Technical Paper

Genotoxicity Detection in Drinking Water by Ames Test, Zimmermann Test and Comet Assay

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Received 29-03-2005

Abstract

Drinking water of good quality is the primary requirement from public health point of view. Over past two decades, many studies have reported the presence of various hazardous compounds in drinking water that may cause long-term health effects, e.g. gastrointestinal and urinary tract cancers. Sources of drinking water contamination are industrial and agricultural activities, disinfection by-products and transportation. Monitoring of drinking water in Slovenia is based on regular physico-chemical and microbiological assays according to European Community legislation. Since physico-chemical analyses do not provide enough information about biological effects of pollutants, we are studying the possibilities of including biological tests into drinking water monitoring. In the present work we tested three genotoxicity assays with water collected at three different sampling points in Ljubljana drinking water region. The Ames test was performed with and without metabolic activation using bacterium *Salmonella typhimurium* TA97a, TA100 and TA1535 strains. The same samples were tested with the yeast *Saccharomyces cerevisiae* strain D7, with and without metabolic activation in Zimmermann test. Parallel genotoxicity evaluation on the samples was carried out as alkaline version of the comet assay performed with human HepG2 cell line. Original and concentrated water samples were tested in all bioassays following the chemical analyses for pesticides and nitrate. There was no indication of genotoxic activity in any of drinking water samples according to Ames and Zimmermann test. On the contrary, the results of the comet assay revealed low genotoxicity in most of the drinking water samples. As only the Comet assay proved to be sensitive enough to detect genotoxicity, we propose to include it in regular biomonitoring of drinking water.

Key words: drinking water, physico-chemical analyses, *in vitro* genotoxicity bioassays

Introduction

Water is a universal solvent and may contain a wide diversity of substances arising from industrial, agricultural and other sources and from supply systems. Water, containing diverse substances, can be modified by treatment and storage. (Geno)toxicological safety of water, especially drinking water, represents an important issue for safeguarding of health and well-being of humans. It should be assumed that there is a potential for all members of population to be exposed to drinking water that might contain possible hazardous contaminants. Therefore, strict quality requirements should be set to protect public health. What is (geno)toxicity, what is risk, and what we wish to do or we are prepared to do about such risks, all these questions are the questions that arise when dealing with safety of the drinking water and which many researchers encounter.[1,2,3] Epidemiological studies have shown that a correlation between genotoxicity of drinking water and increased cancer risks, exists.[4,5,6,7]

The difficulties encountered in performing physico-chemical analyses (i.e. detecting only known chemicals, classifying the chemicals present in a sample and not knowing what their biological effects are, not being able to detect very low quantities of particular chemical, etc.), long term carcinogenicity tests and epidemiological studies have encouraged the analysis of drinking water using short-term mutagenicity biotests.

Another alternative or complementary analytical tool in monitoring of drinking water represent biosensors. Biosensors are analytical devices which use biological interactions to provide either qualitative or quantitative results. They offer the specificity and sensitivity of biologically based assays packed into convenient devices which allow for rapid and superior reaction control.[8] The biosensor technology offers the possibility of identifying and quantifying specific compound directly in water or in air. Biosensors can complement classical analytical methods because they are able to distinguish between bioavailable and unavailable forms of known contaminants present in a sample.[8,9]

Mutagenicity biotests on the other hand are rapid, relatively cheap and predictive of integral mutagenic/carcinogenic activity, and can evaluate the combined action of potentially hazardous compounds present in drinking water as complex mixtures and not only a specific compound like by biosensors. They are able to take into consideration the bioavailability of (geno)toxic compounds, their synergism, aditivism or even antagonism. They provide the answer of an organism/cell, the actual effects, of the whole mixture of (geno)toxic compounds potentially present in drinking water samples or in other environmental samples. Toxic and genotoxic action in fact is the consequence of addition, synergism, antagonism and bioactivation what can be directly shown only by biotests.

