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Detection of Genotoxic Effects on Cells of Liver and Gills of *B. rerio* by Means of Single Cell Gel Electrophoresis

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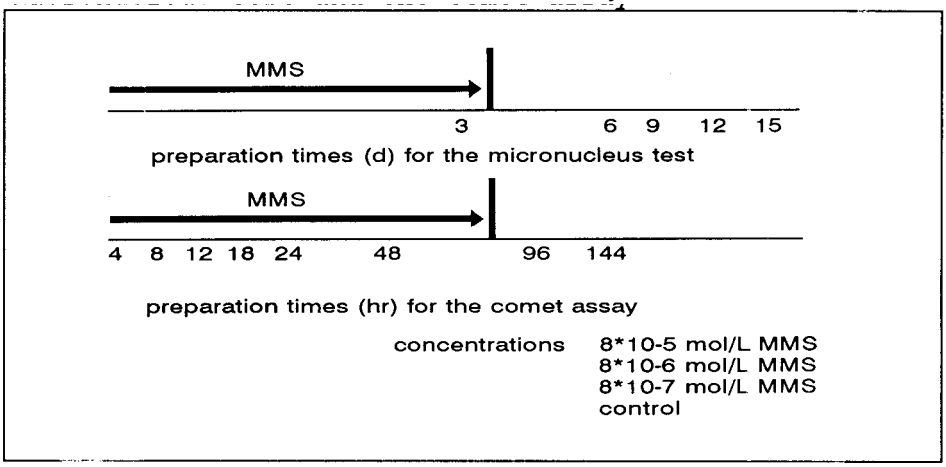
Contamination of surface waters by anthropogenic pollutants has no doubt decreased in the industrial countries during the past years. So concentrations of individual compounds, e.g., of the group of pesticides or chlorinated hydrocarbons have been decreasing continuously for years; in the Upper Rhine, e.g., their concentrations are usually below 1 µg/L now, which is at the limit of detection (Landesanstalt für Umweltschutz 1991). From these individual values, information about the total contamination of surface waters and its biological effect cannot be derived, however. Not all organic compounds present in the water have been determined and only little, if any, is known about possible synergistic effects of different compounds. So novel biological tests are required to determine the total contamination in an integral and sensitive way. The present paper is intended to provide a contribution to the detection of genotoxic effects in surface waters.

Water pollution by genotoxic compounds has so far been determined by established test systems, e.g., the Ames test in water samples or extracts (Omura et al. 1992); sometimes also fish or clams were used as test organisms (Scarpato et al. 1992). Test methods as the micronucleus test (Lehmann 1989) chromosome aberration test (Means et al. (1988) require proliferating cells and are expensive in time and material. The DNA unwinding technique (Herbert 1990) or alkaline filter elution (Sina et al. 1988) are applicable to all eukaryotic cells and independent of a high mitosis rate. As many cells are required, however, pertinent cells of smaller organisms or organs need to be pooled; hence individual differences escape detection. For studies in smaller organisms or organs, single cell gel electrophoresis is a novel method to

Table 1. Scheme of experiments for detecting genotoxicity of MMS in B. rerio; used test systems are the micronucleus test and the comet assay



test a compound or water sample for genotoxic effect on the individual cell level.

Aim of the present work was to test the method of single cell gel electrophoresis for its efficiency in detecting genotoxic pollution in surface waters. In preliminary tests a fish, Brachydanio rerio, was used as test organism; for detection of genotoxic effects both the micronucleus test and single cell gel electrophoresis were applied. Results are presented and discussed.

MATERIAL AND METHODS

Three to six months old, sexually mature fish of both sexes and of 0.5 - 1 g in weight were obtained from Westaquarium Bad Lauterberg, Germany. Males and females were kept in circulating tap water at 25°C and a flow rate of 200 mL/min. Nitrogen was removed and the water was aerated by stripping (Bresch et al. 1990). Water hardness was 360 mg/L, (as $CaCO_3$), pH 7.4, and oxygen concentration was above 80% saturation. The fish were fed TetraMin dry feed and once a week nauplii of Artemia salina. Whole-glass aquaria of 10 L in volume were filled with 6 L water and max. 2 g fish/L. The animal were exposed to the test substance for three d and subsequently kept in circulating tap water for different lengths of time to learn about the effect of the substance and about possible repair of the DNA damage induced (Table 1). The fish were not fed during exposure to the test substance and the test water was exchanged once a day. Test substance was methyl methane sulfonate (MMS) (Sigma, Deisenhofen, Germany), an

