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Kinetics of Inhibition of Methane Oxidation by Nitrate, Nitrite, and Ammonium in a Humisol

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The kinetics of inhibition of CH_4 oxidation by NH_4^+, NO_2^-, and NO_3^- in a humisol was investigated. Soil slurries exhibited nearly standard Michaelis-Menten kinetics, with half-saturation constant [$K_{m(app)}$] values for CH_4 of 50 to 200 parts per million of volume (ppmv) and V_{max} values of 1.1 to 2.5 nmol of CH_4 g of dry soil^{-1} h^{-1}. With one soil sample, NH_4^+ acted as a simple competitive inhibitor, with an estimated K_i of 8 μM NH_4^+ (18 nM NH_3). With another soil sample, the response to NH_4^+ addition was more complex and the inhibitory effect of NH_4^+ was greater than predicted by a simple competitive model at low CH_4 concentrations (<50 ppmv). This was probably due to NO_2^- produced through NH_4^+ oxidation. Added NO_2^- was inherently more inhibitory of CH_4 oxidation at low CH_4 concentrations, and more NO_2^- was produced as the CH_4-to-NH_4^+ ratio decreased and the competitive balance shifted. $NaNO_3$ was a noncompetitive inhibitor of CH_4 oxidation, but inhibition was evident only at >10 mM concentrations, which also altered soil pHs. Similar concentrations of NaCl were also inhibitory of CH_4 oxidation, so there may be no special inhibitory mechanism of nitrate per se.

Methane (CH_4) is an important greenhouse gas that is increasing in atmospheric concentration (13). Much interest has focused on the role of aerobic soils as a sink for CH_4 and on the ecological and land use practices that affect its magnitude (26). Field studies have shown that fertilization with nitrogen, especially in the form of ammonium (NH_4^+) or urea, can reduce CH_4 oxidation rates in soils (5, 10, 15, 19, 24, 28, 30) and sediments (4). In some cases, this is a long-term effect of repeated fertilizer applications rather than an immediate inhibition of methanotrophic bacteria or the methane monooxygenase (MMO) enzyme (17, 30). However, fertilization can also have an immediate effect on CH_4 oxidation in the field (5, 10, 15, 19). In laboratory incubations of soils and sediments, inhibition is caused by NH_4^+ (1, 6, 9, 19, 25, 28), nitrite (NO_2^-) (19, 28), and high (>10 mM) concentrations of nitrate (NO_3^-) (1, 25).

MMO can oxidize a variety of substrates besides CH_4; these should therefore compete with CH_4 for the active site of this enzyme. One such cosubstrate is ammonia (NH_3), which is oxidized to NO_2^- via hydroxylamine (11). Pure culture studies with *Methylococcus capsulatus* (7), *Methylomonas methanica* (14), and *Methylosinus trichosporium* (27) have shown that NH_3 acts as a competitive inhibitor of CH_4 oxidation. Two of these studies (7, 27) noted a significant pH effect on the K_i measured as the NH_4^+ concentration, but the K_i was more constant if NH_3 rather than NH_4^+ was considered to be the inhibitor. The competition between NH_3 and CH_4 for the active sites of MMO in methanotrophs and of ammonia monooxygenase in nitrifiers has led to speculation regarding the contribution of nitrifiers to CH_4 oxidation and of methanotrophs to nitrification in natural environments (2, 31, 32).

Inhibition patterns that are more complex than simple enzymatic competition between CH_4 and NH_3 are occasionally evident in methanotrophs, for example, in low-copper *Methylococcus capsulatus* cells (7). The requirement of MMO for cosubstrates oxygen and NADH can complicate the interpretation of NH_3 inhibition (2). The addition of CH_4 may stimulate rather than inhibit NH_3 oxidation by methanotrophs (20, 21, 23, 27, 28), presumably because of alleviation of NADH limitation. Hydroxylamine (16) and NO_2^- (18, 20, 27) produced through methanotroph oxidation of NH_3 are themselves inhibitors of methanotrophic activity. In a forest soil, the inhibitory effect of NO_2^- was shown to be greater and more enduring than the direct effect of NH_3 (19, 28).

The half-saturation constant [$K_{m(app)}$] for soil CH_4 oxidation of 30 to 50 nM (3) is several orders of magnitude lower than the values (1 to 66 μM) for pure cultures of methanotrophs (2). Known methanotrophs should not be capable of surviving solely on atmospheric CH_4 (8). Those methanotrophs active in aerobic soils might therefore employ a CH_4-oxidizing system unlike that of known methanotrophs. Two forms of MMO, one particulate and one soluble, are known. Although these exhibit different substrate affinities, neither approaches the $K_{m(app)}$ measured in soils (2, 3). This paper presents some experiments on the kinetics of CH_4 oxidation in a humisol designed to examine the competitive strength of NH_3 and to determine the amounts of NH_4^+, NO_2^-, and NO_3^- needed to cause inhibition of CH_4 oxidation.

