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Research Article

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Integral Bio Testing for the Risk Assessment of Crop Production in a Region of Russia with an Uncertain Ecological Well-being

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ABSTRACT

The integral biological testing of soil samples of four districts of the Tula region was performed. The Tula region was selected for the study because it was subjected to radioactive contamination in 1986 but at present, it is considered to be fairly safe for that matter. The districts were selected according to both the presence of industrial pollution and relative ecological safety. The use/non-use of land for crop production was also taken into account (eight sites in total, samples № 1-8). Three different bioassays were used: microorganisms Salmonella typhimurium, cell culture of mammalian Cricetulus griseus, and invertebrates Ceriodaphnia affinis. A relatively high direct mutagenic activity was detected at the sites of the Efremovsky and Shchekino districts (№ 1 and № 3 respectively), where the mutagenic index was 3.3 and 3.9 respectively. Substances contained in the № 2 and № 4 soil extract samples turned out to be pro-mutagens, i.e. induced mutations upon using metabolic activation. The soil samples, such as № 1 and № 3 also showed genotoxicity in Cricetulus griseus cells with the increase of the frequency of chromosomal and chromatid-type aberrations by several times, compared with control. In the experiments on Ceriodaphnia affinis, toxicity was detected in the № 1, № 3, № 5 and № 7 samples, in which the death rate of the crustaceans was 35-45 %, whereas, in the remaining samples, the decrease in the survival rate of the crustaceans did not exceed 15 %. Therefore, the integral bio testing enables detection not only in the presence of ecotoxicants but also it can indicate their origin - industrial or agricultural.

Key words: *plant growing, ecotoxicants in soil, mutagenicity, genotoxicity, cytotoxicity, Ames test, Cricetulus griseus test, Ceriodaphnia affinis test.*

INTRODUCTION

Soil contamination with mutagenic and carcinogenic compounds, including radionuclides, is one of the most important factors of the negative impact on various ecosystems, which poses a real danger to crop production and, consequently, to animals and humans as the food chain links. The continuous increase in the application of fertilizers to soil threatens the sustainability of agricultural frameworks and surroundings [1, 2].

The Tula region is one of the four regions of the Russian Federation most affected by the Chernobyl nuclear power accident in 1986 [3]. As a result of the accident, 17 out of 23 districts of this area were subjected to radioactive contamination to a different extent. Thus, Plavsky, Uzlovsky, Arsenyevsky, and Novomoskovsky districts have been revealed as the most radioactively contaminated.

However, according to the official data of the radiation monitoring authorities, at present, the power of gamma radiation in these and other territories of the Tula region ranges from 10 to 25 μR/hour with a maximum allowable value of 60 μR/hour [4]. Thus, in terms of radioactive contamination, the Tula region is currently considered relatively safe, but it cannot be said about its environmental state as a whole. This is due to the high content of pollutants of technogenic origin in the soil, such as polychlorinated biphenyls, poly-aromatic hydrocarbons (PAHs), cyanides, toxic metals, waste oil products, pesticides, and their end-products.

Following the data of environmental monitoring, including the data of volunteers of the "green" movement, the Efremovsky and Shchekino districts are currently considered as the most industrially polluted areas, while, the Zaoksky and Odoevsky districts are the most prosperous in this respect.

The described above features of the region have suggested the practicability of conducting the research related to the possible adverse effects of the region's soils on local crop production and, ultimately, on the health status of the population consuming it. Therefore, with the help of a set of bio testing methods, the study of the toxicity and genotoxicity of several soils of the Tula region suitable for agriculture (used for both crop production and currently uncultivated) was conducted. The study also included the evaluation of the applicability of various biotests for the assessment of environmental risks and a preliminary analysis of the likely sources of the adverse effects for the evolutionally different organisms.

MATERIALS AND METHODS

Soil sampling. The soil samples were taken from the area of the Tula region suitable for agricultural purposes, both used and unused for crop production during the research period.

Soil samples were taken from the eight sites of four districts of the Tula region, two samples from each site, between June 10, 2018, and June 14, 2018. Two of the four districts (Efremovsky and Shchekinsky) were considered as industrialized, the other two (Zaoksky and Odoyevsky) relatively environmentally friendly. None of these districts were included in the list of those most affected by the nuclear accident in 1986.

Soil sampling and preparation for analysis were carried out according to the standard procedures accepted in Russia [5]. In particular, soil sampling was performed by a sampler with a diameter of 0.10 m taken from a soil depth of 0.00-0.10 m. Soil samples collected from each site were mixed into a combined sample in the laboratory. Then the combined sample was carefully sifted, to remove foreign inclusions and organic materials. Purified samples were mixed by quarrying on a plastic surface and ground.

Water extracts of soil samples were investigated for mutagenicity, genotoxicity, and toxicity using three methods of biological testing.

