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COMPARATIVE ASSESSMENT OF THE MUSSEL MICRONUCLEUS TEST VERSUS BACTERIAL BIOASSAYS FOR GENOTOXICITY TESTING OF BENZO[A]PYRENE

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Polycyclic aromatic hydrocarbons are hazardous compounds to the environment and human health, thus their detection is an important task. In this study the genotoxic effect of benzo[a]pyrene (B[a]P) was examined on a freshwater mussel *Unio pictorum* and results were compared to bacterial tests, such as the Ames test and SOS chromotest. The aim of the study was to calibrate the sensitivity of the mussel micronucleus test to that of the two bacterial tests using B[a]P as a reference chemical. The Ames and the micronucleus tests gave similar response both in sensitivity and in concentration-response pattern. These two tests are proposed to be applied in a battery for genotoxicity testing.

Keywords: micronucleus test, Ames fluctuation test, SOS chromotest, PAH

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous widespread contaminants. The US Environmental Protection Agency (EPA) created a list of priority pollutants that have the greatest concern due to potential exposure and adverse health effects on humans. There are 16 PAHs on this list, of which the most hazardous are acenaphthene, fluoranthene, naphthalene, benzo[a]anthracene and benzo[a]pyrene [1]. These compounds have proven to have carcinogenic and mutagenic effects on animals and humans, hence their regulation is very important [2].

Major sources of PAHs are internal combustion engines, residential heating, incineration, and coke production. There are also natural sources, such as forest fires or volcanoes. PAHs are present in the atmosphere as gas and/or particulate phases and might be transported to other environmental compartments such as soil, sediment, and water *via* dry or wet deposition. Heavier PAHs, such as benzo[a]pyrene (B[a]P), are almost totally adsorbed onto particles. Their further environmental fate in solid compartments is mostly influenced by their low water solubility. However, once taken up by the organism, the detoxification mechanism converts these compounds into more soluble molecules. It was shown that the seawater bivalve, *Mytilus sp*. can activate B[a]P to mutagenic compounds and produce reactive oxygen species (ROS) [3]. For assessing environmental exposure, benzo[a]pyrene seems to be a good indicator, due to the strong correlation between B[a]P and other PAHs [4]. In IARC Monograph Volume 3 it was concluded that benzo[a]pyrene produced tumours in a wide range of animals tested, following exposure by many different routes (oral, dermal, inhalation, intratracheal, intrabronchial, subcutaneous, intraperitoneal, intravenous). It had both a local and a systemic carcinogenic effect [5].

There are various reasons why the genotoxicity of PAHs as well as of other mutagenic compounds is tested on mussels. First of all, bivalves are sedentary creatures, being exposed to both water and sediment contamination. Secondly, their ability to bioaccumulate contaminants is well known and widely used in biomonitoring studies. Actually, the fact that they can not only bioaccumulate waterborne mutagens, but also metabolise them into active forms, on one hand increase their usefulness in these studies but on the other hand may enhance the potential risk to consumers [6].

Of genotoxicity markers, the micronucleus test (MN) is the most widely established, relatively easy-to-perform test. Micronuclei formation indicates chromosomal DNA damage occurring as a result of either chromosome breakage or chromosome mis-segregation during mitosis [7].

BANNI *et al*. used digestive gland cells of the marine mussel *Mytilus galloprovincialis* in an acute test. In this assay, mussels were exposed to 75 nM B[a]P for different exposure time. Micronucleus frequency started to show significant response after 24 hours exposure, reaching the maximum after 72 hours [8]. WOZNICKI *et al.* also tested B[a]P genotoxicity using the MN test, but

on another species, the freshwater *Sinanodonta woodiana* (referred to *Anodonta woodiana* in the original article) [9] in which the time-dependency of ecological effect was demonstrated. Maximum micronucleus formation was experienced after 4 days of exposure, but after it started to decrease, most possibly due to adaptive mechanisms.

The mussel micronucleus (MN) test has also been used in real-world environments, especially for detecting contamination from oil spills. BOLOGNESI *et al.* demonstrated that even 10 years after the wreck of the tanker Haven at the Ligurian coast of Italy, micronucleus frequency in caged oysters was a reliable way to detect the release of genotoxic compounds [10]. MARTINS *et al.* assessed genotoxicity of sediment containing PAHs and metals after dredging operations. The sediment previously was classified as 'trace contaminated', but dredging modified the mobility of pollutants, which was clearly visible in the mussel MN test [11]. In addition to the MN test for eukaryotic organisms, which results in chromosomal damage, several bacterial tests are also available for screening purposes due to their rapid response and short exposure time.

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 as described by QUILLARDET *et al.* [12]. 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 β-galactosidase gene *(lacZ)* is fused to the bacterial *sfiA* SOS operon. Thus, SOS response initiates *lacZ* transcription, and β-galactosidase activity is detected spectrophotometrically by the addition of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) [13, 14].

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 pre-incubation method, and the fluctuation test [15–17].