MATERIALS AND METHODS

The study site, a humisol on the Central Experimental Farm of Agriculture Canada in Ottawa, Canada, has been described previously (12). Soil samples were taken from a depth of 0 to 20 cm on 17 August 1993 and 30 June 1994 and stored at 12°C. The 1993 soil sample was stored for up to 10 months before experiments were performed. The 1994 soil sample was stored for no more than 40 days. Although NH_3 rather than NH_4^+ is thought to be an inhibitor of MMO, we shall refer primarily to measured NH_4^+ concentrations. When given, NH_3 concentrations have been calculated on the basis of average pH values of 6.6 and 6.9 for 1993 and 1994 soil samples, respectively.

To investigate NH_4^+ inhibition of CH_4 oxidation, aggregates were broken by hand and a 1:3 ratio of field-moist soil (81% H_2O, 1993 sample; 136% H_2O, 1994 sample) to distilled deionized H_2O was homogenized 30 min on a magnetic stirrer. Fifteen-milliliter portions (2.5 to 3.1 g of dry soil) were distributed into 60-ml serum vials, and diluted NH_4Cl solutions were added to give final volumes of 20 ml. Of the NH_4^+ added, 60 to 70% was not recoverable from the slurry liquid phase shortly after addition and was assumed to be held on exchange sites. CH_4 concentrations of approximately 2, 30, 75, 100, 150, 200, 300, 400, 500, and

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TABLE 1. Effects of added NH_4Cl on the kinetic coefficients of CH_4 oxidation in humisol slurries

Soil sample and experiment no.	NH_4^+ (μM)[a]	NO_2^- (μM)[a]	Hyperbolic model[b]				Hill model[b]		
			CH_4 range (ppmv)	No. of CH_4 levels	$K_{m(app)}$ (ppmv)	V_{max} (nmol g^{-1} h^{-1})	n_{app}	K_s^* (ppmv)	V_{max} (nmol g^{-1} h^{-1})
1993 soil									
1	12.7	NT[c]	15–730	9	189	1.65	1.06	150	1.47
	20.8[d]	NT	35–730	8	349	2.06	1.19	174	1.42
	34.3	NT	40–730	8	413	1.81	1.22	340	1.21
	94.2	NT	70–730	7	1,016	2.01	1.20	464	1.25
2	16.5	NT	15–730	9	184	2.79	1.05	144	2.22
	35.2[d]	NT	15–730	9	377	2.99	1.12	178	1.89
	47.9	NT	65–730	7	579	3.36	1.14	328	2.47
	121	NT	65–730	7	982	2.89	1.37	409	1.83
3	26.6	29.8	45–750	9	111	1.38	0.91	101	1.31
	57.5	51.6	45–750	9	275	1.35	1.07	256	1.29
	136	65.4	45–750	9	991	2.03	1.23	623	1.17
1994 soil									
4[e]	20.4	20.9	30–450	8	88.7	1.51			
	54.6	54.2	30–450	8	316	1.29			
	138	78.1	50–450	7	370	0.71			
5[e]	27.7	28.0	40–550	9	60.2	1.31			
	52.2	52.7	40–550	9	173	1.27			
	96.2	53.2	70–550	8	153	0.79			
6	24.1	30.8	15–550	16	86.3	1.61	1.16	60.2	1.37
	70.3	48.4	40–550	13	172	1.13	1.39	97.6	0.85
7	19.5	15.8	15–550	14	107	1.64	1.27	56.1	1.22
	48.8	42.3	40–550	11	144	1.16	1.62	68.7	0.80
8	3.4	4.1	15–350	13	86.5	1.48	1.12	63.5	1.28
	94.4	42.2	60–350	10	370	1.52	1.53	119	0.77

[a] NH_4^+ and NO_2^- concentrations are averages of concentrations at the start and end of each incubation.
[b] Kinetic constants were estimated by using the linear portion of Lineweaver-Burk plots for the Michealis-Menten hyperbolic model or by fitting the data to the Hill cooperative model (see reference 29).
[c] NT, not tested.
[d] Added NH_4^+ was completely consumed by the end of the experiment and was not used in K_i calculations or statistics.
[e] The Hill model could not be applied to experiments 4 and 5 because of high data variability.

700 parts per million of volume (ppmv) were added to duplicate vials and incubated on a gyratory shaker at 250 rpm and 25°C. For each experiment, a complete set of CH_4 concentrations was run for control soil samples (no added NH_4^+) and up to three levels of added NH_4^+. Specific details of each experiment are given in Table 1.

Three more rigorous experiments (no. 6, 7, and 8) with the 1994 soil sample used initial CH_4 concentrations of approximately 2, 5, 10, 15, 20, 30, 40, 50, 60, 80, 100, 125, 150, 175, 200, 250, 300, 350, 400, and 500 ppmv (the two highest levels were omitted in experiment 8). Slurries were shaken for 20 to 24 h before NH_4Cl and CH_4 addition in an attempt to alleviate two problems noted in earlier trials. First, slurrying caused transiently elevated NO_2^- levels which could be depleted through nitrification in preincubation. Second, initial net CH_4 production rather than consumption was occasionally observed at 2-ppmv CH_4. This soil has considerable methanogenic capacity (12), and although no methanogenesis should occur in diluted aerobic slurries, a short period of net CH_4 production may result from equilibration of CH_4 already in the soil, perhaps hydrophobically bound to organic matter, with the gas phase of vials.

