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GENOTOXICITY OF EXPLOSIVES-CONTAMINATED SOIL BEFORE AND AFTER BIOREMEDIATION

Two explosives-contaminated soil samples taken in the vicinity of Wrocław (SW Poland) were bioremediated under aerobic and anaerobic conditions. One of the samples (T) was contaminated a few years ago; another (D) underwent a many-year self-cleaning process. The efficiency of their treatment was evaluated based on the change of the contaminant content in the soil. Soil mutagenic activity before and after bioremediation was investigated using the Ames test. The following strains of *Salmonella typhimurium* were used: TA 98, TA 100, YG 1041 and YG 1042. Sample T was more contaminated with organic compounds than sample D (13 980 mg/kg d.m. and 2645 mg/kg d.m.). Bioremediation of sample T was not very effective: contaminants under anaerobic and under aerobic conditions were reduced by 4.5% and 2.8%, respectively. Bioremediation of sample D proved to be highly efficient: reduction of contaminants under anaerobic conditions by 94% and under aerobic conditions by 79%. Contaminants in the soil extracts displayed genotoxic activity. Sample T was more genotoxic than sample D. Direct mutagens predominated in sample T, and indirect mutagens, in sample D. The bioremediation process was conducive to the production of the metabolites whose toxicity was higher than that of initial substrate. Bioremediation under anaerobic conditions caused smaller increase in mutagenic activity of the soil contaminants than bioremediation under aerobic conditions.

1. INTRODUCTION

Contamination of soil by explosives presents a serious environmental problem, especially at the sites formerly occupied by the factories manufacturing explosives and munitions as well as at military sites. Chemically these energetic substances are mostly nitro derivatives of benzene, toluene and phenol as well as heterocyclic compounds containing nitrogen. Many of them are highly toxic to organisms inhabiting different ecosystems [1]. The one especially hazardous is trinitrotoluene (TNT). Permanent exposure of people to TNT can lead to many dangerous illnesses (e.g., anaemia, liver dysfunction, cataracts), and also to the development of neoplastic diseases

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[2]. Nitroaromatic compounds are decomposed by microorganisms, both under aerobic and anaerobic conditions. Under certain conditions microbiological decomposition allows production of some exceptionally toxic metabolites whose molecular structure and biological properties are not yet well known [3].

The aim of this paper was to evaluate the efficiency of both aerobic and anaerobic bioremediation of soil contaminated with explosives. The Ames test was chosen because of its common use in genotoxicity research and its high ability to predict carcinogenicity [4].

2. MATERIALS AND METHODS

Sampling and extraction of samples. Two samples of explosives-contaminated soils taken in the vicinity of Wrocław were examined. The soil of sample T was contaminated quite recently (a few years ago), while sample D underwent a long-lasting self-cleaning process. The samples were taken with a spade from a depth of about 20 cm and transported in plastic bags. Then they were pre-dried under laboratory conditions at room temperature. Dried soil was sieved through the screens of 8, 4, 2 and 1 mm meshes. Soil samples were extracted with dichloromethane in Soxhlet apparatus (6 h). Dichloromethane was evaporated, and the extract dissolved in DMSO so that 1 cm³ of solution corresponded to 5 g of soil. DMSO-dissolved extracts were sterilised before filtration (0.2 µm filter mesh size) and stored at the temperature of -70 °C. Initially a toxicity level test was carried out. It turned out that the samples could be used in the test only after 20-time dilution. There were also performed further 40-, 60- and 80-time dilutions of the extract.

Bioremediation. Bioremediation research was conducted in the pot experiment over a period of 30 days under aerobic and anaerobic laboratory conditions. Native soil microflora was used for the decomposition of contaminants. Soil was enriched with phosphorus in C:P = 100:1 ratio. The reaction took place at the pH range of 6–8, the soil humidity of 60% WHC under aerobic conditions and at 90% WHC under anaerobic conditions. The soil in pots was aerated through periodic stirring (every 24 hours). The soil treated under anaerobic conditions was kept in an anaerostate at an incubation temperature of 25 °C.

Physicochemical analyses. Enzyme immunoassay Field Screening Method for TNT in soil was used. Soils from the immunoassay samples were extracted and analyzed according to the instruction supplied with the D TECH TNT/RDX extraction pack and D TECH TNT explosives test kit. The actual concentration of organic contaminants was measured in soil by gravimetric method using dichloromethane as a solvent. A qualitative analysis of soil contamination was performed earlier in the Department of Water Treatment Technology of Adam Mickiewicz University in Poznań. The analysis re-

vealed that the soil is contaminated not only with TNT, but also with many nitrotoluene derivatives being the by-products of its production (which have not been removed from the main product) and the products of biochemical and photochemical decomposition of TNT (in press).

