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Comparison of the dynamics of natural biodegradation of petrol and diesel oil in soil

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ABSTRACT

Contamination of soil with petroleum products is a major environmental problem. Therefore, one of the issues related to environmental protection is assessment of the ability of soil microbial populations to biodegrade petroleum-derived substances. The aim of the study was to compare the dynamics and fractionation of carbon isotopes during biodegradation of selected petroleum products (petrol and diesel) in soil characterised by optimal humidity for plants. The analyses were performed on soil material sampled from the arable layer of a fertile soil (chernozem) in central Poland. The soil samples were treated with two petroleum substances, i.e. unleaded 95-octane petrol and diesel fuel. The dynamics of changes was assessed by monitoring carbon dioxide content and oxygen content in the headspace over the soil surface. Additionally, the ratio of $\delta^{13}C$ carbon isotopes was measured in the substrates added (petrol and diesel) and in CO_2 emitted to the atmosphere. In summary, it should be concluded that the rate of biodegradation of petrol was higher than that of diesel fuel. In the case of petrol, the process of stabilisation of the CO_2 concentration over the soil surface was completed within several days, whereas in diesel the process lasted at least a few weeks. The Isotope Ratio Mass Spectrometry technique and analysis of changes in $\delta^{13}C$ in the samples indicated selective biodegradation of hydrocarbons contained in petrol and diesel fuel. Lighter fractions (constituting a greater proportion in petrol than in diesel) are metabolised more readily than heavier fractions.

Keywords: Petrol; Diesel; Biodegradation; Soil; CO_2 emission; GC; IRMS

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1. Introduction

Contamination of soil with petroleum products is a major environmental problem. Toxic or xenobiotic substances can disturb vital activity of soil microorganisms and affect functions of the ecosystem [1,2]. However, microbial communities have a unique capability of adjusting to unfavourable conditions and using a broad spectrum of substrates [3]. When organic contaminants, such as petrol or diesel, are accidentally released to the soil, the native soil microbial population will adjust their metabolism in order to use the organic contaminants as carbon and energy sources. If the process is efficient, toxic substances can be converted to neutral compounds. Therefore, one of the issues related to environmental protection is to assess the ability of soil microbial populations to biodegrade petroleum-derived substances, and to understand the transformation of contaminants in the soil environment, which will have considerable benefits necessary for proper assessment of risk and developing remediation strategies [2].

In a microcosms experiment, Dos Santos and co-workers observed that crude oil contamination (2000 or 10,000 mg/kg) decreased the microbial biomass, however, increased respiration and enzyme activities and stimulated growth as well as domination of selected microbial groups [4]. Serrano et al. reported that the diesel fuel spill (1 dm^3 m^{-2}) led to a decrease in soil microbial biomass and enzymatic activities, after which it increased again [5]. In the experiment with soil spiked with diesel and synthetic diesel fuel, Horel and Schiewer observed high variability of respiration rates, depending on soil conditions. In the carbon mass balance, the sum of the diesel range organics recovered from the soil plus the produced CO_2 accounted for approximately 30–85%, while the remaining amount of carbon was either incorporated into biomass or degraded incompletely [6]. Stable isotope fractionation approaches have been developed for assessment of *in situ* biodegradation. Monitoring of the concentration and carbon isotopic composition of greenhouse gases allow to determine the source of gases released from soil [7,8]. On the basis of the CO_2 evolution rate and ^{13}C isotopic signature of respired CO_2, the metabolic response to the addition of some organic compounds may be assayed. When hydrocarbons have carbon isotope characteristics that differ from soil organic components, the ^{13}C–CO_2 characteristics can also be used to monitor their mineralisation [9].

Dilly et al. studied CO_2 evolution and the ^{13}C isotopic signature of respired CO_2 in response to addition of ^{13}C labelled *n*-hexadecane and palmitic acid (which are constituents of fossil fuels) in agricultural and forest topsoils. The CO_2 evolution rate was immediately stimulated in the agricultural soil. In turn, the microbial response was delayed in the forest soil, but developed better in the agricultural soil throughout the subsequent weeks. Consequently, the respiration rate returned back to the initial level for forest soil and the $\delta^{13}C$ of respired CO_2 approached values before hydrocarbon addition. The resilience, defined as the capacity of the soil microbiota to buffer perturbance and to reorganise in response to change resulting in a more desirable system, was higher in the forest ecosystem than in the agricultural soil [3].