CH_4 levels were measured at 1 to 1.5 and 5.5 to 6 h after the addition of NH_4Cl and CH_4 by injections of 2-ml headspace volumes into a Shimadzu gas chromatograph and flame ionization detector equipped with a 1.8-m Porapak Q column and 0.5-ml sample loop. At the same times, 1-ml slurry samples were taken into microcentrifuge tubes and frozen immediately. In calculating CH_4 oxidation rates, compensation was made for the removal of gas during sampling. Vials containing 20 ml of H_2O and several CH_4 concentrations were included as checks on standardization and leakage. In the event of nonzero CH_4 oxidation rates calculated for these vials, linear regression of the calculated rate versus CH_4 concentration was used to correct soil CH_4 oxidation rates.

Inhibition by $NaNO_3$ or $NaNO_2$ was tested by essentially the same procedure. Incubations for experiments with NO_2^- and NH_4^+ supplements were limited to 5 to 6 h because nitrification rapidly depleted these ions, but NO_3^- concentrations remained constant and thus incubation times could be longer. The possibility of phase transfer limitation was examined with triplicate 20-ml slurries containing 1, 2, 3, or 4 g of 1993 soil at 100-ppmv CH_4. To test the reversibility of NO_2^- and NH_4^+ inhibition, vials from representative NH_4^+ and NO_2^- experiments were incubated until all added NO_2^- or NH_4^+ was oxidized and then CH_4 oxidation was retested.

For nitrogen analyses, slurry samples in microcentrifuge tubes were thawed by centrifugation for 5 min at 13,800 $\times$ *g*, resuspended by manual shaking, and centrifuged again for 15 min. NO_3^-, NO_2^-, and NH_4^+ levels in the supernatant were measured colorimetrically by using an automated analysis system (Chem-Lab Instruments, Hornchurch, Essex, England) (23). Initial concentrations were estimated from 2-ppmv CH_4 vials only (this was valid since the CH_4 concentration did not affect the nitrification rate [see Results]). Final concentrations were the averages of at least six vials. pH values were not affected by experimental NH_4^+ additions.

The CH_4, NH_4^+, NO_3^-, and NO_2^- concentrations presented and used in kinetic calculations are the arithmetic means of measurements at the beginning and end of each incubation. The arithmetic mean is an overestimate of the actual average CH_4 concentration over this time, but since maximum CH_4 depletion is 17%, the error incurred should be minor (29). All kinetic and inhibition coefficients were estimated as described by Segel (29). Statistical procedures were performed with SYSTAT (SYSTAT, Inc., Evanston, Ill.). Except when noted, statistical analyses were multivariate analyses of variance (MANOVAs) with soil sample and inhibitor (NH_4^+, NO_2^-, and NO_3^-) concentrations as independent variables and kinetic coefficients as dependent variables. Variance assumptions were checked by using residual plots, and data were occasionally log transformed to satisfy these. Multiple comparisons are Bonferroni contrasts.

RESULTS

Phase transfer limitation and initial rates. CH_4 oxidation rates increased nearly linearly with increasing soil contents in slurries (Fig. 1), indicating that slurries were not limited by phase transfer and diffusion of CH_4. The slight curvature of the plot may result from dilution alterations of slurry pH values and NH_3 concentrations.

Slurries used in inhibition experiments were never incubated

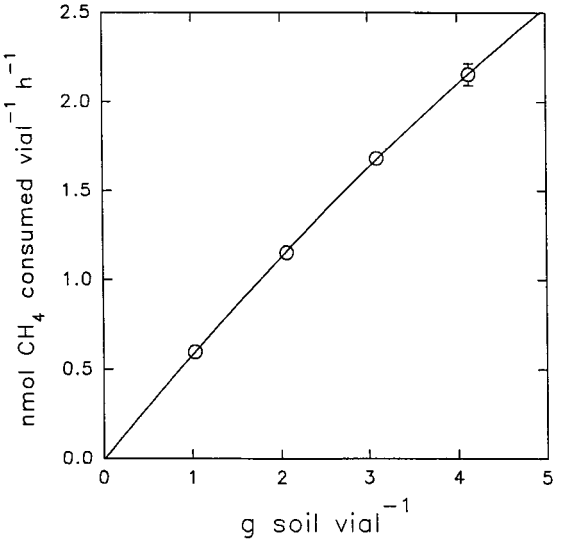

FIG. 1. Oxidation rates of 100-ppmv CH_4 in 20-ml slurries containing different amounts of 1993 soil. Each point is the mean of triplicate vials ± 1 standard error of the mean. When error bars are not visible, they are contained within the symbol.

for more than 24 h (usually <7 h) with added CH_4. During these periods, kinetic coefficients were nearly constant for slurries without added NH_4^+ (Table 2). Methanotrophic activity was stimulated by several orders of magnitude by exposure to 10% CH_4 (data not shown), suggesting that the growth of methanotrophs is not nitrogen limited and should not be affected by NH_4^+ additions. The rates presented in this paper are therefore based on initial enzyme concentrations and are true initial rates, with one potential exception. When significant accumulations of NO_2^- occurred during incubation through oxidation of added NH_4^+, measured rates may have gradually decreased over time. We have attempted to compensate for this in our calculations (see below).

