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Microbial Activity and $^{13}C/^{12}C$ Ratio as Evidence of N-Hexadecane and N-Hexadecanoic Acid Biodegradation in Agricultural and Forest Soils

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The dynamics of microbial degradation of exogenous contaminants, n-hexadecane and its primary microbial oxidized metabolite, n-hexadecanoic (palmitic) acid, was studied for topsoils, under agricultural management and beech forest on the basis the changes in O_2 uptake, CO_2 evolution and its associated carbon isotopic signature, the respiratory quotient (RQ) and the priming effect (PE) of substrates. Soil microbial communities in agricultural soil responded to the n-hexadecane addition more rapidly compared to those of forest soil, with lag-periods of about 23 ± 10 and 68 ± 13 hours, respectively. Insignificant difference in the lag-period duration was detected for agricultural ($t_{lag} = 30 \pm 13$ h) and forest ($t_{lag} = 30 \pm 14$ h) soils treated with n-hexadecanoic (palmitic) acid. These results demonstrate that the soil microbiota has different metabolic activities for using n-hexadecane as a reductive hydrocarbon and n-hexadecanoic acid as a partly oxidized hydrocarbon. The corresponding $\delta^{13}C$ of respired CO_2 after the addition of the hydrocarbon contaminants to soils indicates a shift in microbial activity towards the consumption of exogenous substrates with a more complete degradation of n-hexadecane in the agricultural soil, for which some initial contents of hydrocarbons are inherent. It is supposed that the observed deviation of RQ from theoretically calculated value under microbial substrate mineralization is determined by difference in the time (Δt_i) of registration of CO_2 production and O_2 consumption. Positive priming effect (PE) of n-hexadecane and negative PE of n-hexadecanoic (palmitic) acid were detected in agricultural and forest soils. It is suggested that positive PE of n-hexadecane is conditioned by the induction of microbial enzymes that perform hydroxylation/oxygenation of stable SOM compounds mineralized by soil microbiota to CO_2. The microbial metabolism coupled with oxidative decarboxylation of *n*-hexadecanoic acid is considered as one of the most probable causes of the revealed negative PE value.

Keywords microbial activity, O_2 consumption and CO_2 evolution, respiratory quotient, hydrocarbon contamination, respired CO_2-C isotopic signature, priming effect

INTRODUCTION

The complex petroleum hydrocarbon mixtures, which include crude oil, diesel fuel, and creosote comprising various concentrations of linear and branched alkanes, cycloalkanes, phenolics, aromatics, and polycyclic aromatic hydrocarbons, may continue to be major environmental pollutants, since they remain important inputs in industrial and domestic activities, and together with their by-products, may eventually end up in the different compartments of the biosphere. The soil, although is an important sink for a wide range of substances, pollutant load exceeding certain threshold has the potential of impacting negatively on the capacity of the soil to perform its ecosystem functions with repercussions on sustainability issues such as

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plant growth (Adam and Duncan 2002, 2003; Palmroth et al. 2005). Thus, it is becoming increasingly evident that the fate of anthropogenic hydrocarbons pollutants entering the soil system requires efficient monitoring and control.

Soil microbial communities are able to adjust to unfavourable conditions and to use a broad spectrum of substrates (Jobson et al. 1974; Nikitina et al. 2003). They have unique metabolic systems that allow them to utilise both natural and anthropogenic substances as a source of energy and tissue constituents. These unique characteristics make the microbiota useful tool in monitoring and remediation processes. Bioremediation of soil contaminated with petroleum hydrocarbons has been established as an efficient, economic, versatile, and environmentally sound treatment (van Hamme et al. 2003). Several reports have already focused on the composition of natural microbial populations contributing to biotransformation and biodegradation processes in different environments polluted with hydrocarbons (Hamamura et al. 2008; Juck et al. 2000; Marques et al. 2008).

The bioremediation potential of microbial communities in soil polluted with oil hydrocarbons depends on their ability to adapt to new environmental conditions (Kaplan and Kitts 2004; Mishra et al. 2001). Investigations into how bioremediation influences the response of a soil microbial community, in terms of activity and diversity, are presented in a series of publications (Hamamura et al. 2006; Jobson et al. 1974; Margesin et al. 2007; Margesin and Schinner 2001; Zucchi et al. 2003).

The most widely used bioremediation procedure is biostimulation of indigenous microorganisms by the addition of nutrients, because the input of large quantities of carbon sources (i.e., hydrocarbon contamination) results in rapid depletion of the available pools of major inorganic nutrients such as nitrogen and phosphorus (Alexander 1999; Dilly 1999; Margesin et al. 2007). In this case, the methods of monitoring and characterization of hydrocarbon degrading activity of soil microbiota are of special interest (Abbassi and Shquirat 2008; Margesin and Schinner 2005; Pleshakova et al. 2008).

To determine the pool of soil microorganisms utilizing oil hydrocarbons and to estimate microbial activity, n-hexadecane ($C_{16}H_{34}$) labeled with ^{13}C- or ^{14}C-isotope was used as an analogue of aliphatic hydrocarbon constituents of the complex petroleum hydrocarbon mixtures including crude oil, diesel fuel, creosote, etc. (Dashti et al. 2008; Hamamura et al. 2006; Hassan and Sorial 2008; Marchant et al. 2006). The initial stage of aliphatic hydrocarbons oxidation is carried out by specific microbial enzymes (alkane hydroxylase or oxygenase), which occur only in some microorganisms (Smits et al. 1999; Marchant et al. 2006).

Oxygen limitation that may occur under soil conditions can induce changes in the cytochrome levels (Asperger et al. 1986) and microorganisms are able to produce semi-oxidized metabolites. The n-hexadecanoic (palmitic) acid ($C_{16}H_{32}O_2$) may be one of the primary metabolites produced during microbial degradation of n-hexadecane. In the case of n-hexadecanoic acid, the oxidized end of the molecule is used as the initial site for β-oxidation process, whereas the microbial oxidation of saturated hydrocarbons (n-hexadecane) cannot be initiated as easily as it does for n-hexadecanoic acid. (Metzler 1977; Van Hamme at al. 2003).

Comparing of hydrocarbon consumption by soil microbiota in pristine and oily desert soils showed that alkanes and n-hexadecane are essentially used by bacteria and palmitic acid as a semi-oxidized hydrocarbon is utilized by fungi as additional carbon and energy sources (Dashti et al. 2008). Furthermore, the total content of soil microbial biomass (SMB) in arable soils was lower compared to forest and grassland sites (Ananyeva et al. 2008; Dyckmans et al. 2003). It was suggested that SMB depletion in arable soil compared to forest soil might be due to the decrease of the fungal biomass fraction in soil biomass.

Few studies in this context demonstrate that fungal spores dominated the total fungal biomass in arable soils and the major fraction of SMB was bacterial biomass (Baath and Anderson 2002; Stahl et al. 1999; Susyan et al. 2005; Susyan et al. 2011). The initial degradation of n-hexadecane compared to n-hexadecanoic acid would be expectedly more active in arable soils than in forest soil. Thus, the kinetic studies of microbial consumption of n-hexadecane and n-hexadecanoic acid as test substrates may be of profound importance for investigation of microbial diversity and oil degrading potential in soils.

Hydrocarbon biodegradation and transformation in soils can be monitored by estimating the concentration of pollutant and the formation of respective metabolites. One of the most ubiquitous and universal metabolites is carbon dioxide (CO_2), since respiration is by far the prominent pathway of biologically processed carbon. Soil microbiota can be characterized by means of the substrate-induced respiration (SIR). One of the indices of its activity is the priming effect (PE) of introduced exogenous substrate, which is easily accessible for microorganisms (e.g., glucose, cellulose, amino acids, etc.) (Blagodatskaya and Kuzyakov 2008; Fontaine et al., 2004; Zyakun and Dilly 2005).

The role of exogenous substrate in soil is assessed by the extent of activation of soil microbiota for utilization of native soil organic matter (SOM) and may be represented by the following three indices: (a) positive PE shows that exogenous substrate introduction concurrent with its mineralization increases SOM mineralization to a rate exceeding the previous rate; (b) zero PE shows that CO_2 is produced additionally only as a result of microbial mineralization of introduced substrate without changing the existing rate of SOM mineralization; and (c) negative PE values show that exogenous substrate introduction decreases SOM mineralization rate and CO_2 production is determined mainly by mineralization of the substrate.

Obviously, unambiguous determination of PE by CO_2 production calls for an exogenous substrate different from SOM in carbon isotopes (Dilly and Zyakun 2008; Fontaine et al., 2004; Zyakun et al. 2003). PE determination only by the difference of CO_2 production rate before and after substrate introduction into soil suffers from the known uncertainly of CO_2 sources and does not allow distinguishing between the so-called "real"

and "apparent" PE. (Blagodatskaya et al. 2007; Blagodatskaya and Kuzyakov 2008). Since complete oxidation of hydrocarbons demands high amounts of oxygen, the respiratory quotient (RQ) determined by the ratio of molar quantities of CO_2 produced and O_2 consumed is used as an indicator of microbial activity in soil (Dilly 2001, 2003).

The objectives of this study were (i) to examine the microbial response to n-hexadecane and n-hexadecanoic acid added to soils, (ii) to assess the importance of soil type (agricultural and forest) as determinant for the specific activity of hydrocarbon-degrading microbial population, and (iii) to reveal the relationship between reduced (n-hexadecane) and semi-oxidized (n-hexadecanoic acid) hydrocarbons added to native soils and the kinetics of microbial mineralization of hydrocarbons. The results will supposedly be a basis for estimation of the hydrocarbon bioremediation potential of impacted soils.

The metabolic activities of soil microbial community with respect to substrates (n-hexadecane and n-hexadecanoic acid) was determined from CO_2 evolution rates, the respiratory quotient, and the ^{13}C-CO_2 isotope signature. The level of influence of exogenous hydrocarbons on SOM microbial mineralization was assessed by PE value and direction (positive, negative, or zero value). Nitrogen was added to avoid its limitation (Volke-Sepúlveda et al. 2006). After monitoring the respiratory characteristics, residual hydrocarbons and the changes in the carbon isotope signature of microbial biomass were estimated.

