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# Inhibition of atmospheric methane oxidation by monoterpenes in Norway spruce and European beech soils

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ABSTRACT

Biological oxidation of atmospheric methane ($CH_4$) in forest soils of the northern hemisphere is an important sink in the global $CH_4$ cycle. The effect of tree species on atmospheric $CH_4$ oxidation is not well understood. Previous studies suggest that soils under European beech (*Fagus sylvatica*) consume more atmospheric $CH_4$ than soils under Norway spruce (*Picea abies*). A major difference in the chemical constituents of these tree species is the production of monoterpenes. The objectives of the present study were (i) to quantify monoterpenes in leaves, needles, organic horizons (Oi, Oe, Oa), and mineral soil of a Norway spruce site and an adjacent European beech site of a temperate forest (Steigerwald, Germany) and (ii) to evaluate the potential of abundant monoterpenes to inhibit atmospheric $CH_4$ oxidation. Major compounds were α- and β-pinene, limonene, and camphene. Highest concentrations were measured in Norway spruce samples (up to 63.9 μmol $g_{dw}^{-1}$). In European beech samples, monoterpene concentrations were close to or below the detection limit ($\leq$0.00015 μmol $g_{dw}^{-1}$). For limonene, α- and β-pinene, the dose-dependent inhibition on atmospheric $CH_4$ oxidation was determined. β-Pinene had the highest inhibition efficiency, followed by limonene and α-pinene. Norway spruce roots and mineral soil samples displayed similar monoterpene profiles, suggesting that roots can be considered as a source for inhibitory monoterpenes in Norway spruce soils. Monoterpene addition was always coupled to an increased carbon dioxide production. This indicates that monoterpenes may be microbially mineralized in these soils. In summary, the study revealed that the release of monoterpenes by both roots and litter may be sources in soil and that *in situ* monoterpene concentrations in spruce soil are high enough to explain reduced atmospheric $CH_4$ uptake.



## 1. Introduction

Methane ($CH_4$) has a 25-fold higher global warming potential than carbon dioxide ($CO_2$) (Forster et al., 2007). Atmospheric concentrations of $CH_4$ increased from a pre-industrial level of 0.75 pmmv to a level of 1.75 ppmv in the past 200 years by human activities (Denman et al., 2007). Global sink processes are chemical oxidation in the atmosphere and biological oxidation in aerated soils (Denman et al., 2007). The biological process is primarily mediated by aerobic methanotrophic bacteria (e.g. Conrad, 1996; Hanson and Hanson, 1996). These prokaryotes possess a unique enzyme, the methane mono-oxygenase (MMO) that catalyses the initial step of methane oxidation (Hanson and Hanson, 1996).

On the northern hemisphere, forest soils consume about 30 Tg of atmospheric $CH_4$ per year and, thus, are the most active terrestrial sink (Denman et al., 2007; Smith et al., 2000). Deciduous forest soils consume more $CH_4$ than coniferous ones under same climatic conditions (Borken et al., 2003; Menyailo and Hungate, 2003; Borken and Beese, 2006). European beech (*Fagus sylvatica*) sites exhibited up to three times higher uptake rates than adjacent Norway spruce (*Picea abies*) sites on the same soil type (Borken et al., 2003), indicating that tree species contributes to the site variation in $CH_4$ uptake. Differences in the monoterpene contents of the both tree species are obvious. European beech produces and emits these compounds in very low concentrations (Holzke et al., 2006), whereas Norway spruce contains high concentrations in needles, twigs, bark, and buds (up to 22.0 μmol [g fresh weight]$^{-1}$; Bufler et al., 1990).

