



The Mutagenic Potential of Pesticides Actellic, Rival, Aminopielik and Polybrominated Diphenyl Ethers in Common Carp (*Cyprinus carpio* Linnaeus, 1785) (Cypriniformes: Cyprinidae)

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Abstract: The present study aimed to investigate, under laboratory conditions, the potential mutagenic effects of the pesticides Actellic (pirimiphos-methyl), Rival (propamocarb hydrochloride), and Aminopielik (2,4-dichlorophenoxyacetic acid), as well as the organic contaminants polybrominated diphenyl ethers (PBDEs), in common carp, *Cyprinus carpio*, which was selected as a test organism. The fish were exposed to two selected concentrations of the studied chemicals, as follows: Actellic (1 µl, 6 µl), Rival (3 µl, 6 µl), and Aminopielik (4 µl, 8 µl), and PBDEs according to their maximum allowable concentrations (MAC) of 0.14 µg/l in water and 0.0085 µg/kg in biota. The micronucleus assay was performed to evaluate the genomic DNA damage in the fish erythrocytes. The results showed micronuclei formation and other nuclear abnormalities in the fish erythrocytes, such as lobbed, blebbed, and notched nuclei in the exposed fish compared to the control group. The highest percentages of erythrocytes with DNA damage were observed after treatment with Rival (6 µl) and Aminopielik (8 µl). The exposure to Actellic (1 µl) and PBDEs also revealed mutagenic effects but at lower values.

Key words: Fish, micronuclei, erythrocytes, pirimiphos-methyl, propamocarb hydrochloride, 2,4-D, PBDEs

Introduction

Fish are a suitable model species for studying the mutagenic potential of various anthropogenic pollutants in the aquatic environment, as they can metabolise, concentrate, and bioaccumulate waterborne chemicals. Thus, fish can be a valuable model system to evaluate the potential mutagenic, carcinogenic, and teratogenic risk for humans because they

frequently respond to toxic substances in a manner that is broadly similar to higher vertebrates (AL-SABTI & METCALFE 1995).

The common carp, *Cyprinus carpio* (Linnaeus, 1758), belongs to the order Cypriniformes, family Cyprinidae, which includes about 1300 freshwater fish species. Common carp is a widespread demersal fish with feeding habits that expose it to many environmental pollutants (WILLIAMS et al. 2008, XING et

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al. 2012). The present study chose it as a biomonitor species due to its commercial worldwide value and geographic distribution. The history of carp was first documented around 500 B.C. by a Chinese named Fan Li through his book *Yaneogyong* (Fish Breeding) on fish culture technology in Wushi, Kiangsu Province, eastern China, the first known document on fish culture. Furthermore, common carp is also an excellent model for studying responses at different biological levels (DNA damage, biochemical changes in metabolic and antioxidant enzymes, histopathological lesions, and altered behaviour) when exposed to many different environmental pollutants as shown in previous studies (VINODHINI & NARAYANAN 2009, VAJARGAH et al. 2018, SANNA et al. 2021, ERKMEN et al. 2022, ERGENLER & TURAN 2022, HAMED et al. 2022, TURAN & ERGENLER 2023, WANG et al. 2024).

Biomarkers are important assessment tools because they provide specific information about the biological effects of a toxicant, from molecular to organism level. They can be used for monitoring purposes and to clarify the relationship between the impact on the body and the concentration of the pollutant in health risk assessment and monitoring programs (YANCHEVA et al. 2020, GEORGIEVA et al. 2021).

Thus, the use of the micronucleus test in fish erythrocytes is recommended as a sensitive biomarker for the indication and assessment of water pollution with various mutagenic chemicals (ALI et al. 2008, DA ROCHA et al. 2009, D’COSTA et al. 2018, SHAH et al. 2021, TURAN & ERGENLER 2022, ISLAMY et al. 2023).

