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# THE EFFECT OF A MIXTURE OF SELECTED NANOPOLLUTANTS ON THE LEVEL OF DNA DAMAGE IN THE BLOOD OF *CYPRINUS CARPIO* FRY

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***Abstract.*** *Chemical compounds, widely produced and released into aquatic ecosystems, pose threats to biocenosis and accumulate within food chains. This study examines the impact of four specific pollutants – ibuprofen, SDS, EE2, and DMSO on DNA damage in nine-month-old Cyprinus carpio fry. These pollutants are detected in groundwater, surface water, and drinking water. The study employs the Single Cell Gel Electrophoresis (Comet Assay) to analyze DNA damage levels resulting from exposure to the pollutants. The chosen fish species, serves as a suitable model due to its resilience to diseases and exposure to mutagenic substances. Results indicate a dose-dependent reduction in DNA content in response to pollutant exposure. This research highlights the urgent need to assess the genetic impact of pollutants on aquatic ecosystems and emphasizes the importance of understanding the risks posed by these contaminants.*

## Introducction

Water pollution is a serious ecotoxicological problem. Chemical substances produced on a massive scale end up in aquatic ecosystems, threatening the biocenosis and accumulating at various trophic levels. Scientific studies have confirmed the presence of drugs or detergents in groundwater, surface water and in drinking water [1,2]. Water pollution threatens not only the biocoenosis, but also negatively affects human populations by limiting the possibility of using contaminated water for food purposes. Merely detecting the presence of chemicals in water is not sufficient until their impact on living organisms is determined. These activities are crucial for detecting threats and assessing the risk of their impact on the biocenosis [2, 3]. In the study, we present the effect of ibuprofen, sodium dodecyl sulphate (SDS), 1,7-α- ethinylestradiol (EE2) and dimethyl sulfoxide (DMSO) on the level of DNA damage in nine-month-old Cyprinus carpio fry and the dose-response relationship resulting from the correlation between the concentration of pollutants and damage level. EE2 is the naturally occurring equivalent of the synthetic estrogen found in oral contraceptive pills. It is also used as a feed component supporting the growth of meat mass in animal husbandry [3]. Ibuprofen belongs to non-steroidal anti-inflammatory drugs. According to the World Health Organization, one of the most widespread drugs in the world [4,5]. SDS is commonly used as an ingredient in anionic detergents, widely used in production and processing. Due to its solubilizing properties, DMSO is commonly used as a chemical reagent and solvent [3,5]. These

substances have a genotoxic effect, i.e. they are capable of inducing breaks in a single or double strand of DNA. The selected species of fish is a hybrid of 3/4 carp and 1/4 grass carp. It is a species of freshwater fish from the cyprinid family, dominant in Polish aquaculture and classified as farm fish. It was chosen because of its fast growth, lack of predation and a high rate of resistance to diseases and parasites. Fish are a good material for studying the impact of pollutants on populations because they are exposed to potentially mutagenic substances throughout their lives [2].

The method of testing the mutagenicity of chemical substances on single individuals and determining their impact on the entire population is, Single Cell Gel Electrophoresis in agarose gel, commonly known as SCGE - Comet Assay. This method allows for the analysis of the level of DNA damage in eukaryotic cells by assessing the amount of DNA in the head of the comet (Head DNA) and the amount of DNA in the so-called comet tail (tail DNA). In addition, tail length is measured - the length of the tail in micrometers and the comet tail moment - the product of the length of the comet tail and the fraction of total DNA in the tail [6]. The particular advantages of this test are its sensitivity wich measures 50 to 15,000 damages per cell, speed and simplicity of implementation, low cost and ability to analyze fresh and frozen samples. In addition, this est allows for the analysis of damage at the level of single cells of any type (excluding cells without a cell nucleus) [5,6].

**Methods**

This study used the carp species *Cyprinus carpio* - nine-month-old fry wich is a hybrid of ¾ carp and ¼ grass carp. The fry were bred in the Department of Ichthyobiology and Fisheries Management in Gołysz.

A mixture of compounds of varying importance was used in this study (Table 1). These substances are commonly used, and their presence in the aquatic environment has been proven by scientific research. Among them, pharmaceutical agents are also used, the residues of which in the environment are a huge problem for water treatment technologies, and the ecotoxicological effects of their impact on organisms are still poorly understood. The experiment was conducted at the Department of Ichthyobiology and Fisheries Management in Gołysz. The study consisted of five experimental groups (indicated by consecutive letters) and a control group (K). These samples differed in the concentrations of the tested pollutants, whereas the control group was cultured without contact with the pollutants. The ranges of the substance concentrations in the individual experimental groups are presented in Table 1.

