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# THE EFFECTS OF PAPILIONACEOUS PLANTS AND BIOPREPARATION ON PETROLEUM HYDROCARBONS DEGRADATION IN AGED-POLLUTED SOIL

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## WPŁYW ROŚLIN MOTYLKOWYCH I BIOPREPARATÓW NA USUNIĘCIE ROPOPOCHODNYCH Z GLEB O WIELOLETNIM ZANIECZYSZCZENIU

Zanieczyszczenie substancjami ropopochodnymi stanowi jeden z kluczowych problemów środowiskowych. Jedną z wielu metod stosowanych w remediacji gruntów jest ich bioaugmentacja. Celem tego procesu jest zwiększenie liczebności i aktywności mikroorganizmów degradujących zanieczyszczenia, czego wynikiem jest przyspieszenie i podniesienie wydajności procesów rozkładu zanieczyszczeń. Innym sposobem podniesienia efektywności procesów biodegradacji jest zastosowanie roślin, szczególnie motylkowych, ze względu na ich zdolności symbiotycznego wiązania azotu atmosferycznego. Celem przeprowadzonych badań było porównanie efektywności zastosowania biopreparatów (komercyjnego i naturalnego) oraz koniczyny białej (*Trifolium pratense*) do remediacji gleb o wieloletnim charakterze skażenia substancjami ropopochodnymi. Badania prowadzone były na glebie pochodzącej z terenu rafinerii w Czechowicach-Dziedzicach, która została zaklasyfikowana jako silnie zdegradowana (o stosunku C/N = 100/0,7). W trakcie czternastotygodniowych badań prowadzono między innymi analizy mikrobiologiczne (ogólna liczba bakterii, grzybów, promieniowców oraz bakterii z rodzaju *Pseudomonas*) oraz analizy chemiczne (zawartość frakcji ciężkich, całkowitej zawartości węglowodorów ropopochodnych (TPH) i WWA). Najlepszą modyfikacją okazało się zastosowanie samej koniczyny. Obecność rośliny wpłynęła na polepszenie warunków wzrostu mikroorganizmów, spowodowała wzrost zawartości azotu i efektywności procesów biodegradacji. W próbie tej po 14 tygodniach badań odnotowano usunięcie 63% TPH, 44% frakcji ciężkich oraz 9% i 80% odpowiednio 4–6- i 2–3-pierścieniowych węglowodorów aromatycznych.

Summary

Petroleum pollution is still one of crucial environmental problems. Bioaugmentation is a popular technique used in soil remediation. The aim of soil inoculation is acceleration of decomposition processes or improving the degradation efficiency. Effectiveness of bioaugmentation processes depends on the number and activity of microorganisms adapted to pollutant degradation. Enhancement of microorganisms' activity can be reached by the use of plants. Roots of plants excrete organic substances that stimulate microorganisms' growth. Among different species of plants interesting are papilionaceous plants because of their nitrogen fixation ability in symbiosis with bacteria. The effects of using papilionaceous plants (*Trifolium pratense*), multiplied autochthonous microorganisms and commercial biopreparation in aged-petroleum-polluted soil were studied. The samples of soil were taken from the refinery in Czechowice-Dziedzice (Poland) and classified as heavily degraded with a C/N-ratio of 100:0.7. Investigations were conducted for 14 weeks. Microbiological analysis included: total bacteria, fungi, *Actinomycetes* and *Pseudomonas* counts. Concentration of heavy fractions, TPH (total petroleum hydrocarbons) and PAHs (polycyclic aromatic hydrocarbons) were measured at the start and at the end of the experiment. Presence of papilionaceous plant (*Trifolium pratense*) enhanced the growth of microorganisms, nitrogen concentration and biodegradation processes (removal of 63% of TPH, 44% of heavy fractions, 9% of 4-6 aromatic PAH and 80% of 2-3 aromatic PAH) in polluted soil. An increasing number of *Pseudomonas* species was observed in samples in which pollution removal was more effective.

## INTRODUCTION

The majority of petroleum hydrocarbons contaminating water-soil environment undergo biotransformation or biodegradation processes. The scale and speed of these processes depend on many environmental and microbiological agents e.g. soil properties, age and concentration of pollution, presence of accompanying substances and first of all properties of pollutant [8, 18, 23, 32, 34, 40]. Biological reclamation in soil is possible if petroleum hydrocarbons concentration does not exceed 10% [22].