Monoterpenes are derivates of isoprene and may be linear or cyclic carbon compounds ($C_{10}H_{16}$; Conolly and Hill, 1991). These compounds have various functions including allelopathic effects on other plants and defence against herbivores (e.g. Langenheim, 1990). Furthermore, monoterpenes may inhibit growth and activity of methanotrophic pure cultures (Amaral and Knowles, 1997; Amaral et al., 1998a,b). The methanotroph *Methylosinus trichosporium* OB3b (*Methylocystaceae*) is most strongly inhibited by cyclic

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monoterpenes (Amaral et al., 1998b). The inhibition mechanism has not been resolved on a molecular level, but kinetic studies with pure cultures suggest a non-competitive inhibition of MMO (e.g. Amaral et al., 1998b; White, 1994). Needles, bark, and wood of Norway spruce contain divergent monoterpene profiles (Bufler et al., 1990). Thus, it is not clear which monoterpenes are most abundant in Ah horizon of Norway spruce where high $CH_4$ oxidation activity is located (e.g. Henckel et al., 2000) and, therefore, it is still speculative which monoterpenes are the most effective inhibitors of atmospheric $CH_4$ oxidation in temperate Norway spruce forest soils.

Objectives of the present study were (i) to determine the amounts of monoterpenes in roots, green needles or leaves, organic horizons (Oi, Oe, Oa), and upper mineral soil (Ah, Bw) of a temperate forest with adjacent Norway spruce and European beech stands (Steigerwald; Germany) and (ii) to determine the inhibition efficiency by *in situ* abundant monoterpenes on atmospheric $CH_4$ oxidation.

## 2. Materials and methods

### 2.1. Sampling site and sampling

Soils with adjacent European beech (*Fagus sylvatica*; N49°52′; E10°27′) and Norway spruce (*Picea abies*; N49°51′; E10°27′) stands were used in this study (Steigerwald, Germany). Soils under both tree species were classified as Haplic Cambisols (IUSS Working Group WRB, 2006). The pH values were 4.2 and 3.8 and C/N ratios were 17.0 and 19.4 in the top mineral soil under *F. sylvatica* and *P. abies*, respectively. Soil monoliths ($n = 5$) were randomly taken from the upper 20 cm of both sites for the inhibition experiments with limonene and α-pinene on April 23 and with β-pinene on May 22. The second sampling was necessary because the $CH_4$ oxidation rate of the soil considerably decreased after 3–5 weeks. Atmospheric $CH_4$ oxidation rates and gravimetric water contents of soil samples from different sampling dates were similar (data not shown), and thus, allowed the comparison of the inhibitory effect of three investigated monoterpenes.

Each monolith was stored at 5 °C for 10 days. In the laboratory, Oi and Oe horizons were removed because no net $CH_4$ consumption was detected in these soil horizons (unpublished observations). The Oa horizon and the upper 10 cm of the mineral soil were manually homogenized; visible soil animals, roots, and needles or leaves were removed. The homogenized samples were pooled and stored under a plastic cover at 5 °C for the inhibition experiments. Samples from different soil horizons (Oi, Oe, Oa, Ah, and Bw) and plant organs were sampled for analyses of monoterpene concentrations.

### 2.2. Extraction of monoterpenes from soil and plant samples

Monoterpene concentrations were determined in four replicates of organic horizons (Oi, Oe, Oa), mineral soil (Ah, Bw) and fresh plant samples (leaves, needles, and roots) of *F. sylvatica* and *P. abies*. An extraction protocol based on Kainulainen et al. (1993) and Bowman et al. (1997) was used. At the sampling site, fresh samples were chopped with a pair of scissors. Two gram of sample was filled in a tight flask (30 ml screw cap tube) with 10 ml dichloromethane (DCM; Fluka, Germany) and transported to the laboratory. There, 50 μl of the recovery standard 1-chlorooctane (Fluka, Germany) was added to the flask and then incubated in a horizontal shaker (SM 25, Typ SM-C, Edmund Bühler) for 24 h at room temperature. A silica gel-loaded (Fluka, Germany) chromatography column (Roth, Germany) was preconditioned with DCM and, subsequently, the extract was purified by applying it on the column. The remained monoterpene residues in the samples were re-extracted with 5 ml DCM for 10 min. These extracts were also applied to the column. To elute monoterpene residues from the column it was washed twice with 4.5 ml DCM. The total volume of extract summed up to 24 ml. To this purified extract the quantification standard 1-chloroheptane (50 μl; Fluka, Germany) was added. An aliquot of 1.5 ml of this solution was transferred to a 2-ml crimp top vial (Agilent, Germany) and stored at 5 °C until measurement. For reference, the dry weights of the extracted samples were determined according to Scheffer and Schachtschabel (2002) and organic horizon samples were dried to constant weight at 60 °C. A loss of 10% weight due to extraction with DCM was assumed when dry weights were calculated.

