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# The Waste Oil Resulting from Crude Oil Microbial Biodegradation in Soil

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## 1. Introduction

Environmental pollution by oil and oil products, which occurs at petroleum extraction wells, as a result of spills from oil tankers, pipe line breaks, disposal of refinery waste, leaks at gasoline stations, etc., have caused tremendous damage to ecological systems especially to many plant species (Adam and Duncan 2002; 2003; Palmroth et al. 2005), and a wide array of animals (Khan and Ryan 1991; Tevvors and Sair 2010). According to available data (Wang et al. 2011), the total amount of all major spills in the world was about 37 billion barrels of crude oil pollute soil and water ecosystems. It exceeds the total amount of crude oil consumption for the entire world annually (30 billion barrels in 2006) (Mundi 2010). Consequently, the problem of environmental pollution with anthropogenic hydrocarbons and their influence on natural ecosystems calls for comprehensive investigation. Crude oil consists of a number of rather complicated components, which are toxic and can exert side effects on environmental systems. Oil pool contains aliphatic and polycyclic aromatic hydrocarbons, for example, crude oil consists of alkanes 15 - 60 %, naphthenes 30-60 %, aromatics 3-30% and asphaltenes 6 % by weight ( Speight 1990 ). The extent of oil spills can have a legacy for decades, evens centuries in future (Wang et al. 2011). Toxic effects of oil and oil products on the soil environment include increasing hydrophobicity of soils and disruption of water availability to vegetation, and direct toxicity to plants and microorganisms. At the sub-toxic level, negative effects may include the absorption of low-molecular oil hydrocarbons into plant tissues, and the inhibition or activation of microbial soil processes. 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 plant growth and some non-hydrocarbon utilizing microorganisms. For instance, the aromatics in crude oil produce particular adverse effect to the local soil microbiota. It was found that phenolic and quinonic naphthalene derivatives inhibited the growth of some microbial cells (Sikkema et al. 1995). As follows from the work (Wongsa et al. 2004), the rates of utilization of separate oil fractions may be significantly differed even in case of one and the same strain of hydrocarbon-oxidizing microorganisms. As a result, the influence of microorganisms on crude oil in soil may be accompanied by substantial changes in the initial composition of hydrocarbons, while the rest of hydrocarbons in soil may have absolutely different properties compared to the initial characteristics. The term ‘waste oil’

was used to designate the hydrocarbon tails of crude oil introduced into soil and transformed into the product that lost the original properties (i.e., the quantitative ratio of hydrocarbon components changed and the organic products of microbial biosynthesis appeared, which differ from the initial oil components in metabolic availability for a wide range of soil microorganisms, etc). It has been known that 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 oil 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 (Juck et al. 2000; Hamamura et al. 2008; Marques et al. 2008). It is becoming increasingly evident that the fate of anthropogenic hydrocarbons pollutants entering the soil system requires efficient monitoring and control. The bioremediation potential of microbial communities in soil polluted with oil hydrocarbons depends on their ability to adapt to new environmental conditions (Mishra et al. 2001; Kaplan and Kitts 2004). 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 (Jobson et al. 1974; Margesin and Schinner 2001; Zucchi et al. 2003; Hamamura et al. 2006; Margesin et al. 2007). The methods of monitoring and characterization of hydrocarbon degrading activity of soil microbiota are of special interest (Margesin and Schinner 2005; Abbassi and Shquirat 2008; Pleshakova et al. 2008). Oil hydrocarbon biodegradation and transformation in soils can be monitored by estimating the concentration of pollutant (Tzing et al. 2003) and the formation of respective metabolites. The most ubiquitous and universal metabolites is carbon dioxide ($CO_2$), since respiration is by far the prominent pathway of biologically processed carbon.

The activity of soil microbiota can be characterized by the method of the substrate-induced respiration (SIR) which was used for the measurement of $CO_2$ production and the estimation of soil microbial biomass. When an easily microbial degradable substrate, such as glucose, is added to a soil, an immediate increase of the respiration rate is obtained, the size of which is assumed to be proportional to size of the microbial biomass (Anderson and Domsch 1978). In addition to SIR, the index of the specific microbial activity in soil is the priming effect (PE) of introduced exogenous substrate, which was defined as 'the extra decomposition of native soil organic matter in a soil receiving an organic amendment" (Bingeman et al. 1953). The PE 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. 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). Obviously, unambiguous determination of PE by $CO_2$ production calls for an exogenous substrate different from SOM in carbon isotopes (Zyakun et al. 2003; Dilly and Zyakun 2008; Zyakun et al. 2011). It has been shown that addition to the soil of a substrate easily accessible for microorganisms (e.g., glucose, amino acids, etc.) (Harabi and Bartha1993; Shen and Bartha 1996; Zyakun and Dilly 2005; Blagodatskaya and Kuzyakov 2008), contributes to the increase of SOM mineralization rate 2-3-fold compared to the processes in native soil. Acceleration of SOM degradation (positive PE) was also observed in case of addition of an aliphatic hydrocarbon (n-hexadecane) to the soil. Introduction into soil of n-hexadecanoic acid, the product of n-hexadecane oxidation, resulted in the lower rate of SOM mineralization compared to native soil (negative PE) (Zyakun et al. 2011). In the light of brief presentation of methods characterizing biodegradation and transformation of exogenous organic products entering the soil, the fate of crude oil in soils may be defined by the following parameters: (a) the rate of $CO_2$ production as result of mineralization of crude oil and SOM; (b) activation of mineralization of native soil organic matter by introduced substrate (priming effect); c) the ratio of the quantities of biomass of the microorganisms growing on oil hydrocarbons as a substrate and quantities of SOM mineralized into $CO_2$.

## 2. Methods used to analyze the $CO_2$ microbial production in soil

### 2.1 $CO_2$ sampling

Soil samples, 100 g dry weight, were placed into 700-ml glass vials, hermetically closed and pre-incubated for 3 days at 22 $^0$C. Metabolic carbon dioxide ($CO_2$) formed by microbial mineralization of SOM and test-substrate (crude oil) was collected using glass plates (10 ml) placed the over soil surface, containing 2-3 ml of 1M NaOH solution. Production of $CO_2$ in the course of the experiment in each of the vials was determined by titration of the residual alkali in the plates using an aqueous 0.1M HCl solution. The total amount of $CO_2$ fixed in the NaOH solution was also determined by precipitation with $BaCl_2$ and quantitative retrieval of $BaCO_3$. Barium carbonate was washed with water, precipitated, dried, and the resulting precipitate weighed and used for quantitative calculation of metabolic $CO_2$ production and carbon isotope analysis.

