Menyailo OV, Hungate BA, Abraham WR, Conrad R.. Changing land use reduces soil CH4 uptake by altering biomass and activity but not composition of high-affinity methanotrophs. Glob...

Article *in* Global Change Biology · September 2008

Impact Factor: 8.04 · DOI: 10.1111/j.1365-2486.2008.01648.x

Global Change Biology (2008) **14**, 2405–2419, doi: 10.1111/j.1365-2486.2008.01648.x

Changing land use reduces soil CH_4 uptake by altering biomass and activity but not composition of high-affinity methanotrophs

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Abstract

Forest ecosystems assimilate more CO_2 from the atmosphere and store more carbon in woody biomass than most nonforest ecosystems, indicating strong potential for afforestation to serve as a carbon management tool. However, converting grasslands to forests could affect ecosystem–atmosphere exchanges of other greenhouse gases, such as nitrous oxide and methane (CH_4), effects that are rarely considered. Here, we show that afforestation on a well-aerated grassland in Siberia reduces soil CH_4 uptake by a factor of 3 after 35 years of tree growth. The decline in CH_4 oxidation was observed both in the field and in laboratory incubation studies under controlled environmental conditions, suggesting that not only physical but also biological factors are responsible for the observed effect. Using incubation experiments with $^{13}CH_4$ and tracking ^{13}C incorporation into bacterial phospholipid fatty acid (PLFA), we found that, at low CH_4 concentrations, most of the ^{13}C was incorporated into only two PLFAs, 18:1ω7 and 16:0. High CH_4 concentration increased total ^{13}C incorporation and the number of PLFA peaks that became labeled, suggesting that the microbial assemblage oxidizing CH_4 shifts with ambient CH_4 concentration. Forests and grasslands exhibited similar labeling profiles for the high-affinity methanotrophs, suggesting that largely the same general groups of methanotrophs were active in both ecosystems. Both PLFA concentration and labeling patterns indicate a threefold decline in the biomass of active methanotrophs due to afforestation, but little change in the methanotroph community. Because the grassland consumed CH_4 at a rate five times higher than forest soils under laboratory conditions, we concluded that not only biomass but also cell-specific activity was higher in grassland than in afforested plots. While the decline in biomass of active methanotrophs can be explained by site preparation (plowing), inorganic N (especially NH_4^+) could be responsible for the change in cell-specific activity. Overall, the negative effect of afforestation of upland grassland on soil CH_4 uptake can be largely explained by the reduction in biomass and to a lesser extent by reduced cell-specific activity of CH_4-oxidizing bacteria.

Keywords: afforestation, atmospheric CH_4 oxidation, soil inorganic N cycle, soil methanotrophs, stable isotopes in PLFA

Received 19 October 2007; revised version received 6 February 2008 and accepted 13 February 2008

Introduction

Atmospheric CH_4 is a potent greenhouse gas, second to CO_2, contributing roughly 15–20% to the observed global warming (IPCC, 2001). The abundance of atmospheric CH_4 is controlled by CH_4 emission and CH_4 removal. CH_4 evolves mainly from wetlands including rice paddy fields (Bosse & Frenzel, 1998; Cai *et al*., 2001), swamps (Ueda *et al*., 2000) and marshes (Van der Nat & Middelburg, 2000). The major removal process is the

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reaction of CH_4 with the hydroxyl radical in the troposphere (IPCC, 2001). Aerobic upland soils are the second largest global sink, which accounts for 6–10% of the total CH_4 uptake from the atmosphere, or about $30\,Tg\,CH_4\,yr^{-1}$ (IPCC, 2001). Although the soil sink is relatively small, it is similar in magnitude to the annual CH_4 increase in the atmosphere, and it is strongly affected by human activities (Mosier *et al*., 1991, 1997; Hütsch, 1998). Changes in land use, for example, can alter the strength of the soil CH_4 sink, altering the global CH_4 budget (Mosier *et al*., 1991).

Afforestation is an emerging policy recommended by the Kyoto protocol to increase the terrestrial uptake of atmospheric CO_2 (Schulze *et al*., 2002). It is assumed that forest ecosystems assimilate more CO_2 from the atmosphere and store more carbon in woody biomass than nonforest ecosystems, but the full environmental consequences of afforestation (e.g. fluxes of other greenhouse gases) are rarely considered. An increase in CH_4 consumption after afforestation on drained peat soils in northern Europe was reported in several studies (Fowler *et al*., 1995; Maljanen *et al*., 2001; Ball *et al*., 2002), but the effect of converting upland grasslands into forests is less well understood. Grassland and peat differ considerably, in moisture, aeration regimes and site preparation before afforestation. They play opposite roles in the global CH_4 cycle: peatlands are a globally important CH_4 source, whereas upland grasslands are a sink. For these reasons, conversion of grasslands to forests may cause fundamentally different changes in CH_4 flux compared with conversion of peatlands.

Methane consumption by soil is controlled by soil diffusion when the microbial activity is high, or by microbial activities when the diffusion is large (Ridgwell *et al*., 1999). While many studies have considered the effects of land use change on CH_4 uptake (Mosier *et al*., 1991, 1997; Sanhueza *et al*., 1994; Kruse & Iversen, 1995; Hütsch, 1998; Ball *et al*., 1999; Koga *et al*., 2004), less is known about the mechanisms of such effects (e.g. whether the effect is mediated by changed soil diffusion or by soil biology).

In soils, CH_4 is oxidized by methanotrophs. Currently, 13 genera of methanotrophs are recognized, which can be clustered into Type I and Type II methanotrophs. These differ in intracytoplasmic membrane structure, carbon assimilation patterns, phylogeny and carbon chain length of membrane phospholipid fatty acids (Hanson & Hanson, 1996). All cultured species, belonging to either Type I or Type II, are unable to grow on low CH_4 concentrations (<200 ppm), whereas the methanotrophs responsible for uptake of atmospheric CH_4 by soil have so far not been isolated (Bender & Conrad, 1992; Knief *et al*., 2006). Thus, not all methanotrophs present in soil are active in atmospheric CH_4 uptake, limiting the application of molecular techniques for studying the diversity of atmospheric CH_4 oxidizers, because DNA-PCR-based methods capture both active and inactive methanotrophs. RNA-based studies could potentially shed light on diversity and phylogenetic identity of high-affinity methanotrophs (Lau *et al*., 2007), but the implied existence of high CH_4 concentrations in aggregate micropores even in upland soils complicates their identification. Methanotrophs can be active and produce a lot of RNAs oxidizing high concentration of CH_4 present in anaerobic micropores (Andersen *et al*., 1998) and not from the atmosphere. Because methanotrophs differ in their phospholipid fatty acid (PLFA) composition (Bowman *et al*., 1993; Dedysh *et al*., 2002), studies of ^{13}C-methane assimilation into specific PLFAs provide a fingerprint of the active methanotroph community (Bull *et al*., 2000; Knief *et al*., 2003, 2006; Crossman *et al*., 2005; Maxfield *et al*., 2006). This technique is less specific than nucleic acid analysis in that it does not allow the identification of active bacteria to the genus level; nevertheless, it provides a direct link between diversity and function of soil methanotrophs at the group level and easily distinguishes Type I and Type II methanotrophs. We applied this technique to understand whether afforestation alters methanotroph biomass, community composition or cell-specific activity.

In this work, we utilized a Siberian afforestation experiment, where the six most commonly dominant forests tree species in Siberia were sown on a well-aerated upland grassland 35 years ago (Menyailo *et al*., 2002a). Comparing afforested plots with the remaining adjacent grassland, we estimated the afforestation effect. Using a combination of field and laboratory incubation studies, we distinguished the role of soil physical and biological factors in mediating the effect of afforestation on CH_4 oxidation.

