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TOXIC AND GENOTOXIC POTENTIAL EVALUATION OF SOIL SAMPLES BY BIOASSAYS

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ABSTRACT

Soil samples were obtained from Slovenian industrial region Šaleška dolina and aqueous leachates were prepared. The toxicity and genotoxicity potential of these aqueous fractions have been evaluated by *in vitro* toxicity and genotoxicity bioassays. Freshwater toxicity test (PROTOXKIT F^{TM}) with a ciliate protozoan *Tetrahymena thermophila*, and genotoxicity comet assay using *Tetrahymena thermophila*, Caco-2 and HepG2 cell lines were performed. Biological data were completed through chemical analyses. It has been shown that physico-chemical analyses alone may not be sufficient to characterize soil hazards. To study soil ecotoxcity it is therefore necessary to take into consideration both, the physico-chemical analyses, toxicity and genotoxicity assays.

Key words: microbiology / toxicity / genotoxicity / soil samples / *in vitro* bioassays / Slovenia

VREDNOTENJE STRUPENOSTI IN GENOTOKSIČNOSTI VZORCEV ZEMLJE Z BIOTESTI

IZVLEČEK

Na industrijskem področju Slovenije, v Šaleški dolini, smo vzorčili zemljo in pripravili vodne izlužke zemlje iz štirih vzorčnih mest. Toksični in genotoksični potencial vodnih izlužkov smo ovrednotili z uporabo *in vitro* biotestov za toksičnost in genotoksičnost. Izvedli smo test toksičnosti s sladkovodno praživaljo, migetalkarjem, *Tetrahymena thermophila, s* t.i. PROTOXKIT F^{TM} testom in test genotoksičnosti: kometni test s praživaljo *Tetrahymena thermophila,* in Caco-2 ter HepG2 celičnima linijama. Rezultate biotestov smo dopolnili s fizikalno-kemijskimi analizami. Za relevantnejše ekotoksikološko vrednotenje vzorcev zemlje je pomembno združiti fizikalno-kemijske analize in teste toksičnosti in genotoksičnosti.

Ključne besede: mikrobiologija / toksičnost / genotoksičnost / vzorci zemlje / *in vitro* biotesti / Slovenija

INTRODUCTION

Soils are increasingly becoming sinks for a wide range of hazardous chemicals generated by human activities. These include aromatic compounds coming from black coal and lignite-mining, pesticides from agricultural soil contamination and heavy metals, solvents, and other industry related chemicals. Some are potent carcinogens and because of their solubility tend to accumulate in soils where their decomposition is mainly due to microbial action (Majer *et al.*,

2002). These hazardous compounds and their degradation metabolites endanger human health, soil and aquatic ecosystems by directly affecting soil biota or after runoff or leaking trough soil, they endanger water or groundwater biota. Hazard and risk assessment of polluted soil samples is usually performed by means of physical and chemical measurements, but chemical analyses alone may not be enough due to the fact that:

1) are restricted to a limited list of defined compounds,
2) some measurements are based on sum parameters to determine broader classes of chemical compounds (e.g. total hydrocarbons or PAHs) with a high heterogeneity of toxic properties for environmental targets, and
3) bioavailability of organic and inorganic pollutants can vary (Bispo *et al.*, 1999).

Risk assessment based on ecotoxcity data for the soil compartment is rare and insufficient and as such the risk assessment is highly uncertain. The assessment of synergistic or antagonistic effects of the potentially (geno)toxic substances present in the soil, based on the assessment of the substance concentartions determined by physico-chemical analyse is very difficult (Debus and Hund, 1997). Thus alternative methods are needed such as biological tests (bioassays), which consist of exposing of living organisms to polluted materials. Two ways are already available to assess soil ecotoxicity (Bispo *et al.*, 1999). First, soil biota can be used: bacteria (Majer *et al.*, 2002), earthworms, collembolans (Zang *et al.*, 2000; Žnidaršič *et al.*, 2003, Drobne *et al.*, 2005), and plants (Ma, 1983). The use of these organisms has been standardised to assess the effects of pure substances on soil biota (ISO 11269-1 and -2, 1993: ISO 11268-1 and -2, 1994; ISO 11240-1 and -2, 1997), the draw back being that orgnisms can adapt to polluted soil materials. Bioassays, which use entire solid matrix, are both time and space consuming and therefore expensive. The second way to assess the soil ecotoxicity is to perform bioassays on soil water leachates (Beakert *et al.*, 1999, Bekaert *et al.*, 2002), where a response can be obtained rather quickly.

