Effects of long-term nitrogen fertilization on the uptake kinetics of atmospheric methane in temperate forest soils

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Abstract

To determine whether repeated, long-term NH_4^+ fertilization alters the enzymatic function of the atmospheric CH_4 oxidizer community in soil, we examined CH_4 uptake kinetics in temperate pine and hardwood forest soils amended with 150 kg N ha^{-1} y^{-1} as NH_4NO_3 for more than a decade. The highest rates of atmospheric CH_4 consumption occurred in the upper 5 cm mineral soil of the control plots. In contrast to the results of several previous studies, surface organic soils in the control plots also exhibited high consumption rates. Fertilization decreased in situ CH_4 consumption in the pine and hardwood sites relative to the control plots by 86% and 49%, respectively. Fertilization increased net N mineralization and relative nitrification rates and decreased CH_4 uptake most dramatically in the organic horizon, which contributed substantially to the overall decrease in field flux rates. In all cases, CH_4 oxidation followed Michaelis–Menten kinetics, with apparent K_m ($K_{m(app)}$) values typical of high-affinity soil CH_4 oxidizers. Both $K_{m(app)}$ and $V_{max(app)}$ were significantly lower in fertilized soils than in unfertilized soils. The physiology of the methane consumer community in the fertilized soils was distinct from short-term responses to NH_4^+ addition. Whereas the immediate response to NH_4^+ was an increase in $K_{m(app)}$, resulting from apparent enzymatic substrate competition, the long-term response to fertilization was a community-level shift to a lower $K_{m(app)}$, a possible adaptation to diminish the competitiveness of NH_4^+ for enzyme active sites.
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1. Introduction

Soil biological processes are among the principal regulators of the methane (CH_4) concentration of the atmosphere [1,2]. Our understanding of these processes, which include production and consumption of CH_4 in soils, is incomplete because the nature and identity of the soil microorganisms that consume CH_4 directly from the atmosphere are not well known. Studies employing soil sterilization, specific inhibitors, isotope tracers, and steady-state reaction kinetics demonstrate that bacteria mediate this process via enzymatic oxidation of CH_4 to yield CO_2 and biomass C [3,4]. The organisms active in this process, referred to as high-affinity methanotrophs, generally exhibit much higher enzymatic affinities for CH_4 than methanotrophs examined in pure cultures or those active in CH_4-rich environments (i.e., low-affinity methanotrophs) [5]. Unlike low-affinity methanotrophs, the undescribed atmospheric CH_4 oxidizers exhibit sufficiently high enzymatic affinities for CH_4 that they may be able to use extremely low concentrations (i.e., atmospheric [∼1.8 ppmv]) for growth [6]. Previous studies provide evidence that these organisms assimilate CH_4–C efficiently [4,7], that they may be able to use methanol as an alternative substrate if it is available [8], and that the *pmoA* gene, which encodes subunit A of the particulate methane monooxygenase and is characteristic of all known methanotrophs, was detected by PCR in soil

horizons exhibiting CH_4 oxidation activity [9,10]. Cumulatively, the results of several recent studies suggest that atmospheric CH_4 oxidizers are related to known type I and type II methanotrophs, yet to date no cultured methanotroph has exhibited the same physiological characteristics as atmospheric CH_4 oxidizers in soil, nor has any been linked to active populations in situ. As a result, basic questions remain about the organisms responsible for atmospheric CH_4 consumption in soil, including whether they use atmospheric CH_4 as a major source of C and energy, and whether groups other than methanotrophs, such as chemolithotrophic nitrifiers, may also be involved in the process.

Over the past 15 years, many studies have shown that NH_4^+ fertilization decreases the rate of atmospheric CH_4 consumption by 50–85% in a wide variety of soils [11–18]. Hence, the sensitivity of atmospheric CH_4 oxidizers to human activities likely has accelerated the rate of CH_4 accumulation in the atmosphere [18]. Several in situ flux studies have provided detailed information on the timing and extent of NH_4^+ inhibition of atmospheric CH_4 consumption. Although inhibition often is severe and may not reverse within a decade, complete loss of activity has not been observed, even in agricultural soils fertilized with inorganic N for many decades [19–21]. Moreover, activity recovers slowly taking as long as a century after arable ecosystems have been abandoned to undergo secondary succession [21]. Whereas the physiological mechanism of the initial inhibition response to fertilization has been traced to plausible cellular processes [22–27], information is lacking on any subsequent long-term physiological changes in the CH_4 oxidizer community that might sustain active populations, thus maintaining low rates of activity, and allow gradual recovery of populations and flux rates as soil N dynamics presumably rebound in the direction of the undisturbed soil.

By simply decreasing the size of the active methanotroph population, NH_4^+ fertilization could lower rates of CH_4 consumption. In the absence of an adaptive response, this process could ultimately exclude the methanotroph population, yet complete elimination of activity is rare. Two alternative community-level changes might explain these observations. First, if the active organisms use atmospheric CH_4 as a growth substrate, then long-term exposure to NH_4^+, a competitive inhibitor [23,26], might select for methanotrophs with higher enzymatic affinity and/or substrate specificity for CH_4. Such an adaptation might correspond to a decreased apparent K_m ($K_{m(app)}$) for CH_4 of the active community [28]. Alternatively, NH_4^+ fertilization might effectively eliminate methanotrophic activity, while retaining lower levels of activity by greatly enlarging the autotrophic nitrifier population [19,29,30]. This mechanism is plausible because methanotrophs and autotrophic nitrifiers both possess monooxygenase enzymes that can oxidize CH_4 [3,31]. In this study, we examined steady-state CH_4 oxidation kinetics ($K_{m(app)}$, $V_{max(app)}$) in unfertilized and adjacent soils fertilized repeatedly with NH_4NO_3 for more than 10 years to compare apparent enzymatic affinities [28] of the CH_4 oxidizer community under each condition. The soils are from two northeastern temperate forest ecosystems where the effects of NH_4^+ fertilization have been followed for more than a decade [14,32,33].