A review of different literary sources demonstrates a relatively large number of studies concerning the dynamics and mechanism of biodegradation of organic compounds that are components of petrol and diesel. In contrast, there are relatively few papers on biodegradation of petrol and diesel as such. The aim of the present study was to compare the dynamics and fractionation of carbon isotopes during biodegradation of selected petroleum products (petrol and diesel) in soil characterised by optimal humidity for plants.

2. Materials and methods

The analyses were performed on soil material sampled from the topsoil layer of a fertile soil (chernozem) in central Poland [10]. All measurements were made in three replications. Basic properties of the soil are presented in Table 1. The soil was air dried and sieved through a 2 mm mesh.

Particle size distribution (PSD) was measured with the laser diffraction method (LDM) [11,12]. Mastersizer 2000 with a Hydro G dispersion unit was used. The stirrer speed was 700 rpm and pump speed was 1750 rpm [13]. The soil aggregates were dispersed by maximum power (35 W) of ultrasounds [14]. The Fraunhofer theory was used to convert the reading from detectors into PSD [15]. While interpreting PSD obtained with the LDM, one should bear in mind that this method underestimates the clay fraction content in comparison to sedimentation methods [16].

The organic carbon (C_{org}) content in the soil was determined using a Shimadzu TOC-V CPH analyser equipped with an SSM 5000A unit. The method is based on measurements of the percentage of total carbon (TC) and inorganic carbon (IC) in the soil sample, and next, the calculation of C_{org} as the difference between TC and IN ($C_{org} = TC - IC$). For TC determination, a portion of 300 mg soil covered by ceramic fibre was burned at 900˚C, while for IC determination,

Table 1
Selected soil properties

Site of sampling	Soil	Soil texture class according to FAO/WRB	Particle size distribution (%, diameter mm) Sand 2–0.05	Silt 0.05–0.002	Clay < 0.002	C_{org} (%)
Zlote (Swietokrzyskie Province)	Haplic chernozem	Silt loam	19.55	73.20	7.25	1.06

another 300 mg soil sample was burned in the presence of 0.5 ml 2 M HCl at 200°C. In both cases, the amounts of CO_2 produced were defined by infrared detection (NDIR) and used for calculation of the percentages of TC, IC and finally C_{org} in the soil.

The soil was treated with two crude oil products, i.e. unleaded 95-octane petrol purchased at an Orlen petrol station and diesel from a BP station. Both stations are located in Lublin (central-eastern Poland).

The method of contaminating the soil samples and the experimental scheme were as follows: 10 g of soil (dry weight) with moisture corresponding to pF 2.2 (34% g g^{-1}) was placed in 60 cm^3 brown glass bottles and sealed by septum (therefore, a constant moisture content during entire experiment was assumed). Next, the first series (all analyses were performed in three replicates) was supplemented with 0.2 cm^3 of petrol, the second series with 0.2 cm^3 of diesel and the third series (control) was treated with 0.2 cm^3 of distilled water. The samples were tightly sealed and incubated at 25°C in the dark. The concentration of gases (CO_2 and O_2) in the headspace and the isotopic composition of the carbon dioxide released were analysed. The analyses were performed on day 0, 1, 3, 5, 7, 10, 14, 21, 28, 35 and 42 of incubation.

Gas concentrations were analysed with the use of a Shimadzu GC-14A gas chromatograph equipped with a Thermo Conduction Detector with helium carrier gas [17]. The CO_2 concentration was analysed using a steel column packed with Porapak *Q*, whereas the O_2 concentration was assessed on a column packed with a molecular sieve 5A. Each time, each column was dosed with 50 µl of gases collected at a fixed height (from the bottom) of the samples.