In this study the (geno)toxicity of soil samples was determined by bioassays on soil water leachates. The aim of these preliminary studies was to introduce indirect exposure procedures to assess soil ecotoxicity in combination with physico-chemical analyses.

MATERIAL AND METHODS

Soil samples

Coal and lignite-mining and coal firing, industry, nearby thermal power plant (TPP) and lead smelter (Žerjav), the traffic, and farming, left ecotoxicological consequence in Slovenian industrial-rural region called Šaleška dolina and nearby region, where four soil samples (Silova-sample 1, which is 8.2 km distant from TPP; Mali vrh-sample 2, 4.0 km distant from TPP; Lubela-sample 3, 5.0 km distant from TPP and Andraž-sample 4, which is 6.4 km distant from TPP) were collected according to ISO standard procedure (ISO 11464, 1994; ISO 10381, 1996) at one occasion in december 2003 (9.12.2003). Soil sampling and physico-chemical analysis were performed in the laboratories of ERICo Velenje. Soil samples were dried and sieved according to standard procedure (ISO 11464) and afterwards stored at –20 °C for further analyses. Since the thermal power plant is the main source of heavy metal pollution in the vally, where Pb, Cd, Zn, Hg beside nitrogen oxides and sulphur oxides, are released into the environment as dust particles, heavy metal contents were determined and physico-chemical analyses were performed according to recommended ISO standards (SIST ISO: 5664, 5666, 6468, 10523, 11083, 1996; SIST EN 27888, 1998; SIST EN ISO 10304-2, 1998; SIST ISO: 8245, 9562, 2000; SIST DIN 38406-29, 2000). For biotesting water soil lecahtes were prepared (4.2.2004). Leaching was performed for 24h in a linear shaking apparatus at 25±1 °C and the

suspension was allowed to settle for 24h at 4 °C. The supernatant was then used for biotesting the next day (Békaert *et al.*, 1999).

In vitro comet assay with *Tetrahymena thermophila*

T. thermophila (amicronucleate strain) was originally obtained from Microbiotest (Belgium) as part of the Protox F^{TM} kit. *T. thermophila* cells were cultivated in a semidefined medium for protozoa (Schultz, 1997) at 30 °C for 72h. The cells grow in liquid cultures as motile unicellular ciliates below the surface of the medium. Before incorporation of the cells into the agarose layers, the cells were concentrated by centrifugation in Falcon tubes for 3 min at 300 x *g* and 4 °C.

The dye-exclusion test with Trypan blue (Wilson, 1986) was used to examine the viability of cells before the comet assay was performed.

The modified version of the original Singh *et al.* (1988) protocol, the alkaline comet assay on protozoa was preformed according to the method described by Lah *et al.* (2004). In order to assess genotoxic potential of potentially bioavailable fraction of soil, the cells of *T. thermophila* were exposed to water leachates of soil. The cells were also exposed to 0.9% solution of NaCl (a negative control) and to 500 μM hydrogen peroxide, H_2O_2 (a positive control).

***In vitro* comet assay with Caco-2 and HepG2 cell line**

Epithelial colon cancer cells (Caco-2, obtained from Instituto Zooprofilatico Esperimentale, Brescia, Italy) and human hepatoma cell line (HepG2 cells, obtained from prof. dr. Knasmüller, Institute of Cancer Research of The Univeristy of Vienna) were grown in multilayer culture at 37 °C in hummified atmosphere of 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal calf serum (FCS) and antibiotic (0.1% gentamycin) in culture flasks for 8 days (cell density: 10^6–10^8 cells/mL). Medium was changed every 2 days. Single cell suspensions were prepared with 0.25% trypsin-EDTA solution, passed through injection needle for several times to achieve single cell suspension and finally resuspended in DMEM medium, supplemented with 10% FCS medium (Lah *et al*., 2005).

The dye-exclusion test with Trypan blue (Duthie *et al*., 1997a) was used to examine the viability of cells before the comet assay was performed.

