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# The Effect of Petroleum-Derived Substances and Their Bioremediation on Soil Enzymatic Activity and Soil Invertebrates

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**Abstract:** Petroleum-derived substances (PDSs) as main pollutants of the natural environment can negatively affect the microbiological, biochemical, and biological properties of agricultural soils and, consequently, plant production. The present study aimed to determine the after-effect of PDSs such as petrol, used engine oil, and diesel fuel on the activity of selected soil enzymes (phosphatase, dehydrogenase, and urease) and on the occurrence of soil invertebrates. Moreover, changes in the analyzed parameters in response to bioremediation of the polluted soil by using ZB-01 preparation were investigated. The field experiments were performed four- and five-years post contamination. The results showed that even after five years, PDSs significantly modified the activity of soil enzymes; however, this effect was often varied, depending on the pollutant, enzyme, and time after soil contamination. Dehydrogenase seems to be a good indicator of soil contamination with PDSs, particularly diesel fuel. Engine oil and diesel fuel limited still the occurrence of soil invertebrates, particularly Collembola from the families Hypogastruidae, Isotomidae, and Entomobryidae, even after four and five years of contamination. This finding suggests the usefulness of these organisms in assessing soil pollution and in monitoring the progress of bioremediation. The effect of ZB-01 biopreparation on the activity of selected enzymes was varied. Its effect on the occurrence of soil invertebrates was usually beneficial, which was evident in diesel fuel-contaminated soil.

**Keywords:** agricultural soil contamination; bacterial degradation; petroleum products; soil fauna; soil enzymes



**Citation:** Gospodarek, J.; Rusin, M.; Barczyk, G.; Nadgórska-Socha, A. The Effect of Petroleum-Derived Substances and Their Bioremediation on Soil Enzymatic Activity and Soil Invertebrates. *Agronomy* **2021**, *11*, 80. https://doi.org/10.3390/agronomy11010080

Received: 28 October 2020
Accepted: 29 December 2020
Published: 2 January 2021



## 1. Introduction

Soil biological activity, including enzyme activity, is affected by a range of environmental parameters and perturbations; therefore, it is used to assess disturbed soil [1,2]. Petroleum-derived substances (PDSs) such as diesel oil, petrol, and engine oil can negatively affect the biochemical processes of soil and change soil fertility, which is crucial for plant production [3–6]. The pollutants contained in these substances reduce microbial activity; however, as these substances are mixtures of different hydrocarbons, they also serve as a carbon source for some microorganisms [7]. Numerous studies have shown that the determination of soil enzymatic activity can be used to assess soil pollution with petroleum-derived hydrocarbons [8–10], which have been one of the main pollutants of the natural environment in recent years. Agricultural soils can be affected by different sources of PDSs: machinery use, application of insecticides based on petroleum oil, fertilization of soil using municipal waste composts, discharge of used oils, spills from storage containers, damage to pipelines, road disasters, or industry [11,12].

Among the various enzymes, dehydrogenases are the best ones to determine soil microbial activity. They reflect the level of physiologically active microorganisms in the soil

and thus provide correlative information on biological activities and microbial population of the soil. They also reflect the degree of organic matter decomposition and nutrient availability in the soil. Therefore, the change in dehydrogenase activity can be a reliable indicator to determine changes in soil fertility resulting from the biological oxidation of organic matter. Dehydrogenases play a role in the biological oxidation of organic matter in the soil by catalyzing hydrogen transfer from organic substrates to inorganic acceptors. The activity of dehydrogenases reflects the rate of transformations occurring in the soil [13–16]. Phosphatases are enzymes involved in soil phosphorus transformation, while urease catalyzes the hydrolysis of urea to ammonia and an ammonium ion [17]. Although both phosphatases and urease are less commonly used to determine the effect of pollution on the activity of soil enzymes, many authors stress their importance because they are involved in macronutrient cycles and may provide some insights into the metabolic capacity of the soil, which could enable to assess the potential for transformation of specific sources of energy or nutrients [15]. In addition to viable counts of soil microbiota, biomass, and respiration, enzyme activities have been considered as indices for the biological assessment of soil ecotoxicology [7,18,19]. Enzyme activities are also a useful tool to assess the effectiveness of remediation processes [20].

The biological balance of the soil affected by the toxicity of petroleum compounds has been assessed by ecotoxicological assays using soil invertebrates alongside plants and bacteria [10]. PDSs are particularly harmful to soil invertebrates as these substances can permeate into their bodies. They subsequently accumulate in fat tissues and are released into the blood, and they damage the nervous and reproductive systems by precipitating in body fluids [21]. The toxic potential of hydrocarbons of PDSs results from their ability to bind to nonpolar components of biological membranes, thereby causing their disintegration [22,23]. Therefore, the species structure of invertebrates is often found to be altered in polluted environments, for example, being depleted and showing unusually large numbers representing some dominant species, which indicate modified seasonal population dynamics or dispersion changes. Many studies have confirmed the adverse effects of PDSs on edaphic and epigeic invertebrates [24–27]. It is also noteworthy that soil fauna often shows higher "sensitivity" as a soil PDS pollution indicator than chemical analyzes [10,28,29]. Among soil invertebrates, nematodes (Nematoda), springtails (Collembola), arachnids (Arachnida), and potworms (Enchytraeidae) are often used as bio-indicators of soil pollution with xenobiotics, given the essential role of these organisms in numerous ecological processes, particularly in the nutrient cycle and organic matter decomposition. These organisms respond relatively quickly to all changes in the soil structure and in physical and chemical composition caused by the presence of pollutants in the environment. Furthermore, they occur in large numbers in many environments and are well explored in both taxonomic and ecological terms, which makes them good indicators of soil environment quality [30,31].

Many soil microorganisms (mainly bacteria and fungi) transform petroleum-derived hydrocarbons into nontoxic compounds or perform complete mineralization producing simple nonorganic substances, such as carbon dioxide and water. This natural microbial activity is used in the process of bioremediation, which is one of the most efficient methods of soil purification from petroleum products [15].

Although scientific literature provides information on the effects of PDSs on the activity of both soil enzymes and soil fauna, this information usually pertains to soil environmental changes occurring soon after contamination [2,8,20,32]. There is a lack of data on more long-term consequences of these pollutants. There is also less data on the effect of bioremediation of soil contaminated with petroleum-derived products on the activity of soil enzymes. The purpose of the present study was to determine the aftereffect (i.e., four- and five-years post contamination) of PDSs, i.e., petrol, used engine oil, and diesel fuel, on the activity of selected soil enzymes (phosphatase, dehydrogenase, and urease) and on the occurrence of soil invertebrates. Moreover, changes in the analyzed parameters in response to bioremediation of polluted soil by using ZB-01 preparation were investigated. The present research also aimed at assessing the usefulness of the

abovementioned enzymes and groups of invertebrates for application as bioindicators of soil pollution by PDSs and for determining the rapidness of bioremediation processes. Our studies are related to ERA (Ecological Risk Assessment)—a multistep process aimed to collect and analyze environmental exposure and effect data to estimate the risk of contamination of ecosystems (enzyme activity and occurrence of soil invertebrates for the characterization of the ecological line of evidence at the screening level of ERA) [33,34].

## 2. Materials and Methods

### *2.1. Experimental Setup*

The field study area was in the Experimental Station of the University of Agriculture in Krakow, located in Mydlniki near Krakow (Poland; 50.0815° N, 19.84730° E). The experiment was planned to be long-lasting. Because it acquired a large investment at the time of establishment (cost of containers, works related to setting up the experiment, which were done manually) it had a wide, comprehensive scope, including the influence of PDSs and bioremediation on terrestrial, soil and phytophagous invertebrates, as well as on the condition of the soil and wild and cultivated plants. The results presented in this manuscript are part of this wide project. In November 2009, indigenous soil (loamy sand, detailed characteristics of the soil are given in a previous article [29]), was placed in special containers of 1 $m^3$ volume, retaining the natural arrangement of layers. The containers were sunk in the ground so that their upper edge was at the same level as the surface of the soil. The description of the containers and their arrangement in the experimental field is presented in our previous article [29]. The soil in the containers was left for eight months without any intervention to regain its natural biological functions. In June 2010, the soil surface was artificially contaminated with petrol (P), used engine oil (EO), and diesel fuel (DF) in the quantity of 6000 mg of petroleum product per kg of soil dry mass in the container (i.e., typical for medium-contaminated soils) by pouring it on the soil. The noncontaminated soil in identical containers served as a control. After one week, soil in half the number of containers was subjected to bioremediation by adding ZB-01 biopreparation. The biopreparation contained selected prokaryotic organisms, mainly bacteria such as *Stenotrophomonas*, *Pseudomonas*, *Moraxella*, *Acinetobacter*, *Alcaligenes*, *Ochrobactrum*, *Comamonas*, *Burkholderia*, *Corynebacterium*, and *Oligella*, and was specially prepared for this experiment. The ZB-01 treatment was followed by fertilization using the "Azofoska" compound fertilizer. The method of applying ZB-01 and "Azofoska" has been described in our previous article [29]. The usefulness of the proposed ZB-01 microbial biopreparation for remediating soils contaminated with petroleum products was confirmed earlier [29,35]. After one year, the bioremediation treatment was repeated. For three subsequent years, the soil in the containers was left undisturbed. The experiment was established in four replications in line with the randomized block method (32 containers in total). The effects of PDSs and ZB-01 on soil invertebrates were measured periodically from the day of contamination and the results of these previous data (from years 2010–2013) have been already published [29,36]. After the three years of conducted research, we could see that the effect was still visible, so we decided to continue monitoring also in 2014 and 2015. During this first period post contamination (2010–2013) the soil was maintained without plants and cultivation. Then we decided to try to use the contaminated soil as a place for cultivation of plants. The main reason for that was that we wanted to see the effect of contaminated (and bioremediated) soil not only on soil invertebrates but also on phytophagous insects, feeding on these cultivated plants. Moreover, we wanted to check if the soil after these three years from the contamination could be used for agricultural purposes. Winter wheat was grown on the studied soil in 2014 (i.e., four years post contamination), while broad beans were grown in 2015 (i.e., five years post contamination). The data on the impact of PDSs and ZB-01 on these two plants and the herbivores feeding on them have been already published [6,37]. However, important part of this comprehensive evaluation of the effect of PDSs contamination, bioremediation and cultivation of plants was also assessment

of soil enzymes activity and the condition of soil invertebrates. This is the content of presented manuscript.