2. Materials and methods

2.1. Site description

The control and fertilized plots used in this work are part of the ongoing N addition experiment located at the Harvard Forest LTER site in central Massachusetts. The experimental plots are in a 50+ year-old mixed hardwood stand dominated by black and red oak (*Quercus velutina* Lam.; *Q. rubra* L.) that had regenerated naturally after clear-cutting and in a 70+ year-old red pine (*Pinus resinosa* Ait.) plantation planted in 1926 [34]. Soils are stony-to-sandy loam classified as Typic Dystrochrepts. The hardwood soil is a Canton variant and the pine soil is a Montauk variant. Additional soil characteristics are presented in Table 1.

Table 1
Selected soil characteristics

Soil Depth	Pine soil				Hardwood soil			
	Control			Fertilized %OM[c]	Control			Fertilized %OM
	WHC[a]	%OM[b]	pH		WHC	%OM	pH	
Organic horizon	3.53	40.0	3.3	42.3	3.72	58.4	3.2	41.1
0–5 cm mineral	1.03	10.6	3.8	10.7	1.19	14.9	3.8	11.2
5–10 cm mineral	0.93	8.6	ND[d]	8.7	1.01	11.0	ND	8.1
10–20 cm mineral	0.86	5.3	ND	6.6	0.88	6.8	ND	6.2

[a] WHC (water holding capacity) in g H_2O g^{-1} dry soil; WHC values were assumed to be the same for fertilized soils.
[b] %OM (% organic matter) as% dry soil mass lost following overnight combustion at 500 °C.
[c] Fertilized plots received NH_4NO_3 at an annual rate of 113 kg N ha^{-1} beginning in May 1988 and 150 kg N ha^{-1} in subsequent years.
[d] ND: measurement not done.

Plots (30 m × 30 m) were established in the spring of 1988 and subdivided into 5 m × 5 m subplots. This study was conducted in control plots receiving no fertilizer and in fertilized plots receiving NH_4NO_3 at an annual rate of 113 kg N ha^{-1} beginning in May 1988 and 150 kg N ha^{-1} in subsequent years [34]. Nitrogen additions were applied using a backpack sprayer as six equal applications during the growing season from May through September. Over the past decade, various parameters have been measured at these sites including the rates of atmospheric CH_4 consumption and environmental factors controlling these rates [14,33].

2.2. Soil collection and preparation

Soil samples were collected from five subplots in each treatment plot. The pine site was sampled on June 2, 1998 and the hardwood site was sampled on June 9, 1998. Organic soil samples (Oa horizon) were collected from each subplot by removing the surface litter layer and collecting the organic soil from a 200 cm^2 area. Mineral soils were collected from within this area by coring to 25 cm. Cores were separated into 0–5, 5–10, and 10–20 cm depth intervals. Three cores were collected from each treatment subplot and like depths were pooled in the field to obtain enough soil for analyses. Sub-samples of each depth interval were taken for inorganic N extraction as described below in Section 2.3. Soils for CH_4 oxidation, net N mineralization, and net nitrification were stored for 36 h at 5 °C before adjusting moisture. Samples were sieved through 4 mm screens and water holding capacity (WHC) was determined gravimetrically by saturating soil samples with water, allowing drainage by gravity for 2 h, and drying for 48 h at 105 °C. WHC was related to oven-dried dry weight of soils and field soil moistures were adjusted by drying or wetting with deionized water as necessary to reach 30% WHC, a pre-determined optimum for atmospheric CH_4 oxidation in a wide variety of soils [35]. Adjustment of water content to the optimum in all samples was to insure that differences in CH_4 oxidation rates would not result from differences in water stress or diffusibility among samples [35]. Total soil organic matter was determined by mass loss on ignition at 500 °C.

2.3. Soil nitrogen analysis

Soil extractions for analysis of inorganic N pools were prepared within 24 h of soil collection. Five replicate samples (20 g at field moisture) were weighed into sample cups, to which 100 ml of 2 M KCl were added. Samples were mixed and allowed to extract at room temperature for 20 h. Samples were centrifuged and the supernatant was frozen at −20 °C for future analysis of NH_4^+ and $NO_3^- + NO_2^-$ concentrations. Samples were analyzed on an Alpkem RFA-300 (Clackamas, OR)

autoanalyzer using the manufacturer's RFA^{TM} methodology (methods A303-S020 and A303-S171).

Net N mineralization and net nitrification were estimated from soil samples prepared as described above for N pools and incubated for 10 d at 20 °C. Mineralization and nitrification were calculated by the differences between initial and final extractable NH_4^+ and $NO_3^- + NO_2^-$, respectively. Relative nitrification, expressed as a percentage of total net N mineralization, was calculated based on the beginning and ending concentrations of extractable inorganic N using the equation: Relative nitrification $= 100 \times \delta([NO_3^-] + [NO_2^-])/(\delta[NH_4^+] + \delta([NO_3^-] + [NO_2^-]))$.

