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ORIGINAL PAPER

CHANGES IN THE FUNCTIONAL DIVERSITY OF BACTERIAL COMMUNITIES IN SOIL CONTAMINATED WITH DIESEL OIL*

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Abstract

Monitoring of changes occurring in the microbiota of soil contaminated with Ekodiesel ULTRA diesel oil (DO) is crucial for effective bioremediation. Therefore, the aim of this study has been to determine the functional diversity of bacterial communities, based on an analysis of the metabolic profile in soil contaminated with diesel oil for 270 days. The rate of the degradation of volatile hydrocarbons (BETX) by soil microorganisms was determined. In the assessment, the activity of selected extracellular enzymes was also made. The study revealed that the contamination of soil with Ekodiesel Ultra diesel oil has a lasting effect on the soil environment, as certain groups of organic compounds included in the composition of DO are relatively resistant to biodegradation. They include gasoline fractions (C_6-C_{12}), mineral oil (C_{12}-C_{35}) and xylene isomers found in the composition of BTEX. Ekodiesel Ultra diesel oil increased the counts of most groups of microorganisms as well as raising the enzymatic activity of soil. The catabolic profile of the bacteria, determined on Biolog ECO MicroPlate® microplates, the eco-physiological profile of the bacteria (EP index) and the identification using MALDI-TOF mass spectrometry MS prove that DO causes changes in the structure of bacterial communities. Their diversity decreases and the mutual relationships between the r-strategy and K-strategy bacteria change. The methods applied durign the research ensured good identification of the eco-physiological and catabolic profile of bacteria, which would facilitate optimisation of the biodegradation of diesel oil.

Keywords: bacterial communities, functional diversity, diesel oil, contamination, hydrocarbons, Biolog ECO MicroPlate®.

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INTRODUCTION

The development of civilisation and the technological and economic progress it entails have increased the demand for crude oil and petroleum products. According to the World Energy Outlook (WEO 2017), the demand for crude oil will be increasing until 2030 at an average annual increase rate of 3.4%. The investment decisions being currently made and implemented shape the global outlook and have an impact on the perception of modern strategies and technologies worldwide. Global economic and environmental trends not only create the sense of *contemporaneity* but also enforce a huge demand for coal, crude oil and energy; on the other hand, new environmental hazards are created, which are leading to reduced biological diversity (Global Environment Outlook GEO_5 2012). The most widespread environmental pollutions are due to the contamination with petroleum hydrocarbons, caused by spills during transport and storage, tank and pipeline leakages as well as transport accidents (Das, Chandran 2011). Since these contaminations are usually restrained to the topsoil, the soil ecosystem is regarded as one of the main reservoirs of PAHs (Sivitskaya, Wyszkowski 2013). Soil is also considered to be a good indicator of the degree of environmental pollution (Wang et al. 2012). The hydrocarbons found in contaminated areas not only affect the physicochemical (Griffiths and Philippot 2013, Wyszkowski and Ziółkowska 2013) and biological properties of soil (Wyszkowska et al. 2015, Adam et al. 2017), but they also cause harm to people exposed to these products (Zhang et al. 2015). Therefore, the risk of organic contaminants penetrating into drinking water sources and accumulating in plant tissues and living organisms should never be underestimated (Abdel-Shafya et al. 2016).

For this reason, it is extremely important to remove oil-derived hydrocarbons from the soil in contaminated areas. Common remediation technologies, both mechanical and chemical ones, are still not very economical and result in an incomplete decomposition of contaminants (Das, Chandran 2011). Biodegradation of petroleum products is a good alternative to the removal of these compounds from the contaminated environment. However, there is no information on the degradation of hydrocarbons in soils severely contaminated with petroleum products by autochthonous bacteria, although it is known that microorganisms and the enzymes they produce in a series of metabolic reactions could help biodegrade hydrocarbons (Lipińska et al. 2014, Kaczyńska et al. 2015).

Soil microorganisms in terrestrial ecosystems perform numerous important ecological functions (Kucharski, Jastrzębska 2005). Since the processes of organic contaminant mineralisation are closely linked to the structure and function of soil, it is extremely important to identify the composition of soil microbial populations and their functional diversity in various types of soil under variable climatic conditions. The response of microorganisms

and soil enzymes to the effects of particular petroleum products should be recognised for particular types and brands of these products (Adam et al. 2017). This is essential also because the chemical composition of oil derivatives is modified by the addition of improvers. Improvers are able to modify the effects of particular petroleum products on the quality of soils, with some having an ambiguous effect on the quality of soils (Ramkumar, Kirubakaran 2016). Recognition and understanding of the soil's metabolic response to particular types of oil derivatives will facilitate an application of active soil microorganisms in biotechnological soil remediation, biostimulation or bioaugmentation (Borowik et al. 2017*b*).

The aim of this study has been to determine the counts and functional diversity of bacterial communities, based on an analysis of the metabolic profile in soil contaminated with Ekodiesel ULTRA diesel oil. The above problem was considered in terms of the duration (from 7 to 270 days) of the impact of diesel oil on soil bacterial communities. Moreover, the percentage of volatile hydrocarbons (BETX) degraded by soil microorganisms was determined. The assessment was supported by results of an analysis of the activity of selected extracellular enzymes, i.e. urease, acid phosphatase, alkaline phosphatase, β-glucosidase and arylsulphatase.

MATERIAL AND METHODS

Soil material

The study was carried out on Endocalcaric Cambisol (soil type 3.1: Eutric Cambisols) belonging to Order 3: Brown earths. The soil was sampled at the Experimental Station in Tomaszkowo (NE of Poland, 53.71610 N, 20.41670 E), located in Olsztyn Lake District. For the study, soil samples were taken from the tilled horizon to the depth of 0-20 cm. The grain size composition classified the soil as loamy sand (sand – 81.9%, silt – 16.7%, clay – 1.4%). The soil pH in 1 mol of KCl was 5.2, the carbon nitrogen ratio was 10.7 and the sorption capacity equalled 57.8 mM(+) kg^{-1}.