Micronuclei are chromatin structures positioned in the cell’s cytoplasm originating from chromosome fragments or whole chromosomes left outside the daughter nuclei. The reasons might be chromosome fragmentation leading to acentric fragment output or acentric chromosome (clastogenesis), as well as a defect in the kinetochore and the impossibility of the chromosome attaching to the mitotic spindle, as well as other defects in chromosome sorting during mitosis (aneugeneses). Micronuclei are formed during cell division and are stored in the daughter cells. That is why the frequency of MN is a widely used biomarker to determine the level of chromosomal damage (HEDDLE et al. 1991, JIRAUNGKOORSKUL et al. 2007a, b, PALANIKUMAR & PANNEERSELVAM 2011, BOLOGNESI & FENECH 2013, CHEONG et al. 2013, OBIAKOR et al. 2014, HOVHANISYAN et al. 2017, FENECH et al. 2020, MICHALOVÁ et al. 2020, MILEVA & VELICKOVA 2021, DI BONA & BAKHOUM 2024). Besides the micronuclei, another indicator of genetic damage is the formation

of abnormal-shaped nuclei in erythrocytes of animals exposed to xenobiotics. Many authors report such abnormalities as blebbed nuclei, lobed nuclei, notched nuclei, etc. (JIRAUNGKOORSKUL et al. 2007a, b, AHMAD & AHMAD 2016, MITKOVSKA & CHASSOVNIKAROVA 2020, CANEDO et al. 2021, TRIVEDI et al. 2021, DUTTA et al. 2024).

The present study aimed to investigate the potential genotoxic effects of different priority organic substances – Actellic, Rival, and Aminopielik pesticides and organic contaminants PBDEs – on the common carp via an erythrocyte micronucleus test and compare which one would cause more severe effects.

Materials and Methods

In the present study, different concentrations of three pesticides (insecticide, fungicide, and herbicide) – Actellic, Rival, and Aminopielik- and PBDEs were tested. Actellic (active substance pirimiphos-methyl 500 g/l) is an insecticide applicable for treatment of oats and barley against storage pests such as the Saw-toothed grain beetle (*Oryzaephilus surinamensis*), Grain weevil (*Sitophilus granarius*), Common flour mite (*Acarus siro*), Warehouse moth (*Ephestia elutella*), Flour or Mill moth (*Ephestia kuhniella*), Flour beetles (*Tribolium* spp.), Rust-red grain beetle (*Cryptolestes ferrugineus*) and Cosmopolitan food mite (*Glycyphagus destructor*). The fungicide Rival (active substance propamocarb hydrochloride 722 g/l) is used for tomato and flower growing. Still, the same active substance is present in many other fungicides (Proplant, Infinito, Proksanil, Aksidor, and Rival Duo). These are used against soil pathogens in the cultivation of tomatoes, barley, onion, cucumber, pepper, melon, cabbage, radish, lettuce, turnip, leek, etc. Aminopielik (active substance 2,4-D, 2,4-dichloro phenoxy acetic acid 600 g/l) is a herbicide used against broadleaf weeds for wheat, barley, and maize. However, 2,4-D is also present in the composition of other herbicides (Dikopur, Herbokson Top, Kileo, etc.). PBDEs are a chemical mix of polybrominated diphenyl ethers. As a kind of brominated flame retardant (BFR), PBDEs are frequently used in electronic equipment, furniture, plastics, and textiles. They are ubiquitous environmental contaminants that may harm human health and ecosystems because they are likely to accumulate due to their high octanol-water partition coefficient, Kow (log Kow = 5.8-11). The octanol-water coefficient (Kow) indicates the hydrophobic nature of the chemical compound. According to KARICKHOFF et al. (1979), hydrophobic compounds (low solubility in the water) have a log Kow value > 0.