NO_2^- inhibition. The addition of $NaNO_2$ inhibited CH_4 oxidation in both soil samples (Fig. 2). Although each graph contains curves from two separate experiments, in each case the two control curves are similar. Progressively higher $NaNO_2$ additions resulted in increasingly sigmoidal kinetics, with the highest relative inhibition of CH_4 oxidation occurring at the lowest CH_4 concentrations.

TABLE 2. Kinetic constants calculated at various times after the addition of CH_4 to soil slurries

Soil sample	Incubation time (h)	V_{max} (nmol g^{-1} h^{-1})	$K_{m(app)}$ (ppmv)
1994 soil[a]	3.5–9.0	1.50	68
	9.0–22.5	1.54	92
	22.5–28.5	1.73	92
1994 soil + 74 mM NO_3[a]	3.5–9.0	0.86	43
	9.0–22.5	1.00	77
	22.5–28.5	1.11	60
1993 soil[b]	1.5–7.5	1.12	
	7.5–27.0	1.09	
	27.0–32.0	1.49	

[a] For more details, see results for experiment 11 in Table 3.
[b] Based on duplicate flasks at >400-ppmv CH_4.

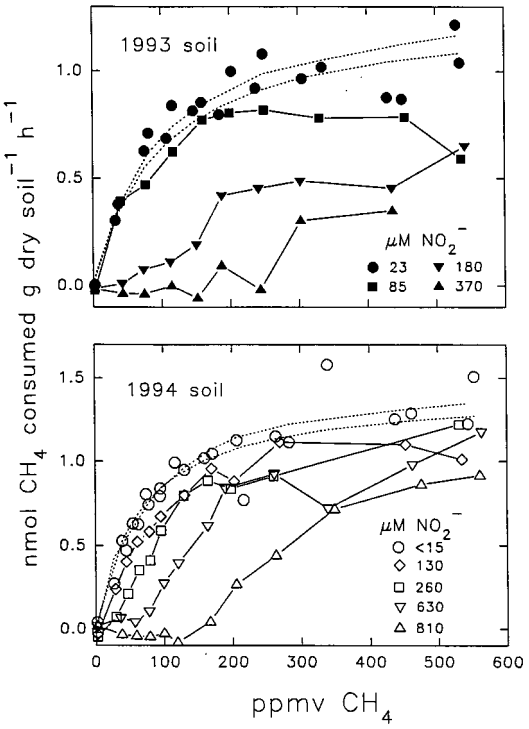

FIG. 2. Effects of $NaNO_2$ additions on the kinetic curves of CH_4 oxidation in two soil samples. Each graph includes curves for two trials; circles represent control rates. The average NO_2^- concentrations during incubations are indicated.

NH_4^+ inhibition. The experiments comparing CH_4 oxidation kinetics with and without added NH_4^+ are summarized in Table 1. The CH_4 oxidation rates in soil slurries without added NH_4^+ agreed well with a Michaelis-Menten hyperbolic model

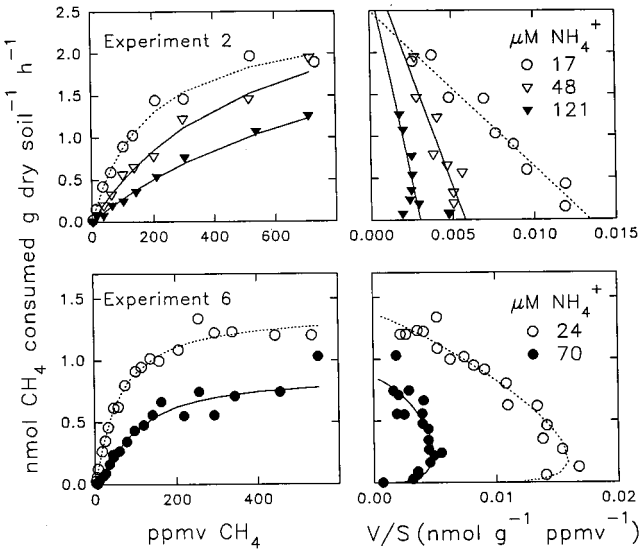

FIG. 3. Representative kinetic curves of CH_4 oxidation in 1993 (experiment 2) and 1994 (experiment 6) soil samples with and without NH_4Cl additions as well as Eadie-Hofstee replots to show deviations from true hyperbolic curves at low CH_4 oxidation rates for 1994 soil. Lines were fit to the curves by using a Michealis-Menten hyperbolic model for 1993 soil and a Hill cooperative model for 1994 soil. Points are means of duplicate vials. The average liquid-phase NH_4^+ contents are indicated.

