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Assessment of the genotoxic potential of soil-associated DDTs and its metabolites using the Salmonella/microsome assay

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Abstract. The study aimed to assess the genotoxicity of different DDT contaminated soils (with different amendment types) in benzene extracts. A *Salmonella/microsome* assay without metabolic activation (-S9) was performed to evaluate the efficiency to initiate the mutations of remediated and non-remediated soils. The number of revertants mutations in his⁺ in TA100 and TA98 strains were obtained, and compared to soils without DDT, and remediated DDT containing soils. The interpretation of the data suggests that, mutagenic activity was high for the non-remediated soils samples Kyzylkairat and Beskainar, and revealed in the scale as a weak mutagen. Soil samples treated with DARCO, ORBO, Sarg<150µm, and Sarg>150µm in Kyzylkairat and Beskainar exhibited a decreasing level of mutagenic activity, which is in line with the decreased environmental availability in these soil. Overall, the study indicates that strains TA100 and TA98 are valuable tools for rapidly assessing the potential mutagenicity of soil contaminated with OCPs.

1 Introduction

Organochlorine pesticides (OCPs), such as dichlorodiphenyltrichloroethanes (DDTs), have been banned for decades. Nevertheless, these environmental contaminants are always present in soil in various geographic areas due to their persistence and capacity to accumulate in biological systems [1]. These substances have been synthetically manufactured and their potential to cause harmful effects on human health and ecosystems is nowadays well established. The main exposure for humans to these POPs is the consumption of contaminated food, particularly fish and fatty food items. Exposure to high levels of DDTs has the potential to lead to the development of cancer, while the effects of lower doses on the general population are still being investigated. Although the use of DDTs have been banned by the Basel, Rotterdam, Stockholm Conventions, there are still stockpiles of OCP

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(organochlorine) pesticides around the world which may contaminate different environmental compartments [2].

Current studies indicated that Kazakhstan is grappling with environmental issues attributed to the discovery of significant quantities of OCPs in the soil surrounding abandoned banned pesticide storage sites across the country [3]. The maximum permissible concentrations of DDT and HCN were substantially exceeded at these places, and soil ingestion would be a primary route of exposure to the cancer risk. This poses a significant challenge not only for agricultural activities in the area but also for human health, underscoring the urgency for proper disposal and remediation to mitigate environmental contamination and associated risks. Consequently, the reduction of the environmental availability of soil-bound POPs such as DDT emerged as a prominent concern due to their inherent toxicity. Various studies are currently implementing methodologies to address the issue of soil contamination, such as phytoremediation, microbial degradation, dehalogenation through chemical reagents, and removal using activated carbon [4]. A recent investigation observed that Sargassum-based biochar (bc) sequestrants and commercial forms of activated carbon (ac) (e.g., ORBO and DARCO) exhibited efficacy in reducing the environmental bioavailability of DDT and its metabolites when applied as a 2% amendment [5] on contaminated surfaces.

Studying the mutagenic effects of soil contaminants allows to assess the potential risks for human health. Furthermore, literature findings have revealed that outdated OCPs and heavy metals originating from industrial waste have the potential to induce genotoxic effects on both soil and water [6]. Various *in vitro* assays can study the mutagenicity potential of soil contaminants. In this regard, bacterial reverse mutation known as the Ames test use commonly *Salmonella typhimurium* and *Escherichia coli* bacterias to assess genotoxicity of contaminants in both soil and water [6]. In this regard the *Salmonella/microsome* test on *Salmonella* bacteria strains TA98 and TA100 with and without metabolic activation (+S9 ; -S9) provides an initial assessment of the situation [7] which may be completed with other tests for an overall environmental assessment.

There is a lack of information regarding the assessment of mutagenicity in DDT-contaminated soils following remediation with sequestrants such as activated carbon and biochar. Therefore, our objective is to evaluate the potential genotoxicity of soils contaminated with DDT and its metabolites using the *Salmonella/microsome* assay system, specifically on two strains (TA98 and TA100) without metabolic activation (-S9). In particular, we will be studying soil samples from two pesticide storage sites in Kazakhstan, Kyzylkairat and Beskainar, as well as treated soil samples using the different types of sequestrants mentioned in the study [5]. This will allow us to assess the mutagenicity of DDT-containing soils and examine the relationship in recovered soils.