2.4. Chamber fluxes

Soil methane fluxes were measured using a static chamber technique, as described previously [14,36], on June 2 and 9, 1998 in the pine and hardwood stands, respectively. Fluxes were measured by attaching a chamber top to permanently installed anchors in each of three subplots in each treatment. Twenty-ml gas samples were withdrawn at 0, 5, 15, and 20 min using gas-tight nylon syringes. Gas samples were returned to Woods Hole, MA and CH_4 concentrations analyzed by gas chromatography within 36 h of collection as described previously [14]. Two certified CH_4 concentration standards (Scott Specialty Gases, Plainfield, NJ) of 0.601 and 4.08 ppmv CH_4 in N_2 were used for calibration. Methane uptake rates were calculated by linear regression of the change in CH_4 concentration against incubation time; later time points were discarded if they deviated from linearity ($R^2 < 0.95$). At least three time points were used to calculate each rate.

2.5. Soil CH_4 profiles

CH_4 concentrations were measured at the atmosphere-litter layer interface and at 2, 5, 10, 30 and 50 cm depths below the litter layer using stainless steel tubes at two locations in each of the control plots and one location in the pine high N plot. One end of the tube was sealed and the other end had a rubber sleeve stopper [32]. One tube was used to sample each depth. Soil air diffused into the tubes through two small holes (0.32 cm) located near the sealed end. Tubes were allowed to equilibrate with soil air for two days before sampling with 10 ml BD syringe. Methane concentrations were analyzed as described above.

2.6. CH_4 uptake kinetic analyses

Twenty g (dry weight equivalent) of each soil were placed in 125 ml sample cups, which were then placed inside plastic storage boxes to prevent moisture loss. Samples were allowed to equilibrate overnight at 20 °C.

The following day, the sample headspace was equilibrated with laboratory air (ambient CH_4 ~1.9 ppmv) for 30 min. Each sample cup was placed inside a gastight 490 ml screw-cap mason jar with a stopcock in the lid for headspace gas sampling. Headspace CH_4 concentrations were adjusted by addition of an appropriate volume of a 942-ppmv standard in air (Scott Specialty Gases) to approximately 1.9 ppmv (no CH_4 added), 5, 10, 15, or 20 ppmv. Three samples from each soil depth were assayed at each starting CH_4 concentration. Samples were assayed for CH_4 uptake at 20 °C. Surface soils that had high rates of CH_4 consumption were incubated for 1 h with 5 ml gas samples taken from each jar at 15-min intervals. Deeper and N amended soils were incubated for 2 h with 5 ml gas samples taken from each jar at 30-min intervals and CH_4 concentrations were measured by gas chromatography (see above). Methane uptake rates were estimated by the linear regression of the change in CH_4 concentration against incubation time; later time points were discarded if they deviated from linearity ($R^2 < 0.95$).

Kinetic parameters were estimated by non-linear regression (least-squares fit; Microsoft Excel 98 Analytical Tools, Redmond, WA) of each uptake rate against its respective midpoint CH_4 concentration ($[CH_4]_{initial} - [CH_4]_{ending}$)/2) using the Michaelis–Menten equation ($v = (V_{max} \times S)/(K_m + S)$) as the regression model [23]. Ideally, the instantaneous rate of CH_4 uptake is related to the initial CH_4 concentration. Determining the instantaneous rate is not feasible in practice and the rate is determined instead based on the rate of change of CH_4 concentration over time. Hence, regressing the rate of CH_4 loss against the midpoint CH_4 concentration minimizes the error that results from the change in headspace CH_4 concentration during the assay [23]. Results from triplicate samples were averaged to obtain the values for $K_{m(app)}$ and $V_{max(app)}$, along with their respective standard errors. Treatment effects on kinetic parameters were analyzed using one-way analysis of variance (ANOVA; Data analysis tool of Microsoft Excel 98) and Bonferroni-adjusted p values for multiple comparisons. Differences were considered significant when $p < 0.05$.

3. Results

3.1. Soil N dynamics

The hardwood control soils generally had the least extractable inorganic N, whereas the organic soil in the hardwood high N plot had the most (Fig. 1). Fertilization significantly increased inorganic N pools at all depths in both stands, except for the NH_4^+ concentration in the pine stand, which did not increase at any depth. The fertilized hardwood soils had the largest increases in inorganic N pools at all depths. ($NO_3^- + NO_2^-$) was barely detectable in control plots but was significantly higher in the fertilized plots of both sites, except for the deepest depths in the hardwood plot.

Regardless of treatment, the highest rates of net N mineralization were measured in the organic horizon and ranged from 15.6 to 77.9 μg N (gram dry weight soil)$^{-1}$ during the 10-day incubation (Fig. 2). Generally, more N was mineralized in the control and fertilized pine soils than in the corresponding hardwood soils. In control plots, some of the deeper soils exhibited net NH_4^+ immobilization, whereas fertilized soils always
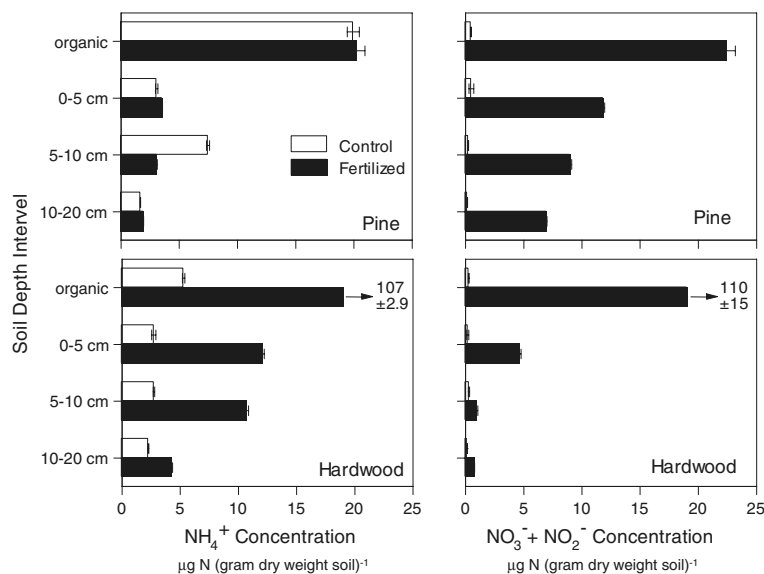

