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THE EFFECT OF RIBWORT (*PLANTAGO LANCEOLATA*) AND ITS MYRORRHIZAS ON THE GROWTH OF MICROFLORA IN SOIL CONTAMINATED WITH USED ENGINE OIL

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Abstract: The aim of the work was to estimate the influence of plants mycorrhizas on the number of hydrocarbons degrading bacteria, saprophytic bacteria and molds fungi during the remediation of the soil samples contaminated with used engine oil. The investigation were carried out in laboratory conditions. Nine modifications of the soil substrate were prepared and divided into three groups: the first one – without used engine oil; the second one – with 10% (w/w) of used engine oil; the third one – with 25% (w/w) of used oil. In each group one sample was sown with ribwort, one was inoculated with living spores of mycorrhizal fungi and sown with ribwort, and the third one was left without plants and mycorrhizal fungi. The sample of the uncontaminated soil was the control. The investigation showed a significant effect of used engine oil, the presence of ribwort and mycorrhizal fungi on the number of soil microorganisms. The increase of the number of hydrocarbons degrading bacteria, in respect to the control sample was observed in the used engine oil contaminated samples. The seeding of soil samples with plants and additional inoculation with spores of mycorrhizal fungi stimulated the increase of the number of microorganisms in the all studied groups.

INTRODUCTION

The soil is a biologically active surface layer of the Earth crust (lithosphere), that finally collects the largest amount of contaminants, including the petroleum–derived compounds. Leakages of petroleum and its products from technological installations, stores and processes of their treatment, degrade not only the ground but also underground waters for a very long time. According to the Polish and many other countries' law regulations, used oils belong to hazardous wastes. The harmfulness of used oils is much more significant than that of oils introduced to the market. From 2400 thousand tons of used oils collected, engine oils constitute more than 70%. In 2000, 1730 thousand tons of used oils were collected in EU countries, which made up 70÷75% of used oils. The remaining 675 thousand tons (25÷30%) were illegally burned or introduced into the environment [11], [12].

The natural attenuation of grounds contaminated with used engine oil is connected with the possibility of their biodegradation by soil microorganisms and with the envi-

ronmental conditions playing the key role in the processes of microbiological decomposition of hydrocarbons. The aim of the bioremediation is to detoxify harmless organic contaminants to undetectable concentrations, or to the admissible level, according to the appropriate standards binding in particular countries [16]. In Poland acceptable value soil pollution levels are specified in the Regulation of the Minister of Environmental Protection of 9th of September 2002 (Dz. U.02.165.1359). One of bioremediation techniques is phytoremediation, i.e. recultivation of soil using plants. The system 'plant – microorganisms' plays an important role in bioremediation of petroleum–derived components. The action of plant roots and microorganisms on organic contaminants present in the soil is one of phytoremediation techniques, called phytodegradation [31].

The proper growth of plants in contaminated areas requires formation of defense and protection mechanisms, in which mycorrhizal fungi, called the biotrophs, play a specific role. This means that they may grow only for defined periods of time without the contact with plant roots. Mycorrhiza is the mutual advantageous coexistence of plants and specific symbiotic fungi, which have a direct contact with roots of the plant – host [1]. The literature sources show the advantageous effect of cultivation of the mycorrhizal plants of rich root systems on decomposition of organic compounds and accumulation of heavy metals. The mycorrhizal fungi stimulate the growth and development of plants in unfavorable environmental conditions, such as, for instance, drought, deficiency of phosphorus, presence of contaminants, etc. They also produce enzymes participating in preliminary and intermediate stages of xenobiotics decomposition. This facilitates the further decomposition of xenobiotics by other organisms living in roots zone (rhizosphaera) [13], [14], [18].

The literature sources [18], [20] inform that the mycorrhizal plants play a significant role in the process of soil bioremediation, particularly in the areas contaminated by petroleum–derived substances. Therefore, it seems reasonable to perform studies on the effect of mycorrhizas of plants, on the number of hydrocarbons degrading bacteria, saprophytic bacteria and molds fungi, during the remediation of soil samples contaminated with used engine oil.

The main aim of this study was to estimate the influence of plants mycorrhizas on the number of hydrocarbons degrading bacteria, saprophytic bacteria and molds fungi during the remediation of soil samples contaminated with used engine oil.

MATERIALS AND METHODS

Materials

In the studies the following materials were used:

- the soil from the „Skalny" dump in Łaziska Górne, from which the spores of mycorrhizal fungi were isolated. The „Skalny" dump is 889 m high above sea level, has cubage of 17 million tons, and occupies the area of 30 ha. This dump was created during the 220 year-exploited Hard Coal Mine «Bolesław Śmiały» [2],
- soil substrate were air dried and then homogenized and sieved through a 2 mm sieve to remove stones and gravel; the physicochemical properties were determined according to [24],
- used engine oil (mineral) of the density of 885 kg/m^3, in which the concentrations of aliphatic [28] and aromatic [29] hydrocarbons were determined,

– seeds of ribwort (*Plantago lanceolata*). This plant belongs to ruderal plants that mycorrhize easily.