Briefly, to achieve a uniform background rough microscope slides were first coated with up to 400 μL of 1% normal melting point agarose (NMP) and left to air dry overnight. The supportive (second) agarose layer (0.6% NMP agarose) was solidified on ice and the collected Caco-2 or HepG2 or protozoa cells were immobilized in the third layer at concentrtion of approximately 1–2 $\times 10^5$ cells/ml. After removing the cover glasses, the slides were covered with 500 μL of 0.5% LMP agarose (the fourth layer).

Four layered slides with incorporated cells were first submerged into water samples, negative control and positive control solutions for 20 min and followed by 1h incubation in alkaline lysis buffer. The slides were submerged in electrophoretic buffer (pH > 13) to unwind the nuclear DNA for 1h and then subjected to electrophoresis in the same buffer. The electrophoresis was carried out at 2V/cm and 300 mA; time of electrophoresis depended on cell type (30minutes for Caco-2 and HepG2 cells, and 5 minutes for *Tetreahymena thermopila* cells). Following electrophoresis the gels were neutralized in 400 mM Tris-HCl pH 7.5 for 15 min. The damaged DNA travelled toward the anode during electrophoresis and formed an image of a “comet” tail. After staining the slides with ethidium bromide (20 μg/mL) the comets were detected and quantified as described below.

Data collection and statistical analyses of the results of the comet assay

Quantitative analyses of nuclear DNA damage in *Tetrahymena thermophila,*Caco-2 and HepG2 cells was done by 20 x objective magnification with an epifluorescence microscope (Olympus BX 50) using a BP 515–560 nm filter and BA 590 nm barrier filter and digital camera (Hammamatsu Orca 2) connected to a computer, and the comets were scored using Komet 5.0 Computer Software (Kinetic Imaging, 2001).

Olive tail moment (OTM) was chosen as the most relevant measure of genotoxicity. Tail length and the percentage of DNA in comet tails and heads were collected. These values were used to calculate OTM, using the relationship: OTM = (tail mean – head mean) X tail % DNA / 100 in arbitrary units (Olive *et al*., 1990).

Images of 50 comets were collected from each of two replicate slides per sample, OTMs were calculated using SAS/STAT statistical software (version 8e; SAS Institute, Cary, NC, 2000) and software package Graph Pad Prism 3.0 (version 3.02, GraphPad Software Inc., 2000). Descriptive statistics were determined by the MEANS procedure. OTM records were tested for normal distribution with the UNIVARIATE procedure. Bauer *et al.* (1998) suggested that the distribution of OTMs obeys a Chi-square (χ^2) distribution. The chi- square distribution, which is a special case of the gamma distribution, fitted well to our data. As a consequence, data were analysed by the GENMOD procedure (Generalised Linear Models) which allows distributions other than a Gaussian one. Statistically significant differences between groups were evaluated by the linear contrast method.

Fresh water toxicity test with ciliated protozoan *Tetrahyemna thermophila* (PROTOXKIT F^{TM})

A 24h growth inhibition test was performed with the ciliate *Tetrahymena thermophila*. The test is based on the turnover of substrate into ciliate biomass. While normal proliferating cell cultures clear the substrate suspension in 24h, inhibited culture growth is reflected by remaining turbidity. Optical density measurements of the turbidity quantify the degree of inhibition. To minimize toxic buffering of e.g. heavy metals and/or reactive and highly lipophilic substances (by binding the organic components in the medium which may interfere with the bioavailability of the toxicants) the PROTOXKIT medium had an organic content more than a factor 100 lower than that of the proteose-pepton media, used in conventional *Tetrahymena* tests (Microbiotest, Protox F^{TM}, Belgium).

