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Changes in microbial dehydrogenase activity and pH during bioremediation of fuel contaminated soil

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Abstract

Commercialization of biodiesel and its blends with diesel oil may lead to the appearance of spills during transport or the leakage of contaminated wastewater into soil. The impact of biodiesel, either pure or blended with diesel oil, on natural habitats has been poorly characterized. The goal of this study was to assess the potential of a bacterial strain, *Gordonia alkanivorans S7*, for remediation of soil contaminated with biodiesel, traditional fossil fuels or their blends (diesel oil, B20 diesel oil/biodiesel blends, P31 petroleum fraction). This was achieved by evaluating the changes in fuel concentration and the activity of extracellular microbial dehydrogenases in soil, as well as measuring the soil's pH under controlled conditions. The removal of biodiesel from contaminated soil, in the event of its 4% initial concentration, was almost complete (99%), but in cases of higher concentrations (5% or 8% w/w) the efficiency of Fatty Acid Methyl Ester (FAME) degradation was 90% and 60%, respectively, after 90 days biodegradation. In soil samples contaminated with biodiesel, the activity of dehydrogenase was very low in the initial stage of the process (only 10 μmol triphenylformazan (TPF) per g of dry weight (g_{dw}^{-1}) after 20h (TPF $g_{dw}^{-1} \times 20\ h^{-1}$) and dropped to 0 after 6 weeks. In soil contaminated with other fuels (diesel oil, B20), the activities of dehydrogenase were higher and reached 40-46 μmol TPF $g_{dw}^{-1} \times 20\ h^{-1}$. The pH of soil contaminated with biodiesel decreased from 6.7 down to 4.9 within 9 weeks. The results of this study demonstrate that the presence of intermediate metabolites of biodiesel degradation may cause significant changes in the environmental conditions and negatively influence the microorganisms present in the environment.

Key words: bacteria, biodiesel, bioremediation, dehydrogenase, fuel, soil

Introduction

Sustainable development requires the constant search for green technologies to treat a wide range of aquatic and terrestrial habitats contaminated by increasing anthropogenic activities. Waste generation is a side effect of consumption and production activities and tends to increase with economic advance (Juwarkar et al., 2010). Extensive fuel consumption from both renewable and non-renewable resources leads to severe contamination of the environment and this is becoming a great threat to the natural habitat. Some substances may reach the environment in small concentrations, but may be subjected to biomagnification or bioaccumulation up the food chain, wherein their concentrations increase as they pass through the food chain (Sharma et al., 2009; Takeuchi et al., 2009; Juwarkar et al., 2010).

In the past few years, a large increase in the use of renewable energy sources, including fuels derived from plant biomass, has been observed. The production of liquid biofuels that have been used as either a fuel itself or a bio-component of blends with petroleum-based fuels has become a rapidly growing branch of the "green energy" sector. Derived from renewable raw materials, methyl esters of fatty acids (fatty acid methyl esters-FAMEs) have been used as a fuel for internal combustion engines (Sharif Hossain et al., 2008). Commercialization of biodiesel and its blends with diesel oil, as well as the tendency to increase the percentage of biodiesel in the total volume of fuel, although in line with European Union regulations (renewable energy directive), may in consequence lead to the appearance of spills during transport or the leakage of contaminated wastewater into soil

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in industrialized areas (Directive 2009/28/EC of the European Parliament and of the Council, European Parliament legislative resolution of 11 September 2013 on the proposal for a directive of the European Parliament and of the Council amending Directive 98/70/EC relating to the quality of petrol and diesel fuels and amending Directive 2009/28/EC).

The impact of biodiesel, either pure or blended with diesel oil, on the natural environment has been poorly characterized. To date, research on biodiesel has mainly focused on the methods of its synthesis (Sekhar et al., 2010; Abduh et al., 2012; Tekade et al., 2012). Other widely discussed subjects include aspects related to the use of fuel blends in the operation of power units, and the emission of gaseous and particulate pollutants into the atmosphere after combustion in engines (Sekhar et al., 2010; Abduh et al., 2012; Tekade et al., 2012). However, the topic of environmental safety in the case of a leakage of diesel and biodiesel mixtures into the environment is still under investigation (Sekhar et al., 2010; Abduh et al., 2012; Tekade et al., 2012).

The literature concerning biofuel biodegradation in soils under aerobic conditions is very scant. Although the higher biodegradability and lower toxicity of fatty acid methyl esters in the environment, when compared to conventional diesel (ON), has been reported by many authors, these results are inconsistent. Besides, the majority of studies have been related to the clean-up of either water or sediment. At the same time, most researchers have used pure, laboratory-produced FAMEs, which, unlike commercial biofuel, do not contain any additives (improving fuel exploitation properties) such as antioxidants (Haws and Randall, 1997; Lapinskien et al., 2006; DeMello et al., 2007; Pinto et al., 2008; Owsianiak et al., 2009; Mańczak et al., 2010; Sôrensen et al., 2011; Meneghetti et al., 2012).

The most important step in reducing the impact of harmful chemicals on the environment, as well as on human and animal health and life, is monitoring their content in all areas of the environment and their efficient removal from ecosystems. The most common method of hydrocarbon removal from contaminated soil is bioremediation. Bioremediation, either as a spontaneous or as a managed strategy, is the application of biological processes for the clean-up of hazardous chemicals present in the environment and often this is the only rational way of restoring polluted soils (Vinas et al., 2002; Chaillan et al., 2004; Nievas et al., 2008). Modern, highly effective remediation technologies outperform natural clean-up processes in the environment. Examples of such technologies are biostimulation that accelerates the biodegradation of petroleum components by enhancing the activity of indigenous microorganisms, and bioaugmentation involving the inoculation of a contaminated area with microbial consortia, effectively degrading hydrocarbons, e.g. benzene or toluene (Hamdi et al., 2007; Alisi et al., 2009; Zeyaullah et al., 2009).

The effectiveness of a bioremediation process depends on many parameters, physical and chemical (e.g. the structure and concentration of impurities, pH, oxygen concentration, temperature) as well as biochemical properties (e.g. enzyme activities, ATP content). The values of these parameters provide information not only about the environmental conditions and metabolic activity of microflora, but also about the efficiency of pollutant degradation. Hydrogen ion concentration (pH) has been revealed to be one of the important environmental factors that influence the bioavailability of contaminants, the availability of other nutrients, the activity of biological processes, and the characteristics of the contaminants with respect to how they interact with a site's geochemical and geological characteristics. Soil pH is a measure of the acidity or alkalinity of water. The pH of an environment may change due to the occurrence of intermediate metabolites scheduled from output impurities which can significantly affect microbial activity and, consequently, the bioremediation rate (Ajoku and Oduola, 2013).

