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Mechanistic Analysis of Ammonium Inhibition of Atmospheric Methane Consumption in Forest Soils†

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Methane consumption by forest soil was studied in situ and in vitro with respect to responses to nitrogen additions at atmospheric and elevated methane concentrations. Methane concentrations in intact soil decreased continuously from atmospheric levels at the surface to 0.5 ppm at a depth of 14 cm. The consumption rate of atmospheric methane in soils, however, was highest in the 4- to 8-cm depth interval (2.9 nmol per g of dry soil per day), with much lower activities below and above this zone. In contrast, extractable ammonium and nitrate concentrations were highest in the surface layer (0 to 2 cm; 22 and 1.6 μmol per g of dry soil, respectively), as was potential ammonium-oxidizing activity (19 nmol per g of dry soil per day). The difference in zonation between ammonium oxidation and methane consumption suggested that ammonia-oxidizing bacteria did not contribute significantly to atmospheric methane consumption. Exogenous ammonium inhibited methane consumption in situ and in vitro, but the pattern of inhibition did not conform to expectations based on simple competition between ammonia and methane for methane monooxygenase. The extent of ammonium inhibition increased with increasing methane concentration. Inhibition by a single ammonium addition remained constant over a period of 39 days. In addition, nitrite, the end product of methanotrophic ammonia oxidation, was a more effective inhibitor of methane consumption than ammonium. Factors that stimulated ammonium oxidation in soil, e.g., elevated methane concentrations and the availability of cosubstrates such as formate, methanol, or β-hydroxybutyrate, enhanced ammonium inhibition of methane oxidation, probably as a result of enhanced nitrite production.

Consumption of atmospheric methane has been reported for a variety of soils (1, 4, 22, 37, 42, 44). Although soil is the only net sink for atmospheric methane, the factors regulating soil methane consumption are still poorly understood.

Nitrogen fertilization has a long-term inhibitory effect on soil methane consumption. Inhibition by ammonium can persist for several years (30), even after the ammonium concentration returns to background levels (31). The mechanism for ammonium inhibition of soil methane consumption remains uncertain. Pure cultures of methanotrophic bacteria cometabolize a variety of substrates, including ammonium, as a result of the broad substrate specificity of methane monooxygenase (2). The ability of methanotrophs to oxidize ammonium is well documented (8, 11, 33, 46). Kinetic studies indicate that methane-oxidizing bacteria have an affinity for ammonium comparable to that of ammonium-oxidizing bacteria; however, maximum ammonium oxidation rates are greater for ammonium-oxidizing bacteria (2). The capacity of methanotrophs to oxidize ammonium and of nitrifiers to oxidize methane (19, 21, 39, 41) has prompted questions about the identity of the organisms responsible for atmospheric methane oxidation and ammonia oxidation in situ. It has been suggested that nitrifiers actively oxidize atmospheric methane in soil (30, 37). On the other hand, Megraw and Knowles (27, 28) observed methane-dependent nitrate production in a cultivated humisol and reported that chemolithotrophic nitrification did not occur in this system. Thus, the interaction between ammonium and methane in soils is complex and involves substrate competition at an enzymatic level as well as various aspects of population dynamics.

We demonstrate here that ammonium inhibition of soil methane consumption increased with increasing methane concentrations, a pattern that contradicts a simple competition mechanism. Our results are consistent with a model in which methane stimulates ammonia oxidation by methanotrophs, with the resultant nitrite causing toxicity. In support of this interpretation, exogenous nitrite was a more effective inhibitor of methane consumption than ammonium. Several nonmethane substrates known to enhance ammonium oxidation by methanotrophs also increased the inhibitory effect of ammonium, providing further support for the model. In addition, depth profiles of methane, ammonium, nitrate, and potential activities of methane and ammonia oxidation indicated that populations of methanotrophs and nitrifiers were spatially distinct and that the inhibition of methane consumption by ammonium was a response of the methanotrophic bacteria, not the ammonium-oxidizing bacteria.

MATERIALS AND METHODS

Forest soil (pH 4) was collected with acrylic tubes (6.4-cm diameter, 30-cm length) from a mixed hardwood-conifer forest adjacent to the Darling Marine Center, Walpole, Maine. Details of the site and sample collection are given in King and Adamsen (23). Depth profiles of methane in intact soil and methane consumption rates at different soil depths were measured by the methods of Adamsen and King (1). For experiments with ammonium, nitrite, nitrate, or carbon additions, soil from a depth interval of 4 to 6 cm was collected and sieved (5-mm pore size) to remove stones and roots. If necessary, the soil was dried to a water content of approximately 25% before aqueous solutions of ammonium chloride, sodium nitrite, sodium nitrate, or organic substrates were sprayed on the soil and distributed throughout the sample by gently mixing. Equal volumes of water were added to all

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parallel assays so that the soil water content was between 20 and 30% (grams per gram dry weight [gdw]); deionized water was added to controls. For most experiments, 10 g of soil was placed into 120-ml glass jars that were sealed with butyl rubber stoppers.