An analysis of the content of PDSs in the soil was conducted for monitoring purposes four and five years after contamination. The level of $C_6$-$C_{12}$ hydrocarbons was measured in accordance with the standard PN-ISO 22155: 2013, while $C_{12}$-$C_{36}$ mineral oil levels were measured in accordance with the standard PN-EN ISO 16703: 2011. Four years after contamination (in 2014) pH values and total carbon content were measured. pH measurement (in $H_2O$ and KCl) was conducted using the potentiometric method, while total carbon content was determined using the high-temperature combustion method with IR detection according to PN-ISO 10694:2002. For the initial soil, the analysis of total carbon content was performed according to PB-01, ed.4.

### *2.2. Soil Enzymatic Activity*

Naturally moist "fresh" soil was used for the analyses and was sampled in early July 2014 and 2015, i.e., four and five years after contamination. The soil was sampled from three regularly distributed sites in each container. Subsequently, the soil was passed through a 2 mm mesh sieve and stored at 4 °C prior to analysis. Acid and alkaline phosphatase activity was measured using the method of Schinner et al. [38]. The concentration of p-nitrophenol (p-NP), which is a product of a phosphatase-catalyzed reaction, was measured by spectrophotometry at the wavelength of 400 nm. Phosphatase activity was expressed as µg p-nitrophenol (p-NP) g d.m.$^{-1}$ h$^{-1}$. The acetone extraction of triphenylformazan (TPF), which is a product of a dehydrogenase-catalyzed reaction, was performed and its concentration was measured by spectrophotometry at the wavelength of 546 nm. Dehydrogenase activity was expressed as µg triphenylformazan (TPF) g d.m.$^{-1}$ 16 h$^{-1}$ [38]. Urease activity was estimated using a colorimetric assay by determining the amount of ammonia produced after the hydrolysis of urea (10% solution) at the wavelength of 630 nm [39]. Urease activity was expressed as µg N g d.m.$^{-1}$ h$^{-1}$. The value of ACR (enzyme activity change ratio) [40] was calculated for each contaminated site to more accurately determine the influence of PDSs and the applied bioremediation process by using the following equation:

$$ACR = \frac{A_h - A_c}{A_c} \times 100\% \quad (1)$$

where:

$A_h$—soil enzymatic activity in the contaminated site,
$A_c$—soil enzymatic activity in the control site.

### *2.3. Soil Invertebrates*

Soil samples used for the study were collected five times from the top layer (depth 0–15 cm) and at the following time points: late September and mid-November 2014, and late June, mid-August, and early October 2015. A split corer was used for sample extraction (according to the ISO standard 23611-2-2006 [41]). The diameter and depth of a sample were 5 cm and 15 cm, respectively. The volume of a sample was 294.52 cm$^3$. Soil was sampled separately from three evenly distributed places in each container. The collected samples were placed in plastic tubes and immediately transported to the laboratory. The extraction of soil fauna was performed by a behavioral method using a Tullgren apparatus [40]. Once deposited in the funnel, the soil was illuminated and heated for seven days by using a 25 W bulb. The obtained invertebrates were pooled and stored in 95% ethanol. Subsequently, their numbers and taxonomic ranks were analyzed using a binocular magnifier. Collembola (given their previously determined potential value in assessing the state of PDS-polluted soils [41]) were identified to the family level by using relevant keys [42,43].

### 2.4. Statistical Analysis

The obtained results were analyzed and checked for normality (Shapiro–Wilk test with Lilliefors correction) and equality of variance (Levene's test), and when necessary, the data were log transformed. The significance of differences between the mean values was tested by one-factor variance analysis (STATISTICA 10.0 software), and the mean values were differentiated by Fisher's LSD test at $p < 0.05$. CANOCO 4.5 was used to perform Principal Component Analysis (PCA) [44]. PCA assessed the similarities and relationships between soil enzyme activity, bioremediation, and soil fauna occurrence. The data were log transformed [Y = log(Y + 1)].

## 3. Results

### 3.1. Selected Soil Parameters: Hydrocarbon Content, pH, and $C_{total}$ Content

The data on hydrocarbon content, soil pH, and $C_{total}$ content have already been published [6,37]. To give a broader background to the results on soil enzyme activity and soil fauna, we have presented here selected information. Four years after contamination, the level of $C_6$-$C_{12}$ hydrocarbons was below 0.8 mg $kg^{-1}$ in all soil samples; however, $C_{12}$-$C_{36}$ mineral oil levels in EO and DF-contaminated soils were still multiple times higher than those in uncontaminated soil (1000 mg $kg^{-1}$ and 750 mg $kg^{-1}$, respectively, in relation to values below 6 mg $kg^{-1}$ in the control soil). The use of ZB-01 biopreparation contributed to a 2- to 3.5-fold reduction in the levels of these substances (530 mg $kg^{-1}$ in EO R and 210 mg $kg^{-1}$ in DF R). $C_{12}$-$C_{36}$ levels in P-contaminated soil were 12 mg $kg^{-1}$ in P 0R and 7 mg $kg^{-1}$ in P R [6]. In 2015, $C_{12}$-$C_{36}$ hydrocarbon levels in the sections contaminated with oils were still higher than those in the control samples, but these levels had decreased by approximately 40% compared to those in the previous year [37]. The analysis of soil pH in 2014 revealed that the PDSs caused an increase in soil acidification, which was particularly evident for EO-contaminated soil, in which the pH value in $H_2O$ was nearly 1 scale lower than that in the initial soil (6.01 and 7.12 for EO 0R and initial soil, respectively). The values of pH in $H_2O$ in the remaining samples ranged from 6.16 (DF 0R) to 6.98 (C 0R). The pH values in KCl for samples of contaminated soil ranged from 4.93 (DF 0R) to 5.78 (P R) versus 6.45 in the initial soil and 6.12 in C 0R treatment. Both EO and DF led to an increase in the levels of total carbon in the contaminated soil compared to those in the initial soil (1.70% and 1.17% for EO 0R and DF 0R treatment, respectively, versus 1.04% for the initial soil and 0.97% for C 0R treatment).

### 3.2. Soil Enzyme Activity

#### 3.2.1. Alkaline Phosphatase, Acid Phosphatase, Dehydrogenase, and Urease

Four and five years after soil contamination (i.e., in 2014 and 2015), EO caused a significant decrease in acid and alkaline phosphatase activity as compared to that in the control soil (Figures 1 and 2). However, in 2014, it contributed to an increase in dehydrogenase (by 19.2 μg TPF g d.m.$^{-1}$ 16 $h^{-1}$) (Figure 3) and urease (by 45.3 μg N g d.m.$^{-1}$ $h^{-1}$) (Figure 4) activity. DF and P generally reduced the activity of the analyzed soil enzymes in both years of research.

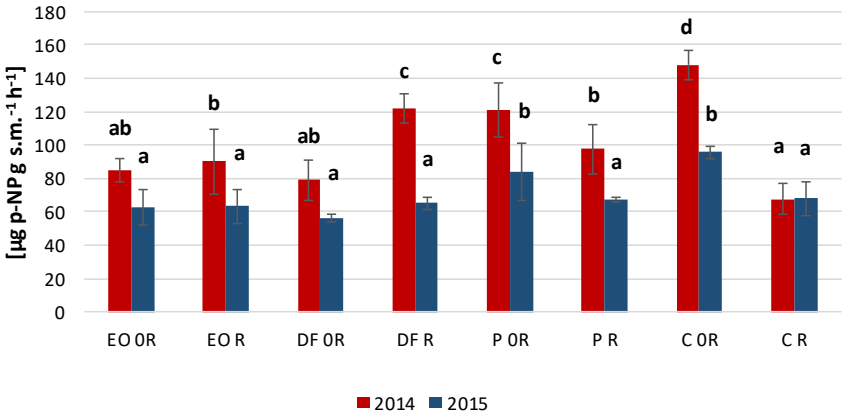


**Figure 1.** Alkaline phosphatase activity after four (2014) and five (2015) years from soil contamination and the use of ZB-01. EO—soil contaminated with engine oil, DF—soil contaminated with diesel fuel, P—soil contaminated with petrol, C—control soil, 0R—without bioremediation, R—with bioremediation. Mean values marked with the same letters for each year separately do not differ significantly according to the LSD test at $p < 0.05$. Vertical bars show mean ± SE.