Fig. 1. Extractable soil NH_4^+ and $NO_3^- + NO_2^-$ concentrations in the control and fertilized plots. Fertilized plots received 150 kg N ha^{-1} y^{-1} as NH_4NO_3. Data represent means ± 1 standard error ($n = 5$).
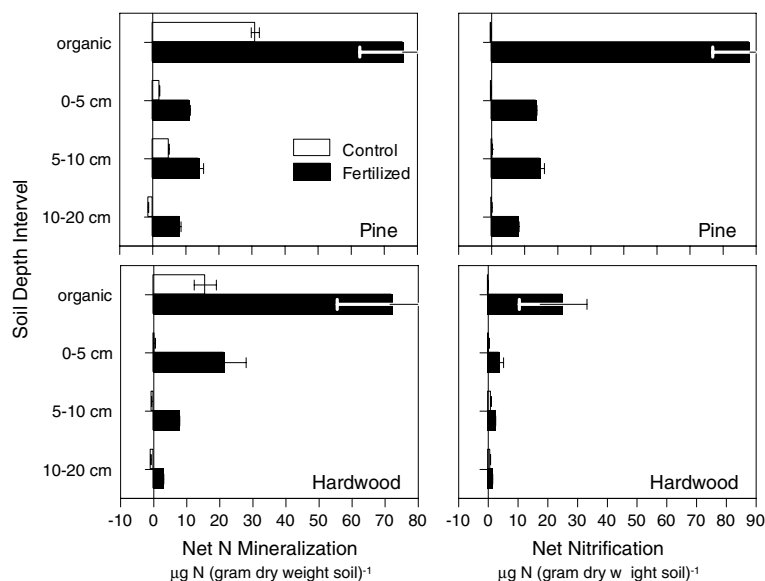
Fig. 2. Net N mineralization and nitrification rates determined in 10-day laboratory incubations. Net N mineralization refers to the accumulation of $NH_4^+ + NO_3^- + NO_2^-$ during the incubation. Net nitrification refers to the accumulation $NO_3^- + NO_2^-$. Negative net N mineralization represents net immobilization of inorganic N. Data represent means ± 1 standard error ($n = 5$).

exhibited significantly greater mineralization than the corresponding control at all depths.

Net nitrification was undetectable or very low in control soils, whereas fertilized soils always exhibited dramatically higher net nitrification at all depths (Fig. 2). The fertilized pine soils exhibited the most net nitrification at all depths. Nitrification rates in all the fertilized soils in the pine plot and the organic horizon of the hardwood plot were significantly greater than the corresponding controls. Relative nitrification, expressed as a percentage of N mineralized, was low in the control soils, except at the 0–5 cm depth in the hardwood, where 82% of mineralized N was nitrified. Depending on sampling depth, 0–50% of the N mineralized was nitrified in the fertilized hardwood soils, whereas essentially all of the N mineralized was nitrified at all depths in the fertilized pine soils. Hence, nitrification was stimulated by fertilization in both sites.

3.2. Soil CH_4 dynamics

The in situ steady-state CH_4 concentration profile measured in the soils of the control plots, was similar in the two soils. In all cases, the maximum concentration ($\sim$1.8 ppmv) occurred at the surface of the organic horizon. The concentration dropped to approximately 0.7 ppmv at the bottom of the organic horizon and reached an apparent threshold of 0.2–0.3 ppmv at about 5 cm mineral soil depth (data not shown). Thus, more than half of the CH_4 consumed in the soil column was oxidized in the organic layer. These results are similar to those reported by Castro et al. [32]. In contrast, the CH_4 concentration at the base of the organic horizon in the

fertilized pine plot was 1.45 ppmv and a threshold concentration (0.3 ppmv) was not reached until a depth of 30 cm in the mineral soil.

In situ flux of CH_4 between the soil and the atmosphere, as measured in static chambers, always exhibited net consumption by the soil. Flux rates were similar in the control plots of both stands, at 3.33 ± 0.36 ($n = 3$; mean $\pm$ SE) and 3.07 ± 0.19 mg CH_4–C m^{-2} d^{-1} for the pine and hardwood, respectively. Consumption rates were significantly lower in the fertilized plots at 0.46 ± 0.07 and 1.56 ± 0.31 mg CH_4–C m^{-2} d^{-1} for the pine and hardwood, respectively. Thus, CH_4 consumption was inhibited relative to flux rates in the control plots by 86% in the pine soil and by 49% in the hardwood soil.