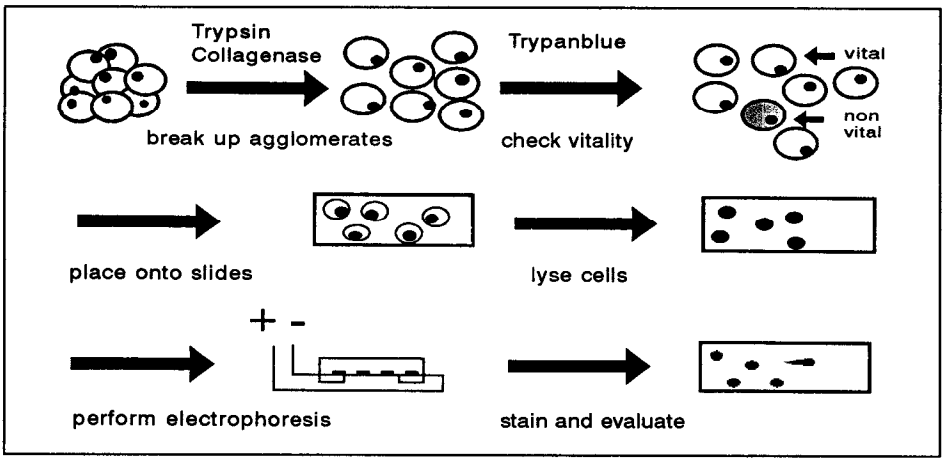


Figure 1. Schematic of single cell gel electrophoresis B.rerio, and the nuclear length of intact cells determined by single cell gel electrophoresis.

Table 2. Isolation of cells of liver and gills of B. rerio, and the nuclear length of intact cells determined by single cell gel electrophoresis.

	Total number of cells	Vitality (%)	Mean nucleus length (μm)
Gill	$4*10^5$	90 min.=76 max.=99	33.8 s=8.2 n=26
Liver	$5*10^5$	89 min.=72 max.=99	25.6 s=5.6 n=26

Isolation: 20 - 25°C; rinse gill and liver in buffer solution: place for 1.5 hr into digestive medium (MEM (Eagle) without L-glutamine and $NaHCO_3$, with 0.025% collagenase D and 0.125% trypsin), centrifuge at 70 g/5 min, wash with erythrocyte lysis buffer (Betti 1993), centrifuge at 70 g/5 min, add to medium (MEM).

alkylating water-soluble compound which had shown positive effects in the micronucleus test in clawed toads (Lehmann 1989) and in the alkaline filter elution in rat hepatocytes (Sina et al. 1988). In our experiments, genotoxic effects were detected by the micronucleus test in erythrocytes and by single cell gel electrophoresis in cells of liver and gills. For the micronucleus test, six animals, and for the single cell gel electrophoresis three animals each of the treated group and of the controls were selected. The micronucleus rate was determined in stained (acridine-

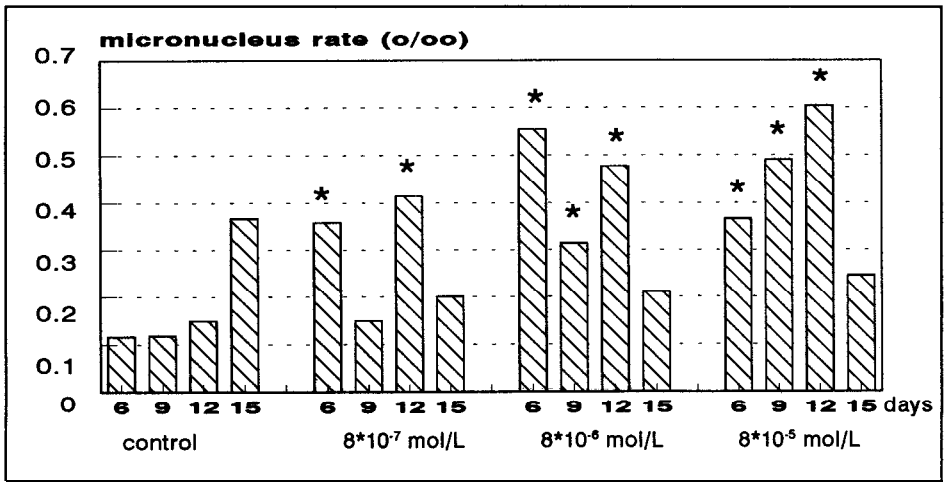


Figure 2. Rate or micronuclei in erythrocytes of B. rerio exposed to different concentrations of MMS for 3 d. Until preparation the fish were kept in control water for different times. * = result significantly different from control according to the Chi-square test ($p < 0.05$). A column represents the mean value of 6 fish.