To isolate the spores of mycorrhizal fungi, 100 g of soil samples from the dump were sieved through in a sieve with the hole size varying from 1 mm to 38 μm, as previously described by Kendrick [15]. The material that was not sieved through the holes of larger diameters (above 50 μm) was washed with water. The material which remained on the sieves of the holes diameter smaller than 50 μm was transferred to the beakers by gentle flushing the sieves with distilled water. The mixture was transferred to 100 cm^3 plastic beakers, which were centrifuged at 3000 rpm for 4 min. The supernatant was decanted. 60% sucrose solution was added to the beakers with the sediment and the mixture was thoroughly mixed. The beakers were closed and centrifuged at 2500 rpm for 1 min. The obtained supernatant, with suspended organic particles and living spores of mycorrhizal fungi, was cautiously poured onto the sieve of a smaller hole diameter (in respect to the holes from which the soil material was transferred into test-tubes), and washed with distilled water for 1–2 min. The final material, containing about 10^7 spores of mycorrhizal fungi was transferred to the pots containing about 1.5 kg of the universal soil.

Nine modifications of the soil substrate samples were prepared (Table 1). The soil samples were placed in the containers (pots), with the diameter of 26 cm and height of 26 cm. Each sample contained 1.25 kg of the universal soil substrate. 10% (w/w) or 25% (w/w) of used engine oil was added to the appropriate substrate samples. Six pots were sown with ribwort (about 200 seeds/0.053 m^2 area). The earlier isolated living spores of mycorrhizal fungi were added to the half of soil samples (Table 1). During the experiment the moisture of 70% WHC (Water Holding Capacity) was maintained in all samples. To eliminate external effects the process was performed in the climate chamber, produced by TIMKO, at 21/18°C (day/night), at 80% air humidity and light intensity of 25000 lm/m^2 area in the cycle of 14/10 hours (day/night). The experiment was carried out in triplicate. The soil material was sampled from the pots in 14 day intervals, during 88 days of experiment. The following factors were determined:

– total number of saprophytic bacteria,
– number of hydrocarbons degrading bacteria,
– number of molds fungi.

Tabela 1. Modification of the samples of soil substrate

Sample	Modification
1	Soil
2	Soil with plants
3	Soil with plants and mycorrhizal fungi
4	Soil wi th 10% crude oil
5	Soil with 10% crude oil and plants
6	Soil with 10% crude oil and plants and mycorrhizal fungi
7	Soil with 25% crude oil
8	Soil with 25% crude oil and plants
9	Soil with 25% crude oil and plants and mycorrhizal fungi

The number of microorganisms was determined by the method of surface inoculation on solid media. Before the experiments, 10 g of soil from the pots was introduced into 90 cm^3 of the sterile isotonic salt solution, and the mixture was shaken for 10 minutes. From this suspension the decimal dilutions were prepared. They were used to inoculate the culture substrate. The quantitative determination of the number of microorganisms was carried out by counting the colonies grown on the culture medium. The total number of saprophytic bacteria was estimated by counting the colonies grown on the MPA agar medium, according to the PN-75/C-04615103 standard [25] and the total number of molds fungi on the Czapek-Doxa medium with chloramphenicol on the basis of PN-75/C-04615103 [26]. The hydrocarbons degrading bacteria were determined on the minimal mineral medium with addition of 1% (w/w) of used engine oil as the sole carbon source [6], [7].

The mathematical analysis of the results was performed using the Statistica software. The following values were computed:

– arithmetic mean,
– standard deviation.

Differences between the control and the contaminated samples were checked for significance by the Dunnett's test.

RESULTS

The physicochemical analysis of soil substrate is shown in Table 2 and the hydrocarbons concentration in used engine oil are presented in Table 3.

Table 2. Physico-chemical analysis of soil substrate

Parameters		Results
pH_{KCl}		6.5
Organic carbon [g kg^{-1} of dry weight]		134.4
Total phosphorus [g $P\text{-}PO_4$ kg^{-1} of dry weight]		2.0
Total nitrogen [g kg^{-1} of dry weight]		20.32
Hydrometer texture [%]	φ – 0.1 mm	38
	φ 0.1 – 0.2 mm	37
	φ < 0.02 mm	25

The influence of used engine oil concentrations on the number of microorganisms in the soil

The influence of used engine oil on the number of microorganisms is presented in Figures 1, 2, 3.