Biological oxidation of organic compounds is largely connected with dehydrogenation carried out by dehydrogenase enzymes. Dehydrogenase activity assays have often been used as an indicator of microbial metabolic activity in contaminated soil (Casida et al., 1964; Riffaldi et al., 2006). This parameter is positively related to microbial respiratory activity measured through evolving CO_2 (Howard, 1972; Riffaldi et al., 2006). On the other hand, as dehydrogenases may be localized only in intact living cells, their activity may be negatively related to some toxic compounds such as phenols. Thus, soil dehydrogenase activity is a useful tool for monitoring the bioremediation of soil contaminated with petroleum hydrocarbons, such as diesel oil. This method has been applied to soil containing fresh (Margesin and Schinner, 1997; Margesin et al., 2000; Margesin, 2005) and aged

contamination (Margesin and Schinner, 1999, 2001). A substantial increase in soil dehydrogenase activity after hydrocarbon contamination reflects the adaptation and exponential growth of hydrocarbon degraders due to the availability of new carbon sources introduced by contamination. Otherwise, soil dehydrogenase activity declines with decreasing hydrocarbon content due to the loss of available compounds as a consequence of biodegradation (Margesin et al., 2000; Margesin 2005).

The objective of this study was to assess the usefulness of a bacterial strain, *G. alkanivorans S7*, for remediation of soil contaminated with different fuels. All the research was performed in 2013 at the Institute of Technical Biochemistry, TUL, Poland.

Materials and methods

Fuels

Four types of fuels were used in this study. Petroleum diesel oil produced according to EN 590:2004 was purchased from a petrol station (PKN Orlen, Poland). Biodiesel (referred to as B100), which was produced from rapeseed oil according to EN 14214, was purchased from BioAgra Oil S.A (Poland). P31 petroleum fraction – a vacuous petroleum fraction – as produced by Płock Refinery. A diesel/biodiesel blend with a biodiesel content of 20% (w/w) (referred to as B20) was obtained by mixing suitable volumetric portions of pure fuels. The fatty acid methyl ester (FAME) profile of the biodiesel used in this study is shown in Table 1.

Selection of microorganisms and a preliminary assessment of fuel biodegradation

Six strains of bacteria (isolated at ITB from petroleum plant sludge or contaminated soil, Poland) from the pure culture collection of the Institute of Technical Biochemistry (ITB) of Lodz University of Technology (TUL) were evaluated for biodegradation of different fuels.

A modified Hanson et al. (1993) method was used for the selection of the bacterial strains. This method consisted of incorporating an electron acceptor such as 2,6-dichlorophenol-indophenol (DCPIP) into the medium to test the ability of the microorganism to utilize the hydrocarbon substrate. This was done by observing the color change of DCPIP from blue (oxidized) to colorless (reduced). Each microtiter-plate well was filled with 250 µl of mineral salt medium (Banerjee et al., 2001), 10 µl of fuel (diesel oil; pure biodiesel – B100; diesel oil/biodiesel blends B20 and P31 petroleum fraction) and 25 µl of each microbial suspension, standardized at 10^8 Colony Forming Unit (CFU) ml^{-1}. All plates were incubated at 30°C. The capacity of microorganisms for fuel degradation was determined based on the change in the color of the culture medium containing DCPIP after 12, 18 and 24 h of incubation (Miranda et al., 2007 and Soares et al., 2009). For each tested bacteria, there were three replicates; 285 µl of uninoculated media were tested for the fuels and used as negative controls.

Bacterial strain used for primary biodegradation tests and inoculum preparation

Bacterial strain *G. alkanivorans S7* from the pure culture collection of the ITB, TUL (Poland) was maintained in "A" medium stocks (glucose 2 $g \cdot l^{-1}$; yeast extract 2 $g \cdot l^{-1}$; anhydrous Na_2HPO_4 1.5 $g \cdot l^{-1}$; NH_4Cl 2.5 $g \cdot l^{-1}$; agar 25 $g \cdot l^{-1}$) at 4°C. To prepare the inoculum, the stock suspension (1 ml) was transferred to a 500-ml flask containing 40 ml of sterile mineral medium "A" and cultivated for 24 h at 30°C on an orbital shaker (120 rpm). Before sterilization by autoclaving (121°C, 20 min), the pH of the medium was adjusted to 6.8.

Soil samples

A sandy loam soil with low organic matter content was used in the bioremediation processes. The soil was collected from an uncontaminated residential area in Lodz province, Poland. The sample was taken from a depth of 10 to 50 cm as a bulk, sieved through 2 mm sieve to remove large debris and ensure homogeneous mixing, and stored at 4°C until use (Carter and Gregorich, 2006). Selected physicochemical and biological characteristics of the soil are shown in Table 2.

The effect of fuel type on biodegradation efficiency

The bioremediation processes were conducted for 9 weeks under laboratory conditions using identical 2 l glass vials containing 1.8 kg portions of soil contaminated with one of the following fuels: P31 petroleum fraction, diesel oil, biodiesel B100 or the mixture of fuels-B20. The soil was inoculated with a one-day liquid culture of *G. alkanivorans S7* (40 ml to 1 kg dry soil, $OD_{600\ nm}$ of 0.3 ± 0.01). The initial concentration of the contaminating hydrocarbons or the FAME fraction in all studied variants was 4% w/w. The P31 petroleum fraction is difficult to degrade; however, in order to maintain similar bioremediation conditions, all contaminants were

Table 1. Fatty acid methyl ester profile of the biodiesel

Fatty acid methyl ester	Content (v/v %)
C16:0 hexadecanoic methyl ester	6
C18:0 octadecanoic methyl ester	1
C18:1 octadec-9-enoic methyl ester	66
C18:2 octadeca-9,12-dienoic methyl ester	23
C18:3 octadeca-9,12,15-trienoic methyl ester	1
C20:0 eicosanoic methyl ester	1.1
C20:1 eicos-11-enoic methyl ester	0.9
C22:0 docosanoic methyl ester	1