orange) blood smears (Lehmann 1989) by Zeiss photomicroscope (1000-fold enlargement). The number of erythrocytes counted without discrimination of maturity stages was 10,000. The results were evaluated statistically by the Chi-square test, $p < 0.05$.

Single cell gel electrophoresis was performed according to Singh (1988) and Pool-Zobel (1992). Figure 1 shows a schematic of the test. Genotoxic damage to the DNA is detected by the extent of DNA migration in the electric field. Single or double strand breaks in the DNA caused by genotoxic compounds are the reason for migration of DNA fragments out of the cell nucleus. So-called comets appear, the length of which measured microscopically is a measure of the extent of damage to the cellular nucleus, compared to the intact cell. Per animal two preparations of 101 cells each are measured and the median and proportion of damaged cells determined. Damaged cells are those whose nuclear length is above the 2.5-fold compared to an intact cell. To isolate cells, tissue of gill and liver was exposed to the enzymes collagenase D (Böhringer, Mannheim, Germany) and trypsin (Difco, Detroit, USA) (Table 2). Cell vitality was determined in parallel by trypan blue to discriminate between cytotoxic and genotoxic damage.

RESULTS AND DISCUSSION

By the micronucleus test and the single cell gel elec-

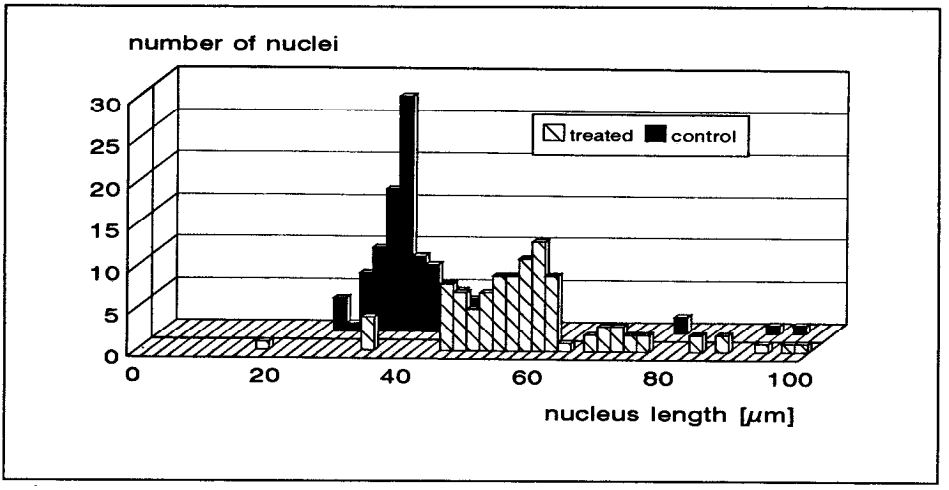


Figure 3. Distribution curve of 101 nuclear lengths (liver cells of B. rerio) after single cell gel electrophoresis; untreated = black columns: treated (= hatched columns) by 8 * 10^{-5} mol/L MMS for 8 hr.

trophoresis, the concentrations 8 * 10^{-5}, 8 * 10^{-6} and 8 * $l0^{-7}$ mol/L MMS were studied in parallel with controls. Preparation times for the micronucleus test were 3, 6, 9, 12 and 15 d (see Table 1). Significant differences compared to controls have been found for all substance concentrations after 6 d (Figure 2). For the preparation times 9 and 12 d a dose-dependent increase of the micronucleus rate was observed. The value obtained at the concentration of 8 * 10^{-7} mol/L MMS after 9 d, which differed insignificantly from the control, cannot be explained. After 15 d the micronucleus rate had decreased for all three concentrations to a level no longer significantly different from controls. After 12 d, an increase in the number of polychromatic erythrocytes and nucleus anomalies was observed at the higher concentrations of 8 x $l0^{-6}$ and 8 * 10^{-5} mol/L MMS which did not correlate with an increase in the number of micronuclei. The high value of the controls at the preparation time 15 d is due to the high number of micronuclei (8 and 9 micronuclei/10,000 erythrocytes) of two animals. However, results after 15 d were not significantly different from controls even when the two increased values of controls are not included. Even the concentration of 8 * 10^{-7} mol/L MMS has still been found significantly positive in the micronucleus test. From the values obtained by single cell gel electrophoresis, a distribution curve of the frequencies of nuclear lengths may be drawn. An example is shown in figure 3, distribution of the lengths of the nuclei of liver cells after exposure of the fish to