Table 3. Concentration of aromatic and aliphatic hydrocarbons in used engine oil

Hydrocarbon	Concentration [mg kg^{-1} of used oil]
Naphtalene	274.0
Acenphtene	2.26
Fluorene	13.3
Phenanthrene	46.6
Anthracene	19.4
Fluoranthene	212.6
Pyrene	29.5
Benzo(a)anthracene	30.8
Chrysene	75.6
Benzo(b)fluoranthene	3.6
Benzo(k)fluoranthene	5.3
Benzo(a)pyrene	17.7
Benzo(ghi)perylene	22.7
Dibenz(a,h)anthracene	12.1
Indeno(1,2,3-cd)pyrene	21.4
Aliphatic hydrocarbons	891000

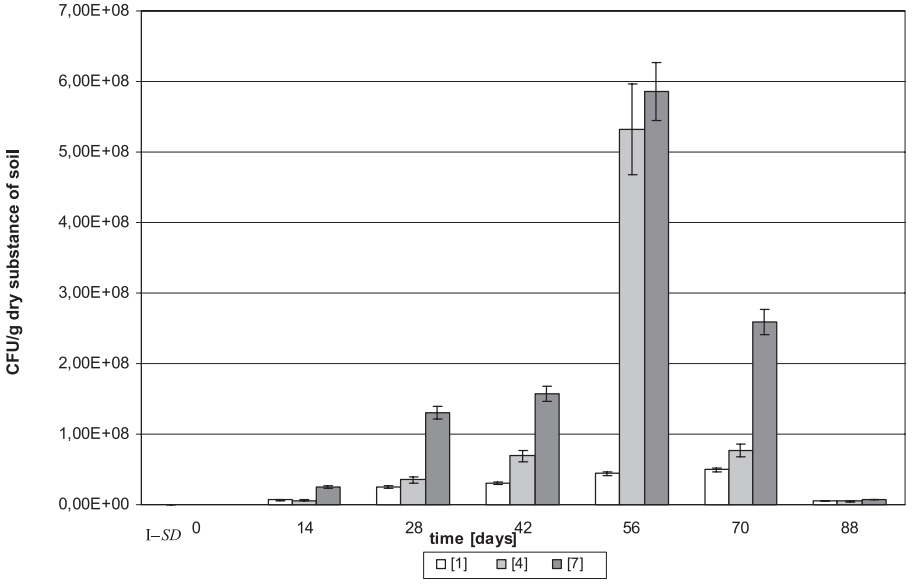

Fig. 1. The total number of saprophytic bacteria in the soil contaminated with used engine oil during natural soil attenuation

SD – error bars stand for SD
CFU – colony forming unit
[1] – soil
[4] – soil with 10% used engine oil
[7] – soil with 25% used engine oil

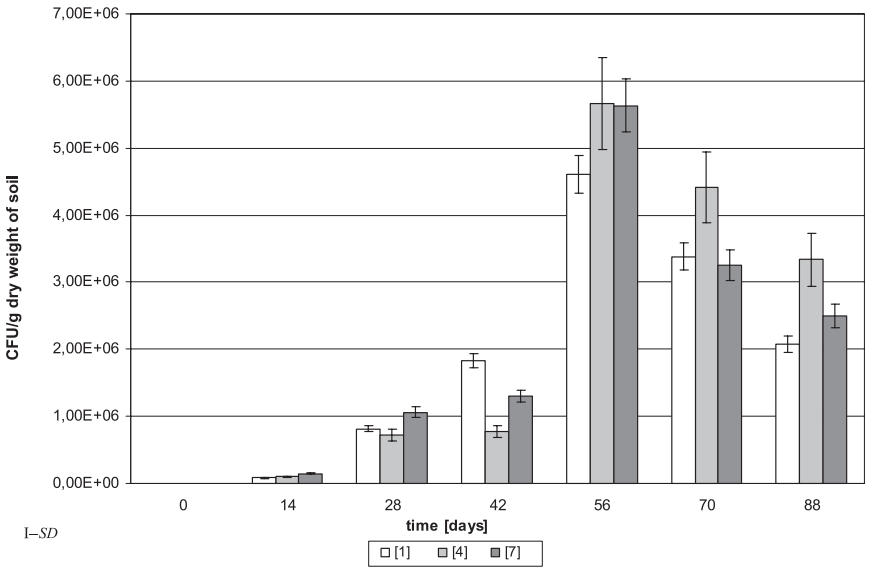

Fig. 2. The number of hydrocarbons degrading bacteria in the soil contaminated with used engine oil during natural soil attenuation

SD – errors bars stand for SD
[1] – soil
[4] – soil with 10% used engine oil
[7] – soil with 25% used engine oil