Characteristics of Ekodiesel ULTRA diesel oil

Ekodiesel Ultra B diesel oil is a mixture of oil-derived hydrocarbons and methyl esters of higher fatty acids. The diesel oil tested was purchased at PKN ORLEN petrol stations. Its detailed characteristics can be found on the website (www.orlen.pl).

Performance of the experiment

In order to verify the research hypothesis predicting that the contamination of soil with diesel oil could change the functional diversity of bacterial communities, an *in vitro* experiment was carried out. The following were

tested: the effect of the duration (7, 30, 60, 90, and 270 days) of the soil's exposure to contamination with diesel oil at an amount of 50 cm^3 per 1 kg soil DM on counts of bacteria, colony development (CD) and eco-physiological diversity (EP) indices for bacteria, functional diversity of bacterial communities, activity of extracellular soil enzymes, and the content of a mixture of hydrocarbons (mineral oil) and volatile hydrocarbons (BETX). A model experiment with three replications was carried out in 1 dm^3 glass beakers, each filled with 1 kg of homogenised soil mixed with diesel oil. The moisture content of the prepared soil samples was increased up to 40% of capillary water capacity, and the beakers were stored in an incubator at a temperature of 22°C for the entire experiment. The moisture content of the soil was monitored for 270 days. Non-contaminated soil served as control.

Isolation and identification of bacteria in the soil

The counts of organotrophic (Org), oligotrophic (Olig), and copiotrophic (Cop) bacteria, *Pseudomonas* (Ps), *Arthrobacter* (Art) and actinomyces (Act) were determined in soil samples, in 5 replications, five times during the experiment, i.e. on days 7, 30, 60, 90, and 270. Details of the procedures for determination of counts of bacteria and the composition of culture media used for the isolation of the above bacteria, are presented by Borowik et al. (2017*b*).

The study presented the effect of Ekodiesel Ultra diesel oil (DO) on particular groups of bacteria as an influence index IF_{DO} calculated from the following formula:

$$IF_{DO} = (Po\text{-}Co)/Co \quad [1]$$

where:

IF_{DO} – influence of diesel oil,

Po – number of c.f.u. in soil contaminated with diesel oil,

Co – number of c.f.u. in soil without diesel oil

The structure and diversity of bacteria were determined based on the number of colony-forming units growing on each day for 10 consecutive days.

Colony development (CD) index was calculated using the formula proposed by Sarathchandra et al. (1997):

$$CD = [N1/1 + N2/2 + N3/3\ldots.. N10/10] \cdot 100 \quad [2]$$

where:

CD – colony development index,

N1, N2, N3, ... N10 – proportions of microbial colonies identified on days 1, 2, 3, ... 10 to all colonies.

The eco-physiological index of bacterial diversity (EP) was calculated using the formula proposed by De Leij et al. (1993):

$$EP = -\Sigma(pi \cdot \log10\ pi) \quad [3]$$

where:

EP – eco-physiological diversity index,

pi – share of individuals of a given species in the community relative to the total number of individuals in the community.

On the last date (day 270), bacteria were isolated from the soil and identified using MALDI-TOF mass spectrometry (MS). The paper discusses only these bacteria whose species were identified.

Bacterial catabolic profile

The catabolic profile was determined in soil samples, in three replications, on particular days of the study, using Biolog ECO MicroPlate® microplates. The results were read with the MicroStation ID (semi-automated) and OmniLog ID (fully automated) systems by Biolog® (http://www.biolog.com). Microplates were incubated at a temperature of 28°C for 216 hours. The consumption of particular carbon sources was measured every 24 hours. Optical density (OD) was measured at λ = 490 nm. For the calculation of Average Well-Colour Development AWCD (Li et al. 2012, Gomez et al. 2004), the Shannon-Weaver index – H (Gomez et al. 2006) and Richness Substrates – R (Gomez et al. 2006) were derived; the OD results obtained at 48 h were used. In order to determine the values of the above indices, the following formulas were applied:

$$AWCD = \sum(C\text{-}R)/31 \quad [4]$$

where:

AWCD – Average Well-Color Development,

C – absorbency in each C-source well,

R – absorbency in the control well.

$$R = C\text{-}R \quad [5]$$

where:

R – Substrate Richness.

R is the well numbers with (C-R) calculated using an OD of 0.25 as the threshold for a positive response.

$$H = -\Sigma p_i(\ln p_i) \quad [6]$$

where:

H – Shannon-Weaver,

pi – the ratio of the absorbency of each well to the absorbency of all wells.

Bacterial activity is presented based on all carbon sources as well as on the grouped sources defined as: CB – Carbohydrate; CA&A – Carboxylic Acids & Acetic Acids; AC – Amino Acids; PY – Polymers; AN – Amines/ /Amides. Based on the OD values, four classes of bacterial consumption of carbon sources were distinguished: high (OD > 0.75); good (OD = 0.51-0.75); average (OD = 0.25-0.50) and low (OD < 0.25).

Soil enzyme activity

On the same days on which the bacterial count and catabolic profile were determined, i.e. on day 7, 30, 60, 90, and 270, the activity of five enzymes belonging to the hydrolase class, i.e. urease (EC.3.5.1.5), acid phosphatase (EC 3.1.3.2), alkaline phosphatase (EC 3.1.3.1), β-glucosidase (EC.3.2.1.21) and arylsulphatase (EC 3.1.6.1), were determined using classical methods described by Borowik et al. (2017*b*). The activity of all soil enzymes under study was determined on a Perkin-Elmer Lambda 25 spectrophotometer (Massachusetts, USA). In order to better illustrate the effect of diesel oil on biochemical activity of the soil, the study presents the activity of particular enzymes as the diesel oil influence index (IF_{DO}). Formula No 1, described in the "Isolation and identification of bacteria in the soil", was applied. The symbol "Po" signifies the activity of particular enzymes in soil contaminated with diesel oil, and the symbol "Co" denotes the activity of enzymes in soil not contaminated with diesel oil.