Data treatment – estimation of the 24h EC50 for PROTOXKIT F^{TM} test

According to the kit instructions (Microbiotest, Protox F^{TM}, Belgium), the mean for the two parallels for each toxicant/sample dilution and the control was calculated. Then the difference between the mean OD at T0 and T24 for each toxicant/sample dilution ($\Delta OD_{C1–Cx}$) and for the control (ΔOD_{C0}) was calculated. The % of inhibition for each toxicant/sample dilution was estimated by the following equation:

$$\% \text{ inhibition}_{(C1–Cx)} = \left(1 - \frac{(\Delta OD\ C1\text{-}Cx)}{(\Delta ODC_0)}\right) \times 100$$

RESULTS AND DISCUSSION

In this study, *T. thermophila* was taken as a test organism because it represents an ideal model for both toxicological and ecotoxicological studies (Sauvant *et al*., 1999) and it can be easily grown in pure cultures. Protozoa have also proved as sensitive organisms to environmental

changes (Nicolau *et al*., 1999, Chen and Leick, 2004). *Tetrahymena thermophila* has been used as a microbial model due to its typical eukaryotic characteristics. Its ultrastructure, cell physiology, development, biochemistry, genetics and molecular biology have been extensively studied (Asai and Forney, 2001) and the organisms are extensively used for toxicity testing because of their sensitivity to heavy metals (Chen and Leick, 2004).

Short-term tests with cultivated mammalian cells are widely used for detection of potential environmental mutagens and carcinogens. Since numerous compounds exert their genotoxic and carcinogenic effects only after metabolic activation, it is important that indicator cell lines possess critical enzymes. For this reason, indicator cells competent in xenobiotic metabolism would be desirable (Glatt *et al.,* 1990). HepG2 cells are a highly differentiated human hepatoma cell line, which has retained many of the specialized functions usually lost upon culturing and as such represent a suitable *in vitro* system for studying drug metabolism and (geno)toxicity in man (Duverger-van Bogaert *et al.*, 1993). The other chosen indicator cell line was Caco-2. Even though this cell line appears not to posses all the desirable xenobiotic metabolising enzymes, it exhibits different degrees of specialisation and enterocyte like functions. The intestinal epithelium permeability, which resembles the Caco-2 cell line, is a critical characteristic that determines the rate and extent of human absorption and ultimately the bioavailability of a xenobiotic compound (Duthie *et al.,* 1997b).

Toxic substances that could be found in soil and water are numerous. Using chemical and physical analyses, we tried to estimate the concentrations of some of the most (geno)toxic substances like heavy metals, pesticides, volatile chlorinated aliphatic carbohydrates and aromatic substances. Some of the general parameters like (pH, specific electric conductivity, etc.) were also estimated, because their contribution to overall (geno)toxicity is great. Sulphate, phosphate, nitrogen and carbon contents are also very important parameters in the field of ecotoxicological evaluation of environmental samples. Studies on toxic effects of trace metals on both soil (micro)organisms and native plants (Glasenčnik *et al.*, 2004; Kugonič *et al.*, 2004) in field conditions are limited and differ from the studies in the laboratory conditions. Studies of the toxic effects of metals should take into consideration: the characteristics of the toxic metal (metal chemical forms) bioavailability, mechanisms of toxic metal action, interactions with other metals, cumulative effects, etc. Some of the main problems in such studies are that soil properties influence the rates of metal transfers to plants and other organisms and no chemical or toxicant interactions are taken into account, etc. (Patra *et al*., 2004).

The total metal concentration of heavy metals in soil samples is not a reliable indicator of metal concentration extracted with water, therefore it is expected that the results of (geno)toxicity assays of complex mixtures like soil are strongly influenced by the method of sample preparation and extraction, because chemical and physical properties of constituents, including genotoxic compounds, in the mixture may differ substantilly. In most of the studies assessing (geno)toxicity, leachates of soil samples were prepared prior to the assay. The leachates from soil samples were generally made by shaking soil samples with aqueous solvents and preferably not filtered (Bekaert *et al.*, 2002), so we followed this step in our study too.

Sample 2 (followed by sample 3, 4 and 1) showed the highest heavy metal contents according to chemical and physical analyses of water soil leachates, presented in Table 1. The concentrations of all parameters for soil leachates measured in table 1 were bellow the detection limit of chosen chemical analytical methods for all sampling locations according to recommended standard method SIST DIN 38406-29 (2000) and ATSDR/CERCLA (2003). However the measurements of As and Cd contents in all four soil samples, exceeded the critical value for As (72.2 mg kg^{-1}) and the warning immision value for Cd (3.6 mg kg^{-1}) only in sample 2. Limit immision value for Ni (62.3 mg kg^{-1}) was exceeded in sample 1 (Table 3, Uredba., Ur. L. RS, 1996; Stropnik, 2003).