The quality control of drinking waters and wastewaters based only on chemical measurements or detection of specific pollutants by biosensors is not sufficient to assess the environmental and human health risks. This measurements are not real measurements of (geno)toxicity effects because (geno)toxicity is a biological response. Therefore only the use of biological assays can provide direct and appropriate measurements of (geno)toxicity. This is important especially in the case where measured pollutants do not exceed maximum allowed concentrations determined by chemical and biosensors based analyses, but where previously mentioned interactions between the pollutants can play an important role in causing (geno)toxicity effects on living beings.[10]

A battery of *in vitro* short-term genotoxicity tests revealing different genetic end-points was used in this study on non-concentrated and concentrated drinking water samples. The following in vitro biotests were performed: the Ames/*Salmonella typhimurium* test[11] using TA97a, TA100 in TA1535 strains in the presence and absence of bio-activation (± S9); the Zimmermann test[12] using yeast *Saccharomyces cerevisiae* diploid D7 strain in the presence and absence of bio-activation (± S9) and the comet assay using human hepatoma cell line (HepG2 cells).[13]

The Ames and the Zimmermann tests are being able to demonstrate nuclear DNA effects such as point mutations, gene reversion and gene conversion to assess the mutation induction after exposure to drinking water samples on bacterial and yeast DNA. *In vitro* alkaline single cell gel electrophoresis or comet assay on HepG2 cells involves detection of cell DNA fragments under alkaline conditions, which during electrophoresis, migrate from the nuclear core, resulting in "a comet" formation. Comet assay is a method for DNA alkali-labile sites and strand breaks detection in individual cells and is one of the major tools in environmental pollution biomonitoring, both *in vivo* and *in vitro*.[13,14,15]

The aim of the present study was to compare the potential genotoxic effects induced by drinking water samples in prokaryotic (*Salmonella*) and lower eukaryotic (yeast) cells and the DNA damage in human hepatoma cell line with the results of physico-chemical analyses. Since nitrates and pesticides enter the water systems by agricultural activities on land surfaces trough fertilization and crop protection processes and since the chosen sampling points of drinking water, based on previous analyses,[16] showed elevated levels of nitrates and pesticides (i.e. atrazine), our interest focused mainly in evaluating the genotoxicity potential of chosen parameters which are also included in regular monitoring of drinking waters according to Slovenian regulations.[17,18,19]

Experimental

Water sampling

Three different parts of Ljubljana drinking water region were selected as sampling sites where tap water samples were taken (samples marked 1, 2, and 3). The regular disinfection process in this area is charcoal filtration. The analysed water samples were the same as those collected for physico-chemical analyses done by the Institute of public health of the Republic of Slovenia (Table 1). Sampling was performed according to the recommended standard method (ISO 5667-5)[20] and was done at one occasion in February 2004. Water samples were transported to the laboratory in 500 mL glass flasks and stored at –20 °C for further testing. Prior to the genotoxicity bioassays 0.9% NaCl was added to the samples in order to avoid hypoosmotic shock, which causes cytotoxic effect resulting in false positive genotoxic results later on. The negative control in all biotests performed was sterile 0.9% NaCl solution prepared from MilliQ water. The concentrating of drinking water samples was done on XAD resins according to the guidance of the producer.[21] The final concentration factor was 1:1000.

Concentrated water samples, diluted with sterile MilliQ water to final concentration 50 ×, and none concentrated water samples were then tested in triplicates.

Ames/*Salmonella typhimurium* test

Ames test was carried out as standard plate incorporation test[11] with *Salmonella typhimurium* strains TA97a, TA100 and TA1535 with and without in vitro microsomal activation (by S9 rat liver homogenate). Strain specific genetic markers were verified prior to use. Mutagenic activities were expressed as induction factors, i.e. as multiples of the background levels. The results were considered positive if the tested sample produced a response which was at least twice as high as the one found with the negative control.[11,22] For positive

controls 4-Nitroquinoline-N-oxide (4NQNO) and 2-amino fluorene (2AF), dimethyl sulfoxide (DMSO) was used as solvent; for methy-methane sulphonate (MMS) and sodium azide, water was used as a solvent. Positive controls for strains TA97a-4NQNO; TA100-MMS and TA1535-sodium azide, were used without metabolic bioactivation and 2AF for all the strains with metabolic bioactivation. After 48h incubation of agar plates at 37 °C counting of bacterial colonies was performed.