Assays of the mixture of hydrocarbons and volatile organic hydrocarbons

The content of gasoline fractions (C_6-C_{12}), mineral oil (C_{12}-C_{35}) and volatile aromatic hydrocarbons (C_6-C_8) was determined in soil samples on particular days of the study. The assays were performed on an Agilent 7890A gas chromatograph coupled with an Agilent 5975C mass spectrometer equipped with an EI/CI ion source. The mineral oil content was determined in accordance with the standard EN ISO 16703 (2011), while the content of gasoline fractions and volatile aromatic hydrocarbons was determined in line with the standard EN ISO 22155 (2016). In order to better illustrate the effect of Ekodiesel Ultra diesel oil on the soil metabolism, the study presents the per cent degradation of the mixture of hydrocarbons and volatile aromatic hydrocarbons on days 7, 30, 60, 90, and 270 of the experiment. The figure does not illustrate the content of gasoline fractions (the total amount of aliphatic and aromatic hydrocarbons containing from 6 to 12 carbon atoms in the molecule), since it was on a constant level (1400 mg kg^{-1}) throughout the experiment (270 days).

Statistical analysis

In order to draw correct conclusions concerning changes occurring in microbiota of soil subjected to the effects of Ekodiesel Ultra diesel oil, all results were statistically processed using Statistica 13.0 software (Dell Inc. 2016). Homogeneous groups were determined using the Tukey's range test and an ANOVA variance analysis. The calculations were made at the significance level of $P < 0.01$. Principal component analysis (PCA) with the use of multi-dimensional exploratory techniques was also employed. Figures 2, 4, 5, 6, 7 and 9 were made using Statistica 13.0 software based on the data obtained from replications, including standard deviations.

RESULTS

Bacterial eco-physiological and species diversity

Diesel oil had a significant impact on the bacterial counts (Figure 1). Positive values of the IF_{DO} index indicate the stimulation of proliferation and

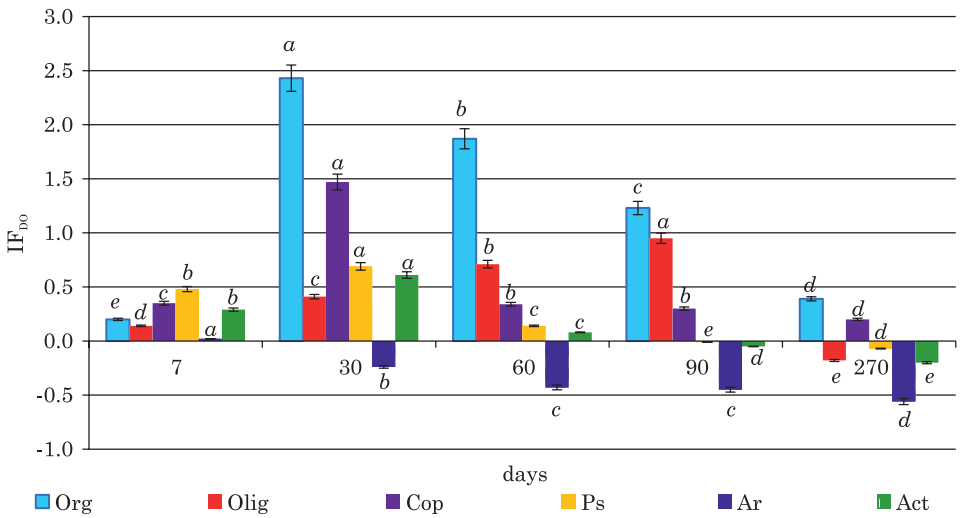

Fig. 1. Effect of diesel oil (IF_{DO}) on counts of bacteria: organotrophic (Org), oligotrophic (Olig), copiotrophic (Cop), *Pseudomonas* (Ps), *Arthrobacter* (Ar) and actinomyces (Act) after 7, 30, 60, 90 and 270 days (C – control without diesel oil).
Different superscript letters show significant differences between objects for individual soil enzymes (One way ANOVA, Tukey test, $P = 0.01$).
Error bars represent standard error of the mean for $n = 3$

negative values point to its inhibition. For the total count of organotrophic bacteria and for copiotrophic bacteria, actinomyces and bacteria of the *Pseudomonas* genus, the highest increase were noted on day 30, while counts of oligotrophic bacteria peaked on day 90. DO had an evidently adverse effect on bacteria of the *Arthrobacter* genus. The reduction in their count ranged from 24% on 30 day to 56% on 270 day. The stimulation of the proliferation of actinomyces and bacteria of the *Pseudomonas* genus for the first 30 days was waning during the later period (day 60-270). The counts of organotrophic, oligotrophic and copiotrophic bacteria decreased considerably with time. On day 270, the proliferation of oligotrophic bacteria was inhibited by 18%, while that of organotrophic bacteria (total count) and copiotrophic bacteria was stimulated by 39% and 20%, respectively.

The colony development (CD) index in the soil contaminated with diesel oil was changing during the experiment (Figure 2*a*). For copiotrophic bacteria, it was the highest on days 7 and 30 (42.05 and 40.95) and the lowest was on day 270 (31.75); for organotrophic bacteria, the highest CD was on