*Table 1*

**Concentrations of the selected compounds from each experimental group.**

| substances [µg] | K | A | B | C | D | E |
|---|---|---|---|---|---|---|
| | 0 | 1x | 3,2x | 10x | 32x | 100x |
| **Ibuprofen** | 0 | 50 | 160 | 500 | 1600 | 5000 |
| **SDS** | 0 | 1000 | 32000 | 10000 | 32000 | 100000 |
| **DMSO** | 0 | 500 | 1600 | 5000 | 16000 | 50000 |
| **1,7α-ethynylestradiol** | 0 | 0,05 | 0,16 | 0,5 | 1,6 | 5 |

Fishes (nine-month-old fry) were exposed to a mixture of compounds for 72 h. Each experimental group was kept in a separate aquarium, ensuring optimal breeding conditions, which were controlled and registered every day of the experiment. The experiment used an alkaline version of SCGE acc. Bilbao and colleagues from 2002, taking into account modifications regarding the composition of buffers, lysis time and electrophoresis parameters [6]. After the fish were delivered to the laboratory, they were individually weighed. After stunning and decapitation, exactly 5 µl of blood was taken

with a chilled heparenized pipette tip and the cells were resuspended in 955 µl of PBS buffer. After suspending the blood sample in PBS, the contents of the tube were thoroughly mixed and then placed in a container protected from daylight, thus obtaining the first dilution of the isolated cells. Then 50 µl of the cell suspension obtained from the first dilution was mixed with 450 µl of PBS buffer to make the final dilution. 50 µl of the obtained solution was mixed with a 50 µl solution of 1% LMP agarose liquefied on the heating block. The whole sample was taken from the test tube with an automatic pipette and applied to the prepared and described slides with the previously prepared solidified first layer of NMP agarose. Each slide was covered with a coverslip and placed on a smooth, ice-cooled plate to solidify the second layer. After clotting, the coverslip was gently slid off and a third layer of 100 µl of 1% LMP solution was applied and covered again with the clean side of the coverslip, then transferred to an ice-cooled plate for final solidification of the last layer. The slides were placed in colins and flooded with the final lysis buffer. Cell lysis took 1.5 h at 4 °C, then the buffer was poured off and the preparations were rinsed three times with distilled water until the residue of the buffer was removed. Slides were placed in a horizontal position with frost to the cathode in the electrophoresis chamber, flooded with chilled buffer and subjected to pre-electrophoresis for incubation lasting 20 minutes. The actual electrophoresis process, lasting 20 minutes, was carried out by setting the initial parameters: electric current intensity 0.3A (as a constant parameter), voltage 25V and power 4W. After the process was completed, the slides were placed on cuvettes with paper towels and rinsed three times by flooding them with neutralizing buffer for 5 minutes. Each slide was then immersed in methanol to dehydrate the slide and then air dried in the laboratory for 24 hours. An important element of the entire procedure, from material collection to neutralization, was to protect the preparations against visible light and high temperature, which is why each stage was carried out in the dark using a red light lamp and maintaining a constant temperature of 18 °C in the laboratory. Microscopic analysis was performed using the Komet 5.5 Kinetic Imaging software. The analysis was performed in a darkened room. Each slide was immersed in distilled water for approximately three minutes. The back of the slide was dried with paper towels. 50 µl of the fluorescent dye DAPI (4',6-diamidino-2-phenylindole) was applied to the obverse of the slides. Analysis was performed using a fluorescence microscope (Olympus BX40 Trinocular) at 40x magnification. Images obtained under the microscope with a camera were transferred to a computer and analyzed. Six slides were analyzed for each of the six experimental groups. While scanning the slide, 50 randomly encountered cell nuclei were measured on each slide. In the case of too intense illumination of a given comet, aperture no. 4 was used.

The computer program allowed for a quick and easy analysis of the most important parameters describing the comet: Head DNA (HDNA), wich determines the amount (%) of DNA in the head of the comet; Tail DNA (TDNA), wich shows the amount (%) of DNA in the tail of the comet; Tail Length (TL), wich describes the length of the comet's tail in micrometers; and the Olive Tail Moment (OTM), which is defined as the product of the tail length and the fraction of total DNA in the tail.

The following descriptive statistics were calculated for each group: N valid measurements of cell nuclei, arithmetic mean, standard deviation, minimum value, maximum value, and measures of variability on which the graphs were based - median and values of the lower (Q25) and upper (Q75) quartiles. Kolomogorov-Smirnov and Shapiro-Wilk tests were used to verify the hypothesis of compliance with the normal distribution. The homogeneity of variance was checked using Levene's test. The obtained results rejected the null hypothesis and allowed us to assume that the distribution of the tested samples was inconsistent with a normal distribution. Further analysis was performed using the non-parametric Kruskal-Wallis test ($p<0.05$). Statistical analysis were performed using Statistica.