A field experiment was conducted with two adjacent plots (3 by 1 m) that were established in a stand of oak and pine. One plot was fertilized by spraying it evenly with 6.25 liters of aqueous 11.4 mM ammonium chloride. This yielded a nitrogen application of 71 mmol m^{-2}, a level comparable to annual atmospheric deposition in areas subjected to agriculturally derived pollution (13) and to the higher fertilization treatment of Steudler et al. (37). The water amount was equivalent to 6 mm of rainfall. The second plot was treated similarly, with deionized water as a control. Within 2 to 3 h after spraying, polyvinyl chloride chambers (inner diameter, 11.5 cm) were deployed in each plot. The chambers were sealed with end caps modified for sampling ports and inserted to a depth of 17 cm, leaving a headspace of about 1 liter. During analysis of methane uptake rates, the chambers were stoppered and samples were removed by needle and syringe for analyses of methane content (for more details, see reference 24).

Methane was analyzed by gas chromatography by the method of King and Adamsen (23). Methane consumption rates for methane concentrations of >100 ppm were calculated from linear regressions of methane depletion over time. Since consumption at methane concentrations of <100 ppm was first order, uptake rates were estimated by linear regression of logarithmically transformed time course data.

For potential nitrification experiments, 5 g of soil was incubated at 30°C with 50 ml of a solution containing 20 mM ammonium chloride and 20 mM sodium chlorate in 250-ml Erlenmeyer flasks that were shaken at 150 rpm. Ammonia oxidation by *Methylosinus trichosporium* OB3b was measured in cell suspensions of 300 μg of protein per ml in 100 mM phosphate buffer (pH 7.0). For some assays, 10 mM β-alanine, 0.01% peptone, or 100 μM N-Serve was added as an aqueous solution or 10% methane was injected into the gas phase. Nitrite was determined colorimetrically by the method of Greenberg et al. (14). Nitrate was measured after reduction to nitrite with a cadmium test kit (Hach Co.). To minimize humic acid interference with nitrite analyses, subsamples of the slurries (700 μl) were acidified with 2 M hydrochloric acid (350 μl), treated with polyvinylpolypyrrolidone, shaken overnight, and centrifuged to sediment the polyvinylpolypyrrolidone. Ammonium in soil was measured spectrophotometrically after extraction with 2 M KCl for 24 h (7).

RESULTS

Depth profiles of methane and methane consumption. Methane concentrations in situ decreased with increasing soil depths (Fig. 1). The initial methane decrease for samples collected on 31 August 1992 was 0.2 ppm cm^{-1} from the surface to 4 cm. Below 4 cm, methane decreased at 0.04 ppm cm^{-1}. The highest in vitro methane consumption rates were measured in the 4- to 8-cm depth interval (1.6 nmol of methane per gdw per h); above and below that depth, methane consumption rates were much lower (Fig. 1). Surface-based uptake rates of <1.6 mg of methane per m^2 per day were measured in situ with static chambers. Rates estimated from the methane gradient in the upper 4 cm of soil were 3.3 mg of methane per m^2 per day (using a D_s of 0.033 cm^2 s^{-1} [26]). Methane uptake was optimal in soils with a water content of 20 to 30% (grams per gdw; data not shown).

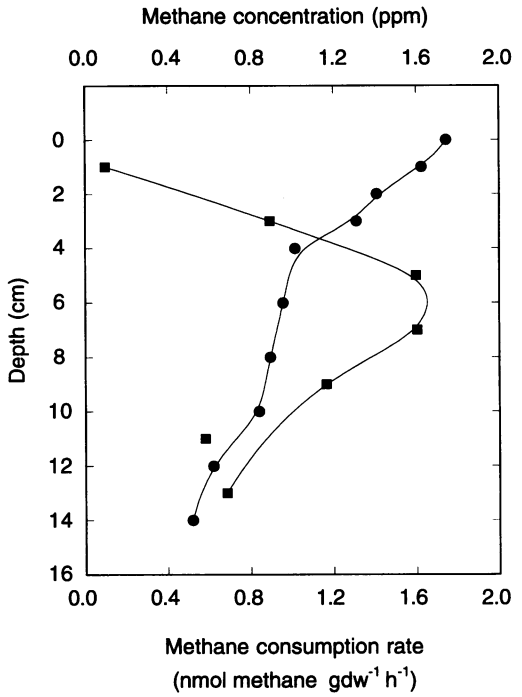

FIG. 1. Profile of methane concentration (●) in forest soil taken 31 August 1992, and in vitro methane-oxidizing activity (■) with atmospheric methane concentration in forest soil incubated at 22°C.