The analyses of the isotopic composition of carbon dioxide were carried out using a Thermo Electronics DELTA V Advantage continuous-flow mass spectrometer for determination of stable isotope ratios (Isotope Ratio Mass Spectrometry, IRMS). The IRMS technique allows to determine a stable isotope ratio. For instance, a vast majority of carbon occurs in nature as isotope ^{12}C. However, carbon occurs as isotopes ^{13}C and ^{14}C as well, although in substantially lower amounts likewise the other stable elements, e.g. hydrogen, nitrogen, oxygen and sulphur. The knowledge of isotope mass ratios of a given substance facilitates the identification of the substance origin and/or processes in which it has participated [18,19].

The $^{13}C/^{12}C$ isotope ratio is usually determined using the so-called "delta" notation ($\delta^{13}C$), in which it is relative to a primary international standard. For isotopes ^{13}C and ^{12}C, the basic standard is V-PDB—Vienna—Pee Dee Bellemnite, in which the absolute $^{13}C/^{12}C$ ratio is 0.0112372. The $\delta^{13}C$ is calculated from the following formula [19,20]:

$$\delta^{13}C = \left[\left(\frac{R_{sample}}{R_{standard}}\right) - 1\right] 1000[‰] \quad (1)$$

where R_{sample} is the ratio of $^{13}C/^{12}C$ for the sample and $R_{standard}$ is the ratio of $^{13}C/^{12}C$ for the standard (V-PDB).

During the GC and IRMS analyses, the samples were dosed with a VICI 100 µl manual dispenser (shut-off valve) side-port syringe with a full-tip needle, which prevented the gas sampled from mixing with laboratory air.

Analyses of $\delta^{13}C$ of petrol and diesel added to the soil were performed at the Mass Spectrometry Laboratory, Institute of Physics, UMCS, Lublin, with the use of a modified MI-1305 mass spectrometer. The values of $\delta^{13}C$ of the substrates are presented in Table 2; the results are the means from nine replications.

The total value of $\delta^{13}C$ does not offer a possibility of determining the content of individual hydrocarbons or groups of these compounds, as $\delta^{13}C$ differ in the range from −24 to ca. −48‰ [21,22].

Table 2
Values of stable isotope ratios of carbon substrates

Substrate	$\delta^{13}C$ [‰, V-PDB]	σ
Petrol	−31.12	0.10
Diesel	−31.06	0.11

3. Results and discussion

Changes in the concentration of gases in the headspace over soil contaminated with petroleum derivatives and the control are presented in Fig. 1.

The analysis of changes in concentrations of gases (CO_2 and O_2) during successive days of incubation (Fig. 1) demonstrates three stages in the study period. The first stage, the so-called lag phase, is the period of adaptation of micro-organisms to a new, artificially established ecosystem (in the bottle). This stage lasted approximately one day, which is indicated by a low rate of increase in the CO_2 concentration and relatively low dynamics of O_2 decline. In the second stage, the dynamics of microbial metabolism was higher, and there was a substantial increase in the CO_2 concentration and a considerable decrease in the O_2 level. The third stage consisted in achievement of a certain equilibrium and stabilisation of the concentrations of both gases. Another conclusion that can be drawn from the analysis of both graphs (Fig. 1) is the obvious difference in the dynamics of changes in gas concentrations in the case of soil contaminated with petrol and diesel. Biodegradation of petrol exhibits significantly higher rates than biodegradation of diesel. In the case of petrol, micro-organisms "coped" with an available experimental biodegradable contamination within only 7 d, after which the CO_2 concentration remained at a constant level. The lack of an increase in the CO_2 concentration and a decline in the O_2 level does not imply that all petrol supplied has been metabolised by micro-organisms. Given other investigation results, from several up to a few dozen per cent could be expected [23,24]. In the case of diesel, stabilisation of CO_2 in the headspace of diesel was first visible only after 40–42 d. Simultaneously, CO_2 production in this system was significantly higher, as its concentration at the end of the incubation was 5% higher than in the case of petrol. Microbial activity leading to CO_2 production was substantially higher in the samples treated with both the petroleum derivatives rather than in the control ones (without addition of petroleum derivatives). During the incubation, some volatilization of light gaseous hydrocarbons occurred, but only in the petrol-polluted soil (up to 10% v/v in the headspace, mainly C3 and C4 chains), while no volatile hydrocarbons were detected in the diesel-polluted soil.