Bioaugmentation is a recommended cleaning technology in order to increase petroleum hydrocarbons removal from polluted soil. With this technique multiplied autochthonous microflora or ready to use commercial products are used. Biopreparation is based on the application of microorganisms that are adapted to and selected for the degradation of the contaminant under scrutiny. The purpose of introduction of biopreparation into soil is an increase in the number and activity of autochthonous microflora [8, 14, 30, 36, 43, 44, 47]. It is reported that a lot of bacteria species can degrade petroleum hydrocarbons. Between them *Pseudomonas* species are very effective in biodegradation of petroleum hydrocarbons [30, 43].

Another remediation technique is phytoremediation. This technology uses plants properties to enhance biodegradation of contaminants. Removal of pollutants is possible by uptake and subsequent volatilization of certain organic compounds, transformation or by supporting microbiological processes in the rhizosphere [2, 15, 27, 39, 43]. Plant roots exude many organic substances such as organic acids, phytohormones, and carbohydrates. The presence of an additional bioavailable carbon sources promotes growth and activity of microorganisms and can be helpful during the co-metabolic degradation. Special attention is paid to usage of nitrogen-fixating organisms. In petroleum hydrocarbon polluted soil C/N ratio is always lower than the ratio required for efficient bioremediation process. Nitrogen-fixating organisms, such as Papilionaceous plants, enrich soil with nitrogen in cooperation with bacteria that grow in nodules. The amount of nitrogen fixed by such symbiosis is higher than can be used by plants so nitrogen-fixating plants do not enlarge the nitrogen deficit in the soil as other groups can do [21, 27, 41]. Deficit of nitrogen in soil can be reduced by mineral fertilization. Uncontrolled and improper usage of mineral fertilizers can cause additional changes in the environment such as eutrophication, especially at water environment (as a result of soil rising) [25, 45, 46].

The structure of hydrocarbons polluted soil is destroyed because of conglomeration of soil particles and hence air and water penetration in this environment is limited. Advantages of phytoremediation are connected with the structure of soil. Plant roots penetrating soil improve oxygen conditions in the soil. Plants can limit the soil erosion, water loss by evaporation and provide the biomass profitable for soil processes [31, 37, 39].

The aim of this study was the comparison of the effect of using different modifications in studied aged-polluted refinery soil samples. Used modifications were: bioaugmentation by multiplied autochthonous microorganisms (natural biopreparation), microorganisms from a commercial biopreparation and seeding papilionaceous plant (*Trifolium pratense L.*) together with biopreparation and separately. Increase of microbial number and activity as well as effectiveness of hydrocarbons removal was expected.

We supposed that in aged-polluted soil rich in TPH (total petroleum hydrocarbons containing brunched and non-brunched $C_{12}$-$C_{35}$ hydrocarbons) and heavy fractions (dry residue after extraction in $CCl_4$ and evaporation of light volatile hydrocarbons), microorganisms adapted for this kind of contamination should be present and active. We decided to isolate them, multiply and introduce them into polluted soil. For comparison a commercial product recommended for oil polluted sites reclamation was tested. In aim to improve the soil properties (enhance C/N ratio, improve air-water and microorganisms growth conditions) clover (*Trifolium pratense* L.) was used.

## MATERIALS AND METHODS

### Soil samples

Soil samples were collected from the refinery in Czechowice-Dziedzice in Poland. All samples were taken at 0–30 cm depth in the area which was not covered by plants. The soil was classified as a loamy soil (granular analysis). Initial analysis detected that the main contaminants were heavy fractions – 16% and TPH 1.9 %. Aromatic hydrocarbons (PAH) with 2–6 rings were in concentration 32.1 mg/kg of dry soil matter what was about 0.0032% (Tab. 1). Proportion of C/N was 100:0.7. Soil samples were sieved, mixed, moistened with water and then put into plastic pots (300 g per pot). During the experiment the humidity was maintained at 25% of weight.

Table 1. Contents of petroleum hydrocarbons in clay soil at the beginning of the experiment (TPH, heavy fractions, PAH)

| TPH [g/kg dry soil matter] | Heavy fractions [g/kg dry soil matter] | PAH [g/kg dry soil matter] | |
|---|---|---|---|
| 19.11 | 159.11 | 0.032 | |
| | | 2–3 aromatic PAH | 4–6 aromatic PAH |
| | | 0.011 | 0.021 |