Depth profiles of ammonium, nitrate, and potential ammonia oxidation. Ammonium and nitrate concentrations in the soil were both highest at the surface (0 to 2 cm; 22 μmol of NH_4^+ per gdw and 1.6 μmol of NO_3^- per gdw) and decreased exponentially with depth (Fig. 2A). Nitrite was never detected in any sample (detection limit, <1 nmol/gdw). The highest rates for potential ammonia oxidation in slurry incubations at in situ pH (4 to 5) were also measured in the surface layer (Fig. 2B). Rates for potential ammonia oxidation decreased exponentially with depth from 19.5 nmol/gdw/day at the surface interval of 0 to 2 cm to 0.9 nmol/gdw/day at 10 to 12 cm of soil depth. Only nitrate was observed from ammonia oxidation; nitrite was never detected. No nitrate or nitrite production occurred in phosphate-buffered soil slurries at pH 7.5 within 35 days.

Addition of β-alanine, peptone, or N-Serve to soil slurries did not affect nitrate production. Also, methane in the gas phase did not affect nitrate production within 21 days. A cell suspension of *Methylosinus trichosporium* OB3b added to soil slurries showed a nitrite production rate of 0.7 nmol/min/mg of protein. The same ammonia oxidation rate was measured in cell suspensions without soil added, indicating that the soil per se did not inhibit the nitrification activity of *M. trichosporium* OB3b.

Effect of nitrogen addition on methane oxidation. Both ammonium and nitrite additions to soil inhibited atmospheric methane consumption, but nitrite was more potent (Fig. 3). At 1 μmol of ammonium per gram fresh weight (gfw) of soil, methane uptake was inhibited by 41.8%; nitrite at the same concentration inhibited uptake by 58.7%. Nitrate additions (2 μmol/gfw of soil) had no effect. Nitrite addition to soil showed a biphasic kinetic of methane consumption, with very slow initial methane consumption rates. This lag time increased with increasing nitrite concentrations from 0.05 to 1 μmol/gfw of soil (Fig. 4A) but was not observed at ≤0.05 μmol/gfw of

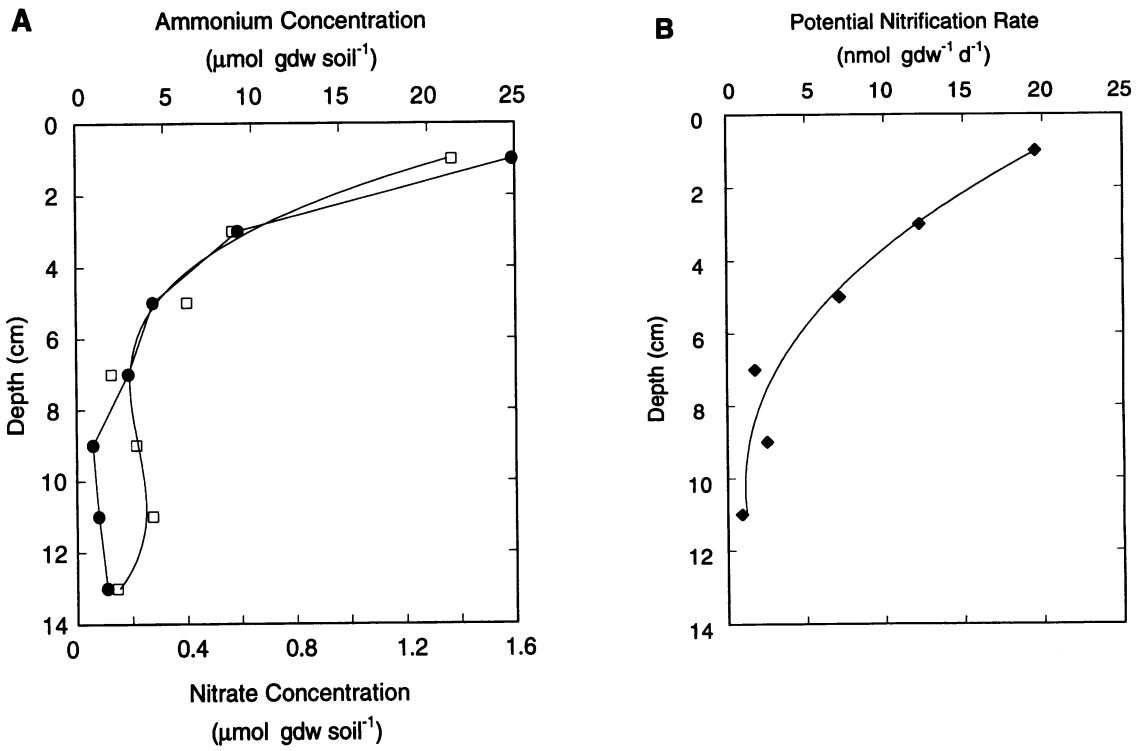

FIG. 2. (A) Profile of KCl-extractable ammonium (□) and water-extractable nitrate (●). (B) Profile of potential ammonia-oxidizing activity measured in soil slurries.

soil. After the lag time, methane consumption rates became first order, but rate constants decreased progressively with increasing nitrite concentrations, from 0.01 to 1 μmol/gfw of soil (Fig. 4B).