### 2.2 The kinetics of $CO_2$ respiration

Specific $CO_2$ evolution rates (μ) of soil microorganisms after crude oil addition to soil were estimated from the kinetic analysis of substrate-induced respiration ($CO_2(t)$) by fitting the parameters of equation [1]:

$$CO_2(t)=K+r\cdot\exp(\mu\cdot t) \quad (1)$$

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 which total respiration coupled with ATP generation and cell growth, and $t$ is time (Panikov and Sizova 1996; Stenström et al. 1998; Blagodatsky et al. 2000). 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.

$$t_{lag}=\ln(K/r)/\mu \quad (2)$$

According to the theory of microbial growth kinetic (Panikov 1995; Blagodatskaya et al. 2009), the lag period was calculated by using the parameters of approximated curve of respiration rate of microorganisms with [2].

### 2.3 Carbon isotopic analysis

The metabolic activity of soil microbial community with respect to substrate (crude oil hydrocarbons) was determined from $CO_2$ evolution rates and the $^{13}C$-$CO_2$ isotope signature. The characteristics of abundance ratios of carbon isotopes $^{13}C/^{12}C$ in SOM, crude oil, and metabolic $CO_2$ (as $BaCO_3$) were measured using by isotopic mass-spectrometry (Breath MAT-Thermo Finnigan) connected with a gas chromatograph via ConFlow interface. Isotope analysis of metabolic $CO_2$ was performed using about 3-4 mg of obtained $BaCO_3$ [M = 197.34], which then was degraded to $CO_2$ by orthophosphoric acid in a 10-ml container. For the analysis of carbon isotope contents of organic matter, SOM and crude oil samples were combusted to $CO_2$ in ampoules at 560 $^0C$ in the presence of copper oxide.

The ratios of peak intensities in $CO_2$ mass spectra with m/z 45 ($^{13}C^{16}O_2$) and 44 ($^{12}C^{16}O_2$) were used for quantitative characterization of the content of $^{13}C$ and $^{12}C$ isotopes in the analyzed samples. According to the accepted expression [3], the amount of $^{13}C$ isotope was determined in relative units $\delta^{13}C$ (‰):

$$\delta^{13}C = (R_{sa}/R_{st} - 1)\ 1000\ ‰ \quad (3)$$

where $R_{sa}=(^{13}C)/(^{12}C)$ represented the abundance ratios of isotopes $^{13}C/^{12}C$ in a sample and $R_{st}=(^{13}C)/(^{12}C)$ was the ratio of these isotopes in the International Standard PDB (Pee Dee Belemnite) (Craig 1957). Each $CO_2$ sample was analyzed in three repeats; standard error was about ± 0.1‰. The $\delta^{13}C$ values are characteristics of stable isotope composition or the $^{13}C/^{12}C$ abundance ratio in the analyzed compounds. Negative values indicate the $^{13}C$ depletion; positive values indicate $^{13}C$ enrichment relative to PDB standard.

### 2.4 Mass isotope balance

Metabolic carbon dioxide produced in the experiments and controls was accumulated during the appropriate time intervals (1-3 days) followed by determination of its quantity and carbon isotope characteristics. The average weighed carbon isotope composition of metabolic $CO_2$ ($\delta^{13}C_{ave}$), which was obtained in detached time intervals, was determined using the expression [4]:

$$\delta^{13}C_{ave}= (\sum q_i, \cdot \delta^{13}C_i )/\sum q_i, ‰ \quad (4)$$

where $q_i$ and $\delta^{13}C_i$ were $CO_2$ production rate and carbon isotope composition at *i*–intervals, respectively.

Determination of mass isotope balance is based on the suggestion that the characteristics of carbon isotope content ($\delta^{13}C$) of $CO_2$ produced during microbial mineralization of hydrocarbons will inherit the $\delta^{13}C$ value of crude oil with an accuracy of isotopic fractionation effect. According to (Zyakun et al. 2003), the $\delta^{13}C$ value of metabolic $CO_2$

produced during oxidation of n-hexadecane and aliphatic hydrocarbons was less by 1-3 ‰ compared to the isotope characteristics of substrates used. It means that the $\delta^{13}C$ value of $CO_2$ produced during microbial degradation of oil hydrocarbons was estimated by $\delta^{13}C$ equal to the value over a rang of -28 to -31 ‰, where $\delta^{13}C$ of the crude oil was about of $\delta^{13}C_{oil}$ = –28,4±0,2 ‰. It is rather different from $CO_2$ resulting from soil organic matter (SOM) mineralization ($\delta^{13}C_{SOM}$ is equal to -23,5±0,5 ‰ for the soil). Thus, after addition of the oil hydrocarbon to soil, the mass isotope balance for $CO_2$ evolved during microbial mineralization of SOM and the exogenous substrate (SUB) was calculated using equation [5]:

$$\delta^{13}C_{SOM} \times Q_{SOM} + \delta^{13}C_{SUB} \times Q_{SUB} = \delta^{13}C_{MIX} \times (Q_{SOM} + Q_{SUB}) \quad (5)$$

where $\delta^{13}C_{SOM}$ and $\delta^{13}C_{MIX}$ are isotopic characteristics of $^{13}C$ content in $CO_2$ 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 test substrate; $Q_{SOM}$ and $Q_{SUB}$ are $CO_2$ quantities resulted from microbial mineralization of SOM and added substrate in the soil samples, respectively.

Here the shares of $CO_2$ formed by mineralization of SOM ($F_{SOM}$) and oil hydrocarbons ($F_{SUB}$) are presented, by definition, as [6] and [7]:

$$F_{SOM} = Q_{SOM} / (Q_{SOM} + Q_{SUB}) \quad (6)$$

$$F_{SUB} = (1-F_{SOM}) = Q_{SUB} / (Q_{SOM} + Q_{SUB}) \quad (7)$$

Using carbon isotope characteristics of total $CO_2$ formed by microbial mineralization of SOM and oil hydrocarbons ($\delta^{13}C_{tot}$) (in experiments) and $CO_2$ formed by mineralization of only SOM ($\delta^{13}C_{SOM}$) (in controls) and assuming that $CO_2$ produced by oil mineralization inherits its isotope composition ($\delta^{13}C_{oil}$), respectively, the share of $CO_2$ formed by mineralization of SOM ($F_{SOM}$) in experiments was calculated by expression [8].

$$F_{SOM} = (\delta^{13}C_{tot} - \delta^{13}C_{oil}) / (\delta^{13}C_{SOM} - \delta^{13}C_{oil}) \quad (8)$$