Zimmermann test

For the *Saccharomyces cerevisiae* test the procedures described by Zimmermann et al.[12] and Stehrer-Schmid[23] were used. Colonies of strains D7 were grown to saturation according to Zimmermann[12] and stored at 4°C. During storing phase spontaneous reversion rates were determined. Cultures with the lowest spontaneous background (30–60 convertants/10^5 cells and 10–20 revertants/10^6 cells) were grown at 28 °C to exponential phase of growth (6–8×10^7 cells/mL) and washed cells were suspended in 125mM phosphate buffer pH = 7.2. Exponential cultures were used, because these cells responded with higher sensitivity to mutagenic/carcinogenic action, according to Zimmermann.[12] Cells were exposed to drinking water samples for 4h at 28 °C and washed cells were plated on appropriate media to detect revertants, convertants and survival rate. The controls followed the same experimental protocol and were prepared with cells treated with 0.1 and 0.01 mM ethil-methane-sulphonate without and with metabolic bioactivation (–S9 and +S9). Five plates in each category were incubated at 28 °C for 3 days for survival rates and frequency of convertants determination and 6–8 days for frequency of revertant determination. In every test the actual colony count were related to 10^5 for convertants and revertants cells surviving the treatment with the sample.

Statistical analysis for the Ames and Zimmerman test and interpretation of the results

Genotoxic activities for both tests were expressed as induction factors (induction factor of reversions and conversions) i.e. as multiples of the background levels. Statistical significance was evaluated with Kruskal-Walis test (non-parametric ANOVA) for differences between treatment groups and Dunnett's C multiple comparison for differences to the negative control.

The interpretation of the Ames test results followed OECD 471 guidelines[24] and EPA Health Effects Tests Guidelines (OPPTS 870.5265)[25] for genotoxicity testing of chemicals. The results of the Zimmermann test were interpreted according to the EPA Health Effects Tests Guidelines (OOPTS 870.5575) and as described by Zimmermann.[12,26]

***In vitro* comet assay with HepG2 cell line**

Modified version of the alkaline protocol described by Uhl et al.[13] was performed with drinking water samples. Human hepatoma cell line (HepG2 cells) was obtained from prof. dr. Knasmueller, Institute of Cancer Research of The Univeristy of Vienna. Cells were grown in multilayer culture at 37 °C in humified atmosphere of 5% CO2 in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal calf serum (FCS) and antibiotic (0.1% gentamycin) in well plates for 7 days (cell density: 10^6–10^8 cells/mL). Medium was changed every 2 days. Seven days old cells were exposed to water samples, negative and positive controls (500 μM hydrogen peroxide) for 20 min and afterwards 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. The dye-exclusion test with Trypan blue was used to examine the viability of cells before the comet assay was performed.[27]

Rough microscope slides were used for the comet assay, they were first coated with up to 400 μL of 1% normal melting point agarose (NMP) the day before the test and left to air dry overnight. The supportive (second) agarose layer (0.6% NMP agarose) was solidified on ice and the collected HepG2 cells were immobilized in the third layer. Approximately 2×10^4 cells were mixed with 0.7% low melting point agarose (LMP) and spread over the slides as the third layer. After removing the cover glasses, the slides were covered with 500 μL of 0.5% LMP agarose (the fourth layer) to prevent nuclear DNA escaping during cell lysis and electrophoresis. One-hour incubation in alkaline lysis buffer followed. The slides were submerged in electrophoretic buffer (pH >13) to unwind the nuclear DNA for 1hr and then subjected to electrophoresis in the same buffer. The electrophoresis was carried out at 2V/cm and 300 mA; for 30 min. Following electrophoresis the gels were neutralized in 400 mM Tris-HCl pH 7.5 for 15 min. The damaged DNA traveled 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 analysis of the comet assay results

For quantitative analysis of nuclear DNA damage HepG2 cells, the slides were viewed at 200× magnification with an epifluorescence microscope (Olympus BX 50) using a BP 515–560 nm filter and BA 590 nm barrier filter. Microscopic images of comets were captured by a digital camera (Hammamatsu Orca 2) connected to a computer, and the comets were scored

using Komet 5.0 Computer Software.[28] Among the parameters available for analysis of the comets, 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.[29] Analysis was restricted to OTM as this parameter takes into consideration the intensity profile of the DNA signal and percentage of DNA in the comet heads and tails.

Images of 50 comets were collected from each of two replicate slides per sample, OTMs were calculated, and the significance of treatment-related differences tested using SAS/STAT statistical software version 8e.[30] Descriptive statistics was performed by the MEANS procedure. OTM records were tested for normal distribution with the UNIVARIATE procedure. The chi- square distribution, which is a special case of the gamma distribution, fitted well to our data. As a consequence, data were then analysed by the GENMOD procedure (Generalised Linear Models). Statistically significant differences between groups were evaluated by the linear contrast method.