Methane uptake rates for both water- and nitrite-treated soils increased with increasing methane concentrations, and the inhibitory effect of nitrite decreased (Fig. 5). However, the inhibitory effect of ammonium increased continuously with increasing methane concentrations, from 1.4 ppm to 1.1% methane. The methane uptake rates of ammonium-treated soils increased much more slowly with increasing methane concentrations from 1.4 to 430 ppm than those of water-treated control soils (Fig. 6), and the ratios of rates from the ammonium- and water-treated soils decreased. At methane concentrations of ≥430 ppm, rates of methane uptake decreased in the ammonium-treated soil; no methane uptake was detectable at 1% methane within 3 days. Consequently, the inhibition by ammonium increased from 59% at 1.4 ppm methane to >99% at 1.1% methane (Fig. 7).

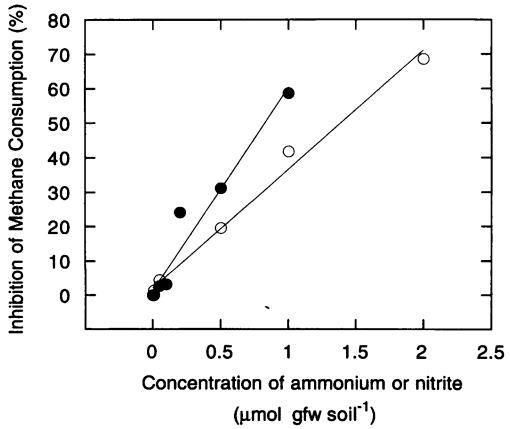

FIG. 3. Inhibition of atmospheric methane consumption by ammonium (○) and nitrite (●) addition (both 1 μmol/gfw of soil). Inhibition is expressed by the ratio of the rate constants of methane uptake by ammonium- or nitrite-treated soil and the rate constants of the water controls.

A single treatment with ammonium had a long-term inhibitory effect on soil methane consumption. In soil samples treated with 1 μmol of ammonium per gfw of soil inhibition of atmospheric uptake rates was unchanged within 39 days (Fig. 8B), even though the uptake rates of both water- and ammonium-treated soils decreased over that time period because of desiccation (Fig. 8A). In a field experiment, the uptake rates for atmospheric methane of chambers in the water-treated control plot and the ammonium-treated plot increased slightly over a time period of 12 days, and inhibition of methane uptake in the ammonium plot stayed constant (Fig. 8C and D).

The addition of organic substrates had a minor effect on methane consumption at atmospheric methane concentrations. However, the addition of both organic substrates and ammonium inhibited methane oxidation to an even greater extent than did ammonium alone (Table 1). For example, the addition of methanol and ammonium (1 μmol of each per gfw of soil) inhibited methane uptake by 71%, compared with 38% inhibition for ammonium only. Formate and β-hydroxybutyrate also enhanced the inhibitory effect of ammonium by 19 and 17%, respectively, compared with inhibition by ammonium only.