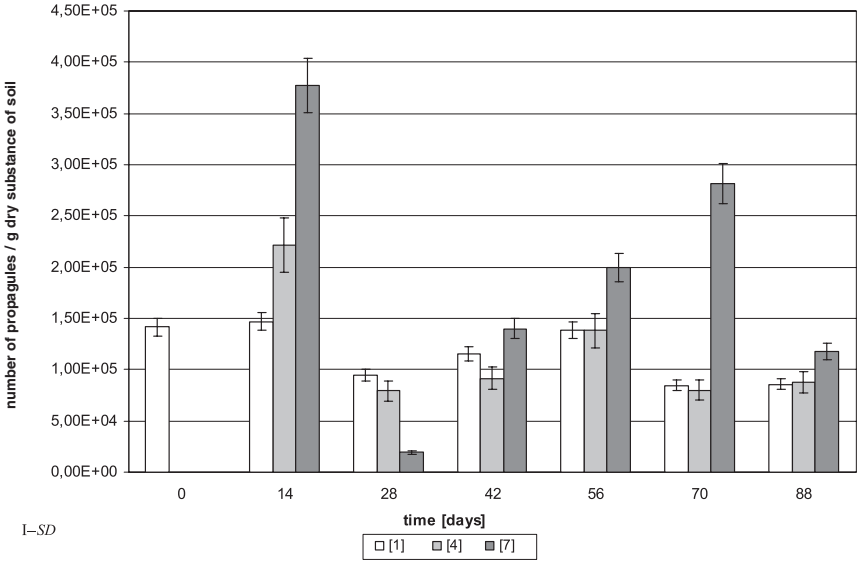

Fig. 3. The number of molds fungi in the soil contaminated with used engine oil during natural soil attenuation

SD – errors bars stand for SD
[1] – soil
[4] – soil with 10% used engine oil
[7] – soil with 25% used engine oil

Our studies showed a larger number of soil microorganisms in the soil samples contaminated with used engine oil in comparison with the control samples (Figs 1–3). The largest number of bacteria was found in all analyzed samples on the 56th day of natural soil attenuation (Figs 1 and 2). The number of saprophytic bacteria in the soil samples contaminated with 10% (w/w) and 25% (w/w) of used engine oil was 12 and 13 times larger than the number of bacteria in the control sample (Fig. 1). The soil contamination by oil products stimulated also the growth and development of hydrocarbons degrading bacteria (Fig. 2). The increase of the number of molds fungi was also observed. Their number increased by ca. 30%, in the soil sample, contaminated with 25% (w/w) of used engine oil, in comparison with the control sample (Fig. 3).

The test of significance showed that the addition of 10% (w/w) of used engine oil affected significantly the increase of the number of saprophytic bacteria on the 14th, 28th, 42nd, 70th, and 88th day of the studies. In the case of bacteria capable to decompose hydrocarbons the significant effect of the addition of 10% (w/w) of used engine oil on the increase of the number of these bacteria in respect to the uncontaminated sample, on the 56th, 70th, and 88th day of the studies, was shown. On the other hand, the addition of 25% (w/w) of used engine oil affected significantly the growth and development of molds fungi during the total period of the studies, in comparison with the control sample.

The influence of plants on the number of soil microorganisms in soil contaminated with used engine oil

Figs 4–6 present the number of microorganisms in the soil contaminated with used engine oil during the phytoremediation process.

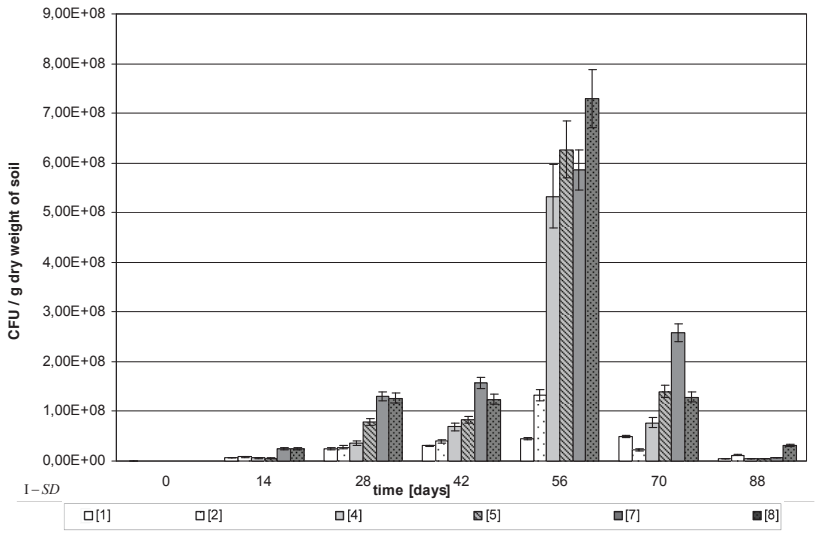

Fig. 4. The total number of saprophytic bacteria in the soil contaminated with used engine oil during the phytoremediation process