MATERIALS AND METHODS

Soil characteristics and sampling. Two soils (A horizon) were sampled at the Bornhöved Lake District in the northern Germany (54°06′N, 10°14′E). The first classified as eutri-cambic Arenosol (IUSS Working Group WRB, 2006) is an agricultural soil under Lolium perennial grass since 2003, after regular crop rotation and has a pH [H_2O] of 6.4, organic C and total N contents of 14.4 mg C and 1.4 mg N g^{-1} soil, respectively. The second, which is a dystric-cambic Arenosol according to IUSS Working Group WRB (2006), developed under beech forest under a matured stratified litter layer, and is characterized by pH [H_2O] of 4.1, an organic C of 33.8 mg C g^{-1} soil, and a total N content of 2.4 mg N g^{-1} soil.

Three soil replicates, each composed of more than 10 subsamples bulked together were collected at a distance of approximately 20 m from each other at both the agricultural and forest sites. Plant materials were carefully removed, and the soils were sieved to 2 mm and stored at 4°C for no longer than one month before the analysis. The soil moisture content was adjusted before incubation experiments and it was varied in the range of 40 to 70% of the water-holding capacity during incubations.

The microbial activities were estimated using three replicates of agricultural and forest soils which were amended with n-hexadecane and n-hexadecanoic acid, respectively. There were twelve independent soil samples in experimental measurements.

Preparation of soil samples and measurements. The hydrophobic compound was applied as follows: n-hexadecane or n-hexadecanoic (palmitic) acid (200 μl of n-hexadecane corresponding to 156 mg and 177 mg of palmitic acid) were added to 1 g of dried and dispersed soil together with 20 mg NH_4Cl (5 mg N) and then 1 g of the soil was mixed with fresh moist soil equivalent to 100 g of dry material. The final substrate concentration was 1.3 mg C g^{-1} soil with a C/N-ratio of 25. Nitrogen was added to favour microbial metabolism (Dilly 1999) and n-hexadecane degradation (Volke-Sepúlveda et al. 2006). Nitrogen was applied as ammonium chloride since nitrate may be denitrified (Chayabutra and Ju 2000).

Moist soil corresponding to 100 g dry weight was pre-incubated for 7 to 10 days (BAS, basal respiration) prior to the addition of n-hexadecane or n-hexadecanoic acid with carbon isotopic signatures different from that of SOM to determine CO_2 sources in the experiments on substrate-induced respiration (SIR).

The oxygen uptake by soil microbiota and total volume of CO_2 produced during the experiments was determined according to the techniques presented early (Dilly and Zyakun 2008; Zyakun and Dilly 2005).

The hydrocarbon substances present in the native soils were quantified as follows: 5 g of soil was extracted with 10 mL of acetone-hexane (1:1) mixture for 2 hours on a shaker; the flask was filled up with 50 mL of deionised water. n-Hexadecane was determined in the hexane phase by gas chromatography with a flame ionisation detector (Column: BPX5 0.25 μm 60 m × 0.25 mm ID, Column flow: 2 mL He min^{-1}, temperature programme: 100°C, 2 min hold, 20 K/min to 240°C, 12 min hold, 30 K/min to 300°C, 17 min hold, Injection: 1 μL splitless 300°C, Detector: 300°C). The n-hexadecanoic (palmitic) acid in the soil was quantified as follows: 40 μL of the hexane phase and 40 μL of the internal standard (0.1 μg mL^{-1} heptadecanoic acid in hexane), was evaporated to dryness and dissolved in 40 μL chloroform plus 40 μL TMSH solution (0.25 M in methanol, Fluka 92732) and analysed as described above. A FAME-mixture (Supelco 47885-U) was used as the external standard.

Carbon isotopic analysis. The ratios of $^{13}C/^{12}C$ abundances of microbial respired CO_2-C in $BaCO_3$ were determined using the Thermo Finnigan Delta V Advantage Isotope Ratio Mass Spectrometer (IRMS) coupled with the Element Analyzer Vario EL (Elementar Instruments). The temperatures in the oxidation and reduction oven were 1150 and 850° C, respectively. Approximately 0.09 mg C, corresponding to 1.8 mg of the dried $BaCO_3$, mixed with about 0.5 mg wolframate for effective combustion, was used for the analysis of the $^{13}C/^{12}C$ ratio. The ratio between the peak intensities at m/z 45 ($^{13}C^{16}O_2$) and 44 ($^{12}C^{16}O_2$) was a quantitative characteristic of the ^{13}C and ^{12}C contents. By convention, the carbon isotope characteristics are expressed by equation [1]:

$$\delta^{13}C = (R_{sa}/R_{st} - 1)1000‰, \quad [1]$$

where $R_{sa} = ([^{13}C]/[^{12}C])_{sa}$ and $R_{st} = ([^{13}C]/[^{12}C])_{st}$ are isotope abundance ratios of a sample and the international Pee Dee Belemnite (PDB) standard (Craig 1957), respectively.

Mass isotopic balance for n-hexadecane. The test-substrate of n-hexadecane with $\delta^{13}C$ value of +47.8±0,2 ‰ was prepared by mixing 30 mg of n-hexadecane-1,2 $^{13}C_2$ (where 99 Atom % ^{13}C ware in 1 and 2 sites) and the remainder of 14 C atoms have natural ^{13}C isotope content (i.e., 1.0960 (±0.0002) Atom% ^{13}C) (Sigma 485799)) with 5000 mg of natural n-hexadecane (HEX) ($\delta^{13}C = -28 \pm 0{,}2$ ‰ relative to the PDB standard or $R_{HEX} = 0.010922(\pm,2{\cdot}10^{-6})$ and $R_{st} = 0.0112372$ (Craig 1957).

The carbon isotope abundance ratio of n-hexadecane-1,2 $^{13}C_2$, 99 Atom% (HEX*) was equal to $R^*_{HEX} = 0.153855$ ($\pm 5{\cdot}10^{-6}$) and was calculated as a ratio of $[^{13}C]_{HEX} = 14{\cdot}0.01096(\pm 2{\cdot}10^{-5}) + 2{\cdot}0.99 = 2.13344(\pm 3{\cdot}10^{-4})$ to $[^{12}C]_{HEX} = 14{\cdot}0.98904(\pm 2{\cdot}10^{-5}) + 2{\cdot}0.01 = 13.86656$ ($\pm 3{\cdot}10^{-4}$) (note that $[^{13}C]^* + [^{12}C]^* = 1$). As is shown by mass isotopic balance, the mixture (MIX) of 30 mg of HEX* with $R^*_{HEX} = 0.153855(\pm\ 2{\cdot}10^{-5})$ and 5000 mg of natural n-hexadecane with $R_{HEX} = 0.010922(\pm\ 0.2{\cdot}10^{-6})$ is characterized by the average carbon isotope abundance ratio equal to R_{MIX} ($0.011774(\pm\ 0.2{\cdot}10^{-6})$ or $\delta^{13}C_{MIX} = +47.8 \pm 0.2$‰ relative to the PDB standard.

When n-hexadecane was used as aliphatic hydrocarbon, it was assumed that the initial and subsequent stages of microbial mineralization of this substance to CO_2 are independent of the presence and sate position of ^{13}C atoms in the molecule. The point is that, the pathways of aliphatic hydrocarbon degradation are represented at the initial stages of this process as molecules containing alcohol – aldehyde – acid groups (Delasse and Nyns 1974; Van Hamme et al. 2003). Oxidation of the mono-alcohol group into an aldehyde group (Tauchert et al. 1978) and a carboxyl group is accompanied by appearance of the key metabolite: n-hexadecanoic (palmitic) acid.

Then Acetyl CoA, which is formed as a result of step by step β-rupture of the C−COO^- bond in n-hexadecanoic acid, is metabolized into CO_2 molecules (Kawamoto et al. 1978). In this case, the characteristics of carbon isotope content ($\delta^{13}C$) of CO_2 pool produced during microbial n-hexadecane mineralization will inherit the carbon isotopic characteristics of the n-hexadecane pool. It means that the $\delta^{13}C$ value of CO_2 pool produced during microbial degradation of n-hexadecane (the average value of $\delta^{13}C_{HEX} = +47.8 \pm 0.2$‰) is rather different from CO_2 pool resulting from soil organic matter (SOM) mineralization ($\delta^{13}C_{SOM}$ is equal to -26.8 ± 0.2‰ and -26.2 ± 0.2‰ for agricultural and forest soils, respectively). These values of $\delta^{13}C$ can be used as specific parameters for mass isotopic balance calculations and PE substrate estimations.