### Biopreparations

Studied samples were inoculated with natural biopreparation or microorganisms from commercial product. As a natural biopreparation, a cultured mixture of autochthonous bacteria isolated from the studied soil was used. Classification of microorganisms was not done. Characteristic of growth on solid media suggested mixture of different species. Microorganisms were isolated on liquid mineral medium ($KH_2PO_4$ – 1.56 g, $Na_2HPO_4$ – 2.13 g, $(NH_4)_2SO_4$ – 0.5 g, $MgSO_4 \cdot 7H_2O$ – 0.2 g, $CaCl_2 \cdot 2H_2O$ – 0.02, 1000 $cm^3$ distilled water pH 7.2 – autoclaved at 121°C 1.2 atm. for 20 minutes) with crude oil as the only carbon source (1% v/v). Incubation was done for 14 days at temperature 26°C. Biomass was centrifuged (5 000 rpm) and washed with physiological salt solution three times. Subsequently biomass was resuspended in physiological salt solution and introduced to the soil. The suspension of microorganisms introduced to soil included 6.5 x $10^5$ cfu/g dry soil matter (evaluation was done by plate method on MPA (Meat-Pepton Agar) medium). As a second biopreparation microorganisms from the commercial product, recommended for removing petroleum hydrocarbons from soil and water (BioActiv HGS 208 - POCh), were applied. Species composition of the product was not characterized by manufacturer and was not verified during experiment. Biomass introduced to the soil was measured and was on the level of 7.2 x $10^5$ cfu/g dry soil matter (the same method as for natural biopreparation). The plant used for phytoremediation was clover (*Trifolium pratense* L.) whose seeds were bought at a local market. These modifications (biopreparations and plants) were used together or separately. Control soil sample was not modified. Modifications of soil during experiment: control (contr.), soil with natural biopreparation (s + n.b), soil with commercial biopreparation (s + c.b), soil with clover (s + cl.), soil with clover and natural biopreparation (s + cl + n.b) and soil with clover and commercial biopreparation (s + cl + c.b). To each pot 20 seeds were added. Samples were put into phytothron (climatic chamber) in order to ensure stabile term and light conditions (temperature 16° at night and 21°C during the day). Studies were conducted for a 14 week period. All samples were done in triplicate.

**Chemical analysis**

Performed analytical analyses included concentration in soil: heavy fraction of petroleum hydrocarbons (weight method), PAH (HPLC with fluorescent detection) and TPH (gas chromatography GC) at the beginning and the end of experiment. All extractions were done with $CCl_4$. TPH is defined as total petroleum hydrocarbons containing brunched and non-brunched $C_{12}$-$C_{35}$ hydrocarbons and heavy fractions are defined as dry residue after extraction in $CCl_4$ and evaporation of light volatile hydrocarbons. Organic carbon (Tiurin method), total nitrogen (Kjeldahl method) and pH (with hydrogen-electrode method) were analyzed at the beginning, in the $4^{th}$ week, in the $8^{th}$ week and at the end of experiment [29].

**Microbiological analysis**

Microbiological analysis (spread method) included determination of total bacteria number on nutrient agar medium (MERCK 105450.0500), fungi number on Czapek-Dox agar medium (MERCK 105460.0500), *Actinomycetes* number on medium with arginine (glycerol – 12.5 g, arginine L – 1.0 g, NaCl – 1 g, $K_2HPO_4$ – 1 g, $MgSO_4 \cdot 6H_2O$ – 0.5 g, $Fe_2(SO_4)_3 \cdot 6H_2O$ – 0.01 g, $CuSO_4 \cdot 5H_2O$ – 0.001 g, $ZnSO_4 \cdot 7H_2O$ – 0.001 g, distilled water 1000 $cm^3$, pH 6,8–7,0) and *Pseudomonas* plating on Oxoid selective medium (Pseudomonas C-F-C selective supplement, Code SR 103E). Petri-plates were incubated at 26°C. Last group (*Pseudomonas*) was chosen for investigation on the basis of literature studies confirming meaningful participation of this group in elimination of petroleum hydrocarbons contaminations. Statistical analysis was done with Statistica 5.1.

## RESULTS

**Removal of petroleum hydrocarbons**

The studied soil was taken at the refinery from the open place exposed to heavy pollution for a long time. The high pollution of the surface made plant growth impossible. Initial hydrocarbons concentration was: TPH 19.11 g/kg dry soil matter, heavy fractions – 159.11 g/kg of dry soil matter and PAH 2–3 aromatic and 4-6 aromatic 0.011 and 0.021 g/kg of dry soil matter respectively (Tab. 1). The results of petroleum hydrocarbons removal after 14 weeks of experiment are shown in Figure 1. The highest elimination of TPH was observed in the sample with clover as modification (63.0%) and clover with commercial biopreparation (21.6%). In other samples loss of petroleum hydrocarbons was not observed. Removal of heavy fractions of petroleum hydrocarbons was also the highest in the sample with clover (44.0%), good result was observed in the sample with clover and natural biopreparation (34.0%). In the sample with clover and commercial biopreparation elimination of heavy fractions was on the level of 16.7%, the same in the sample with commercial biopreparation (16.0%) and they did not differ from the control (14.9%). At the same time in the sample with natural biopreparation elimination of heavy fractions it was 10%.