It is noteworthy that the changes in the CO_2 and O_2 concentrations occurring during successive days correlated well with each other—the graphs are virtually mirror images. Therefore, a conclusion can be drawn that C-substrates were metabolised by aerobic organisms [25].

The analysis of Fig. 1 may bring up a question which is difficult to answer based on the conducted experiment. In the case of petrol, the final O_2 concentration (which changed slightly between day 7 and 42) indicated that the dynamic stage of biodegradation was accomplished, i.e. organic compounds that were more easily available for micro-organisms were supposed to be utilised by that time (relatively high levels of oxygen, i.e. ca. 6–7%, remained in the headspace and it did not decline during the following days). In contrast, it was impossible to assess whether the same stage of diesel biodegradation ceased due to depletion of C-substrates or oxygen, or, alternatively, the chemical composition of the diesel determined the possibility of its biodegradation by multiplying microbes. From day 28, O_2 concentration over the soil samples was stabilised at ca. 2%, which practically indicates anaerobic conditions in soil suspension. Nevertheless, it is widely known that biodegradation

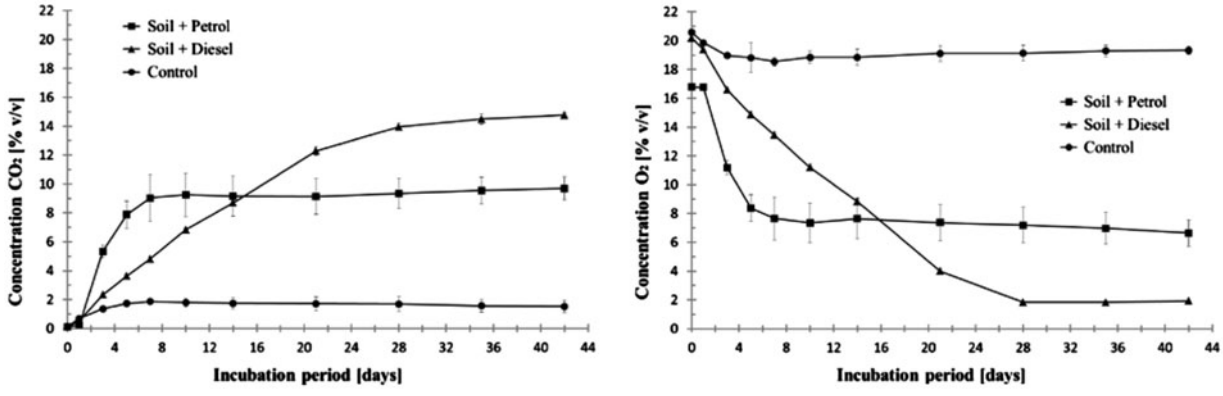

Fig. 1. Changes in the gas concentration in the headspace over soil contaminated with petroleum derivatives during successive days of incubation: (a) CO_2; (b) O_2 (mean values ± SE).

of aliphatic and aromatic compounds proceeds in hypoxic [26,27] or even in anoxic conditions, although its rate is considerably slower in the presence of oxygen [28]. Therefore, it is difficult to identify clearly the cause of the "flattening" of the O_2 concentration graph observed after day 28 of the incubation. The short stage of adaptation of the soil microflora to utilisation of an additional carbon source should be taken into account; this may have been related to the relatively low level of petroleum derivative addition. Labud et al. [29] investigated the effect of contamination of sandy and clayey soil with diesel oil, petrol and crude petroleum (added in the amount of 5 and 10% w/w) on the activity of soil micro-organisms. The authors observed a 20 d lag phase. An addition of crude petroleum and diesel oil stimulated respiration, whereas the effect of petrol was relatively low (petroleum 10% > petroleum 5% > diesel 10% > diesel 5% > petrol 10% = petrol 5% = control soil). However, contamination reduced the microbial biomass, with the highest reduction in the sandy soil incubated with petrol, and having an ambiguous effect on the enzymatic activity. The authors emphasise that the toxicity of organic additives highly depends on soil properties. Higher content of organic matter and the silty fraction in clayey soil may serve a "protective" function for micro-organisms, e.g. through adsorption of contaminants, decreasing the concentration in the solution and gas phase [29]. Diverse response to contamination with crude oil, both in terms of CO_2 emission and soil microbial biomass, was reported by Franco et al. [30]. During 60 d incubation of soils (Inceptisols, Mollisols, Entisols), below 2% of crude oil was only mineralised to CO_2, while 36–50% was irreversibly adsorbed, and 10–33% was lost by volatilisation [30].