Thus, after addition of the n-hexadecane as a test substrate to soil, the mass isotopic balance for CO_2 evolved during microbial mineralization of SOM and the substrate (SUB) was calculated using equation [2]:

$$\delta^{13}C_{SOM} \cdot Q_{SOM} + \delta^{13}C_{SUB} \cdot Q_{SUB} = \delta^{13}C_{MIX} \cdot (Q_{SOM} + Q_{SUB}), \quad [2]$$

where $\delta^{13}C_{SOM}$ and $\delta^{13}C_{MIX}$ are isotopic characteristics of ^{13}C content in CO_2 pool before and after substrate addition to the soil; $\delta^{13}C_{SUB}$ is the isotopic characteristic of ^{13}C content in CO_2 produced during microbial mineralization of the substrate; Q_{SOM} and Q_{SUB} are CO_2 quantities resulted from microbial mineralization of SOM and added test substrate in the soil samples, respectively. Using the mass isotopic balance, the CO_2 fractions of SOM (F) and of the test substrate (1-F) in the total quantity of CO_2 formed after exogenous substrate addition to the soil were estimated by equation [3]:

$$F_{SOM} = (\delta^{13}C_{MIX} - \delta^{13}C_{SUB})/(\delta^{13}C_{SOM} - \delta^{13}C_{SUB}) \quad [3]$$

Here by definition $F_{SOM} = Q_{SOM}/(Q_{SOM} + Q_{SUB})$ and $1\text{-}F_{SOM} = Q_{SUB}/(Q_{SOM} + Q_{SUB})$

Mass isotopic balance for n-hexadecanoic acid. The test substrate of n-hexadecanoic acid with ^{13}C value of +29.6(±0.2) ‰ was prepared as follows: the 18.5 mg of ^{13}C labeled n-hexadecanoic (palmitic) acid (*PA) containing one ^{13}C in the carboxylic group $^{13}COOH$ about 99 Atom% ^{13}C and the rest of 15 C atoms with the $^{13}C/^{12}C$ ratio of $R_{C15} = 0.010960$ ($\pm 2{\cdot}10^{-6}$) was mixed with 2000 mg of natural n-hexadecanoic acid (PA) ($^{13}{}_{PA} = -26.0$ (±0.2)‰ relative to PDB standard or $R_{PA} = 0.010945(\pm 2{\cdot}10^{-6})$. The average carbon isotope abundance ratio of n-hexadecanoic acid-$^{13}C_1$ labeled in ^{13}C was equal to $R^*_{PA} = 0.07776(\pm 2{\cdot}10^{-5})$ and calculated as a ratio of $[^{13}C]^* = 0.99{+}15{\cdot}0.01096(\pm 2{\cdot}10^{-6}) = 1.1544(\pm 3{\cdot}10^{-5})$ to $[^{12}C]^* = 15{\cdot}0.98904(\pm 2{\cdot}10^{-6}){+}0.01 = 14.8456(\pm 3{\cdot}10^{-5})$ (note that $[^{13}C]^* + [^{12}C]^* = 1$).

The test substrate prepared as a mixture (MIX) of 18.5 mg PA* with $R^*_{PA} = 0.07776(\pm 2{\cdot}10^{-5})$ and 2000 mg of natural n-hexadecanoic acid with $R_{PA} = 0.01096(\pm 2{\cdot}10^{-6})$ was characterized by the average carbon isotope abundance ratio equal to $R_{PA(MIX)} = 0.011570(\pm 2{\cdot}10^{-6})$ or $\delta^{13}C_{PA(MIX)} = +29.6(\pm 0.2)$ ‰ relative to the PDB standard. It means that the $\delta^{13}C$ value of CO_2 pool produced during microbial degradation of the test substrate (n-hexadecanoic acid pool with the average value of $\delta^{13}C_{HEX} = +29.6(\pm 0.2)$ ‰) is rather different from CO_2 pool resulting from soil organic matter (SOM) mineralization ($\delta^{13}C_{SOM}$ is equal to -26.6 ± 0.3 ‰ and -26.2 ± 0.4 ‰ for agricultural and forest soils, respectively). Thus, the values of $\delta^{13}C$ (SOM) and $\delta^{13}C$ (PA) can be used as specific parameters for mass isotopic balance calculations and PE estimations (Eqs. 2 and 3).

Furthermore, it should be remembered that the test substrate PA represents the pool of PA molecules with nonhomogeneity ^{13}C distribution in the carbon sites of molecule. This is due to fact that the PA test substrate is a mixture of 18.5 mg of PA-$^{13}C_1$ (99% ^{13}C) and 2000 mg of PA-$^{13}C_1$ (1.095% ^{13}C) and the probability to detect the ^{13}C isotope in the C_1-site (-COOH) will be higher than in the $C_{2\text{-}16}$-sites of the test substrate pool. According to the mass isotopic balance, we determined that the content of ^{13} in the sate of C_1 (carboxyl group) of the test substrate pool will characterized by $R(C_1) = [^{13}C/^{12}C] = 0.01992$, while the

rest part of carbon atoms of C_2-C_{16}-sates will be $R(C_2\text{-}C_{16}) = 0.01095$.

Respiratory quotient (RQ). To test for microbial activity, the respiratory quotient (RQ), defined as a molar ratio of CO_2 evolution per O_2 consumption during certain time intervals (Δt_i) of microbial growth in soils, was expressed by RQ_i = [mol CO_2]/[mol O_2] (Dilly 2001). The average value of RQ_{av} for microbial activity throughout the total Δt-period defined as $\Delta t = \Sigma(\Delta t_i)$ was calculated by the equation

$$RQ_{av} = \sum (RQ_i \cdot \Delta t_i) / \sum (\Delta t_i) \quad [4]$$

According to the schemes of chemical reactions, the theoretical estimation of RQ values for quantitative aerobic oxidation of n-hexadecane ($C_{16}H_{34}$) and n-hexadecanoic acid ($C_{16}H_{32}O_2$) to CO_2 are equivalent to 0.65 mol CO_2 $mol^{-1}O_2$ and 0.70 mol CO_2 $mol^{-1}O_2$, respectively.

The Kinetics of Substrate-Induced Respiration

Specific CO_2 evolution rates (μ) of soil microorganisms after n-hexadecane or n-hexadecanoic acid addition to soil were estimated from the kinetic analysis of substrate-induced respiration ($CO_2(t)$) by fitting the parameters of equation [5]:

$$CO_2(t) = K + r \cdot \exp(\mu \cdot t) \quad [5]$$

where K is the initial respiration rate uncoupled from ATP production, r is the initial rate of respiration by the growing fraction of the soil microbiota coupled with ATP generation and cell growth, and t is time (Blagodatsky et al. 2000; Panikov and Sizova 1996; Stenström et al. 1998). The lag period duration (t_{lag}) was determined as the time interval between substrate addition and the moment when the increasing rate of microbial growth-related respiration $r \cdot exp(\mu \cdot t)$ became as high as the rate of respiration uncoupled from ATP generation. According to the theory of microbial growth kinetic (Blagodatskaya et al. 2009; Panikov 1995), the lag period was calculated by using the parameters of approximated curve of respiration rate of microorganisms with [6]:

$$t_{lag} = ln(K/r)/\mu \quad [6]$$

Cumulative CO2 resulted from hydrocarbon mineralization. Cumulative CO_2 produced during the microbial substrate oxidation was calculated as follows. The ΔQ_i quantity of CO_2 evolved during the Δt_i-time interval (i = 1,2, ... ,n) was estimated as $\Delta Q_i = \Delta t_i \cdot v_i$, where the v_i-value is the rate of CO_2 evolved during the time interval Δt_i. Using $\delta^{13}C_{soil}$, $\delta^{13}C_{Subst}$ and $\delta^{13}C_{CO2(mix)(i)}$, the fraction of CO_2 resulting from the exogenous substrate (n-hexadecane or n-hexadecanoic acid) oxidation during Δt_i can be calculated as [7]:

$$\Delta Q_{Subst(i)} = (1 - F_i) \cdot \Delta Q_i \quad [7]$$

where F_i value can be estimated using equation [3]. The cumulative CO_2 quantity ($Q_{Subst(CO2)}$) resulting from microbial oxidation of the substrates in soils was presented by [8]:

$$Q_{Subst(CO2)} = \sum \Delta Q_{Subst(i)} \quad [8]$$

where i varied from 1 to n.

Calculation of priming effects. The addition of exogenous test substrates to soil was accompanied by the change in soil microbiota activity: the rate of CO_2 production initially increased as a result of substrate and probably SOM mineralization and then, on depletion of the substrate, gradually decreased. The amount of CO_2 evolved was divided by means of mass isotopic balance into two fractions: from the substrates (n-hexadecane or n-hexadecanoic acid) and from SOM mineralization.

Thus, the difference between CO_2 evolved from SOM mineralization in n-hexadecane and n-hexadecanoic acid amended soil (C_{*SOM}) and in the control soil (C_{SOM}) relative to the control (in percentage) was used to estimate the magnitude of the priming effect (PE) induced by n-hexadecane or n-hexadecanoic acid (denoted as SUB). The PE value was determined in two notations as *kinetic* $PE(\Delta t_i)$ [9] and *total* PE(*total*) calculated as a weighted average value for the whole period of observation using equation [10].

$$PE(\Delta t_i)[\%] = 100 \times \left(C_{*SOM(i)} - C_{SOM(i)}\right) / C_{SOM(i)}, \quad [9]$$

$$PE(total)[\%] = \sum (PE(\Delta t_i) \cdot \Delta t_i) / \sum (\Delta t_i) \quad [10]$$

where $C_{*SOM(i)} = F_i \times C_{(SUB+SOM)I}$; $C_{(SUB+SOM)i}$ is the total C evolved as CO_2 in the amended soil during Δt_i-time; and F_i is the share of CO_2-C resulting from the SOM in n-hexadecane and n-hexadecanoic acid amended soil in Δt_i-time, which was calculated by equation [3].

RESULTS

Basal respiration and hydrocarbon contents. The native hydrocarbon contents in agricultural and forest soils used in the experiments were measured by gas chromatography (Table 1). There are significant differences in the basal amounts of n-hexadecane (HEX) between agricultural and forest soils: n-hexadecane makes up 2.57% of total hydrocarbons in the agricultural soil (equal to 1.5 μg C-HEX g^{-1} soil) but is absent in the forest soil. At the same time, n-hexadecanoic (palmitic) acid (PA) contents are close for both soils: about 7.02% and 7.61% of total hydrocarbons for agricultural and forest soils, respectively (C16:0, Table 1).