Table 2. Contents of total nitrogen and organic carbon and pH in contaminated soil

| Modification | Total N [g/kg dry soil matter] | | | | Organic C [g/kg dry soil matter] | | | | pH | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 week | 4 week | 8 week | 12 week | 0 week | 4 week | 8 week | 12 week | 0 week | 4 week | 8 week | 12 week |
| contr. | | 1.06 | 1.13 | 1.18 | | 182 | 164 | 201 | | 6.3 | 6.6 | 6.0 |
| s + cl. | | 1.33 | 1.37 | 1.31 | | 161 | 179 | 133 | | 5.8 | 6.2 | 5.9 |
| s + n.b | 1.24 | 1.17 | 1.18 | 1.16 | 175 | 165 | 236 | 182 | 5.8 | 6.4 | 6.3 | 6.5 |
| s + cl. + n.b | | 1.43 | 1.63 | 1.55 | | 179 | 201 | 157 | | 6.1 | 6.3 | 5.9 |
| s + c.b | | 1.10 | 1.14 | 1.18 | | 159 | 189 | 170 | | 6.3 | 6.5 | 6.4 |
| s + cl. + c.b | | 1.44 | 1.59 | 1.55 | | 177 | 177 | 152 | | 6.1 | 6.1 | 5.9 |

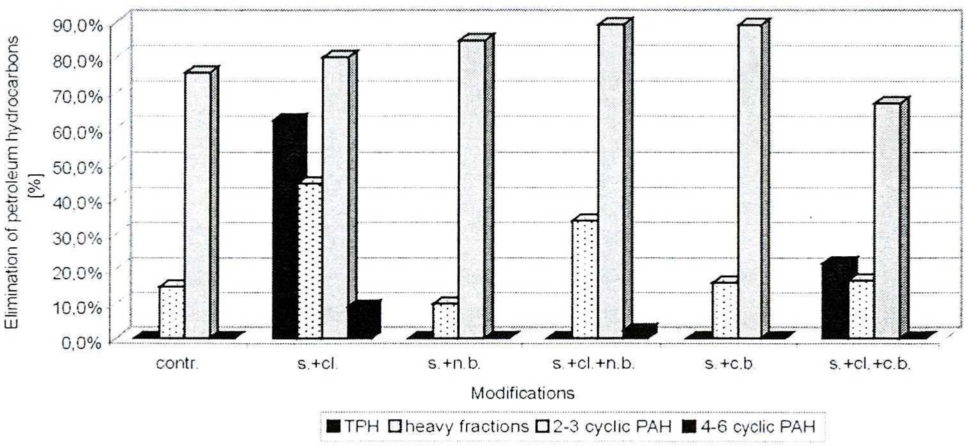


Fig. 1. Removal of petroleum hydrocarbons in modified soil

Efficiency of PAH removal was connected with number of aromatic rings. Loss of 2–3 aromatic PAHs in samples with clover, with natural biopreparation, with clover and natural biopreparation and with commercial biopreparation was close to 80–90%. In the control sample elimination was on the level of 75.6% what can suggest participation of evaporation of light hydrocarbons in pollutants losses and activity of natural microflora. The lowest removal was observed in the sample with clover and commercial biopreparation (67%). The highest elimination of 4–6 PAHs (9%) was observed in soil with clover. In the sample with clover and natural biopreparation 2.4% of this fraction was removed. In other samples loss of 4–6 PAHs was not observed.

**Microbiological analysis**

Fluctuations of bacteria number are shown on Figure 2. In the control sample a slow decrease of total bacteria number was observed during experiment ($2.5 \cdot 10^7$–$3 \cdot 10^5$ cfu/g of dry soil matter). In the sample with natural biopreparation tendencies were the same but decrease was slower. During the experiment significant fluctuations were observed in other samples. The highest number of bacteria was measured in the samples with clover and with clover and the commercial biopreparation. At the beginning of experiment the highest number was observed in soil with clover ($6.7 \cdot 10^7$ cfu/g). In the $10^{th}$ week a total bacterial number decreased (from $6.7 \cdot 10^7$ up to $2.2 \cdot 10^7$ cfu/g of dry soil matter). Next weeks bacteria number was increasing and reached $1.5 \cdot 10^8$ cfu/g dry soil matter at the end of experiment. In the sample with clover and commercial biopreparation from the $10^{th}$ week a total bacteria number dramatically increased (from $7.0 \cdot 10^6$ at $8^{th}$ week up to $1.6 \cdot 10^8$ cfu/g of dry soil matter in the $14^{th}$ week).