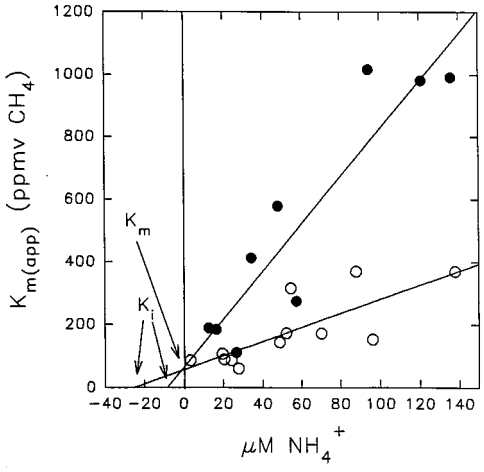

FIG. 4. $K_{m(app)}$ values of CH_4 oxidation, estimated by using Lineweaver-Burk replots, plotted against average liquid-phase NH_4^+ concentrations in 1993 (●) and 1994 (○) humisol slurries. The K_m of CH_4 oxidation in an NH_4^+-free system and the K_i of NH_4^+ for CH_4 oxidation are estimated from axis intercepts.

(Fig. 3). However, when NH_4^+ was added, kinetic curves often became sigmoidal and curvature of Lineweaver-Burk and Eadie-Hofstee plots was evident at low velocities, especially for the 1994 soil sample (Fig. 3). Replots used to estimate the Michaelis-Menten kinetic coefficients in Table 1 were linearized by deleting the lowest substrate concentrations. This procedure gives valid estimates when curves of v plotted against s are only slightly sigmoidal, as was the case with the 1993 soil sample. However, it is not an ideal treatment of sigmoidal kinetics (29) and gives biased estimates for the 1994 soil sample. Therefore, $1/v$-versus-$1/s$ plots were also fit to the Hill cooperative model (29) by using the curve-fit function of SigmaPlot 5.1 (Jandel Scientific, San Raphael Calif.). Because of weighting toward low-velocity points, the lowest points were deleted until an unbiased agreement with the equation was obtained (in practice, this usually meant deleting any velocity that was <0.1). Representative experiments showing good agreement with the hyperbolic model for the 1993 soil sample and agreement with the Hill model for the 1994 soil sample are given in Fig. 3.

Ammonium acted as a simple competitive inhibitor in the 1993 soil sample. The $K_{m(app)}$ values for CH_4 increased with increases in the NH_4^+ concentration ($P < 0.001$; log-transformed data), but the V_{max} values were unaffected ($P = 0.003$; log-transformed data). Plot curvature was minor, as shown by low n_{app} values (Table 1). Replots of $K_{m(app)}$ versus NH_4^+ concentration were used to estimate the K_i of NH_4^+ and the true K_m of CH_4 oxidation in the absence of NH_4^+ (Fig. 4). These estimates were a K_i of 8.3 μM NH_4^+ (18.5 nM NH_3) and a K_m of 63.5 ppmv for CH_4 (88.2 nM dissolved).

The 1994 soil sample shows a more complex response to NH_4^+ addition. The V_{max} decreased, whether estimated by the hyperbolic or Hill model ($P = 0.004$), while the $K_{m(app)}$ (and the K_s^*) again increased ($P < 0.001$) with increasing NH_4^+ concentrations (Table 1). Unlike those for the 1993 soil sample, the $K_{m(app)}$ estimates for the 1994 soil sample are not particularly reliable because of sigmoidal v-versus-s plots. These estimates would give a K_i of 25.2 μM NH_4^+ (112 nM NH_3) and a K_m for CH_4 of 56.7 ppmv (78.7 nM dissolved). Methanotrophs in the 1994 soil sample seemed to have a lower affinity for NH_4^+ than those in the 1993 soil sample.

At levels below about 50-ppmv CH_4, measured CH_4 oxidation rates were less than predicted by a strictly Michaelis-Menten hyperbolic model, resulting in sigmoidal kinetics. n_{app} values increased with increasing NH_4^+ concentrations (Table 1), especially in the 1994 soil sample, whose kinetic plots were often strikingly sigmoidal (Fig. 3). The competitive model of NH_4^+ inhibition is therefore conservative, underestimating inhibition at low CH_4 concentrations. The n_{app} parameter should probably be considered simply a measure of plot curvature rather than in its correct sense as a measure of cooperativity, since we hypothesize that the sigmoidal curves result from NO_2^- toxicity. While the data could result from an allosteric enzyme, the Hill model might fit because (i) NO_2^- is more inhibitory of CH_4 oxidation at low CH_4 concentrations and (ii) if NH_4^+ is competitively oxidized by MMO, NO_2^- production decreases with increasing CH_4 concentrations. This system may thereby mimic a cooperative system whereby increasing CH_4 concentrations result in optimal oxidation.