Results and discussion

Chemical analyses

Chemical analyses of nitrates and pesticides were performed as part of regular drinking water monitoring by The national institute of health of Republic of Slovenia, Department of sanitary chemistry – the accredited institution for chemical analyses of drinking water in Slovenia. The concentrations of all measured parameters in Table 1 were bellow the maximum allowed concentration (MAC) of chosen chemical analytical methods for all water samples according to Slovenian regulations [17,18,19]

Analyses (Table 1) were performed by The institute of public health of the Republic of Slovenia, Department of sanitary chemistry. Methods of the chemical analyses (Table 1) are part of the Annex to the Accreditation Certificate No. L-052 (LP-029). Nitrates were analyzed by molecular absorption spectrometric standard method (SM-NO_3-4500B)[31] pesticides and their metabolites were analyzed by gas chromatography with mass spectrometry detection (SIST EN ISO 10695).[32]

Table 1. Concentrations of nitrates, pesticides and their degradation products in drinking water samples.

	Sample 1	Sample 2	Sample 3	MAC
Nitrates (mgNO_3/L)	21.5	14.3	12.9	50
Pesticides (μg/L)				
Atrazine (μg/L)	0.09	<0.05	<0.05	0.1
Desetilatrazine (μg/L)	0.09	<0.05	<0.05	0.1
Desizopropilatrazine (μg/L)	<0.05	<0.05	<0.05	0.1
Propazine (μg/L)	<0.05	<0.05	<0.05	0.1
Prometrine (μg/L)	<0.05	<0.05	<0.05	0.1
Simazine (μg/L)	<0.05	<0.05	<0.05	0.1
Terbutilazine (μg/L)	<0.05	<0.05	<0.05	0.1
Terbutrine (μg/L)	<0.05	<0.05	<0.05	0.1
Bromacile (μg/L)	<0.05	<0.05	<0.05	0.1
Metolachlor (μg/L)	<0.05	<0.05	<0.05	0.1
Ametrin (μg/L)*	0.05	0.05	0.05	0.1
2,6-Dichlorobenzamide (μg/L)*	0.09	0.05	0.05	0.1

MAC »Maximum allowed concentration«, * The institute of public health of the republic of Slovenia is not accredited for the analyses of this analyte.

The results of the three biotests

Short-term mutagenicity test are widely applied in the analyses of complex environmental mixtures as sensitive tools for the detection of trace amounts of contaminants or unknown components with genotoxic properties. Among environmental samples, water has been extensively investigated, especially drinking water, where the occurrence of genotoxic contaminats and chlorine by-products has intensively been studied with bacterial tests, because drinking water has a potential to impact human health.[3,33,34]

Since the presence of potentially genotoxic components in drinking water is expected to be very low, very sensitive test methods should be used or water samples should be concentrated. We should be aware however, that the method used for sample concentration can have an important bearing on study results. The concentration of the water samples allows efficient detection of trace amounts of organic genotoxic components trough their concentration on proper absorbents and components otherwise untestable can be proven by the bioassays.[35] In our study the concentration of water samples was performed on XAD4 columns containing copolymer of styrene divinil benzene in order to be able to detect possible presence of pesticides or herbicides.[21]

The results of the bacterial reversion assay with three nonconcentrated and 50 x concentrated drinking water samples are presented in Table 2. According to the EPA and GenPharmTox guidelines, a mutagenic potential of a test item, tested with Ames test, is confirmed if the mutant frequency is 2.0 or higher.[22,24] A dose effect relationship could underline this conclusion. A possible mutagenic potential is assumed if the quotient ranges between 1.7 to 1.9 in combination with dose effect relationship. No mutagenic potential is assumed if all quotients range between 1.0 (and lower) to 1.6. A nonexistent dose effect relationship could underline

Table 2. Results of the Ames test with the strains TA97a, TA100 and TA1535 of three nonconcentrated (1, 2, 3) and 50× concentrated (1C, 2C, 3C) drinking water samples expressed as revertants/plate and induction factors (i.e. multiple of negative control).