The Ames fluctuation test is a microplate adapted version of the Salmonella reverse mutation assay with a pH change indicated colorimetric endpoint. This method is suitable for the screening of large numbers of samples, and because of its sensitivity it is ideal for water sample testing [18].

The ability of both the SOS chromotest and the Ames test to detect genotoxicity of B[a]P has been long established [19, 20]. B[a]P was also used as a reference chemical for calibrating the newt micronucleus test or Jaylet test [21].

The correlation between the genotoxic substances and the number of micronuclei in an organism has been used in water toxicological tests since the 1980's [22]. The micronuclei are small bodies containing DNA parts that appear near the nucleus as a result of chromosome breakage or mitotic spindle dysfunction. This process can occur without external factors as well, but the effect of genotoxic substances made a far greater number of micronuclei than normal. Therefore the micronuclei frequency may characterise the extent of genetic damage that accumulate over the life of the individual [23]. The MN test, performed on freshwater mussel species, is widely distributed for assessing genotoxic effects triggered by environmental pollutants [24, 25].

Still, the micronucleus test has gained relatively low attention in Hungary, therefore a native freshwater mussel, *Unio pictorum* was introduced as test organism. Main aim of the study was to calibrate this test also using B[a]P as reference chemical, by comparing its sensitivity to that of the bacterial assays.

Materials and Methods

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.

Test conditions and treatment

The assay was performed based on the protocol described by WOZNICZKI *et al.* with some modifications. A B[a]P stock solution was prepared in acetonitrile in 1 mg cm^{-3} concentration for the following series: 70 µg dm^{-3}, 175 µg dm^{-3}, 350 µg dm^{-3} and 700 µg dm^{-3}. For solvent control 0.07% acetonitrile was used, and solvent quantity was adjusted to 0.07% in each treatment. *U. pictorum* specimens with length of 5–8 cm were used. Treatments were performed in triplicates. For each concentration and for the controls, the volumes of the aquaria were 3 l. They were aerated during the experiment and temperature was set at 22 ^{o}C. Exposure time was 4 days.

Micronucleus test

After 4 days, haemolymph was taken from the posterior adductor using the non-lethal technique described by GUSTAFSON *et al.* [26]. A 1 ml aliquot 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 and the pellet containing the cells was smeared onto a microscope slide and allowed to dry. After that the slides were fixed in 80 % methanol, dried and stained with 5 % Giemsa in distilled water for 20 minutes.

Photos were taken by a Zeiss AxioScope A1 microscope with an AxioCam ICC1 camera and Zen 2011 program at 400x magnification. Micronuclei were

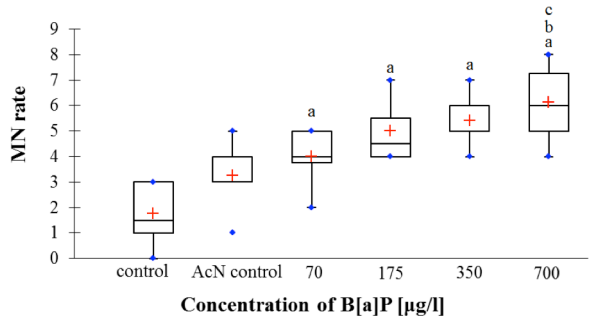

Figure 1: Result of the MN test with B[a]P showing significant difference compared to control (a), to AcN control (b) and to 70 µg dm^{-3} B[a]P (c)

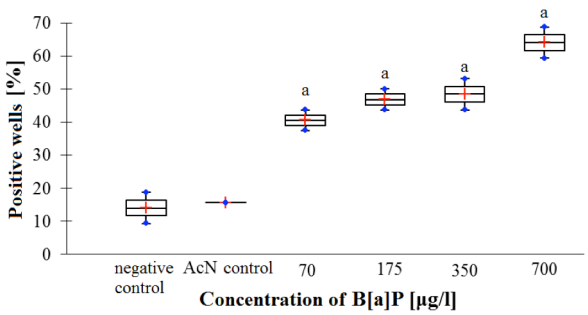

Figure 2: Result of the S9 supplemented fluctuation Ames test with B[a]P (significant difference compared to control (a))

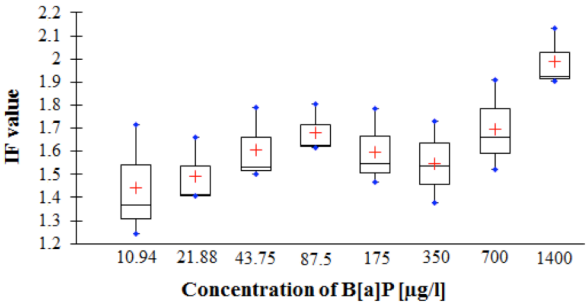

Figure 3: Result of the S9 supplemented SOS chromotest with B[a]P

identified according to FENECH [27]. 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.