Table 1. Heavy metal contents and other physico-chemical prameters of water soil leachates

Preglednica 1. Prikaz rezultatov vsebnosti težkih kovin in fizikalno-kemijskih analiz vodnih izlužkov zemlje

		sample point			
PARAMETER	units	Sample 1	Sample 2	Sample 3	Sample 4
Arsenic – As	µg/L	1.23	1.82	1.39	1.40
Cobalt – Co	µg/L	0.98	2.83	1.91	0.26
Chromium – Cr	µg/L	0.89	1.29	0.87	0.75
Molybdenum – Mo	µg/L	1.06	15.00	2.45	1.38
Selenium – Se	µg/L	2.01	2.17	2.01	1.54
Silver – Ag	µg/L	0.54	0.55	0.59	0.53
Copper – Cu	µg/L	5.17	9.67	4.87	13.20
Zinc – Zn	µg/L	4.32	6.69	6.50	10.60
Cadmium – Cd	µg/L	0.66	0.73	0.70	0.65
Nickel – Ni	µg/L	5.74	2.72	1.75	4.02
Lead – Pb	µg/L	3.25	3.75	3.73	3.61
Specific Electric Conductivity – SEC (T= 25.0 °C)	µs/cm	391.0	240.0	235.0	86.0
T (SEC)	st.C	20.0	20.6	20.0	19.6
Volatile chlorinated CH – VCCH	µg/L	<0.5	<0.5	<0.5	<0.5
1,1,1-trichloroethane	µg/l	<0.5	<0.5	<0.5	<0.5
1,1,2,2-tetrachloroethilene	µg/l	<0.5	<0.5	<0.5	<0.5
1,1,2-trichloroethilene	µg/l	<0.5	<0.5	<0.5	<0.5
Dichloromethane	µg/l	<0.5	<0.5	<0.5	<0.5
Tetrachloromethane	µg/l	<0.5	<0.5	<0.5	<0.5
Trichloromethane	µg/l	<0.5	<0.5	<0.5	<0.5
Chloride	mg/L	2.92	1.56	1.40	1.25
Nitrate – nitrogen	mg-N/L	<0.2	<0.2	<0.2	<0.2
Nitrite – nitrogen	mg-N/L	<0.3	<0.3	<0.3	<0.3
Sulphate	mg/L	2.98	3.44	1.81	1.31
pH	/	7.76	8.21	8.20	7.71
T (pH)	°C	19.0	19.7	19.2	19.9
Chromium – Cr (VI)	mg/L	<0.01	0.02	0.01	<0.01
Volatile aromatic CH – BTX	µg/L	<10	<10	<10	<10
Benzene	µg/L	<10	<10	<10	<10
Ethilbenzen	µg/L	<10	<10	<10	<10
Xylene	µg/L	<10	<10	<10	<10
Toluene	µg/L	<10	<10	<10	<10
Trimethilbenzene	µg/L	<10	<10	<10	<10
Ammonia nitrogen	mg N/L	1.7	2.1	2.3	1.1
Mercury – Hg	µg/L	<0.5	<0.5	<0.5	<0.5
total organic carbon – TOC	mg C/L	47.8	40.5	23.1	20.5
Adsorptive Organic Halogens – AOX	µg Cl /L	11.00	11.00	<10	<10
Total cyanide	mg/L	<0.05	<0.05	<0.05	<0.05

A set of bioassays was performed on the leachates obtained from four soil samples to asses the toxicity and genotoxicity. Because of the problems of extractability of heavy metals and other possible genotoxic compounds mentioned above, different results from different bioassays using different cell types were expected. The results of the toxicity assay PROTOXKIT F^{TM} test with water leachates presented in Table 2, showed the estimated EC50 value of 69.99% for sample 4. For all the other samples the EC50 values were not detected due to low toxicity.