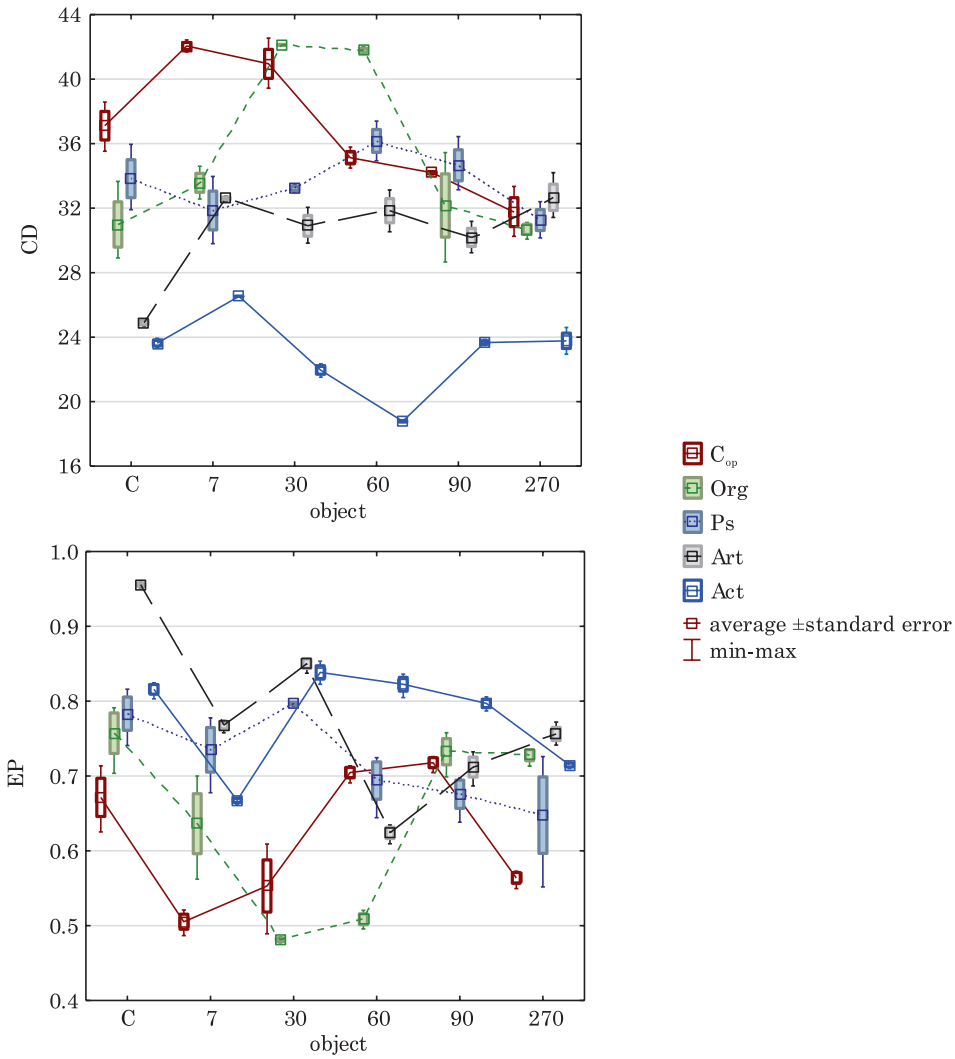

Fig. 2. The colony development (CD) and eco-physiological diversity (EP) indices for bacteria: copiotrophic (Cop), organotrophic (Org), *Pseudomonas* (Ps), *Arthrobacter* (Art) and actinomyces (Act), isolated from soil contaminated with diesel oil (DO) after 7, 30, 60, 90 and 270 days (C – control without diesel oil). Error bars represent standard error of the mean for $n = 3$

days 30 and 60 (42.12 and 41.78), and the lowest one was on day 270 (30.68); for bacteria of the *Pseudomonas* genus, the CD value was the highest on days 60 and 90 (36.17; 34.65) and the lowest on days 270 (31.26), while for bacteria of the *Arthrobacter* genus 270 and actinomyces, it was the highest on day 7 (32.60 and 26.53, respectively). Different CD values indicate a change in the proportions between slowly and rapidly growing bacteria.

The values of the eco-physiological index of bacterial diversity (EP) were inverse to the values of the CD index (Figure 2*a*,*b*), particularly on the days on which diesel oil caused the greatest increase in the CD index. This occurred at the expense of the reduction in eco-physiological diversity. Irrespective of the days of the study, lower values of the EP index were noted in the soil contaminated with DO than in the non-contaminated soil. An exception was the case of actinomyces, whose eco-physiological diversity was only disturbed on day 270 of the study (EP 0.71).

Our analysis of the protein profile of microorganisms and their comparison to a model set of proteins of reference microorganisms, enabled us to identify species of the isolated bacteria (Figure 3). Bacteria belonging to the

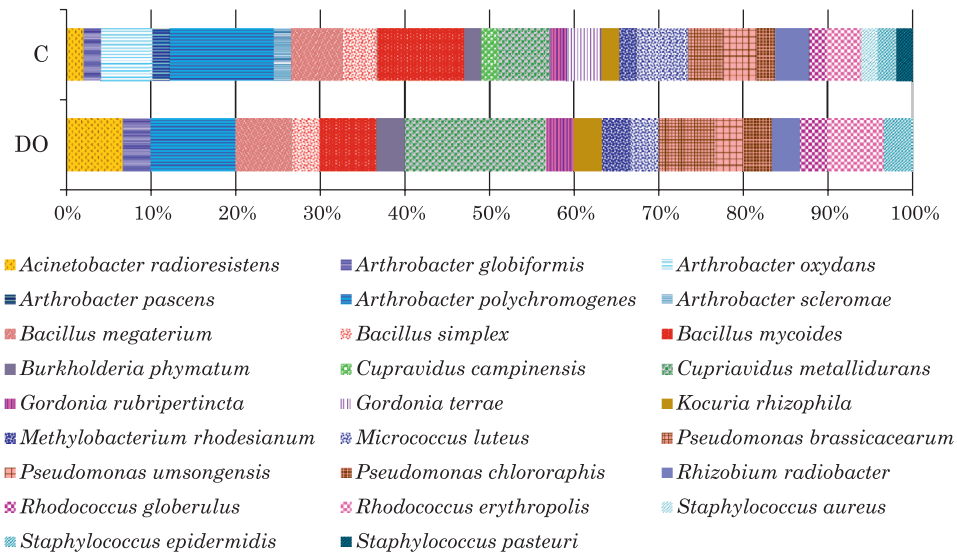

Fig. 3. Percentages of bacterial species isolated from soil unpolluted (C) and diesel oil polluted (DO) following the MALDI-TOF method

genera of *Acinetobacter, Arthrobacter, Bacillus, Burkholderia, Cupriavidus, Gordonia, Kocuria, Methylobacterium, Micrococcus, Pseudomonas, Rhizobium, Rhodococcus* and *Staphylococcus* were isolated from both the non-contaminated soil and soil contaminated with diesel oil. It is noteworthy that although the same genera were isolated from both soils, significantly fewer species were found in the soil contaminated with DO.