2 Materials and methods

2.1 Sampling

In this study, sampling and remediation were not performed but instead relied on previously collected soil samples from France (L2A, University of Lorraine-INRAe, Nancy, France) and Kazakhstan (Institute of Genetics and Physiology, Almaty, Kazakhstan). A total of 10 soils were included in the analysis. Two Kazakh soils, namely Kyzylkairat (K) and Beskainar (B), were selected to investigate the mutagenic effects of DDTs and its metabolites (DDD-DDE-DDT- 4.4', DDT-2.4') residues. Additionally, the mutagenic activity of soils B and K was studied when these soils were treated with sequestrants such as activated carbons (ORBO

and DACRO), as well as biochars made from Sargassum particles sieved at sizes <150 µm and >150 µm, at an amendment rate of 2%.

2.2 Obtaining benzene extracts from soil samples

Samples of 10 g of sieved soil were added in 300 mL of benzene, shaken for 6 h, and filtered through a de-isol filter "red tape." The solution was evaporated in a vacuum evaporator at 40-50°C. The precipitate was dissolved in 2 mL of dimethyl sulfoxide (DMSO) and used for analysis. Thus, benzene extracts of soil samples at a concentration of 0.5 g/mL were used to determine mutagenicity.

2.3 Ames test (Salmonella/microsome without metabolic activation)

The total mutagenic activity of the samples was investigated by the method of counting his⁺ revertant mutations in the Salmonella/microsomes assay without metabolic activation (-S9) [8]. The test strains *Salmonella typhimurium* TA100 (detect base-pair substitutions) and TA98 (detect frameshift mutations) were provided by the Institute of Genetics and Physiology (Institute of Molecular Genetics, Almaty, Kazakhstan). Overnight culture was grown in nutrient broth at 37°C, precipitated by centrifugation, and twice reconstituted at 5000 rpm for 15 min. As positive controls for the strains TA98 and TA100, 2,7-diamino-4-dioxy-4,5,9,10-tetrahydro-6,9-diazopyrene (DDTDP) and nitrosomethylurea (NMM) were used. For the negative control (untreated or solvent), distilled water or dimethyl sulfoxide is used. Benzene extracts of soil were incubated in solvent (DMSO) for 3 h with constant shaking. Samples were extracted with DMSO in the amount of 0.1 mL of solution per Petri dish. Plates were incubated at 37°C for 48 h. All the assays were done in triplicate. The revertant bacteria colonies were hand-counted. The presence of mutagenic effect in the studied preparations was considered by induction of reverse mutations from auxotrophy by histidine to prototrophy. The presence of mutagenic activity was judged by the mutagenic index (MI), which is the excess number of his⁺ revertants in samples over the level of spontaneous mutation (Mean±SE).

$$\text{Mutagenic index (MI)} = \frac{\text{number of of his}^+ \text{ revertants treated with soil extracts}}{\text{number of spontaneous his}^+ \text{ revertant in negative control}} \quad (10)$$

Exceeding the level of his⁺ revertants above the control (O/C) can be categorized as follows:

- 2 times - absence of mutagenic effect (-);
- > 2-10 times - weak mutagen (+);
- > 10-100 times – moderate mutagen (++);
- > 100-1000 times - strong mutagen (+++).

2.4 Statistical analysis

Data were statistically analyzed using R (version 4.3.2) program (Rstudio2023.12.1-402) for Windows. Normality testing for the variances of TA98 and TA100 was done using the Shapiro-Wilk test at p>0.05 (W=0.9) leading to acceptance of the null hypothesis indicating normal distribution for both strains in B, K soils. In addition, a comparison between treated groups of soil in two strains was tested with the Applying the library (doebioresearch), the one-way ANOVA test was performed on the variables number of his⁺ revertants at the significant level of p<0.001, considering the soil as a factor. The number of revertant

mutations in each strains were compared individually using a Duncan's Multiple Range test (MRT) in order to assess the effect of the different soil groups, untreated and remediated.

3 Results and Discussion

The majority of samples TA98 (frameshift mutagens) and TA100(base pair substitutions) showed mutagenic activity in samples with two soils K and B, also in the group with Sarg sequestrant in the absence of S9 mixture and gave positive result for mutagen induction.