Table 2. Selected physico-chemical and biological properties of the soil

Properties	Results	References
Clay (%) (≤ 2 μm)	16	Bouyoucos, 1962
Silt (%) (2-50 μm)	19	Bouyoucos, 1962
Sand (%) (≥ 50 μm)	65	Bouyoucos, 1962
Bulk density (g cm^{-3})	1.52	Brady, 1984
pH (1:3), H_2O	5.5	Reeuwijk, 2002
EC (mS cm^{-1})	0.62	Reeuwijk, 2002
TOC (%)	2.1	Nelson and Sommers, 1982
Total N (%)	0.14	Franzluebbers, Hons, Zuberer, 1994
C:N	15:1	Franzluebbers, Hons, Zuberer, 1994
Avail. P (mg · kg^{-1})	37.7 ± 0.26	Pietrzyński, 2000
Ca (cmol · kg^{-1})	2.00 ± 0.15	Grant, 1982
Mg (cmol · kg^{-1})	1.00 ± 0.10	Grant, 1982
K (cmol · kg^{-1})	0.45 ± 0.01	Grant, 1982
Na (cmol · kg^{-1})	0.11 ± 0.01	Grant, 1982
Al^{3+} (cmol · kg^{-1})	0.95 ± 0.03	Sparks et al., 1996

used in the amount of 4% w/w. Control soil samples were contaminated with the same hydrocarbon fractions or FAME (4% w/w), but were not inoculated with *G. alkanivorans S7*.

The effect of biodiesel concentration and inoculum amount on biodegradation efficiency

Bioremediation processes were conducted in laboratory conditions using identical 2 l glass vials containing 1.8 kg portions of soil contaminated with B100 supplemented with the tested antioxidant: tert-butylhydroquinone (TBHQ). The soil was inoculated with a one-day liquid culture of *G. alkanivorans S7*. The concentrations of the biodiesel in the soil were 3%, 5% or 8% w/w. The control soil samples contained the same concentrations of biodiesel, but were not inoculated with *G. alkanivorans S7*. Due to the higher concentration of impurities (B100) in the samples, the duration of the experiment was extended to 90 days.

Control of the bioremediation process

The parameters controlled during the four or three month bioremediation processes were as follows: a) water content in the soil, which was maintained at 20% or 10% through the replenishment of evaporated water; b) mass of the soil, which was checked every 2-3 days and aeration the soil texture – along with the addition of water (Das and Chandran, 2011). In addition, soil sam-

ples in the vials were supplemented once a week with NH_4NO_3 (ammonium ions were the nitrogen source) at doses ensuring the maintenance of the N:C ratio at 10:1 (Boopathy, 2000).

Chromatographic analysis of hydrocarbons and FAMEs

Each sample of contaminated soil was sonicated prior to 2 h extraction with dichloromethane in a Soxhlet apparatus. After evaporation of the solvent, the impurities were dissolved in hexane (1.5 ml) and analyzed by gas chromatography (GC). GC of hydrocarbons was performed using a Hewlett-Packard gas chromatograph (model 5890) equipped with a DB1 capillary column (30 m × 0.53 mm × 0.25 μm) and a flame-ionization detector (FID). The analysis conditions were: the solvent-hexane, nitrogen as a carrier gas, injector temperature of 300°C, 1 μl injection volume, temperature program: 60°C to 260°C at a rate of 4°C/min. Split/splitless injector and detector (FID) temperatures were 300°C and 260°C, respectively. GC of FAMEs and the blend B20 was performed using a Trace GC Ultra Thermo Scientific gas chromatograph equipped with a Stabilwax capillary column (Restek, 30 m × 0.32 mm × 0.25 μm) and a flame-ionization detector (FID). The analysis was performed under the following conditions: solvent-hexane; carrier gas-nitrogen, a flow rate gradient: 1 ml/min for 60 min, 10 ml/min for 1 min, 3 ml/min for 32 minutes, injection temperature – 250°C; detector temperature – 250°C, temperature program – an increase from 50°C to 205°C at a rate of 10°C/min for 13 minutes, 205°C to 250°C at a rate of 15°C/min for 60 minutes, 250°C to 260°C at a rate of 20°C/min, injection volume of 1 ml.

pH measurment

Changes in the pH of the soil during bioremediation were measured using the potentiometric method (Reeuwijk, 2002).

Dehydrogenase activity assay

To determine the activity of soil dehydrogenases, a modified (reaction time of 20 h) Lester Earl Casida method (1964) with TTC (2,3,5 triphenyltetrazoline chloride) was used.

Mathematical and statistical analysis

The data were processed using STATISTICA 10.0 software (StatSoft Inc., Tulsa, USA). One-way ANOVA was carried out to compare the means of results of different treatments. When significant F values were obtained, differences between individual means and the control mean were tested using the Tukey's test. Significance was set at $P = 0.05$.

Results and discussion

Selection test

Only one strain, *G. alkanivorans S7*, was selected for the experiments. After 12 and 18 h of incubation at 30°C, the strain almost completely discolored the culture medium. Other strains discolored the culture medium after 24 h or more (Table 3). In our previous study (Kwapisz et al., 2006; Kwapisz et al., 2008; Romanowska et al., 2010), the results showed that *G. alkanivorans S7* was an efficient degrader of fuel oil hydrocarbons and can simultaneously utilize oxygen and nitrate as electron acceptors. The significant flexibility of *G. alkanivornas S7* metabolism probably resulted in the efficient degradation of not only hydrocarbons but also fatty acid methyl esters.

Bioremediation of soil – the effect of fuel type on biodegradation efficiency

The progress of fuel biodegradation was estimated by monitoring the decrease in the level of contamination, changes in the activity of microbial dehydrogenases in the soil and the soil pH. For the purposes of the estimation of the effect of fuel type on biodegradation efficiency, two series of experiments were performed. One aimed at a comparison of the dynamics of microbial degradation of pure biodiesel, the mixture of B20 fuels containing 20% biodiesel and 80% ON, and traditional fuel products (diesel oil and hardly degradable P31 petroleum fraction). Our results provide evidence of significant differences in the effectiveness of the processes, which depended on the chemical composition of the fuels subjected to biodegradation. Within the first 4 weeks of biodegradation, the decrease in contamination levels was similar (approximately 30%) in all monitored soil samples, including the controls. However, after 6 weeks of bioremediation, some significant differences in the extent of degradation of pollutants were observed. The largest decrease in their content was observed after 63 days of bioremediation of soil contaminated with either diesel oil (78%) or a mixture of B20 fuels (67%).