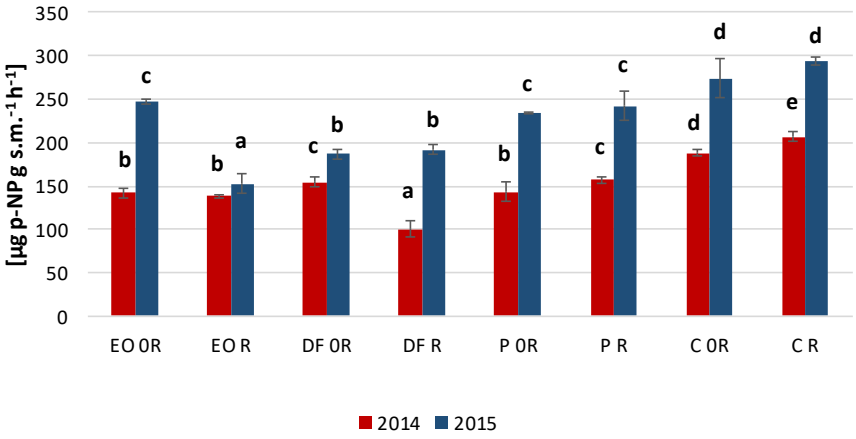


**Figure 2.** Acid phosphatase activity after four (2014) and five (2015) years from soil contamination and the use of ZB-01. Mean values marked with the same letters for each year separately do not differ significantly according to the LSD test at $p < 0.05$. Symbols are the same as those given in Figure 1. Vertical bars show mean ± SE.

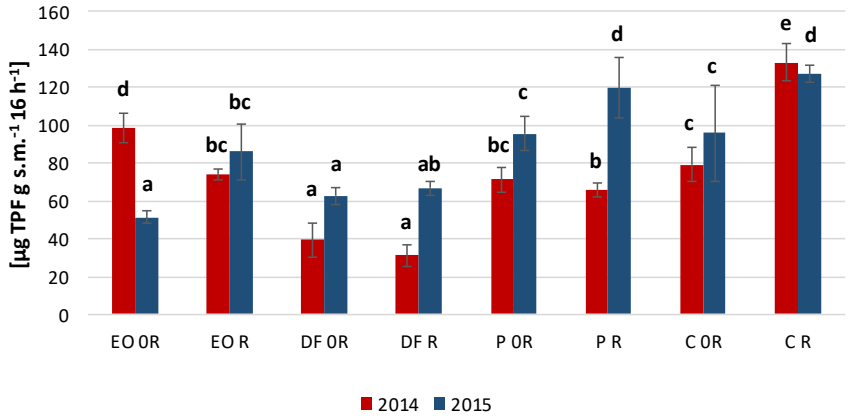


**Figure 3.** Dehydrogenase activity after four (2014) and five (2015) years from soil contamination and the use of ZB-01. Mean values marked with the same letters for each year separately do not differ significantly according to the LSD test at $p < 0.05$. Symbols are the same as those used in Figure 1. Vertical bars show mean ± SE.

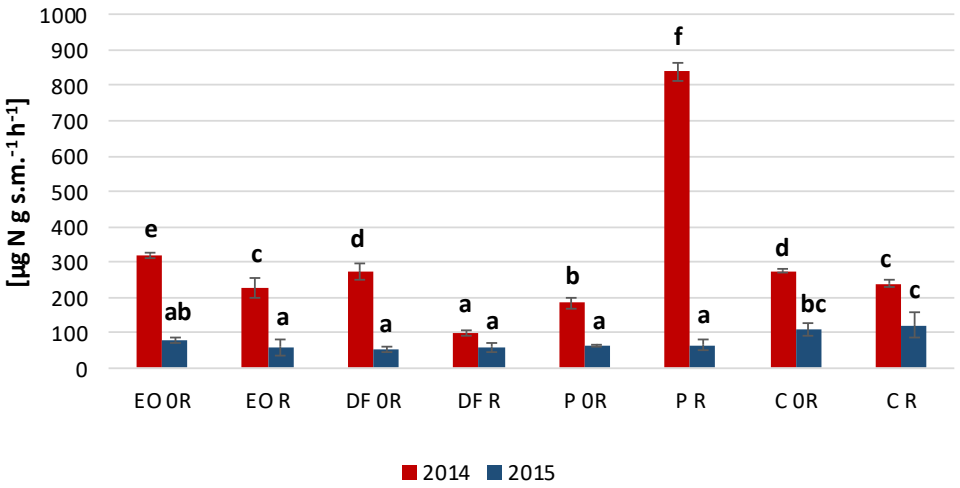


**Figure 4.** Urease activity after four (2014) and five (2015) years from soil contamination and the use of ZB-01. Mean values marked with the same letters for each year separately do not differ significantly according to the LSD test at $p < 0.05$. Symbols are the same as those used in Figure 1. Vertical bars show mean ± SE.

The effect of ZB-01 biopreparation on the activity of selected soil enzymes was varied and depended on the type of contamination, the type of enzyme analyzed, and the year of study. The biopreparation applied to EO-contaminated soil led to reduced dehydrogenase and urease activity four years after soil contamination as compared to that in the control soil. On the other hand, it caused an increased dehydrogenase activity after five years (by nearly 35 µg TPF g d.m.$^{-1}$ 16 h$^{-1}$). In the DF-contaminated soil, the biopreparation caused an increase in alkaline phosphatase activity four years after soil contamination by approximately 35%, while also causing a decrease in acid phosphatase and urease activity. The application of the biopreparation to P-contaminated soil decreased alkaline phosphatase activity four and five years after soil contamination by nearly 20% and increased urease activity, but only in 2014. In the control section, the biopreparation usually increased the activity of the analyzed soil enzymes, except alkaline phosphatase.

### 3.2.2. ACR Indicator

Four years from the moment of soil contamination, EO in the sections without biopreparation use contributed to an increased dehydrogenase and urease activity, which was evident from the positive ACR values (higher activity than that in the control soil), while also lowering phosphatases activity, as indicated by the negative values of the analyzed ratio (Table 1). In the second year, soil enzymatic activity was found to decrease due to the presence of PDSs in the soil (both in the sections with biopreparation use and those without it).

**Table 1.** Values of the enzyme activity change ratio (ACR) indicator after four (2014) and five (2015) years from soil contamination and the use of ZB-01.

| Treatment | Alkaline Phosphatase | | Acid Phosphatase | | Dehydrogenase | | Urease | |
|---|---|---|---|---|---|---|---|---|
| | 2014 | 2015 | 2014 | 2015 | 2014 | 2015 | 2014 | 2015 |
| EO 0R | −42.77 $^{a*}$ | −34.57 $^{a}$ | −24.45 $^{c}$ | −9.65 $^{d}$ | 24.28 $^{d}$ | −46.30 $^{ab}$ | 16.50 $^{d}$ | −27.61 $^{b}$ |
| EO R | 33.53 $^{b}$ | −6.70 $^{b}$ | −33.27 $^{b}$ | −47.91 $^{a}$ | −44.37 $^{b}$ | −32.60 $^{b}$ | −4.60 $^{c}$ | −51.63 $^{a}$ |
| DF 0R | −46.60 $^{a}$ | −41.41 $^{a}$ | −17.89 $^{d}$ | −31.95 $^{b}$ | −50.14 $^{b}$ | −34.51 $^{ab}$ | −0.02 $^{c}$ | −51.45 $^{a}$ |
| DF R | 80.08 $^{c}$ | −4.30 $^{b}$ | −51.50 $^{a}$ | −34.65 $^{b}$ | −76.65 $^{a}$ | −47.66 $^{a}$ | −58.04 $^{a}$ | −51.35 $^{a}$ |
| P 0R | −18.30 $^{a}$ | −12.46 $^{b}$ | −23.90 $^{cd}$ | −14.72 $^{cd}$ | −9.82 $^{c}$ | −0.23 $^{c}$ | −32.76 $^{b}$ | −40.45 $^{ab}$ |
| P R | 44.11 $^{b}$ | −1.0 $^{b}$ | −24.27 $^{c}$ | −17.65 $^{c}$ | −50.52 $^{b}$ | −5.98 $^{c}$ | 249.90 $^{e}$ | −46.05 $^{a}$ |

* Mean values in columns marked with the same letters do not differ significantly according to the LSD test at $p < 0.05$. Symbols are the same as those used in Figure 1.