Materials and methods

Siberian afforestation experiment

The research plots are located 50 km Northwest from Krasnoyarsk and were established by the Laboratory of Soil Science of the Institute of Forest, Siberian Branch of the Russian Academy of Sciences (Menyailo *et al*., 2002a). The experimental plots were established on a well-aerated grassland. The grassland consists of approximately 35 grass and grass-like species belonging to 16 families. The dominant species of the first vegetation layer (0–15 cm tall) are *Poa pratensis* L., *Trifolium repens* L., *Plantago media* L. and *Ranunculus repens* L. The second vegetation layer (40–50 cm) is dominated by *Calamagrostis epigeios* (L.) Roth, *Phleum pratense* L., *Dactylis glomerata*

L., *Carum carvi* L., *Achillea millefolium* L. and *Lathyrus pratensis* L. The plant species composition is nearly homogeneous over the studied area. In 1970, the upper 0–50 cm of soil of 1.5 ha area was removed, mechanically homogenized to reduce the vertical and spatial heterogeneity of soil chemical, physical and biological properties and subsequently returned to the site before experimental planting. During 1971–1972, 2–3-year-old seedlings of spruce (*Picea abies*), birch (*Betula pendula*), Scots pine (*Pinus sylvestris*), aspen (*Populus tremula*), larch (*Larix sibirica*) and Arolla pine (*Pinus cembra*) were sown into individual plots, each occupying 2400 m^2. No fertilizers were applied before or after afforestation. An area of 9600 m^2 was left for grassland as a control and the soil under grass was not mechanically homogenized. The region is characterized by continental climatic conditions, with average rainfall of 500 mm yr^{-1} and average daily summer temperature of 20 °C (at 12:00 hours). The soil is the gray forest type according to the Russian Soil Classification System and Greyzem according to FAO (1990). Field CH_4 fluxes were assessed with closed chamber techniques during the two vegetative seasons: from May to September of 2004 and 2005.

Collection of soil samples

Soil samples for incubation studies were collected in July 2005. For this, each plot was sub-divided into three parts: A, B and C. From each subplot, two trees were randomly chosen and four soil samples (0–10 cm) were taken at 50 cm apart of the stem of each tree. The depth of the organic layers (litter layer) in forest soil was 2–4 cm, relatively shallow because of the short time since establishing the forest canopy (35 years). The maximum CH_4 oxidation activity occurred in the mineral soil at 5–10 cm depth in the forests and at 0–5 cm in the grassland (data not shown). Thus, rates for the 0–10 cm depth that we report capture the maximum activity for both cover vegetation types. Soil samples from each subplot were mixed. The total number of soil samples was 21: six tree species and grassland by three subplots. Field moist soil samples were brought to the laboratory in a box filled with ice. All samples were sieved (1 mm) and gravimetric soil moisture was determined in each sample. Some chemical characteristics of the soil samples are presented in Table 1.

Field CH_4 flux measurements

In each of the subplots, one PVC collar, 25 cm tall and 30 cm in diameter, was installed approximately 5 cm into the soil and left intact for the growing season (May–September). CH_4 uptake was measured between 10:00 and 13:00 hours on a weekly basis using the static chamber technique, placing a PVC lid with gas sampling ports onto each collar, and removing headspace samples over a 40-min period (0, 10, 20 and 40 min). Samples were taken using 10 mL gas-tight plastic syringes which were closed with stopcocks. The CH_4 concentrations were analyzed in the laboratory on a gas chromatograph (Agilent 6890N, Agilent Technologies, Palo Alto, CA, USA), equipped with a flame ionization detector (FID). Repeated measurements of CH_4 standard (2 ppm) resulted in a precision of 2.0% (range of variation observed in five repeat injections). The CH_4 fluxes were calculated, using chamber air temperature, barometric air pressure and the slope of the temporal change in CH_4 concentration within the chamber headspace. The CH_4 flux is reported as mg CH_4 m^{-2} day^{-1}. Parallel to the flux measurements, soil temperature at 10 cm depth was recorded. With each measurement event, samples of mineral soil (0–10 cm, one per plot) were collected for gravimetric water determination. The water-filled pore space (WFPS) was estimated using the volumetric water content and volume of pores. The volume of pores and bulk density were estimated separately for grassland and for forest soils.

Labeling with ^{13}C-CH_4 in incubation experiments

Soil samples (3 g) were placed in 120 mL flasks sealed with rubber stoppers. Distilled water was added to adjust soil moisture to 60% of water holding capacity (WHC). The incubation was carried out during 1 month at three levels of CH_4 addition (30, 200 and 1000 ppm). The 30-day incubation was necessary to obtain detectable

Table 1 Chemical characteristics of soil samples under forest stands ($n = 6$) and grassland ($n = 3$) for 0–10 cm depth

Ecosystem	pH	NH_4^+ (mg kg^{-1})	NO_3^- (mg kg^{-1})	N (%)	C (%)	C/N	DON (mg kg^{-1})	DOC (mg kg^{-1})
Forest	**5.9 (0.0)**	9.7 (2.0)	3.4 (0.8)	0.2 (0.0)	3.5 (0.3)	**15.6 (0.3)**	16.8 (0.5)	**271.5 (9.9)**
Grassland	**6.1 (0.2)**	7.9 (2.6)	4.2 (1.8)	0.3 (0.1)	4.3 (1.0)	**13.8 (0.4)**	18.6 (3.5)	**197.5 (33.4)**
Afforestation effect, *P*-value	**0.030**	0.745	0.694	0.173	0.424	**0.028**	0.294	**0.014**

Mean values and standard errors (in parentheses) are given.
Chemical parameters which were significantly different between grassland and forest soils are shown in bold.

amount of ^{13}C incorporated into PLFAs. Half of the soil samples were incubated with unlabeled CH_4 (with ^{13}C at natural abundance) and half with $^{13}CH_4$ (99.9 at.%). Every 3 days, the flasks were opened to avoid anaerobic conditions for 30 min, after this they were sealed and CH_4 was injected again through syringe at the concentrations given above. Five milliliters of headspace was sampled every day to measure CH_4 concentration, using a gas chromatograph equipped with a flame-ionization detector (SRI Instruments Inc., Torrance, CA, USA) and the rate was estimated for every 3 days using a linear regression and expressed as nmol CH_4 g soil^{-1} day^{-1}. This experiment allowed estimating CH_4 oxidation rates at three levels of CH_4 concentration and to label PLFAs in the soils treated with ^{13}C-CH_4.

PLFA extraction and ^{13}C determination

After the incubation, lipids were extracted from 3 g of soil by a modified Bligh and Dyer method (Bligh & Dyer, 1959) and fractionated on silica columns (CUSIL15Z; ICT, Bad Homburg, Germany). PLFAs were subjected to mild alkaline methanolysis. Separation, identification and quantification of fatty acid methyl esters (FAMEs) was performed by gas chromatography mass spectrometry (GC-MS) on a 5% phenyl methyl silicone capillary column (length 50 m, inside diameter 0.2 mm, HP Ultra 2, Hewlett-Packard, Palo Alto, CA, USA) using the temperature program: 2 min at 150 °C, increase of 4 °C min^{-1} to 280 °C and finally 280 °C for 11 min (Abraham *et al*., 1998). The positions of double bonds were determined by analysis of dimethyl disulfide adducts (Nichols *et al*., 1986). Carbon isotope ratios of the individual FAMEs were determined by GC-IRMS on a Finnigan MAT 252 isotope ratio mass spectrometer (Finnigan, Bremen, Germany) in triplicate. It was coupled via a combustion interface with a HP 5890 gas chromatograph (Hewlett Packard). The FAMEs were separated with a Restek Rtx-2 column (Restek Corp., Bellefonte, PA, USA; 60 m, 0.32 mm inner diameter, 0.25 μm film thickness). The column effluent was combusted on-line in an oxidation oven (copper, nickel, platinum catalyst, 980 °C), passed through a reactor with elemental copper (600 °C) for reducing NO_x and removing surplus O_2. Combustion gas was dried by a water-permeable membrane (Nafion). To calculate isotope ratios ($\delta^{13}C$) for the PLFAs, $\delta^{13}C$ values of the FAMEs were corrected with a mass balance for the carbon atom of the methyl group that was added during methanolysis (Abrajano *et al*., 1994).