Table 3. Time needed for different bacterial strains to decolorize the culture medium containing a DCPIP indicator (in hours)

Microorganism / Fuel	Diesel oil	B100	B20	P31 petroleum fraction
G. alkanivorans S7	14	12	12.5	18
Sarcina spp.	26	24	30	34
Pseudomonas sp G-4B	29	33	34	36
Bacillus subtilis P31	24	38	28	47
Acientobacter spp.	49	72	52	77
Ochrobactum anthropi R51	25	29	18	26

Table 4. Changes in the pH of soil contaminated with different fuels (means ± SD, $n = 3$) during microbial bioremediation. Values designated with the same letter are not significantly different ($P < 0.05$) according to Tukey's test

Week		Blends			
		Diesel Oil	P31	B20	B100
0	assay	6.7 ± 0.056^{a}	6.7 ± 0.050^{a}	6.7 ± 0.090^{a}	6.7 ± 0.020^{a}
	control	6.9 ± 0.047^{a}	6.6 ± 0.035^{a}	6.8 ± 0.029^{a}	6.5 ± 0.026b
2	assay	6.5 ± 0.058^{b}	6.5 ± 0.101^{b}	6.3 ± 0.030^{b}	$5.9 \pm 0.006^{c}\downarrow$
	control	6.8 ± 0.013^{a}	6.6 ± 0.032^{a}	6.7 ± 0.035^{a}	6.4 ± 0.015^{b}
4	assay	6.4 ± 0.010^{b}	6.4 ± 0.040^{b}	6.2 ± 0.041^{b}	$6.0 \pm 0.066^{c}\downarrow$
	control	6.8 ± 0.071^{a}	6.6 ± 0.390^{a}	6.3 ± 0.055^{b}	6.2 ± 0.046^{b}
6	assay	6.4 ± 0.076^{b}	6.5 ± 0.049^{b}	$5.6 \pm 0.026^{c}\downarrow$	$5.7 \pm 0.046^{c}\downarrow$
	control	6.7 ± 0.021^{a}	6.6 ± 0.023^{a}	$5.9 \pm 0.043^{c}\downarrow$	$5.9 \pm 0.102^{c}\downarrow$
9	assay	6.2 ± 0.006^{b}	6.4 ± 0.023^{b}	5.3 ± 0.059^{d}	4.9 ± 0.045^{e}
	control	6.7 ± 0.043^{a}	6.4 ± 0.055^{b}	5.5 ± 0.055^{d}	5.2 ± 0.037^{d}

$\downarrow$ $CaCO_3$ – 2 mg 1000 g^{-1} of wet soil

In both of these two variants, the loss of hydrocarbons was about 20-30% higher when compared with the controls. This difference suggests more efficient utilization of the hydrocarbons by the *G. alkanivorans S7* strain as compared with the indigenous microflora of the soil. The degree of degradation of the other fuels was different. After 63 days of bioremediation of soil contaminated with either pure biodiesel or petroleum fraction P31 treated with *G. alkanivorans S7*, the decrease in the content of hydrocarbons was 48% and 26%, respectively, as in the control samples (44% and 27%, respectively). Thus, the compounds contained in these two fuels were not degraded any faster by *G. alkanivorans S7* than by the indigenous microflora. This effect was surprising; given the fact that biodiesel is thought to be more easily degraded than conventional diesel oil.

The intensity of fatty acid methyl ester metabolism in soil is influenced by a number of factors, including environmental conditions (soil properties, temperature, oxygen) and contamination (concentration, bioavailability). Optimization of the environmental factors affecting the progress of bioremediation is necessary (Aleksander, 1999). The hydrogen ion concentration (pH) has been found to be one of crucial factors that influence the bioavailability of contaminants, the availability of other nutrients, the dynamics of biological processes, and the characteristics of the contaminants with respect to their interplay with a site's geochemical and geological characteristics. The pH of the environment can significantly affect the microbial activity and hence the bioremediation rate. Most microorganisms thrive within a neutral pH range. Laboratory and field bioremediation studies have demonstrated that a pH ranging from 6.5 to 7.5 is sufficient for the optimal growth of bacteria with the ability for degradation of contaminants (Nitschke and Pastore, 2002; Millioli et al., 2009; Ajoku and Oduola, 2013).

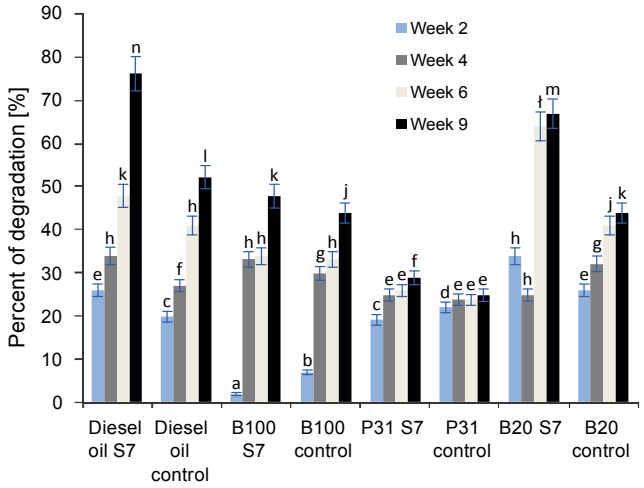

Fig. 1. The decrease in the content of pollutants during bioremediation of soil using *G. alkanivorans* S7 and/or indigenous microflora (means ± SD, $n = 3$). Values designated with the same letter are not significantly different ($P < 0.05$) according to Tukey's test; *P31-P31 – petroleum fraction, B100 – biodiesel, B20 – diesel oil/biodiesel blends