**Results**

The analysis of the data obtained in the experiment made it possible to estimate the effect of the mixture of selected compounds on the level of DNA damage in the blood of *Cyprinus carpio* fry after a 72-hour exposure and to determine the relationship between the dose of the mixture of these substances and the potential genotoxic effect on the organism.

Considering the grouping variable of the tested samples, the statistical analysis showed that there were statistically significant differences in the level of damage to the genetic material between the experimental groups. Exposure of carp fry for 72 hours is sufficient to generate damage to the genetic material. It was observed that an increase in the concentration of the mixture of pollutants in the analyzed samples significantly reduced the content of genetic material in the head of the comet compared to the control group. For the analyzed HDNA parameters, the Kruskal-Wallis test showed homogeneity of the results for the erythrocytes of individuals in samples A and B, together with the control group. The homogeneity of the results was also observed for samples C and D. A significantly higher level of DNA damage was also noted in carp erythrocytes from the sample exposed to the highest concentration of the tested pollutants (group E). The average amount of DNA in the head of group E comets was 74% compared to the control group, in which the average amount of genetic material in the head of comets was 88% (Fig. 1). To confirm the obtained results, the data obtained for the TDNA parameters were subjected to statistical analysis (Fig. 2). For the TL parameter, that is the length of the comet tail, homogeneity of the results was also observed for carp from groups A, B, and the control group. However, the statistical analysis for the remaining groups was different, in which homogeneity was demonstrated for individuals from samples C and E, while the carp from the tested sample D did not show homogeneity with any other group (Fig. 3). In the case of the OTM parameter, the statistical analysis showed a large variation in the results, which was related to the combination of data on the length of the comet tail and the percentage of genetic material in it. Homogeneity occursed among individuals from groups K and B; A and B, and carps from groups C and E (Fig. 4)(Tab. 2).

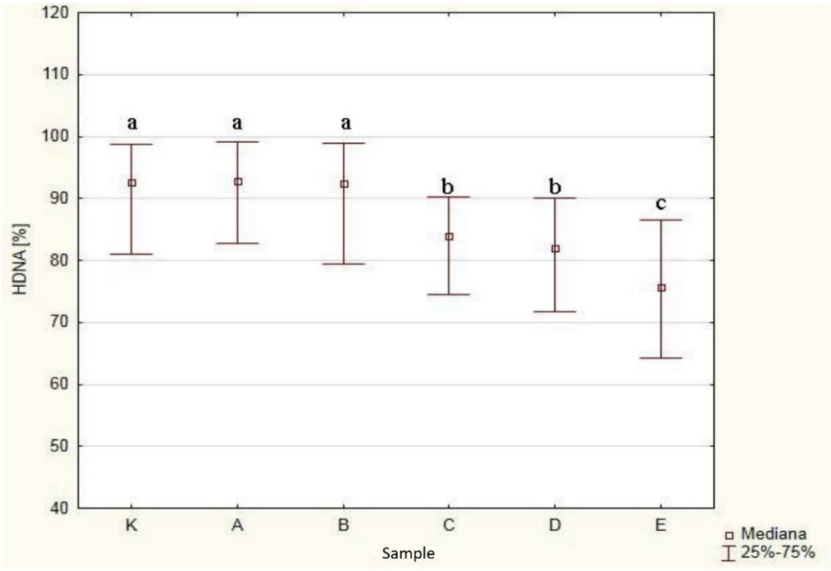


Fig. 1. **Head DNA content (HDNA; %) in carp blood cells after exposure to a mixture of nanoparticles. The same letters denote homogeneous groups (Kruskal-Wallis test, taking into account the grouping variable - test sample; p > .05). Explanations: K - (control), A, B, C, D, E - tested samples. (See table 1)**

*Table 2*

**Average, standard deviation (SD), median, lower quartile (Q 25), upper quartile (Q 75) for the parameter HDNA in carp erythrocytes after 72 h of exposure to pollutants. Explanations: K, A, B, C, D, E – tested samples; N - number of analyzed cell nuclei; Max, (Min) - maximum (minimum) value of the HDNA parameter of the analyzed nuclei.**

| Sample | Parameter Head DNA | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | N | Avereage | Mediana | Max | Min | Q25 | Q75 | SD |
| K | 250 | 88,18 | 92,65 | 100,0 | 0,05 | 81,05 | 98,79 | 13,64 |
| A | 300 | 88,67 | 92,79 | 100,0 | 32,61 | 82,75 | 99,15 | 13,51 |
| B | 279 | 87,39 | 92,45 | 100,0 | 23,50 | 79,48 | 98,98 | 14,09 |
| C | 284 | 81,10 | 83,94 | 100,0 | 11,40 | 74,39 | 90,29 | 13,96 |
| D | 272 | 79,77 | 82,06 | 100,0 | 24,03 | 71,75 | 90,09 | 14,15 |
| E | 127 | 74,45 | 75,77 | 100,0 | 31,82 | 64,31 | 86,51 | 14,52 |