### *3.3. Soil Invertebrates*

#### 3.3.1. Total Number of Soil Invertebrates Four- and Five-Years Post Contamination

In the 2014 season, i.e., after four years of soil contamination, soil invertebrates captured using the Tullgren apparatus were scarce in both the contaminated and control soils. Statistical analysis demonstrated that none of the studied PDSs had a significant effect on the overall numbers of soil invertebrates (Figure 5). However, it is noteworthy that the number of captured invertebrates in oil-contaminated soils was one sixth of that of the control one. In 2015, soils contaminated with all xenobiotics showed significantly lower number of captured soil invertebrates than the control soil. EO reduced the abundance of the analyzed organisms to the greatest extent, causing them to be 10 times fewer than that in the uncontaminated soil.

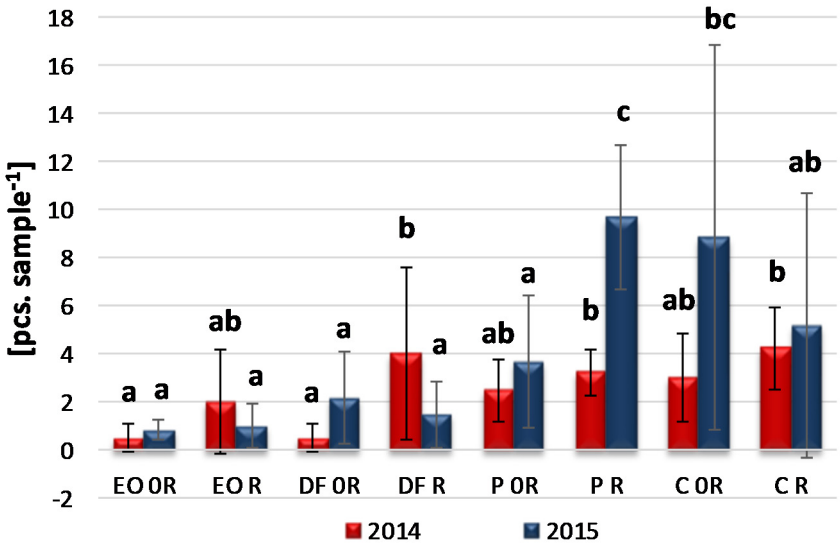


**Figure 5.** Occurrence of soil invertebrates (pcs. sample$^{-1}$). Mean values marked with the same letters for each year separately do not differ significantly according to the LSD test at $p < 0.05$. Symbols are the same as those used in Figure 1. Vertical bars show mean ± SE.

Four years after soil contamination and the application of biopreparation, the numbers of soil fauna specimens grew in all cases, while significant differences were found in the section contaminated with DF, where ZB-01 caused a nearly eightfold increase in the number of captured invertebrates. In 2015, the biopreparation caused a significant (nearly threefold) augmentation of the number of captured organisms only in the P-contaminated section.

#### 3.3.2. Occurrence of Enchytraeidae, Collembola, Nematoda, and Arachnida

The detailed data including the individual dates of observations and the individual groups of invertebrates are provided in Tables 2 and 3. Collembola was one of the groups captured in the largest numbers. Five years after contamination (in late June), Collembola individuals were significantly more scarcely captured in all the contaminated sections than in the control one. Furthermore, the presence of Enchytraeidae, Nematoda, and Arachnida was noted. Nonetheless, PDSs usually had no significant effect on the numbers of these organisms and only periodically reduced the number of Arachnida (EO and DF in November 2014, and EO in June 2015).

**Table 2.** The occurrence of soil invertebrates after four years from soil contamination and the use of ZB-01 (pcs. sample$^{-1}$).

| Treatment | Soil Invertebrates | | | | |
|---|---|---|---|---|---|
| | Total | Enchytraeidae | Collembola | Nematoda | Arachnida |
| | | | **29.09** | | |
| EO 0R | 1.0 (±0.0) $^{a*}$ | 0.0 (±0.0) $^{a}$ | 0.0 (±0.0) $^{a}$ | 1.0 (±0.0) $^{ab}$ | 0.0 (±0.0) $^{a}$ |
| EO R | 2.5 (±0.7) $^{ab}$ | 0.0 (±0.0) $^{a}$ | 0.5 (±0.7) $^{ab}$ | 0.0 (±0.0) $^{a}$ | 0.0 (±0.0) $^{a}$ |
| DF 0R | 1.0 (±0.7) $^{a}$ | 0.5 (±0.7) $^{a}$ | 0.0 (±0.0) $^{a}$ | 0.0 (±0.0) $^{a}$ | 0.0 (±0.0) $^{a}$ |
| DF R | 7.0 (±1.4) $^{b}$ | 0.0 (±0.0) $^{a}$ | 0.0 (±0.0) $^{a}$ | 1.5 (±0.7) $^{b}$ | 5.5 (±0.7) $^{b}$ |
| P 0R | 2.5 (±2.1) $^{ab}$ | 0.0 (±0.0) $^{a}$ | 2.0 (±1.4) $^{b}$ | 0.5 (±0.7) $^{ab}$ | 0.0 (±0.0) $^{a}$ |
| P R | 3.0 (±1.4) $^{ab}$ | 0.0 (±0.0) $^{a}$ | 1.5 (±0.7) $^{ab}$ | 1.0 (±0.0) $^{ab}$ | 0.5 (±0.7) $^{a}$ |
| C 0R | 3.0 (±1.4) $^{ab}$ | 0.5 (±0.7) $^{a}$ | 1.5 (±0.7) $^{ab}$ | 1.0 (±0.0) $^{ab}$ | 0.0 (±0.0) $^{a}$ |
| C R | 4.0 (±3.5) $^{ab}$ | 2.5 (±2.1) $^{b}$ | 0.0 (±0.0) $^{a}$ | 1.0 (±1.4) $^{ab}$ | 0.0 (±0.0) $^{a}$ |
| | | | **18.11.** | | |
| EO 0R | 0.0 (±0.0) $^{a}$ | 0.0 (±0.0) $^{a}$ | 0.0 (±0.0) $^{a}$ | 0.0 (±0.0) $^{a}$ | 0.0 (±0.0) $^{a}$ |
| EO R | 1.5 (±0.7) $^{ab}$ | 0.5 (±0.7) $^{a}$ | 0.0 (±0.0) $^{a}$ | 0.5 (±0.7) $^{a}$ | 0.5 (±0.7) $^{ab}$ |
| DF 0R | 0.0 (±0.0) $^{a}$ | 0.0 (±0.0) $^{a}$ | 0.0 (±0.0) $^{a}$ | 0.0 (±0.0) $^{a}$ | 0.0 (±0.0) $^{a}$ |
| DF R | 1.0 (±0.0) $^{ab}$ | 0.0 (±0.0) $^{a}$ | 0.5 (±0.7) $^{ab}$ | 0.0 (±0.0) $^{a}$ | 0.5 (±0.7) $^{ab}$ |
| P 0R | 2.5 (±0.7) $^{abc}$ | 0.0 (±0.0) $^{a}$ | 0.5 (±0.7) $^{ab}$ | 1.0 (±0.0) $^{a}$ | 1.0 (±0.0) $^{b}$ |
| P R | 3.5 (±0.7) $^{bc}$ | 0.5 (±0.7) $^{a}$ | 2.0 (±1.4) $^{b}$ | 1.0 (±1.4) $^{a}$ | 0.0 (±0.0) $^{a}$ |
| C 0R | 3.0 (±1.4) $^{bc}$ | 0.0 (±0.0) $^{a}$ | 0.5 (±0.7) $^{ab}$ | 0.5 (±0.7) $^{a}$ | 1.0 (±0.0) $^{b}$ |
| C R | 4.5 (±2.1) $^{c}$ | 0.0 (±0.0) $^{a}$ | 1.5 (±0.7) $^{ab}$ | 1.0 (±1.4) $^{a}$ | 0.5 (±0.7) $^{ab}$ |

* Mean ± SE values in columns marked with the same letters do not differ significantly according to the LSD test at $p < 0.05$. Symbols are the same as those used in Figure 1.

#### 3.3.3. Collembola Families

The analysis of taxonomic ranks of the captured Collembola showed that individuals from the Hypogastruidae family were most frequently captured both after four and five years of soil contamination (Table 4). Specimens of the families Isotomidae and Entomobryidae were also noted to occur. Collembola individuals were sporadically captured in the sections contaminated with EO and DF, whereas the proportions of specimens belonging to each family in the P-contaminated section were similar to that in the control soil. The biopreparation had no significant effect on the occurrence of members of the given Collembola families in the sections contaminated with EO and DF. However, it caused an increase in the number of Hypogastruidae, Isotomidae, and Entomobryidae members in the P-contaminated section five years after its application and soil contamination.

### *3.4. The Relationships between PDSs, Bioremediation, Soil Enzyme Activity, and Soil Fauna Occurrence*

To determine the relationships between contamination with PDSs, bioremediation, soil enzyme activity, and soil fauna occurrence, the PCA analysis was performed (Figures 6 and 7). The gradient represented by axis 1 accounts for 92.5% of the variability for contaminated soils without ZB-01 application. This variability is explained by the content of PDSs in the soil. The analysis shows that soil contamination with DF and EO had the most significant influence on the activity of enzymes and soil fauna occurrence. This effect persisted even after 4- and 5-years post contamination. The most significant differences in enzyme activity, together with an increase in the interval from the moment of contamination, were observed for urease and dehydrogenase. Changes in soil fauna provided an equally strong response, especially to soil contamination with oils. After the application of ZB-01, the effect of PDSs on the tested soil properties decreased, as demonstrated by the PCA analysis (Figure 7). The gradient represented by axis 1 accounts for 49.7% of the variability for soils. The analysis showed that ZB-01 had the most significant effect on the number of invertebrates, especially in soils contaminated with DF and P.