As shown in Table 4 during 9 weeks of bioremediation of soil samples contaminated with B20 and B100 (either treated with *G. alkanivorans S7* or not), a decrease in the pH of the soil was observed along with a gradual degradation of pollutants (shown in Fig. 1). The pH of samples contaminated with B100 and treated with *G. alkanivorans S7* decreased to 5.9 after 2 weeks and to 4.9 at the end of the process, despite additions of calcium carbonate to the soil. In samples of soil contaminated with B20, a decrease in the pH to 5.6 was observed after 6 weeks and later the pH dropped to 5.3 (to 5.5 in the control). Such significant changes in the pH were not observed during the degradation of other fuels: diesel oil and P31 petroleum fraction, for which after 9 weeks the pH was 6.2 and 6.4, respectively. In the controls, where the process was conducted by the indigenous microflora, the pH decreases were slightly lower for each of the fuels. These data indicate that the decrease in the soil pH was correlated with the intensification of fuel biodegradation by the *G. alkanivorans S7* strain. The gradual decrease in the pH observed throughout the clean-up of soil contaminated with either B100 or B20 was a result of the degradation of fatty acid methyl esters and hydrocarbons, yielding acidic intermediate and ultimate products. These results are consistent with the report of Bücker et al. (2011) who monitored changes in the pH during the biodegradation of diesel oil and biodiesel blends using yeasts and filamentous fungi. However, these authors did not observe a significant decrease in the pH during the degradation of biofuels. By contrast, our experiments revealed that microbial biodiesel biodegradation caused an apparent decrease in the pH of the soil. The discrepancies between the reported results may be ascribed to the different species of microorganisms used in the studies, the different initial concentrations of pollutants and the different chemical compositions of soils used in experiments. Our results are consistent with those of Bento et al. (2004), who reported that, when *A. fumigatus* was grown in Bushnell and Hass mineral medium (Atlas, 2005) containing diesel oil as the carbon source, the pH of the aqueous phase was reduced from 7.0 to 4.8 after 60 days. This phase contained propionic acid and other soluble metabolites (including alcohols and ketones). Apart from the microbial metabolites produced during the growth on hydrocarbons, products of cell lysis or organic acids generated during abiotic degradation of diesel or biodiesel may also contribute to the decrease in the pH of the aqueous phase.

The dynamics of biodegradation of organic compounds such as hydrocarbons and FAMEs are also affected by many factors. Therefore, not only the direction and intensity of chemical reactions (reduction of contamination but also nitrogen and phosphorus levels, and changes in the soil pH), but also biological parameters (e.g. the respiratory activity, the intensity of nitrogen fixation, the activity of dehydrogenases, lipases or oxygenases) should be monitored during the whole process. An analysis of soil biological parameters provides data about the presence of microorganisms and their enzymes, and enables evaluation of the conditions of microflora in polluted environments. In this study, changes in the activity of dehydrogenases were determined, because these oxidoreductases play important roles in the metabolism of organic contaminants by soil microorganisms. There is a close relationship between the activity of dehydrogenases and the content of organic matter in soil, microbial abundance and respiration activity (absorption of O_2, CO_2 evolution). Dehydrogenases constitute a numerous group of oxidoreductases located in the cytoplasm or specific structures created from cytoplas mic membranes. Regardless of the state of soil oxygenation, dehydrogenases are an element of the respiratory metabolism that is strictly connected with the genera-