Table 2. Optical density measurements (OD at 440 nm) and estimation of EC50 value with PROTOXKIT F^{TM}

Preglednica 2. Prikaz meritev optične gostote (OD pri 440 nm) in EC50 s testom PROTOXKIT F^{TM}

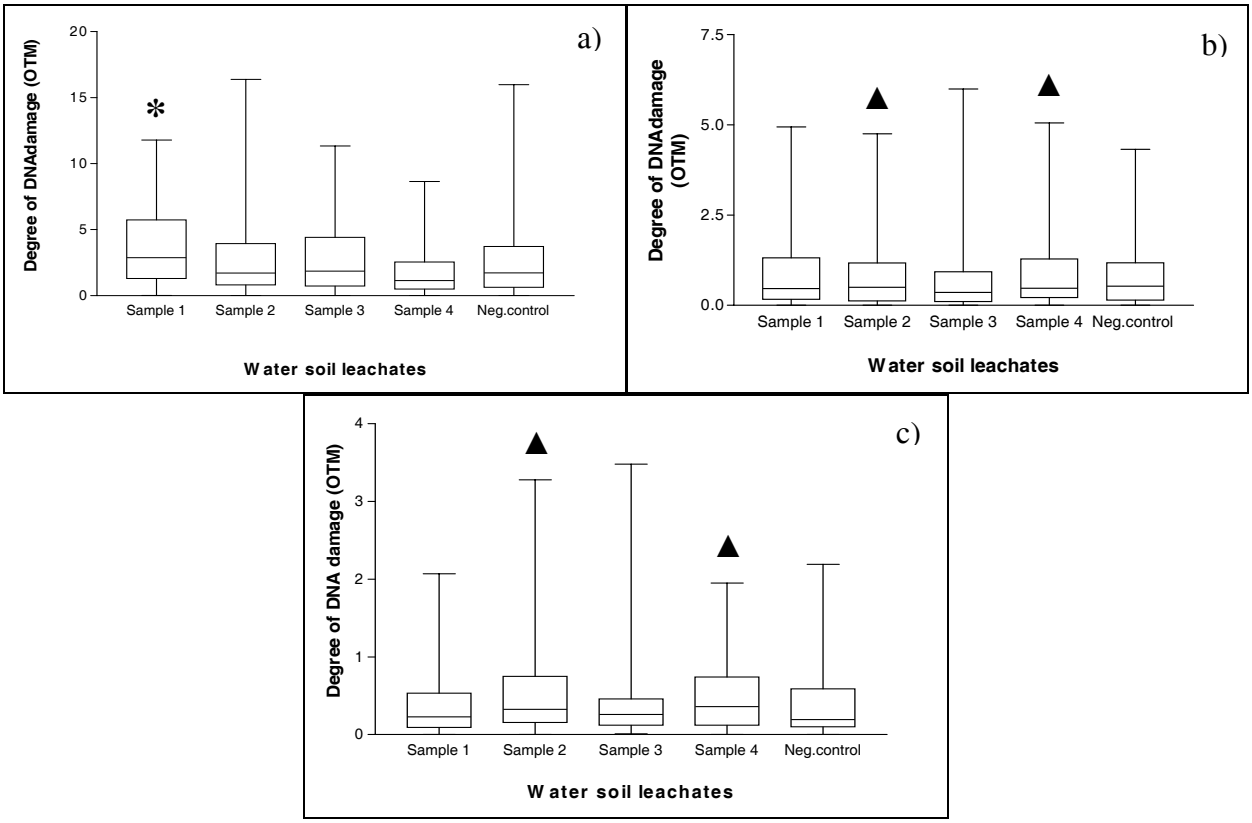
Sample point	Sample concentration %	OD(»a«)			OD (»b«)			% inhibition	EC50
		T0	T24	T48	T0	T24	T48		
Sample 1	control	0.613	0.567	0.167	0.683	0.571	0.159	/	ND
	100	0.634	0.634	0.223	0.655	0.595	0.208	11.55	
	50	0.690	0.604	0.203	0.678	0.567	0.201	0.62	
	25	0.725	0.614	0.184	0.607	0.566	0.188	1.03	
	12.5	0.656	0.610	0.196	0.646	0.576	0.188	5.36	
	6.25	0.688	0.585	0.420	0.688	0.600	0.164	18.35	
Sample 2	control	0.685	0.579	0.154	0.682	0.588	0.185	/	ND
	100	0.662	0.496	0.159	0.685	0.506	0.176	4.18	
	50	0.742	0.555	0.150	0.616	0.579	0.187	0.68	
	25	0.675	0.547	0.165	0.683	0.551	0.145	–1.95	
	12.5	0.646	0.534	0.206	0.673	0.550	0.159	7.20	
	6.25	0.678	0.535	0.227	0.714	0.577	0.174	3.60	
Sample 3	control	0.641	0.549	0.167	0.648	0.518	0.125	/	ND
	100	0.711	0.505	0.157	0.643	0.519	0.149	–5.12	
	50	0.685	0.513	0.159	0.662	0.533	0.137	–5.42	
	25	0.689	0.534	0.150	0.690	0.564	0.179	–5.32	
	12.5	0.656	0.582	0.165	0.705	0.525	0.227	3.81	
	6.25	0.639	0.556	0.169	0.666	0.528	0.257	11.84	
Sample 4	control	0.666	0.557	0.167	0.675	0.578	0.159	/	69.99%
	100	0.818	0.727	0.697	0.797	0.750	0.578	65.91	
	50	0.686	0.611	0.318	0.762	0.677	0.403	34.58	
	25	0.677	0.596	0.331	0.649	0.580	0.326	34.09	
	12.5	0.668	0.550	0.261	0.712	0.579	0.345	23.74	
	6.25	0.635	0.526	0.237	0.699	0.550	0.292	20.69	