Sample	TA97a		TA100		TA1535	
	Revertants/plate	Induction factor	Revertants/plate	Induction factor	Revertants/plate	Induction factor
–S9						
Negative control	33±6	1	313±13	1	14±4	1
	Mean±SD	IF±SD	Mean±SD	IF±SD	Mean±SD	IF±SD
1	32±10	0.97±0.31	313±23	0,99±0.07	13±3	0.98±0.20
1C	33±14	1.01±0.43	315±14	1.00±0.04	11±3	0.81±0.13
2	34±14	1.02±0.23	319±33	1.01±0.10	10±3	0.70±0.22
2C	34±9	1.01±0.28	311±29	0.99±0.09	12±4	0.79±0.27
3	33±5	1.00±0.15	351±25	0.96±0.06	12±4	0.80±0.26
3C	33±7	1.00±0.22	3016±21	1.02±0.08	12±3	0.80±0.23
4NQO (50 µg/plate)	142±37	4.31±1.10	–	–	–	–
MMS (2.5 µg/plate)	–	–	1060±106	3.16±0.75	–	–
Sodium azide (1.5µg/plate)	–	–	–	–	151±13	10.88±0.64
+S9						
Negative control	37±8	1	169±10	1	10±3	1
	Mean±SD	IF±SD	Mean±SD	IF±SD	Mean±SD	IF±SD
1	37±7	0.99±0.19	158±16	0.98±0.10	9±2	1.09±0.27
1C	38±6	0.99±0.15	151±12	0.99±0.08	9±3	0.92±0.42
2	37±8	0.99±0.21	152±13	1.10±0.09	8±3	1.07±0.46
2C	39±8	0.99±0.20	155±14	0.99±0.09	8±3	0.99±0.31
3	37±9	0.99±0.25	156±8	1.10±0.05	10±4	1.07±0.38
3C	38±8	0.99±0.22	160±13	0.99±0.08	11±3	0.99±0.29
2AF (10 µg/plate)	153±13	4.08±0.35	524±93	2.01±0.13	160±12	16.70±0.94

SD-standard deviation.

Table 3. Results of the Zimmermann test: survival, mean number, standard deviation (SD) and frequency of conversions and reversions of three nonconcentrated (1, 2, 3); 50× concentrated (1C, 2C, 3C) drinking water samples and controls (negative and positive).

			Conversion		Reversion	
	sample	survival (%)	mean±SD	freq. (10^{-5})	mean±SD	freq. (10^{-5})
– S9	NC	100.00	112.4 ± 14.2	6.460	77.6 ± 16.1	4.460
	PC	89.79	46.6 ± 21.5	10.747	9.1 ± 9.1	9.764
	1	100.00	110.2 ± 13.7	7.011	66.4 ± 19.9	3.816
	1C	98.28	113.8 ±15.1	5.801	79.6 ± 13.9	4.655
	2	99.65	113 ± 21.6	6.355	76.0 ± 12.7	4.383
	2C	97.93	98.2 ± 11.6	6.678	81.6 ± 22.3	4.789
	3	100.69	99.2 ± 12.8	6.450	74.6 ± 21.5	5.258
	3C	100.34	121.8 ± 15.5	5.624	59.6 ± 21.6	3.414
+ S9	NC	100.00	116.4 ± 15.2	8.255	93.4 ± 10.3	6.624
	PC	90.67	113.6 ± 7.2	12.486	92.2 ± 7.8	8.442
	1	84.25	112.4 ± 20.7	9.461	101.6 ± 25.7	8.552
	1C	105.53	123.2 ± 16.7	8.279	106.8 ± 12.2	7.177
	2	94.47	106.6 ± 16.4	8.003	95.2 ± 7.7	7.147
	2C	101.28	120.4 ± 13.3	8.431	104.0 ± 7.3	7.283
	3	99.15	122.4 ± 12.5	8.755	97.6 ± 11.9	6.981
	3C	103.83	111.2 ± 11.8	7.595	90.6 ± 11.9	6.188

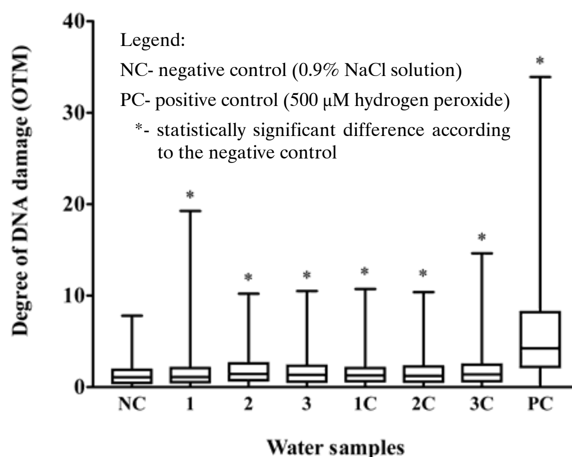
NC-negative control (0.9% NaCl solution). PC-positive control (0.1 mM ethil-methan-sulphonate –S9 and 0.01 mM ethil-methan-sulphonate +S9).