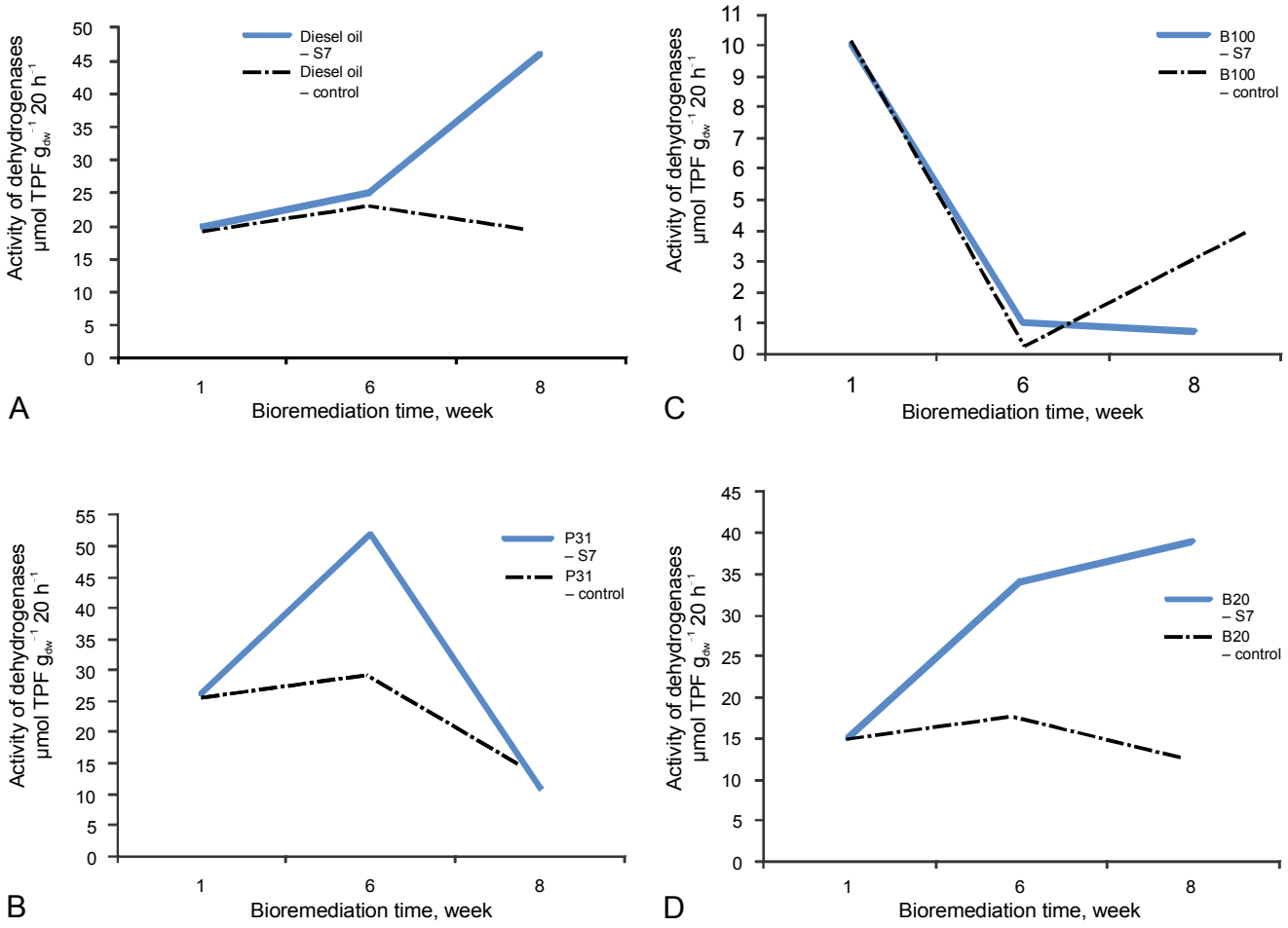

Fig. 2. Changes in the activity of dehydrogenases during bioremediation of soil contaminated with different fuels (A – diesel oil, B – P31 fraction, C – biodiesel B100, D – biodiesel/diesel blend B20) conducted with *G. alkanivorans S7* strain and/or with indigenous microflora (n = 3); P31-P31 – petroleum fraction, B100 – biodiesel, B20 – diesel/biodiesel blends

tion of biologically available energy – ATP (Wyszkowska et al., 2006, Ziółkowska and Wyszkowski, 2010).

Our results provide evidence of considerable differences in the activity of dehydrogenases, which depend on the fuel added to soil and the phase of bioremediation (Fig. 1). In soil samples contaminated with biodiesel, this activity was very low at the initial stage of the process (only 10 µmol TPF $g_{dw}^{-1} \times 20\ h^{-1}$) and was reduced to 0 after 6 weeks, both in the soil inoculated with *G. alkanivorans S7* and in the control samples (Fig. 2C). In the samples of other bacterial strains, this phenomenon was not observed. Only in the soil contaminated with P31 petroleum fraction was the activity of dehydrogenases decreased from 50 to 20 µmol TPF $g_{dw}^{-1} \times 20\ h^{-1}$ after 8 weeks (Fig. 2B). In the soil contaminated with diesel oil, the activity of these enzymes increased from 20 to 46 µmol TPF $g_{dw}^{-1} \times 20\ h^{-1}$ (Fig. 2A), while in the soil contaminated with a mixture of diesel/biodiesel B20, the activity increased from 15 to 40 µmol TPF $g_{dw}^{-1} \times 20\ h^{-1}$ (Fig. 2D). In the control samples (Fig. 2 – variant A, B and D), the activity of soil dehydrogenases fluctuated around 20 µmol TPF $g_{dw}^{-1} \times 20\ h^{-1}$ throughout the whole bioremediation process. The results obtained in the present study show that sometimes the decrease in microbial activity may be associated with factors other than the original toxicity of the contamination (P31 petroleum fraction). In trials contaminated with B100, the decrease in pH values was probably caused by the accumulation of metabolic intermediates. This could be the reason for the decline in microbial activity.

Our results provide evidence that the *G. alkanivorans S7* strain can degrade various organic substrates, but the process must be monitored closely. Additionally, changes in environmental conditions during biofuel biodegradation also require modifications of existing bioremediation strategies.

Some results described here (Fig. 1 and 2) are inconsistent with findings of other authors (Haws and Randall, 1997; Lapinskien et al., 2006; DeMello et al., 2007; Fernandez-Alvarez et al., 2007; Pinto et al., 2008; Owsianiak et al., 2009; Soares et al.,2009; Mańczak et al., 2010; Sôrensen et al., 2011, Gassen et al., 2015) who suggest that biofuels are more biodegradable than traditional petroleum products and less toxic to plants and microorganisms. This discrepancy may be caused by differences in experimental conditions. For instance, the correlation between the initial biodiesel concentration and the activity of dehydrogenases was evidenced by the cited authors based on the activity measurements that were conducted just after the addition of biodiesel to the soil. Only a few authors have described changes of this parameter during the whole process of diesel oil/biodiesel mixture biodegradation, but only in cases of their low initial concentrations. Fatty acid methyl esters are more easily degraded, but products of their metabolism (e.g. free fatty acids, methanol, H_2O_2, aldehydes) can be toxic to microorganisms (Mcdonnell, 2006; Desbois and Smith, 2010; Barah, 2013; Chudobova et al., 2013). Changes in environmental conditions (soil moisture, pH, soil structure, nutrients and oxygen concentration) may inhibit bacterial activity and therefore affect process efficiency. These results show that dehydrogenase activity measurements can be an effective tool for monitoring processes of biofuel bioremediation and may be an appropriate tool to evaluate the metabolic condition of microflora which have the capacity for biofuel degradation.

The effect of biodiesel concentration and inoculum amount on biodegradation efficiency

To verify the negative impact of biodiesel or intermediate metabolites of biodiesel degradation on microorganisms, in a second series trials on bioremediation processes only the biodiesel was added into the soil (at 3 different concentrations). Additionally, a higher amount of microorganisms was introduced to the soil. Also, the previous ratios of the inoculum to pollutant were checked to ensure that they were sufficient for effective biodegradation.

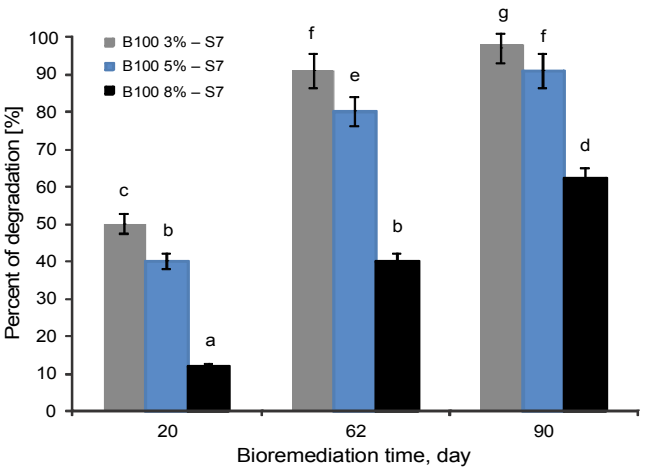

Fig. 3. Decrease in FAME content in soil (initial biodiesel concentrations of 3%, 5%, 8% v/w) during soil bioremediation using *G. alkanivorans S7* and indigenous microflora (means ± SD, $n = 3$). Values designated with the same letter are not significantly different ($P < 0.05$) according to Tukey's test; *B100 – biodiesel