To measure atmospheric CH_4 consumption rates throughout the soil depth profile, we incubated soils from each depth interval of the cores (see Section 2.2) in the laboratory at ambient ($\sim$1.9 ppmv) CH_4 concentration (Fig. 3). In each site, the greatest rates occurred in control soils from 0 to 5 cm mineral soil depth followed by the organic horizons. Rates dropped precipitously in the mineral soils below 5 cm. In samples from the fertilized plots, the greatest relative decrease in CH_4 consumption was observed in the organic and 0–5 cm mineral soils, where the rates were significantly reduced by 62–96% relative to controls. The effect of fertilization was observed to 20 cm depth in the pine soil but only to 10 cm in the hardwood plot. The relative decrease in CH_4 uptake calculated from the soil profile rates measured in the laboratory incubations, agreed closely with the in situ flux rate, with incubated samples showing depth-integrated decreases of 88% and 53% in the fertilized pine and hardwood soils, respectively.
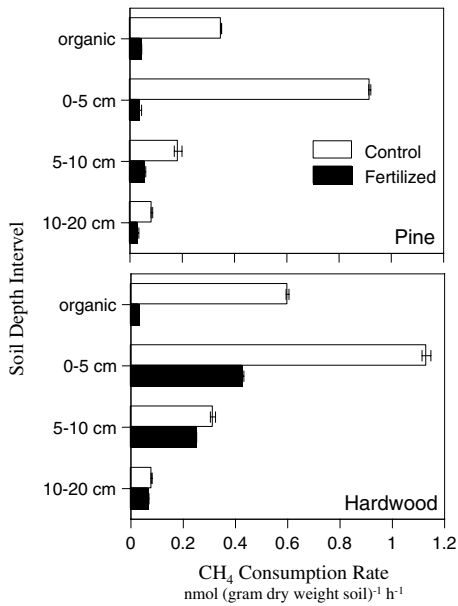
Fig. 3. Capacity for atmospheric CH_4 consumption in control and fertilized soils sampled from different depths. "Capacity" refers to the rate of oxidation when soils from all depths are exposed to atmospheric CH_4 concentration. Data represent means $\pm$ 1 standard error ($n = 3$).

Unlike what has been observed in several other forest soils [3], we found that the organic horizons in both sites oxidized a substantial proportion of the atmospheric CH_4 consumed in the entire soil column. Atmospheric CH_4 consumption was inhibited by fertilization most strongly in this horizon, thus accounting for about half of the overall reduction in field flux rates.

3.3. CH_4 uptake kinetics

CH_4 oxidation followed standard Michaelis–Menten saturation kinetics as observed previously in the unfertilized soils from the Harvard Forest [23]. In general, $K_{m(app)}$ decreased with depth in the mineral soils of the control plots (Fig. 4). $K_{m(app)}$ was always highest in the 0–5 cm mineral soils, where the highest atmospheric CH_4 uptake rates occurred, than in the underlying 5–10 cm mineral soils. In the hardwood soil, $K_{m(app)}$ increased again at 10–20 cm, whereas in the pine soil it continued to decrease at this depth. Overall, ANOVAs indicated that $K_{m(app)}$ differed ($p < 0.05$) between the corresponding organic horizon and 0–5 cm mineral soils in the controls and that both $K_{m(app)}$ and $V_{max(app)}$ were significantly lower in the fertilized soils than in the corresponding controls (Fig. 4).

4. Discussion

4.1. Soil CH_4 dynamics

We measured CH_4 flux rates across the soil surface during the eleventh year of fertilizer application in the Harvard Forest high N plots. At 86% and 49%, respectively, the inhibition levels in the pine and hardwood sites were substantially greater than those (57% and 38%) observed five years earlier in the same plots [33], indicating a persistent and increasing effect of N additions on atmospheric CH_4 consumption. This comparison is based on a June sampling that had comparable soil moistures and nearly the same surface
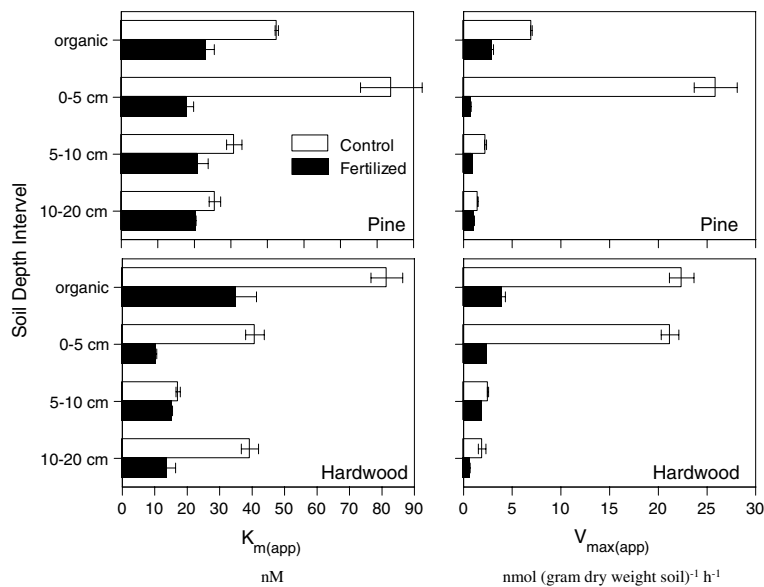

Fig. 4. Michaelis–Menten kinetic parameters for CH_4 consumption in the control and fertilized soils. Data represent means $\pm$ 1 standard error ($n = 3$).

soil temperatures as our measurements. These results are consistent with a progressive change in the size, composition, and/or the physiology of the CH_4 consumer community over time in response to ongoing N fertilization.