### 2.3. Quantification of monoterpenes

Monoterpenes were quantified according to Kainulainen et al. (1993). They were detected using a gas chromatograph coupled to a mass spectrometer (GC–MS; Hewlett Packard HP 6890 Series Mass Selective Detector, USA). Separation of analytes was done on an Equity-5-column (30 m × 0.25 mm, 0.25 μm film thickness; Supelco, Germany) using helium as carrier gas. The oven temperature was programmed from 50 to 70 °C at a rate of 1 °C $min^{-1}$, then at 20 °C $min^{-1}$ to 300 °C held for 10 min. The compounds were quantified using pure monoterpenes at five different concentrations as standards ((−)-α-pinene, Fluka; (−)-β-pinene, Fluka; 3-carene, Fluka; (*S*)-(−)-limonene, Fluka; (+)-camphene, Supelco). Additional monoterpenes were identified based on their retention time according to Merck (unpublished observations, 1988, doctoral thesis, Technical University of Munich). Routine measurements were done in selected ion monitoring (SIM) mode to provide for a detection limit of ≤0.00015 μmol $g_{dw}^{-1}$. Data acquisition and analysis was done with MSD ChemStation Software (Hewlett Packard, USA).

### 2.4. Inhibition experiments

The effect of different monoterpene (α- and β-pinene, limonene) concentrations on $CH_4$ oxidation and $CO_2$ production was studied by using pooled soil samples from the beech and spruce stand. Untreated soils were simultaneously incubated and served as a control. For each replicate ($n = 4$), 80 g soil was put in a 1-l-brown glass flask (Roth, Germany) corresponding to a soil thickness of 1–2 cm in the incubation vessel. The headspace in the flask of about 1 l was precisely calculated considering the volume of the soil sample. The flask was closed with a gas dispersion fitting (Roth, Germany) and connected to a device that allowed flushing, injection and sampling of monoterpenes or gas samples. Before incubation, the headspace was flushed with purified air for 1 h at 15 °C at a rate of 135 ml $min^{-1}$. Then a defined volume of a monoterpene was added by injection into the headspace using a precision syringe (Hamilton, Switzerland). The experiment was performed at 15 °C with atmospheric $CH_4$ concentration. After 0, 12, 24, 36, and 60 h, 20-ml gas samples were taken for analysis of $CH_4$ and $CO_2$ concentrations. Gas samples and four standards were injected by a headspace auto sampler (DANI HSS 1000, Germany) into a gas chromatograph (Shimadzu GC-14A, Germany) equipped with a FID and ECD detector. Data were analysed using Clarity Lite software (Data Apex ltd., UK). Atmospheric $CH_4$ oxidation rates were calculated from first order kinetics and presented in percent of the average value from the controls at same time point.

After 60 h, the remaining monoterpene concentration in the headspace was measured. The gas sampling vial was exchanged with an activated charcoal-packed adsorption tube (ORBO™ 32 Standard Charcoal Tube, Supelco, Germany) and coupled to a vacuum pump (KNF Typ N89 KNDC, Neuberger, Germany). The headspace was flushed for 20 min with a flux of 0.15 l $min^{-1}$