Lower dynamics of microbiological activity in diesel, compared with the one in petrol, corresponds well with data reported in other research papers. For instance, Rachman and co-workers observed some stabilisation of petrol biodegradation after ca. 15 d [23], while Horel and Schiewer reported a distinct increase in CO_2 concentration in soil contaminated with diesel after 120 d and no stabilisation was observed [5]. Van De Steene and Verplancke [31] observed significantly higher O_2 consumption and CO_2 production in loamy sand soil contaminated with diesel fuel (in doses of 1000, 5000 and 10,000 mg kg^{-1} d.w. soil) than in the control soil. Significantly, higher CO_2 emission in the soil bulked from around a car park and incubated with 5% petrol, in comparison with the control soil and elevated emission from the soil treated with diesel oil was reported by Obire and Nwaubeta [32].

In order to determine $\delta^{13}C$ values for CO_2 released from the control soil, and the soil contaminated with petroleum derivatives, the values obtained on day 5 of incubation were adopted. It was assumed that on that day a maximum of $\delta^{13}C$ relationship presented in Fig. 2 was achieved, and there was an equilibrium between $\delta^{13}C$–CO_2 from atmospheric air and $\delta^{13}C$–CO_2 released from the soil (the maximum for the control soil was achieved a few days later but the difference between the maximum and the $\delta^{13}C$ value on day 5 of the incubation was slight). After the maximum was reached, changes in $\delta^{13}C$ were observed in all the experimental variants. These changes observed in the control variant and that with petrol addition are related to equilibrium isotope effects, since CO_2 emission practically ceases after 5–7 d of incubation (Fig. 1). Changes in $\delta^{13}C$ in the variant with diesel were most likely due to CO_2 emission, which unlike in the control and the petrol addition variants persisted until the day 42 of incubation (although the $\delta^{13}C$ value most probably resulted from CO_2 emission and equilibrium isotope processes). Therefore, $\delta^{13}C$ in CO_2 was −22.37‰ for petrol, −29.43‰ for the control and −32.58‰ for diesel oil.

The IRMS technique and analysis of $\delta^{13}C$ changes (Fig. 2) in samples allow to identify selective biodegradation of various hydrocarbons contained in petrol and diesel. The changes in $\delta^{13}C$ in time for both petrol and diesel differed significantly from the changes in

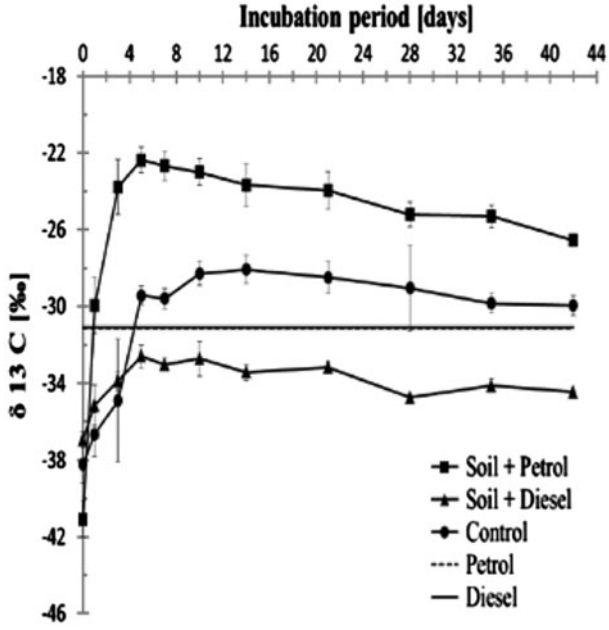

Fig. 2. Values of $\delta^{13}C$ in the samples in successive incubation days and substrate delta. Straight lines represent $\delta^{13}C$ of pure substrates (Table 2): the continuous line denotes diesel oil, and the dashed line represents petrol (mean values ± SE).