At the beginning of experiments the CO_2 evolution rates were not significantly different in agricultural and forest soils (Fig. 1, A, curve 1; D, curve 5; and Fig. 2, A, curve 1; D, curve 5). At the same time, the values of respiratory quotients (RQ) were rather

TABLE 1
The relative quantities of hydrocarbons and fatty acids in soils (in % of sum of total peak areas)

Number	Retardation time [min]	Peak name	Agricultural soil Relative area [%]	Forest soil Relative area [%]
1	8.67	C 10:0	1.23	−**
2	9.91	−*	1.02	1.18
3	10.68	−*	2.36	2.86
4	11.03	n-hexadecane	2.57	−**
5	11.83	−*	1.09	−**
6	12.19	−*	2.38	2.4
7	13.10	−*	1.86	3.69
8	14.03	C 16:1	2.01	2.37
9	14.19	C 16:0	7.02	7.61
10	14.54	−*	3.62	2.53
11	16.74	−*	1.88	1.72
12	16.95	C 18:2n6/C18:1n9	3.95	5.42
13	17.34	C 18:0	1.64	2.68
14	19.59	−*	0.99	1.22
15	22.20	C 20:0	1.18	1.33
16	22.68	−*	1.18	1.53
17	24.61	C 22:2	3.29	11.12
18	25.40	C 22:0	2.66	6.21
19	26.90	C 23:0	1.51	2.51
20	28.43	C 24:0	4.51	4.52

*Components could not be identified.
**No experimental peak was registered.

different: 0.73 ± 0.05 and 0.87 ± 0.05 mol CO_2 mol^{-1} O_2 for agricultural and forest soils, respectively (Fig. 1, C and F). The δ^{13}C-CO_2 value during basal metabolism was -26.88 ± 0.08 ‰ and -26.15 ± 0.18‰ for agricultural and forest soil (Table 2, Time = 0) and was not significantly different from the carbon isotope signature of soil organic matter (SOM) (data not shown) except that the δ^{13}C-CO_2 of agricultural soil was depleted by 0.7‰ relative to the bulk soil during the first 3 days.

Microbial consumption of n-hexadecane. The addition of the test substrate to soils at a rate of 6.77 μM n-hexadecane per g soil or 1300 μg C g^{-1} soil stimulated the CO_2 evolution rate in agricultural soil within 24 hours (Fig. 1, A, curve 1). In the forest soil (Fig. 1, D, curve 5), no significant increase of CO_2 evolution rate was observed during the same period of time.

Parameterization of Equation [5] showed the response of growing microorganisms to the test substrate addition: with n-hexadecane added to the soils, the discrepancy of specific CO_2 production rates (μ) obtained for agricultural vs. forest soil was within the experiment error of analysis, but fraction of growing microorganisms (parameter *r*) was substantially higher in agricultural vs. forest soil (Table 3). Besides, a significant change in lag-period duration was revealed between agricultural and forest soil treated with n-hexadecane (Table 3). In 10–30 and in 45–65 hours, n-hexadecane introduced into agricultural and forest soils, respectively, caused an exponential increase in the CO_2 emission rate indicating microbial growth after the lag-phase (Fig. 3).

In agricultural soil (Fig. 1, A, curve 1), two CO_2 respiration peaks were registered at 2.74 μg CO_2-C g^{-1} soil h^{-1} and 2.20 μg CO_2-C g^{-1} soil h^{-1} in 43 and 165 hours (or 1.8 and 6.9 days) from the moment of n-hexadecane addition, respectively. In forest soil (Fig. 1, D, curve 5), only one peak of the CO_2 emission rate was detected at 4.14 μg CO_2-C g^{-1} soil h^{-1} in 120 hours (or 5 days) from the moment of n-hexadecane addition. On the basis of mass isotopic balance CO_2 fractions, produced during microbial mineralization of n-hexadecane (F_{HEX}), in agricultural and forest soil were calculated (Table 2).

Using F_{HEX} values, the rates of total CO_2 production after addition of n-hexadecane to agricultural and forest soil were divided into two parts: production due to mineralization of n-hexadecane (Fig. 1, A, curve 2; D, curve 6) and due to mineralization of SOM in experiments (Fig. 1, B, curve 3; E; curve 7). For comparison the rates of CO_2 production prior to addition of n-hexadecane to the same soil (control) are given (Fig. 1, B, curve 4; E, curve 8). Simultaneously with the change of rates of CO_2 production alterations of respiratory coefficient which reflected the ratio of CO_2 produced by soil microbiota to consumed oxygen herewith were registered (Fig. 1, C and F).

At the end of 17-day (414-hour) experiments with n-hexadecane, the total metabolic quantities of CO_2 about 631 ±

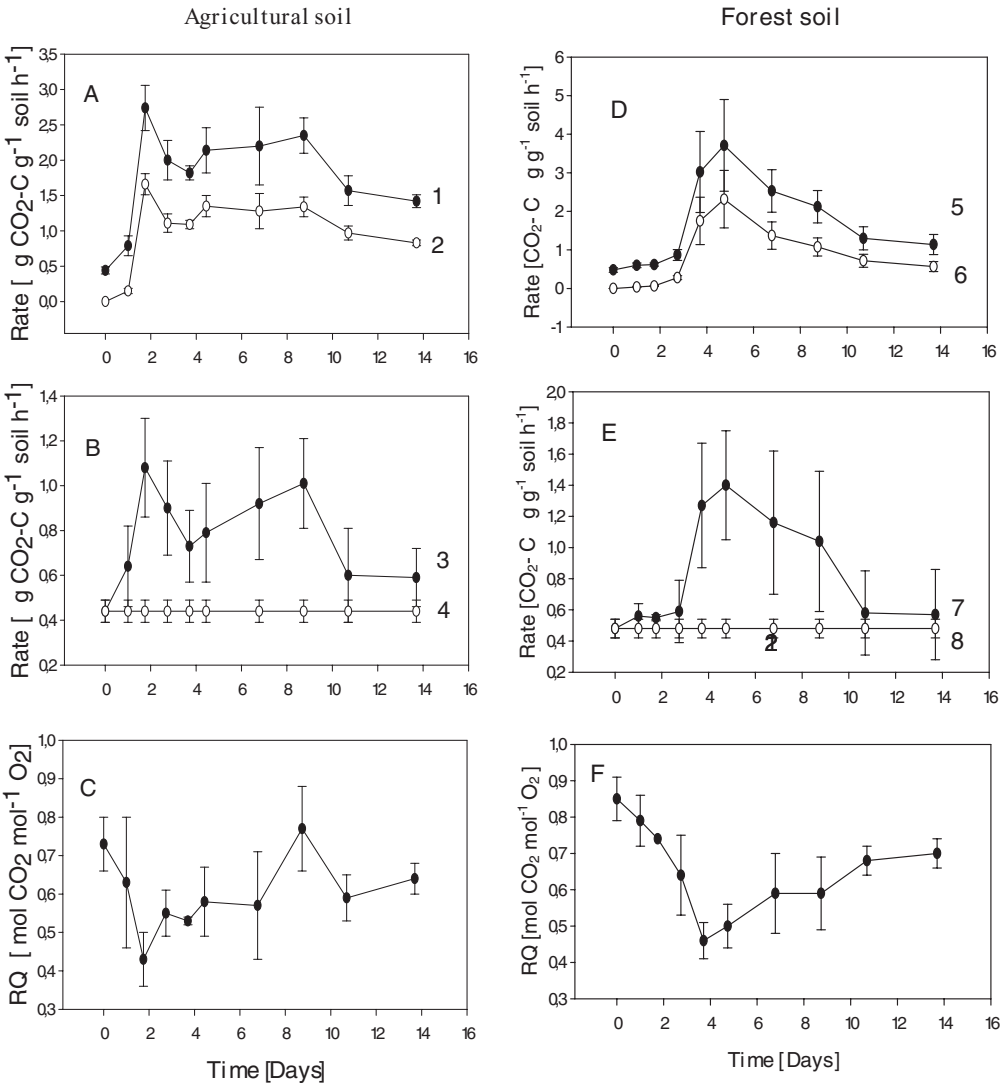

FIG. 1. Carbon dioxide evolution rate and respiratory quotient (RQ) in experiments with n-hexadecane added to agricultural and forest soils: A—(1) total CO_2 rate after n-hexadecane addition to the agricultural soil; (2) rate of CO_2 from n-hexadecane mineralization; B—(3) rate of CO_2 from SOM mineralization after n-hexadecane addition; (4) rate of CO_2 from SOM (control); D—(5) total CO_2 rate after n-hexadecane addition to the forest soil; (6) rate of CO_2 from n-hexadecane mineralization; E—(7) rate of CO_2 from SOM mineralization after n-hexadecane addition; (8) rate of CO_2 from SOM mineralization (control); C and F—respiratory quotient values in agricultural and forest soils; bars extend to confidence limits at $p < 0.05$.

42 μg C-CO_2 g^{-1} soil and about 587 $\pm$ 55 μg C-CO_2 g^{-1} soil were evolved from agricultural and forest soils, respectively (Table 4, Q_{CO2}). Based on the mass isotopic balance [Eq. 2], the quantities of CO_2 resulting from microbial mineralization of n-hexadecane were estimated at about 349 ($\pm$26) μg C-CO_2 g^{-1} soil and 291($\pm$32) μg C-CO_2 g^{-1} soil in the experiments with agricultural and forest soils (Table 4, $Q_{CO2(SUB)}$). At the same time, the total quantities of CO_2 derived from SOM mineralization after the n-hexadecane addition to agricultural and forest soils were 284($\pm$26) μg C-CO_2 g^{-1} soil and 296($\pm$32) μg C-CO_2 g^{-1} soil, respectively (Table 4, $Q_{CO2(SOM)}$).

After the 17-day period, CO_2 production by microbiota from soil without the addition of exogenous substrates (control) was estimated at about 180($\pm$25) μg C-CO_2 g^{-1} soil and 196($\pm$24) μg C-CO_2 g^{-1} soil for agricultural and forest soil, respectively (Table 4, $Q_{CO2(contr)}$). The results of calculated average PE are presented in Table 4: the positive priming effect of n-hexadecane ($PE_{agr} = +57(\pm29)\%$ and $PE_{forest} = +51(\pm29)\%$) were estimated for agricultural and forest soil, respectively. Concurrently to the stimulation of respiratory response by n-hexadecane, the kinetics of the positive priming production of CO_2 from SOM mineralization relative to native process was determined for agricultural soil and for forest soil (Fig. 1, B, curves 3 relative to curve 4: E, curves 7 relative to curve 8).