The Ames test. The *Salmonella typhimurium* strains TA 98 and TA 100 were given us by Professor B. Ames (Laboratory Department of Biochemistry, University of California). The strains YG 1041, YG 1042 were given us by Professor M. Watanabe (Division of Mutagenesis, National Institute of Hygienic Science in Tokyo).

All the strains are histidine-requiring ones (His^-) and have the following genetic markers: rfa, Δ (uvrB, bio), +R (pKM101) [6], [7], [8]. The strains YG 1041 and YG 1042 were obtained from the strains TA 98 and TA 100 by introducing to their genomes the plasmids carrying genes that code for nitroreductase and *O*-acetyltransferase (pYG233) [9], [10], [11].

The procedure described by MARON and AMES [5] was used. All samples were tested in 5 replications. For metabolic activation the S9 mix was used based on the homogenate of rat liver induced with Aroclor 1254. Positive controls included: 2,4,7-trinitro-9-fluorenone (for TA 98 and YG1041) and sodium azide (for TA 100 and YG1042) as direct mutagens (without S9 mix) and for all the strains used 2-aminofluorene as indirect mutagen (with S9 mix).

According to the procedure, the sample was considered mutagenic when its mutagenicity ratio satisfied $\text{MR} \geq 2$ and when it demonstrated a linear dose-response relationship [5]. The mutagenicity ratio means the ratio of an average number of revertants induced by the sample per plate to an average number of spontaneous revertants.

3. RESULTS AND DISCUSSION

3.1. RESULTS OF PHYSICOCHEMICAL ANALYSES

Dichloromethane extractable compounds in the soil samples were different. Sample T was more contaminated and contained 5.2 times more pollutants than sample D, which underwent self-cleaning process over the period of many years. Toxic effect of pollutants present in soil T on soil microorganisms was likely to be the reason for slow biodegradation process. The reduction in the content of dichloromethane extractable compounds after 30 days of bioremediation was below 5%, whereas biological decomposition of pollutants in soil D was exceptionally efficient. Under aerobic conditions the substrate reduction was as great as 79%, while under anaerobic conditions it was even bigger than 90% (the table).

Table

Content of dichloromethane extractable contaminants
in soils before and after bioremediation

Sample	Soil contamination [mg/kg d.m.]		
	Before bioremediation	After bioremediation	
		Anaerobic conditions	Aerobic conditions
T	13979.94	13352.38	13584.61
D	2644.52	166.42	550.63

Initial concentration of TNT determined by immunoassay method was also higher in soil T than in soil D and amounted to 500 mg/kg and 150 mg/kg of dry matter, respectively. The TNT reduction after bioremediation under aerobic conditions exceeded 85% and 90% under anaerobic conditions in sample T and was almost complete in sample D.

3.2. EVALUATION OF SOIL GENOTOXICITY

Contaminants present in soil extracts displayed genotoxic activity and increased the reversion of the *Salmonella typhimurium* strain TA 98 in all of the concentration tested (figures 1 and 2). Sample T showed higher genotoxicity than sample D. A correlation was found between the number of the revertants produced and the dilution of soil extract. Diversification of test strain responses corresponded to the results of instrumental analysis. The results of the tests in which the sample was activated by S-9 mix fraction or it was not activated showed that the direct mutagens predominated in soil sample T. Probably higher concentration of TNT in sample T than in sample D was responsible for this phenomenon.

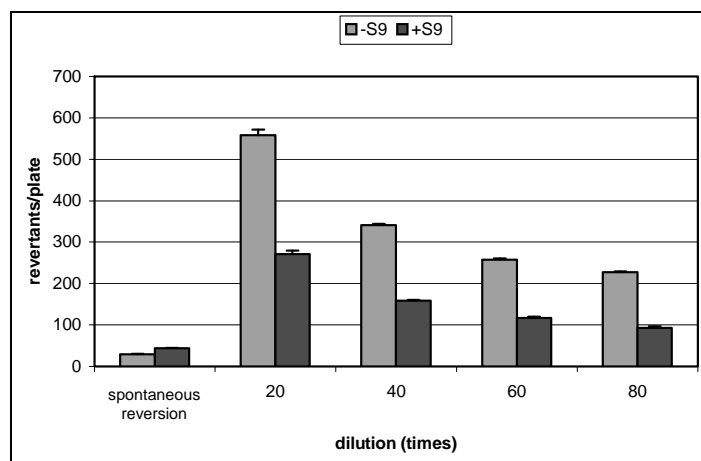


Fig. 1. The effect of the soil extract T on the number of the revertants of *Salmonella typhimurium* TA 98 (the number of spontaneous revertants per plate: 29.0 ± 1.6 (without S9 fraction) and 43.6 ± 1.7 (with S9 fraction))