### 2.5 Cumulative $CO_2$ resulted from hydrocarbon mineralization

Cumulative $CO_2$ produced during the microbial substrate oxidation was calculated as follows. The $\Delta Q_i$ quantity of $CO_2$ evolved during the $\Delta t_i$-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 $\Delta t_i$. 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 (crude oil hydrocarbons) oxidation during $\Delta t_i$ can be calculated as [9]:

$$\Delta Q_{Subst(i)} = (1-F_{SOM(i)}) \cdot \Delta Q_i \quad (9)$$

where $F_{SOM(i)}$ value can be estimated using equation [8]. The cumulative $CO_2$ quantity ($Q_{Subst(CO2)}$) resulting from microbial oxidation of the substrates in soils was presented by [10], where *i* varied from 1 to *n*:

$$Q_{Subst(CO2)} = \Sigma \Delta Q_{Subst(i)} \quad (10)$$

### 2.6 Calculation of priming effects

The addition of exogenous test substrate (oil hydrocarbons) 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 isotope balance into two fractions: from the substrates (oil hydrocarbons) and from SOM mineralization. Thus, the difference between $CO_2$ evolved from SOM mineralization in oil hydrocarbons 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 oil hydrocarbons (denoted as SUB). The PE value was determined in two notations as *kinetic* PE($\Delta t_i$) calculated as a value for $\Delta t_i$–time intervals using equation [11] and the PE(*total*) calculated as a weighted average value for the whole period of observation using equation [12].

$$PE(\Delta t_i)\ [\%] = 100\times(C_{*SOM(i)} - C_{SOM(i)})/C_{SOM(i)} \quad (11)$$

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 $\Delta t_i$-time; and $F_i$ is the share of $CO_2$-C resulting from the SOM in crude oil amended soil in $\Delta t_i$-time, which was calculated by equation [8].

$$PE(total)\ [\%] = \Sigma(PE(\Delta t_i)\cdot\Delta t_i)/\Sigma(\Delta t_i) \quad (12)$$

where PE($\Delta t_i$) was calculated according to Eq. [11].

## 3. Degradation of oil hydrocarbons by soil microbiota and laboratory bacteria introduced into soil

### 3.1 Soil samples

Arable soil samples from the Krasnodar region of Russia were used in the experiment after they had been cultivated with corn ($C_4$-plant). Soil samples were sieved through a 2 mm sieve and then moistened to 60 % of field capacity. The initial organic matter content was about 4.9 % of dry soil (DS) weight or 19.6 mg C $g^{-1}$ DS. The carbon isotope composition in the initial SOM was characterized by a $\delta^{13}C$ value of -23.01 ± 0.2 ‰, typical of soils vegetated by $C_4$-plants.

### 3.2 Crude oil test-substrate

The crude oil as hydrophobic compound was applied as follows: crude oil (4 ml of oil corresponding to 3200 mg) was added to 10 g of dried and dispersed soil and then 10 g of the soil was mixed with fresh moist soil equivalent to 100 g of dry material. The final substrate concentration was 27.43 mg C $g^{-1}$ soil. Since the content of SOM in the initial dry soil sample was about 19.6 mg C/g DS, the share of oil hydrocarbons introduced into the soil exceeded 1.4-fold the quantity of SOM. Assuming that the major part of crude oil spilled over the soil is contained in the upper 10-cm layer, we find that the supposed degree of soil pollution will be about 32 tons per 1 ha.

The carbon isotope composition of the oil hydrocarbons used in these experiments was characterized by a $\delta^{13}C$ value of -28.4 ± 0.1 ‰, the light and heavy oil hydrocarbon

fractions having values -28.9 %o and -27.2 %o, respectively. The isotopic characteristics ($\delta^{13}C$) of the oil used in the experiments were found to be close to the samples of crude oil from oilfields of the Arabian region, where the $\delta^{13}C$ value was –27.5 ± 0.5 ‰ for oil, -28 ± 0.5 ‰ for alkane fraction, and -26.5 ± 1.5 ‰ for the fraction containing mainly aromatic hydrocarbons, respectively (Belhaj et al. 2002).

### 3.3 Microorganisms

To estimate the potential of microbial mineralization of oil hydrocarbons polluted soils, the $CO_2$ production was determined in 12 glass vials with tested soils (three replicates of each experiment and control) (Table 1). In Experiment 1, crude oil was introduced into vials with native soil containing only native soil microorganisms; in Experiment 2, the laboratory strain *Pseudomonas aureofaciens* BS1393(pBS216) (Kochetkov et al. 1997) was additionally introduced into the same soil with oil. Native soil without oil and the same soil with the strain BS1393(pBS216) were used as controls 1 and 2, respectively (Table 1).

The strain *Pseudomonas aureofaciens* BS1393(pBS216) bears the plasmid pBS216 that controls naphthalene and salicylate biodegradation, is able to utilize aromatic oil hydrocarbons, and has an antagonistic effect on a wide range of phytopathogenic fungi (Kochetkov et al. 1997). The ability of the strain to synthesize phenazine antibiotics and thus staining its colonies bright-orange on LB agar medium allowed its use as a marker of quantitative presence of the above microorganisms in soil in the presence of aboriginal microflora ( Sambrook, et al. 1989].

| | |
|---|---|
| *Control* 1: Native soil with soil microbiota (three of glass vials) | *Control* 2: Native soil with soil microbiota + *Pseudomonas aureofaciens* BS1393(pBS216) (three of glass vials) |
| *Experiment* 1: Native soil with soil microbiota + crude oil (three of glass vials) | *Experiment* 2: Native soil with soil microbiota + crude oil+ *Pseudomonas aureofaciens* BS1393(pBS216) (three of glass vials) |

Table 1. Scheme of experiments and controls

The introduced strain was previously grown in liquid LB medium till stationary phase (28°C, 18 h) and then uniformly introduced into soil to a concentration of $10^6$ cells $g^{-1}$ soil. The control of the bacteria strain growth was accomplished weekly during 67 days. A composite soil sample was collected from three separate sub-samples from the vial and analyzed for bacterial quantities. Approximately one g of the composite soil sample was suspended in 10 ml of 0.85% NaCl on "Vortex", soil particles were precipitated, and 1 ml of supernatant was used for making dilutions (10×-10000×). Volume of 0.1 ml of the

corresponding dilutions was inoculated onto Petri dishes with LB medium. The colony-forming units (CFU) on the plates were counted and their mean values in the control and experiments were calculated.