The capacity to consume atmospheric CH_4 was greatest in the 0–5 cm depth of the mineral soils of the control plots (Fig. 3), with perhaps the highest reported rates for upland forest soils [9,37,38]. Although many studies report negligible CH_4 consumption in the organic horizon [26,37–40], in agreement with at least four other studies [41–44] we observed relatively high rates in this horizon of both sites (Fig. 3). Moreover, depth profiles of in situ steady-state CH_4 concentration in control plots indicated that almost half of the total CH_4 consumed in situ was oxidized in the organic horizon, despite the highest capacity for CH_4 consumption occurring in the underlying mineral soil (data not shown; cf. [32]). Dramatic decreases in CH_4 oxidation in the organic horizon resulting from fertilization had a major effect on overall soil CH_4 consumption.

As observed previously at these sites [14,33], the relative decrease in atmospheric CH_4 consumption due to fertilization was greater in the pine soils than in the hardwood soils. This contrast may result from inherent differences in N cycling in the two sites. In the laboratory incubations, net N mineralization in controls was 2–15 times greater in the pine soils than in the hardwood soils, depending on depth (Fig. 2), whereas relative nitrification, expressed as a percent of net N mineralization, was negligible in the control soils from both sites. In the fertilized plots, however, net N mineralization was similar in the two sites, but relative nitrification was several-fold higher in the pine soils than in the hardwood soils (Fig. 2). These laboratory results agree with the pattern of in situ net N mineralization and nitrification measured previously in the same sites [45]. Mosier [46] suggested that high rates of N turnover, regardless of whether a soil has been fertilized, might decrease rates of CH_4 consumption. Consistent with this suggestion, our results and those of Steudler et al. [30] point to a pattern of lower CH_4 consumption rates in a variety of forest and agricultural soils with high nitrification rates. Increases in relative nitrification rates indicate the extent to which NH_4^+ is biologically available to nitrifiers and might therefore indicate the extent to which other soil microorganisms, including methane consumers, are exposed to NH_4^+. If so, the higher relative nitrification rate in the fertilized pine site compared to the fertilized hardwood site is consistent with the higher degree of NH_4^+ inhibition in the pine site.

The $K_{m(app)}$ values for CH_4 uptake in the control soils fell within the range of values reported for other forest soils, but were 1–3 orders of magnitude lower than values reported for CH_4-emitting soils and CH_4-consuming cultures (Fig. 4; Table 2).

In the fertilized soils, $V_{max(app)}$ values were similar to those reported for agricultural soils and acidic forested soils in Germany. Hence, the effects of NH_4^+ fertilization in this study are comparable to those of several other studies (Table 2) and suggest that our interpretations may apply to soils other than those examined here. Other studies reporting K_m and V_{max} with soil depth in side-by-side control and fertilized plots are not available for comparison to this study. Clearly, comparable studies are needed in a variety of other ecosystems to determine whether our results and interpretations can be generalized.

4.2. Physiological implications of CH_4 uptake kinetics

Whether atmospheric CH_4 consumption in soil provides energy for growth remains a fundamental question regarding the physiology of the active organisms. In nature, most bacteria must survive and grow in an oligotrophic environment, and enzymatic affinity for growth substrate is the fundamental factor limiting population growth [47]. If some soil methanotrophs use atmospheric CH_4 for growth, then a correlation between enzymatic affinity and in situ steady-state substrate supply might be expected in soils that are supplied only (or mostly) with CH_4 from the atmosphere. In turn, such a correlation would suggest that the active organisms do rely on atmospheric CH_4 as a growth substrate.

In the control plots of the pine and hardwood sites in the Harvard Forest, we observed a consistent pattern in which $V_{max(app)}$ and $K_{m(app)}$ were significantly lower in the 5–10 cm mineral soils compared to the shallower and more active 0–5 cm mineral soils (Fig. 4). Because the steady-state CH_4 concentration decreases with depth [32], a decrease in V_{max} can be expected for a CH_4-dependent population, as reduced substrate supply would support a smaller CH_4 consumer population. Consistent with this scenario, the pattern of decreasing $K_{m(app)}$ with depth suggests population-level adaptation of enzyme kinetics to the steady-state substrate supply (i.e., enzymatic affinity for CH_4 increases as the ambient CH_4 concentration decreases), as the K_m should be independent of enzyme number (or population size; [28]). Although the increase in $K_{m(app)}$ at 10–20 cm in the control hardwood soil breaks this pattern, all other kinetic data from this study, including the entire depth profile in the control pine soil, support this concentration-adaptation hypothesis. We do not consider the lower $K_{m(app)}$ in the pine organic horizon to break the pattern because the soil characteristics do not allow direct comparison between the organic and mineral horizons due to differing diffusion kinetics [23]. Moreover, the CH_4 uptake kinetics in the pine organic horizon may be dictated by substantially greater NH_4^+ availability compared to the other soil horizons, rather than by CH_4 supply (see Section 4.3). Further investigation will determine whe-

Table 2
Kinetic parameters for CH_4 oxidation in soils and methanotrophic cultures