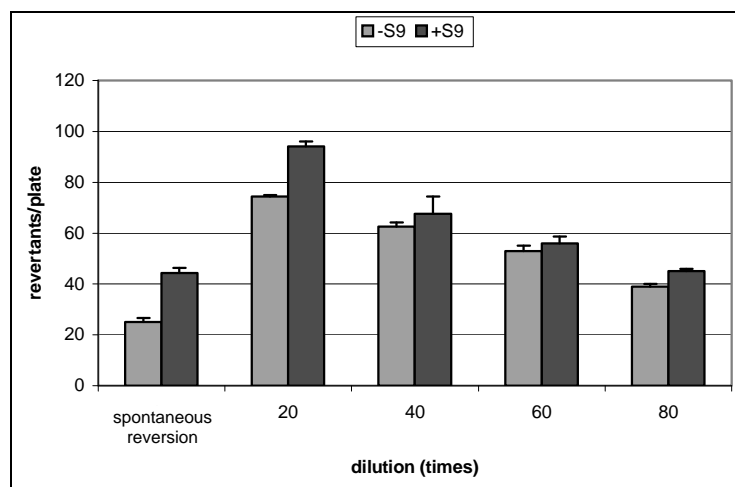


Fig. 2. The effect of the soil extract D on the number of the revertants of *Salmonella typhimurium* TA 98 (the number of spontaneous revertants per plate: 25.0 ± 1.6 (without S9 fraction) and 44.3 ± 2.0 (with S9 fraction))

Then the genotoxicity of the samples having undergone bioremediation was evaluated and compared with the genotoxicity of the initial sample (figures 3–6). Test strains TA 98, TA 100, YG 1041 and YG 1042 were used in this test. Soil extracts being 80-times diluted were tested. The tests revealed that the bioremediation process was conducive to the production of metabolites more genotoxic than the initial substrate. This phenomenon was observed in both samples, although the biological decomposition in sample T was of low efficiency. The contaminants of sample T caused frame-shift mutations (this could be detected with *Salmonella typhimurium* strains TA 98 and YG 1041) and base-substitution mutations (this could be detected with *Salmonella typhimurium* strains TA 100, and YG 1042). However, base-substitution mutations occurred only in the cells of strains of higher activity of nitroreductase and *O*-acetyltransferase. Due to the bioremediation of soil T under aerobic conditions a distinct increase in the activity of the frame-shift mutagens was observed. The bioremediation of soil T under anaerobic conditions also resulted in an increase in mutagenic activity, but to a lesser extent.

Biological decomposition of soil D resulted in a significant increase of its mutagenic activity and the presence of mutagens causing a base-pair substitution. However, the mutagenicity of biodegradation products in soil D was several times lower than that in soil T. Also in the case of soil D the mutagenic activity was found to be lower after bioremediation under anaerobic conditions.

Diversification of mutagenicity factors in the test with and without activation with S-9 mix fraction proves that in the soil samples tested, also after biodegradation, both direct and indirect mutagens were present. Sample T was richer in indirect

mutagens, while sample D – in direct mutagens which have the ability to affect strain YG 1041.

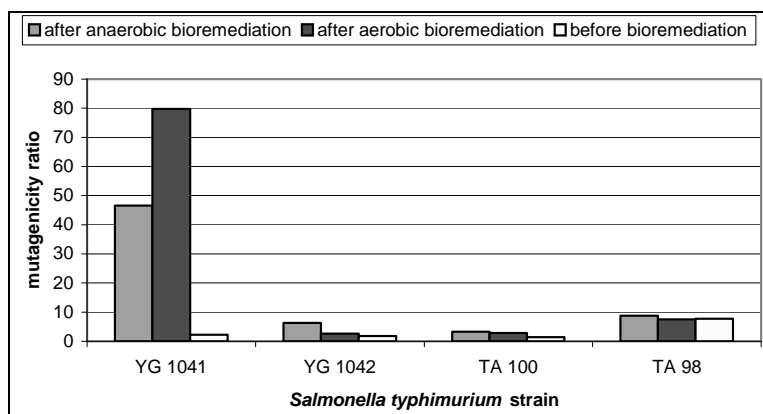


Fig. 3. The effect of bioremediation on a direct mutagenicity ($-S_9$) of soil T (the number of spontaneous revertants of the strains used: 235 ± 17.2 , 134 ± 16.5 , 114 ± 11.7 and 29 ± 1.6 per plate, respectively)