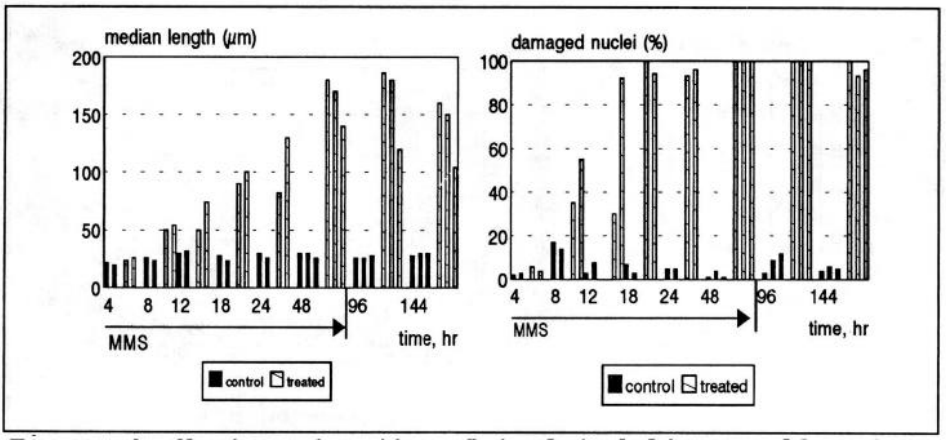


Figure 4. Nuclear lengths of isolated liver cells of B. rerio after exposure to 8 * 10^{-5} mol/L MMS, until preparation at the times 96 and 144 hours, the animals were kept after 72 hours exposure in control water. Results are shown as median length (µm) and share of damaged cells (%).

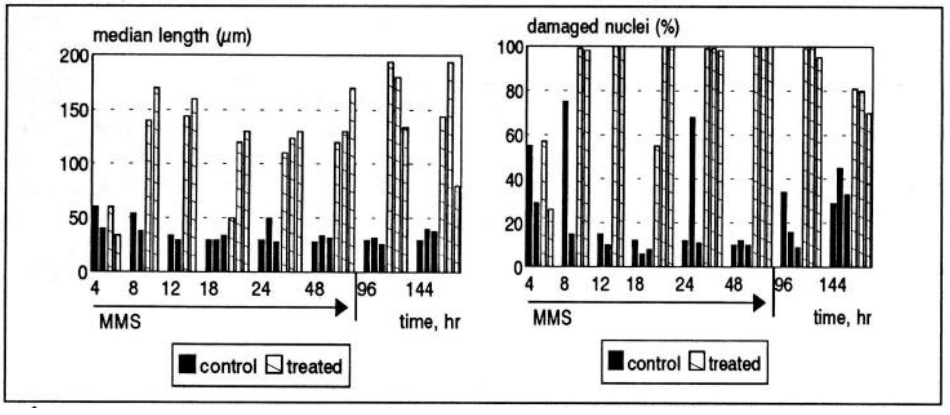


Figure 5: Nuclear lengths of isolated gill cells of B. rerio, after exposure to 8 * 10^{-5} mol/L MMS, until preparation at the times 96 and 144 hours, the animals were kept after 72 hours exposure in control water. Results are shown as median length (µm) and share of damaged cells (%).

8 x $l0^{-5}$ mol/L MMS for 8 hr. Initially the number of nuclei with a comet increases while the median shifts only little. In the course of further genotoxic damage, however, all nuclei lengthen and the median increases. For single cell gel electrophoresis, fish were exposed to MMS and prepared at the preparation times 4, 8, 12, 18, 48, 96 and 144 hr (see Figure 1). For the preparation times 96 and 144 hr the fish were exposed to MMS for 72 hr and kept in control water until preparation. Figures 4 and 5 show the results for liver

and gill cells at a concentration of 8 * 10^{-5} mol/L MMS as the median of the distribution curve and the percent share of damaged cell nuclei. The vitality of liver and gill cells was above 75% in all tests.

