C (Table 1). A temperature increase from 20 to 30°C resulted in a minor increase in atmospheric methane oxidation, whereas the carbon assimilation decreased (Table 1). The apparent Q₁₀ (5 to 20°C) was 1.9 for oxidation and assimilation of atmospheric methane. This compared to a Q₁₀ of 1.7 for the overall microbial respiration in the same soil samples (total CO₂ production). The carbon conversion efficiency for atmospheric methane did not change with increasing temperatures up to 20°C (30 to 42%), whereas a further increase from 20 to 30°C resulted in a 52% decrease in carbon conversion efficiency (Table 1).

Effect of methane concentration. Soil methane oxidation and carbon assimilation increased with increasing methane concentrations up to 10,000 ppm (Table 2). In contrast, the carbon conversion efficiency decreased with increasing methane concentrations; the greatest relative carbon assimilation (43%) was measured at 5 ppm methane (Table 2). At 10,000 ppm methane, only 20% of the methane oxidized was converted into microbial biomass (Table 2).

The kinetics of methane oxidation estimated from Eadie-Hofstee plots and nonlinear regression analysis showed an apparent V_{max} and K_m for methane uptake (1.7 to 1,000 ppm) of 188 ± 1.2 pmol of CH₄ cm⁻³ h⁻¹ and 21 ± 0.6 ppm, respectively (Fig. 2). A K_m of 21 ppm corresponds to approximately 31 nM CH₄ in the soil water. The apparent threshold for methane uptake by the atmospheric methane oxidizers was ≤0.2 nM CH₄.

Long-term exposure (days to weeks) of soil samples to 10,000 ppm methane resulted in a change in methane oxidation kinetics (activation of low-affinity methanotrophic bacteria). As a result, the apparent K_m and V_{max} increased, whereas oxidation rates at atmospheric methane concentrations remained almost unaltered (data not shown).

Stratification. The greatest assimilation of atmospheric methane in Klosterheden Forest soil occurred at the organic

TABLE 2. Effect of methane concentration on oxidation and assimilation of methane by soil methane oxidizers

Methane concn (ppm)	Methane oxidation (pmol of C cm ⁻³ h ⁻¹)	Methane assimilation (pmol of C cm ⁻³ h ⁻¹)	Carbon conversion efficiency (%)
5	35.5	15.3	43
10	60.6	22.8	38
100	153.2	49.3	32
1,000	184.9	62.5	34
10,000	531.8	104.2	20

FIG. 2. Effect of different methane concentrations on soil methane oxidation rates. The kinetic parameters were determined from Eadie-Hofstee plots and nonlinear regression analysis.

poor mineral soil horizon at 12- to 18-cm depths (Fig. 3A). This depth distribution of assimilation activity was determined by using intact soil cores incubated with ¹⁴CH₄ in the headspace. The zone with maximum assimilation corresponds to the depth with maximum potential atmospheric methane oxidation activity (Fig. 3B). The latter was determined by measuring the oxidation activity for undisturbed subsamples (slices) at ambient methane concentrations.

Approximately 1.0% of the ¹⁴C-labelled microbial biomass was turned over each day in soil samples from 12- to 18-cm depths (measured as ¹⁴CO₂ production after labelling) (Fig. 4). In contrast, soil samples from depths with low oxidation and assimilation potential showed a much more rapid turnover (Fig. 4). The initial respiration of the ¹⁴C-labelled biomass was 20 to 38 times greater at 0 to 3 and 21 to 24 cm than at 12 to 18 cm.

Effect of disturbances. Atmospheric methane oxidation and assimilation rates were greatest for undisturbed soil samples (163.5 and 50.3 pmol of C cm⁻³ h⁻¹, respectively) (Fig. 5A). Physical disturbance (mixing or sieving) decreased the atmospheric methane oxidation rate by approximately 50% (Fig.

FIG. 3. Stratification of atmospheric methane oxidizers in Klosterheden Forest soil. (A) Depth-specific assimilation of ¹⁴C in an intact core incubated with ¹⁴CH₄ in the headspace (sectioned after the incubation). (B) Potential atmospheric methane oxidation rates in soil samples from the same depths.