**Table 3.** The occurrence of soil invertebrates after five years from soil contamination and the use of ZB-01 (pcs. sample$^{-1}$).

| Treatment | Soil Invertebrates | | | | |
|---|---|---|---|---|---|
| | Total | Enchytraeidae | Collembola | Nematoda | Arachnida |
| | | | **22.06.** | | |
| EO 0R | 1.0 (±0.0) $^{a*}$ | 0.0 (±0.0) $^{a}$ | 0.5 (±0.7) $^{ab}$ | 0.5 (±0.7) $^{a}$ | 0.0 (±0.0) $^{a}$ |
| EO R | 1.5 (±0.7) $^{a}$ | 1.5 (±0.7) $^{b}$ | 0.0 (±0.0) $^{a}$ | 0.0 (±0.0) $^{a}$ | 0.0 (±0.0) $^{a}$ |
| DF 0R | 4.0 (±2.8) $^{a}$ | 0.5 (±0.7) $^{ab}$ | 2.0 (±1.4) $^{ab}$ | 1.0 (±1.4) $^{a}$ | 0.5 (±0.7) $^{ab}$ |
| DF R | 1.0 (±0.7) $^{a}$ | 0.0 (±0.0) $^{a}$ | 0.5 (±0.7) $^{ab}$ | 0.0 (±0.0) $^{a}$ | 0.5 (±0.7) $^{ab}$ |
| P 0R | 5.5 (±3.5) $^{a}$ | 0.0 (±0.0) $^{a}$ | 3.5 (±3.5) $^{ab}$ | 1.5 (±0.7) $^{a}$ | 0.5 (±0.7) $^{ab}$ |
| P R | 9.5 (±3.5) $^{ab}$ | 0.0 (±0.0) $^{a}$ | 9.5 (±3.5) $^{bc}$ | 0.0 (±0.0) $^{a}$ | 0.0 (±0.0) $^{a}$ |
| C 0R | 17.5 (±9.1) $^{b}$ | 0.5 (±0.7) $^{ab}$ | 14.0 (±9.9) $^{c}$ | 1.0 (±0.0) $^{a}$ | 2.0 (±1.4) $^{b}$ |
| C R | 3.0 (±0.7) $^{a}$ | 0.5 (±0.7) $^{ab}$ | 0.5 (±0.7) $^{ab}$ | 0.5 (±0.7) $^{a}$ | 1.0 (±0.0) $^{ab}$ |
| | | | **18.08.** | | |
| EO 0R | 0.5 (±0.7) $^{a}$ | 0.0 (±0.0) $^{a}$ | 0.0 (±0.0) $^{a}$ | 0.0 (±0.0) $^{a}$ | 0.0 (±0.0) $^{a}$ |
| EO R | 0.5 (±0.7) $^{a}$ | 0.0 (±0.0) $^{a}$ | 0.5 (±0.7) $^{a}$ | 0.0 (±0.0) $^{a}$ | 0.0 (±0.0) $^{a}$ |
| DF 0R | 1.0 (±0.0) $^{ab}$ | 0.0 (±0.0) $^{a}$ | 0.5 (±0.7) $^{a}$ | 0.0 (±0.0) $^{a}$ | 0.5 (±0.7) $^{a}$ |
| DF R | 0.5 (±0.7) $^{a}$ | 0.5 (±0.7) $^{a}$ | 0.0 (±0.0) $^{a}$ | 0.0 (±0.0) $^{a}$ | 0.0 (±0.0) $^{a}$ |
| P 0R | 4.0 (±2.8) $^{abc}$ | 0.0 (±0.0) $^{a}$ | 2.0 (±2.8) $^{a}$ | 0.0 (±0.0) $^{a}$ | 2.0 (±0.0) $^{b}$ |
| P R | 8.0 (±2.8) $^{c}$ | 0.0 (±0.0) $^{a}$ | 8.0 (±2.8) $^{b}$ | 0.0 (±0.0) $^{a}$ | 0.0 (±0.0) $^{a}$ |
| C 0R | 5.0 (±4.2) $^{bc}$ | 0.0 (±0.0) $^{a}$ | 3.0 (±2.8) $^{a}$ | 0.0 (±0.0) $^{a}$ | 0.5 (±0.7) $^{a}$ |
| C R | 4.0 (±1.4) $^{abc}$ | 0.5 (±0.7) $^{a}$ | 1.5 (±0.7) $^{a}$ | 0.5 (±0.7) $^{a}$ | 2.0 (±1.4) $^{b}$ |
| | | | **06.10** | | |
| EO 0R | 1.0 (±0.0) $^{a}$ | 0.5 (±0.7) $^{a}$ | 0.0 (±0.0) $^{a}$ | 0.0 (±0.0) $^{a}$ | 0.5 (±0.7) $^{a}$ |
| EO R | 1.0 (±1.4) $^{a}$ | 0.0 (±0.0) $^{a}$ | 1.0 (±1.4) $^{ab}$ | 0.0 (±0.0) $^{a}$ | 0.0 (±0.0) $^{a}$ |
| DF 0R | 1.5 (±0.7) $^{a}$ | 0.0 (±0.0) $^{a}$ | 0.5 (±0.7) $^{a}$ | 0.0 (±0.0) $^{a}$ | 1.0 (±1.4) $^{a}$ |
| DF R | 3.0 (±1.4) $^{ab}$ | 0.0 (±0.0) $^{a}$ | 3.0 (±1.4) $^{ab}$ | 0.0 (±0.0) $^{a}$ | 0.0 (±0.0) $^{a}$ |
| P 0R | 1.5 (±0.7) $^{a}$ | 0.0 (±0.0) $^{a}$ | 0.5 (±0.7) $^{a}$ | 0.0 (±0.0) $^{a}$ | 1.0 (±0.0) $^{a}$ |
| P R | 11.5 (±3.5) $^{b}$ | 1.5 (±2.1) $^{a}$ | 10.0 (±5.7) $^{b}$ | 0.0 (±0.0) $^{a}$ | 0.0 (±0.0) $^{a}$ |
| C 0R | 4.0 (±0.0) $^{ab}$ | 0.5 (±0.0) $^{a}$ | 3.0 (±0.0) $^{ab}$ | 0.0 (±0.0) $^{a}$ | 0.5 (±0.7) $^{a}$ |
| C R | 8.5 (±9.9) $^{ab}$ | 0.0 (±0.7) $^{a}$ | 8.0 (±9.9) $^{ab}$ | 0.0 (±0.0) $^{a}$ | 0.0 (±0.0) $^{a}$ |

* Mean ± SE values in columns marked with the same letters do not differ significantly according to the LSD test at $p < 0.05$. Symbols are the same as those used in Figure 1.

**Table 4.** The effect of petroleum-derived substances and the bioremediation process on the occurrence of individuals belonging to collembola families (Collembola) (pcs.).

| Treatment | Collembola Familiy | | | | | |
|---|---|---|---|---|---|---|
| | Hypogastruidae | | Isotomidae | | Entomobryidae | |
| | 2014 | 2015 | 2014 | 2015 | 2014 | 2015 |
| EO 0R | 0.0 (±0.0) $^{a*}$ | 1.2 (±0.4) $^{a}$ | 0.0 (±0.0) $^{a}$ | 0.0 (±0.0) $^{a}$ | 0.0 (±0.0) $^{a}$ | 0.0 (±0.0) $^{a}$ |
| EO R | 1.3 (±0.5) $^{a}$ | 3.4 (±0.8) $^{a}$ | 0.0 (±0.0) $^{a}$ | 0.0 (±0.0) $^{a}$ | 0.0 (±0.0) $^{a}$ | 0.0 (±0.0) $^{a}$ |
| DF 0R | 0.0 (±0.0) $^{a}$ | 4.8 (±0.8) $^{a}$ | 0.0 (±0.0) $^{a}$ | 1.2 (±0.4) $^{a}$ | 0.0 (±0.0) $^{a}$ | 0.0 (±0.0) $^{a}$ |
| DF R | 1.3 (±0.5) $^{a}$ | 6.1 (±1.3) $^{a}$ | 0.0 (±0.0) $^{a}$ | 1.2 (±0.4) $^{a}$ | 0.0 (±0.0) $^{a}$ | 0.0 (±0.0) $^{a}$ |
| P 0R | 2.8 (±1.0) $^{a}$ | 9.6 (±2.0) $^{a}$ | 1.3 (±0.5) $^{a}$ | 2.3 (±0.5) $^{ab}$ | 1.3 (±0.5) $^{a}$ | 0.0 (±0.0) $^{a}$ |
| P R | 3.5 (±0.6) $^{a}$ | 40.8 (±2.4) $^{b}$ | 3.3 (±0.5) $^{b}$ | 8.5 (±0.8) $^{c}$ | 0.0 (±0.0) $^{a}$ | 4.8 (±1.0) $^{b}$ |
| C 0R | 2.8 (±0.5) $^{a}$ | 33.6 (±6.3) $^{b}$ | 1.3 (±0.5) $^{a}$ | 4.8 (±0.8) $^{b}$ | 0.0 (±0.0) $^{a}$ | 1.2 (±0.4) $^{a}$ |
| C R | 0.5 (±0.6) $^{a}$ | 19.2 (±5.3) $^{ab}$ | 2.3 (±0.5) $^{a}$ | 1.2 (±0.4) $^{a}$ | 0.0 (±0.0) $^{a}$ | 0.0 (±0.0) $^{a}$ |

* Mean ± SE values in columns marked with the same letters do not differ significantly according to the LSD test at $p < 0.05$. Symbols are the same as those used in Figure 1.