this conclusion. In our study non of the results of the Ames test (+S9 and –S9) exceeded the critical value 2.0 and all the quotients ranged below 1.6, therefore no mutagenic activity was observed in any of drinking water samples. However an increase in induction factor can be seen in presence of S9 homogenate (+S9), but the statistical significances of genotoxic potentials in any of the water samples according to the negative control were not proven ($p > 0.05$).

According to EPA guidelines and Zimmermann recommendations[26,12] the evaluation and presentation of Zimmermann test results (Table 3), a frequency greater than at least two-fold over the control frequency in the same experiment, is judged as a positive response. None of the results of the Zimmermann test (+S9 and –S9) exceeded this frequency (except positive control) therefore no mutagenic activity was observed in any of drinking water samples.

Many mutagens relevant to human exposure are biologically inert unless they are metabolically activated to their mutagenic or cancerogenic forms. This activation is usually achieved *in vitro* by employing a microsomal fraction from rodent liver (S9 microsome) as it was done in Ames and Zimmermann tests. Since the metabolite can be generated directly in target cells, the human hepatoma cell system has lately received major attention in short-term screening tests and is recommended as a suitable in vitro metabolic activation assay.[36,37] We used the human hepatoma (HepG2 cells) cell line for testing potential genotoxicity of drinking water and the cells acted as metabolic activation source as well as the target for DNA damage assessment by comet assay. The results of the comet assay with HepG2 cells are presented in Graph 1. All the water samples (1, 2, 3 and 1C, 2C, 3C) showed an increase of genotoxicity according to negative control, which was statistically significant (0.0416, <0.0001, <0.0001, 0,0002, <0.0001,<0.0001; p-values respectively for water samples). This could be explained by the possible interactions (synergism, additivism) between the individual compounds in the whole water samples, although the concentrations of nitrates, pesticides and their degradation products in drinking water samples were bellow the MAC values (Table 1). Such interactive effects play an important role in the cumulative response of a sample, which could be proven only by bioassay assessment.

These results confirm the fact that comet assay is a very sensitive and rapid technique for measuring DNA damage in individual cells. It is in fact more sensitive than short-term test using prokaryotic organisms (Ames test) and even yeast cells (Zimmermann test). This could be explained by the fact that bacteria do not posses internal metabolic activation enzyme system, responsible for xenobiotic metabolism and the addition of an exogenous system was not effective enough to



Graph 1: Nuclear DNA damage in HepG2 cells (represented as OTM) treated with nonconcentrated (1,2,3) and concentrated (1C,2C,3C) water samples. Results from 100 comets for each water 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.

activate potentially genotoxic compounds in drinking water present at very low concentrations. Yeasts, on the other hand, do posses the metabolic enzymes responsible for bioactivation processes, but the content of the enzymes depend on the growth state and here we must focus on another possible draw back of the system: the complex cell wall. For this purpose special strains of yeasts with higher permeability of cell wall (D7 *ts1*) are used in order to increase the sensibility of the test. Our study showed that the comet assay with HepG2 cells is very quick, simple and most sensitive method in comparison to both other tests. These advantages of the comet assay, offer the assay to become one of the tests of a larger battery of tests, which are used in genotoxicity evaluations of environmental samples, especially drinking water samples.

While talking about the presence of potential genotoxins in water samples, arising mainly from the anthropogenic activities (i.e. industrial chemicals, biocides, agrochemicals, pharmaceuticals, etc.), we must take into consideration the (geno)toxic compounds arising from different water treatment strategies, especially disinfection of drinking water by chlorination.[38] New strategies for reduction of genotoxins in drinking water (like granular activated carbon, filtration, chemical destruction, ozone, chlorine dioxide and monochloramine) have to be considered. Granular activated carbon treatment has been found to be effective for removal mutagens from drinking water. All disinfectant chemicals appear to have the capacity of forming mutagenic chemicals during water treatment.[1,2] It has been shown that the levels of mutagenicity formed

with the alternative disinfectants have been generally less than those seen with chlorine and especially in the case of ozone.[38] So the resolution of the question how best accomplish avoiding chlorination by-products in drinking water and maintaining the microbiologically safe drinking water lies in alternative treatment methods like ozonation and others, which on behalf of certain public safety, need to be tested by bioassays.