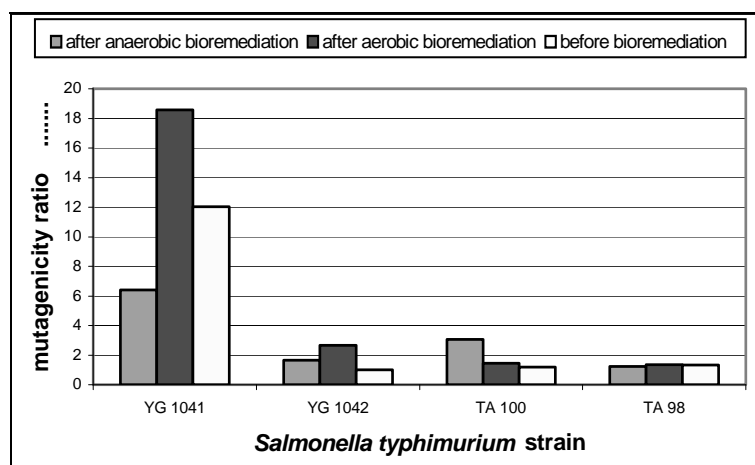


Fig. 4. The effect of bioremediation on a direct mutagenicity ($-S_9$) of soil D (the number of spontaneous revertants of the strains used: 235 ± 17.2 , 134 ± 16.5 , 114 ± 11.7 and 25 ± 1.6 per plate, respectively)

Sensitivity diversification of individual test strains testifies to a significant share of the frame-shift mutagens, among them probably nitro derivatives of PAHs, in mutagenicity of the soils tested. Our results and the results obtained by other authors [12], [13] reveal that *Salmonella typhimurium* strains demonstrate greater suitability for detecting a frame-shift mutation than for a base-pair substitution mutation. Such muta-

tions are caused most often just by chemical compounds from aromatic hydrocarbon group. Of the *Salmonella* strains employed in the tests presented in this paper the most sensitive proved to be the strain YG 1041. It is derivative of a TA 98 strain of increased activities of nitroreductase and *O*-acetyltransferase enabling the detection of base-substitution mutations, with a special attention given to the mutations caused by PAH nitro derivatives and also PAH amino derivatives in the presence of microsomal S-9 mix fraction.

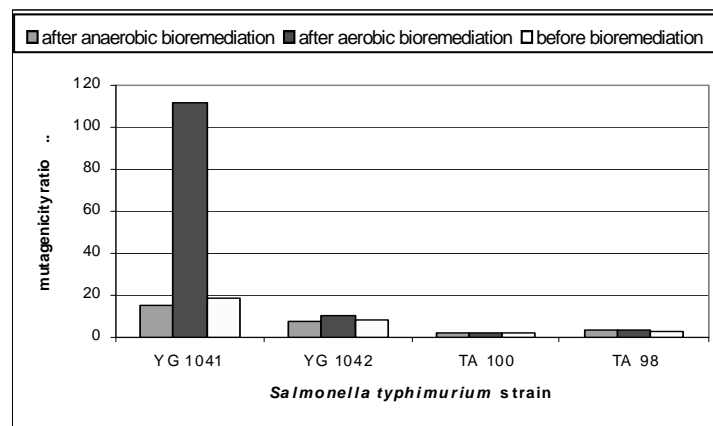


Fig. 5. The effect of bioremediation on an indirect mutagenicity (+S9) of soil T (the number of spontaneous revertants of the strains used: 254±42.7, 115±25.1, 140±7.8 and 43.7±1.7 per plate, respectively)

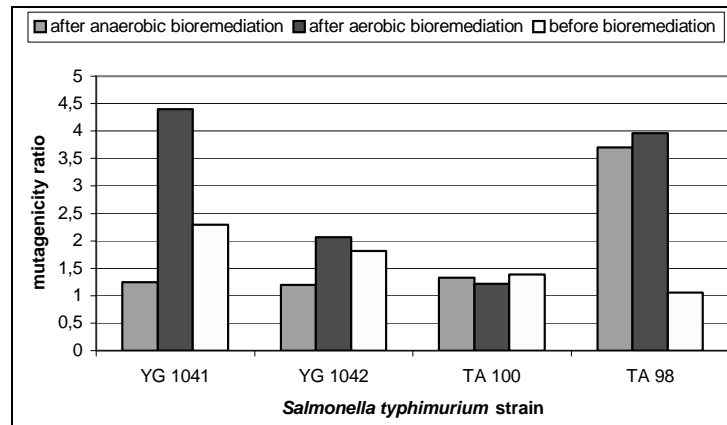


Fig. 6. The effect of bioremediation on an indirect mutagenicity (+S9) of soil D (the number of spontaneous revertants of the strains used: 254±42.7, 115±25.1, 140±7.8 and 44.3±2.1 per plate, respectively)