FIG. 4. Depth-specific turnover of ^{14}C -labelled biomass. $^{14}\text{CO}_2$ production was measured for soil samples incubated in ambient air after incubation with $^{14}\text{CH}_4$.

5A). Addition of 0.1 or 1.0 μmol of NH_4^+ gfw of soil $^{-1}$ decreased atmospheric methane oxidation by 70 and 98%, respectively. The natural background of KCl-extractable NH_4^+ was 0.15 μmol of NH_4^+ gfw of soil $^{-1}$.

The decreases in methane oxidation rates resulted in decreases in methane assimilation (Fig. 5B). However, the pattern of inhibition was different for the two types of manipulation. Physical disturbances decreased the overall carbon assimilation (Fig. 5B) but not the carbon conversion efficiency (Fig. 5C). In contrast, ammonia addition decreased both oxidation activity (Fig. 5A) and conversion efficiency (Fig. 5C). The combined inhibitory effects of ammonia on the soil methane oxidizers resulted in a greater relative decrease in carbon

assimilation than observed after physical disturbances (Fig. 5B). Addition of 1.0 μmol of NH_4^+ gfw of soil $^{-1}$ decreased the overall methane assimilation by 99% (from 50.3 to 0.3 pmol of $\text{C cm}^{-3} \text{ h}^{-1}$). In addition, ammonia fertilization altered the methane assimilation pattern so that a greater relative proportion was assimilated into low-molecular-weight compounds and less into nucleic acids, polysaccharides, and protein. For example, 31% more methane-derived carbon was recovered as labile low-molecular-weight compounds (metabolites) in ammonia-treated soils than in untreated controls.

The turnover of assimilated methane was also seriously affected by ammonia additions (Fig. 5D). After labelling the cells with $^{14}\text{CH}_4$ and flushing the soil samples with ambient air, the release of $^{14}\text{CO}_2$ was measured over 4 days. In samples with 1.0 μmol of NH_4^+ gfw of soil $^{-1}$ added, approximately 40% of the recently assimilated methane was quickly respired. In contrast, there was no apparent effect of soil mixing or sieving on the turnover of the assimilated methane (Fig. 5D). In undisturbed samples, 1 to 3% of the ^{14}C -labelled biomass was respired per day (Fig. 5D). This apparent endogenous respiration did not change during methane starvation (<0.1 ppm CH_4) or during incubations at an elevated methane concentration (1,000 ppm CH_4). Similarly, the potential for oxidation of atmospheric methane did not change significantly in samples incubated without methane for 4 days compared with parallel samples incubated at 1.7 or 1,000 ppm methane.

DISCUSSION

Although oxidation of atmospheric methane has been reported for a range of terrestrial environments, very little is

FIG. 5. Effect of soil mixing, sieving, or ammonia addition on atmospheric methane oxidation (A), carbon assimilation (B), carbon conversion efficiency (C), and carbon turnover after labelling (D). Mixing, mechanical homogenization with a spoon; sieving, sieved through a 2-mm mesh; + NH_4^+ , addition of 0.1 or 1.0 μmol of NH_4Cl gfw of soil $^{-1}$. The turnover of assimilated methane-derived carbon was determined subsequent to the incubation with $^{14}\text{CH}_4$.

known about the fate of the carbon metabolized. It has been suggested that atmospheric methane is incorporated into bacterial biomass during the oxidation process, but the available data are scarce. On the basis of direct and indirect techniques, assimilation of atmospheric methane has been quantified for some North American tundra and forest soils (e.g., see references 32, 39, 40, and 42). These investigations suggest an upper carbon conversion efficiency of 34 to 54% for atmospheric methane oxidation in nonagricultural soils. In the present study, a comparable fraction of the atmospheric methane oxidized was assimilated into microbial biomass (31 to 43%). The methane-derived carbon was incorporated into all major cell fractions (Fig. 1), and the assimilation pattern was similar to that of methanotrophic bacteria during balanced growth at elevated methane concentrations (29, 41). This suggests that atmospheric methane was used as a true source of cell carbon by the soil bacteria with no apparent bias toward production of certain cell constituents.