As seen from Table 2, in one day after introduction of the strain *P. aureofaciens* BS1393(pBS216) experiments (soil with oil) and controls (soil without oil) showed a decrease of the quantity of cells of this strain from $10^6$ cells $g^{-1}$ soil to $10^4$ cells $g^{-1}$ soil measured as colony-forming units (CFU). However, in 7 days after the beginning of the experiment, the CFU number of the bacteria introduced in the experiment with oil was about $2.7 \times 10^6$ cells $g^{-1}$ DS, i.e. more than 17-fold higher than the CFU of the same bacterium in the control soil without oil (Table 2). These results indicate the ability of the strain introduced for biodegradation of oil hydrocarbons to utilize them as a growth substrate. In 14-21 days, the CFU of the introduced strain noticeably decreased again and by day 28 reached the initial level of $10^4$ cells $g^{-1}$ DS.

| Variants | Colony-forming units ($x10^4$)/g of soil | | | | | |
|---|---|---|---|---|---|---|
| | *1 d | 7 d | 14 d | 21 d | 28 d | 35 d |
| Control | 8.0 (1.7) | 15.7 (5.8) | 4.0 (0.9) | 1.6 (0.6) | 3.1 (3.5) | 2.2 (0.9) |
| Experiment | 4.7 (3.4) | 268.0 (149) | 31.7 (18.7) | 21.1 (14.4) | 2.4 (1.4) | 1.5 (0.5) |

*Times after bacteria culture was introduced into soil. (Standard deviations from 3 parallels are given in parenthesis)

Table 2. Growth of *Pseudomonas aureofaciens* BS1393(pBS216) without (control) and with crude oil hydrocarbons (experiment) to a concentration of $10^6$ colony-forming units $g^{-1}$ of soil introduced into arable soil.

### 3.4 Microbial $CO_2$ production in soil

Total rates of microbial mineralization of SOM and oil hydrocarbons in soil were determined by the rate of $CO_2$ production (μg C-$CO_2$ $g^{-1}$ DS $h^{-1}$).

In controls 1 and 2, the rates of SOM mineralization both by aboriginal soil microorganisms and the mixture of these microorganisms plus introduced strain *P. aureofaciens* BS1393(pBS216) were within the range of $0.2 \pm 0.02$ μg C-$CO_2$ $g^{-1}$ DS $h^{-1}$ and practically did not change during the 67-day observation (Fig. 1, control 1 and control 2). In soil with added oil hydrocarbons (experiments 1 and 2), the rate of mineralization of total organic carbon significantly increased and reached the maximum value of about 3.2 μg C-$CO_2$ $g^{-1}$ DS $h^{-1}$ on days 7-8 after the beginning of the exposure (Fig. 1, Exp. 1 and Exp. 2). In experiment 2, with the bacterium P. *aureofaciens* BS1393(pBS216) added to the indigenous microbiota, there are two maximums of $CO_2$ production rate: the first in 3 days and the second one in 8 days after the beginning of exposure (Fig. 1, Exp. 2). In the experiment 1 with aboriginal microbiota (Fig. 1, Exp. 1) only one maximum of $CO_2$ production rate was observed in 7 days after the beginning of exposure. It is supposed that this special feature was responsible for the availability of the introduced bacteria P. *aureofaciens* BS1393(pBS216) to consume the oil hydrocarbons.

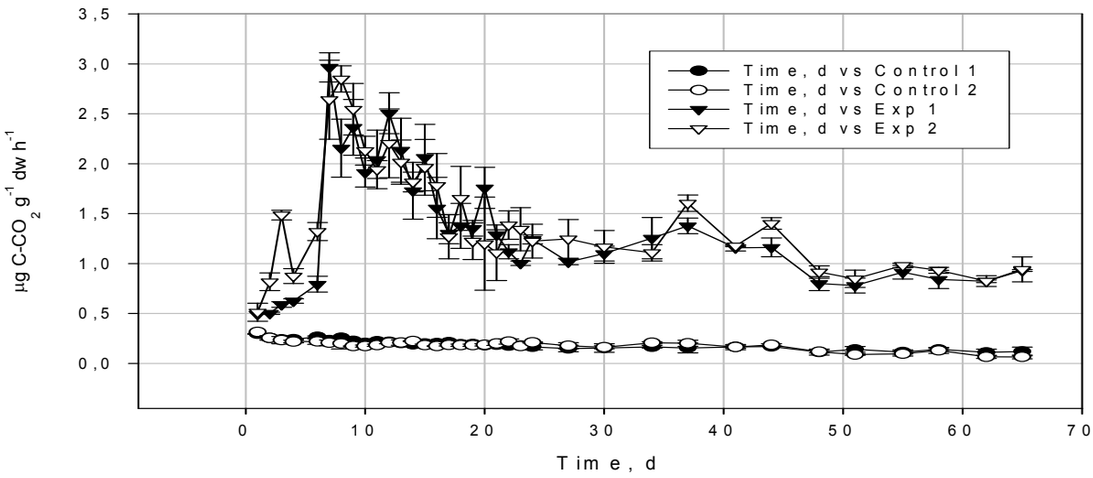


Fig. 1. Rates of $CO_2$ production by microbial mineralization of substrates in experiments simulating microbial utilization of oil hydrocarbons. Control 1 (aboriginal microflora); Control 2 (aboriginal microflora + introduced bacteria); Experiment 1 (aboriginal microflora + oil); Experiment 2 (aboriginal microflora + introduced bacteria + oil)

Two to 3 days (Exp. 2) and 5 to 6 days (Exp. 1) days after the start of exposure, the crude oil introduced into agricultural soil caused an exponential increase in the $CO_2$ emission rate indicating microbial growth after lag-phase (Fig. 2).