Bacterial functional diversity

The Average Well-Color Development (AWCD) index in the soils contaminated with diesel oil ranged from 0.116 on day 270 to 0.748 on day 90 (Figure 4*a*). Initially, from day 7 to day 60, it decreased to 0.641-0.649, then it increased significantly on day 90 and dramatically decreased on day 270. For 90 days, the number of the carbon substrates used, which were 31 organic

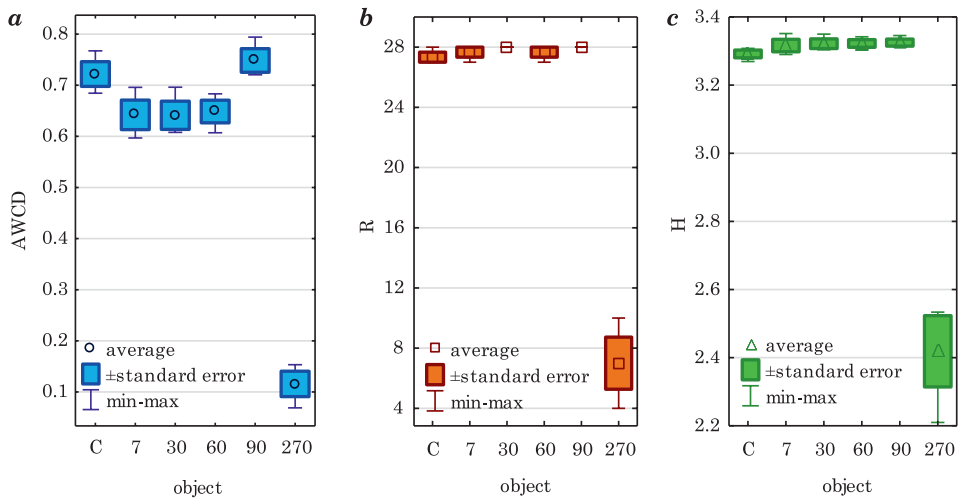

Fig. 4. The biodiversity indices of bacterial community of soil polluted with diesel oil, depending on the duration of soil exposure to diesel oil: 7, 30, 60, 90, 270 days, C – control without diesel oil, following Biolog ECO MicroPlates®, AWCD – Average Well-Color Development (a), R – Richness of carbon substrates (b), H – Shannon-Weaver index (c)

compounds, ranged from 27 to 28 (Figure 4*b*). Only on day 270, it decreased to 7 sources. Certainly, this result caused a significant decrease in the Shannon-Weaver diversity index (Figure 4*c*). The value of this index was almost unchanged for 90 days and ranged from 3.29 to 3.32, but on day 270. it decreased to 2.42. These results are visualised in the thermal map (Figure 5), where the breakdown of the use of carbon sources on day 270 is presented, and in Figures 6 and 7, which document the AWCD values of particular groups of organic compounds. The current study showed (Figure 7) that the best carbon sources for bacteria are carbohydrates and carboxylic acids and acetic acid, while amino acids and polymers are much worse and amines and amides are the worst. The bacteria utilise the best D-Mannitol (D2), Pyruvic Acid Methyl Ester (B1), D-Xylose (B2), L-Serine (D4), Tween 80 (D1), L-Asparagine (B4), D-Glucosaminic Acid (F2), Itaconic Acid (F3) and 4-Hydroxy Benzoic Acid (D3) – Table 1. For these compounds, the OD value at 48 h was higher than 0.75. The least degraded compounds were *β*-Methyl--D-Glucoside (A2) and D-Galactonic Acid *γ*-Lactone (A3). For these compounds, the OC at 48 h was 0.03-0.04.

Enzyme activity

Diesel oil increased the activity of soil hydrolases, which is indicated by the positive values of the IF_{DO} index (Figure 8). The highest values of the IF_{DO} were noted on day 30, and the lowest ones were on day 270. The activity of urease (average IF_{DO} of 2.84) and alkaline phosphatase (average IF_{DO} of 2.53) was stimulated most strongly, while the activity of acid