The mutagenic activity of the soils contaminated with explosives was demonstrated by many authors [14], [15]. To the best of our knowledge, the influence of the bioremediation process on the mutagenicity of this kind of soils is not firmly established. GRIEST et al. [16] using the Ames test observed rapid reduction of mutagenic activity of soil after its remediation by a composting process. On the other hand, JARVIS et al. [17] using the Mutatox assay found, as the authors of this study, a significant increase in soil mutagenic activity after its bioremediation. The differences may arise from the different sensitivity of the tests used and different characteristics of the soils tested and bioremediation processes. In the present study, the Ames test was used, but in comparison with the investigations described by Griest et al., the more sensitive strains were applied.

The increase in soil mutagenic activity after its bioremediation may be explained by the formation of more mutagenic by-products during biodegradation processes stimulated by different strains of microorganisms present in soil. For example, TAN et al. [18] and HONEYCUTT et al. [19] found that the microbial metabolites of TNT are as mutagenic as, or even more than, TNT itself.

4. CONCLUSIONS

The investigations revealed the suitability of the Ames test for the bioindication of soil contaminated with explosives. The strains from YG series are particularly effective as their plasmids carry the genes responsible for synthesis of nitroreductase and *O*-acetyltransferase. It was found that in order to detect the mutagenicity of nitro derivatives, it seems more reasonable to use the strains that detect frame-shift mutations (strains TA 98 and YG 1041) than the strains that detect base-substitution mutations (strains TA 100 and YG 1042).

A significant reduction in the content of organic pollutants in soil D treated under anaerobic and aerobic conditions leads to a successful bioremediation of soil contaminated with explosives. The efficiency of the biological treatment depends on the initial concentration of nitro derivatives in the soil – the high concentration inhibits their biological decomposition. Biological decomposition of explosives leads to the formation of metabolites more hazardous than the initial substances. Therefore, it is not advisable to carry out soil treatment under *in situ* conditions, as the process can lead to propagation of the environment contamination. Furthermore, the results obtained suggest that the bioremediation of soil contaminated with explosives should proceed under anaerobic conditions which make the process more efficient and the mutagens less frequent than during aerobic processes.

It should be stressed that soil mutagenicity can increase during the bioremediation and that the process should last long enough to allow metabolic reactions to be finished.

ACKNOWLEDGEMENT

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GENOTOKSYCZNOŚĆ GLEBY ZANIECZYSZCZONEJ MATERIAŁAMI WYBUCHOWYMI PRZED I PO BIOREMEDIACJI

Próbki gleby z okolic Wrocławia zanieczyszczone materiałami wybuchowymi poddano bioremediacji w warunkach tlenowych i beztlenowych. Jedna z próbek (T) była zanieczyszczona przed kilku laty, druga (D) przeszła wieloletni okres samooczyszczania. Oceniono wydajność bioremediacji na podstawie zmian stężenia zanieczyszczeń w badanej glebie. Za pomocą testu Amesa zbadano aktywność mutageną gleby przed i po bioremediacji. Zastosowano następujące szczepy *Salmonella typhimurium*: TA 98, TA 100, YG 1041 i YG 1042. Próbka T była bardziej zanieczyszczona związkami organicznymi niż próbka D (odpowiednio: 13980 mg/kg s. m. i 2645 mg/kg s. m.). Bioremediacja próbki T była mało efektywna: redukcja zanieczyszczeń w warunkach beztlenowych wyniosła 4,5%, a w warunkach tlenowych – 2,8%. Bioremediacja próbki D była bardzo wydajna: redukcja zanieczyszczeń w warunkach beztlenowych osiągała 94%, a w warunkach tlenowych – 79%. Zanieczyszczenia obecne w ekstraktach glebowych wykazywały aktywność genotoksyczną. Próbka T była bardziej genotoksyczna niż próbka D. W próbce T dominowały mutageny bezpośrednie, a w próbce D – mutageny pośrednie. Proces bioremediacji sprzyjał powstawaniu metabolitów o toksyczności większej niż toksyczność substratów wyjściowych. Bioremediacja w warunkach beztlenowych powodowała mniejszy wzrost aktywności mutagennej gleby niż bioremediacja w warunkach tlenowych.