The results presented in the Figures 1 and 2 illustrates the average number of his+ revertants per dish on *Salmonella* strains TA98 and TA100 for the K and B sites, with a different variation of sequestrant materials such as activated carbon (ORBO, DARCO) or biochar *Sargassum* (<150µm; >150 µm). According to the data, regardless of the strain, the number of his+ revertants in all tested samples followed a hierarchical order based on the soil types (B or K) and treatment types (Sarg >150 µm and <150 µm, ORBO, DARCO). Therefore, the groups are separated into three based on the treatment responses, independently from the positive and negative controls: 1. Untreated soil (B and K); 2. Soil with Sarg >150 µm and <150 µm; 3. Soil with ORBO and DARCO. In total, there were six groups for soils. ANOVA analysis revealed significant differences in group of mean revertant values per dish of soil depending on the type of treatment, between the first, second, and third ($p < 0.001$). The negative control and DMSO groups exhibited no significant differences observed between them. The mean values of revertants were 22 and 20 for TA100, as well as 17 and 14 for TA98 respectively. For the positive controls the HHM for TA100 was 544, while the DDDTDP for TA98 was 380 number of revertants.

3.1 TA98 strain response

The distribution of the average number of revertants per dish showed that in strain TA98 with K soil, the number of mutations in the first group with untreated soil was recorded at 75 ± 2 (Mean revertants \pm SE). Contrastingly in the second group with Sarg >150 and <150 µm type sequestrant there were introduced a reduction of number of revertants observed, decreasing to 50 ± 1 and 47 ± 2 , respectively. Further, the mitigation of revertants number was noted in the the third group with ORBO and DARCO sequestrants, where the mean number of his+ mutations dropped to 28 ± 2 and 26 ± 2 , respectively. Similarly, in the second soil with B, the average number of histidine-dependent revertants exhibited a declining trend across the treatment groups. In the first group without remediation number of mutations was recorded at 54 ± 1 . While, in the second group with Sarg >150 and <150 µm reduction of mutagenicity were noted with the decrease of mean number to 46 ± 1 and 40 ± 3 . The third group with ORBO and DARCO showed further reduction in mutagenicity, in number of revertants reaching 37 ± 1 and 24 ± 2 , respectively.

3.2 TA100 strain response

The TA100 strain from soil in K exhibited an average of 101 ± 1 revertants in the untreated first group. Test groups with Sarg >150 and <150 µm type sequestrant had 70 ± 2 and 64 ± 1 revertants respectively, while those treated with ORBO and DARCO sequestrants showed decreased number of revertants 40 ± 2 and 37 ± 1 , respectively. In the case of B soil, the average mutation response was 82 ± 2 in the untreated soil group. The second group, introduction with Sarg >150 and <150 µm sequestrant, led to reduce mutagenicity with mean values of 55 ± 2 and 46 ± 1 respectively. Meanwhile, the third group treated with ORBO and DARCO, similarly decreased mutagenicity, with the average values of 35 ± 2 and 32 ± 1 .

Further, according to the mutagenic index (MI) calculated by [10], the mutagenic potential of soils is presented in Figure 3 as a heat map for strains TA98 and TA100. The MI values for soils with DDT and its metabolites ranged from 2 to 5, which is considered as a weak mutagenicity (+). In the first group of soils without sequestrant amendment, was the MI >4 for K and MI >3 for B.

The treatments in the second group with Sarg <150 μm and >150 μm had a mean reaction value of MI response below 3 for K and B.

Meanwhile, absence of soil mutagenic potential was considered as MI<2 and was observed in group three, with ORBo and DARCO for both strains. Moreover, the ORBO soil treatment for TA98 showed significant differences in MI>2 from strain TA100, where it showed a weak mutagenicity level.