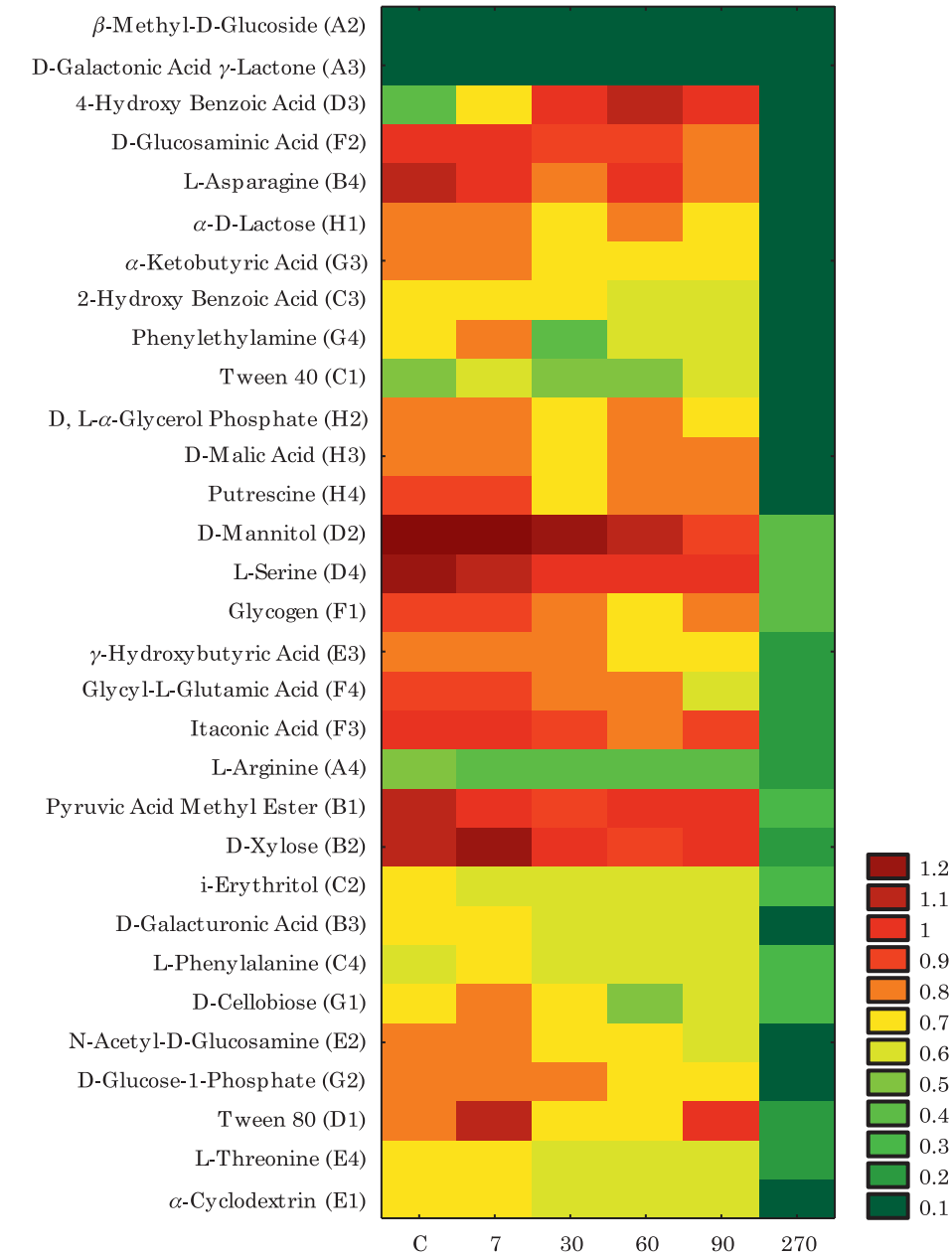

Fig. 5. Utilisation of different carbon sources located on Biolog ECO MicroPlates® by a bacterial community of soil polluted with diesel oil depending on the duration of soil exposure to diesel oil: 7, 30, 60, 90, 270 days, C – control without diesel oil

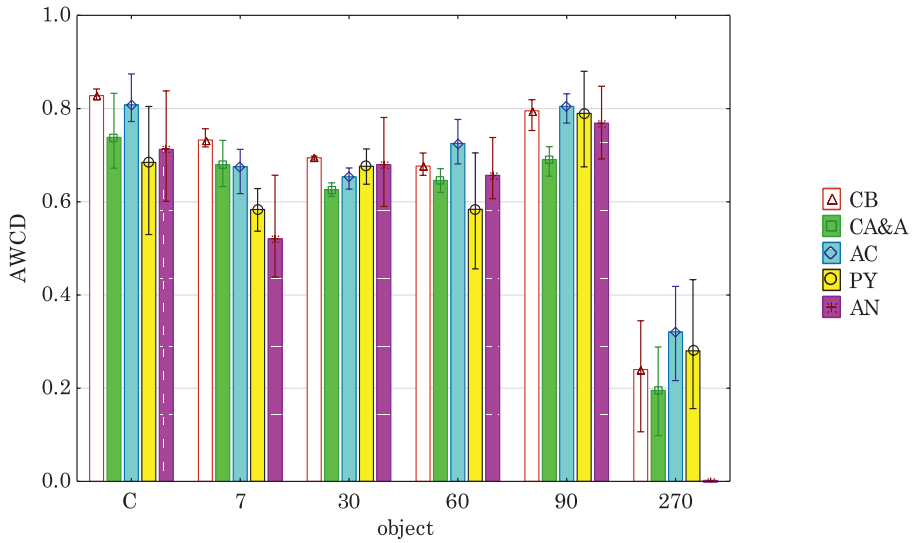

Fig. 6. Utilisation of different carbon sources groups located on the Biolog ECO MicroPlates® by bacterial community of soil polluted with diesel oil, depending on the duration of soil exposure to diesel oil: 7, 30, 60, 90, 270 days, C – control without diesel oil, CB – Carbohydrate, CA&A – Carboxylic Acids & Acetic Acids, AC – Amino Acids, PY – Polymers, AN – Amines/ Amides, AWCD – Average Well-Color Development

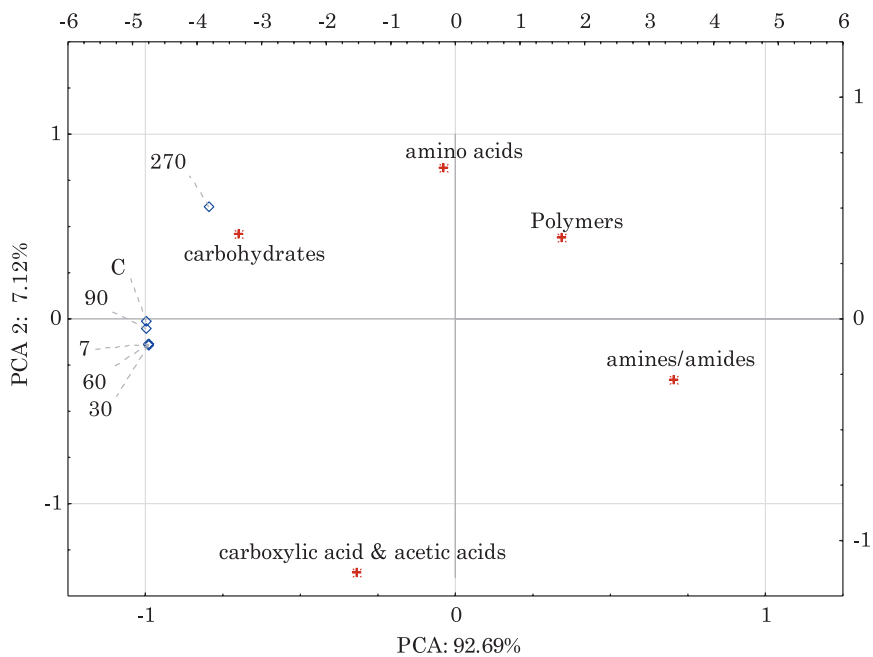

Fig. 7. Utilisation of different carbon sources groups by a bacterial community in soil polluted with diesel oil depending on the duration of soil exposure to diesel oil and carbon sources: 7, 30, 60, 90, 270 days, C – control without diesel oil, based on the Biolog ECO MicroPlates®