The carbon conversion efficiency estimated for atmospheric methane oxidation approaches the upper efficiency limits (29 to 62%) calculated for methanotrophic bacteria in cultures (22). The carbon conversion efficiencies for atmospheric methane oxidation were also comparable to values observed for landfill soils incubated at elevated methane concentrations (39,000 ppm) (41). This is somewhat impressive because the estimated carbon conversion efficiencies for methanotrophs in culture are based on an unlimited supply of primary substrates (oxygen and methane) (3, 22). In contrast, atmospheric methane oxidizers in Klosterheden Forest exist at methane concentrations (≤ 1.7 ppm) which are well below their apparent K_m value of 21 ppm. At these suboptimal substrate concentrations, cell maintenance requirements may pose a relatively greater burden on the cells' energy budget and thereby tend to lower growth yields (7, 30). Maintenance energy requirements have not been determined for atmospheric methane oxidizers, but the turnover of labelled cell carbon suggests an endogenous metabolism that consumes approximately 1 to 3% of the cell biomass per day (Fig. 4 and 5D). Although this rate may represent a minimum estimate due to potential isotopic dilution by unlabelled carbon (e.g., see references 18 and 20), the turnover of biomass is comparable to values reported for apparent maintenance requirements in other bacteria (2, 25, 30).

On the basis of data obtained in Klosterheden Forest, we have calculated a theoretical microbial biomass production with atmospheric methane as the sole carbon source. Using data obtained with soil from the zone with maximum methanotrophic activity, we estimate that methane-derived carbon could support the production of approximately 0.8×10^5 bacteria $\text{cm}^{-3} \text{day}^{-1}$ (assuming an atmospheric methane oxidation rate of $100 \text{ pmol cm}^{-3} \text{h}^{-1}$, a 40% carbon conversion efficiency, a 1% carbon turnover day^{-1} , and a bacterial carbon content of $1.5 \times 10^{-13} \text{ g of C cell}^{-1}$). These approximations suggest that the potential for population growth on atmospheric methane alone is relatively limited regardless of the initial population density (e.g., 10^6 to 10^8 cm^{-3}). Even a modest biomass turnover of 0.01 day^{-1} may become a severe limitation for populations with low growth potentials (e.g., $<0.1 \text{ day}^{-1}$). Some methanotrophs may be stimulated by methane produced in anoxic soil aggregates which could support higher growth rates in certain environments (8, 42). However, a potential for methane production has not been observed for all soils with atmospheric methane oxidation (e.g., see references 5 and 27).

The growth potential calculated above was apparently not limited by microbial predation as judged by the moderate turnover of labelled biomass in the zone with maximum oxidation

activity (Fig. 4). Significant microbial predation would likely have resulted in greater $^{14}\text{CO}_2$ production. In contrast, a dramatic carbon turnover was observed in soil above and below the zone with maximum methanotrophic activity (Fig. 4). Methane oxidizers at these soil depths likely experienced physiological stress perhaps in combination with an increased microbial predation (see also below). Such factors may partly determine the distinct stratification of atmospheric methane oxidizers observed in many forest soils.