The possibility of a false competitive oxidation curve simulated by increased NO_2^- production with increasing CH_4 concentrations, a trend noted elsewhere (20), exists. However, this was not evident from our nitrogen measurements and would in any case require increased enzyme activity at high CH_4 concentrations. Enzyme activity did not increase during our incubations.

Nitrification. The maximum nitrification rate, measured as the production of NO_3^- and NO_2^- in slurries with NH_4^+ added (>30 μM), was 0.18 to 0.25 μmol g^{-1} h^{-1} for the 1994 soil sample (experiments 5 through 8) and 0.11 to 0.25 μmol g^{-1} h^{-1} for the 1993 soil sample (experiments 1 and 2 and data from reference 12). The control 1993 soil sample also accumulated NO_3^- and NO_2^- at 0.03 to 0.15 μmol g^{-1} h^{-1}, while nitrification was undetectable in the control 1994 soil sample (<0.03 μmol g^{-1} h^{-1}), indicating a lower rate of NH_4^+ mineralization in the latter. The net NH_4^+ depletion rate in the 1993 soil sample with added NH_4^+ was only 0.08 to 0.10 μmol g^{-1} h^{-1}. Nitrification was rapid enough to consume a large proportion of the added NH_4^+ during incubation, introducing error into the determination of its K_i for CH_4 oxidation. However, the arithmetic mean should be a valid estimate of the slurry NH_4^+ concentration since measured nitrification rates were nearly constant regardless of the amount of NH_4^+ added [i.e., the $K_{m(app)}$ of soil nitrification was probably lower than 30 μM (data not shown)].

The effects of CH_4 concentrations on nitrification were examined by measuring postincubation NO_3^- and NO_2^- concentrations in all vials from experiment 3 and the 94.4 μM NH_4^+ treatment of experiment 8. A two-way MANOVA with the CH_4 concentration as a regression factor and NH_4^+ addition as a categorical factor revealed no significant effects of CH_4 concentrations on total nitrification (final concentration of NO_2^- and NO_3^-, $P = 0.16$), but the effects on net NO_2^- production were significant ($P = 0.014$; overall Pillai trace, $P = 0.025$). This difference probably simply reflects the more precise measurement of NO_2^- than of NO_3^- (NO_2^- was typically <50 μM, while the pool of NO_3^- was >800 μM). While statistically more NO_2^- was produced in slurries with low CH_4 concentrations, the difference was small compared with absolute NO_2^- concentrations. The average regression slope was only −0.015 μM NO_2^- ppmv CH_4^{-1}, and the effects of CH_4 concentrations on total nitrification were therefore minute.

$NaNO_3$ inhibition. Three experiments investigated $NaNO_3$ inhibition (Table 3); one of them is shown in Fig. 5. A noncompetitive mechanism of inhibition [the $K_{m(app)}$ was unaffected, and the V_{max} decreased] is indicated. Statistical tests were not performed because of the paucity of curves, but the 1993 soil sample appeared to be more sensitive to $NaNO_3$ inhibition than the 1994 soil sample was. An increase in plot

TABLE 3. Effects of added $NaNO_3$ on kinetic coefficients of CH_4 oxidation in humisol slurries

Soil sample and experiment no.	Incubation time (h)	NO_3^- (mM)	NO_2^- (μM)	pH	Hyperbolic model				Hill model		
					CH_4 range (ppmv)	No. of CH_4 levels	$K_{m(app)}$ (ppmv)	V_{max} (nmol g^{-1} h^{-1})	n_{app}	K_s^* (ppmv)	V_{max} (nmol g^{-1} h^{-1})
1993 soil, 9	5.5	1.17	26.0	6.70	30–500	8	158	1.57	1.46	61.0	0.99
		37.8	72.5	6.47	70–550	7	141	0.80	1.38	86.7	0.64
		58.7	105	6.46	70–550	7	195	0.53	2.27	86.7	0.34
1994 soil											
10	7.0	0.31	<3.0	NT[a]	30–350	9	65.3	1.21	1.25	52.3	1.07
		18.2	11.9	NT	30–350	9	77.2	1.24	1.49	48.4	0.98
		33.9	29.6	NT	30–350	9	71.4	1.07	1.57	44.8	0.85
11	13.5	0.44	<3.0	7.14	25–500	9	91.7	1.54	1.11	74.5	1.41
		74.2	37.4	6.74	25–550	9	82.0	1.07	1.18	61.6	0.96
		102	57.8	6.70	45–550	8	73.1	0.64	1.73	52.0	0.53

[a] NT, not tested.

curvature (n_{app}) also accompanied $NaNO_3$ addition, probably because of NO_2^- contained as a contaminant or produced through NO_3^- reduction (Table 3).