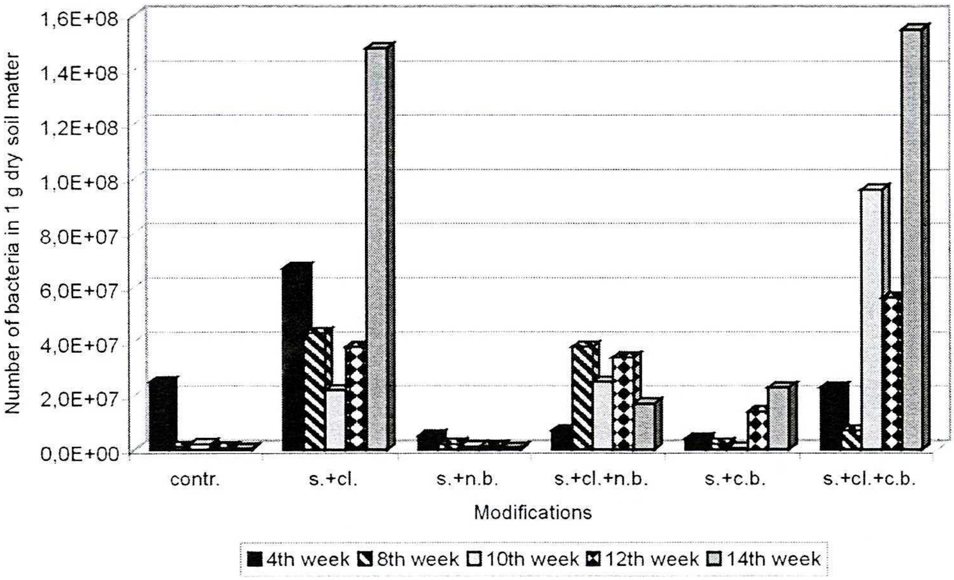


Fig. 2. Influence of used modifications on changes of total bacteria number

Similar tendencies were observed in the *Pseudomonas* counts (Fig. 3). The highest number of these bacteria was observed in samples with clover, and clover with commercial biopreparation. Percentage of *Pseudomonas* bacteria in total bacteria number (Tab. 2) was the lowest in the control sample, (0.7–5.0%), soil with natural biopreparation (1.6–6.0%) and soil with commercial biopreparation (0.1–5.0%). In the sample with clover participation of *Pseudomonas* group was from 2.2% (in the $4^{th}$ week) to 26.4% (in the $10^{th}$ week). In soil with clover and natural biopreparation contribution of *Pseudomonas* group in total bacteria number was the highest at the beginning of experiment (30% in the 4th week) and slowly decreased up to 16.5% at the end of experiment. The highest percentage of *Pseudomonas* in total bacteria number was in the 8th week (40%) in sample with clover and commercial biopreperation. During the experiment these bacterial counts varied from 10% to 40%.