SD – errors bars stand for SD
[1] – soil
[2] – soil with plants
[4] – soil with 10% used engine oil
[5] – soil with 10% used engine oil and plants
[7] – soil with 25% used engine oil
[8] – soil with 25% used engine oil and plants

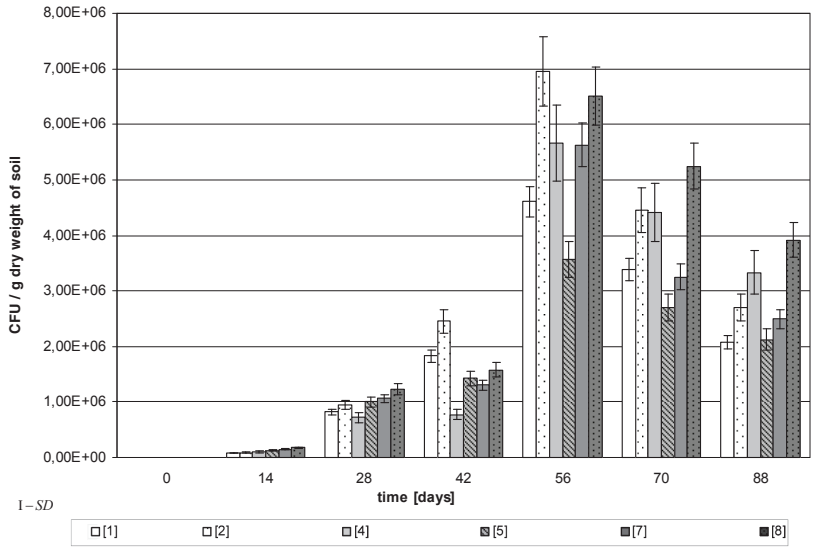

Fig. 5. The number of hydrocarbons degrading bacteria in the soil contaminated with used engine oil during the phytoremediation process

SD – errors bars stand for SD
[1] – soil
[2] – soil with plants
[4] – soil with 10% used engine oil
[5] – soil with 10% used engine oil and plants
[7] – soil with 25% used engine oil
[8] – soil with 25% used engine oil and plants

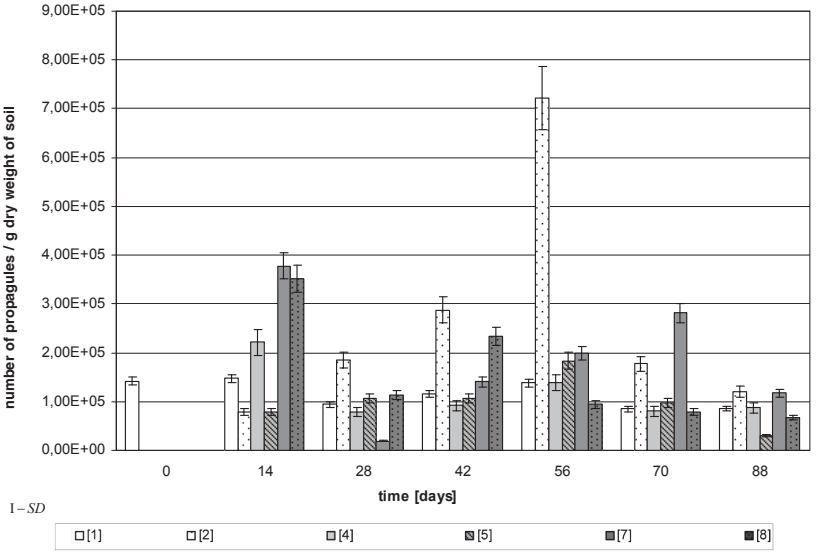

Fig. 6. The number of molds fungi in the soil contaminated with used engine oil during the phytoremediation process

SD – errors bars stand for SD
[1] – soil
[2] – soil with plants
[4] – soil with 10% used engine oil
[5] – soil with 10% used engine oil and plants
[7] – soil with 25% used engine oil
[8] – soil with 25% used engine oil and plants

This study showed that the presence of ribwort had the significant effect on the number of microorganisms. In the case of the total number of saprophytic bacteria (Fig. 4) on the 56th day of the natural attenuation the sample of soil sown with ribwort and the samples of soil contaminated with 10% and 25% of used oil and sown with ribwort possessed larger numbers of bacteria than the same samples without plants.

The number of saprophytic bacteria in the control soil sample sown with ribwort was almost 3 times larger than in the control soil sample without plants. In the soil samples contaminated with 10% and 25% of oil and sown with ribwort the total number of saprophytic bacteria was respectively 14 and 16 times larger than in the control sample (Fig. 4).

In the soil sample with addition of 25% of oil and sown with ribwort, the number of hydrocarbons degrading bacteria was 30% larger than in the control sample (Fig. 5).