This noncompetitive inhibition is not necessarily due to NO_3^- per se. Slurry pH values were altered by $NaNO_3$ additions, although greater than 40 mM increases continued to decrease the V_{max} values without further affecting the pH values (Table 3). Similar concentrations of NaCl also inhibited CH_4 oxidation. In experiment 9, the CH_4 oxidation rates (at 160-ppmv CH_4) in slurries supplemented with 35 or 55 mM NaCl were not significantly different from those in slurries with 38 or 59 mM $NaNO_3$, respectively (two-way analysis of variance [ANOVA] with four Bonferroni contrasts, $P = 0.009$). The pH of slurries with added NaCl (6.49) was nearly the same as that of slurries with added $NaNO_3$. Smaller NaCl additions (up to 10 mM) did not significantly affect CH_4 oxidation rates at 2-, 8-, 32-, and 120-ppmv CH_4 in the 1994 soil sample (two-way ANOVA of log-transformed data, $P = 0.61$); therefore, dissolved salts should not have affected the results of NH_4Cl or $NaNO_2$ inhibition experiments.

Reversibility of inhibition. After 70 h, 0.7 or 1 mM of added NO_2^- (1994 soil) reached background, control levels. In 7-h incubations at 2-, 50-, 85-, 200-, and 380-ppmv CH_4, these slurries then had the same CH_4 oxidation rate as slurries that had never received NO_2^- additions (two-way ANOVA, $P = 0.70$). In experiment 5, the effects of NH_4^+ additions on the CH_4 oxidation rate were also not significant after the NH_4^+ was nitrified in 5-h incubations at 2-, 120-, 275-, and 450-ppmv CH_4 (two-way ANOVA of log-transformed data, $P = 0.30$). Inhibition by both NH_4^+ and NO_2^- at these concentrations was fully reversible.

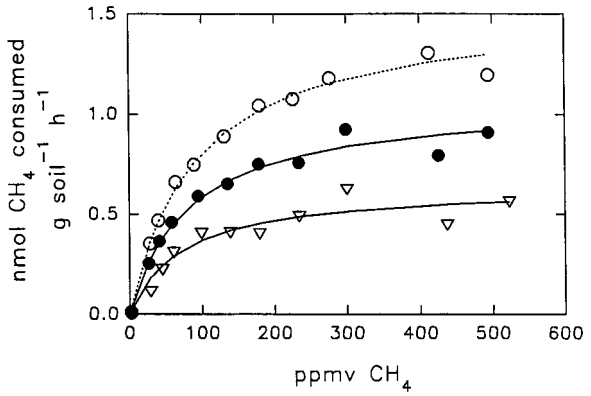

FIG. 5. Effects of $NaNO_3$ addition on the kinetics of CH_4 oxidation in the 1994 soil sample (experiment 11 [Table 3]). The average NO_3^- concentrations are 0.4 (○), 74 (●), and 102 (▽) mM.

DISCUSSION

It should be stressed that the statistically significant effects of NH_4^+ and NO_3^- on kinetic coefficients of CH_4 oxidation do not justify the basic assumption that the kinetic response of MMO is being measured. Measuring the kinetic coefficients of a preparation as crude and ecologically complex as soil is problematic. However, the CH_4 oxidation rates measured in control soil slurries were true initial rates, there was no diffusion limitation, and *v*-versus-*s* curves usually fit a hyperbolic model well. CH_4 oxidation is the initial step in an oxidative pathway, and CH_4 is freely diffusible across the cell membrane. This system may therefore be relatively simple and allow for a kinetic interpretation. If this is accepted, then we can make some conclusions on the mechanisms of inhibition and the strengths of various inhibitors.

Ammonium acted as a competitive inhibitor of CH_4 oxidation in the 1993 soil sample. Our kinetic plots cannot distinguish between simple competitive inhibition and partial competitive inhibition (29). However, since the CH_4 oxidation rate was driven to zero with very high levels of NH_4^+ additions (>1 mM [data not shown]), the mechanism is probably simple competition. The two samples produced similar K_m estimates (about 60-ppmv CH_4). Nevertheless, while the results of the 1993 soil sample were easily interpretable as simple competition between CH_4 and NH_4^+, the results of the 1994 soil sample were more complex, with decreased V_{max} values and strongly sigmoidal kinetics at high NH_4^+ concentrations. NH_4^+ also probably acted as a competitive inhibitor in this sample, and although accurate estimation of the K_i was impossible, this soil seemed less sensitive to NH_4^+.

Explaining the differences between the two samples is difficult. There were differences in storage time and experimental protocol (experiments 6 to 8 included 24 h of preshaking and more intense sampling at lower CH_4 values). Sampling variations may have influenced the microbial flora present, and if measured $K_{m(app)}$ values resulted from the activities of several enzymes rather than the activity of one, their ratios could have varied between samples. The more rapid NH_4^+ depletion rate of the 1994 soil sample might also have protected methanotrophs from the inhibitory effects of NH_4^+. Considering NH_3 to be the inhibitor instead of NH_4^+ increases the dispar-

ities in K_i values, but the pH could certainly affect the enzyme system in ways that are distinct from its effects on the NH_4^+-to-NH_3 ratio.