Source	Habitat	$K_{m(app)}$[a]	$V_{max(app)}$[b]	References
Upland forest and agricultural soils	Organic horizon pine	42.6	7.0	This study
	Organic horizon hardwood	81.5	22.4	This study
	0–5 cm mineral pine	76.5	25.9	This study
	0–5 cm mineral hardwood	42.3	21.2	This study
	10 cm forest cambisol	29.7	3.6	[6]
	10 cm cultivated cambisol	50.6	0.7	[6]
	3–6 cm mineral forest	24	7.4	[53]
	0–20 cm agric. humisol	90	1.5	[22]
	10–20 cm mineral aspen	37	0.75	[54]
	20–30 cm mineral spruce	124	8.6	[54]
	4–6 cm mineral hardwood	10	1.0	[50]
	12–18 cm mineral spruce	31	0.19	[4]
	0–10 cm mineral pine	9.8	2.59	[23]
	0–10 cm mineral hardwood	22	5.81	[23]
	4–8 cm mineral hardwood	14.7	0.23	[7]
	Mineral spruce	78.4	2.8	[55]
	Organic horizon hardwood	69	13	[43]
	0–4 cm mineral hardwood	9	0.3	[43]
	5–20 cm mineral hardwood	9	0.84	[56][c]
	5–20 cm mineral coniferous	4	0.04	[56]
Organic and landfill soils	Black spruce bog soil	1.1×10^3	223.1	[54]
	Agricultural humisol	66×10^3	NR[d]	[57]
	3–12 cm landfill cover soil	$\sim 5 \times 10^3$	156	[58]
	Landfill cover soil microcosm	1.6×10^3	258	[24]
	Above after CH_4 enrichment	32×10^3	998	[24]
Isolates and enrichment cultures	<275 ppm CH_4 enrichment	~ 100	0.006	[52]
	CH_4-utilizing mixed culture	1.7×10^3	NR	[59]
	Methylocystis strain LR1	$\sim 2.9 \times 10^3$	0.24	[60]
	Above when starved of CH_4	~ 300	2.04	[60]
	Methylomicrobium album BG8	6.3×10^3	648	[8]
	M. album BG8 (CH_3OH grown)	1.2×10^3	133	[8]
	Methylosinus trichosporium OB3b	2×10^3	NR	[61]
	Methylococcus sp.	44×10^3	NR	[62]
	Methylococcus capsulatus Bath sMMO	71×10^3	NR	[63]
	M. capsulatus Bath sMMO	73×10^3	NR	[27]
	M. capsulatus Bath pMMO	23×10^3	NR	[27]
	Pseudomonas methanica	15×10^3	NR	[64]

[a] $K_{m(app)}$ is expressed in nM in soil solution and was converted from headspace ppmv to nM by multiplying by 1.45, a conversion factor derived form the Bunsen coefficient for CH_4 solubility.

[b] $V_{max(app)}$ is expressed for soils as nmol (gram dry weight soil)$^{-1}$ h^{-1} and for cultures as nmol (10^8 cells)$^{-1}$ h^{-1}.

[c] Mean of forested sites with pH < 4.6.

[d] NR: not reported.

ther a general correlation exists between in situ CH_4 concentration and apparent enzyme affinity across a variety of aerobic soils. If so, this relationship may reflect enzyme adaptation to varying degrees of growth limitation by substrate supply and may offer a foundation for understanding the long-term effects of NH_4^+ fertilization on atmospheric CH_4 consumption in soil.

4.3. Physiological effects of long-term N fertilization

The relative changes in $K_{m(app)}$ and $V_{max(app)}$ due to long-term fertilization were greatest in the most active soils (i.e., organic and 0–5 cm mineral soils), likely because these soils were the shallowest and would have received the greatest amount of fertilizer NH_4^+. The

decrease in $K_{m(app)}$ in response to years of N fertilization differs fundamentally from the immediate response to NH_4^+ additions observed previously in the Harvard Forest unfertilized pine and hardwood soils [23]. In short-term incubations, $K_{m(app)}$ increased immediately upon addition of $(NH_4)_2SO_4$ to laboratory microcosms, as NH_4^+ competitively inhibited CH_4 consumption at the enzyme level [23]. Competitive inhibition persisting over years would likely stress the atmospheric CH_4 consumers, eventually leading to a declining population, a shift to a different dominant CH_4 consumer population (e.g. nitrifiers), the adaptation of an extant methanotroph population to excess NH_4^+, or some combination of these possible long-term responses.

The values of $K_{m(app)}$ and $V_{max(app)}$ for the fertilized soils in our study were lower than those for the corresponding controls and values reported for many other unfertilized soils (Table 2). If we assume that nitrifiers would exhibit higher $K_{m(app)}$ values than typical atmospheric CH_4 consumers (cf. [3,31]), then this fertilizer-driven decrease in $K_{m(app)}$ suggests that nitrifiers are not likely the dominant atmospheric CH_4 consumers in the fertilized plots. A more powerful line of evidence stems from the observed concurrent decrease in both the $K_{m(app)}$ and the rates of in situ CH_4 consumption in the fertilized soils. All else being equal, at a given substrate concentration, an unsaturated enzyme will turnover substrate faster as the K_m decreases [28]. We can infer from increased nitrification (Fig. 1 and [34]) that the nitrifier population and, hence, the ammonia monooxygenase (AMO) pool were strongly enriched by long-term NH_4^+ fertilization. If we assume that this enlarged nitrifier population outnumbered the CH_4 consumer population in the control plots, yet exhibited a lower K_m for CH_4, it should have generated a higher rate of atmospheric CH_4 consumption than in the control plots, which is opposite to the results observed. Based on these assumptions, a decreased methanotroph population with a lower K_m is more consistent with the observed kinetics. A caveat of this interpretation is that we must assume that K_m reflects the specific affinity (V_{max}/K_m normalized for population size or enzyme concentration) of the dominant population [47]. However, some nitrifiers exhibit K_m values for CH_4 that are similar to cultured methanotrophs, but with much lower specific affinities [31] (i.e. similar K_m but lower V_{max}). Hence, an alternative interpretation of our results is that a nitrifier population with a lower K_m and a much lower specific affinity for CH_4 than the active population in the control plots dominates CH_4 consumption in the fertilized plots. This scenario would permit a larger nitrifier population to exhibit a lower K_m for CH_4 concurrent with lower in situ consumption rates.