Table 1

Use of different carbon sources by bacteria incubated on Biolog ECO MicroPlate®

IOD > 0.75		IOD 0.51-0.75				IOD 0.25-0.50		IOD < 0.25	
C	IOD	C	IOD	C	IOD	C	IOD	C	IOD
D2	1.14	*F1*	0.75	*H3*	0.65	*A4*	0.36	*A2*	0.03
B1	0.94	*G2*	0.72	*H1*	0.64	*C1*	0.50	*A3*	0.04
B2	0.99	*H2*	0.72	*G1*	0.63				
D4	0.96	*E3*	0.72	*B3*	0.60				
D1	0.91	*H4*	0.71	*C2*	0.58				
B4	0.84	*F4*	0.70	*C3*	0.58				
F2	0.85	*G3*	0.67	*C4*	0.58				
F3	0.83	*E2*	0.65	*E1*	0.58				
D3	0.79			*E4*	0.58				
				G4	0.56				

C – symbol of carbon source on Biolog ECO MicroPlate®; IOD – intensity of optical density
Degree of use of a carbon source: IOD < 0.25 – low; IOD = 0.25-0.50 – medium;
IOD = 0.51-0.75 – good; IOD > 0.75 – high

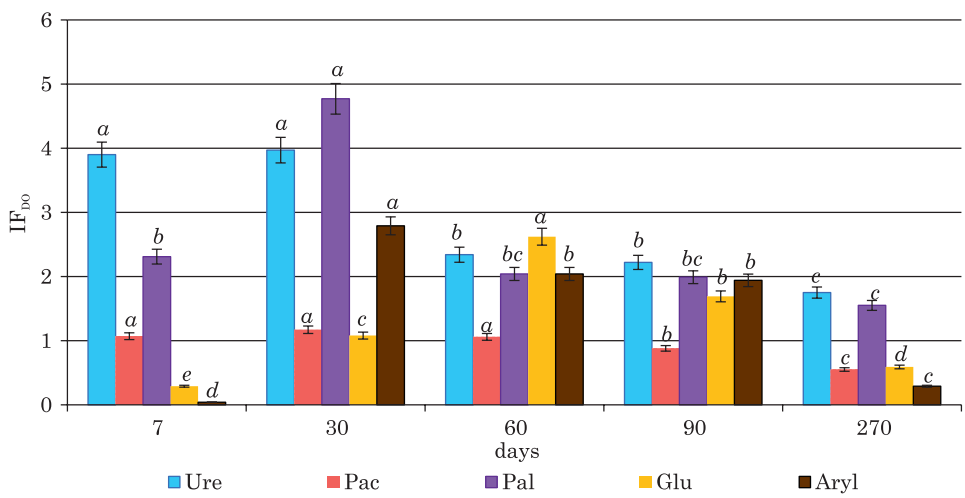

Fig. 8. Effect of diesel oil (IF_{DO}) on the activity of urease (Ure), acid phosphatase (Pac), alkaline phosphatase (Pal), β-glucosidase (Glu) and arylosulfatase (Aryl) after 7, 30, 60, 90 and 270 days. Different superscripts show significant differences between objects for individual soil enzymes (One way ANOVA, Tukey test, $P = 0.01$).
Error bars represent standard error of the mean for $n = 3$

phosphatase was the least stimulated by DO (average IF_{DO} of 0.95). Indirect stimulation by diesel oil was noted for arylsulphatase (average IF_{DO} 1.42) and β-glucosidase (average IF_{DO} of 1.25).

Hydrocarbon degradation

The degradation of hydrocarbons in the soil depended on their type and the duration of the experiment (Figure 9). The longer the study lasted,

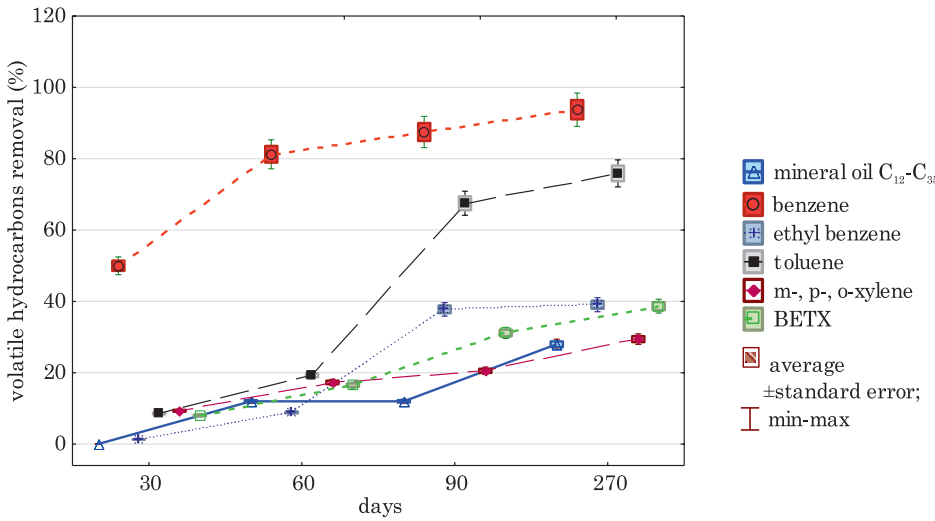

Fig. 9. Hydrocarbon removal (%) from soil contaminated with diesel oil after 30, 60, 90 and 270 days

the more hydrocarbons were transformed. Benzene was removed from the soil most rapidly and in the greatest amounts, while mineral oil (C_{12}-C_{35}) and m-, p-, and o-xylenes (C8) were removed in the smallest amounts. After 270 days, there was 94% less benzene (C6) in the soil, 76% less toluene (C7), 39% less ethylbenzene (C8), 29% less xylene isomers and 28% less mineral oil. Despite the relatively high differences in the degradation rate of particular hydrocarbons, monoaromatic hydrocarbons (BTEX) were relatively stable, as the BTEX content decreased by only 39% after 270 days.