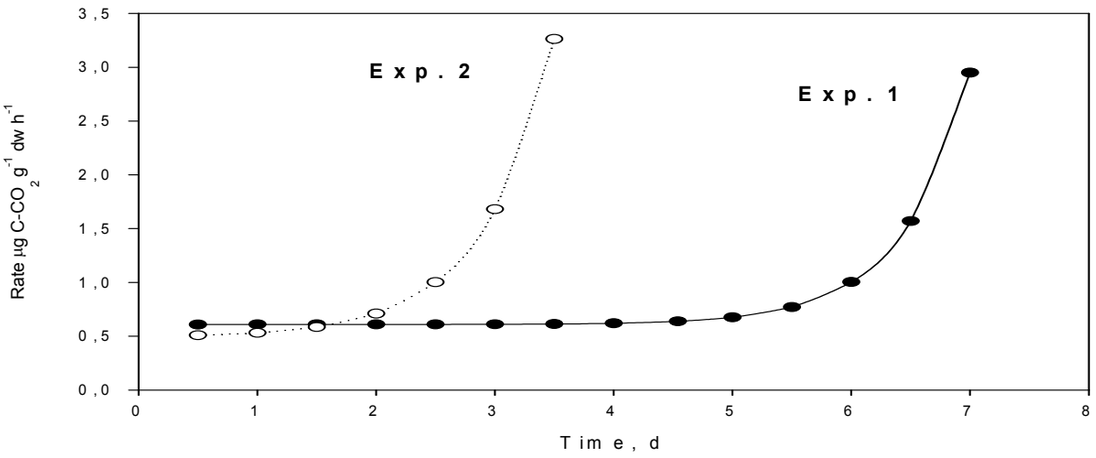


Fig. 2 Substrate-induced respiratory response of the microbial community during incubation of soil treated with crude oil hydrocarbons: 1 - the initial $CO_2$ emission by growth of native soil microbiota and 2- the initial $CO_2$ emission by growth of mixture of native soil microbiota with strain *P. aureofaciens* BS1393(pBS216)

At the initial stages of microbial oil mineralization in experiments 1 and 2, the specific rates of metabolic $CO_2$ emission (μ) were determined using the approximating equation [1] and lag periods ($t_{lag}$) were calculated by the equation [2] (Table 3). The values of parameters K as an index of catabolism of microbial cells in soil were calculated from the analysis of $CO_2$ production at the initial stages of microbial oil mineralization. The values of parameters K (Table 3) show the close rates of initial production of metabolic $CO_2$ in these experiments. At the same time, parameter *r* indicating the presence of growing microorganisms in soil is higher by three orders of magnitude in experiment 2 with introduced bacteria compared to experiment 1 with native microbiota in soil. Parameter μ showing specific rates of $CO_2$ production in experiments 1 and 2 has close values within the measurement error. As one would expect, the lag period of test substrate consumption and $CO_2$ production in experiment 2 with the introduced bacterium P. *aureofaciens* BS1393(pBS216) was about 2,5±03 days, i.e., significantly less than in experiment 1 with native microbiota only (the lag period of 6,2±0,5 days).

| Type of soil | μg $CO_2$-C $g^{-1}$ soil $h^{-1}$ | | | $t_{lag}$, d |
|---|---|---|---|---|
| | K | r | μ | |
| Native soil microbiota (Experiment. 1) | | | | |
| Agricultural soil | 0.6085 | $8.991 \cdot 10^{-6}$ | 1.7814 | 6.2 (0.5) |
| Native soil microbiota + *P. aureofaciens* BS1393(pBS216) (Experement. 2) | | | | |
| Agricultural soil | 0.4906 | $7.445 \cdot 10^{-3}$ | 1. 6913 | 2.5 (0.3) |

Table 3. Parameters of the equations [1] and [2] characterized the respiration rates of native soil microbiota (Experiment 1) and mixture microbiota after bioagmentation with strain *P. aureofaciens* BS1393(pBS216) (Experiment 2) after crude oil addition to the agricultural soil. Standard deviation intervals are in brackets

Beginning from day 25 to day 67 from exposure, the rate of $CO_2$ production in experiments 1 and 2 decreased slightly and stabilized at a level of 1.25 ± 0.25 μg C-$CO_2$ $g^{-1}$ DS $h^{-1}$ (Fig. 1). Total $CO_2$ production in controls (control 1 and 2) for the 47-day and for 67-day periods of observation was 24.8 ±1.2 mg C-$CO_2$ and 35.5 ± 1.2 mg C-$CO_2$ (Table 4).

| Experiment | Mean Production rate, μg C-CO2 g-1 DS h-1 | *Total production, mg C-CO2 | **Time, days |
|---|---|---|---|
| Control 1 | 0.228(0.013) | 25.7 (0.6) | 47 |
| Control 1 | 0.228(0.013) | 36.7 (0.6) | 67 |
| Control 2 | 0.213(0.013) | 24.03 (0.6) | 47 |
| Control 2 | 0.213(0.013) | 34.25 (0.6) | 67 |
| Experiment 1 | 1.480(0.122) | 167 (6) | 47 |
| Experiment 1 | 1.480(0.122) | 238 (6) | 67 |
| Experiment 2 | 1.546(0.100) | 174 (5) | 47 |
| Experiment 2 | 1.546(0.100) | 251 (5) | 67 |

*Total production $Q_{total}=(24 \cdot v_{average}$ (μg C-$CO_2$ $g^{-1}$ DS $h^{-1}) \cdot t$ (days))x100 g DS

**Time after the crude oil addition to soil. Standard deviations of three parallel determinations are given in brackets.

Table 4. Mean rates of $CO_2$ emission (μg C-$CO_2$ $g^{-1}$ DS per h) and total production of C-$CO_2$ during the time experiment (mg C-$CO_2$ per 100 g DS)

The absence of any significant differences in $CO_2$ production in controls 1 and 2 was considered as an evidence of insignificant additional mineralization of SOM attributable to the introduced strain of *P. aureofaciens* BS1393(pBS216 ). In the case of oil-containing soils, the amounts of metabolic $CO_2$ in experiments 1 and 2 exceeded 6.8-fold that of controls 1 and 2, being 167.0 and 238 mg C-$CO_2$ (Exp. 1) and 174.0 and 251 mg C-$CO_2$ (Exp. 2) during 47- and 67-day exposure, respectively (Table 4). The data also showed that the additional introduction of the hydrocarbon-oxidizing strain *P. aureofaciens* BS1393(pBS216) into oil-containing soil (Exp. 2) promoted the increase of metabolic $CO_2$ amount (4 - 13 %) compared to the aboriginal soil microorganisms.

Total $CO_2$ production in experiments 1 and 2 included microbial mineralization of SOM and oil hydrocarbons, therefore the share of $CO_2$ formed by mineralization of each of the above substrates was determined by measuring values $\delta^{13}C$, both in the carbon isotope characteristics of SOM and oil products and in the metabolic carbon dioxide formed during this process.

### 3.5 Analysis of the origin of soil $CO_2$ using $^{13}C/^{12}C$ ratios

In experiments 1 and 2, the $\delta^{13}C$ values of the metabolic $CO_2$ released from soil in the 3 days before oil hydrocarbons introduction into soil were –23.53 ± 0.21 ‰ and –23.56 ± 0.25 ‰, respectively, and actually identical to the isotopic characteristics of $CO_2$ in the controls (Fig. 3).