Estimation of relative incorporation

For each PLFA, the incorporation of ^{13}C (I, expressed as picograms of ^{13}C per gram of total PLFAs) was calculated as follows: $I = (F_l - F_u) \times (A_x)$, where A_x is the peak area of $PLFA_x$ divided by the sum of the peak areas of all of the PLFAs. F is the fraction of ^{13}C in $PLFA_x$ of samples incubated with ^{13}C (F_l) or samples incubated with ^{12}C (F_u) and was calculated as follows: $F = {}^{13}C/({}^{13}C + {}^{12}C) = R/(R + 1)$. The carbon isotope ratio (R) was derived from the measured $\delta^{13}C$ values as follows: $R = (\delta^{13}C/1000 + 1) \times R_{VPDB}$, with $R_{VPDB} = 0.0112372 \pm 0.0000090$.

Experiment with N addition

To test the effect of inorganic N on high-affinity CH_4 oxidation, we conducted an incubation experiment with six soil samples from the afforested plots (one composite sample for each tree species) and two soil samples from grassland (subplots A and B). N was added either as $(NH_4)_2SO_4$ or KNO_3 at five rates: 0 (control), 5, 25, 100 and 500 mg N kg^{-1}. Incubation was performed at 30 ppm of CH_4 over 1 week. The total number of flasks in the N addition experiment was 80, 8 soil samples by two forms of N by five N concentration levels.

Statistical analysis

The effect of afforestation was assessed by comparing grassland ($n = 3$) with aggregated forest group ($n = 6$) consisting of six mean values for each six tree species. For each tree species, we had three replicates (subplots A, B and C); mean values were estimated so that tree species were used as replicates. The data were tested for homogeneity of variance and normality of distribution. The effect of afforestation on field CH_4 flux was tested with one-way repeated-measures ANOVA. For the laboratory incubation experiments, the effects of afforestation and CH_4 concentrations (three levels) and their interactions were estimated using two-way ANOVAs, where CH_4 oxidation rates (mean rates for whole incubation time), total extracted PLFAs amount, and the relative abundances of 18 : 1ω7 and 16 : 0 were the dependent variables and afforestation and treatment CH_4 concentrations were the independent factors. For the N-addition experiment, the relative response was estimated by normalizing all data to the CH_4 oxidation rate without N addition (control) for both grassland and forest soils. After N addition, the reduced rate is estimated as a percentage of the rate observed under the control conditions. For this experiment, we used a three-way ANOVA design with relative CH_4 oxidation rate the dependent variable and ecosystem (grassland or forest), form of N (NH_4^+ or NO_3^-) and the rate of N application (five levels including control) as independent factors. Spearman correlation analysis was used to assess relationships between (1) field CH_4 consumption rate and soil temperature and moisture, (2) CH_4 uptake rates in

grassland and forests, and (c) the level of ^{13}C incorporation into PLFAs and the amount of CH_4 oxidized. All effects and correlations were considered significant at $P<0.050$. All statistical procedures were performed using the STATISTICA package.

Results

Field flux

CH_4 uptake in the field showed a clear temporal pattern (Fig. 1a): most of the CH_4 was consumed in the summer (main effect of time, $P<0.001$), when soil moisture was lowest (Fig. 1b) and temperature was highest. The effect of afforestation was significant ($P<0.050$), but varied over time (afforestation × time interaction, $P<0.010$), because the difference in CH_4 uptake between grassland and forest soils peaked in the middle of the summer. The rate of CH_4 uptake in the field was more strongly correlated to soil moisture (negative correlation, $r^2=0.37$–0.74) than to soil temperature (positive correlation, $r^2=0.25$–0.37) (Fig. 2). Soil CH_4 uptake rates in grassland correlated in both years to those in forest soils, indicating climatic control over CH_4 fluxes (Fig. 3). CH_4 uptake rates in grassland were nearly three times higher than in forest soils both years, despite higher WFPS in grassland than in forests (Fig. 1b). During the growing season (May–September), the grassland consumed 3.7–3.9 kg CH_4 ha^{-1}, while the forest soils consumed only 1.3–1.8 kg CH_4 ha^{-1}. Higher WFPS in the grassland could not explain the difference in CH_4 uptake, suggesting that some other factor(s) differing between forests and grassland caused the observed difference in CH_4 uptake rates, possibly the biological characteristics of the methane-oxidizing community. The effect of tree species on the field CH_4 flux was small but significant (data not shown). Only two species deviated significantly from the other four: spruce had 20–25% lower and Arolla pine 10–20% higher consumption rates than all other species.

Incubation studies

CH_4 oxidation rates were constant over time at 30 and 200 ppm, but increased over time at 1000 ppm, indicating that this rather high CH_4 concentration initiated the growth of methanotrophic microorganisms, thus increasing the amount of CH_4 oxidized (Fig. 4). Both afforestation ($P<0.001$) and initial CH_4 concentration ($P<0.001$) affected the CH_4 oxidation rate, and these effects were interacting ($P<0.010$). At 30 and 200 ppm, grassland soils showed higher CH_4 oxidation rates compared with forest soils, but this effect disappeared at the highest CH_4 concentration (1000 ppm). Likely, growth of methanotrophic organisms at 1000 ppm masked the effect of afforestation (Fig. 4).

In the laboratory incubations at near-atmospheric concentrations (30 ppm) and at constant moisture and temperature, the difference between grassland and forest in CH_4 consumption was larger than the difference observed in the field.

^{13}C incorporation into PLFAs

The patterns of ^{13}C incorporation into PLFAs in grassland and forests at 30 and 200 ppm were similar: most of ^{13}C (98%) was incorporated into only two PLFAs, 18 : 1ω7 and 16 : 0 (Fig. 5). At 1000 ppm, on the other hand, more PLFAs became labeled (Fig. 5), and these PLFAs were different from those labeled at 30 and 200 ppm, suggesting that microorganisms oxidizing CH_4 at low and high concentrations are different. Because the major scope of this work was high-affinity CH_4 oxidizers, we further concentrated on the 30- and 200-ppm treatments. Even though soils originated from different ecosystems and exhibited different CH_4 oxidation rates, at 30 and 200 ppm, the labeling pattern of the PLFAs was constant: ca. nine times more ^{13}C was incorporated into 18 : 1ω7 than in 16 : 0 (Fig. 5).

The amount of ^{13}C incorporated into PLFAs increased with the amount of CH_4 oxidized (Fig. 6). In the forest soil samples ($n=18$), the ^{13}C concentration in PLFAs was significantly and positively correlated to the amount of CH_4 oxidized (Fig. 6), indicating a strong relationship between CH_4 oxidation and biomass production of the active methanotrophic community. Furthermore, this relationship suggests a constant PLFA concentration among forest soils, because changes in CH_4 oxidation activity (x-axis) are directly correlated with the ratio of newly labeled to total PLFA mass (y-axis). In support of this idea, the total PLFA area among forest soils was constant across samples and species (data not shown). Hence, the biomass of methanotrophs appears to have been equal under all tree species.