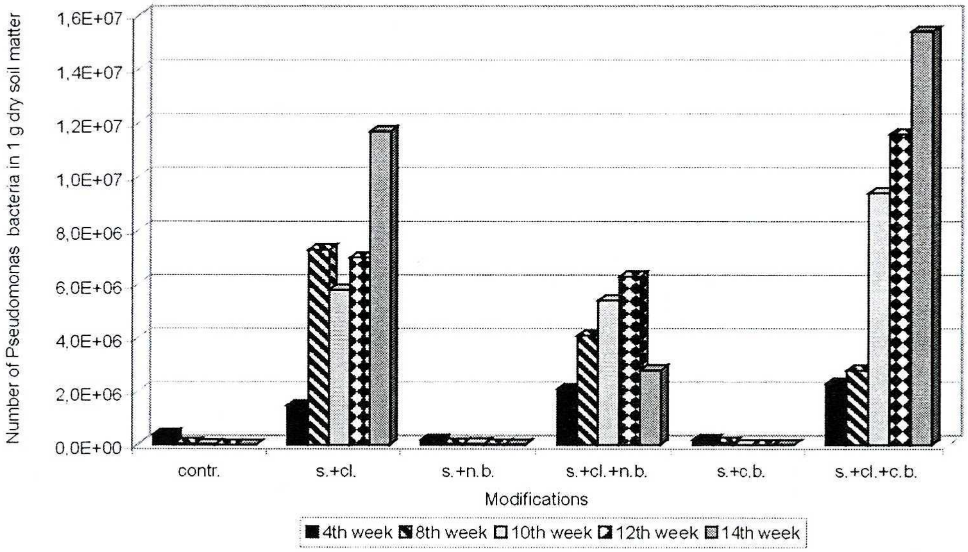


Fig. 3. Number of *Pseudomonas* bacteria in 1 g dry soil matter

Table 3. Percentage of *Pseudomonas* bacteria in total bacteria number

| Percentage of *Pseudomonas* bacteria in total bacteria number | | | | | | | |
|---|---|---|---|---|---|---|---|
| Modifications | | contr. | s + cl. | s + n.b | s + cl. + n.b | s + c.b | s + cl. + c.b |
| Week of experiment | 4 | 1.6% | 2.2% | 4.0% | 30.0% | 5.0% | 10.0% |
| | 8 | 5.0% | 17.0% | 2.0% | 10.7% | 4.0% | 40.0% |
| | 10 | 1.5% | 26.4% | 4.5% | 21.6% | 0.5% | 9.8% |
| | 12 | 0.7% | 18.0% | 1.6% | 18.5% | 0.1% | 20.1% |
| | 14 | 0.7% | 7.9% | 6.0% | 16.5% | 0.1% | 9.9% |

Total fungi number is shown in Figure 4. The highest fungi number was observed in samples with clover and with clover with commercial biopreparation ($8.0 \cdot 10^3$ to $5.2 \cdot 10^4$ and $3 \cdot 10^3$ to $4.8 \cdot 10^4$ propagules/g of dry soil matter respectively). A high number of fungi were also observed in soil with clover and natural biopreparation ($5.0 \cdot 10^3$–$2.5 \cdot 10^4$ propagules/g of dry soil matter respectively). The number of fungi was increasing during experiment and reached the highest value in the 14th week. In the other samples (control, soil with natural biopreparation and soil with commercial biopreparation) the number of these microorganisms was much lower and almost at the same level during the whole experiment.

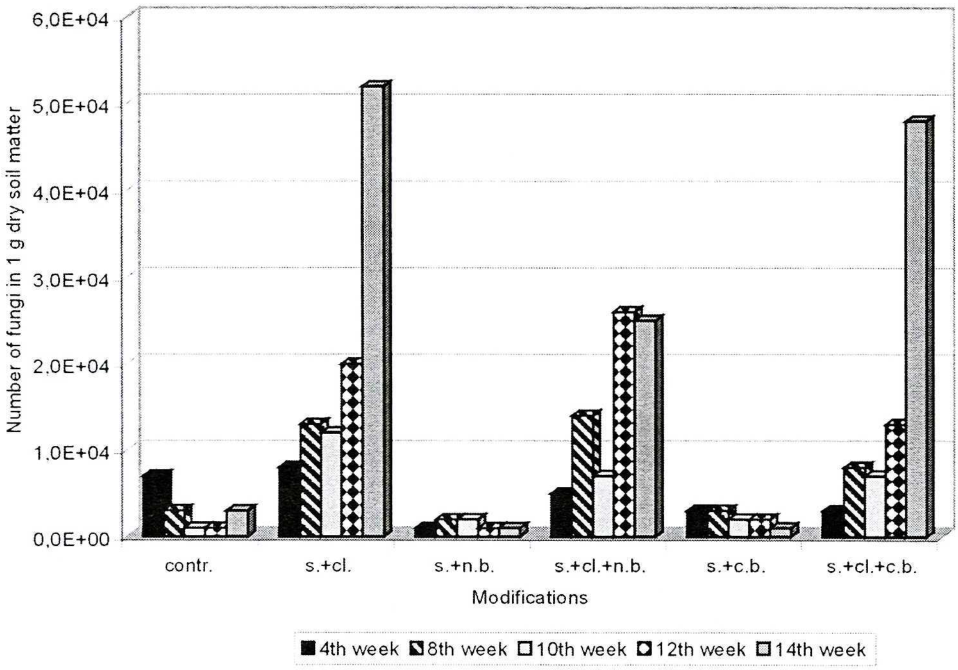


Fig. 4. Influence of used modifications on changes of total fungi number

**Physico-chemical properties of soil**

Changes in physico-chemical properties of the soil were observed (Tab. 2). The pH increased during the experiment and varied between 5.8 at the beginning of the experiment and 6.6 (in the 8$^{th}$ week). At the same time an increase in total organic carbon concentration was observed. The highest value was measured in the 8$^{th}$ week. The highest increase was observed in samples with low degradation and low microorganisms number (control, soil natural biopreparation, soil with commercial biopreparation). In samples with high microorganism's number a total organic carbon decreased (from 175 g/kg at the beginning to 133 g/kg at the end of experiment in sample with clover). Presence of clover in soil caused the increase of nitrogen in samples. In soil with clover the N content increased from 1.24 g/kg to 1.31 g/kg at the end. The highest concentration of nitrogen at the end of experiment was in samples with clover and biopreparations (both, commercial and natural). In both samples final concentration of nitrogen was 1.55 g/kg.