Microbial consumption of n-hexadecanoic acid. Experimentally, the addition of n-hexadecanoic (palmitic) acid to soils

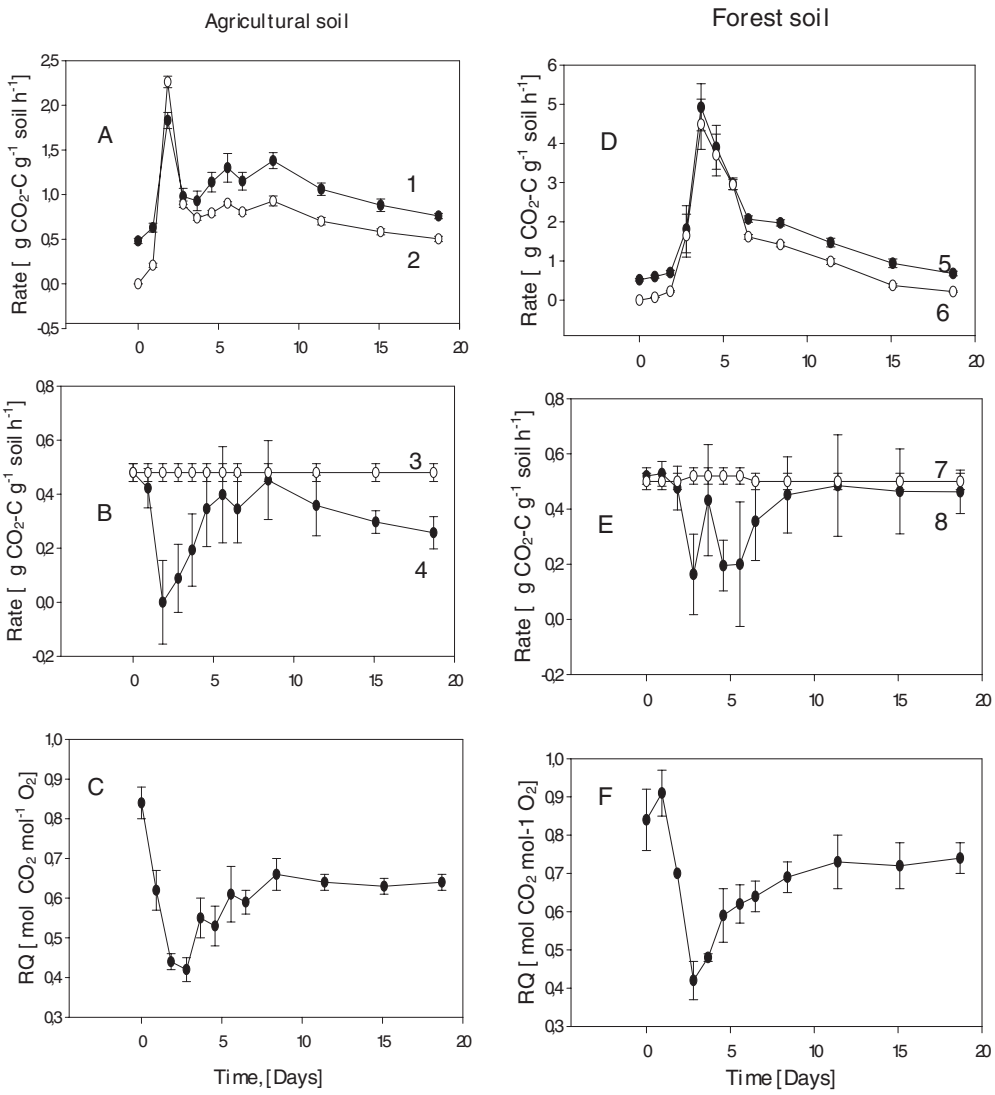

FIG. 2. Carbon dioxide evolution rate and respiratory quotient (RQ) in experiments with n-hexadecanoic acid added to agricultural and forest soils: A—(1) total CO_2 rate after n-hexadecanoic acid addition to the agricultural soil; (2) rate of CO_2 from n-hexadecanoic acid mineralization; B—(3) rate of CO_2 from SOM (control); (4) rate of CO_2 from SOM mineralization after n-hexadecanoic acid addition); D—(5) total CO_2 rate after n-hexadecanoic acid addition to the forest soil; (6) rate of CO_2 from n-hexadecanoic acid mineralization; E—(7) rate of CO_2 from SOM mineralization (control); (8) rate of CO_2 from SOM mineralization after n-hexadecanoic acid addition; C and F—respiratory quotient values in agricultural and forest soils; bars extend to confidence limits at $p < 0.05$.

at a rate of 6.77 μM n-hexadecanoic acid g^{-1} soil or 1300 μg C g^{-1} soil stimulated CO_2 evolution in agricultural soil within 24 hours, while in forest soil CO_2 evolution was delayed within this period (Fig. 2, A, curve 1; and D, curve 5). Besides, two CO_2 respiration peaks were registered in agricultural soil at 1.8 μg CO_2-C g^{-1} soil h^{-1} and 1.4 μg CO_2-C g^{-1} soil h^{-1} in 24 and 120 h (or 1.0 and 5.0 days) after n-hexadecanoic acid addition (Fig. 2, A, curve 1), respectively. In forest soil, only one peak of CO_2 emission rate was detected at 4±1 μg CO_2-C g^{-1} soil h^{-1} in 84 h (or 3.5 days) after n-hexadecanoic acid addition (Fig. 2, D, curve 5).

By analogy with n-hexadecane, parameterization of Equation [5] revealed the response of growing microorganisms after the addition of n-hexadecanoic acid to agricultural and forest soils. The parameter (r) for CO_2 production by agricultural soil was four times lower than for the forest soil, but specific CO_2 production rates (μ) were twice higher for agricultural than for forest soil (Table 3). The durations of lag-periods for CO_2 emission in agricultural and forest soils after n-hexadecanoic acid treatment were close to each other within the accuracy of analysis and estimated at about 30 hours (Table 3).

At the end of the 16-day experiments with n-hexadecanoic acid, the total quantities of metabolic CO_2 about 387 ± 30 μg C-CO_2 g^{-1} soil and about 548 ± 35 μg C-CO_2 g^{-1} soil were evolved from agricultural and forest soils, respectively (Table 4, Q_{CO2}).

TABLE 2
Carbon isotope indicators of metabolic CO_2 ($\delta^{13}C_{CO2}$, ‰) before and after n-hexadecane addition to soils and CO_2 fractions (F_{HEX}) produced during mineralization of n-hexadecane

	Agricultural soil		Forest soil	
Time [days]	$\delta^{13}C_{CO2}$ [‰]	$^*F_{HEX}$	$\delta^{13}C_{CO2}$ [‰]	$^*F_{HEX}$
0.00	-26.88 ± 0.08	0.000	-26.15 ± 0.18	0.000
1.00	-13.60 ± 2.70	0.178 ± 0.036	-21.40 ± 0.79	0.064 ± 0.024
1.75	18.07 ± 1.03	0.604 ± 0.014	-18.00	0.110 ± 0.020
2.74	14.20 ± 1.29	0.552 ± 0.017	-2.95 ± 2.48	0.315 ± 0.034
3.71	17.51 ± 1.18	0.596 ± 0.016	16.65 ± 4.18	0.580 ± 0.057
4.47	19.87 ± 0.90	0.628 ± 0.012	19.87 ± 0.90	0.624 ± 0.012
6.78	16.12 ± 1.58	0.577 ± 0.021	13.82 ± 5.56	0.542 ± 0.075
8.74	14.90 ± 2.42	0.561 ± 0.033	11.33 ± 3.30	0.508 ± 0.045
10.7	18.90 ± 1.00	0.615 ± 0.013	14.70 ± 1.00	0.554 ± 0.014
13.7	16.70 ± 1.50	0.585 ± 0.008	10.60 ± 1.40	0.498 ± 0.020

$^*F_{HEX} = (\delta^{13}C_{CO2} - \delta^{13}C_{SOM})/(\delta^{13}C_{HEX} - \delta^{13}C_{SOM})$ is the fraction of CO_2 produced during microbial mineralization of n-hexadecane, where $\delta^{13}C_{HEX} = +47.8$ ‰ and $\delta^{13}C_{SOM}$ for CO_2 evolved from agricultural and forest soil were equal to -26.88 (0.08) ‰ and -26.15 (0.18) ‰, respectively.

Value ± standard deviation.

The carbon isotope indicator ($\delta^{13}C$) of CO_2 evolved from soils after the test substrate addition to the soils was measured during Δt_i time intervals (Table 5). Using of the test substrate as a mixture of n-hexadecanoic (PA) acid fractions labeled and unlabeled in C_1-^{13}C, the substrate content of isotope is characterized by different probability of ^{13}C detection on separate carbon sates of the PA molecule in the pool of molecules, i.e., there is intramolecular isotope heterogeneity at the level of molecular pool (see section of *Mass isotopic balance for n-hexadecanoic acid)*. In the present view, the succession of inclusion of C_1 – C_{16} atoms of the PA substrate into the microbial cell reaction is strictly determined and isotopic characteristic of PA fraction in CO_2 pool should be depended on the number carbon atoms of substrate molecules mineralized. It is easily comprehended that the change in carbon isotope characteristics of CO_2 ($\delta^{13}C_{CO2}$) as a result of PA mineralization will take place only at the initial stage of PA uptake (lag period). It should be specially emphasized that these considerations are significant only for presentation of the possible kinetics of CO_2 production from PA and SOM and are not used for estimation of the total value of priming effect.