However, soil samples from grassland deviated from the linear relationship observed for the forest soils: the concentration of ^{13}C in PLFAs was approximately three times lower per unit of CH_4 oxidized in grassland than in forests (Fig. 6). The similar PLFA profile of the methanotrophs in grasslands and forests (Fig. 5) suggests little difference in carbon use efficiency. Thus, the lower ^{13}C concentration in the PLFAs in grasslands is most simply explained by a threefold higher standing biomass of methanotrophs in the grassland compared with the forest soils. Consistent with this conclusion, the total amount of PLFAs expressed as a sum of all peak

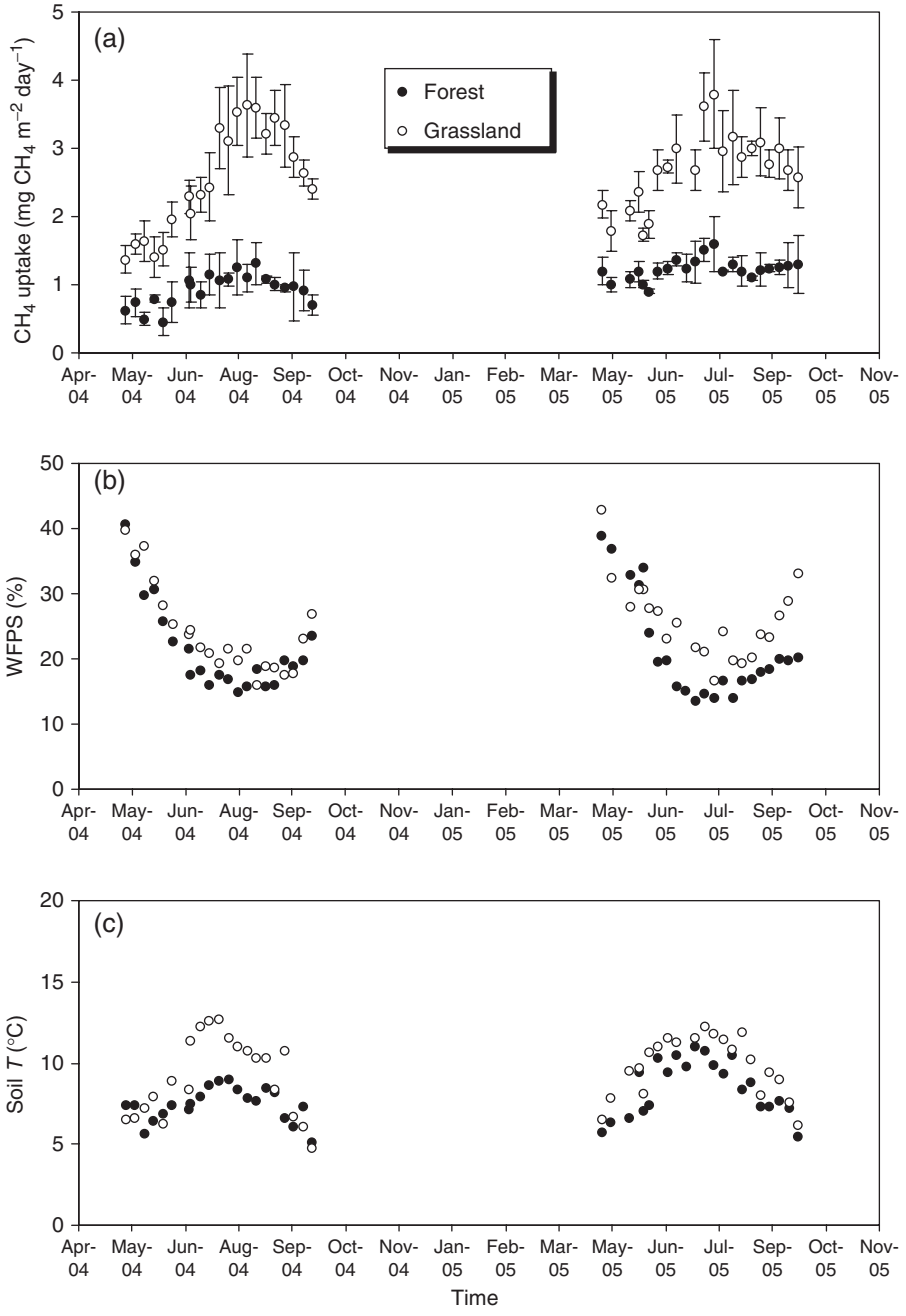

Fig. 1 CH_4 consumption in the field (a), soil moisture (b) and temperature (c) in the Siberian afforestation experiment for May–September of 2004 and 2005. Values are means (and standard errors for CH_4 uptake rate) for six replicates for forests and three replicates for grasslands.

area (Fig. 7a) was three times higher in grassland than in forest soils ($P<0.001$). Thus, afforestation reduced total methanotroph biomass by a factor of 3.

The PLFA profiles did not indicate major changes in the composition of the methanotroph community. The abundance of 16:0 PLFAs as a proportion of total PLFAs was slightly (1–4%) higher in forest than in grassland (Fig. 7b, $P=0.002$), but the abundance of 18:1ω7 PLFAs was not different between grassland and forest (Fig. 7c; $P>0.050$).

Because the activity of CH_4 consumption in the laboratory differed between forest and grassland by a factor of 5, and biomass only by a factor of 3, methanotrophs in grassland should have ca. 40% higher cell-

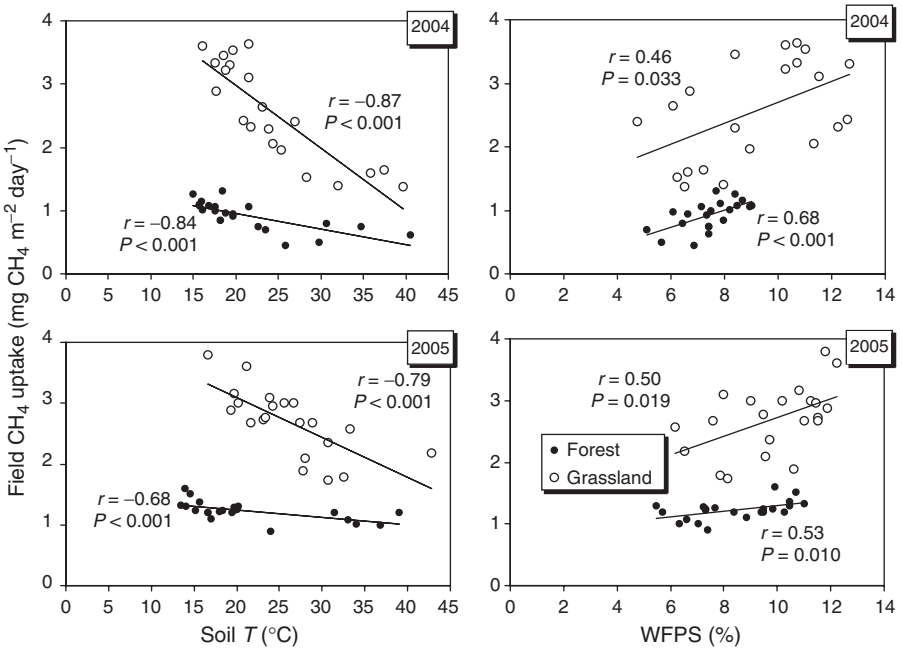

Fig. 2 Relationships between soil moisture and temperature and CH_4 uptake rates during the two growth seasons for forests (dark circles) and grassland (open circles).

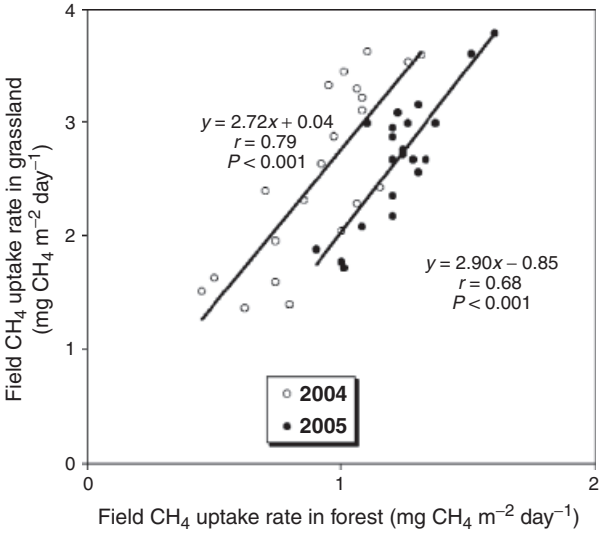

Fig. 3 Relationship between field CH_4 uptake rates in grassland and forests for 2004 (open circles) and 2005 (dark circles). Compared with forest soils, grasslands consumed CH_4 at rates 2.72 times higher in 2004 and 2.9 times higher in 2005.

specific activity, a parameter that can be affected by many environmental variables. Given that many of them (temperature, moisture, O_2 and CH_4 supply) were standardized in our laboratory incubations, changes in N supply due to different net rates of nitrification and net N mineralization (Menyailo *et al*., 2002b) may explain the differences in cell-specific CH_4 oxidation rate.