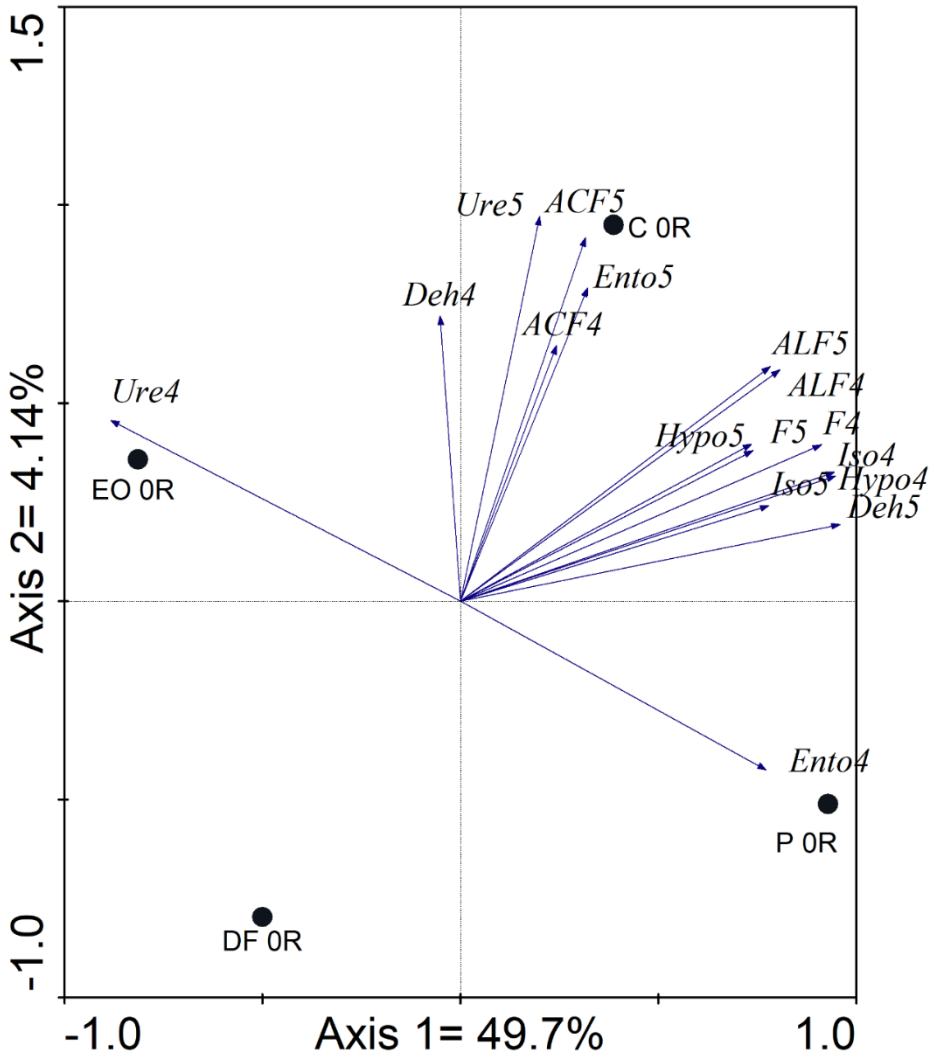


**Figure 6.** Principal component analysis of soil enzyme activity and soil fauna occurrence in soils without ZB-01 application. ACF4—acid phosphatase activity after 4 years, ACF5—acid phosphatase activity after 5 years, ALF4—alkaline phosphatase activity after 4 years, ALF5—alkaline phosphatase activity after 5 years, Deh4—Dehydrogenase activity after 4 years, Deh5—dehydrogenase activity after 5 years, Ure4—urease activity after 4 years, Ure5—urease activity after 5 years, F4—occurrence of soil invertebrates after 4 years, F5—occurrence of soil invertebrates after 5 years, Hypo4—occurrence of Hypogastruidae individuals after 4 years, Hypo5—occurrence of Hypogastruidae individuals after 5 years, Iso4—occurrence of Isotomidae individuals after 4 years, Iso5—occurrence of Isotomidae individuals after 5 years, Ento4—occurrence of Entomobryidae individuals after 4 years, Ento5—occurrence of Entomobryidae individuals after 5 years.

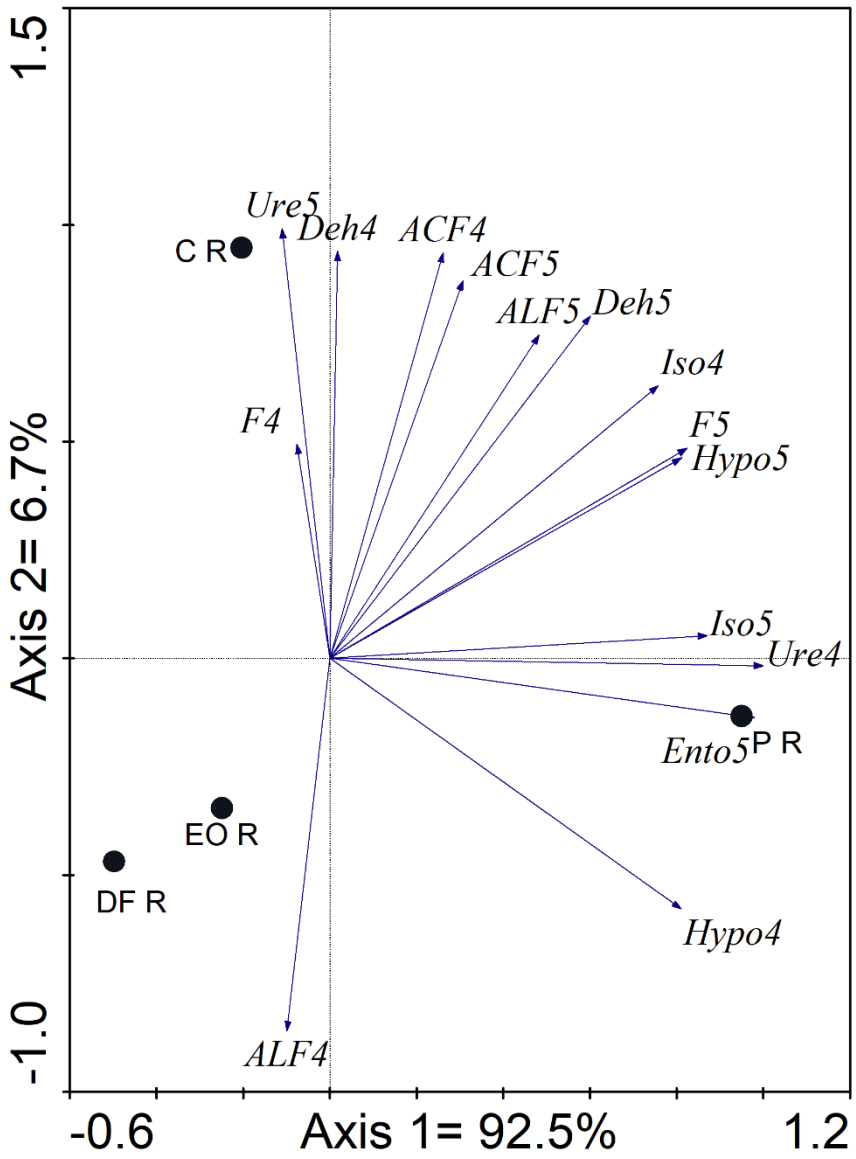


**Figure 7.** Principal component analysis of soil enzyme activity and soil fauna occurrence in soils with ZB-01 application. ACF4—acid phosphatase activity after 4 years, ACF5—acid phosphatase activity after 5 years, ALF4—alkaline phosphatase activity after 4 years, ALF5—alkaline phosphatase activity after 5 years, Deh4—Dehydrogenase activity after 4 years, Deh5—dehydrogenase activity after 5 years, Ure4—urease activity after 4 years, Ure5—urease activity after 5 years, F4—occurrence of soil invertebrates after 4 years, F5—occurrence of soil invertebrates after 5 years, Hypo4—occurrence of Hypogastruidae individuals after 4 years, Hypo5—occurrence of Hypogastruidae individuals after 5 years, Iso4—occurrence of Isotomidae individuals after 4 years, Iso5—occurrence of Isotomidae individuals after 5 years, Ento4—occurrence of Entomobryidae individuals after 4 years, Ento5—occurrence of Entomobryidae individuals after 5 years.

