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Assessment of municipal wastewater genotoxicity using the Ames fluctuation test, the SOS Chromotest and the mussel micronucleus test: a comparison

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Abstract—**Municipal wastewaters may contain a variety of genotoxic compounds, including drugs or their metabolites, PAHs, etc. Bacterial genotoxicity assays use DNA impairment as end point while micronucleus tests, conducted on eucaryotes, assess chromosome aberrations. As relatively few comparative studies exist, in this study results of the micronucleus test using the painter's mussel (*Unio pictorum*) are compared to those of two bacterial assays, the Ames test and the SOS Chromotest. Both the Ames test and the micronucleus test showed clear concentration-response pattern, however, the Ames test proved more sensitive. Of the two bacterial assays, the SOS Chromotest gave positive result only for the most concentrated sample.**

Keywords—**municipal wastewater, genotoxicity, mussel micronucleus test, Ames test, SOS Chromotest**

I. Introduction

In municipal wastewaters, most often drug residues and/or their metabolites might exert genotoxic effect [1]. Another potentially genotoxic group of chemicals are polycyclic aromatic hydrocarbons (PAHs), in areas where rainwater and municipal wastewater are collected together (as rainwater might wash PAHs from the roads) [2]. The monitoring of the occurrence of genotoxic compounds might require rather costly analytics, especially in case of drug residues. Genotoxicity bioassays, on the other hand, are estimating the aggregate genotoxicity of the sample and might be relatively cost-effective. These tests cover assays on different taxonomic levels: prokaryotes (bacterial tests) and eukaryotes (most often micronucleus test). Bacterial tests include, among others, the SOS Chromotest [3], and the reverse mutation Ames test.

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The SOS Chromotest is a short-term, enzymatic colorimetric assay for the detection of the presence of genotoxic compounds using *Escherichia coli* PQ 37 strain. The SOS system is a complex, DNA-damage activated response under the regulation of the SOS promoter. In *E. coli* PQ 37 the only functioning b-galactosidase gene (*lac Z*) is fused to the bacterial sfiA SOS operon. Thus, SOS response initiates *lacZ* transcription, and b-galactosidase activity is detected spectrophotometrically by the addition of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) [4].

The Ames bacterial reverse mutation assay applies genetically engineered strains of *Salmonella typhimurium*. The method is based on the chemical triggered reversion of histidine producing ability of the strains, enabling them to grow on histidine free medium. Several different methods have been developed, including the plate incorporation assay, the preincubation method, and the fluctuation test [5] [6] [7]. Both tests have been widely applied for detecting genotoxicity in wastewater samples [8] [9].

The micronucleus test has been used for assessing the genotoxicity of individual compounds or complex environmental matrices since the 1980's [10]. MN formation indicates mitotic chromosome breakage or chromosome mis-segregation [11]. Several studies use mussel MN test: though this has not been standardized yet, well described, step-by-step test protocols are available [11] [12]. Mussels are sedentary, filter-feeding organisms, and have proven sensitive for a wide range of environmental contaminants. In addition to laboratory experiments, they can be used in situ, for detecting mutagen compounds in surface waters [13] [14].

As bacterial tests and the micronucleus test represent (1) different taxonomic levels (prokaryotes vs. eukaryotes) and different end points (DNA vs. chromosomal damage), in this study the sensitivity of the mussel micronucleus test is evaluated in comparison with two bacterial tests, the Ames fluctuation test and the SOS Chromotest, using pre-treated municipal wastewater.

II. Materials and methods

Raw wastewater sample was collected from the municipal treatment plant of Veszprém. Capacity of the plant is 12000 m^3/day. The micronucleus test was initiated directly after sampling. The sample was kept at -18°C until the bacterial tests were performed.

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A. *Test organisms*

Unio pictorum specimens were collected from Lake Balaton and were kept in a flow-through aquarium. Water source was Lake Balaton water, therefore not only proper oxygenation was ensured but a constant food supply as well. Animals were acclimatized for 4 weeks prior to testing (t=18-24°C, DO=85-93%).