The potential for growth on atmospheric methane may easily be attenuated by various soil manipulations, such as physical disturbance or ammonia addition (Fig. 5). Several studies have suggested that atmospheric methane oxidation is sensitive to nitrogen fertilization and that the effect may persist after the nitrogen concentration has decreased to background levels (1, 19, 23, 24, 34). King and Schnell (19, 31) proposed that the inhibitory effects of ammonia on atmospheric methane oxidizers were due to several mechanisms, including a competitive inhibition of methane monooxygenase and an inhibition of cell metabolism by oxidation products such as hydroxylamine and nitrite. The present study supports these findings and suggests that not only methane oxidation but also carbon conversion efficiency may decrease after ammonia addition. The resulting negative effect on the overall carbon assimilation was accompanied by a dramatic increase in carbon turnover in the presence of elevated ammonia concentrations (up to 30% turnover of labelled carbon day^{-1} ; Fig. 5). As a result, the potential for utilization of atmospheric methane as a growth substrate decreased 85 to 99% after ammonia fertilization (Fig. 5B). Further studies will show whether such decreases in the potential for atmospheric methane metabolism after ammonia fertilization are due to a persistent change in cell-specific methanotrophic activity or perhaps a result of cell death followed by a limited capacity for population recovery. Similarly, the mechanisms underlying the inhibitory effects of soil mixing on the atmospheric methane oxidizers are unclear. A possible explanation for the mixing effects may be sought in transient post-treatment increases in ammonia concentrations due to increased mineralization or perhaps increases in evaporation resulting in critical decreases in the soil water content. The latter possibility appeared plausible in the sandy Klosterheden soil where the in situ water content was relatively low (7 to 16%, wt/wt). Other studies have shown that methane oxidation activity in soils is sensitive to water stress and may decrease substantially at water contents below 25% (33).

The above results suggest a limited capacity for microbial growth on atmospheric methane, although it remains difficult to evaluate whether the observed assimilation of methane-derived carbon can lead to net population growth per se. Attempts to stimulate or enrich for atmospheric methane oxidizers using methane, other C_1 compounds, complex substrates, or a combination of these have so far been unsuccessful (4, 27, 32). Exposure of soil to elevated methane concentrations ($\geq 1,000$ ppm) resulted in stimulation of methane consumption at the elevated concentrations mainly, whereas atmospheric methane oxidation rates did not change correspondingly (4, 32). It was concluded that methane addition had resulted in enrichment of methanotrophic bacteria with kinetic characteristics similar to those of the known methanotrophs. A cell-specific change in methane affinity in a single group of methanotrophs appeared less likely (4). These findings led the investigators to suggest that two physiologically distinct populations of methanotrophic bacteria exist in soils: one group of known methanotrophs with a high affinity but low capacity and a second group, the unknown atmospheric methane oxidizers,

FIG. 6. Outline of possible schemes for microbial oxidation of atmospheric methane. X, Y, and Z represent carbon and/or energy sources other than atmospheric methane (see text for details).

with a low capacity but a high affinity for methane oxidation (4, 8, 32).

These observations have prompted speculations about the possible physiological mechanisms underlying atmospheric methane oxidation by the unknown high-affinity methane oxidizers. Some of the possible schemes for atmospheric methane oxidation and assimilation have been summarized in Fig. 6. The figure illustrates a simplification of potential metabolic interactions. Scheme 6A represents a situation where methane is used as the sole source of cell carbon, similar to what has been shown for known methanotrophic bacteria (albeit with different kinetic characteristics). Scheme 6B represents a situation where atmospheric methane oxidation is dependent on the simultaneous cooxidation or metabolism of another substrate. In both cases (Fig. 6A and B), a fraction of the atmospheric methane oxidized is assimilated into cell biomass by the organism. In scheme 6C, atmospheric methane is oxidized but not assimilated by the methane oxidizer. Instead, one or several metabolites are excreted or leaked and subsequently assimilated by a second organism. Although each of the three oxidation schemes may theoretically account for atmospheric methane oxidation, the controls of the oxidation and assimilation activity will be somewhat different. In general, the results of the present study suggest an oxidation scheme that appears best explained by model A or B. Some of the results may also be accounted for by model C, but certain observations are difficult to explain. For example, the relatively high carbon conversion efficiency and the inhibition pattern by ammonia appeared inconsistent with model C. According to this scheme, an inhibitory effect of the cosubstrate NH_3 and its oxidation products (e.g., NH_2OH and NO_2^-) would decrease methane oxidation in organism 1 and the assimilation of methane-derived carbon in organism 2 but have a minor effect on the carbon conversion efficiency per se (in the latter organism). However, the carbon conversion efficiency decreased substantially after ammonia addition to soil samples (Fig. 5B). This suggests that methane and ammonia metabolism likely took place in the same organism. In support of this, the elevated carbon turnover following inhibition by ammonia (Fig. 5D)

also indicated that methane oxidation and assimilation occurred in one organism.