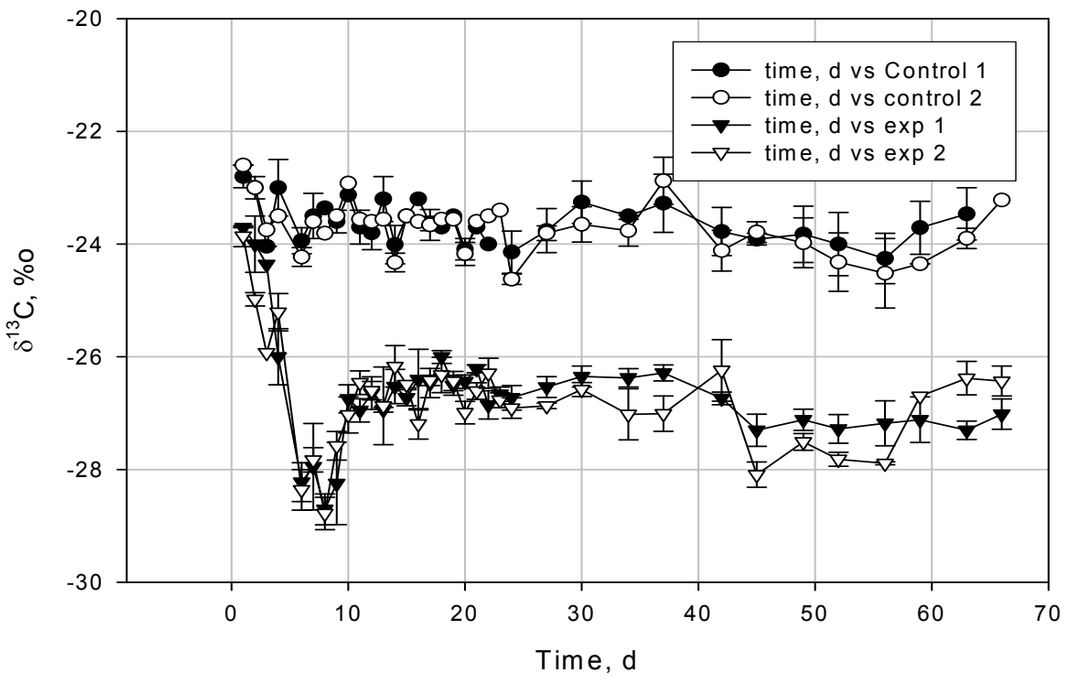


Fig. 3. Carbon isotope characteristics ($\delta^{13}C$, ‰) of $CO_2$ produced in experiments of microbial mineralization of SOM and oil hydrocarbons introduced into soil: Control 1 (aboriginal microflora); Control 2 (aboriginal microflora + introduced bacteria); Experiment 1 (aboriginal microflora + oil); Experiment 2 (aboriginal microflora + introduced bacteria + oil)

After oil hydrocarbons addition to soil, the share of $^{13}C$ isotope in metabolic carbon dioxide abruptly dropped, which was an evidence of $CO_2$ production partly from oil hydrocarbons containing less $^{13}C$ isotope compared to SOM. The maximum depletion of $^{13}C$ isotope in metabolic $CO_2$ was registered during the days 11-15 from the beginning of exposure in experiments 1 and 2. This was considered a result of the mineralization of mainly alkane oil fractions. Our assumption that the major part of aliphatic hydrocarbons from the introduced crude oil had already been utilized by that period is evidenced by the carbon isotope characteristics of the metabolic carbon dioxide with the value of $\delta^{13}C$ = -28.5 ± 0.5 ‰ (Fig. 3). After 15 days and until the end of the experiment (67 days), the isotopic characteristic of $CO_2$ was at around the value of $\delta^{13}C$ = -26.8 ± 0.5 ‰. Using equation [4], the average weighted isotope composition of $CO_2$ produced by microbial mineralization of total organic products (oil hydrocarbons and SOM) in experiments 1 and 2 during 67-days was characterized by $\delta^{13}C$ values about of -26.6 ± 0.1 ‰, which significantly differed from the carbon isotope characteristics of oil ($\delta^{13}C$ = -28.4 ± 0.2‰) and SOM ($\delta^{13}C$ = -23.01 ± 0.2 ‰,). It can be said with confidence that metabolic $CO_2$ was produced during microbial mineralization of a part of SOM and a part of oil hydrocarbons.

### 3.6 Priming effect of oil hydrocarbons

The *kinetic* PE was calculated by comparing $CO_2$ amounts generated by microbial mineralization of SOM and oil products (Exp. 1 and 2) to $CO_2$ amounts generated in the controls in the corresponding periods of observation [Eq. 11].

In order to quantify both the extent and direction of PE of oil hydrocarbons, we have compared the rates of $CO_2$ production by microbial mineralization of SOM before and after introduction of oil hydrocarbons into soil at the initial period of maximum microbial activity, i.e., during 15 days after addition of crude oil to soil (Fig. 4). As shown in Figure 4 (A), the activation of the metabolism of aboriginal hydrocarbon-oxidizing soil microorganisms in experiments 1 took about 6 days from the introduction of the hydrocarbon substrate, when microbial rate of $CO_2$ production increased to a rate closer to that of experiment 2 with the *P. aureofaciens* BS1393(pBS216) addition. The mass isotope balance data showed that during these days in experiment 1 the mineralization of oil hydrocarbons was insignificant and the rate of SOM mineralization was less the rate in control (negative PE) (Fig. 4, C PE_1). Experiment 2, in contrast to experiment 1, showed the utilization of oil hydrocarbons in the initial period of exposure was accompanied by a noticeable increase of SOM mineralization rate compared to the initial value (positive PE) (Fig. 4, C PE_2). However, PE became negligible in both experiments during 6-8 day exposure; it is possibly the mineralization time of aliphatic hydrocarbons or their partially oxidized products. The negative PE has been demonstrated previously in the processes of the microbial mineralization of n-hexadecanoic acid introduced into soil (Zyakun et al. 2011). At the next period of the exposure, the PE values demonstrate the positive values of 300 % in experiment 1 and about 400 % in experiment 2. On completion experiments, the total PE has been calculated using Eq. {12}. Taking into account the $CO_2$ quantity registered in experiments 1 and 2 during the whole period of exposure (Table 4, $Q_{total}$) and the share of $CO_2$ under microbial utilization of SOM (Table 5, $F_{SOM}$), we find the quantity of $CO_2$ form as a result of SOM mineralization in the experiments (Table 5, $[CO_2]_{SOM}$)