DISCUSSION

At the agricultural areas acceptable concentration of crude oil in upper part of soil (0–0.3 m) is 50 mg•kg of dry soil matter (Dz. U. Nr 165, poz. 13559). Fundamental criterion in evaluation of soil degradation level is C/N ratio [40]. Optimal C/N ratio accepted for biodegradation is 250/10 but some authors recommend 100/10 [26, 40, 46]. In studied soil proportion C:N was 100/0.7. Absence of flora in soil samples collecting place indicated the high destructions of that area.

The most intensive biodegradation processes were observed in sample with clover. This sample was characterized by the highest removal of all fractions of hydrocarbons. In other samples the most eliminated fractions were 2–3 PAHs. Low molecular mass and high vapor pressure of 2 aromatic PAH could be a reason of considerable participation of abiotic processes in contaminants losses [12, 34, 42].

The highest elimination of petroleum hydrocarbons in the sample with clover corresponded with high number of bacteria and fungi. The number of these microorganisms increased during experiment more than 100 times relatively to the control sample. Clover represents Papilionaceous group, which is more and more used in bioremediation processes. Thanks to the symbiosis with root nodule bacteria, growth of Papilionaceous plants is not strongly dependent on the nitrogen level in soil. These plants do not participate in increase of nitrogen deficit and through root secretions enrich soil with biogenic elements in environment [1, 19, 34, 38, 40, 41]. In samples with plants an increase of total nitrogen was observed.

Overlapping fluctuations of bacteria and fungi number were described by Cerniglia [10] what could confirm possible cooperation of both groups of microorganisms in soil detoxification. Fungi can metabolize hard degradable hydrocarbons and supply easier degradable semi-finished products of decomposition for bacteria [11]. Bacteria through production of biosurfactants can enlarge bioavailability of hydrocarbons and their desorption from soil [4, 5, 24, 28].

The efficiency of biodegradation processes depends on numerous factors, for example on number and activity of native microflora with potential for pollutant degradation. In some cases soil enrichment with microorganisms (bioaugmentation) is a prerequisite step for intensification of contaminant decomposition [7, 20, 40, 45).

In our experiment natural microflora such as multiplied biomass of mixed autochthonic population was applied as a biopreparation. This mixed culture was used in conditions they were adapted to. Numerous authors show advantages of such intervention [6, 7, 36, 45]. Another modification was usage of commercial biopreparation recommended for removal of petroleum hydrocarbons from soil. Biomass of biopreparations introduced to soil responded to (for natural biopreparation) – $6.5 \cdot 10^5$ cfu/g dry soil matter and for commercial biopreparation $7.2 \cdot 10^5$ cfu/g dry soil matter. Kanska *et al.* [20] proved that increasing number of introduced bacteria cells from $2 \cdot 10^3$ to $2 \cdot 10^7$ cfu/g dry soil matter affected on pollution elimination from 39% to 94%.

Introduction of biopreparations (natural and commercial) to studied soil did not cause increase of TPH and 4–6 aromatic PAHs elimination. Also the loss of heavy fractions in sample with commercial biopreparation did not differ from the control sample (15–16%). It was surprising that inoculation with natural biopreparation caused lower elimination of this fraction (10%). Such effect is difficult to explain. In soil with an aged pollution (dozens of years), selection and adaptation of microorganisms should occur. Such selection is connected with biological equilibrium and homeostatic mechanisms settlement. Homeostatic mechanisms counteract any outside intervention and microbiocenosis enlargement too, which was emphasized by Alexander [1]. Natural biopreparation used contained only a part of microorganisms representing natural microflora. Maybe artificial multiplying of microorganisms with the same pathways of metabolism as that existing in soil caused increase of competition between all of soil microorganisms and elimination of part of them. The confirmation of this phenomenon is a decrease of total bacteria number and

*Pseudomonas* group. It is also possible that dead biomass became an easily available carbon source and caused lower removal of hydrocarbons. Characteristic and age of pollution probably exceeded metabolic abilities of microflora present and introduced with natural biopreparation. Lack of easily available carbon source, deficit of nutrients in the control sample as well as in samples with natural biopreparation could lead to decrease of microorganisms' number. Especially in control soil there was no modification of physico-chemical conditions, no aeration even by mixing of soil. Because of that, after four weeks a deficit of oxygen and aggregation of soil particles covered by hydrocarbons film could occur and limit microorganism's growth.