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 [28]. B[a]P concentrations were 1400 µg dm^{-3}, 700 µg dm^{-3}, 350 µg dm^{-3}, 175 µg dm^{-3}, 87.5 µg dm^{-3}, 43.75 µg dm^{-3}, 21.88 µg dm^{-3}, 10.9 µg dm^{-3}, 0 µg dm^{-3}. Acetonitrile concentration was adjusted to 0.07% in each sample, and an additional DMSO solvent control was also used. 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 [29]. Samples with 1.5 or higher IF were considered genotoxic.

Ames test

The fluctuation Ames test was performed according to HUBBARD with slight modification. In short, *Salmonella typhimurium* TA100 cells were pre-cultured overnight in nutrient broth (Oxoid) on 37 ºC. 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 cm^{-3} histidine, 0.1 mg cm^{-3} D-biotin) and cell number was adjusted to 10^6 cells cm^{-3}. B[a]P was added to the samples in 700 µg dm^{-3}, 350 µg dm^{-3}, 175 µg dm^{-3}, 70 µg dm^{-3} and 0 µg dm^{-3} and acetonitrile concentration was adjusted to 0.07%. 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 cm^{-3} concentration NaN_3 were also applied. Plates were incubated in humid chamber for 72 hours in 37 ºC. On the day of evaluation 20 µl of 2 mg cm^{-3} aqueous solution of bromcresolpurple 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 producer's guide (S9 activation simulates metabolic processes in the liver of higher organisms). For positive control 2-amino-antracene was used in 100 µg cm^{-3} concentration. For the evaluation of mutagenic effect the χ^2-test was applied with 95% confidence level [30].

Results and Discussions

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. Significant difference between the control and all treatments was observed in case of micronucleus numbers (ANOVA: $F = 12.015$; $df = 5$; $P < 0.00001$, Tukey post hoc: $P < 0.002$); however, only the highest concentration treatment differed from the AcN-control (Tukey post hoc $P = 0.02$). The difference between the lowest (70 µg dm^{-3}) and highest (700 µg dm^{-3}) concentrations was also indicated *(Fig.1)*.

χ^2-square tests indicated significant differences between the control and all treatments in case of ratios of Ames fluctuations test ($P < 0.0017$) *(Fig.2)*. The results of SOS chromotest are shown in *Fig.3*.

To date no assessment has been published for comparing the sensitivity of the mussel micronucleus test and bacterial genotoxicity assays. There are a few comparative works; however, those are based on amphibian micronucleus tests. One protocol uses *Xenopus laevis* embryos and the end-point of the test is number of micronucleated erythrocytes per thousand. The test is standardised, international and some national

test protocols apply, e.g. the AFNOR NFT 90-325 procedure [31]. MOUCHET *et al.* tested genotoxicity of PAH-contaminated soil leachates on the amphibian MN test and two bacterial tests (Ames and Mutatox) [30]. The latter test developed by the Microbics Company (now Azur Environmental) uses dark mutants of the luminescent bacterium *Vibrio fischeri*. In the presence of mutagenic compounds, these mutants can revert and recover their luminescence, which is easily measurable by a luminometer. It was found that the MN test was able to detect genotoxicity, while the Ames test was not. Sensitivity of the Mutatox test was intermediate. It should be noted that chemical analysis of both soil and leachate samples revealed much lower individual PAH concentration in leachates than in the soil samples.

LE CURIEUX *et al.* used the SOS chromotest, the Ames fluctuation test and another amphibian, the newt *Pleurodeles waltl* for a comparative assessment of 7 chemicals, including B[a]P. In their study, the newt micronucleus test was the most sensitive, the fluctuation test and the SOS chromotest gave practically similar but lower response [33].

In our study all three tests gave positive response, but analysis of the concentration-response graphs shows somewhat different patterns. Bacterial tests gave positive response only with S9 activation. Ideal concentration-response graphs were found for the MN test and the S9 supplemented Ames test. Ideal concentration-response curve is observed when the response steadily increases for each higher effluent concentration [34]. Main difference is the response given in the AcN control, which elucidated micronucleus formation but the Ames test gave practically the same response for both controls. Notably, WOZNICZKI *et al.* did not find concentration-response relationship when tested B[a]P on *Sinanodonta woodiana*.

In the SOS chromotest after S9 activation, positive response was given for the lowest concentration, but no clear concentration-response relationship could be established. In general, sensitivity of the SOS chromotest is considered lower than that of the Ames test. For example, there are mutagenic compounds that do not induce the SOS response, such as benzidine, cyclophosphamide, acridines, and ethidiumbromide [13].

Conclusions

The very similar response of the Ames test and the micronucleus test (considering both sensitivity and concentration-response pattern) indicate that B[a]P elucidates both chromosomal aberrations and point mutation, and is genotoxic for prokaryotes and eukaryotes as well; however, this is not necessarily the case for all potentially genotoxic chemicals. As such, for testing genotoxicity of either individual compounds or environmental samples, application of both tests can be advised, defining the minimum necessary battery as the MN and Ames tests.

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