The mutagenicity of the soil sample extracts was determined using the modified Ames Salmonella/microsomes test with the metabolic activation system based on the S9 microsomal fraction obtained from the rat liver and induced by the Aroclor 1254 solution [6]. The *Salmonella typhimurium* TA-98 and TA-100 histidine auxotrophic strains were used as indicator strains. Two mutation types, such as reading frame shift and base-pair substitution were registered in TA-98 and TA-100 strains respectively [7]. The mutagenicity was evaluated by the frequency of reversions to histidine (His+) prototrophy detected on plates with the minimal medium. The direct mutagenic effect of the studied soil samples was evaluated in the experiments without the metabolic activation (-MA). The mutagenicity of metabolic products (pro-mutagenic effect) of compounds present in the soil samples was detected in the experiments with the metabolic activation (+MA).

To obtain extracts, 50 g of each sample was extracted three times with a fivefold volume of methyl chloride. The extracts were evaporated to dryness in a film evaporator, then dissolved in 5 ml of dimethyl sulfoxide (DMSO). 100 μl of the test sample extract was added to each Petri dish, then 100 μl of DMSO was additionally added. 100 μl of DMSO per plate without sample extract was used as the universal control. The known mutagen 2-aminoanthracene (0.5 μg per dish) was used as a positive control. The universal control was tested on both strains in five cycles, the remaining samples were tested in three cycles. The results were presented as a mutagenic index (MI): the ratio of the experimental colony number of His+ revertants (average number of colonies per plate in the presence of the test substance) to control (average number of colonies on DMSO plates).

MI equal to 2.0 or more was taken as an indication of the presence of a mutagenic effect in the TA-98 strain in at least one of the options: +MA, -MA. The toxicity of the extracts was determined by the degree of the TA-100 strain growth inhibition on a full-fledged nutrient medium in the presence of the test extract, compared to that in control. MI equal to 0.4 or less was taken as an indication of the presence of a toxic effect in the TA-100 strain in at least one of the options: +MA, -MA, as described in [8]. The significance of variations was evaluated using Student's criterion of the "Statistics" program. The standard deviation of the experimental colony number was $p < 0.001$, compared with control.

To investigate the genotoxicity and toxicity of the selected soil samples, water extracts were prepared. Thus, 200 g of soil from each sample were mixed with 800 ml of distilled water and shaken with a shaker for 2 hours. The obtained solution was left to sit for 30 minutes, then the supernatant was siphoned and filtered through a white ribbon filter paper.

Genotoxicity analysis was performed on the mammalian cell culture using the *Cricetulus griseus* test (Chinese hamster), according to the procedure described in [9-11]. The genotoxicity was determined by the quantitative criterion, i.e the increase in the proportion of aberrant cells and/or frequency of chromosomal aberrations, compared to control (exposure to the dMEM nutrient medium).

The toxicological analysis was carried out in the laboratory culture of *Ceriodaphnia affinis*, according to the method [12]. The acute toxicity was measured during 48 hours. The chronic toxicity was measured after 7 days. The decrease in the lifespan of *Ceriodaphnia* was taken as the quantitative criterion of the toxicity of the test extract. Each extract was tested four times. When processing the results, Z-statistics was used as a criterion of significance without additional assumptions about the variances of the studied quantities for the analysis of genotoxicity and toxicological analysis. The excess over control at the level of 95 % ($p<0.05$) by one-sided criterion was manifested when $Z>1.65$.

RESULTS

Table 1 presents the characteristics of the test soil samples of the Tula region agricultural lands.

Table 1: Properties of soil, in terms of geographical location, economic use, and environmental safety

Sample №	City/District	Use in crop production	Degree of environmental safety
1	Efremovsky district	used, medium loamy	industrially polluted
2	Efremovsky district	unused, medium loamy	industrially polluted
3	Shchekino district	used, medium loamy	industrially polluted
4	Shchekinsky district	unused, medium loamy	industrially polluted
5	Zaoksky district	used, medium loamy	non-contaminated
6	Zaoksky district	unused, medium loamy	non-contaminated
7	Odoyevsky district	used, medium loamy	non-contaminated
8	Odoyevsky district	unused, medium loamy	non-contaminated

The obtained results of the mutagenicity and toxicity of soil extracts were presented in Table 2.