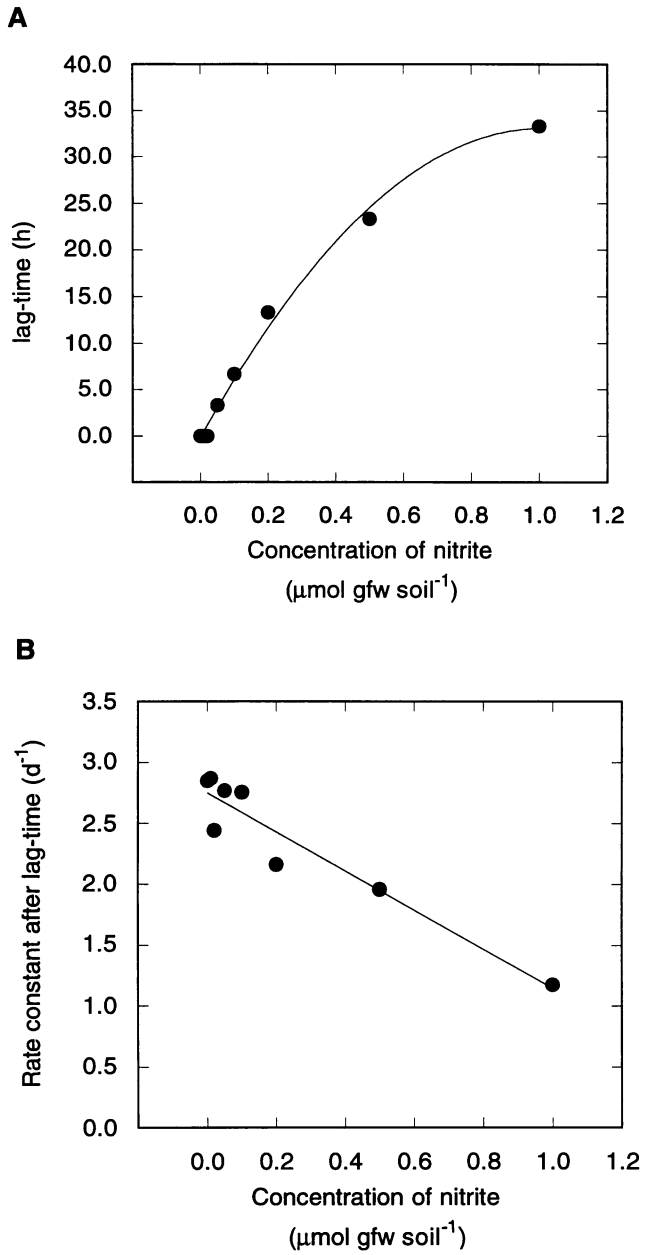

FIG. 4. Inhibition of atmospheric methane consumption by nitrite addition. (A) Lag time of methane consumption at nitrite concentrations between 0.01 and 1 μmol/gfw of soil. (B) Rate constants of methane consumption after the initial lag phase at various nitrite concentrations.

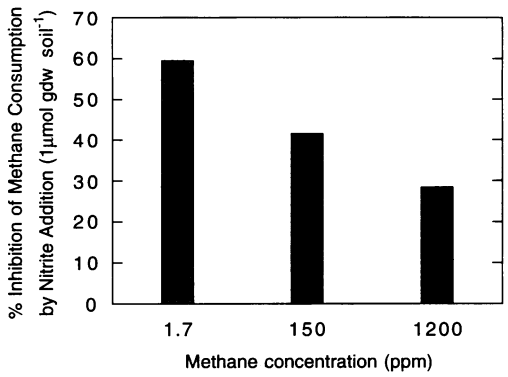

FIG. 5. Inhibition of methane consumption by nitrite (1 μmol/gfw of soil) at three different methane concentrations (ambient [1.7 ppm] plus 150 and 1,200 ppm). Inhibition is expressed by the ratio of rates with and without nitrite.

DISCUSSION

Maximal methane-consuming activity is typically found in the subsurface mineral soil layer of the A horizon (1, 5, 26) (Fig. 1), where methane concentrations are usually less than atmospheric levels. The subsurface localization of atmospheric methane consumption indicates that edaphic factors in or near the surface are suboptimal or even inhibitory. Were this not the case, maximal activities proximate to the highest methane concentrations would be expected. Low activity in surface soils might result from inhibitory concentrations or fluxes of ammonium, nutrient limitation due to competition between methanotrophs and other microbes, or rates of bacterivory that exceed methanotrophic growth rates. At present, the roles of these factors are unknown, although it is evident that the highest ammonium concentrations often occur in the soil surface (e.g., Fig. 2A) (36), which is the locus of atmospheric ammonium deposition. In contrast to temperate forests and grasslands, depth profiles of Alaskan tundra (42) show highest activity at the soil surface; a comparison of these various systems could provide important insights about the regulation of the distribution of atmospheric methane consumption. Irrespective of the causal factor(s), one of the important consequences of subsurface zonation is that diffusion limits the rate of atmospheric methane consumption (5, 23). This implies that the determinants of the depth distribution of methanotrophs regulate the magnitude of the global soil methane sink.

Unlike the profiles for atmospheric methane consumption, potential ammonia oxidation rates were maximal at the soil surface (Fig. 2), where concentrations of ammonium and nitrate are greatest because of organic matter mineralization and deposition of ammonium from wet and dry precipitation (32). In our study, nitrate was the only product from ammonia oxidation, even when soil was incubated with chlorate, which inhibits nitrite oxidation (3). The absence of nitrite is consistent with heterotrophic nitrification, a process that is insensitive to chlorate (35). During heterotrophic nitrification, organic nitrogen sources, such as β-alanine and peptone, are metabolized to nitrate. This process occurs in fungi, e.g., *Aspergillus flavus* (35) and *Absisia cylindrospora* (38), and in some bacteria (27, 28, 34, 40). However, the addition of neither β-alanine, peptone, nor methane stimulated nitrate production in our assays. As a result, there is some uncertainty about both the populations and process(es) that oxidize ammonium in the soils described here. Nitrate production could be explained by a nonenzymatic oxidation of nitrite by manganese oxides (38), but such a process seems of doubtful significance for the upper, organic horizons. Irrespective of these uncertainties, the ammonium oxidation patterns indicate that ammonia-oxidizing bacteria play a minor role, if any, in atmospheric methane consumption in the Darling Marine Center forest soils. On the other hand, ammonia-oxidizing bacteria may play a more significant role in agricultural soils (18) or forest soils that are subjected to regular ammonium fertilization (9). Ammonium inputs might not only inhibit methanotrophs, but enhance populations of ammonium-oxidizing bacteria as well.