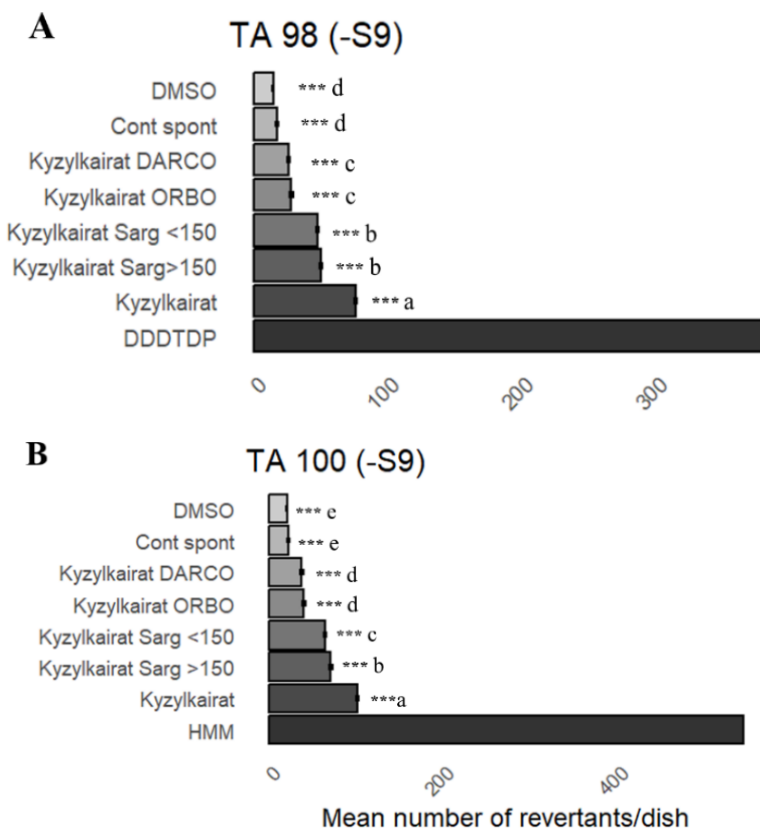


Fig. 1. The number of his+ revertants per plate in Salmonella/ microsome assay without S9 activation, using TA98(A) and TA100(B) strains, was determined in a soil samples from Kyzylkairat, and remediated forms according to the different sequestrant types (Sarg >150, Sarg <150, ORBO, DARCO). (Mean±SE) n=3, error bars represent the standard error (SE) of the mean. ANOVA test indicated overall significant differences between non-remediated and remediated soils at p<0.001 level. Post-hoc analysis using Duncan's test revealed specific differences between individual treatment groups denoted by letters (a,b,c,d,e,f) at the p<0.05 level; treatments with the same letter are not significantly different from each other at this specified significance level

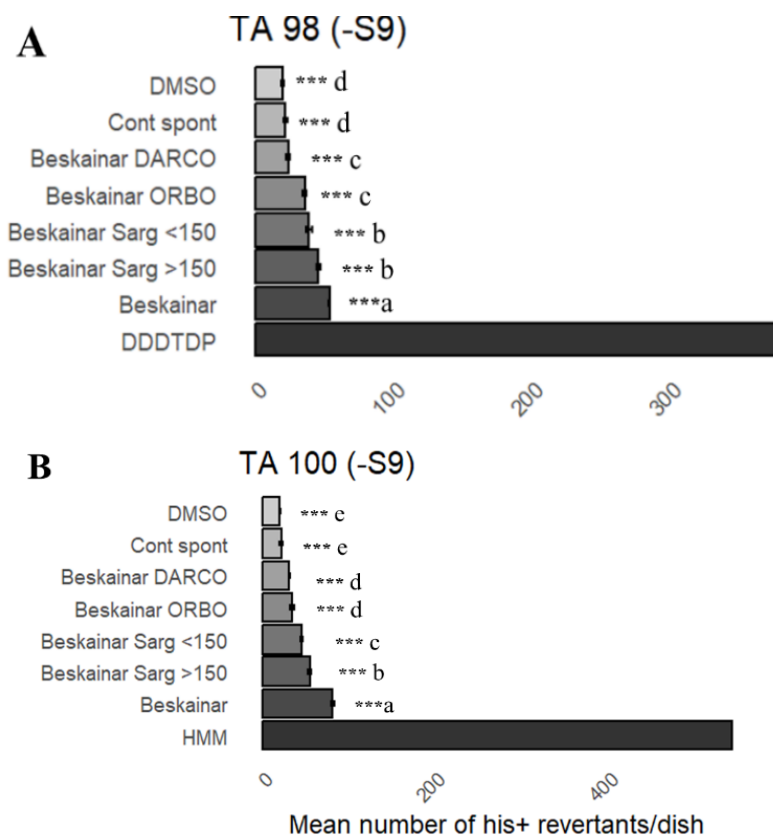


Fig. 2. The number of his+ revertants per plate in Salmonella/ microsome assay without S9 activation, using TA98 (A) and TA100 (B) strains, was determined in a soil samples from Beskainar, and remediated forms according to the different sequestrant types (Sarg >150, Sarg <150, ORBO, DARCO). (Mean±SE) n=3, error bars represent the standard error (SE) of the mean. ANOVA test indicated overall significant differences between non-remediated and remediated soils at p<0.001 level. Post-hoc analysis using Duncan's test revealed specific differences between individual treatment groups denoted by letters (a,b,c,d,e,f) at the p<0.05 level; treatments with the same letter are not significantly different from each other at this specified significance level.