N addition

Addition of both NO_3^- and NH_4^+ reduced CH_4 oxidation (Fig. 8; main effect of N concentrations – $P < 0.001$; Table 2). The effect of $(NH_4)_2SO_4$ was the same as KNO_3 (Table 2). The relative decline of CH_4 consumption in grassland was significantly stronger than in forests (main effect of ecosystem, $P = 0.003$), and this difference in ecosystem responses to elevated N was most pronounced at higher N concentrations (interaction of ecosystem and N concentration). The results indicate higher sensitivity of CH_4 uptake to N addition in grassland compared with forest soils.

Discussion

Afforestation effects on CH_4 uptake by soil

We demonstrated that converting grassland to forest strongly reduces CH_4 uptake by soil. During the vegetative season (May–September), the grassland consumed 2.2–2.8 times more CH_4 than the forest soils. The values of annual CH_4 uptake varied between 1.3–1.8 kg CH_4 $ha^{-1}\,yr^{-1}$ for forest and 3.7–3.9 kg CH_4 ha^{-1} yr^{-1} for grassland and were in the range of annual CH_4 uptake by different European soils: 0.1–5.5 kg CH_4 $ha^{-1}\,yr^{-1}$

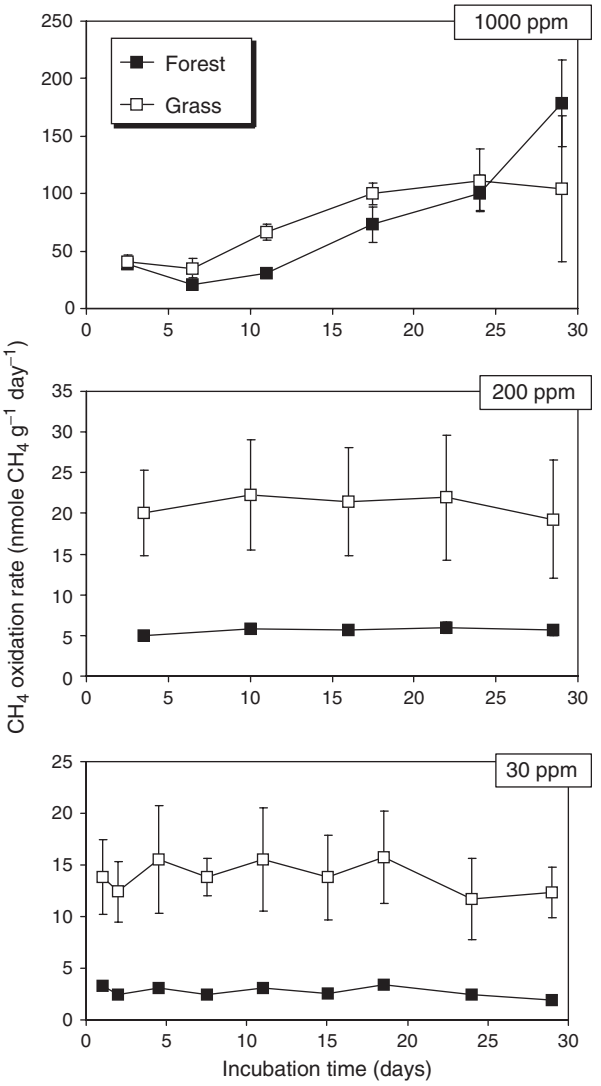

Fig. 4 Dynamics of the CH_4 oxidation rates measured during 1-month laboratory incubations at three CH_4 concentrations. Mean values and standard errors are given for three replicates for grassland and six replicates for forests. Rates of CH_4 oxidation were constant during the incubation at 30 and 200 ppm but not at 1000 ppm of CH_4.

(Smith *et al.*, 2000). We found that the field CH_4 uptake rate was well correlated to soil moisture, in accordance with previous work showing that WFPS and associated soil diffusivity are the major factors controlling CH_4 uptake rate in the field (Smith *et al.*, 2000, 2003) and soil temperature is just a covariate (Brumme & Borken, 1999).

There are several studies where a strong effect of tree species on the field CH_4 fluxes was observed (Borken *et al.*, 2003; Reay *et al.*, 2005; Borken & Beese, 2006). The effect of afforestation thus might depend not only on the site being afforested but also on the tree species selected. Because the variation in the field CH_4 fluxes between different tree species on our sites was not large, we considered the effect of afforestation regardless of the tree species.

The effect we observed is opposite to that found for afforestation of drained peat soils in Northern Europe, where planting trees followed by peat draining increased CH_4 uptake (Fowler *et al.*, 1995; Maljanen *et al.*, 2001; Ball *et al.*, 2002). This difference is not surprising, as peatlands are waterlogged anaerobic soils, in which CH_4 production dominates over consumption. Draining before afforestation aims to lower the water table to allow tree growth. The reported positive effects may thus be due primarily to draining rather than to the direct effects of afforestation. In contrast with peatlands, upland grasslands are well aerated and require no draining before afforestation.

Temperate upland grassland soils are an important global CH_4 sink. Temperate terrestrial ecosystems are responsible for 20 Tg yr^{-1} CH_4 consumption or ca. 66% of global CH_4 consumption by soils (Ojima *et al.*, 1993). Globally, the area covered by temperate grasslands is nearly equal to the area covered by temperate forests (Ojima *et al.*, 1993). Yet, undisturbed temperate forests consume ca. 40% more CH_4 than undisturbed grasslands (Ojima *et al.*, 1993). Our results suggest that, even 30 years after conversion, artificially afforested sites do not oxidize CH_4 at rates comparable to undisturbed forests. If our results are general, such that artificially converting all temperate grasslands to forests could reduce the CH_4 sink by a factor of 3, the temperate CH_4 sink would decline by 20–25% and the global methane soil sink by 12–15%.

But how important is the reduction of the CH_4 sink due to afforestation for the overall budget of greenhouse gases? Given that 1 kg of CH_4 equals 23 kg of CO_2 during a 100-year time scale (IPCC, 2001) and accounting for the mass of C in CO_2 (0.2727), the maximum difference in carbon equivalent methane consumption after afforestation is 12.5 kg C ha^{-1} yr^{-1}. This is a negligible amount compared with carbon storage in woody biomass: the average for all tree species annual biomass accumulation at the Siberian afforestation experiment is near 4000 kg C ha^{-1} yr^{-1} (Vedrova, 2005). Assuming that our measurements of CH_4 consumption are representative for the entire 35-year afforestation period, we can conclude that reduced CH_4 oxidation under forest offsets the total carbon gain of afforestation by only 0.3%. This value could be higher if the costs of afforestation and other consequences of afforestation are considered. We reported, for example, that afforestation alters potential activities of the two steps of denitrification N_2O production and reduction, favoring N_2O as the dominant final product of denitrification in afforested