Errors in determining $K_{m(app)}$ values mean that the K_i of 8 to 25 μM NH_4^+ (18 to 112 nM NH_3) should be considered a rough estimate only, but it does suggest that in situ soil NH_4^+ concentrations inhibit CH_4 oxidation. Similar to the finding that the $K_{m(app)}$ values for soil CH_4 oxidation are much lower than those for pure cultures of methanotrophs (2, 3), our soil K_i is lower than the K_i values of 1.7 to 18 mM NH_4^+ at pH 6 to 7 (8 to 56 μM NH_3) determined for pure cultures (2, 7, 14, 27). However, our results also agree with those of King and Schnell (19, 28) that the inhibition caused by the addition of NH_4^+ may not be simply competitive dilution but may also be the result of production of toxic NO_2^-.

Kinetic curves became increasingly sigmoidal with increased NH_4^+ or NO_2^- additions. This pattern would result if MMO exhibited cooperative behavior which was influenced by NO_2^- or NH_4^+. It could also result from NO_2^- toxicity. The relative inhibition caused by NO_2^- was highest at low CH_4 concentrations, a pattern also noted for another soil (28). This pattern, combined with the shift in competitive balance of MMO as the ratio of NH_4^+ to CH_4 increases, could contribute to the sigmoidal CH_4 oxidation curves of soils with NH_4^+ added. In other words, more NO_2^- is produced in soil at low CH_4 concentrations, at which it is also inherently more inhibitory of CH_4 oxidation. Nitrite is an inhibitor of formate dehydrogenase and can contribute to NADH limitation (18), explaining its greater influence on CH_4-limited cells. A high metabolic rate might also be necessary to export toxic, cellular NO_2^-. The NO_2^- concentrations measured in NH_4^+ inhibition experiments were slightly lower (<100 μM) than the levels of exogenously added NO_2^- required to cause inhibition of CH_4 oxidation, but if methanotrophs themselves produce NO_2^-, intracellular concentrations would be higher.

In this soil, as in many other soils (22), nitrification was not affected or was only slightly affected by up to 800-ppmv CH_4. Methanotrophs can occasionally contribute to soil nitrification (2), and a methane-dependent nitrifying consortium was isolated from a similar humisol (23), but methanotrophs probably contributed little to nitrification in our humisol. Slightly elevated soil NO_2^- concentrations were noted as CH_4 concentrations decreased. This indicates a competitive effect of CH_4 on NH_4^+ oxidation by nitrifiers or methanotrophs, but the effect was too small to greatly affect the overall nitrification rate.

The competitive nature of NH_4^+ inhibition in our soil, whereby inhibition decreased at high CH_4 levels because of competitive dilution, is the complete opposite of a trend found in a forest soil (28). The same authors also showed that NO_2^- production from NH_4^+ in methanotroph cultures increased with increasing CH_4 concentrations (20) and advanced this as an explanation of the ammonium inhibition pattern observed in soil (28). Other reports (21, 23, 27) also note that while large amounts of added CH_4 may inhibit NH_4^+ oxidation by methanotrophs, small amounts of added CH_4 may actually stimulate it. The former effect is presumably competitive, with the latter stemming from energetic limitation of methanotrophs at low CH_4 concentrations. Increased NH_4^+ oxidation and NO_2^- production can result from increased MMO activity, through the alleviation of NADH limitation or induction of enzyme production and population growth. In the study of forest soil noted above (28), the authors make no mention of the linearity of their rates over time at high CH_4 levels; it seems likely that the methanotrophic population was stimulated. Our short incubations provide a look at initial rates based on initial enzyme concentrations, a "snapshot" indicating that NH_4^+ or NH_3 probably acts as a competitive inhibitor. It is perhaps also noteworthy that the initial CH_4 concentrations in our soil were greater than ambient-air CH_4 concentrations, presumably because of methanogenesis in anaerobic soil microsites, so methanotrophs may not have suffered from NADH limitation. The slurrying of soil, as well as other disturbances (such as sonication), results in transient CH_4 efflux.

There is a discrepancy between the complete reversibility of NO_2^- and NH_4^+ inhibition in our humisol and the persistence noted in other studies (19, 25). Perhaps the absolute concentration and time of exposure affect the ability of methanotrophs to recover from NH_4^+ and NO_2^- inhibition, and perhaps the extremely high natural nitrification rate of this humisol shields methanotrophs from NO_2^- and NH_4^+. The levels of our NH_4^+ additions were much lower than those used in other studies (19, 25), although in a parallel field study, we also found no effects of the addition of 100 kg of urea ha^{-1} on CH_4 oxidation (12).

These studies demonstrate the competitive nature of NH_4^+ inhibition of soil CH_4 oxidation and further implicate NO_2^- as a significant inhibitor, as previously noted (20, 28). Methanotrophic activity in soil has a higher affinity for NH_4^+ than that noted for pure methanotroph cultures. As found in other studies (1, 25), inhibition by $NaNO_3$ was evident only at concentrations at which salts had a similar inhibitory effect. This inhibition was not purely attributable to pH, and only the maximum catalytic rate of the enzyme was affected.

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