If atmospheric methane was used as the only source of energy and carbon by the soil methanotrophs, one would expect a decrease in oxidation capacity and carbon turnover in the absence of methane (methane starvation). Such a starvation response has been observed previously for obligate methanotrophic bacteria in cultures (28, 29) and for atmospheric methane oxidizers in a forest soil (32). A somewhat different pattern was seen for methane oxidizers in the Klosterheden Forest soil. We observed a carbon turnover (endogenous metabolism) that did not change significantly with changes in methane concentration. In addition, the capacity for oxidation of atmospheric methane was relatively insensitive to short-term methane starvation. These observations suggest that the atmospheric methane oxidizers were not solely dependent on methane for maintaining their metabolic activity (Fig. 6B). Potential cosubstrates may include inorganic compounds, complex organics, or perhaps other C_1 compounds, such as methanol or formic acid, as has been suggested by King (17) and Jensen et al. (14). A regulatory role of other compounds or substrates may explain why methane enrichment alone is not sufficient for stimulation of atmospheric methane oxidation in soils.

Several authors have indicated a potential role for autotrophic ammonia-oxidizing bacteria (nitrifiers) in atmospheric methane oxidation (e.g., see references 6, 23, 34, and 35). A cooxidation of methane by such organisms could explain the inability to enrich for atmospheric methane oxidizers by using methane as the sole substrate. However, the relatively high carbon conversion efficiency observed for atmospheric methane oxidizers (31 to 43%) is not consistent with direct methane assimilation by autotrophic nitrifiers. In general, nitrifiers assimilate only a minor fraction of the methane they cooxidize (15). An exception appears to be the marine nitrifier *Nitrosococcus oceanus*, which has been reported to assimilate 25 to 47% of the methane oxidized (38), although a substantially lower value (1 to 2%) has also been reported (15). Methane assimilation by nitrifiers may take place directly as an organic metabolite or at the level of CO_2 after complete oxidation of CH_4 . If CO_2 fixation was a significant mechanism for CH_4 assimilation in our forest soils, $^{14}\text{CH}_4$ -derived $^{14}\text{CO}_2$ would probably become substantially diluted by CO_2 already present in the soil environment (dilution by exogenous and/or endogenous CO_2). This dilution would likely result in lower methane assimilation and carbon conversion efficiency than observed in the present study. Furthermore, the long-term inhibition of atmospheric methane oxidation by ammonia addition is not consistent with what is currently known for ammonia-oxidizing bacteria (16, 31). For example, methane oxidation by nitrifiers is stimulated by low concentrations of ammonia not inhibited, whereas elevated methane concentrations inhibit rather than stimulate oxidation activity (15, 38). These findings are supported by recent analyses of the phospholipid fatty acid composition of atmospheric methane oxidizers, which suggest a limited role of nitrifiers in oxidation of atmospheric methane in forest soils (12, 27).

In conclusion, the present study demonstrates a capacity for bacterial growth on atmospheric methane. Our results suggest that oxidation and assimilation of atmospheric methane likely occur in the same organism and not in a microbial consortium. This implies that the responsible organism may be defined as a true methanotroph. However, factors other than the methane concentration per se appeared to regulate oxidation and growth potential. The pattern observed for soil methane metabolism was not consistent with the physiology of known

methane- or ammonia-oxidizing bacteria. This supports the idea that novel methanotrophic bacteria are responsible for oxidation of atmospheric methane in soils.