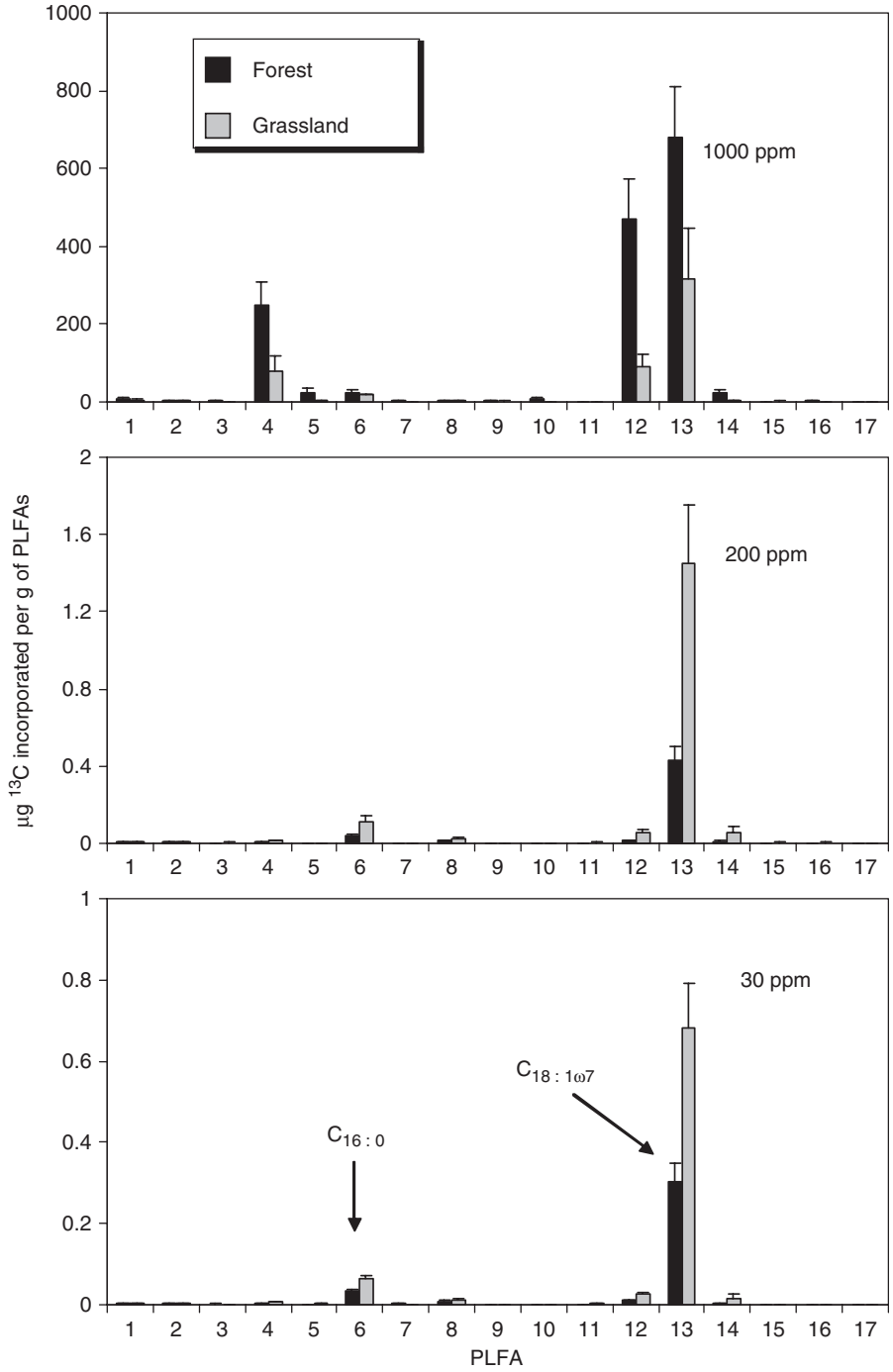

Fig. 5 ^{13}C incorporation into individual PLFAs in soil samples incubated under 30, 200 and 1000 ppm of CH_4. Numbers on the *x*-axis indicate the following PLFAs: (1) i15 : 0, (2) a15 : 0, (3) i16 : 0, (4) 16 : 1ω7c, (5) 16 : 1ω5c, (6) 16 : 0, (7) 10me-16 : 0, (8) i17 : 0, (9) a17 : 0, (10) cy17 : 0ω7, (11) 18 : 2ω6,9c, (12) 18 : 1ω9c, (13) 18 : 1ω7c, (14) 18 : 0, (15) 10me-18 : 0, (16) cy19 : 0ω7 and (17) 20 : 0. Branched fatty acids are indicated by i (iso), a (anteiso), me (methyl) and cy (cyclo). Mean values and standard errors are presented for three replicates (grassland) and six replicates for forest.

soils (Menyailo & Huwe, 1999; Menyailo *et al*., 2002b; Menyailo, 2006). Reduced albedo with afforestation may have far larger impacts on radiative forcing than changes in trace gas budgets, particularly in boreal and northern temperate regions (Liu *et al*., 2005; Randerson *et al*., 2006). In addition, there are consequences beyond radiative forcing such as soil acidification and salinization caused by afforestation (Jackson *et al*., 2005).

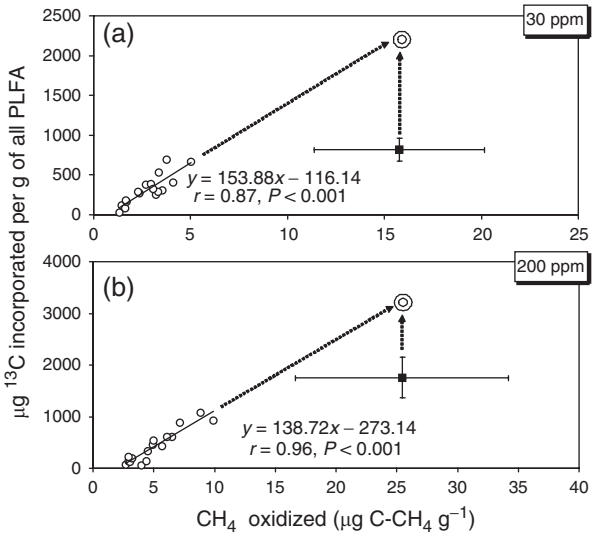

Fig. 6 Plot of ^{13}C incorporated into PLFAs vs. CH_4 oxidized during 1 month of incubation for two concentrations of CH_4: (a) 30 ppm and (b) 200 ppm. Open circles represent forest soils and dark squares represent soil samples from grassland. Arrows and rings indicate putative ^{13}C incorporation into PLFAs in grassland soils if the biomasses of high-affinity methanotrophs were equal between forest and grassland.

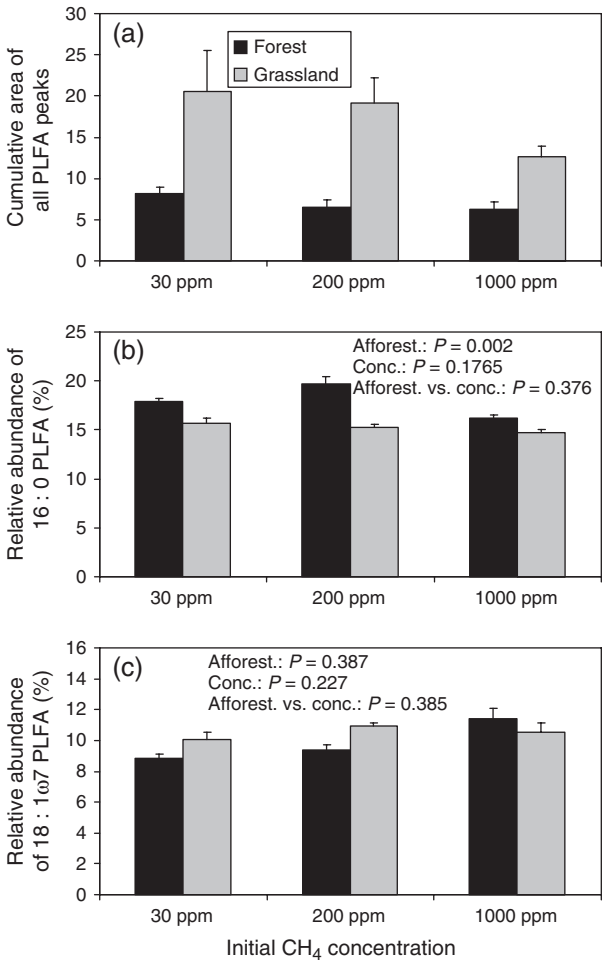

Fig. 7 Cumulative area of all PLFA peaks (a), relative abundance of 16 : 0 (b) and 18 : 1ω7c (c) PLFAs for grassland and forest soils incubated at three concentrations of CH_4.