Table 2: Results* of the soil extracts investigation for mutagenic and toxicological activity by the Ames Salmonella/microsomes test as with the metabolic activation system (+ MA), as without it (-MA)

Sample №	Strain TA-98				Conclusion	Strain TA-100				Conclusion
	+MA		-MA			+MA		-MA		
	QC	MI	QC	MI		QC	MI	QC	MI	
1	101	2.0	39	3.3	M	32	0.4	24	0.3	T
2	97	2.9	41	1.9	PM	89	1.0	102	1.1	No
3	99	2.1	49	3.9	M	34	0.4	34	0.4	T
4	86	2.8	38	1.4	PM	90	1.0	101	1.1	No
5	75	1.6	35	2.6	M	93	0.9	99	1.2	No
6	64	1.2	29	1.5	No	99	0.8	106	0.6	No
7	59	1.3	27	2.8	M	101	0.7	95	0.5	No
8	51	1.1	26	1.2	No	94	0.6	97	0.5	No

DMSO	31	1.0	22	1.0	No	89	1.0	95	1.0	No
2-aminoanthracene	540	16.0	33	2.7	M	405	4.6	65	1.0	No

Note: *QC is the number of colonies per plate, the calculated average of 3 cycles, MI is the mutagenic index; "No" – without mutagenic activity or non-toxic, M – mutagen; PM – pro-mutagen, T – toxicant.

The toxic effect, as the growth inhibition of Salmonella colonies, was detected in TA-100 strain, in samples № 1 and 3. None of the samples tested in the TA-100 strain showed mutagenic or pro-mutagenic effects.

Samples number 1, 3, 5, and 7 showed direct mutagenic activity (-MA) in the strain TA-98. Samples № 1 and № 3 showed the highest mutagenic activity with MI of 3.3 and 3.9 respectively.

According to the TA-98 strain test results presented in Table 2, the substances contained in the soil extracts of the samples № 2 and 4 turned out to be pro-mutagens (PM), i.e. they induced mutations of frameshifting with metabolic activation. This means that the metabolites of this strain were more genotoxic than the original soil pollutants. Likewise, most industrial eco-toxicants, including PAHs and polychlorinated biphenyls possess such genotoxic properties [13].

The data analysis presented in the Tables 1 and 2 has revealed that, in the TA-98 strain, MI corresponded with both the presence in the soil of industrial pollutants along with agrochemicals (insecticides, fertilizers) with direct mutagenic activity (samples № 1, 3, 5 and 7), and with the industrial pollution (pro-mutagenic samples 2 and 4) in the absence of agrochemicals. However, the overall results did not allow making a conclusion about which pollutants were the cause of the pro-mutagenic and mutagenic activity observed in the test strains.

It should be noted that, in samples 6 and 8, no mutagenic activity was detected in either of the TA-100 or the TA-98 strains, as it was expected. These samples were taken at the sites with a low-level of industrial pollution, temporarily not used for crop production (uncultivated).

To evaluate the effect of pollutants in mammalian cells, chromosomal and chromatid type aberrations were investigated. The results obtained were presented in Table 3.

Table 3: Genotoxicity of soil sample extracts obtained from different districts of the Tula region, as indicated by the test of induction of chromosomal aberrations in *Cricetulus griseus* cells

Samp-le №	Cell №	Proportion of aberrant cells, %	Frequency of aberrations/100 cells							
			chromosomal				chromatid			Total frequency (sum)
			dicentrics	paired fragments	centered rings	total	single fragments	exchanges	total	
Cont-rol	1594	0.69	0.44	0.00	0.00	0.44	0.00	0.25	0.25	0.69
1	200	4.60*	2.00	0.25	0.50	2.75	0.00	2.00	2.00	4.75*
2	200	2.00	1.00	0.00	0.00	1.00	0.00	1.00	1.00	2.00
3	200	4.00*	1.00	0.50	0.70	2.20	0.00	1.90	2.10	4.20*
4	200	1.50	0.50	0.00	0.00	0.50	0.00	1.00	1.00	1.50
5	200	1.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00	1.00
6	200	0.50	0.50	0.00	0.00	0.50	0.00	0.00	0.00	0.50
7	200	1.00	0.50	0.00	0.00	0.50	0.00	1.00	1.50	2.00
8	200	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Note: *95 % difference between the test sample and control.

The presence of chromosome type aberrations indicates the chromosome damage at the pre-synthetic stage (phase g1), when the chromosome is a single-stranded structure, while chromatid-type aberrations occur, when the chromosome is damaged at the double-stranded stage, i.e. in phase S and g2. Relying on the differences in the formation mechanisms of chromosomal and chromatid-type aberrations, it is possible to assume the nature of the mutagenic factor.

The results showed the significant mutagenic effect in cells with the increase in the frequency of chromosomal and chromatid-type aberrations by several times in the № 1 and № 3 extract samples, compared with control. The samples differed from control in both the proportion of aberrant cells and the total frequency of aberrations with 95 % statistical significance. Moreover, these results have corresponded with the mutagenic and toxic effects identified by the Ames test.

Soil extract samples № 6 and 8 did not show a mutagenic effect in *C. griseus* cells, the frequency of aberrations was at the control level. Soil extract samples № 2, 4, 5, and 7 showed the total frequency of aberrations above the control level, but the increase was statistically insignificant.