The experimental pesticide concentrations were prepared based on the recommended concentrations used in plant protection, the amount of their active substances, and indicated LC_{50} values. Due to the high toxicity of the tested chemicals in our preliminary experiments, which showed high mortality rates for common carp, an additional dilution and a corresponding recalculation for 50-L aquaria were performed. Therefore, the applied concentrations of the tested pesticides for 50-L were as follows: Actellic – 1 μ l and 6 μ l (respectively pirimiphos-methyl 10 μ g/L and 60 μ g/L), Rival – 3 μ l and 6 μ l (respectively propamocarb hydrochloride 40 μ g/L and 80 μ g/L), and Aminopielik – 4 μ l and 8 μ l (respectively 2,4-D 50 μ g/L and 100 μ g/L). The PRIORITY SUBSTANCES DIRECTIVE (2013/39/EU) also sets out maximum allowable concentration EQSs (MAC EQSs) for PBDEs of 0.14 and 0.014 μ g/l for inland surface waters and other surface waters, respectively and the biota environmental quality standard (EQS) set in 2013 (2013/39/EU) for PBDEs in fish were 0.0085 micrograms per kilogram (μ g/kg) wet weight. Therefore, we applied two tested concentrations based on the EU legislation, which for 50-L aquaria resulted in a total of 7 μ g and 0.425 μ g. The PBDEs were presented as a mixture of 2,4,4'-tri brominated diphenyl ether (PBDE 28), 2,2',4,4'-tetrabromodiphenyl ether (PBDE 47), 2,2',4,4',5-pentabromodiphenyl ether 60348-60-9 (PBDE 99), 2,2',4,4',6-pentabromodiphenyl ether (PBDE 100), 2,2',4,4',5,5'-hexabromodiphenyl ether (PBDE 153), and 2,2',4,4',5,6'-hexabromodiphenyl ether (PBDE 154).

Common carp juveniles ($n = 135$ total), healthy, with no pathological lesions, and with similar average weight and length: 12.5 ± 2.5 g and 8.5 ± 1.5 cm, were provided by the Institute of Fisheries and Aquaculture (Plovdiv, Bulgaria). After transportation, the fish were left to acclimatise in evaporation-dechlorinated tap water in a 100-L glass tank attached to oxygen pumps. The fish were fed (1.0%–1.5% of fish weight) daily with commercial fish food, but feeding was suspended before the experiment. The experiment followed the protocol by KOVACHEVA et al. (2022). Briefly, the fish were maintained in a 12 h/12 h (dark/light) cycle and randomly divided into 9 glass tanks ($n = 15$ for each treatment), including a toxicant-free tank, which served as control, and exposed for 96 hours to different concentrations of the tested chemicals.

After dissection, blood smear preparations were performed according to MEGARANI et al. (2020) (fixation in methanol and stained with 10% Giemsa (Merck, Darmstadt, Germany). From each treatment, including the control group, 10 individuals were examined, and 1 microscopic slide was prepared for

each fish. The slides were analysed under a light microscope (Leica DM 2000, Leica Microsystems, Wetzlar, Germany) (10 (ocular) \times 100 (objective) magnification) using immersion oil, and the number of erythrocytes with micronuclei and abnormal nuclei, as well as the total number of scored erythrocytes, were registered. The micronuclei (MN) and nuclear anomalies were identified according to the criteria by FENECH (2000) and CARRASCO et al. (1990). The requirements for the identification of MN were as follows: MN must be separated, not linked or connected to the main nuclei; must be smaller than one-third of the main nuclei; they should be non-refractile and can therefore be readily distinguished from artefacts, such as staining particles; they must be coloured in the same colour as the main nucleus; MN may touch but not overlap the main nuclei and the micronuclear boundary should be distinguishable from the nuclear boundary (FENECH 2000).

Abnormal-shaped nuclei were scored into one of the following categories: blebbed nuclei, lobed nuclei, or notched nuclei. Blebbed nuclei possess a relatively small evagination of the nuclear membrane. The nuclei with larger evaginations, which could have several lobes, are classified as lobed nuclei. Nuclei with depth grooves formed were recorded as notched nuclei (CARRASCO et al. 1990).