a, b – parallels of dilutions of water leachates; ND- EC 50 not detected

The results of the comet assay on three different cell types are presented in Figure 1. The comet assay results with *Tetrahymena thermophila* cells showed the statistically significant increase of genotoxic potential according to negative control ($p < 0.005$) for sample 1. The comet assay with Caco-2 and HepG2 cell lines showed increases of genotoxic potential according to negative control for samples 2 and 4, which however were not statistically significant. The negative results obtained by the comet assay on human cell lines and also in toxicity testing with PROTOX F^{TM} test for samples 1, 2 and 3 could be explained by low extractability of heavy metals and other possible genotoxic compounds (like pesticides) by water.

Observations of potential metal genotoxicity in soil are rare and majority of metal (geno)toxicity are focused on soil-plant interactions trough metallic salts. Most metallic salts are effective poisons at particular concentrations, because they are able to bind to thiol groups and

induce spindle disturbances in the cells which can be detected by micronuclues assay on plant cells.



* statistically significant

▲ increased degree of DNA damage according to negative control, but not statistically significant

Figure 1. Nuclear DNA damage in *T. thermophila* (a), Caco-2 (b) and HepG2 (c) cells (represented as OTM) treated with water soil leachates. Results from 100 comets for each soil sample are shown as box-and-whiskers plots. The OTM values are shown as boxes that include 50% of the data. The top and bottom of the boxes mark 25th and 75th percentiles; the inner line marks the median value. 25% of the data above the 75th percentile and 25% of the data below the 25th percentile are marked as "whiskers" limited by the maximum or minimum values.

Slika 1. Poškodbe jedrne DNK na T. thermophila (a), Caco-2 (b) and HepG2 (c) celicah, izražene z RM-Olive (Repni Moment po Olivu) po izpostavitvi vodnim izlužkom zemlje. Rezultati 100 ocenjenih celic so prikazani z okvirji z ročaji. 50 % podatkov vrednosti RM-Olive so prikazane v okvirjih. Zgornji in spodnji ročaj predstavljata 25. in 75. percentil; notranja meja, pa mediano vrednost. 25 % podatkov se nahaja nad vrednostjo 75. percentila in 25 % pod vrednostjo 25. percentila, ki sta omejeni z maksimalno in minimalno vrednostjo RM-Olive.

Many researchers including Bekaert *et al.* (1999) agree that it is hard to establish the relationship between the contaminants and (geno)toxicity results because of interactions phenomena such as synergistic and antagonistic effects. While interpreting the data, the influence of natural substances extracted from the soil studied should not be excluded. A negative control soil with the same composition as samples studied would have been needed to distinctly state the genotoxic effects of present soil contaminates. As negative control with an identical composition to the soil sample is rarely found in environmental studies, the

identification of the kinds of pollutants responsible for (geno)toxicity is always difficult and can only be suggested. It can be frequently observed that genotoxic respones are registered in bioassays, while no (geno)toxic chemicals can be identified in the fraction studied. Many authors report on mutagenicity found in environmental samples, although no mutagenic compound could be detected following standard analytical methods (Maron in Ames, 1983; Donnelly *et al*. 1995), this underlines the fact that physical and chemical analyses are not always extensive and that other pollutants than the measured ones may be present in the tested sample and may be responsible for the (geno)toxicity observed.