As already noted, after all 16 carbon atoms of the first portions of consumed PA molecules are oxidized to CO_2; the isotope characteristics of CO_2 produced from the PA fraction will stabilize at a level of the mean value of isotopic indices of the PA molecule pool. In our case, it corresponds to $R = 0.011572$ or $\delta^{13}C_{PA} = +29.6$‰. The ^{13} value (Table 5), which exceeds the mean ^{13} at the initial stage of PA mineralization, is evidence of the existing mechanism of microbial mineralization of PA, i.e., PA mineralization begins with CO_2 production as a result of breakage of the -COOH group. We believe that at the end of the lag period the mean value of CO_2 formed during

TABLE 3
Parameters of the equations $CO_2(t) = K + r \cdot \exp(\mu \cdot t)$ and $t_{lag} = \ln(K/r)/\mu$ characterized the rates of n-hexadecane and n-hexadecanoic acid induced CO_2 respiration and microbial growing in the agricultural and forest soils

	K	r	μ	t_{lag}
Treatment	[μg CO_2 g^{-1} soil h^{-1}]			[hour]
n-hexadecane				
Agricultural soil	0.4263 ± 0.0513	0.0289 ± 0.0160	0.1055 ± 0.0126	23 ± 10
Forest soil	0.5910 ± 0.0282	0.0009 ± 0.0005	0.0915 ± 0.0069	68 ± 13
n-hexadecanoic acid				
Agricultural soil	0.4726 ± 0.0360	0.0205 ± 0.0126	0.0953 ± 0.0132	30 ± 13
Forest soil	0.3978 ± 0.0830	0.0975 ± 0.0257	0.0427 ± 0.0028	30 ± 14

Value ± standard deviation.

TABLE 4
Microbial mineralization of substrates (SUB) (n-hexadecane and n-hexadecanoic (palmitic) acid) added to the agricultural and forest soils and soil organic matter (SOM) and priming effects of substrates

	n-hexadecane		n-hexadecanoic acid	
Parameters	Agricultural soil	Forest soil	Agricultural soil	Forest soil
Time (t), [hour]	414	414	385	385
Q_{CO2}, μgC-CO_2 g^{-1} soil	631 ± 42	587 ± 55	387 ± 30	548 ± 35
$\delta^{13}C_{av}$,‰	$+14.4 \pm 0.5$	$+10.5 \pm 0.5$	$+12.7 \pm 0.3$	$+15.7 \pm 0.5$
$X_{(SUB)}$	0.553 ± 0.005	0.496 ± 0.008	0.697 ± 0.013	0.751 ± 0.016
$Q_{CO2(SUB)}$, μgC-CO_2 g^{-1} soil	349 ± 26	291 ± 32	270 ± 26	412 ± 35
$Q_{CO2(SOM)}$, μgC-CO_2 g^{-1} soil	284 ± 26	296 ± 32	117 ± 26	136 ± 35
$Q_{CO2(contr)}$ μgC-CO_2 g^{-1} soil	180 ± 25	196 ± 24	184 ± 25	200 ± 27
$PE_{average}$,%	$+57(\pm 29)$	$+51(\pm 29)$	$-36(\pm 28)$	$-32(\pm 30)$

$t = \Sigma(\Delta \mathbf{t_i})$, (hour) is the time interval of measurements;

$\mathbf{Q_{CO2}} = \Sigma(\Delta \mathbf{t_i} \cdot \mathbf{v_i})$, ($\mu$g C-$CO_2$ g^{-1} soil) is a total CO_2 produced during the time t where v_i (μg C-CO_2 ^{1} soil h^{-1}) is a rate of CO_2 evolved from soil in the time interval of Δt_i;

$\delta^{13}\mathbf{C_{av}} = \Sigma(\delta^{13}\mathbf{C_i} \cdot \Delta \mathbf{t_i} \cdot \mathbf{v_i})/\mathbf{Q_{CO2}}$, (‰) is an average value;

$\mathbf{X_{(SUB)}} = (\delta^{13}\mathbf{C_{av}} - \delta^{13}\mathbf{C_{t=0}})/(\delta^{13}\mathbf{C_{substr}} - \delta^{13}\mathbf{C_{t=0}})$ is a carbon fraction derived from substrates (n-hexadecane or n-hexadecanoic acid) in the total CO_2 respired and $\delta^{13}C_{t=0}$ is the carbon isotopic characteristic of CO_2 before substrates addition to soil;

$\mathbf{Q_{CO2(SOM)}} = \mathbf{(1\text{-}x)} \cdot \mathbf{Q_{CO2}}$, ($\mu$g C-$CO_2$ g^{-1} soil) is a quantity of CO_2 evolved from soil amended with n-hexadecane or n-hexadecanoic acid;

$\mathbf{Q_{CO2(contr)}} = \mathbf{t} \cdot \mathbf{v_{contr}}$, ($\mu$g C-$CO_2$ g^{-1} soil) is a total CO_2 produced during time of experiment, where v_{icontr} (μg C-CO_2 g^{-1} soil h^{-1}) is a rate of CO_2 evolved from the agricultural and forest soils without the substrate addition;

$\mathbf{PE} = \mathbf{100} \cdot (\mathbf{Q_{CO2(SOM)}} - \mathbf{Q_{CO2(contr)}})/\mathbf{Q_{CO2(contr)}}$, (%) is the average priming effect of microbial mineralization of substrates (n-hexadecane or n-hexadecanoic acid);

Value $\pm$ standard deviation.

mineralization of the PA test substrate approaches the value of $C = +29‰$.

According to the pattern of experiments, the $\delta^{13}C$ value of the test substrate (n-hexadecanoic acid) amounted to as average of $+29.6‰$ and was rather different from $\delta^{13}C$ values of the SOM of agricultural and forest soils ($\delta^{13}C$-CO_2 values which were equal to -26.6 ± 0.3 ‰ and -26.2 ± 0.4 ‰ for agricultural and forest soil, respectively)

The weighted average values of C_{av} of the respired CO_2 pools for agricultural and forest soils were $+12.7 \pm 0.3$ and

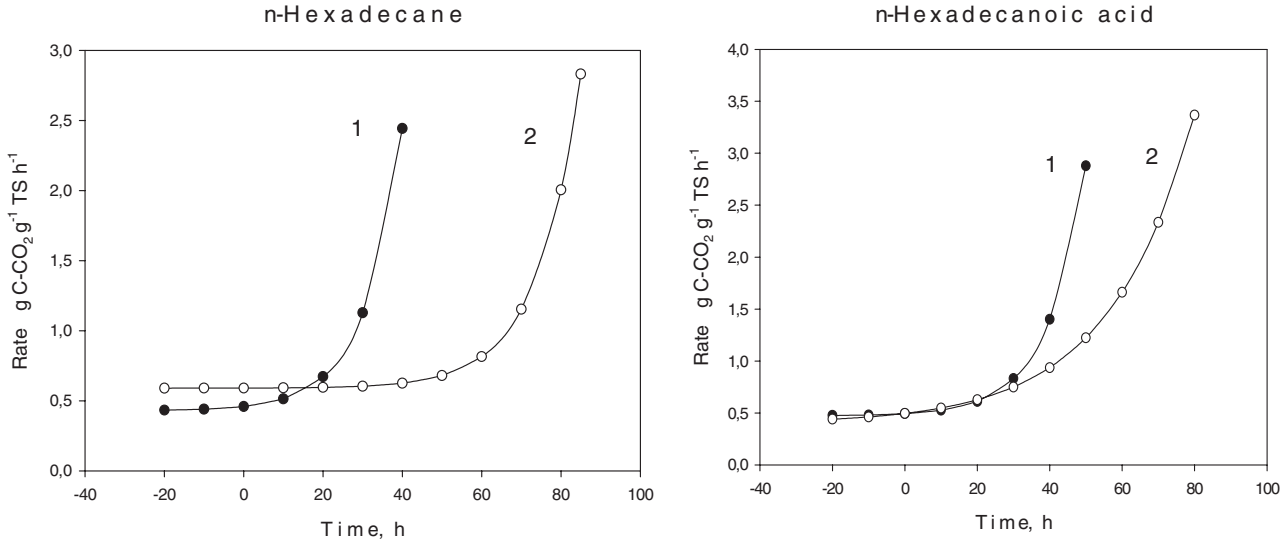

FIG. 3. Kinetics of substrate-induced respiratory responses of microbial communities during periods of slow mineralization of n-hexadecane (left) and n-hexadecanoic (palmitic) acid (right) in the agricultural (1) and forest (2) soils: rates of microbial CO_2 production before substrate addition to soils are labeled as "−" value of time, h. (Experimental data are shown as symbols and model simulation as curves according to Equation 1 with parameters presented in Table 3).

TABLE 5
Carbon isotope indicators of metabolic CO_2 ($\delta^{13}C_{CO2}$, ‰) before and after n-hexadecanoic (palmitic) acid addition to soils and CO_2 fractions (F_{PA}) produced during mineralization of n-hexadecanoic acid

Time [days]	Agricutural soil		Forest soil	
	$\delta^{13}C_{CO2}$ [‰]	F_{PA}	$\delta^{13}C_{CO2}$ [‰]	F_{PA}
0.00	-26.6 ± 0.3	0.00	-26.2 ± 0.4	0.00
0.92	-10.0 ± 1.8	0.30 ± 0.04	-20.3 ± 0.68	0.11 ± 0.02
1.84	36.2 ± 1.8	<1.0	-10.0 ± 0.70	0.29 ± 0.02
2.80	20.0 ± 1.8	0.83 ± 0.04	19.4 ± 2.98	0.82 ± 0.06
3.67	13.6 ± 1.3	0.72 ± 0.03	31.1 ± 3.05	<1.0
4.58	8.7 ± 1.3	0.63 ± 0.03	21.6 ± 1.08	0.86 ± 0.03
5.57	8.6 ± 0.7	0.63 ± 0.02	16.1 ± 4.92	0.76 ± 0.09
6.49	8.9 ± 1.1	0.63 ± 0.02	12.8 ± 3.74	0.70 ± 0.07
8.40	7.5 ± 2.1	0.61 ± 0.04	9.9 ± 2.97	0.65 ± 0.06
11.40	7.0 ± 1.8	0.60 ± 0.04	7.5 ± 2.00	0.60 ± 0.04
15.10	7.0 ± 1.9	0.60 ± 0.05	-6.0 ± 3.00	0.36 ± 0.06
18.70	7.0 ± 2.0	0.60 ± 0.05	-10.0 ± 3.50	0.29 ± 0.07

*$F_{PA} = (\delta^{13}C_{CO2} - \delta^{13}C_{SOM})/(\delta^{13}C_{PA} - \delta^{13}C_{SOM})$ is the fraction of CO_2 produced during microbial mineralization of n-hexadecanoic acid, where $\delta^{13}C_{PA} = +29.6 \pm 0.2$ ‰ and $\delta^{13}C_{SOM}$ for CO_2 evolved from agricultural and forest soil were equal to -26.6 (± 0.3) ‰ and -26.2 (± 0.4) ‰, respectively.