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REFERENCES

- Adamsen, A. P. S., and G. M. King. 1993. Methane consumption in temperate and subarctic forest soils: rates, vertical zonation, and response to water and nitrogen. *Appl. Environ. Microbiol.* **59**:485–490.
- Anderson, T.-H., and K. H. Domsch. 1985. Maintenance carbon requirements of actively-metabolizing microbial populations under in situ conditions. *Soil Biol. Biochem.* **17**:97–203.
- Anthony, C. 1986. Bacterial oxidation of methane and methanol. *Adv. Microb. Physiol.* **27**:113–210.
- Bender, M., and R. Conrad. 1992. Kinetics of CH₄ oxidation in oxic soils exposed to ambient air or high CH₄ mixing ratios. *FEMS Microbiol. Ecol.* **101**:261–270.
- Bender, M., and R. Conrad. 1994. Methane oxidation activity in various soils and freshwater sediments: occurrence, characteristics, vertical profiles, and distribution on grain size fractions. *J. Geophys. Res.* **99**:16531–16540.
- Castro, M. S., W. T. Peterjohn, J. M. Melillo, and P. A. Steudler. 1994. Effects of nitrogen fertilization on the fluxes of N₂O, CH₄, and CO₂ from soils in a Florida slash pine plantation. *Can. J. For. Res.* **24**:9–13.
- Conrad, R. 1984. Capacity of aerobic microorganisms to utilize and grow on atmospheric trace gases (H₂, CO, CH₄), p. 461–467. *In* M. J. Klug and C. A. Reddy (ed.), *Current perspectives in microbial ecology*. American Society for Microbiology, Washington, D.C.
- Conrad, R. 1995. Soil microbial processes involved in production and consumption of atmospheric trace gases. *Adv. Microb. Ecol.* **14**:207–251.
- Crill, P. M. 1991. Seasonal patterns of methane uptake and carbon dioxide release by a temperate woodland soil. *Global Biogeochem. Cycles* **5**:319–334.
- Dobbie, K. E., K. A. Smith, A. Priemé, S. Christensen, A. Degorska, and P. Orlanski. 1996. Effect of land use on the rate of methane uptake by surface soils in Northern Europe. *Atmos. Environ.* **30**:1005–1011.
- Hanson, R. S., and T. E. Hanson. 1996. Methanotrophic bacteria. *Microbiol. Rev.* **60**:439–471.
- Holmes, A. J., P. Roslev, N. Iversen, K. Henriksen, and J. C. Murrell. Unpublished data.
- Iversen, N., and T. H. Blackburn. 1981. Seasonal rates of methane oxidation in anoxic marine sediments. *Appl. Environ. Microbiol.* **41**:1295–1300.
- Jensen, S., A. Priemé, and L. Bakken. 1996. Methanol improves methane uptake in starved methanotrophic bacteria, p. 56. *In* Abstracts of substrate use for characterization of microbial communities in terrestrial ecosystems. University of Innsbruck, Innsbruck, Austria.
- Jones, R. D., and R. Y. Morita. 1983. Methane oxidation by *Nitrosococcus oceanus* and *Nitrosomonas europaea*. *Appl. Environ. Microbiol.* **45**:401–410.
- King, G. M. 1992. Ecological aspects of methane oxidation, a key determinant of global methane dynamics. *Adv. Microbiol. Ecol.* **12**:431–469.
- King, G. M. 1993. Ecophysiological characteristics of obligate methanotrophic bacteria and methane oxidation in situ. *In* J. C. Murrell and D. P. Kelly (ed.), *Microbial growth on C1 compounds*. Intercept, Andover, United Kingdom.
- King, G. M., and T. Bearman. 1984. Potential effects of isotopic dilution on apparent respiration in ¹⁴C heterotrophy experiments. *Mar. Ecol. Prog. Ser.* **19**:175–180.
- King, G. M., and S. Schnell. 1994. Effect of increasing atmospheric methane concentrations on ammonium inhibition of soil methane consumption. *Nature (London)* **370**:282–284.
- Koch, A. L. 1991. Quantitative aspects of cellular turnover. *Antonie van Leeuwenhoek* **60**:175–191.