In isolated liver cells the genotoxic effect of MMS was visible after as soon as 8 hr of exposure. The median of the treated group was twice as high as that of the control group. The effect increased with exposure time; after 48 hours, the median of the treated group had increased to about the sevenfold compared to controls. After 18 hours of exposure, nearly all nuclei were clearly damaged. One day after completion of the 3 days' treatment (at the preparation time 96 hr, see Table 1) by MMS genotoxic effect on the liver cells is still visible, although the median of nuclear lengths had somewhat decreased. The DNA damage provoked seems to be repaired.

In the isolated gill cells genotoxic effects also appear after 8 hours of exposure. The effect increases with increasing time of exposure but not as linearly as in the liver cells. As soon as after 8 hours of exposure nearly all gill cells were damaged. One day after completion of the treatment by MMS repair of the provoked DNA damage was observed. The concentration of 8 * $l0^{-6}$ mol/L MMS could not be tested. At 8 * 10^{-7} mol/L MMS no effect was detected in cells of liver and gills.

The results have shown that, when the micronucleus test is applied, effects on the erythrocytes were still visible at a concentration as low as 8 * $l0^{-7}$ mol/L MMS, but only as late as 6 days after initiation of the treatment. By single cell gel electrophoresis effects visible on cells of liver and gills were visible already after 8 hours. Whether this test would also reveal effects on erythrocytes remains to be found out. Preliminary studies in erythrocytes have failed so far as the erythrocytes of the control group contained many damaged nuclei. So the micronucleus test, because of the accumulating measured values, seems more suitable to detect long-time effects of low concentrations of genotoxic compounds or waste water, however, only after longer times of exposure and provided the fish used for the experiment respond in a similar way as zebrafish. Single cell gel electrophoresis is a promising screening test to estimate the genotoxic potential of compounds or waste water within a short time, provided the concentrations of toxic compounds are not too low. The tests allows to test different organisms comparatively or to use primary or permanent cell cultures for reasons of animal protection. Practicability of the two methods for the surveillance

of surface waters will be tested in rainbow trout in natural lakes and rivers.

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REFERENCES

Betti C, Barale R, Pool-Zobel B L (1993) Comparative studies on cytotoxic and genotoxic effects of two organic mercury compounds in lymphocytes and gastric mucosa cells of Sprague-Dawley Rats. Environ Molec Mutag 22:172-180

Bresch H, Beck H, Ehlermann D, Schlaszus H, Urbanek M (1990) A longterm toxicity test comprising reproduction and growth of zebrafish with 4-chloraniline. Arch Environ Contam Toxicol 19:419-427

Herbert A (1990) Monitoring DNA damage in Mytilus galloprovincialis and other aquatic animals. Angew Zool 2:143-149

Landesanstalt für Umweltschutz Baden-Württemberg (1991) Organische Mikroverunreinigungen in den Fließgewässern in Baden-Württemberg:1-80

Lehmann M (1989) Entwicklung und Erprobung eines aquatischen in vivo-Mutagenitätstests mit Xenopus laevis. Inst. Wasser-, Boden- und Lufthygiene des Bundesgesundheitamtes Berlin, WaBoLu-Hefte l/1989

Means J C, Daniels C B (1989) Development of in vivo genotoxicity tests in estuarine fish and their application to aquatic toxicology. Mar Environ Res 24:327-331

Omura M, Inamasu T, Ishinishi N (1992) Mutagenic activity of the leachate of municipal solid waste landfill. Mut Res 298:125-129

Scarpato R, Migliore L, Alfinito-Cognetti G, Barale R (1990) Induction of micronuclei in gill tissue of Mytilus galloprovincialis exposed to polluted marine waters. Mar Poll Bull 21:74-80

Sina J F, Bean C L, Dysart G R, Taylor V I, Bradley M O (1983) Evaluation of the alkaline elution/rat hepatocyte assay as a predictor of carcinogenic/mutagenic potential. Mut Res 113:357-391

Singh N P, McCoy M T, Tice R R, Schneider E L (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res 175:184-191