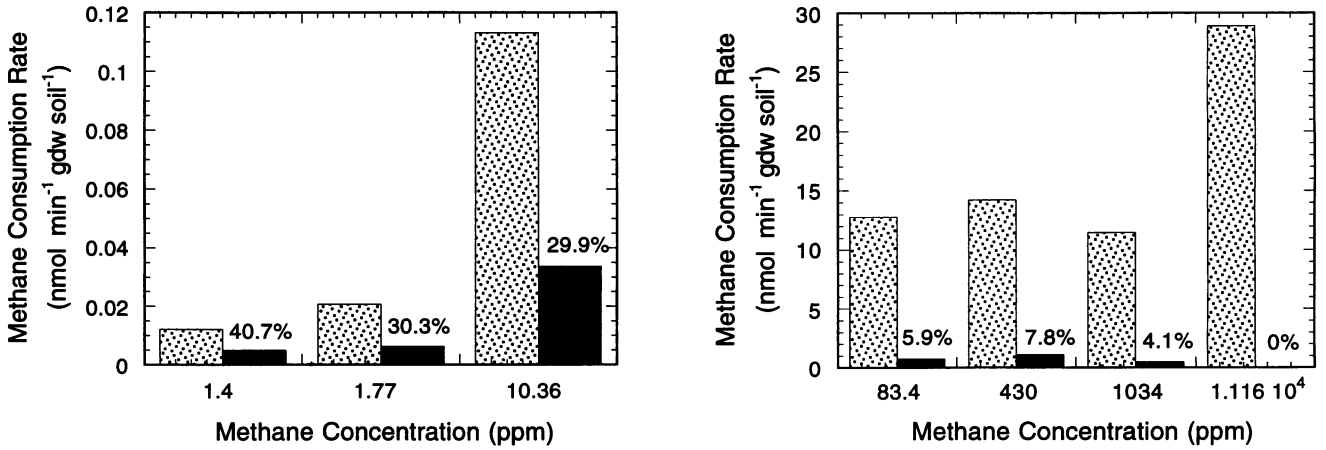

FIG. 6. Comparison of rates for methane consumption in water-treated (stippled bars) and ammonium-treated (solid bars) soils (1 μmol/gfw) at methane concentrations between 1.4 ppm and 1.1%. The ratios of rates from ammonium-treated and water-treated soils are given above the bars.

Soil methane consumption was rapidly inhibited by ammonium additions of ≥10 nmol/gfw of soil (Fig. 3). The extent of ammonium inhibition progressively increased with increasing methane concentrations from 1.4 ppm to 1.1% (Fig. 6 and 7). The absolute rates of methane uptake increased with increasing methane in both control and ammonium-treated soils, but the changes in absolute rates were less in the ammonium-treated soils. The addition of substrates metabolized by methanotrophs, e.g., methanol, formate, and β-hydroxybutyrate, stimulated methane consumption slightly compared with untreated controls. However, when added simultaneously with ammonium, inhibition of methane consumption was much greater than that caused by ammonium alone, e.g., the addition of methanol and ammonium increased the inhibition by 33% (Table 1).

These responses are consistent with the results of O'Neill and Wilkinson (33), who showed that ammonium oxidation by *M. trichosporium* OB3b was stimulated by substrates that were co-oxidized. King and Schnell (25) have also shown that methane enhances ammonium oxidation by *M. trichosporium* OB3b and *Methylobacter albus* and that inhibition of methane uptake by ammonium is dependent on the methane concentration. The ability of methanotrophic substrates to enhance ammonium oxidation and to increase ammonium inhibition can be explained by the requirement for methane monooxygenase (MMO) activity for reductant. The oxidation of methanol, formaldehyde, formate, and β-hydroxybutyrate generates NADH plus H^+ and thereby increases the reductant availability, which facilitates ammonium oxidation by MMO. This is evident in cultures of *M. albus* and *M. trichosporium*, where increasing methane concentrations between 1.7 and 1,000 ppm increased nitrite production from ammonium oxidation (25).

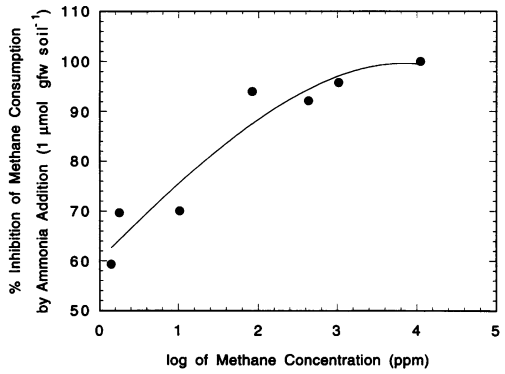

FIG. 7. Inhibition of soil methane consumption by ammonium addition (1 μmol/gfw of soil) as a function of increasing methane concentration. Note the logarithmic scale for methane concentration.