The frequencies of micronuclei (MN) and nuclear abnormalities (NA) were calculated according to the formulas proposed by OBIAKOR et al. (2021) and presented in percentages as shown below:

$$\% \text{ MN} = \frac{\text{Number of micronucleated erythrocytes}}{\text{Total number of erythrocytes examined}} \times 100$$

$$\% \text{ NA} = \frac{\text{Number of erythrocytes with abnormal nuclei}}{\text{Total number of erythrocytes examined}} \times 100$$

The results were presented as an average number of analysed erythrocytes, micronuclei, and abnormal nuclei per tested pesticide and PBDE concentrations. Comparisons between the treated and the control samples were submitted to a statistical analysis using Student's t-test to assess significant differences ($p < 0.05$).

Results

In sum, the following aberrations were observed in fish erythrocytes following the exposure to the different tested chemical concentrations: micronu-

clei and/or abnormal nuclei as blebbed, notched, or lobed formations (Figs. 1a, b, c, d).

Table 1 presents the data on the total number of analysed erythrocytes, the observed micronuclei, and total nuclear abnormalities.

Micronuclei and abnormal nuclei were detected in all treated samples in statistically significant numbers compared to the control group (except for PBDEs – 0.425 µg, which equals the maximum permissible concentration in biota by law for 50-L glass tanks in our experiment).

Significantly higher frequencies of micronuclei were observed in the fish erythrocytes after treatment with Actellic (6 µl), Rival (both tested concentrations), and Aminopielik (both tested concentrations). Still, the highest values were registered in the more concentrated solutions of the fungicide Rival (1.47%) and herbicide Aminopielik (1.70%) compared to the control group (Table 2).

In addition, the highest frequencies of abnormal nuclei were observed in the fish after exposure to the herbicide Aminopielik (8 µl) (0.98%, Table 2).

The frequencies of micronuclei and nuclear abnormalities were compared, and micronuclei formation was predominant in all treated samples (Fig.2).

Lastly, we determined that the PBDEs concentration of 7 µg (which equals MAC in water by law for 50-L glass tanks in our experiment) possesses a higher mutagenic effect than the maximum allowable PBDEs concentration in biota and provokes micronuclei and abnormal nuclei in the erythrocytes of common carp.

Discussion

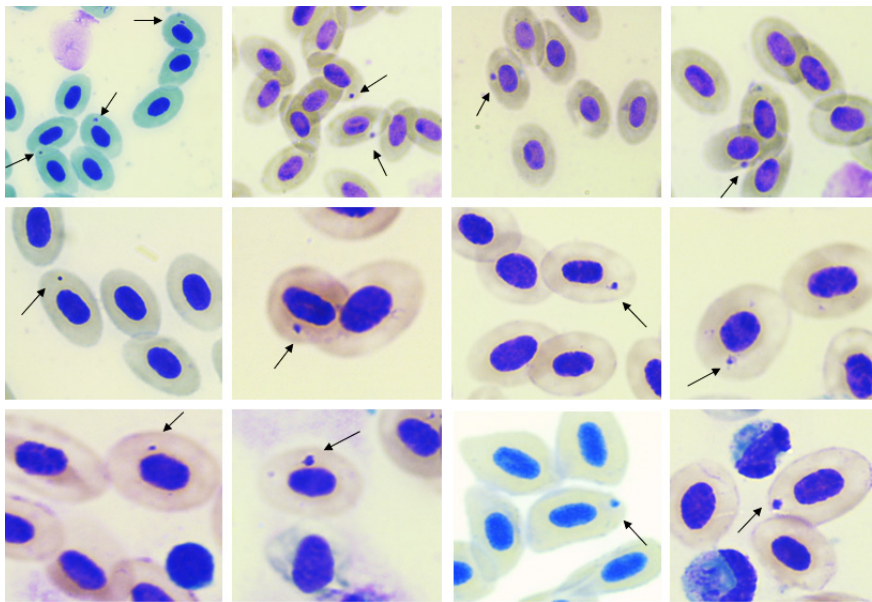
The micronucleus (MN) term, also known as Howell–Jolly bodies (KRISHNA & HAYASHI 2000), was introduced in 1951, and it is related to acentric or centric fragments thrown out from the nucleus at late stages of anaphase (KIRSCH-VOLDERS et al. 2003). The micronucleus assay is a widely applied method for genetic monitoring due to its simplicity, sensitivity, reliability, and proven suitability for many species. Initially, it was developed for application in mice (BOLLER & SCHMID 1970, SCHMID 1975, HEDDLE 1973), but it was modified subsequently for application in fish (HOOFMAN & RAAT 1982). The subcellular processes, such as chromosomal breaks (clastogenesis) or disruption, as well as malfunction of the cell spindle (aneugeneses), lead to MN formation, which is detectable via the micronucleus test (HEDDLE et al. 1991, BONASSI et al. 2007, SAMANTA & DEY 2012).