Almost a similar situation was observed with commercial biopreparation. Low loss of hydrocarbons was connected with low number of bacteria especially at the beginning of an experiment. Such situation can suggest that this biopreperation was not accepted by natural soil microflora what could lead to limitation of growth of "strange" microorganisms. Such situation was noticed as well by Bento et al. [7], Atlas and Bartha [3] and Vogel [45]. In opposite to natural biopreparation commercial product contained microorganisms with another metabolic abilities and characteristic. It resulted in difficulties in adaptation at the beginning of experiment connected with needed time for adaptation to new conditions. After the 12$^{th}$ week an increase of number of microorganisms in this sample was noticed and could be a confirmation of this process.

The use of plant in combination with biopreparations resulted in higher elimination of heavy fractions (34%), 2–3 aromatic PAHs (89%) and 4–6 aromatic PAHs (2%) than observed in control and sample with biopreparation only. Such treatment gave worse results than seeding with clover only. It seems that introduction of biopreperation to samples with clover had negative influence on effectively of pollutant removal. In the case of using clover with commercial biopreparation a high elimination of TPH correlated with high number of microorganisms was noticeable. Connection of clover and commercial product gave surprisingly worse results in 2–3 aromatic PAHs removal than usage of these two modifications separately.

The results described above show that microorganism's amount did not affect the effectiveness of contaminants degradation but it is the result of plants stimulation and food competition between organisms. In the sample with natural biopreparation results of hydrocarbons degradation were worse than in soil with commercial biopreperation. It suggests that commercial biopreparation contained microorganisms with better potential for petroleum hydrocarbons removal and probably with wider spectrum of abilities.

Many researches show high activity of *Pseudomonas* bacteria group in petroleum hydrocarbons biodegradation [5, 9, 22, 35, 40, 45]. An increased number of this group was observed in oil-polluted sites. Bacteria from *Pseudomonas* group can produce biosurfactants and are very helpful in decomposition of hydrophobic substances such as petroleum hydrocarbons [5, 13, 16, 17, 33]. Our research showed an increase of *Pseudomonas* group counts especially in samples characterized by very effective degradation processes. This confirms that this group is important in hydrocarbon biodegradation. The highest percentage of *Pseudomonas* group in total bacteria number was observed in samples with clover and clover and biopreparations. Probably the presence of clover creates up good conditions for growth of this group of bacteria.

In the majority of samples very low concentrations (about $10^1$ spore/g dry soil matter) of *Actinomycetes* were observed. This group of organisms was absent in the sample with clover. Probably *Actinomycetes* did not have a meaningful influence on the investigated processes. High food competition (result of dynamically developing bacteria microflora or antibiosis of fungi numerously presented in soil) and low pH value could lead to inhibition of growth of this group.

Seeding with clover had a positive effect on concentration of N in soil and C/N ratio. This parameter is very important during petroleum hydrocarbons biodegradation. There was observed some correlation between addition of clover to soil, C/N ratio and removal of hydrocarbons (in samples with clover correlation between TPH removal and nitrogen concentration was $R^2 = 0.88$ and correlation between 4–6 aromatic PAHs and nitrogen concentration was $R^2 = 0.94$). Additionally, clover roots can exude some bioavailable organic compounds that can stimulate growth of microorganisms and also can be helpful during co metabolism processes [2 45].

## CONCLUSION

Bioaugmentation with multiplied autochthonous microorganisms, the same as commercial biopreparation, did not enhance and accelerate pollutant degradation. The reason of such results could be a specific balance and homeostasis in studied aged-polluted soil. Presence of Papilionaceous plants (*Trifolium pratense* L.) appeared to be the most beneficial modification. Clover changed soil properties and made them better for growth of microorganisms and removal of hydrocarbons. Degradation of all forms of hydrocarbons was on the highest level in the sample with clover only. Degradation rate was correlated with the highest bacteria and fungi number and also high contribution of *Pseudomonas* group. Chemical analysis showed positive plants influence on nitrogen concentration in degraded soil. Probably this was the main reason of such good results.

Introduction of biopreparations and plants resulted in lower removal of petroleum hydrocarbons (especially after introduction of natural biopreparation). Results were better than in samples with biopreparations only but worse than in the sample with clover only.

In order to achieve good bioremediation results in situ technique it is necessary to do laboratory researches, followed by field studies which can show that our choice is correct.

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