## 4. Discussion

### *4.1. Hydrocarbons Degradation*

Despite the passage of 4 or 5 years from soil contamination by DF and EO, the $C_{12}$-$C_{36}$ hydrocarbon levels were still several hundred times higher than those found in the uncontaminated soil, which proves their persistence. Comparison of the levels of these hydrocarbons in soil contaminated with P and the control soil, both with or without biopreparation application, suggests that the contamination with P was neutralized by evaporation, and the obtained concentrations of organic compounds showed naturally

occurring organic substances in the soil (e.g., humic acids and other compounds). The process of petroleum derivative biodegradation is determined by the type of xenobiotics that have caused this contamination. The susceptibility to biodegradation decreases with the increasing complexity of molecular structure of hydrocarbons according to the following pattern: alkanes → branched-chain alkanes → branched-chain alkenes → alkyl derivatives of monocyclic aromatic hydrocarbons → cycloalkanes → polycyclic hydrocarbons → asphaltenes [45]. In the present experiment, studies conducted in the earlier period, i.e., after 2 years from the moment of contamination, confirmed different biodegradation dynamics for each of the three types of pollutants (P, DF, and EO). The TPH (total petroleum hydrocarbons) concentration decreased the fastest in P-contaminated soil and the slowest in EO-contaminated soil [29]. EO (PLATINUM Classic Semisynthetic 10W-40), which was used in the experiment, is a mixture of mineral and synthetic base oils and enriching additives (detailed description provided in Material Safety Data Sheet: https://www.orlenoil.pl/oodownload/1117.pdf?), which are among the ingredients that decompose slowly. In addition, EO acquires a number of additional components from engine wear (heavy metals such as lead, chromium, and cadmium as well as other components such as naphthalene, chlorinated hydrocarbons, and sulfur) during use, the impact of which on the environment may be long-lasting [46]. Used EO is more slowly biodegradable even as compared to crude oil. The biostimulation of degradation by the addition of wastewater sludge in this type of pollution was found to be ineffective [47]. In our experiment, however, biopreparation ZB-01 caused decrease in $C_{12}$-$C_{36}$ hydrocarbon content by 470 mg $kg^{-1}$ and 80 mg $kg^{-1}$ in fourth- and fifth-year post contamination, respectively [6,37].

### *4.2. The Effect of PDSs on Alkaline Phosphatase, Acid Phosphatase, Dehydrogenase, and Urease*

PDSs, and particularly PAHs (polycyclic aromatic hydrocarbons) they contain, show toxic properties toward soil microorganisms by strongly inhibiting their development and metabolic activity [48]. Additionally, by coating the mineral and organic surfaces of soil particles and cell surfaces, they block the interaction between enzyme active sites and soluble substrates, thus exerting a negative effect on enzyme activity [49].

Wyszkowska and Kucharski [50] showed that phosphatases are more resistant to contaminants than other enzymes, while doses of P higher than 4 $cm^3$ $kg^{-1}$ of soil can lower acid phosphatase activity by approximately 20%. A decline in phosphatase activity driven by the presence of PDSs in the soil was also demonstrated by other authors [2,51]. In our experiment, oils particularly contributed to reduction in alkaline phosphatase activity, whereas acid phosphatase showed lowered activity in response to all PDSs. Joniec et al. [52] demonstrated that compared to soil enzymes such as catalase, protease, and urease, acid phosphatase exhibited the weakest stimulation by reclamation treatments (addition of post-flotation lime, mineral fertilization, sewage sludge, and mineral wool). In our study, the pH of PDS-contaminated soil varied from 4.93 to 6.12 (pH in KCl). We obtained positive correlation coefficient values between pH and soil enzyme activity, which were statistically nonsignificant (except for values for alkaline phosphatase: 0.75 and 0.79). The pH of the soil may affect soil enzyme levels in different ways such as by changes in the ionic form of the active sites of the enzyme, by altering the three-dimensional shape of the enzyme, and by affecting the affinity of the substrate to the enzyme [53]. Soil enzyme activities are usually correlated to soil pH, and positive, negative, or no correlations have been reported [54]. In our study, the activity of acid phosphatase was higher than that of alkaline phosphatase. Earlier studies have confirmed that alkaline phosphatase activity was not predominant in neutral or alkaline soils, which agrees with the finding of our study [55].

A reduction in the activity of soil enzyme, such as dehydrogenase, urease, amylase, and protease, caused by the presence of PDSs in the soil was demonstrated [56]. In our experiment, the addition of P, DF, and EO to the soil was both stimulatory and inhibitory to the analyzed enzymes, depending on the nature of the petroleum substances and the type

of enzyme as well as the time elapsed from the moment of contamination. In comparison to the control probes, P contributed to an approximately 50% decrease in urease activity after four and five years of contamination, while DF lowered the activity of this enzyme after five years. Dehydrogenase activity was reduced by DF in both four and five after contamination. Wyszkowska and Kucharski [50] also demonstrated that soil contamination with P at the dose of 6 $cm^3$ $kg^{-1}$ of soil decreased urease activity fivefold 49 days after contamination. These authors found similar patterns in their later research [51,57]. Alrumman et al. [2] found that soil contamination with hydrocarbons lowered dehydrogenase activity 10 weeks after contamination. Serrano et al. [58] also obtained negative correlation coefficients between PDSs and dehydrogenase and urease activities, similar to Gianfreda et al. [54] who reported a decrease in dehydrogenase activity in the soil polluted for a long time (over 50 years) with petroleum and showing very high levels of PAHs (100 mg $kg^{-1}$). Significant and negative correlation coefficients between phenanthrene content and dehydrogenase and urease activities were also obtained in the latter cited studies, which suggests that these two enzymes are the most sensitive ones to this kind of pollution.

A stimulatory effect of PDSs on soil enzymatic activity was also demonstrated in some earlier studies [8,9,59]. Similar dependencies were also noted periodically in the present experiment: EO increased urease and dehydrogenase activities four years from contamination. The increase in enzyme activity can result from the ability of microorganisms to adapt to pollutants and use them as sources of carbon and energy [60,61]. PDSs and their metabolites may be used as substrates for increasing soil biomass, which leads to an increased enzymatic activity [62]. Furthermore, changes in microbial population and composition during biodegradation of PDSs can improve soil enzyme activity [63]. Additionally, as suggested by Baran et al. [59] and Dindar et al. [15], there is an increase in respiration intensity, an increase in enzyme activity, growth of microorganisms, and a gradual decomposition of pollutants after a period of stress. During the subsequent period, the microorganisms utilize pollutants as sources of carbon and energy due to increased enzyme activity.

Taken together, the results of our study suggest that of all the enzymes, the activity of dehydrogenase can be used as an indicator of soil biochemical quality, especially for DF-contaminated soil. It is also noteworthy that apart from dehydrogenase, the other investigated enzymes are not purely intracellular and are not dependent on cellular activity.

### *4.3. The Effect of ZB-01 on Alkaline Phosphatase, Acid Phosphatase, Dehydrogenase, and Urease*

The addition of specialized bacteria after three years from the moment of soil contamination with PDSs usually enhances soil enzymatic activity [49]. Shen et al. [64] conducted a 40-day investigation of soil contaminated (low and moderate concentration) with crude oil and inoculated with mixed bacterial suspension and showed substantially higher enzyme activities, but a slight downward trend was observed in the inoculated soil. In our experiment, the effect of ZB-01 biopreparation on soil enzyme activity varied and depended on the type of pollutant, the type of enzyme, and the time elapsed from contamination.

The biopreparation applied to DF-contaminated soil usually caused alkaline phosphatase activity to increase while resulting in its decrease in the P-contaminated soil. The effect of the biopreparation on acid phosphatase activity was variable and depended on the year of study and the type of pollutant. Available literature contains very little data on the effect of various techniques of soil decontamination from PDSs on the activity of these enzymes. Tejada et al. [65] demonstrated that an addition of compost, poultry manure, or sewage sludge to soil polluted with P causes alkaline phosphatase activity to increase after one day of contamination and the addition of organic substances.

Dehydrogenase activity during the remediation of soils polluted with PDSs may exhibit major fluctuations, because it is correlated to oil removal and respiration [8]. Experiments analyzing early effects (soon after introducing remediation) have confirmed the above assertion, e.g., an addition of *Paracoccus* to soil polluted with PAHs caused more than 35% increase in dehydrogenase activity after 28 days [62]. However, in a 40-

day experiment on the bioremediation of crude oil-contaminated soil, it was observed that after increased dehydrogenase activity, the values became stable [64]. In our studies, after four years of ZB-01 biopreparation application, the effect was observed as lowered dehydrogenase activity in soils contaminated with EO and P as compared to the control soil, while the activity of this enzyme in the same soils was enhanced after one more year. Moreover, dehydrogenase activity reflects a broad range of microbial oxidative activities. Its level may result not only from the presence of PDSs but also from inorganic nutrient concentrations in the soil [8]. Kaczyńska et al. [16] observed that compost was useful for stimulating dehydrogenases in soils contaminated with petroleum products as these products constitute a source of necessary cosubstrates and nutrients for microorganisms.