Value ± standard deviation.

$+15.7 \pm 0.5$‰ (Table 4, $\delta^{13}C_{av}$), respectively, and considered as an evidence of simultaneous metabolism of the introduced test substrate and SOM. Taking into account the isotopic characteristic of CO_2 evolved from soil with $\delta^{13}C_{av\ (PA+SOM)}$ and substrate introduced into soil with $\delta^{13}C = +29.6 \pm 0.2$ ‰, the carbon fractions of n-hexadecanoic (palmitic) acid and SOM was calculated using Eq. 3.

The quantities of CO_2 resulting from microbial mineralization of n-hexadecanoic acid were estimated at about 270 (±26) μg C-CO_2 g^{-1} soil and 412 (±35) μg C-CO_2 g^{-1} soil in the experiments with agricultural and forest soil (Table 4, $Q_{CO2(SUB)}$), respectively. The total quantities of CO_2 derived from SOM mineralization after the n-hexadecanoic acid addition to agricultural and forest soils were estimated about 117 (±26) μg C-CO_2 g^{-1} soil and 136 (±35) μg C-CO_2 g^{-1} soil, respectively.

Comparing the quantities of CO_2 produced with and without test substrate addition to soils, the priming of SOM mineralization in experiments was evaluated. The results of average PE calculation are presented (Table 4, PE(average)): the negative priming effects of n-hexadecanoic acid ($PE_{agr} = -36(\pm 28)\%$ and $PE_{forest} = -32(\pm 30)\%$) were detected for agricultural and forest soil, respectively. Concurrently to the stimulation of respiratory response by *n*-hexadecanoic acid, the kinetics of the production of CO_2 from SOM mineralization in soils amended with n-hexadecanoic acid relative to native process was determined for agricultural soil and forest soil (Fig. 2, B, curves 4 relative to curve 3: E, curves 8 relative to curve 7).

Microbial O_2 consumption and respiratory quotient. The kinetics of RQ variation in the soils with *n*-hexadecane (Fig. 1, C and F) and *n*-hexadecanoic acid (Fig. 2, C and F) were determined within certain periods (Δt_i) of about 8–12 h at the initial stages and 20–24 h at the final stages of the experiment. Following substrates addition, the respiratory quotient (RQ_i) decreased to 0.4 mol CO_2 mol^{-1} O_2, which was lower than the theoretically expected values of 0.65 and 0.70 mol CO_2 mol^{-1} O_2 for the complete oxidation of n-hexadecane and n-hexadecanoic acid and indicated relatively high O_2 requirement. The rapid increase of respiration rates in agricultural and forest soils at the initial stage of the experiments was reflected in the earlier lowering of the RQ_i value (Fig. 1, C and F and Fig. 2, C and F) and the ^{13}C-CO_2 enrichment (Table 2, $\delta^{13}C_{MIX}$ and Table 5, $\delta^{13}C_{MIX}$).

Henceforth, RQ_i continuously increased in proportion to substrate depletion, approaching the value observed prior to substrate addition into soil. Table 6 gives the amounts of oxygen taken up by microorganisms and CO_2 produced during the experiment with agricultural and forest soils after the addition of *n*-hexadecane and *n*-hexadecanoic acid, respectively. It is obvious that the ratios of CO_2 and molar quantities observed during the whole experimental period represent the average value of respiratory quotient (RQ_{av}) in the tested agricultural and forest soils, which is about 0.60(±0.03) and 0.61(±0.04) mol CO_2 mol^{-1} O_2 in the experiments with *n*-hexadecane and 0.57(±0.02) and 0.64(±0.03) mol CO_2 mol^{-1} O_2 in the experiments with *n*-hexadecanoic acid.

The carbon isotope characteristics were significantly modified in both the fumigation (microbial biomass) and non fumigated extracts by the hexadecane and palmitic acid addition

TABLE 6
The quantities of O_2 taken up and CO_2 produced by soil microbiota in agricultural and forest soils with the test amounts of *n*-hexadecane and *n*-hexadecanoic acid

Treatment	O_2 consumed [μM O_2 g^{-1} soil]	CO_2 produced [μM CO_2 g^{-1} soil]	RQ_{av} [mol CO_2 mol^{-1} O_2]
n-hexadecane			
Agricultural soil	90 ± 5	54 ± 3	0.60 ($\pm$0.03)
Forest soil	82 ± 5	50 ± 5	0.61 ($\pm$0.04)
n-hexadecanoic acid			
Agricultural soil	56 ± 3	32 ± 3	0.57 ($\pm$0.02)
Forest soil	81 ± 4	52 ± 3	0.64 ($\pm$0.03)

Value $\pm$ standard deviation.

after 17 and 35 days, respectively (Table 7). The initial $\delta^{13}C$ values ranged at $-28‰$ and the maximal values at $-6‰$ for the hexadecane treatment in the fumigated soil after 17 days. Thus, the highest values occurred 17 days after the hexadecane addition in the microbial C pool. The carbon isotope signature of microbial biomass and soil extract returned to -20 and $-26‰$, respectively, after 28 days palmitic acid addition. The maximal difference between the extracellular and intracellular $\delta^{13}C$ values was 8‰.

DISCUSSION

Basal soil respiration. The presence of n-hexadecane in native agricultural soil and its absence in forest soil (Table 1) can be considered as a result of difference in the contents of bacterial and fungal biomasses in soils (Ananyeva et al. 2008; Ladygina et al. 2006) and the associated different microbial biosynthesis of hydrocarbons. The obtained soil characteristics (Table 1) explain the fact that the RQ value during microbial oxidation of soil organic matter (SOM) and hydrocarbons in native agricultural soil (i.e., $RQ_{init} = 0.73 \pm 0.05$ mol CO_2 mol^{-1} O_2) is lower than the value during mineralization of SOM and partially oxidized hydrocarbons (e.g., fatty acids) in forest soil (i.e., $RQ_{init} = 0.87 \pm 0.05$ mol CO_2 mol^{-1} O_2).

TABLE 7
Carbon isotope signature in bulk soil and in the non-fumigated (- Fum) and fumigated (+ Fum) soil extracts in the agricultural and forest soil after n-hexadecane and n-hexadecanoic (palmitic) acid mineralization

Treatment	Soil, $\delta^{13}C$ [‰]	$-$Fum, $\delta^{13}C$ [‰]	+ Fum, $\delta^{13}C$ [‰]	Fractionation, biotic [‰]
Agricultural soil				
Control	-26.3	-28.3	-28.2	0.1
+ Hexadecane	-24.3	-14.6	-6.3	8.2
+ Palmitic acid	-25.3	-26.8	-19.9	7.0
Forest soil				
Control	-26.6	-28.6	-27.2	1.3
+ Hexadecane	-25.4	-23.1	-19.2	3.9
+ Palmitic acid	-26.0	-27.8	-21.7	6.1

n-Hexadecane consumption. The addition of n-hexadecane stimulated microbial respiration in both agricultural and forest soils (Fig. 1, A, curve 1 and D, curve 5). The detection of two peaks of microbial CO_2 production rate in agricultural soil and one peak in forest soil after n-hexadecane addition may be treated as an evidence of essential difference in the microbial pools of these soil systems. This fact suggests that some of the hydrocarbon-oxidizing microorganisms may be present in soils in the active state (K-strategists), while others are in the "dormant" state (r-strategists).

Obviously, the presence of native n-hexadecane in agricultural soil (Table 1) contributes to the maintenance of some part of hydrocarbon-oxidizing microbiota in the active state, whereas its absence in forest soil may have kept this group of microbiota in the "dormant" state. Exogenous n-hexadecane added to agricultural soil is consumed by the active part of microbiota (K-strategists) and then, after a certain period of time, the "dormant" part of microbiota (r-strategists) is activated. Soil microbial communities in agricultural soil responded to n-hexadecane addition more rapidly compared to those of forest soil, with the lag-periods of about 23 ± 10 and 68 ± 13 hours, respectively (Table 3). The initial fraction of active microbial biomass capable of immediate growth on applied n-hexadecane in agricultural soil (parameter r) were higher by the order of magnitude than in forest soil (Table 3). The CO_2 production rates (μ) obtained for agricultural soil compared to forest soil were close to each other within the error limit of analysis (Table 3).

Previously it has been demonstrated with the growth of bacteria *Burkhalderia* sp. on n-hexadecane, the carbon quantities of metabolic CO_2 and cell biomass were estimated by the ratio of 1.0 to 1.2 (Zyakun et al. 2003). It means that about 50% of the n-hexadecane carbon consumed by microbiota can be contained in the cell biomass. Like the ratio between cell biomass and metabolic CO_2 was presented in experiments with the yeast *Candida tropicalis* growth on n-hexadecane (Gmünder et al. 1981). Using techniques presented by Potthoff et al. 2003, the $\delta^{13}C$ signatures of soil organic extracted samples of fumigation (+Fum) and non-fumigated (-Fum) with and without addition of n-hexadecane to the agricultural and forest soils were used to demonstrate the inclusion of n-hexadecane carbon into the microbial cell biomass (Table 7).

Assuming that the carbon yield of microbial biomass during n-hexadecane utilization by microorganisms is not higher than the yield of , the total amount of substrate utilized in agricultural and forest soils may be 698 ± 52 μg C g^{-1} soil and 582 ± 64 μg C g^{-1} soil or about 54% and 45% of the 1300 μg C g^{-1} soil of n-hexadecane introduced into soils, respectively. In accordance with this estimation, about 46% (agricultural

soil) and about 55% (forest soil) of introduced *n*-hexadecane may be adsorbed on soils and/or present as partially oxidized exometabolites.