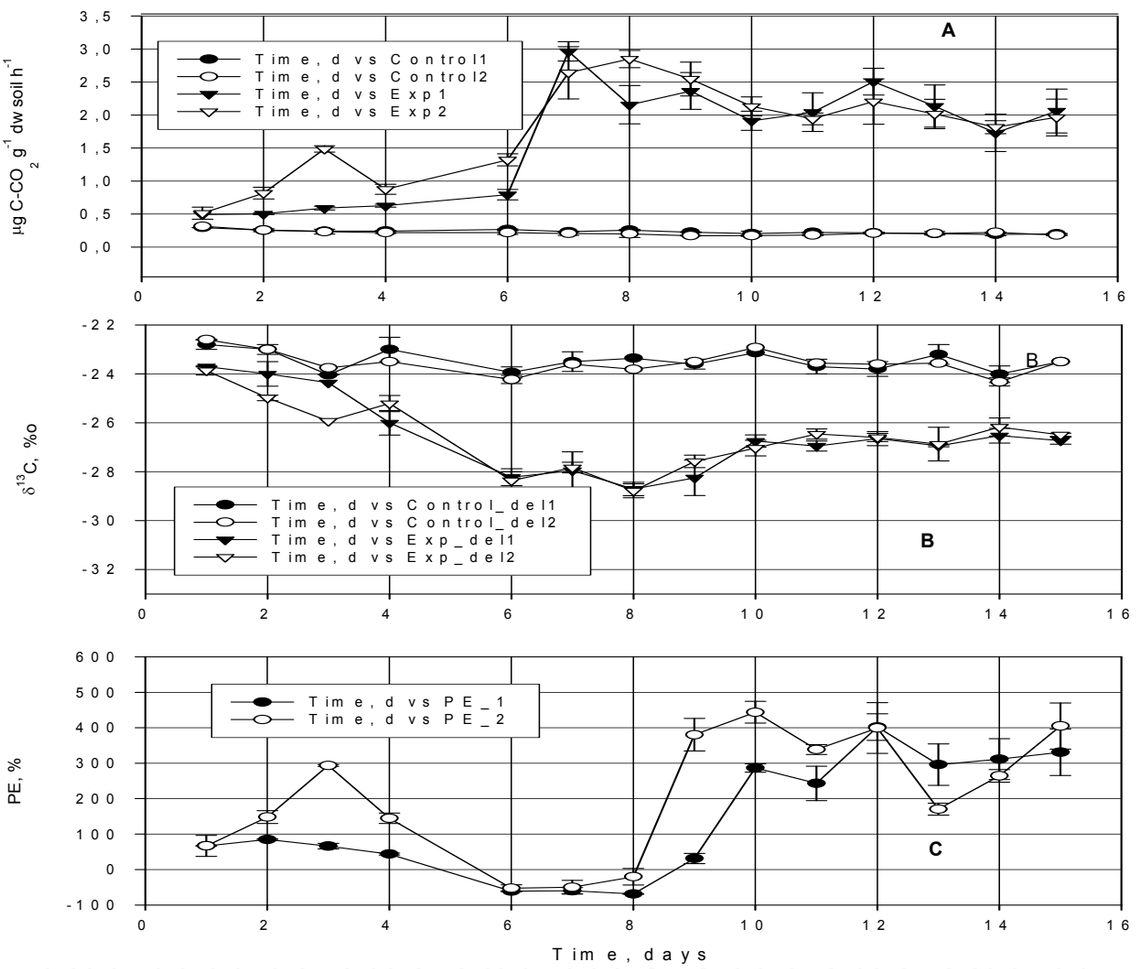


Fig. 4. Rates (A) and isotopic characteristics of $CO_2$ resulting from SOM and oil hydrocarbons mineralization (B), and priming effect (C) for 15 days after oil introduction into soil: control 1 (aboriginal microflora); control 2 (aboriginal microflora + introduced bacteria); experiment 1 (aboriginal microflora + oil); experiment 2 (aboriginal microflora + introduced bacteria + oil).

| Experiment | *$\delta^{13}C_{ave}$, ‰ | **$F_{SOM}$, % | $[CO_2]$(SOM) mg C-$CO_2$ | #PE, % | ##Time, days |
|---|---|---|---|---|---|
| Control 1 | -23.70 (0.1) | 100 | 25.7 (0.6) | 0 | 47 |
| Control 1 | -23.70 (0.1) | 100 | 36.7 (0.6) | 0 | 67 |
| Control 2 | -23.77 (0.1) | 100 | 24.03 (0.6) | 0 | 47 |
| Control 2 | -23.77 (0.1) | 100 | 34.25 (0.6) | 0 | 67 |
| Experiment 1 | -26.59 (0.2) | 38.5 (1.7) | 64 (3) | 150 (13) | 47 |
| Experiment 1 | -26.59 (0.2) | 38.5 (1.7) | 92 (3) | 151 (13) | 67 |
| Experiment 2 | -26.63 (0.2) | 38.2 (1.6) | 67 (3) | 177(15) | 47 |
| Experiment 2 | -26.63 (0.2) | 38.2 (1.6) | 96 (3) | 180 (15) | 67 |

*$\delta^{13}C_{ave}$ is an average weighted of isotope characteristic of $CO_2$ was calculated [Eq. 4]
**$F_{SOM}$ is a share of metabolic $CO_2$ formed by microbial mineralization of SOM; # PE is a priming effect was calculated according to [ Eq. 11]; ##Time after the crude oil addition to soil. Standard errors of three parallel calculations are given in brackets.

Table 5. Average weighted characteristics ($\delta^{13}C_{ave}$) of carbon isotope composition and fraction of $CO_2$ formed by SOM mineralization and priming effect (PE) in experiments 1 and 2 relative to controls

Using the equation [12], we calculate the value of PE(total) by comparing $CO_2$ production during microbial SOM utilization in the experiments and controls. As follows from Table 5, during 67-day exposure of oil hydrocarbons in soil the PE value reached 150 % in experiment 1 with native soil microbiota and 180 % in experiment 2 with mixed microbiota (soil microorganisms and the bacterium strain *P. aureofaciens* BS1393(pBS216)). Thus, addition of crude oil to the soil activates to a large extent the microbial mineralization of native soil organic matter.