Table 5. Changes in the pH of soil contaminated with biodiesel at different concentrations (3%, 5%, 8% v/w) during bioremediation using *G. alkanivorans S7* and/or indigenous microflora (means ± SD, $n = 3$). Values designated with the same letter are not significantly different ($P < 0.05$) according to Tukey's test

Day		B100 concentration		
		3%	5%	8%
2	assay	6.7 ± 0.043^{a}	6.6 ± 0.228^{a}	6.6 ± 0.032^{a}
	control	6.8 ± 0.040^{a}	6.7 ± 0.038^{a}	6.7 ± 0.010^{a}
20	assay	5.5 ± 0.032^{d} ↓	5.8 ± 0.059^{c} ↓	6.5 ± 0.020^{a}↓
	control	6.4 ± 0.016^{b} ↓	6.5 ± 0.010^{a} ↓	6.7 ± 0.043^{a}
62	assay	4.9 ± 0.017^{e} ↓	5.2 ± 0.064^{d}↓	6.0 ± 0.054^{c}↓
	control	6.1 ± 0.036^{b} ↓	6.4 ± 0.026^{b}↓	6.3 ± 0.020^{b}↓
90	assay	6.2 ± 0.070^{b}	6.3 ± 0.051^{b}	6.3 ± 0.025^{b}
	control	6.4 ± 0.030^{b}	6.4 ± 0.065^{b}	6.5 ± 0.045^{a}

↓ $CaCO_3$ – 2 mg 1000 g^{-1} of wet soil; B100 – biodiesel

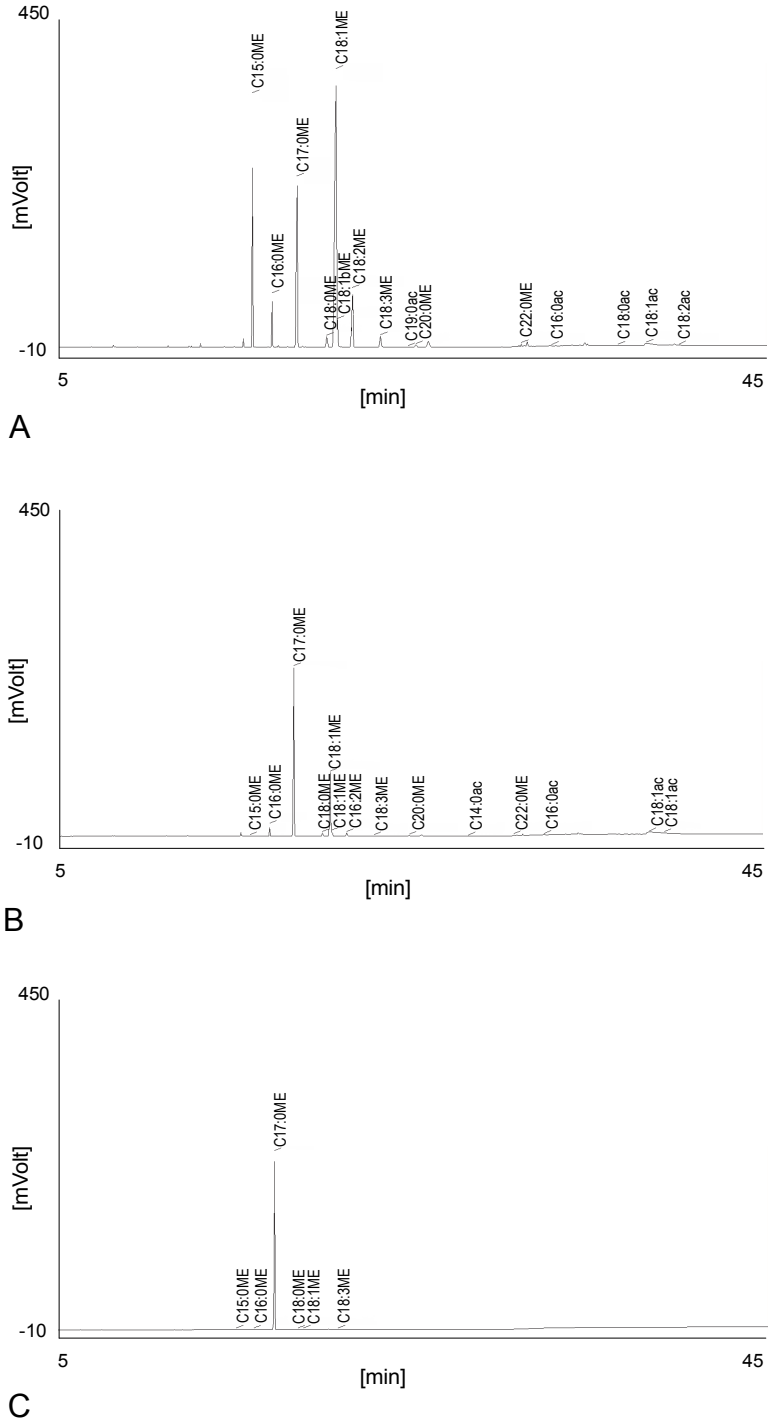

Fig. 4. Results of GC analysis of soil contaminated with 5% v/w biodiesel B100 after: 7 (A), 50 (B) and 90 (C) days of bioremediation; *C15:0 – pentadecanoic ME(metyl esters)-internal standard, C17:0 – heptadecanoic ME-internal standard, C16:0 – hexadecanoic ME, C18:0 – octadecanoic ME, C18:1 – octadec-9-enoic ME, C18:2 – octadeca-9,12-dienoic ME, C18:3 – octadeca-9,12,15-trienoic ME, C20:0 – eicosanoic ME, C22:0 – docosanoic ME, C14:0AC – tetradecanoic acid, C16:0AC – hexadecanoic acid, C19:0AC – nonadecanoic acid, C18:0AC – octadecanoic acid, C18:1AC – octadec-9-enoic acid C18:2AC – 9,12-octadecadienoic acid

The results presented here provide evidence that the degree of biodiesel removal from soil depends on its initial concentration (Fig. 3). It was almost completely degraded (99%) within 90 days when its initial concentration was the lowest (3%) but for the two higher concentrations (5% or 8% w/w) the efficiency of FAME degradation was 90% and only 60%, respectively. We have also shown that the implemented amount of *G. alkanivorans S7* inoculum must be greater in order to achieve effective biodiesel biodegradation (3.5%). Biodegradation of greater concentrations of FAME in the environment performed using selected bacteria strains was less effective. In control trials, in which bioremediation was performed by autochthonic microflora, the efficiency of biodiesel biodegradation was as follows: for B100 in 3% concentration – 48%; for B100 in 5% concentration – 39%; for B100 in 8% concentration – 21% (data not shown).