In this experiment, the biopreparation applied to contaminated soils usually decreased urease activity, except for P-treated soil after four years from contamination. Fluctuations in the activity of this enzyme over time after the moment of adding specialized bacteria aimed at purifying crude oil-contaminated soil were also demonstrated by Shen et al. [64]. The reason underlying the depletion of soil enzyme activity in contaminated and bioremediated soil, besides the exhaustion of nutrient sources, may also entail the formation of intermediate products with strong toxicity throughout the purification process [66].

### *4.4. The Effect of PDSs on Soil Invertebrates*

Many authors emphasize that soil invertebrates show a higher "sensitivity" as PDS soil pollution indicators than chemical tests [10,28]. Gospodarek et al. [29] found that an analysis of TPH performed on soils polluted with P and DF eight months after contamination showed no differences when compared with uncontaminated soil; however, differences in soil fauna density were still observed at that time. In the present experiment, EO and DF exerted the most harmful effect on the analyzed invertebrates, which is confirmed in the studies by Jaworska and Gospodarek [67].

The Tullgren funnel-captured fauna included Enchytraeidae, Collembola, Nematoda, and Arachnida. Pirhonen [68] showed that Enchytraeidae are particularly sensitive to soil contamination with heating oil. The results of our study confirm this finding, as a very low number of Enchytraeidae individuals were noted in all contaminated soils.

A significant PDS-driven decrease in the number of Collembola individuals was noted only at one of the three time points throughout the observation, in 2015, i.e., five years from contamination. Studies on the early effects of pollutants indicate a high sensitivity of these invertebrates to the presence of PDSs in their surroundings. Kireeva et al. [32] demonstrated that pollution with Tyumen oil at the dose of 2% causes a 90% increase in Collembola mortality and shortens their lifespan by over 5 days, while the doses of 5% and 10% cause complete mortality of these insects. The negative effect of PDSs on the occurrence of Collembola in field conditions may linger from five weeks to even five months after the moment of inducing contamination [69]. In the present experiment, the after-effect of soil contamination with PDSs was investigated, spanning four and five years after contaminating the soil; therefore, the negative effect of the used substances on the studied invertebrates was no longer visible. However, the sole fact of noting any changes after such a long period of time following contamination confirms their high vulnerability to this type of pollution. Rusin and Gospodarek [36] demonstrated that members of various Collembola families show different sensitivities to soil contamination with PDSs. The authors revealed that Collembola of the families Entomobryidae and Isotomidae are most sensitive to this kind of contamination. In the present experiment, PDSs limited the presence of individuals of the abovementioned families even after four and five years of contamination, which also included the family Hypogastruidae. This suggests that they are suitable for assessing the rate of bioremediation, both natural and supported by using biopreparations. However, it should be noted that even individual species of Collembola exhibit different sensitivities to soil pollution with PDSs [10,70]. Their bodies are protected by thick epidermis, which may partly shield them from the adverse effect of PDSs [10],

and some species have the ability to remove harmful substances (including heavy metals) from their bodies while moulting [71].

Nematodes are typically far more sensitive to soil contamination with PDSs than Collembola and Arachnida [30]. PDSs, and particularly the PAHs that they contain, exhibit fungicidal properties, which may limit food sources for Nematoda that feed on fungi, thus reducing the number of these invertebrates [72]. Ropek and Gospodarek [73,74] and Elarbaoui et al. [75] demonstrated that soil pollution with PDSs adversely affects the occurrence of entomopathogenic nematodes. No significant effect of PDSs on the occurrence of Nematoda was found in the present experiment; however, these invertebrates were noted very rarely in all soils (including control).

Both EO and DF periodically inhibited the abundance of soil Arachnida in this experiment. Arachnids frequently take up harmful substances together with food (from their prey), but they have the ability to detoxify them. This ability increases the chance of survival for these invertebrates in contaminated soil environments; however, this kind of protective mechanism may slow down their growth and development as well as disrupt their reproduction processes [76]. For Arachnida, which are highly mobile animals, contact avoidance with contaminated soils can represent another defensive mechanism. Nonetheless, as shown in previous studies, these animals from the group of epigeic organisms do not respond by number reduction to the presence of these kinds of substances [60,77], while some pollutants can even boost their abundance on soil surface. This suggests a major difference in PDS-induced response between epigeic arachnids and those inhabiting the inside soil. The effect of soil contaminants on the occurrence of Arachnida largely depends on the type of pollutant and may vary widely, from a decrease in numbers and species diversity to the stimulation of their occurrence, which has been demonstrated by many authors investigating the effect of heavy metals [78,79]. This can be explained by the fact that xenobiotic-driven environmental changes may result in the formation of new ecological niches that may be suitable for new arachnid species [80], or—as suggested in previous studies—PDSs may contain attractants for these organisms [77].

### *4.5. The Effect of ZB-01 on Soil Invertebrates*

Research on the effect of the supported bioremediation process on the occurrence of soil invertebrates is quite scanty. Gospodarek et al. [29] demonstrated that microbial-supported bioremediation showed different effects depending on the group of fauna studied and the type of pollutant as well as time elapsed from contamination (the analysis covered 0–28 months from contamination), which is also reflected in the present experiment. The authors stressed that the said process accelerated the colonization of DF-contaminated soil by epigeic invertebrates, while in EO-contaminated soil, some of these invertebrates (mainly Collembola) occurred more scarcely in the section subjected to supported bioremediation, the latter effect being evident only as late as two years after contamination. In the present experiment, the use of ZB-01 product on DF-contaminated soil contributed to the growth of a number of captured soil invertebrates four years after contamination, and five years after contamination in P-contaminated soil.

Regarding Collembola, the applied preparation usually had no effect on their numbers, and their recorded number was larger when the biopreparation was applied only in the section contaminated with P five years from contamination. Kireeva et al. [32] showed that applying Basispecin biopreparation (containing *Bacillus* sp. 739 strains) to soil contaminated with small doses of PDSs has a beneficial effect on the survival rate of Collembola. As early as after three days of incubation, the insect survival rate was 40% higher in the sections where the preparation was used than in those unexposed to this procedure. Rusin and Gospodarek [36] found that the bioremediation of PDS-contaminated soil accelerates the process of soil recolonization by the members of Collembola families Isotomidae and Hypogastruidae, especially in the DF-contaminated section, 23 months from the moment of soil contamination and the use of the biopreparation. In the present experiment, the use of ZB-01 preparation in soil contaminated with P augmented the number of specimens

from the families Hypogastruidae, Isotomidae, and Entomobryidae five years after soil contamination and the application of the biopreparation.

Ropek and Gospodarek [73] found that the use of a microbial preparation had a beneficial effect on the occurrence of entomopathogenic nematodes, as they were isolated earlier than in the sections undergoing the process of remediation naturally. In our study, the ZB-01 biopreparation usually had no impact on the occurrence of Nematoda, except in DF-contaminated soil. Nematodes appeared in the contaminated soil four years after soil contamination with DF (in late September) and the use of the biopreparation, while there were still none reported in the sections unexposed to the biopreparation.

Gospodarek et al. [77] found that the bioremediation of PDS-polluted soil does not usually affect the occurrence of Arachnida, and it can only limit their presence in a section contaminated with P. Similar trends were observed in the present experiment. In most cases, the biopreparation had no effect on the number of arachnids captured, while only in the section contaminated with P where the preparation was applied, their number was periodically observed to be smaller. However, it should be noted that the effect of supported bioremediation on the occurrence of Arachnida largely depends on their taxonomic position [36].

## 5. Conclusions

- PDSs even after the lapse of five years from the moment of contamination significantly modified the activity of soil enzymes analyzed; however, this effect was often varied, depending on the pollutant, enzyme, and time elapsed after soil contamination. Dehydrogenase seems to be a good indicator of soil contamination with PDSs, particularly DF.
- Of the analyzed PDSs, oils (DF and EO) restricted the occurrence of soil invertebrates, particularly Collembola from the families Hypogastruidae, Isotomidae, and Entomobryidae, even after four and five years of contamination. This proves the usefulness of these groups of organisms in assessing soil pollution with PDSs and in monitoring the progress of bioremediation.
- The effect of bioremediation using ZB-01 biopreparation on the activity of selected enzymes was variable and depended on the type of contaminant, the type of enzyme analyzed, and the time elapsed after contamination and the use of the preparation. The effect of the application of the biopreparation on the occurrence of soil invertebrates was usually beneficial, which was particularly evident for DF-contaminated soil.

**Author Contributions:** Conceptualization, J.G. and A.N.-S.; Investigation, J.G., M.R. and A.N.-S.; Methodology, J.G. and A.N.-S.; Writing—original draft, J.G., M.R., G.B. and A.N.-S. All authors have read and agreed to the published version of the manuscript.

**Funding:** Scientific publication financed from the funds for science in 2009–2013 as a research project (N N305 151537) and by the Ministry of Science and Higher Education of the Republic of Poland.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy restrictions.

**Conflicts of Interest:** The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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