Table 3. Heavy metal contents in soil samples for chosen locations accordnig to Slovenian regulations (Uredba., Ur. L. RS, 1996)

Preglednica 3. Vsebnosti težkih kovin v tleh na izbranih vzorčnih lokacijah in imisijske vrednosti težkih kovin v tleh povzeto po veljavnem predpisu (Uredba., Ur. L. RS, 1996)

	Cd mg kg^{-1}	As mg kg^{-1}	Ni mg kg^{-1}
Limit immision value	1	20	50
Warning immision value	2	30	70
Critical Immision value	12	55	210
Sample 1	0.6	9.1	62.3
Sample 2	3.6	72.2	37.9
Sample 3	1.8	18.8	27.6
Sample 4	0.5	3.0	16.1

CONCLUSIONS

A combined testing protocol for risk assessment using both biological and physico-chemical analyses provides more accurate information than the use of either method alone. Our future plan is to introduce a plant bioassay, *Tradescantia* micronucleus assay, for (geno)toxicity testing of soil samples and soil water extracts. This may give more relevant information on soil ecotoxicity. Since the extractability of heavy metals with water is expected to be very low, the tests on soil samples and water extracts need to be done in order to compare the results and to determine the most sensitive battery of bioassays suitable for soil ecotoxicity evaluations.

POVZETEK

Onesnaženje zemlje vpliva tudi na vodni ekosistem in posledično na človeka. Vnos strupenih snovi (kot so: težke kovine, pesticidi, ipd.) v zemljo postaja vedno večji in mnoge od teh snovi imajo potencialni (geno)toksični in/ali kancerogni vpliv na živa bitja. Kemijske analize vzorcev zemlje in vodnih izlužkov zemlje nam ne povedo veliko o dejanskih bioloških učinkih prisotnih onesnaževal v okolju, zato je uporaba biotestov v spremljanju stanja okolja zelo pomembna. Le biotesti nam lahko ponudijo odgovore o možnem sinergizmu, aditivizmu in drugih učinkih onesnaževal na živa bitja, iz česar lahko tudi izpeljujemo ocene tveganja človeka, ki se nahaja v takšnem okolju.

V Šaleški dolini, kjer je v preteklih letih prihajalo do onesnaženja zaradi premogovništva, bližine termoelektrarne in toplarne in danes prihaja do onesnaženja v glavnem zaradi prometa in kmetijstva, smo vzorčili zemljo. Pripravili smo vodne izlužke zemlje. Ker nam fizikalno-kemijske analize izlužkov pokažejo le prisotnost določenih snovi in njihove koncentracije, smo

želeli v tej študiji preveriti še geno(tokičen) potencial izlužkov zemlje z biotesti. V ta namen smo izvedli test strupenosti s *Tetrahymeno thermophilo* (PROTOXKIT F^{TM}) in kometni test (test genotoksičnosti) z istim organizmom in dvema celičnima linijama (Caco-2 in HepG2).

Fizikalno-kemijske analize niso pokazale preseženih mejnih koncetracij težkih kovin, pesticidov in njihovih presnovnih produktov ter drugih organskih spojin v vodnih izlužkih, glede na lestvico ATSDR/CERCLA (2003). Biotesti pa so pokazali strupenost enega vzorca (vzorec 4) in povečano stopnjo poškodb DNA glede na negativno kontrolo, ki je bila statistično značilna v primeru uporabe *Tetrahymene thermophila* kot testnega organizma, in statistično neznačilna za vzorca 2 in 4 v primeru uporabe celičnih kultur.

Menimo, da je potrebno za relevantnejše ekotoksikološko vrednotenje vzorcev zemlje in drugih vzorcev iz okolja, združiti fizikalno-kemijske analize okoljskih vzorcev s testi toksičnosti in genotoksičnosti.

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