- Kruse, C. W., and N. Iversen. 1995. Effect of plant succession, ploughing, and fertilization on the microbiological oxidation of atmospheric methane in a heathland soil. *FEMS Microbiol. Ecol.* **18**:121–128.
- Leak, D. J., and H. Dalton. 1986. Growth yields of methanotrophs. *Appl. Microbiol. Biotechnol.* **23**:477–481.
- Mosier, A., D. Schimel, D. Valentine, K. Bronson, and W. Parton. 1991. Methane and nitrous oxide fluxes in native, fertilized and cultivated grasslands. *Nature (London)* **350**:330–332.
- Nesbit, S. P., and G. A. Breitenbeck. 1992. A laboratory study of factors influencing methane uptake by soils. *Agric. Ecosyst. Environ.* **41**:39–54.
- Ohtonen, R. 1994. Accumulation of organic matter along a pollution gradient: application of Odum's theory of ecosystem energetics. *Microb. Ecol.* **27**:43–55.
- Reeburgh, W. S., S. C. Whalen, and M. J. Alperin. 1993. The role of methylophony in the global methane budget, p. 1–14. *In* J. C. Murrell and D. P. Kelly (ed.), *Microbial growth on C₁ compounds*. Intercept, Andover, United Kingdom.
- Roslev, P., N. Iversen, and K. Henriksen. Unpublished data.
- Roslev, P., and G. M. King. 1994. Survival and recovery of methanotrophic bacteria starved under oxic and anoxic conditions. *Appl. Environ. Microbiol.* **60**:2602–2608.
- Roslev, P., and G. M. King. 1995. Aerobic and anaerobic starvation metabolism in methanotrophic bacteria. *Appl. Environ. Microbiol.* **61**:1563–1570.
- Russel, J. B., and G. M. Cook. 1995. Energetics of bacterial growth: balance of anabolic and catabolic reactions. *Microbiol. Rev.* **59**:48–62.
- Schnell, S., and G. M. King. 1994. Mechanistic analysis of ammonium inhibition of atmospheric methane consumption in forest soils. *Appl. Environ. Microbiol.* **60**:3514–3521.
- Schnell, S., and G. M. King. 1995. Stability of methane oxidation capacity to variations in methane and nutrient concentrations. *FEMS Microbiol. Ecol.* **17**:285–294.
- Schnell, S., and G. M. King. 1996. Response of methanotrophic activity in soils and cultures to water stress. *Appl. Environ. Microbiol.* **62**:3203–3209.
- Steudler, P. A., R. D. Bowden, J. M. Melillo, and J. D. Aber. 1989. Influence of nitrogen fertilization on methane uptake in temperate forest soils. *Nature (London)* **341**:314–316.
- Steudler, P. A., R. D. Jones, M. S. Castro, J. M. Melillo, and D. L. Lewis. 1996. Microbial controls of methane oxidation in temperate forest and agricultural soils, p. 69–84. *In* J. C. Murrell and D. P. Kelly (ed.), *Microbiology of atmospheric trace gases*. Springer, Berlin, Germany.
- Striegl, R. G., T. A. McConnaughey, D. C. Thorstenson, E. P. Weeks, and J. C. Woodward. 1992. Consumption of atmospheric methane by desert soils. *Nature (London)* **357**:145–146.
- Voroney, R. P., J. P. Winter, and E. G. Gregorich. 1991. Microbe/plant/soil interactions, p. 77–99. *In* D. C. Coleman and B. Fry (ed.), *Carbon isotope techniques*. Academic Press, San Diego, Calif.
- Ward, B. B. 1990. Kinetics of ammonia oxidation by a marine nitrifying bacterium: methane as a substrate analogue. *Microb. Ecol.* **19**:211–225.
- Whalen, S. C., and W. S. Reeburgh. 1990. Consumption of atmospheric methane to subambient concentrations by tundra soils. *Nature (London)* **346**:160–162.
- Whalen, S. C., W. S. Reeburgh, and V. A. Barber. 1992. Oxidation of methane in boreal forest soils: a comparison of seven measures. *Biogeochemistry* **16**:181–211.
- Whalen, S. C., W. S. Reeburgh, and K. A. Sandbeck. 1990. Rapid methane oxidation in a landfill cover soil. *Appl. Environ. Microbiol.* **56**:3405–3411.
- Yawitt, J. B., D. M. Downey, G. E. Lang, and A. J. Sextstone. 1990. Methane consumption in two temperate forest soils. *Biogeochemistry* **9**:39–52.