An increased frequency of micronucleated polychromatic erythrocytes (MNPCEs) indicates chromosomal damage (FENECH & MORLEY 1985, KRISHNA &

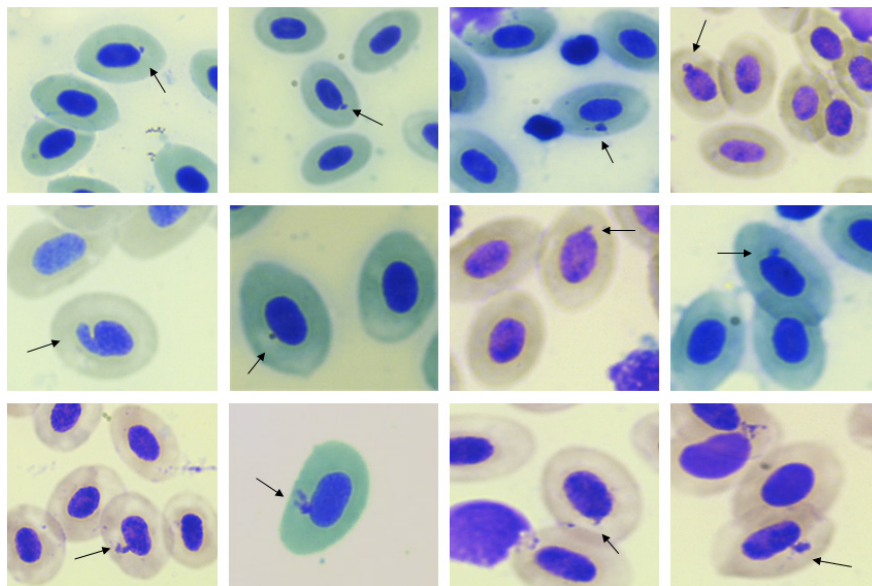
HAYASHI 2000). However, the MNs represent chromosomal losses and may result from DNA amplification (SAMANTA & DEY 2012). The amplification of DNA is commonly observed in the oncogenic process. The increased amount of DNA moves out of the nucleus, forming micronuclei removed from the main cell nucleus, originating MNs (SHIMIZU et al. 2000, SAMANTA & DEY 2012). The MN expelling is associated with the loss of allele dose, contributing to carcinogenesis (BONASSI et al. 2007, TERRADAS et al. 2010).