The relationship between ammonium inhibition and methane concentrations reported here for soils and the relationships reported elsewhere for cultures (25) indicate that simple kinetic models based on competitive interactions between substrates are insufficient for describing the observed patterns. Both the intermediate and final products of ammonium oxidation can inhibit methane consumption. Hydroxylamine inhibits MMO activity (17), while nitrite can affect metabolism generally and formate dehydrogenase activity specifically (20, 33, 45). Inhibition of formate dehydrogenase by nitrite is particularly important since this enzyme is a key source of NADH plus H^+. Exogenous nitrite appears to be a more potent inhibitor of soil atmospheric methane consumption than ammonium (Fig. 3). Nitrite-treated soils not only show lower methane consumption rates than ammonium- or water-treated soils, but also show a lag time for methane consumption which increases with increasing nitrite concentrations (Fig. 4). However, nitrite inhibition decreases with increasing methane concentrations (Fig. 5), while ammonium inhibition increases (Fig. 6 and 7). The different response patterns of ammonium and nitrite inhibition might be due in part to the fact that exogenous nitrite is less effective as an inhibitor than nitrite generated endogenously from ammonium oxidation.

Nitrite and perhaps hydroxylamine must play a role not only in the relationship between ammonium inhibition and methane concentrations, but also in the persistence of the inhibitory effect. The observed increasing lag time of atmospheric methane consumption with increasing nitrite concentrations (Fig. 4) could reflect a detoxification mechanism by methanotrophic bacteria and/or other soil bacteria (e.g., nitrifiers and denitrifiers). Another possible sink for nitrite in acidic soil could be the disproportionation of nitrous acid into nitric acid and NO. However, in our soil with a pH of 4 to 4.5, the loss of nitrite is not expected to be large, as the pK_a for the nitrite-nitrous acid