Mechanisms of afforestation effects on the CH_4 flux – the role of soil biology

The mechanisms by which afforestation and other land use changes affect soil CH_4 uptake are largely unknown. Hypotheses include (1) changes in soil diffusion due to altered porosity caused by plowing (Mosier *et al.*, 1997; Hütsch, 1998; Ball *et al.*, 1999; Koga *et al.*, 2004) or to shifts in moisture regimes caused by different water uptake by different plants; (b) changes in soil inorganic N availability due to N application or to internal N cycling (Steudler *et al.*, 1989); and (c) soil microbial properties, or specifically the biomass, species composition and cell-specific activity of methanotrophic bacteria (Kolb *et al.*, 2003, 2005; Gulledge *et al.*, 2004). The role of soil microbial properties is the least understood factor controlling CH_4 uptake. We used a combination of field observations and laboratory experiments, in which the influence of moisture, temperature and diffusivity is minimized, in order to assess the biological influence on CH_4 consumption in response to afforestation.

In the incubations at near-atmospheric concentrations, the difference between grassland and forest in methane consumption was even larger than in the field. This larger difference under standardized laboratory conditions suggests that factors other than moisture and temperature contribute to the higher CH_4 oxidation rates in grassland, again suggesting the potential importance of differences in soil biological properties. The effect of ecosystem was dependent on the initial CH_4 concentration: differences were larger at lower CH_4 concentrations. Similar results were observed in a previous study (Menyailo & Hungate, 2003), where soil samples were exposed to 10 000 ppm CH_4 and no effect of afforestation was observed. High-affinity methanotrophs consuming atmospheric CH_4 appear to be more sensitive to changes in land use than low-affinity methanotrophs active at higher CH_4 concentrations.

Furthermore, we used ^{13}C labeling of uncultured soil methanotrophs active at different CH_4 concentrations to test whether high- and low-affinity methanotrophs are different microorganisms. At the high CH_4 concentration (1000 ppm), more and different peaks were labeled including those labeled at lower CH_4 concentrations (Fig. 5). This suggests that high-affinity methanotrophs, oxidizing CH_4 at low concentrations, could still be

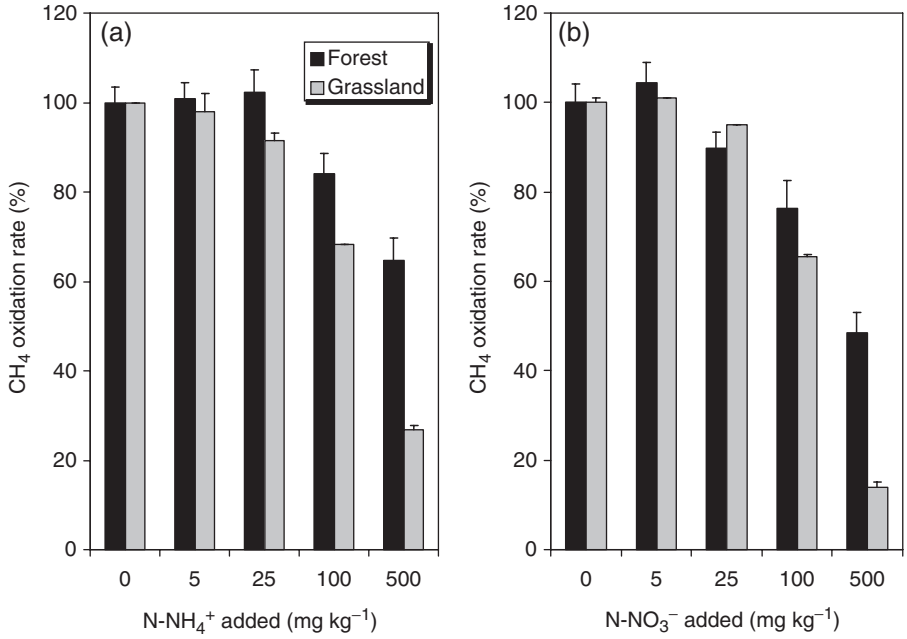

Fig. 8 Effects of five rates of NO_3^- and (a) NH_4^+ or (b) NO_3^- addition on soil CH_4 oxidation in grassland and forest soils. Values (means and standard errors) are expressed as a percentage of rates observed under control conditions; $n = 6$ for forests, $n = 2$ for grasslands.

Table 2 Results of three-way ANOVA with three independent factors – form of N, ecosystem and the rate of N application and one dependent parameter – standardized CH_4 uptake from Fig. 8

Factors	Significance of the main effects, *P*-level
Form of N (NH_4^+ or NO_3^-)	0.247
N concentration (0, 5, 25, 100, 500 mg N kg^{-1})	<0.001
Ecosystem (forest or grassland)	0.003
Form of N × N concentration	0.600
Form of N × Ecosystem	0.511
N concentration × Ecosystem	0.011
Form of N × N concentration × Ecosystem	0.954

active in oxidation of CH_4 at high concentration (1000 ppm), but it also indicates that other additional taxa of methanotrophs become active at 1000 ppm. Because the major scope of our study is atmospheric CH_4 consumption, we further concentrated on high-affinity methanotrophs.

It was surprising that at low CH_4 concentrations, $^{13}CH_4$ incorporation was apparent into only two PLFAs: 18 : 1ω7 and the 16 : 0. While 16 : 0 and 18 : 1ω7 PLFAs are not specific to methanotrophs (Bowman *et al*., 1993; Dedysh *et al*., 2002; Knief *et al*., 2006), the strong correlation between the concentration of ^{13}C incorporated into these two PLFAs and the amount of CH_4 oxidized (Fig. 6) suggests that these PLFAs represent methanotrophs in our soils. The taxa of the methanotrophs whose PLFAs became labeled at low CH_4 concentrations (30 and 200 ppm) cannot be determined exactly, but labeling of 18 : 1ω7 and the 16 : 0 PLFAs is characteristic for type II methanotrophic bacteria, and more exactly to *Methylocapsa acidophila* (Dedysh *et al*., 2002; Crossman *et al*., 2005; Maxfield *et al*., 2006). The observed labeling pattern was similar to that observed in other European soils (Roslev & Iversen, 1999; Knief *et al*., 2003, 2006; Crossman *et al*., 2005; Maxfield *et al*., 2006), where labeling of 18 : 1ω7 and 16 : 0 PLFAs at near atmospheric CH_4 concentrations was associated with the presence of uncultivated methanotrophs of upland soil cluster alpha (USCα) (Knief *et al*., 2003, 2006) identified on the basis of sequences of the gene (*pmoA*) coding for subunit A of the particulate methane monooxygenase (Holmes *et al*., 1999). USCα is considered an uncultured methanotroph responsible for the oxidation of atmospheric CH_4 in upland soils of different geographic regions (Holmes *et al*., 1999; Knief *et al*., 2006) and has been reported to be a dominant methanotroph in upland soils (Kolb *et al*., 2005). Metagenomic data recently showed that USCα is closely related to *M. acidophila* (Ricke *et al*., 2005). Our results suggest that USCα is also a dominant CH_4 consumer in Siberian soils.

Identification of active microorganisms to the species level is possible with the stable isotope probing – SIP (Radajewski *et al*., 2000), in which nucleic acids (either DNA or RNA) of active organisms are labeled with ^{13}C and are physically separated by centrifugation from nucleic acids of inactive organisms. The two fractions of nucleic acids belonging to either active or inactive organisms are further analyzed with traditional molecular biology techniques (e.g. PCR, cloning and sequencing). The SIP is feasible only for rapidly growing bacteria, in which more than 10% of the C in nucleic acids becomes ^{13}C (Mainfield *et al*., 2002). Our data on ^{13}C incorporation into the PLFAs of high-affinity methanotrophs (Fig. 5) suggests that the ^{13}C incorporation is very low, constituting only up to 1 mg of ^{13}C g^{-1} PLFA or 0.1%, 100 times lower than that necessary for SIP. Therefore, ^{13}C labeling of the PLFAs remains the best currently available technique to apply to high-affinity methanotrophs.