The specific index of possible state of *n*-hexadecane introduced into soils is respiratory quotient (RQ) representing the ratio of molar concentrations of CO_2 produced and oxygen consumed during microbial metabolism of the substrate. Table 6 (RQ_{av}) gives the value of the average respiration quotient RQ_{av} for the 17-day experiment, equal to 0.60(±0.03) and 0.61(±0.04) mol CO_2 mol^{-1} O_2 during *n*-hexadecane metabolism in agricultural and forest soils, respectively. Because some part of oxygen is utilized for cell biomass production and biosynthesis of exometabolites, the resultant value was a little lower than the theoretical value ($RQ_{av} = 0.65$ mol CO_2 mol^{-1} O_2).

The changes in RQ value during *n*-hexadecane uptake by soil microbiota, reached 0.4 mol CO_2 mol^{-1} O_2 (Fig. 1, C and F). This demonstrates that during the time Δt_i some part of the consumed is utilized for the formation of semi-oxidized products, from which CO_2 is formed later on. This fact is confirmed by the values indicating substantial contribution of the carbon of introduced substrate into CO_2 production at the final stage of the experiment (Table 2,).

n-Hexadecanoic acid consumption. The addition of *n*-hexadecanoic acid to soils under study is accompanied by the increase of metabolic activity of soil microbiota (Fig. 2, A, curve 1 and D, curve 5). We believe that two peaks of CO_2 production for agricultural soil and one peak for forest soil demonstrate the existence of two states of microorganisms (active and dormant) relative to the *n*-hexadecanoic acid consumption. Microbial activity represented by the rate of CO_2 production in forest soil is almost 4-fold higher compared to agricultural soil.

It is considered as an evidence of the known prevalence of fungal microflora in forest soil, for which *n*-hexadecanoic acid is an easily metabolized substrate (Ananyeva et al. 2008; Dashti et al. 2008). As follows from the analysis of parameters reflecting the kinetics of microbial metabolism of *n*-hexadecanoic acid in soils (Table 3), the initial specific rates of substrate uptake and CO_2 production (μ) in agricultural soil are twice higher than in forest soil, i.e., agricultural soil contains microorganisms capable for higher growth on n-hexadecanoic acid compared to forest soil. At the same time, value *r* as a metabolic index of the fraction of growing microorganisms in forest soil is 4-fold higher compared to agricultural soil, demonstrating the higher metabolic potential of microbial population resident in the forest soil with reference to *n*-hexadecanoic acid as a substrate. It is supposed that the same lag periods of microflora activation after substrate introduction into agricultural ($t_{lag} = 30 \pm 13$ h) and forest ($t_{lag} = 30 \pm 14$ h) soils is considered as an evidence of its consumption by fungal microbiota present in these soils.

The quantity of CO_2 formed due to mineralization of exogenous n-hexadecanoic acid during 16 days of exposure was 270 ± 26 μg C g^{-1} soil and 412 ± 35 μg C g^{-1} soil in agricultural and forest soil (Table 4, $Q_{CO2(SUB)}$), respectively. Similarly to the n-hexadecane microbial consumption, the $\delta^{13}C$ signatures of soil organic extracted samples of fumigation (+Fum) and non-fumigated (-Fum) with and without addition of n-hexadecanoic acid to the agricultural and forest soils were used to detect the inclusion of n-hexadecanoic acid carbon into the microbial cell biomass (Table 7).

Assuming that the carbon yield of microbial biomass during n-hexadecanoic acid metabolism is not higher than the yield of CO_2, the total amount of substrate utilized in agricultural and forest soils may be 540 ± 52 μg C g^{-1} soil and 824 ± 70 μg C g^{-1} soil or 42% and 63% of the 1300 μg C g^{-1} soil of introduced n-hexadecanoic acid, respectively. In accordance with this estimation, about 48% (agricultural soil) and about 37% (forest soil) of introduced n-hexadecanoic acid may be adsorbed on soils and/or present as partially oxidized exometabolites.

During the microbial metabolism of *n*-hexadecanoic acid in the course of 16-day experiment (Table 6), the value of total respiratory quotient in agricultural soil ($RQ_{av} = 0.57(\pm 0.02)$ mol CO_2 mol^{-1} O_2) was noticeably less than in forest soil ($RQ_{av} = 0.64(\pm 0.03)$ mol CO_2 mol^{-1} O_2), i.e., in agricultural soil the consumed oxygen is utilized to a greater extent for production of semi-oxidized metabolites and to a lesser extent for mineralization of the substrate to CO_2. As in the case of *n*-hexadecane, in the initial time (2–3 days) after substrate introduction (Fig. 2, C and F) RQ reached the value of about 0.4 mol CO_2 mol^{-1} O_2 and then, in 7 days, approached the theoretical value, i.e., RQ = 0.7 mol CO_2 mol^{-1} O_2, in the experiments with forest soil, but remains at a level of 0.65 mol CO_2 mol^{-1} O_2 in agricultural soil.

Thus, the addition of both n-hexadecane and n-hexadecanoic (palmitic) acid induced intensive oxygen requirement (higher than expected theoretically) at the initial stages. The RQ values of the two soils decreased to 0.4 mol CO_2 mol^{-1} O_2 and were even lower than observed for the *Aspergillus niger* strain (Volke-Sepúlveda et al. 2003). Similarly, excessive O_2 requirement was observed with single species cultures (Chayabutra and Ju 2000). The RQ value was about 0.52 mol CO_2 mol^{-1} O_2 during the growth of the yeast *Candida tropicalis* in chemostat culture, but this value changed from about 0.5 to 0.65 mol CO_2 mol^{-1} O_2 depending on growth condition (O_2 concentration, C/N ratios, growth rate) (Gmünder et al. 1981). This phenomenon may be caused by different times of O_2 consumption for oxidation of H_2-equivalents/hydroxylation of alkane substrates and CO_2 production as the result of substrate mineralization in the citric acid cycle reactions.

Priming effects of substrates. The initial stage of *n*-hexadecane utilization by soil microbiota is its enzymatic hydroxylation and/or oxygenation by means of molecular oxygen (Metzler 1977; Smits et al. 1999; Marchant et al. 2006). The enzyme systems induced during this process, along with hydrocarbon substrate modification, are capable of hydroxylation of some part of SOM molecules, which are then used in microbial metabolism (Fontaine et al. 2004) Positive PE in the case of *n*-hexadecane is conditioned by the induction of microbial

enzymes that perform hydroxylation/oxygenation of stable SOM compounds mineralized by soil microbiota to CO_2.

The situation is different during the microbial uptake of *n*-hexadecanoic acid in the same soils. In the experiments with 16-day observation, negative PE values of this substrate were recorded after its addition to soil, i.e., introduction of exogenous substrate decreases the rate of SOM mineralization, and consequently, CO_2 production is mainly due to biodegradation of the added substrate.

The special feature of n-hexadecanoic acid as compared to n-hexadecane lies in the fact that the oxidized end of the acid molecule is used as the initial site for β-oxidation process (van Hamme et al. 2003) and the microbial oxidation of saturated hydrocarbons (n-hexadecane) can not be initiated as easily as it does for n-hexadecanoic acid (Metzler 1977). The increased amount of CO_2 during production at the initial stage of substrate utilization as compared with calculated average content is an experimental evidence of this mechanism. As follows from Table 5, the maximal value of CO_2 reached +(31–36) ‰, whereas the calculated mean value was only +29.6(±0.2) ‰. Since the ^{13}C atom was in the carboxyl group of *n*-hexadecanoic acid (Experimental part), the share of isotope in production at the first stages of test substrate degradation will be higher than it has been calculated for the average of n-hexadecanoic acid pool.

Nevertheless, under complete mineralization of molecule of *n*-hexadecanoic acid (i.e., 16 stages of successive removal of the COOH group) the carbon isotope composition of the formed total CO_2 from PA mineralization will inherit the average content of in the test substrate. In the case of mineralization of more than 50% of substrates introduced (Table 4, $X_{(SUB)}$) it is absolutely justified to believe that the characteristics of carbon isotope composition of the formed CO_2 from PA mineralization will correspond to the calculated average value of isotopic characteristics for the substrate used.

Consequently, the mass isotopic balance was a basis for determining the production of CO_2 formed as a result of SOM mineralization after the introduction of substrate into soil. Comparison of this CO_2 production in the experiments with n-hexadecane and n-hexadecanoic acid and in controls made it possible to determine PE values with a high degree of reliability. It can be assumed that the microbial metabolism coupled with oxidative decarboxylation (β-oxidation) of *n*-hexadecanoic acid provides energy for growth of the main part of microbial cells in soils and it is considered as one of the most probable causes of the revealed phenomenon (i.e. negative PE of *n*-hexadecanoic acid with microbial mineralization of SOM).

Thus, in line with the research tasks mentioned, the following geomicrobiological index of soils should be noted: a) when exogenous hydrocarbons (n-hexadecane and n-hexadecanoic acid) are added to agricultural and forest soil, the metabolic activity of native soil microbiota noticeably increases. The enhancement of the rate of CO_2 production, consumption of molecular oxygen and the change of respiratory coefficient are indicative of microbial mineralization of organic substances in soils. b) The ^{13}C isotope signatures of metabolic CO_2 are incontestable evidence of the microbial use of test hydrocarbons and native soil organic matter as substrates; c) Quantitative comparison of rates of mineralization of SOM prior to and after addition of test hydrocarbons to agricultural and forest soils showed that microbial degradation of n-hexadecane or n-hexadecanoic acid occurs with positive and negative priming-production of CO_2 at the expense of SOM, respectively. d) In agricultural and forest soils the microbial consumption of n-hexadecanoic acid is different from n-hexadecane and does not contribute to the activation of microbial SOM mineralization as strong as might be seen for n-hexadecane.

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