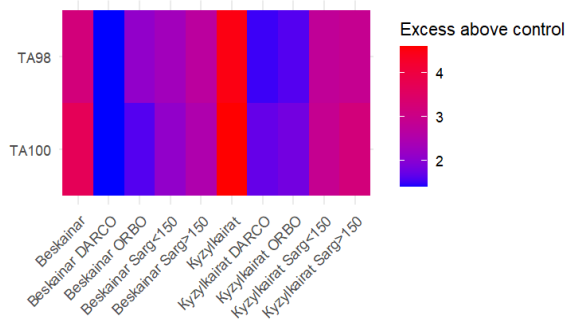


Fig. 3. Heatmap of mutagenicity representations on strains of *Salmonella* TA98 and TA100 without metabolic activation (-S9) according to the excess above control (O/C) for: soils with DDTs: 1. Beskainar, Beskainar DARCO, Beskainar ORBO, Beskainar Sarg <150, Beskainar Sarg>150 at 2% rate amendment; 2. Kyzylkairat, Kyzylkairat ORBO, Kyzylkairat Sarg <150, Kyzylkairat Sarg>150 at 2% rate amendment

The total concentrations of DDTs and its metabolites were at 10.07 and 0.343 $\mu\text{g/g}$ respectively for soils from K and B sites according to previous studies [5]. Similarly, another study focused on a contaminated site in the village Kyzylkairat found OCPs, including DDTs and HCHs, in all analyzed soil samples, with concentrations ranging from 0.0014 to 11.1 $\mu\text{g/g}$ for DDT and its metabolites which is respectively close for the K and B sites [9].

As it can be noticed from the results of mutagenic activity of the DDT soils without remediation K and B reveals as a mutagenic, particularly the soil K as it contains higher concentration levels of DDTs compared to the B. The observed differences for the K and B soils in a TA98 and TA100 strains were statistically significant, suggesting difference in the response among the untreated and various forms of remediated groups as biochar Sargassum (<150 μm ; >150 μm) and activated carbon (ORBO, DARCO). And multiple comparisons between the groups for each strain and soil type was significant at $p < 0.05$ according to the Duncan test. Consequently, the availability of DDT and its metabolites in the environment was lower with DARCO, ORBO and Sargassum (<150 μm ; >150 μm) sequestered materials than in the soil itself [5]. Which might explain the higher mutagenic potential of untreated soils with B and K in both strains TA100 and TA98.

The literature indicates that the mutagenic potential is dose dependent and responds differently depending on the origin of the contaminants as well as their concentration. In this regard, the present results show that the remediation procedures used in this study were effective in reducing mutagenic activity in contaminated soils.

Thus, although the Salmonella/microsome assay is widely used and effective for mutagenic detection, some limitations regarding sensitivity to heavy metals and the influence of the extractor solvent should be considered [8]. Similarly, variations in extraction parameters may affect the efficiency and accuracy of mutagenicity assessment of DDT present in soil samples, as well as the interaction of other contaminants and bacteria from soil. Among all soil extracts tested, strains TA100 and TA98 were sensitive to mutagen detection, showing mutagenic index against each soil extract tested depending on its characteristics.

4 Conclusion

The present study confirms that Salmonella/microsome analysis without metabolic activation is a suitable method for the initial demonstration of the mutagenic potential of contaminated soils after remediation. Our findings indicate that soils from the Kyzylkayrat and Beskainar sites in Kazakhstan, contaminated with DDT and its metabolites, induce mutations in Salmonella strains TA98 and TA100, as observed in previous studies conducted at these sites. These soils are identified as weak mutagens compared to the positive control. Furthermore, fewer his⁺ revertant mutations were observed in the remediated soils: DARCO > ORBO > Sarg150 μm , in descending order, which corresponds with a lower environmental bioavailability level. However, additional studies are needed to fully understand the specific mechanisms of DNA damage and the interrelationships contributing to the mutagenicity.

Author Contributions

Conceptualization, R.T., S.J., M.D., B.B.; Methodology, G.K., S.Z., R.T.; Software, R.T.; Formal analysis, G.K., S.Z., R.T.; Investigation, R.T.; Resources, B.B.; Writing-Original Draft Preparation, R.T.; Writing-Review & Editing, S.J., R.T., B.B.; Visualization, R.T.; Supervision, S.J., M.D., and B.B.; Project Administration, B.B.; Funding Acquisition, B.B.

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