### 3.7 Microbial utilization of oil hydrocarbons and SOM transformation

As follows from Table 6, the oil hydrocarbons introduced into soil were mineralized to $CO_2$ to the extent of about 4.59 (0.2) and 4.81 (0.15) mg C-$CO_2$ g-1 DS or 16.7 and 17.5 percents of the initial crude oil quantities in the soil over the course of 67-day exposure in experiments 1 and 2, respectively.

| Variants of analysis | Initial $C_{org}$, (SOM + Oil) mg C $g^{-1}$ DS | [a]C-SOM mineralized, mg C-$CO_2$ $g^{-1}$ DS | Crude oil metabolized $C_{oil}$, mg C $g^{-1}$ DS | | | [b]R |
|---|---|---|---|---|---|---|
| | | | $CO_2$ | Biomass | [c]Total | |
| Experiment 1 | 19.6+ 27.43 | 2.87(0.2) | 4.59 (0.2) | 4.59 (0.2) | 9.18 (0.2) | 1.60 |
| | | [c]14.6 % | [d]16.7 % | [d]16.7 % | 33.4 % | |
| Experiment 2 | 19.6+ 27.43 | 2.98(0.15) | 4.81 (0.15) | 4.81 (0.15) | 9.62 (0.15) | 1.61 |
| | | 15.2 % | 17.5 % | 17.5 % | 35.0 % | |

*The $CO_2$ evaluation from SOM calculated as $Q_{CO2(SOM)} = v_{CO2(SOM+SUB)} \cdot \Delta t \cdot F_{SOM}$
[b]R= (Q biomass + exometabolites from oil carbon) / ($Q_{SOM}$ mineralized of SOM);
[c]Parts (%) of the initial amount of SOM and crude oil mineralized to $CO_2$ in soil [d]Parts of the initial amount of crude oil (in percents) consumed by microorganisms producing $CO_2$ and organic substances (biomass and exometabolites). Standard deviations are given in brackets.

Table 6. The quantities of SOM mineralization and crude oil consumption by microbiota during the 67-day exposure in soil.

Previously (Zyakun et al. 2003), it was shown that during the growth of microbial cells on hydrocarbons the ratio of biomass and $CO_2$ carbon quantities was corresponding 1:1. In view of the above, we believe that the quantity of oil hydrocarbons taken up for the biosynthesis of cell biomass and organic exometabolites in soil during the 67-day exposure will be close to the carbon quantity of $CO_2$ production and make no less then 16.7 and 17.5 percents of the oil introduced in experiments 1 and 2, respectively. By this is meant that the oil hydrocarbon consumption by microbial pool in soil amounts up 33.4 and 35 percent of total oil, respectively.

Extrapolation of the obtained data (Table 6) to a 6-month season, when the temperature conditions in the Krasnodar region provide for the metabolic activity of soil microbiota, shows that the uptake of crude oil hydrocarbons by native soil microbiota may reach no more than 92±2 % of the total oil hydrocarbon quantity in the oil. At a positive PE of oil hydrocarbons in soil, there is more intensive microbial degradation of SOM compared to the processes in native soil. On the other hand, oil hydrocarbons consumed by microorganisms are spent both for $CO_2$ production and for the biosynthesis of biomass and organic exometabolites, which then are included in SOM and transform the structure of soil. The newly synthesized metabolites and microbial biomass components can be used by other biological systems (plants, macro- and microorganisms) that are incapable of direct utilization of oil hydrocarbons. The quantitative and isotopic data obtained in the experiments were used as a basis for estimation of the degree of replacement of part of SOM mineralized to $CO_2$ by the newly synthesized products under microbial utilization of oil hydrocarbons. Table 6 shows the rates of microbial degradation and production of cell biomass and organic exometabolites in model experiments with microbial utilization of crude oil as a substrate. As a result of oil consumption both by native soil microbiota (Exp. 1) and introduced the bacterium strain *P. aureofaciens* BS1393(pBS216) (Exp. 2), the quantity of the newly synthesized organic products (carbon of cell biomass and exometabolites) nearly 1.6-fold exceeds the carbon quantity of SOM taken up for the $CO_2$ mineralization (Table 6, R). It means that microbial transformation of oil hydrocarbons into products available as substrates for other living systems may be a peculiar source of organic fertilizers. In addition, there is more and more evidence that the bioremediation of oil-polluted soils is companied by plant growth stimulation.

## 4. Conclusion

With the proviso that crude oil carbon content no more than 1.4-fold higher than the SOM carbon amount, the soil microbiota is able to mineralize up to 17 % of crude oil hydrocarbons and 15 % of SOM during the 67-day experiments. Using mass isotope balance and differences between the $\delta^{13}C$ values of SOM and oil hydrocarbons, the quantities of $CO_2$ produced during microbial mineralization of SOM and oil hydrocarbons have been determined. According to the highest depletion of $^{13}C$ in $CO_2$ evolved from soil during the initial time of the exposure with crude oil, it is suggested that at this time the aliphatic oil fraction predominantly participates in mineralization. Microbial consumption of oil hydrocarbons activates the process of SOM mineralization and demonstrates the presence of PE of oil hydrocarbons. During a 67-day period of the crude oil exposure in soil, the average values of PE reached over 150 % in soil with native soil microbiota and over 180 % in soil with the mixture of native microbiota and introduced bacteria *P. aureofaciens*

BS1393(pBS216) containing the plasmid pBS216 which controls naphthalene and salicylate biodegradation and able to utilize aromatic oil hydrocarbons. It has been found experimentally that in the total emission of carbon dioxide from soil to atmosphere, about 38 % $CO_2$ was produced as a result of SOM mineralization and about 62 % was formed from oil hydrocarbons as anthropogenic pollutant. The soils polluted with oil hydrocarbons undergo the change of SOM by replacement of part native organic substances on the newly synthesized products in the course of oil biodegradation and the increase of the residual oil share in the total pool of organic matter in soil. Within 6-month time, the quantity of the microbial newly synthesized organic products (carbon of cell biomass and exometabolites) nearly 1.6-fold exceeds the carbon quantity of SOM taken up for the $CO_2$ microbial mineralization. After partially microbial consumption of oil hydrocarbons, the substrate characteristics of residual oil are rather different from crude oil and can be considered as *waste oil* in the soil.

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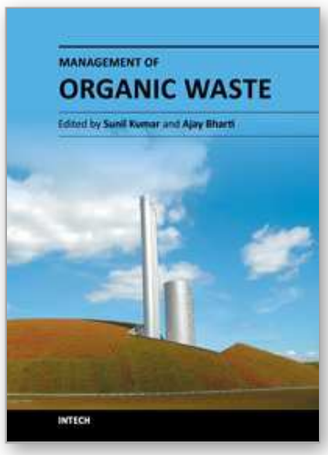


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This book reports research on the utilization of organic waste through composting and vermicomposting, biogas production, recovery of waste materials, and the chemistry involved in the processing of organic waste under various processing aspects. A few chapters on collection systems and disposal of wastes have also been included.

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