Our work differs from other ^{13}C labeling studies (Knief *et al*., 2003, 2006; Crossman *et al*., 2005; Maxfield *et al*., 2006), because we had more numerous and different types of soil samples labeled. This allowed finding a stable pattern of ^{13}C incorporation into PLFAs at low CH_4 concentrations: at 30 and 200 ppm, nine times more ^{13}C was incorporated in 18 : 1ω7 than in 16 : 0, and the ratios of ^{13}C18 : 1ω7 to ^{13}C16 : 0 were independent of the ecosystem. This pattern may have eluded others analyzing fewer soil samples. Because pure cultures of known methanotrophs vary widely in PLFA composition (Bowman *et al*., 1993; Dedysh *et al*., 2002), it is unlikely that the community composition of methanotrophs differed much in our soils. The most plausible explanation for the similar PLFAs and the stable ratio is that the same species of methanotrophs were oxidizing the labeled CH_4 in both grassland and forest soils. Thus, the similarity of PLFAs labeled together with the constant ratio of ^{13}C incorporated into the two individual PLFAs indicates that the methanotrophs responsible for uptake of CH_4 likely belonged to the same taxa in both ecosystems. This argues against the hypothesis that afforestation alters CH_4 uptake by altering composition and diversity of high-affinity methanotrophs. In contrast to our study, afforestation in New Zealand caused detectable changes in labeled PLFAs (Singh *et al*., 2007). Thus, afforestation in different geographic regions can have distinct effect on soil methanotrophs. The effect of afforestation and other land use changes on active methanotrophs should be further tested with molecular biology techniques, once they will become sensitive enough to resolve the diversity of high-affinity methanotrophs.

Afforestation reduces biomass by a factor of 3

Two lines of evidence indicate that the biomass of methanotrophs oxidizing CH_4 at atmospheric concentrations was negatively affected by afforestation. First, afforestation reduced the total microbial biomass (total mass of PLFAs) by a factor of 3, but had almost no effect on the proportion of the PLFAs belonging to high-affinity methanotrophs, which was constant in both grassland and forest. Second, the relative rate of incorporation of ^{13}C into PLFAs was three times lower in grassland per unit of CH_4 oxidized, which is best explained by a higher standing biomass of methanotrophs in the grassland soil. Because we found similar methanotroph PLFA profiles in forests and grasslands (Fig. 5), we assume that they are also similar in carbon use efficiency. If so, the lower ^{13}C labeling in grassland must be due to proportionally higher methanotroph's PLFA amount and higher biomass of high-affinity methanotrophs. Together, these two lines of evidence indicate that afforestation reduces the biomass of active CH_4 oxidizers by a factor of 3. One possible mechanism is that mixing of biomass with those in deeper soil layers by plowing during the site preparation reduced the biomass of methanotrophs in the upper soil layer, where the highest CH_4 oxidation rate is usually reported (e.g. Gulledge *et al*., 2004).

The effect of plowing on the activity of methanotrophs was reported to be negative (Mosier *et al*., 1997; Hütsch, 1998; Ball *et al*., 1999; Koga *et al*., 2004) or neutral (Sanhueza *et al*., 1994; Kruse & Iversen, 1995). Mosier *et al*. (1997) observed that cultivated soils that had been converted back into grassland attained about one-third of the CH_4 uptake rate of natural grassland after 50 years. If the decline in biomass of high-affinity methanotrophs in forest soils was caused by plowing, the low recovery of biomass (35 years in our study and 50 years in Mosier *et al*., 1997) suggests extremely slow growth rates of high-affinity methanotrophs. Lower biomass could be the major reason for reduced CH_4 uptake rate in the field because both of these parameters were three times lower in forests than in grassland. However, in the incubation studies, the difference in CH_4 oxidation rates between grassland and forest soils was even larger, suggesting higher cell-specific activity of methanotrophs in grassland. Differences in biomass may thus only partially explain the effects of afforestation.

Possible effect of inorganic N

The N-addition experiment tested whether the communities of methanotrophs in the two different ecosystems differed in their responses to the availability of NO_3^- and NH_4^+. Here, we hypothesized that methanotrophs showing weaker response to N addition have adapted to higher N supply originating from higher soil N turnover rates. The basis for this hypothesis stems from the work of Mosier *et al*. (1991), who suggested that high

rates of N turnover might decrease soil CH_4 consumption and from the work showing that long-term and short-term N additions have opposite effects on CH_4 consumption by forest soil (Gulledge & Schimel, 1998; Gulledge *et al*., 2004), suggesting adaptation of soil methanotrophs to high NH_4^+ supply.

As expected, we found that CH_4 oxidation declined as N application increased. Surprisingly, both forms of N equally inhibited the CH_4 oxidation. Several studies found both positive and negative effects of NH_4^+ and NO_3^- on CH_4 oxidation (reviewed by Bodelier & Laanbroek, 2004), but none of the possible mechanisms has been experimentally clarified. The mechanism of N effects is difficult to study, because inorganic N can be applied only in the form of a salt and the microbial community can, thereafter, be affected not only by N but also by accompanied cations or anions (or salt effect). Therefore, the effects of NH_4^+ or NO_3^- (compare Fig. 8a and b) are potentially confounded by the possible side effects of SO_4^{2-} or K^+ ions. However, we found a stronger response of CH_4 oxidation in grassland to both salts compared with forests. The difference in ecosystem response to N was most evident at the highest N concentration, $500\,mg\,kg^{-1}$ (Fig. 8). The lower rate of CH_4 consumption in forest soils and their lower sensitivity to N addition might be due to the suppression of CH_4 oxidation by higher rates of N mineralization resulting in higher NH_4^+ concentrations in forest soils.

Soils under forests and grassland differed in C/N ratios, concentrations of dissolved organic carbon (DOC) and soil acidity (Table 1), but the strongest differences were in net N mineralization and net nitrification (Menyailo *et al*., 2002b). More importantly, forests and grasslands differed in net production of NH_4^+ and NO_3^-: while both N forms accumulated in forest soils, only NO_3^- accumulated in grassland. Because NH_4^+ is known to strongly inhibit CH_4 oxidation at the enzymatic level (Steudler *et al*., 1989), higher NH_4^+ abundance in forest soils than in grassland might be responsible for low CH_4 oxidation and low sensitivity to N addition in forest soils. We have thus demonstrated that soils with higher NH_4^+ availability and lower rates of CH_4 consumption respond more weakly to N addition, corroborating the idea of Gulledge *et al*. (2004) that methanotrophs can adapt to a long-term elevated NH_4^+ supply. We only considered N as a possible factor affecting cell-specific activity; however, other factors such as production of gaseous organic compounds (e.g. monoterpenes), which could inhibit CH_4 oxidation (Amaral & Knowles, 1997) should also be considered in the future.

Conclusions

In this work, we demonstrated a strong negative effect of upland grassland afforestation on soil CH_4 uptake. The effect is fundamentally different compared with afforestation of peat soils in northern Europe, but the net effect of methanotrophs on greenhouse gas fluxes caused by afforestation is small: trees store much more carbon ($4000\,kg\,C\,ha^{-1}\,yr^{-1}$) in wood biomass than the decline in CH_4 uptake (in CO_2-carbon equivalent $12.5\,kg\,C\,ha^{-1}\,yr^{-1}$). We also showed that the decline in CH_4 consumption after afforestation is mainly attributed to a reduction in both the biomass and cell-specific activity of methanotrophs, possibly due to higher net NH_4^+ production compared with grassland soils. Using a ^{13}C profiling of PLFAs, we demonstrated that only a narrow group of so far uncultured methanotrophs is capable of CH_4 consumption at atmospheric concentrations. We found that the composition and diversity of this group was not affected by afforestation.

Acknowledgements

We thank Esther Surges for the isotope ratio measurements, V. Menyailo and V. Novikov for the help with field flux measurements, A. Pimenov for botanical description of the grassland and P. Frenzel for discussion of the data. We are deeply grateful to the staff of Soil Science Department of the Institute of Forest in Krasnoyarsk for creation and maintaining the afforestation experiment over the last 35 years. The work was funded by the US Civilian Research and Development Foundation (USA) and by the Alexander von Humboldt Foundation (Germany).

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