Thus, the result analysis of using a *C. griseus* cells cytogenetic test has enabled the identification of two samples with the pronounced genotoxicity. The extract samples № 1 and № 3 demonstrated the highest total frequency of aberrations, with the over 6-fold increase above control. In these two samples, the high total frequency of aberrations, as well as the ratio between aberration types have indicated the likely presence of toxic factors not only industrial but also of a different nature, for example, pesticides or mineral fertilizers.

The evaluation of the acute toxicity of soil extracts was performed by a common bio testing method based on the survival of crustaceans (*Ceriodaphnia affinis*). The results obtained were presented in Table 4.

Table 4: The survival of crustaceans *Ceriodaphnia affinis* in the soil samples extracts *

Sample №	Experiment duration/days					Survival rate, % of control
	1	2	3	5	7	
Control	20	20	20	20	20	100
1	20	19	19	14	11	55±2*
2	20	20	19	19	17	85±3
3	20	19	19	15	12	60±2*
4	20	20	20	19	18	90±4
5	20	20	20	16	13	65±2*
6	20	20	20	20	20	100±3
7	20	20	20	16	13	65±2*
8	20	20	20	20	20	100±3

Note: *Significance of difference from control $p<0.05$

In the experiments, where the crustaceans were kept in the test medium up to 48 hours, all the studied samples did not show acute toxicity, the death rate of the crustaceans did not exceed 5 %. In the seven-day experiments, the toxicity was revealed in the samples № 1, № 3, № 5 and № 7, the death rate of crustaceans was 35-45 %, whereas, in the remaining samples, the decrease in the survival rate of crustaceans did not exceed 15 %. Statistical analysis has shown that the overall difference between the test results of the № 1, № 3, № 5, and № 7 samples and control was statistically significant ($p<0.05$). Notably, the toxicity observed in these samples reflected the soil composition of the areas exposed to agrochemicals.

DISCUSSION

Three evolutionally different organisms, such as microorganisms, mammalian cells, and lower crustaceans – *Ceriodaphnia affinis* were used in the experiments. This approach allowed not only to integrally determine the potential risk of using the studied soils for crop production but also to compare different methods of biotesting. Noteworthy, the TA-100 strain was not able to detect mutagenic activity that corresponded with the similar observations published in [13].

Therefore, using the three bioassays has enabled identification not only the most dangerous districts (№ 1 and № 3) and detection of the pro-mutagenic activity of pollutants (№ 2 and № 4) but also to assume the sources of this activity, considering the contribution of agrochemical components in the first case and the industrial pollution – in the second. Since the radioactive contamination level was declined in several territories of the Tula region, the regular agroindustrial activities with intensive farming have been resumed. That lead to the further accumulation of various pesticides, as well as other pollutants in the soil that have been already present in these sites from the earlier use of DDT in agricultural practice.

As for industrial pollution, PAH derivatives, such as aromatic amines, oxidized PAH, and S-heterocyclic compounds [14], as well as heavy metal salts serve as its sources [15, 16].

Genotoxic agents are known to have the ability to interact with DNA and induce chromosomal aberrations leading to the development of abnormalities in the genome [17, 18]. Testing soil genotoxicity in the mammalian cell

culture, such as the Chinese hamster can be an informative method for both its hazard in general and the particular contribution of various components to it (ecotoxins of industrial and agrochemical origin).
Finally, the study of the acute toxicity of the soil in *Ceriodaphnia affinis*, to some extent, explained and complemented the results of the two previous tests. № 1, № 3, № 5, and № 7 samples with the toxic effect were obtained from the sites with intensive farming, where agrochemicals have been already present in the soil. Probably, the additional detrimental effect in daphnia caused by industrial pollutants present in the soil is likely to occur thanks to metabolic activation, as shown by the Ames test indicating the pro-mutagenic effect. It is worth mentioning that there is evidence that a bioassay with *D. magna* does not allow identification of the environmental hazards [19].

CONCLUSION

This study has confirmed the view that integral bio testing can be a useful approach for the assessment of the toxicity caused by environmental factors [20]. Such an approach based on the responses of the different organisms to environmental factors due to the differences in their metabolism is the most informative hazard assessment of pollutants. Also, it can be helpful to detect the presence of unknown compounds that can be mutagenic.
Thus, the bio testing of soil samples from several districts of the Tula region using three evolutionally different organisms (TA-98 and TA-100 strains of microorganisms, *C. griseus* cells, and *C. affinis*) revealed the presence of genotoxic agents that pose a potential hazard for crop production in these territories, even after the soil recovery over more than 30 year period since the Chernobyl accident. Further studies of the ecological toxicity of soils, as well as studies that would help to develop the advancements of the current bio testing methods and methods for the physico-chemical identification of natural and industrial pollutants, are needed.

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