The total number of molds fungi in the sample of the uncontaminated soil sown with ribwort was about 5 times larger than in the control sample. In the sample of the soil contaminated with 10% of oil and sown with ribwort the number of fungi increased slightly, in the sample contaminated with 25% of oil with ribwort the number of molds fungi decreased ca. two times in comparison to the soil sample with addition of 25% of oil, in the absence of plants (Fig. 6).

The seeding soil with ribwort improved the conditions of growth of microorganisms in the soil sample contaminated with used oil. The significance test showed the relevant effect of ribwort on the increase of the number of all studied microorganisms. The applied modification of soil (10% (w/w) of oil + ribwort) caused the significant effect on the number of saprophytic bacteria in respect to the samples that were not sown with plants (excluding the 14th and 88th day of the studies). A similar relationship was observed for the hydrocarbons degrading bacteria up to the 42nd day. Moreover, a significant effect of the soil modification (25% of oil + ribwort) on the number of bacteria capable to decompose hydrocarbons was also observed during the whole period of the studies, in relation to the samples in the absence of plants. The significance test showed the relevant effect of the applied modification – 10% of oil + ribwort – on the number of molds fungi on the 28th, 42nd, 56th, and 70th day of the studies, in relation to the modification in the absence of the plant.

The effect of plant mycorrhizas on the number of soil microorganisms in the samples of soils contaminated with used engine oil

Figures 7, 8 and 9 present the variations of the number of microorganisms in the soil contaminated with used engine oil during the phytoremediation process with use of mycorrhizal plants.

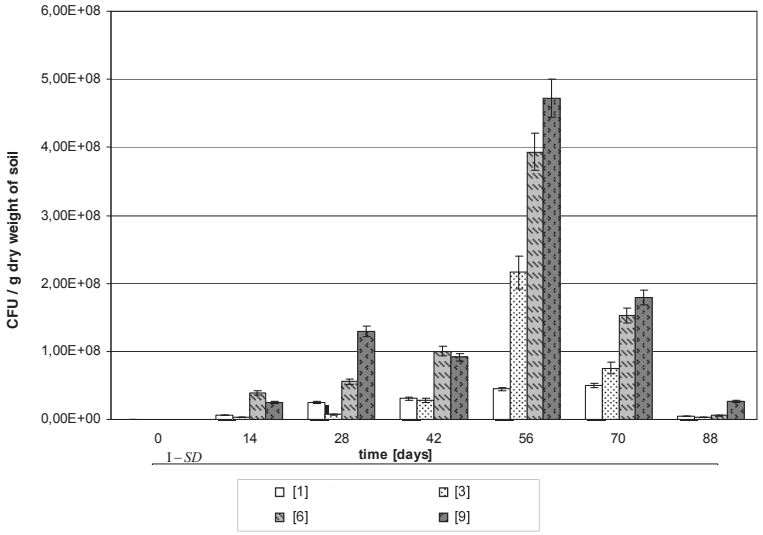

Fig. 7. The total number of saprophytic bacteria in the soil contaminated with used engine oil during the phytoremediation process with use of mycorrhizal plants

SD – errors bars stand for SD
[1] – soil
[3] – soil with plants and mycorrhizal fungi
[6] – soil with 10% used engine oil and plants and mycorrhizal fungi
[9] – soil with 25% used engine oil and plants and mycorrhizal fungi

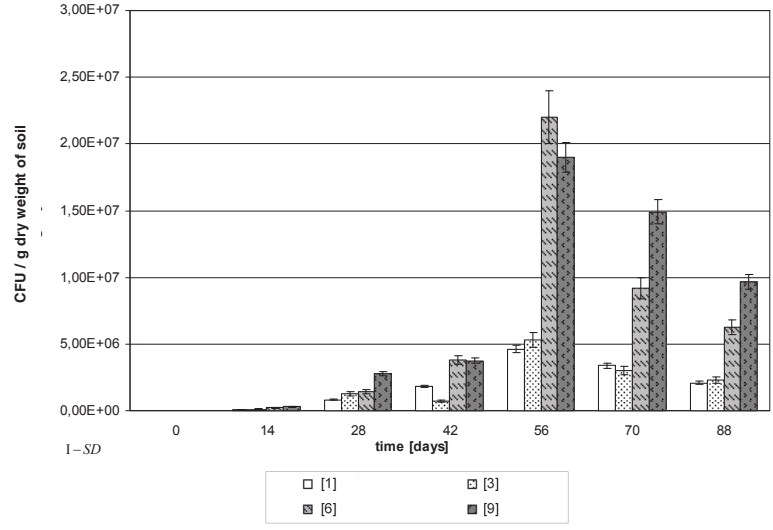

Fig. 8. The number of hydrocarbons degrading bacteria in the soil contaminated with used engine oil during the phytoremediation process with use of mycorrhizal plants

SD – errors bars stand for SD
[1] – soil
[3] – soil with plants and mycorrhizal fungi
[6] – soil with 10% used engine oil and plants and mycorrhizal fungi
[9] – soil with 25% used engine oil and plants and mycorrhizal fungi