B. *Test conditions and treatment*

The assay was performed based on the protocol described by Wozniczki et al (2004), with some modifications. *U. pictorum* specimens with length of 5-8 cm were used. Treatments were performed in 3 replicates. For each concentration as well as for the controls, aquaria of 3 l volume were used. Aquaria were aerated during the experiment, temperature was set at 22 °C. Exposure time was 4 days. Dilution series was set as follows: 10x, 20x, 30x and 40x dilution. For dilution, as well as for the control, Lake Balaton water was used. Considering the fact that municipal wastewater contains degradable organic compounds, a semi-static test was conducted, that is, test solution was changed in the middle of the test, after 2 days.

C. *Micronucleus test*

After 4 days, haemolymph was taken from the posterior adductor muscle using the non-lethal technique described by Gustafson et al (2005). 1 ml of haemolymph was mixed with 0.3 ml 10% acetic acid in methanol as a fixative and centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded and the rest was fixed in 1 ml 80 % ethanol. In this way the sample can be kept refrigerated for several weeks. For processing the samples, refrigerated samples were centrifuged again at 1000 rpm for 5 minutes. The supernatant was discarded, the pellet which contained the cells in a more concentrated form, was smeared onto a microscope slide and allowed to dry. After that the slides were fixed in 80 % methanol, air dried and stained with 5 % Giemsa in distilled water for 20 minutes. [15]

Photos were taken by a Zeiss AxioScope A1 microscope with an AxioCam ICC1 camera and Zen 2011 program at 400x magnification. Micronuclei were identified according to Fenech (1992) [16]. For each animal 250 cells were counted. One-way ANOVA with Tukey post hoc test was used to compare the mean MN numbers between the treatments.

D. *SOS Chromotest*

For SOS Chromotest the SOS Chromotest TM kit (EBPI - Environmental Bio-detection Products Inc.) was used according to the manufacturer's instructions, and in compliance with the OECD guidelines No 471:1977. The absorbance of samples was detected on 615 and 405 nm with DiareaderELx800 ELISA device. The SOS repair system induction was measured by the calculation of induction factor (IF) and induction potential (SOSIP) according to Krifaton (2012). Samples with 1.5 or higher IF were considered genotoxic [17].

E. *Ames test*

The fluctuation Ames test was performed according to Hubbard (1984) with slight modification [18]. Shortly: *Salmonella typhimurium* TA100 cells were precultured overnight in nutrient broth (Oxoid) on 37°C. Cells were washed twice in Davis minimal medium (67.4 mM PO_4^{3-}, 8.38 mM SO_4^{2-},15.1 mM NH_4^+, 5.1 mM Na^+, 98.1 mM K^+, 0.83 mM Mg^{2+}, 1.7 mM citrate, 139 µM glucose 10 µg/ml histidine, 0.1 mg/ml D-biotin) and cell number was adjusted to $10*10^5$ cells/ml. Samples were distributed in 200 µl volumes to 96 well microplates. Cell free control, a solvent free negative control, and a positive control with 0.5 µg/ml concentration sodium-azide were also applied. Plates were incubated in humid chamber for 72 hours in 37 °C. On the day of evaluation 20 µl of 2 mg/ml concentration bromcresolpurple (aqueous) solution was added to each sample. Purple colour signified negative, yellow positive (cell growth) result. Intermediate shades were regarded positive. The experiment was also performed with S9 activation, in which case 10 ml suspension contained 2.5 ml S9 mix (EBPI) assembled according to the producers guide (S9 activation simulates metabolic processes in the liver of higher organisms). For positive control 2-amino-antracene was used in 100 µg/ml concentration. For the evaluation of mutagenic effect the χ^2 test was applied with 95 % confidence level [19].

III. Results and Discussion

Genotoxic response is expressed as number of micronuclei/250 cells in case of the mussel micronucleus test, percentage of positive wells in case of the Ames test and IF value in case of the SOS Chromotest. *Fig. 1.* shows results of the micronucleus test. Genotoxic response was already observed in the lowest concentration (40x dilution) and a clear concentration-response curve was given.