DISCUSSION

Organic compounds contained in Ekodiesel Ultra diesel oil are a source of carbon and energy for most bacteria, even though some of them are highly toxic. Therefore, an increase in the count of all bacteria except those of the *Arthrobacter* genus could be noted, particularly up to day 30 since the contamination of soil with the product, in which our results were similar to the ones reported by Chaineau et al. (2005), in addition to the increasing activity of enzymes. The same trend regarding the effects of diesel oil (DO) on soil enzymes was observed by del Carmen Cuevas-Díaz et al. (2017), Kucharski and Jastrzębska (2006) and Wyszkowska et al. (2006). The adverse effect of DO on bacteria of the *Arthrobacter* genus was also confirmed by results

of the identification analysis based on MALDI-TOF mass spectrometry. In this case, fewer species of this genus were isolated from the soil contaminated with DO than from the non-contaminated soil. This probably results from the deteriorating physicochemical conditions in the soil (Griffiths, Philippot 2013, Kauppi et al. 2011, Semrany et al. 2012), to which particular groups of bacteria responded variously, for the number of actinomyces and bacteria of the *Pseudomonas* genus increased under the influence of DO only up to day 30, while the total number of organotrophic bacteria and copiotrophic bacteria, while decreasing with time, remained detectable up to day 270. For oligotrophic bacteria, their number significantly decreased during this last period and it is a highly adverse phenomenon, because this group of bacteria is relatively stable in the soil, in contrast to copiotrophic bacteria, which respond vigorously to the inflow of new organic substance (Fierer et al. 2007).

The CD index proves that during the first period after contamination of soil with DO, r-strategy bacteria, i.e. the rapidly growing ones, were dominant, but with time they were displaced by K-strategy bacteria, i.e. the more slowly growing organisms (Fierer et al. 2007). On the other hand, the EP index proves that their eco-physiological diversity decreased under the influence of DO. The adverse effect of DO on bacterial diversity is a long-term phenomenon, which is proven by the lowest values of the following indices during that period: Average Well-Color Development (AWCD), Substrate Richness (R), and Shannon-Weaver (H). Attention was also drawn to this issue by Shahsavari et al. (2013). This study confirms the negative influence of DO on bacterial diversity through the differences in the bacterial species isolated from DO contaminated and non-contaminated soil, to the advantage of the former. *Arthrobacter oxydans, Arthrobacter pascens, Arthrobacter scleromae, Cupravidus campinensis, Gordonia terrae, Staphylococcus aureus, Staphylococcus pasteuri* were not isolated from contaminated soil, in contrast to non-contaminated one. This proves that the above species did not adapt to the soil environment contaminated with DO, while those which thrived well (Figure 3) can be used in bioaugmentation (Tyagi et al. 2011). These bacteria include ones that belong to *Pseudomas* (*P. brassicacearum, P. umsongensis* and *P. chlororaphis*), which were isolated from both DO contaminated soil and non-contaminated soil. Fatima et al. (2015) also noted that bacteria of the *Pseudomonas* genus could efficiently decompose crude oil. Moreover, other bacteria could also effectively degrade petroleum products, including *Acinetobacter, Bacillus* and *Staphylococcus* (Fatima et al. 2015, Mnif et al. 2015). In the current study, most bacteria isolated from the soil contaminated with DO were those of the genera *Arthrobacter* (13%), *Bacillus* (17%), *Pseudomonas* (13%) and *Rhodococcus* (10%). It should be therefore assumed that they can be effective in biodegradation of organic compounds included in the composition of DO. In soil subjected to the pressure of Ekodiesel Ultra diesel oil, the pollutant had a similarly adverse effect, not only on the functional diversity of bacteria but also on the functional diversity of fungi (Borowik et al. 2017*a*).

The disturbance of bacterial diversity caused by DO is attributed to the deteriorating physical properties of soil, direct impact of DO components on bacteria, change in their structure, and formation of intermediate compounds during their biodegradation (Chaineau et al. 2005). The most stable organic compounds were gasoline fractions, mineral oil and xylene isomers, while the amounts of benzene and toluene decreased rapidly. In a study by Xu and Lu (2010) of the biodegradation of crude oil hydrocarbons under the conditions of biostimulation and bioaugmentation, 26% to 61% of hydrocarbons were removed over a period of 84 days. Even better results of bioaugmentation were obtained by Suja et al. (2014). The efficiency of hydrocarbon removal reached 97%. In this own study, which lasted for 270 days, but did not involve either biostimulation or bioaugmentation, the efficiency of hydrocarbon removal was considerably lower.

CONCLUSIONS

1. The contamination of soil with Ekodiesel Ultra diesel oil has a long-term effect on the soil environment.

2. Certain groups of organic compounds included in the composition of DO are relatively resistant to biodegradation. They include gasoline fractions (C_6-C_{12}), mineral oil (C_{12}-C_{35}) and xylene isomers found in the composition of BTEX.

3. Ekodiesel Ultra diesel oil increased the counts of most groups of microorganisms as well as the activity of urease, acid phosphatase, alkaline phosphatase, β-glucosidase and arylsulphatase, although it had an adverse effect on bacteria of the *Arthrobacter* genus.

4. The catabolic profile of the bacteria, determined on Biolog ECO MicroPlates®, the eco-physiological profile of the bacteria (EP index) and the identification using MALDI-TOF mass spectrometry MS prove that DO causes changes in the structure of bacteria, namely their diversity decreases and the mutual relationships between the r-strategy and K-strategy bacteria change.

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