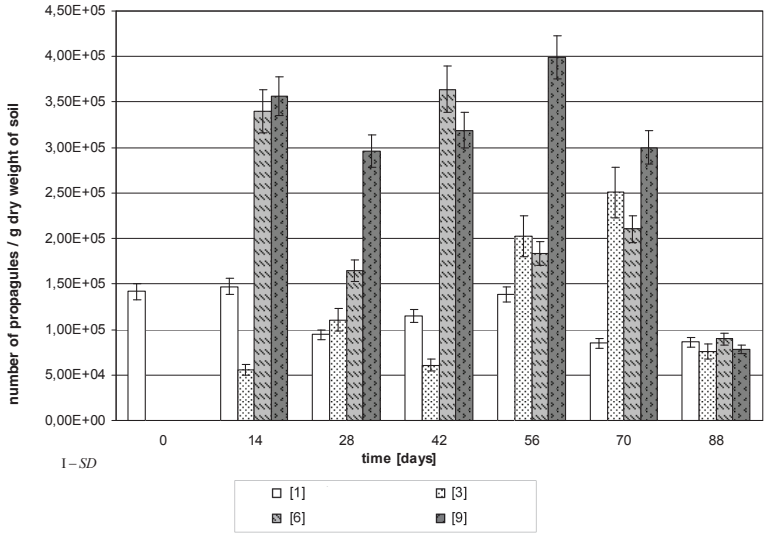

Fig. 9. The number of molds fungi in the soil contaminated with used engine oil during the phytoremediation process with use of mycorrhizal plants

SD – errors bars stand for SD
[1] – soil
[3] – soil with plants and mycorrhizal fungi
[6] – soil with 10% used engine oil and plants and mycorrhizal fungi
[9] – soil with 25% used engine oil and plants and mycorrhizal fungi

The application of soil modification, by using the mycorrhizal plants increased the number of microorganisms in the majority of samples, as compared with the samples without fungi. The largest number of bacteria was noted on the 56th day of phytoremediation (Figs 7–9). The number of saprophytic bacteria in the soil samples contaminated with 10% and 25% of oil, in the presence of the mycorrhizal plants was about 9 and 11 times larger than the number of bacteria in the control sample (Fig. 7). The number of hydrocarbons degrading bacteria in the same time and in the same samples was about 3 and 5 times larger than the number of bacteria capable to decompose hydrocarbons in the control sample (Fig. 8). While the number of molds fungi in the soil sample contaminated with 25% of engine oil, in the presence of the mycorrhizal plants was almost 3 times larger in relation to the control sample (Fig. 9).

The significance test showed that the application of the mycorrhizal ribwort had the relevant effect on the number of saprophytic bacteria on the 56th and 70th day of the studies. Moreover, a significant effect of the application of the modification – 10% and 25% of oil + mycorrhizal ribwort – on the number of these microorganisms on the 28th, 56th and 70th day was observed in respect to the samples that did not contain used engine oil. The significance test performed for hydrocarbons degrading bacteria showed that the modification – 10% of oil and mycorrhizal plants – affected significantly the number of these microorganisms in the total period of the studies, in respect to the samples that did not contain the oil. Whereas, in the case of molds fungi, the modification – 10% of oil + mycrorrhizal ribwort – affected significantly the increase of the number of molds fungi up to the 42nd day.

DISCUSSION

The influence of various concentrations of used engine oil on the number of microorganisms in the soil.

The performed analysis revealed a higher number of the soil microorganisms in the samples contaminated with used engine oil, in comparison with control soil samples (Fig. 1–3). The significant increase of the number of the hydrocarbons degrading bacteria, saprophytic bacteria and molds fungi in contaminated soil samples may indicate that the petroleum–derived products, present in the used engine oil, are used as nutrient substrates.

A similar relationship was observed by Cheung *et al*. [5], Ghazali *et al.* [10] and Gaskin and Bentham [8]. Basing on the literature data [11], [20], [22], [32], it may be assumed that decomposition of hydrocarbons in soils proceeds in two stages. Initially, when hydrocarbons are easily accessible to microorganisms, a relatively high biodegradation rate is controlled by the rate of the intake of contaminants and the rate of their metabolism by soil microorganisms. During the biodegradation process, the easily accessible hydrocarbons are gradually declined. Therefore, the biodegradation rate diminishes and the whole process is limited by the rate of desorption or washing out of the contaminants from soil colloids. The effect of this process might be the gradual decrease of the number of saprophytic bacteria and hydrocarbons degrading bacteria during the natural attenuation of the soil contaminated with used engine oil, lasting longer than 56 days (Figs 1–3). The same effect was observed by Chaineau *et al.* [4].