the control samples and $\delta^{13}C$ from the added substrates. However, the amount of the heavier isotope (^{13}C) in CO_2 caused by petroleum biodegradation was higher than that in the control (as well as to $\delta^{13}C$ of added substrates), while smaller than in diesel fuel. On the basis of the results, it is difficult to determine unequivocally which hydrocarbons and/or other compounds contained in both the petroleum derivatives were metabolised. Available data from literary sources concerning the $^{13}C/^{12}C$ isotope ratio report different values for the same hydrocarbons/hydrocarbon groups [21,22]. That is not surprising as the contents of the heavier carbon isotope may vary significantly depending on the location of crude oil extraction. It is evident that lighter fractions (constituting a greater proportion in petrol than in diesel) are metabolised more readily. That is confirmed by higher dynamics of CO_2 concentration increase during incubation (Fig. 1(a)) and a higher value of $\delta^{13}C$ on the day 5 of incubation of soil contaminated with petrol.

A more detailed analysis of changes shown in Fig. 2 requires additional experiments since the carbon source in a soil is a mixture of different compounds rather than a homogeneous substance, including these with diverse carbon isotopic composition, e.g. the residues of C3 and C4 plants [33]. Additionally, the result of degradation of any substrate, both homogeneous and heterogeneous, is associated with multiple overlapping processes. The study performed by Auffret et al. on the degradation of diesel oil, gasoline and a mixture of hydrocarbons by bacterial strains with capacities to use a broad spectrum of various hydrocarbons emphasised the positive role of co-metabolism in the biodegradation of a complex mixture of chemicals, and the problem of the inhibitory effects of some compounds on the degradation of other compounds when present in complex mixtures [34]. The fate of the mixtures of different macromolecules in soil may differ considerably from that of individual components, because (1) a substrate mixture may result in competition, inhibition and *priming effects* that change microbial degradation kinetics, and (2) some of the substrates may not be easily accessible to microbes due to chemical or physical protection mechanisms within the soil structure [35]. Therefore, respiration occurring in field conditions and the isotope ratio of released CO_2 are the results of degradation of a heterogeneous substrate, irrespective of whether native organic matter or organic soil contaminant is degraded.

A review of literary sources shows that currently the IRMS technique for analysis of biodegradation of petroleum derivatives in soil and/or water is not widely applied. Majority of papers are focused on investigating the biodegradation of individual components of petroleum derivatives, e.g. aromatic hydrocarbons and chlorinated solvents [28,36,37]. For example, Spence and co-workers investigated the space distribution (in profiles) and isotopic composition of petroleum fuel contaminants in groundwater of the Chalk aquifer beneath a petroleum hydrocarbon spill site [38]. Medina-Bellver et al. proved that the microorganisms present in the contaminated sea water were readily able to transform components of crude oil into IC [39]. In turn, Jewell and Wilson studied biodegradation of crude oil derivatives, but they were focused on its specific product, i.e. methane [40]. Therefore, it was difficult to find papers that would combine the analysis of stable isotopes and determine the dynamics of biodegradation of petroleum derivatives reflected by changing concentrations of CO_2 and O_2. The main aim of the use of IRMS analysis in our study was to define the fractionation of carbon isotopes during biodegradation of petrol and diesel. The results showed that such fractionation occurred, and the $\delta^{13}C$ of produced CO_2 significantly differed from that in the initially added substrates. However, we still do not know whether the mechanism of the fractionation is biological or physico-chemical in nature, and whether it proceeds inside or outside the microbial cell.

4. Conclusions

(1) The dynamics of changes in the CO_2 concentration over a contaminated soil sample can be a measure of the dynamics of biodegradation of petroleum derivatives in soil.
(2) Biodegradation of petrol proceeds faster than biodegradation of diesel. In the case of petrol, the first phase (reflected by stabilisation of the CO_2 concentration above the soil surface) lasted several days, while in the case of diesel, it lasted for at least several weeks.
(3) The IRMS technique and analysis of $\delta^{13}C$ changes indicated selective biodegradation of hydrocarbons contained in petrol and diesel fuel. Lighter fractions (constituting a greater proportion in petrol than in diesel) were metabolised more readily than heavier fractions.

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