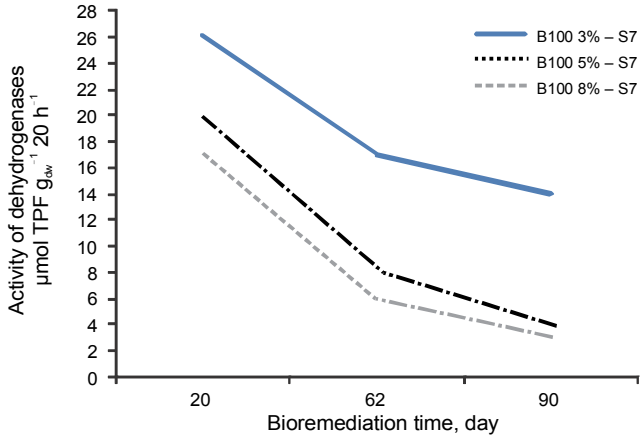

Fig. 5. Changes in the activity of soil dehydrogenases during bioremediation of soil contaminated with pure biodiesel (concentration of 3%, 5%, or 8% v/w) using *G. alkanivorans S7* strain and indigenous microflora

The greatest decrease in pH was observed in the variant in which the soil was contaminated with the lowest concentration of biofuel (3%) (Table 5). A drastic drop in pH after 20 days, from an initial value of 6.7 to 5.5, was probably caused by the intensive metabolism of FAME by the microorganisms (Fig. 3). At the end of the process, after the addition of calcium carbonate to the soil and the exhaustion of carbon sources (FAMEs and free fatty acids), which is shown in the chromatograms (Fig. 4), an increase in pH to 6.2 was observed. In the other soil samples, containing either 5% or 8% v/w of FAMEs, it was observed that a higher initial concentration of biodiesel caused a smaller decrease in pH. For instance, in the sample containing 8% of FAMEs, the lowest pH of the soil (6.0) was observed after 62 days of bioremediation. These results suggest that when the concentration of FAMEs was relatively high, the rate of their degradation by *G. alkanivorans S7* bacteria strain was lower (Fig. 3). Acidic intermediates (fatty acids) released into the soil probably caused a decrease in the pH value (Table 5). Changes in the activity of soil dehydrogenases are shown in Figure 5. In the soil containing 3% w/w FAMEs, this activity fluctuated around 17 µmol TPF $g_{dw}^{-1} \times 20\ h^{-1}$, while in the other samples it was lower-especially in the second trials of bioremediation processes (about 5 and 3 µmol TPF $g_{dw}^{-1} \times 20\ h^{-1}$ for 5% and 8% w/w, respectively). Elevated biodiesel concentrations in soil (above 3% w/w) negatively affected the metabolic activity of the *G. alkanivorans S7* strain. This result is consistent with the findings of Hawrot-Paw et al. (2010) and Hawrot-Paw and Martynus (2011) who observed the negative effect of biodiesel on the indigenous microflora of soil. The results of our study suggest that, when the activity of microbial dehydrogenases during biodegradation is low, the efficiency of biological treatment of soil contaminated with biofuels is also low. In the work of Meyer et al. (2014) on natural attenuation strategies, dehydrogenase activity showed a tendency to decrease, especially for B100, over the 60 days of incubation. On the other hand, bioaugmentation/biostimulation led to increased dehydrogenase activity (Meyer et al., 2014). These results were confirmed by Kaczyńska et al. in 2015. The literature data presented here imply that the metabolic potential of *G. alkanivorans S7* used in our experiments in the case of B100 degradation is rather low.

Kandeler et al. (1996) showed that the composition of the microorganism population determines the potential of this population for the production of enzymes. Any changes in the population composition, under the influence of environmental factors, find expression in the level of soil enzyme activities. The low dehydrogenase activity in soil treated with organic pollutants may be associated with a decrease in the metabolic activity of microorganisms living in a contaminated environment. Dehydrogenases freed from microbial cells are degraded quite quickly, since they have no ability to accumulate in the soil. Soil enzymes, in the form of free molecules, in general display short-term activity, since they either undergo rapid denaturation or degradation or their activity is irreversibly suppressed (Marx et al., 2005).

Conclusions

Bacteria are the main agents responsible for the degradation of diesel oil. The present project describes a study on the biodegradation of soil contaminated with different fuels. According to the results from ex-situ degradation studies, the *G. alkanivorans S7* strain is capable of growing and effectively biodegrading diesel oil, P31 petroleum fraction, B20 fuel blends and biodiesel (in high amounts of 4% and 5% w/w). When higher concentrations (8% w/w) of biodiesel fuel were added to the soil, *G. alkanivorans S7* yield was lower. Indirectly, the results obtained within the scope of this study show that the presence of biodiesel or intermediate metabolites of biodiesel degradation in the environment can influence

the indigenous microflora and microorganisms that are adapted to the degradation of hydrocarbons such as the *G. alkanivorans S7* strain. FAMEs easily undergo oxidation processes involving oxygen, water and microbial enzymes due to their chemical structure (the high content of unsaturated bonds in the molecules renders them reactive). Monitoring the pH of the soil throughout the course of biodiesel degradation revealed that it became significantly decreased, despite the addition of $CaCO_3$. The *G. alkanivorans S7* strain cannot grow in an acidic environment and, therefore, in the final phase of the process the activity of dehydrogenases in the soil was low. Furthermore, biofuels easily undergo oxidation and therefore manufacturers of such products use antioxidants (which have a deleterious effect on microorganisms) which may be an additional factor lowering the efficiency of the clean-up process.

Further research into the microbial degradation of biofuels, encompassing the screening of microorganisms for effective degradation of biodiesel (yeast and filamentous fungi) and an investigation of the effect of antioxidants used to stabilize biofuels is necessary, as is a detailed study of the intermediate metabolites of biodiesel degradation. The bacterial strain studied in this project may be useful in the bioremediation of sites which are highly contaminated with traditional fuels (diesel oil, heavy fraction, crude oil).

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