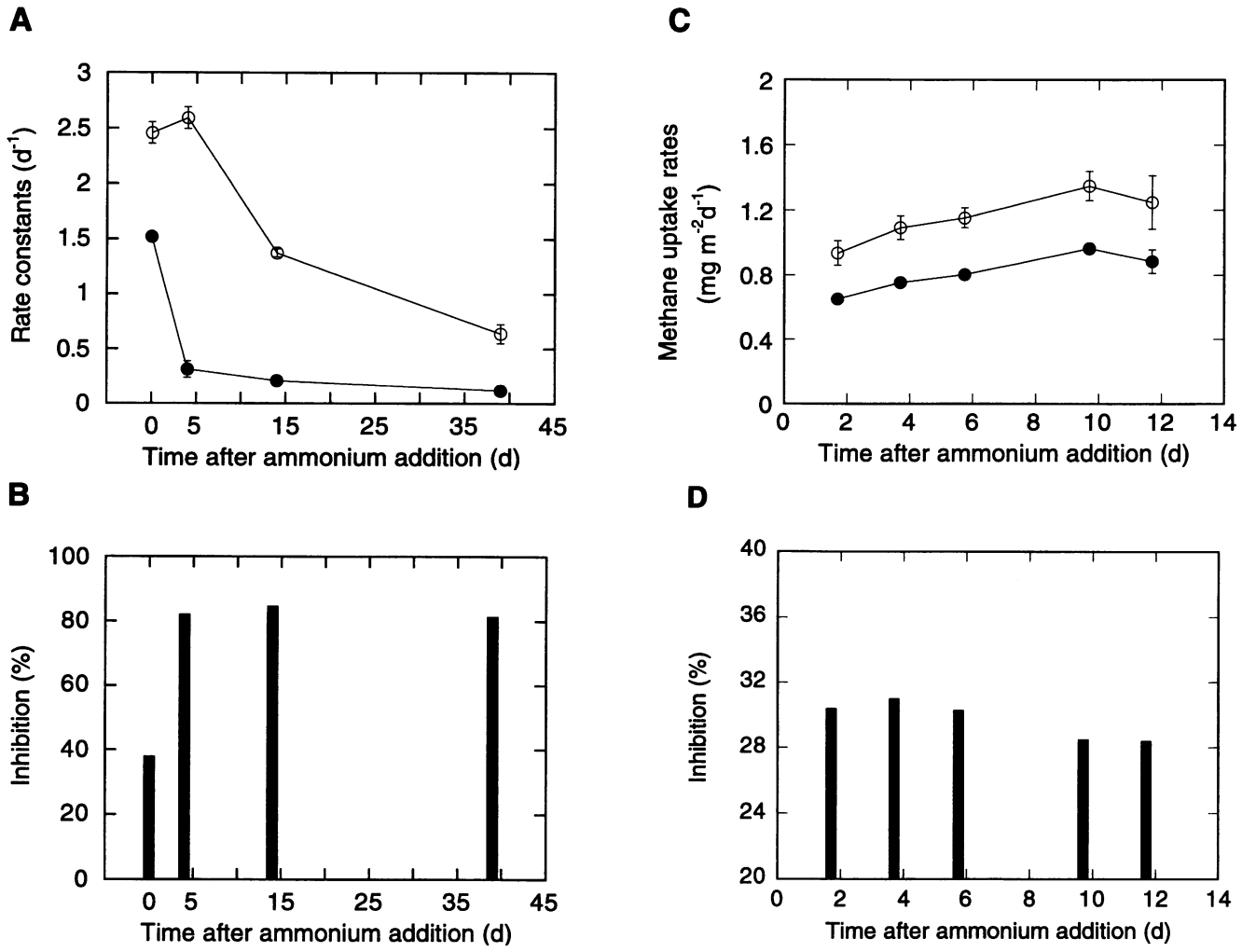

FIG. 8. Persistence of ammonium inhibition. (A) Ammonium chloride was added to fresh soil as an aqueous solution (1 μmol/gfw of soil); uptake of atmospheric methane was monitored for 39 days (●) and compared with that by water-treated control soils (○). (B) Inhibition of ammonium for the time points in panel A, calculated as [1 − (*K* for ammonium/*K* for control)] × 100. (C) In situ uptake rates of atmospheric methane in a water-treated control plot (○) and an ammonium-treated plot (●) over a period of 12 days. (D) Inhibition of methane consumption in situ by a single treatment with ammonium, expressed as in panel B.

couple is 3.4. In vitro assays by Nesbit and Breitenbeck (31) and field studies by Mosier et al. (30) show that inhibition from a single application of ammonium can persist for days to months, even after the added ammonium is no longer detectable. In our study, ammonium-induced inhibition persisted unabated for 12 days under in situ conditions and 39 days during an in vitro incubation (Fig. 8). These results, and similar observations by others, are best explained by processes other than substrate competition at the level of MMO; the most likely processes involve hydroxylamine or nitrite toxicity. Although the inhibitory effects of these compounds appear minimal or are reversible at high methane concentrations in cultures and soil (e.g., Fig. 5), the very low rates of atmospheric methane uptake rates by soil even in the absence of inhibitors may substantially limit the ability of methanotrophs to recover from physiological stresses or cell damage. Regardless of the mechanisms involved, the persistence of ammonium-related inhibition is an important phenomenon, since short-term anthropogenic disturbances (e.g., ammonium fertilization) have long-term consequences.

Ammonium inhibition could play an important role as a regulating factor for methane consumption in situ. In unfertilized soils, ammonium concentrations between 1 and 20 μmol/g have been reported (12, 29, 36) (Fig. 2). Though much of this ammonium is adsorbed and not in the soil solution per se, dissolved ammonium concentrations must exceed dissolved methane concentrations, which are about 2.2 nM in equilibrium with gas phase methane concentrations of 1.7 ppm, by at least 10- to 1,000-fold. The substantial concentration difference between ammonium and methane in situ, together with the similar affinities of methanotrophs for both substrates (2), strongly suggest that ammonium is an important cosubstrate for methane oxidizers in soil. However, ammonium oxidation by soil methanotrophs is not energy yielding but results in inhibition of methane oxidation due to substrate competition for the MMO and secondary toxic effects of the ammonia oxidation products. Inhibition of methane consumption by ammonium has been reported for fertilized forest soils (9, 25, 30, 31, 37) and for agricultural soil (18) but may also occur in undisturbed soil. The subsurface distribution of methane con-

TABLE 1. Rate constants for atmospheric soil methane consumption in the presence of ammonium with and without organic substrates

Substrate	K (day^{-1})	% of control activity	% Inhibition by ammonium[a]
Water (control)	2.46	100	
Ammonium	1.52	62	38
Methanol	3.04	124	
Methanol + ammonium	0.88	36	71
Formate	3.11	126	
Formate + ammonium	1.34	54	57
β-Hydroxybutyrate	2.26	92	
β-Hydroxybutyrate + ammonium	1.01	41	55

[a] Inhibition of methane consumption by ammonium in the presence of an organic substrate calculated as [1 − (K for substrate plus ammonium/K for substrate)] × 100. All substrates were added at 1 μmol/gfw of soil.

sumption (1, 5, 26) (Fig. 1) may reflect this naturally occurring inhibition. Methane fluxes from aquatic systems are also partly controlled by interactions between ammonium and methane, since ammonium concentrations in the zone of active methane consumption (1 to 100 μM [15, 16]) equal or exceed methane concentrations. Inhibition of methane oxidation by ammonium has also been reported for the surface soil of a flooded rice field (10) and the surface layer of a littoral sediment (6).

In conclusion, ammonium inhibition of atmospheric methane consumption by soils is a complex process, probably involving competitive interactions between ammonium and methane for MMO, as well as toxic effects resulting from hydroxylamine and nitrite. The depth distribution of ammonium oxidation and atmospheric methane consumption in soils, as well as the similar response of soils and pure cultures of methanotrophs to exogenous ammonium, indicates that the primary agents for methane consumption in undisturbed soils are methanotrophic bacteria. In addition, increased susceptibility of soil methane consumption to ammonium inhibition with increasing methane concentrations indicates that anthropogenic disturbances resulting in increased atmospheric methane and increased ammonium deposition in soils have both affected the soil methane sink in the past and will likely do so for the foreseeable future.

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