Conclusions

The results of short-term genotoxicity/mutagenicity tests on *Salmonella typhimurium* did not show the presence of genotoxic compounds in drinking water samples (neither nonconcentrated nor concentrated). The differences between prokaryotic and eukaryotic cells can affect the sensitivity to a variety of substances. Gene conversion frequences and recombination events in *Saccharomyces cerevisiae* (Zimmermann test) were also not increased after exposure to water samples.

Since the statistically significant results of the comet assay revealed the presence of potentially genotoxic/mutagenic compounds in water samples, we can state that this assay is more sensitive than bacterial and yeast tests. Detection of low levels of genotoxicity in drinking water is very important because of the daily intake of drinking water and possible loading of genotoxic burden in our bodies. Since correlations between genotoxicity of drinking water and increased cancer risk are proven by epidemiological studies, it is necessary to use sensitive test methods, like comet assay.[4,5,6,7]

The approach proves to be very helpful in environmental monitoring, since these biological assays (in our case especially the comet assay) appear more sensitive and relevant than the physico-chemical parameter measurements alone. Nevertheless, further investigations are necessary for more complete detections of genotoxic compounds in environmental samples, like use of bioassays with organisms of different trophic levels.

In conclusion, the results of this study confirm that the comet assay is applicable to genotoxic load assessment of environmental samples, such as nonconcetrated drinking water with hopefully very low genotoxic loads in comparison with classical short-term mutagenesis tests. The use of other human cell lines like Cancer colon (Caco-2) cell line could give further information regarding environmental genotoxic load. Higher plants have a long tradition of use in mutation research and the inclusion of the comet assay to plant system or inclusion of other standard mutagenesis assays into a battery of tests could extend the utility of mentioned tests in *in situ* monitoring of environment.

Acknowledgements

This study was supported by Municipality of Ljubljana Town, VO-KA Company Ljubljana and Ministry of Education, Science and Sport of the Republic of Slovenia.

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Povzetek

Neoporečna pitna voda je izjemno pomembna za zdravje ljudi. V zadnjih dvajsetih letih številne študije poročajo o prisotnosti različnih nevarnih snovi v pitni vodi, ki lahko vplivajo na človekovo zdravje, npr. sprožijo rakava obolenja gastrointestinalnega in urinarnega trakta. Viri onesnaženja pitne vode so industrijske in kmetijske dejavnosti, stranski produkti dezinfekcije in transport pitne vode po ceveh. Javni monitoring pitne vode temelji na rednih fizikalno-kemijskih in mikrobioloških analizah, ki ustrezajo evropski zakonodaji. Ker iz fizikalno-kemijskih analiz ni mogoče sklepati na biološke učinke onesnaževal, raziskujemo možnosti za uvrstitev bioloških testov v monitoring pitnih voda. V tej študiji smo preizkusili tri teste genotoksičnosti na vzorcih pitne vode iz treh vzorčnih mest na območju Ljubljane. Test Ames smo opravili brez in z metabolno aktivacijo z bakterijo *Salmonella typhimurium*, sevi TA97a, TA100 in TA1535. Enake vzorce smo testirali *s* kvasovko *Saccharomyces cerevisiae*, sevom D7, z in brez metabolne aktivacije s testom Zimmermann. Vzporedno smo genotoksičnost vzorcev ugotavljali z alkalno različico kometnega testa s humano celično linijo HepG2. S temi testi smo testirali nekoncentrirane in koncentrirane vzorce pitne vode, predhodno so bile opravljene kemijske analize pesticidov in nitratov. S testoma Ames in Zimmermann v nobenem primeru nismo dokazali genotoksičnosti. Nasprotno rezultati kometnega testa dokazujejo majhno genotoksičnost pri večini testiranih vzorcev pitne vode. Na osnovi statističnih analiz lahko sklepamo, da je za ugotavljanje genotoksičnosti vzorcev pitne vode kometni test bolj občutljiv kot testa Ames in Zimmermann.