Fig. 2. shows the results of the Ames test, with S9 activation. Here the lowest concentration (40x dilution) already gave a significant positive response. The 10x and 20x dilutions do not seem genotoxic: however, in these concentrations cytotoxic effect appeared, killing the test bacteria. Without S9 activation, no genotoxic effect could be observed. In case of the SOS Chromotest, only the highest concentration gave positive response, after S9 activation.

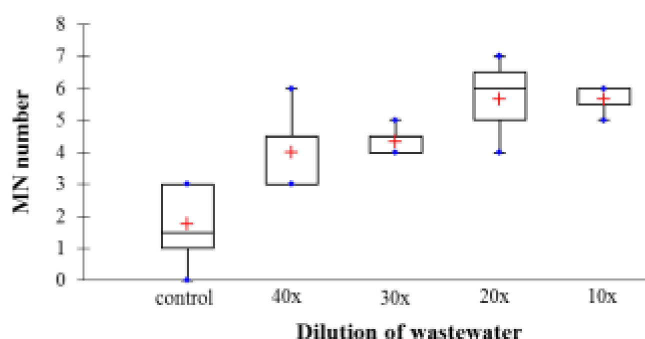

Figure 1. *Unio pictorum* micronucleus numbers (expressed as MN/250 cells)

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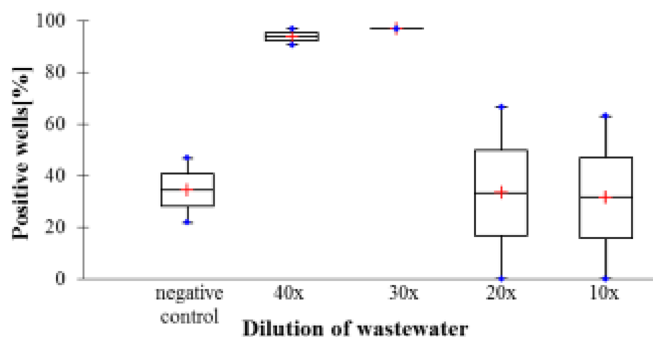

Figure 2. Ratio of positive wells in the Ames test (%)

Several reports are available to compare the sensitivity of the Ames test and SOS Chromotest. Isidori et al. (2006) assessed the genotoxic effect of the pharmaceuticals Furosemide and its photoproduct and found that while these compounds proved negative in the SOS Chromotest, a variability among the mutagenic responses was observed in the Salmonella mutagenicity assay [20]. The Ames test proved more sensitive than the SOS Chromotest for detecting genotoxicity in hospital wastewaters [21] and in municipal wastewaters [22].

Results of the present study are in concordance with literature data: with S9 activation, the Ames fluctuation test gave the highest response, showing cytotoxicity in the 10x and 20x dilutions, and maximum genotoxic response in the 40x dilution. The SOS Chromotest gave positive response only in the highest concentration (10x dilution), also after S9 activation.

Relatively few reports are available on the comparison of the micronucleus test and bacterial genotoxicity assays. Monarca et al. (2004) applied a complex battery to evaluate the genotoxicity of disinfected drinking water. They found that while none of the bacterial tests gave positive answer, the mollusc micronucleus test was able to detect genotoxicity in raw water (in their study, *Dreissena polymorpha* was employed) [23].

However, as bacterial genotoxicity assays detect DNA aberration and the MN test detects chromosomal damage, the genotoxic risk screened by the bacterial tests does not necessarily overlap with the MN test results [24]. The same authors therefore suggest the application of both the MN test and a bacterial test. In their study, however, a flow-cytometry based MN test was used, which significantly increases effort and cost of the test.

IV. Conclusions

Based on our results, it can be also suggested that for the characterisation of a wastewater sample, the two sensitive tests, Ames test and the micronucleus test should be performed in parallel. However, taking into consideration the methodological constraints of the micronucleus test (relatively long exposure which requires the renewal of the sample and also, the assay needs relatively expensive equipment), for screening purposes the Ames test seems sufficient.

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