In the present study, micronuclear formation was observed following exposure to the tested concentrations of insecticide Actellic, fungicide Rival, and herbicide Aminopielik, as well as MAC for PBDEs in the biota. The study of abnormal nuclei is applied as an additional biomarker related to chromosomal instability and loss of genetic material. In identifying abnormal nuclei on microscopic preparations, as opposed to micronuclei, a relationship is established between the main nucleus in the cell and the additional genetic material (STANKEVIČIŪTĖ et al. 2022). The results presented in Tables 1 and 2 showed statistically significant differences between the fish treated with pesticides and PBDEs and the control ($p < 0.05$). The relative values of this indicator represent a directly proportional trend between the value of the percentage of cells with abnormal nuclei and the concentration of the three studied pesticides and PBDEs. The highest percentage of erythrocytes with abnormal nuclei was found at the Aminopielik concentration of 8 µg. The results obtained in this study revealed the predominance of micronuclei formation compared to the formation of abnormal nuclei in all tested samples. In contrast, MITKOVSKA and CHASSOVNIKAROVA (2020) reported that low-level exposure to the organophosphate pesticide chlorpyrifos exposure did not induce elevated levels of micronuclei in common carp but significantly increased the frequency of total nuclear abnormalities (notched, blebbed, lobed, and eight-shaped nuclei, nuclear buds, nuclear bridges, and binucleated cells).

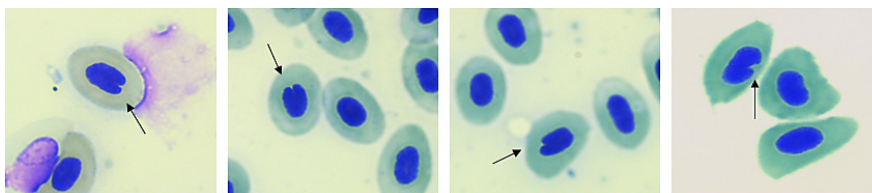
We confirmed the results of other authors who also studied the genotoxic effects of the tested chemicals. Pirimiphos-methyl, the active substance in Actellic, was genotoxic and provoked micronucleus formation in mice (ALABI et al. 2014). The genotoxic effect and micronuclei induction of propamocarb hydrochloride (the active substance in Rival) were reported in Swiss Albino mice by NOORI et al. (2016). Furthermore, 2,4-dichloro phenoxyacetic acid (the active substance in Aminopielik) was reported as genotoxic and leading to micronucleus production in the erythrocytes of catfish (*Clarias batrachus* Linnaeus, 1758) (ATEEQ et al. 2002).



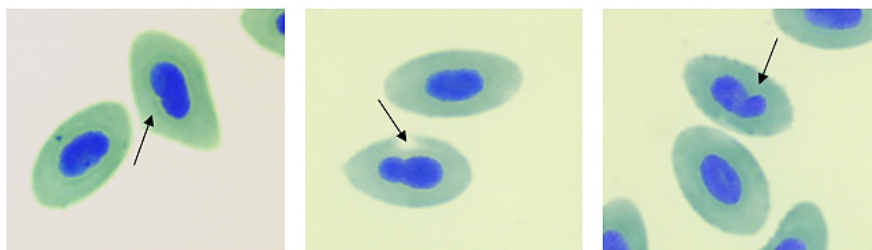
a)



b)



c)



d)

Fig. 1. Micronuclei (a) and abnormal nuclei (b – blebbed; c – notched; d – lobed) induced in common carp erythrocytes treated with different concentrations of Actellic, Rival, Aminopielik, and PBDEs.

Table 1. Total analyzed cells, cells with micronuclei and abnormal nuclei (X±SD) in common carp treated with different concentrations of Actellic, Rival, Aminopielik, and PBDEs.