Which of the two foregoing scenarios is more plausible hinges on whether or not the specific affinity of the dominant population in the fertilized plot is much lower than that of the dominant population in the control plots, which currently we have no means to determine. However, a theoretical distinction between the two hypotheses is that one can envisage potential selective forces (e.g., persistent competitive inhibition by NH_3) acting on methanotrophs to drive an adaptive decrease in K_m (i.e. a more selective enzyme) for CH_4, whereas it is difficult to envisage a mechanism for or benefit of the same result in nitrifiers. In other words, it seems possible to explain why the apparent K_m of methane monooxygenase in methanotrophs would decrease over time due to persistent competitive inhibition, but not why the apparent K_m of ammonia monooxygenase in nitrifiers would be so low for CH_4,

especially in soils with abundant NH_4^+. Hence, we propose that the inverse relationship between nitrification and CH_4 consumption in the fertilized soils in the Harvard Forest results from NH_4^+ stimulation of nitrifier growth, combined with simultaneous but independent NH_4^+ inhibition of methanotroph activity, and that CH_4 consumption is dominated by a diminished, high-affinity methanotroph population, rather than an enlarged, low-affinity nitrifier population. This interpretation is consistent with some studies that have used non-physiological approaches [38,48] and therefore have not observed possible shifts in methanotroph enzyme function.

Investigators have speculated that a population-level shift in the physiology of the CH_4 oxidizers would explain the inhibition dynamics observed in long-term field experiments [3,37]. The decreased $K_{m(app)}$ values observed without exception in Harvard Forest soils fertilized for more than a decade provide evidence for such a change. Moreover, the pattern of increasing apparent affinity for CH_4, both with soil depth (as in situ CH_4 supply decreases) and in NH_4^+-fertilized plots (with elevated concentrations of a competitive inhibitor), is consistent with the possibility that CH_4 consumers in these soils use atmospheric CH_4 for growth and thus adapt to altered substrate supply through mechanisms of natural selection of enzyme function. Whether or not atmospheric CH_4 is an essential resource for the organisms that consume it has been an enigma. Various attempts to stimulate population changes by CH_4 enrichment or starvation have met with mixed results (cf. [49–52]). The results presented here suggest that in situ CH_4 supply acts as a selective force on the atmospheric CH_4 consumer population, thus offering an evolutionary corollary to atmospheric CH_4 serving as an essential resource for these organisms.

5. Conclusions

This report presents the first detailed analysis of how the kinetics of atmospheric CH_4 consumption changes with depth in the soil profile and in response to long-term N fertilization. The results of this work lead to some new questions and possible insights about the biology of CH_4 uptake in the soils of the Harvard Forest, where atmospheric CH_4 consumption has been studied for more than a decade. Our interpretation of the data presented here leads to the following conclusions or hypotheses:

(1) In the Harvard Forest, surface organic soils play an important role in atmospheric CH_4 consumption and NH_4^+ inhibition acts strongly on this component of the soil CH_4 sink. Organic horizons should be examined as possible sinks for atmospheric CH_4 in future studies.

(2) The affinity of the CH_4-oxidizing enzyme(s) in these soils increases as a function of a shift in the population-level physiology of the CH_4 consumer community in response to decreasing CH_4 supply with depth in the soil profile and in response to long-term NH_4^+ fertilization.

(3) The physiology of the methane consumer population in soils fertilized for several years is distinct from the short-term response to NH_4^+ addition. Whereas the immediate response to NH_4^+ is an increase in $K_{m(app)}$, resulting from apparent enzymatic substrate competition, the long-term response to ongoing fertilization is a population-level shift to a lower $K_{m(app)}$, a possible adaptation to diminish the competitiveness of NH_4^+ for enzyme active sites.

(4) The concomitant decrease in the rate of atmospheric CH_4 consumption and the $K_{m(app)}$ for CH_4 uptake in response to NH_4^+ fertilization suggests that nitrifiers are not likely the dominant atmospheric CH_4 consumers in the fertilized soils in the Harvard Forest. More likely, a diminished, high-affinity methanotroph population dominates.

The changes in uptake kinetics observed in the present study may represent an increase in the enzyme affinity for CH_4 of the methanotroph population in response to long-term NH_4^+ fertilization. Given that the growth of the active population may be limited by CH_4 supply (Section 4.2) and that NH_4^+ is a potent competitive inhibitor of CH_4 oxidation in these soils [23], a lower K_m for CH_4 in fertilized soils might reflect a logical adaptation leading to greater substrate selectivity and a higher proportion of enzyme active sites bound by CH_4 rather than NH_3. Examining CH_4 uptake kinetics in a wider variety of paired control and fertilized soils will reveal whether the type of physiological change observed in the atmospheric CH_4 consumer community in the Harvard Forest is a general phenomenon. Ultimately, a reliable quantitative marker that allows the specific affinity of the active organisms to be estimated is needed to test this adaptation hypothesis. Evidence supporting this hypothesis would suggest that oligotrophic soil methanotrophs use atmospheric CH_4 as a growth substrate despite its extremely low concentration. Moreover, the ability to adapt to fertilizer application would provide a mechanism for survival during periods of population decline, thus allowing long-term recovery as soil N dynamics recover from disturbance over several decades.

Acknowledgements

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