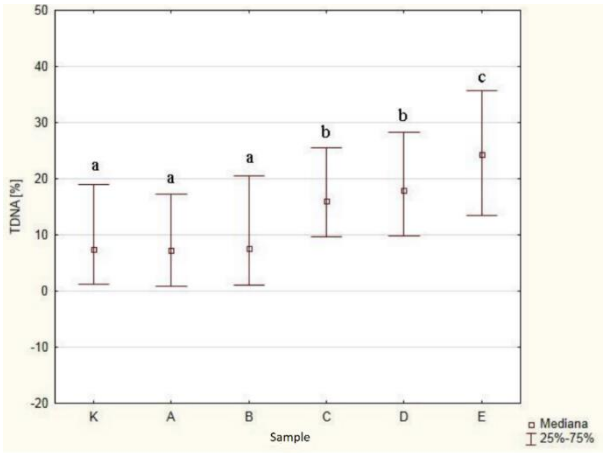


Fig. 2. **Tail DNA content (TDNA; %) in carp blood cells after exposure to a mixture of nanopollutants. The same letters denote homogeneous groups (Kruskal-Wallis test, taking into account the grouping variable - the study sample; p> 0.05). Explanations: K - (control), A, B, C, D, E - tested samples. (See table 1).**

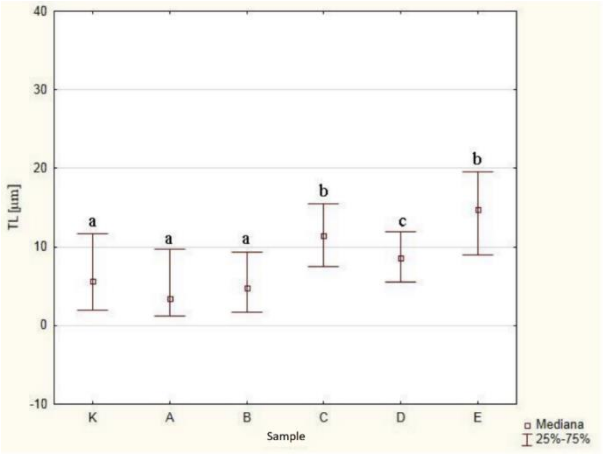


Fig. 3. **Comet tail length (TL; µm) in carp blood cells after exposure to a mixture of nanopollutants. The same letters denote homogeneous groups (Kruskal-Wallis test, taking into account the grouping variable - the study sample; p> 0.05). Explanations: K - (control), A, B, C, D, E - tested samples. (See table 1).**

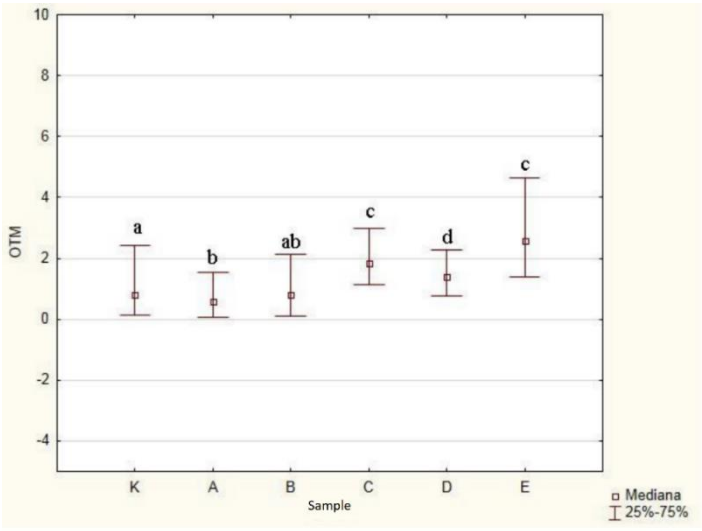


Fig. 4. **Comet tail moment (OTM) in carp blood cells after exposure to a mixture of nanopollutants. The same letters denote homogeneous groups (Kruskal-Wallis test, taking into account the grouping variable - the study sample; p> 0.05). Explanations: K - (control), A, B, C, D, E - tested samples. (See table 1).**

**Conclusion**

The mixture of impurities in nano amounts used in the experiment showed genotoxic potential in relation to erythrocytes of the blood of fry. This is a direct reflection of the risk posed by the presence of the mixture various pharmaceutical compounds and detergents in the environment natural. An increase in the level of material damage has been demonstrated correlated with an increasing dose of pollutants.

The exposure time of carp fry for 72 hours is sufficient to produce the effect genotoxic. It is sufficient to penetrate the mixture through the body's protective barriers. Moreover, these compounds (even in nano-concentrations) when they last long enough, they contribute to damage genetic material.

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