The decrease of the number of bacteria along the progress of the natural attenuation might be also caused by the hydrocarbons metabolism by fungi (Fig. 3) and the appearance of the intermediate reaction products in the soil environment requiring bacteria adaptation, for instance, by the production of appropriate enzymes. Microbial adaptation to the contamination is most often linked with the changes of metabolism or genetic changes [3], [27].

The effect of the plant mycorrhizas on the number of soil microorganisms in the soil samples contaminated with used engine oil

On the basis of the obtained results, it was stated that the presence of ribwort has a significant effect on the number of microorganisms. In the soil samples seeded with the plant, the number of saprophytic bacteria, hydrocarbons degrading bacteria and molds fungi was significantly higher in comparison with the control samples (Fig. 4–6). A similar phenomenon was observed by Gaskin and Benath [9] and Kirk *et al.* [17]. In their research, they proved that hydrocarbons present in the soil stimulated the number of microorganisms and together with the plants the effect was significantly increased. The literature studies [21], [23], [30], [33] show that the plant roots create favorable conditions for the growth and development of hydrocarbons degrading bacteria, by the plant root exudates (aminoacids, organic acids, plant growth hormones and soluble proteins).

The mycorrhizal fungi are one of the main groups in *rhizosphere* (next to bacteria). They take a particular place among the rhizosphere microorganisms, because they are widely spread. The ability of the root system to be associated with microorganisms, especially to create mycorrhizas, lets the plants survive and accept the environmental stress caused by soil pathogens, drought, soil pH changes and contamination. It is connected with the changes of roots physical features. Mycorrhiza modifies the root architecture:

hormone-like secondary metabolites clearly stimulate roots expansion [1], [19]; the root system is more bifurcated, it possess more lateral roots, it occupies larger volume of the ground.

Ribwort mycorrhization stimulated the growth and development of all studied microbial groups in comparison with samples without fungal partner. It seems to be particularly important that the number of hydrocarbons degrading bacteria in the soil samples seeded with mycorrhizal plants was 3–4 times higher than in the samples with plants without mycorrhiza.

CONCLUSIONS

The contamination of soil with used engine oil influenced significantly the number of soil microorganisms. The number of saprophytic bacteria in the contaminated samples was ca. 12 times higher than in the control sample. The presence of plants in the contaminated soil samples stimulated the growth and development of saprophytic bacteria, hydrocarbons degrading bacteria and molds fungi in comparison with samples without plants. The plants mycorrhization additionally stimulated the increase of microorganisms number, mainly of those in the the group of hydrocarbons degrading bacteria. The number of hydrocarbons degrading bacteria in the soil samples with mycorrhizal plants was ca. 5 times higher than their number in samples without mycorrhiza. The number of hydrocarbons degrading microorganisms increased together with used engine oil concentration.

Developing successful phytoremediation strategies is dependent on selecting plants that are most effective for removing or stabilizing the potential contaminant, e.g. used engine oil, from the soil or waters over a long period of time. Results may have extensive application nationwide to the problems associated with hydrocarbon contaminated sites.

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WPŁYW MIKORYZACJI BABKI LANCETOWATEJ NA WZROST MIKROFLORY W GLEBIE ZANIECZYSZCZONEJ PRZEPRACOWANYM OLEJEM SILNIKOWYM

Celem pracy było określenie wpływu mikoryzacji roślin na liczbę bakterii degradujących węglowodory, bakterii saprofitycznych oraz grzybów pleśniowych podczas remediacji próbek gleb zanieczyszczonych przepracowanym olejem silnikowym. Badania prowadzono w warunkach laboratoryjnych. Przygotowano dziewięć modyfikacji próbek podłoża glebowego, które podzielono na trzy grupy: pierwsza – próbki gleby bez dodatku przepracowanego oleju silnikowego, druga – z dodatkiem 10% wagowych przepracowanego oleju silnikowego oraz trzecia – z olejem w stężeniu 25%. W każdej z grup jedną próbkę obsiano babką lancetowatą, jedną – zaszczepiono żywymi sporami grzybów mikoryzowych i obsiano babką lancetowatą, a jedną pozostawiono bez roślin i grzybów mikoryzowych. Próbka gleby nieskażonej bez roślin i grzybów mikoryzowych była próbką kontrolną. Przeprowadzone badania wykazały istotny wpływ wprowadzonego oleju silnikowego, obecności babki lancetowatej oraz grzybów mikoryzowych na liczebność mikroorganizmów glebowych. W próbkach gleb skażonych przepracowanym olejem silnikowym zaobserwowano wzrost liczebności bakterii degradujących węglowodory w porównaniu z próbką kontrolną. Obsianie próbek gleb roślinami oraz dodatkowo zaszczepienie ich sporami grzybów mikoryzowych wpływało stymulująco na liczbę mikroorganizmów wszystkich badanych grup.