Sample	Analyzed cells		Micronuclei		Abnormal nuclei		
	Total number	Mean ± SD	Total number	Mean ± SD	Total number	Mean ± SD	
Control	16691	2086.38±59.65	7	0.88±2.47	0	0±0	
Actellic	1 µl	21545	2154.5±101.39	66	6.6±2.17**	48	4.8±3.68***
	6 µl	22947	2294.7±146.71	163	16.3±4.9***	141	14.1±11.24***
Rival	3 µl	23968	2396.8±74.34	170	17±8.25***	134	13.4±3.24***
	6 µl	23217	2321.7±51.23	341	34.1±7.11***	142	14.2±9.46***
Aminopielik	4 µl	23070	2307±68.98	133	13.3±4.85***	116	11.6±5.36***
	8 µl	22467	2246.7±50.22	381	38.1±11.53***	220	22±9.32***
PBDEs	0.425 µg	20522	2052.2±30.46	8	0.8±1.48	5	0.5±0.98
	7 µg	20431	2043.1±23.88	73	7.3±8.14*	55	5.5±6.13*

p<0.05*, p<0.01**, p<0.001***

Table 2. Incidence of different types of aberrations (micronuclei or abnormal nuclei) in common carp treated with different concentrations of Actellic, Rival, Aminopielik, and PBDEs (%).

Sample	Analyzed cells	Micronuclei, %	Abnormal nuclei, %
Control	16691	0.04	0.00
Actellic	1 µl	21545	0.31
	6 µl	22947	0.71
Rival	3 µl	23968	0.71
	6 µl	23217	1.47
Aminopielik	4 µl	23070	0.58
	8 µl	22467	1.70
PBDEs	0.425 µg	20522	0.04
	7 µg	20431	0.36

Conclusion

In summary, we can conclude that the insecticide Actellic caused mutagenic damage in the common carp erythrocytes at the applied concentrations, as well as the formation of micronuclei and abnormal nuclei. Similarly, the PBDEs exposure following the permitted concentration in water exhibited a genotoxic effect as those of the insecticide Actellic (1 µl). The fungicide Rival showed significantly high-

er mutagenic and genotoxic effects than the control group and the other tested toxicants, but the herbicide Aminopielik produced the highest mutagenic effects.

The common carp, a species of significant economic importance in Bulgaria, is also a bioaccumulative species that feeds on benthic organisms. Therefore, it should continue to be used in additional studies employing other toxicological approaches.

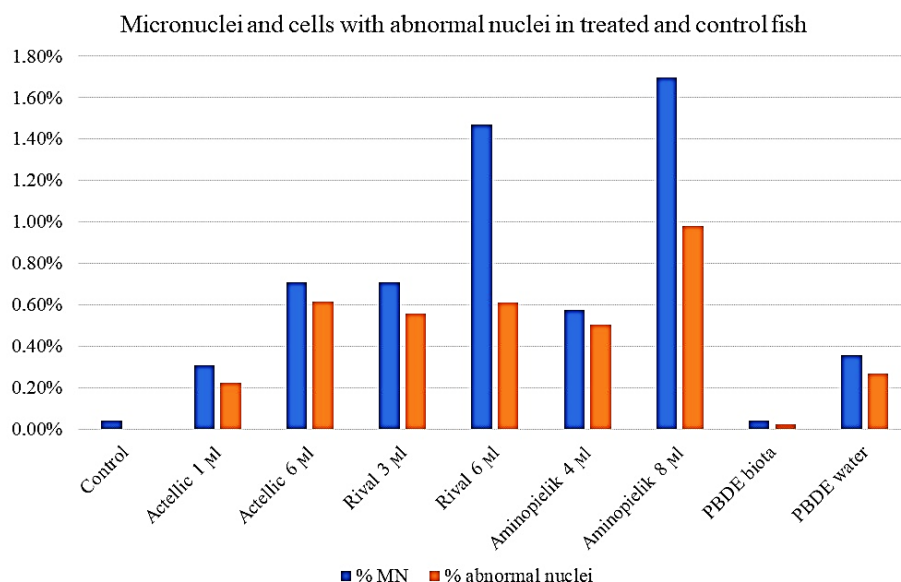


Fig. 2. Frequencies of micronuclei or abnormal nuclei in common carp treated with different concentrations of Actellic, Rival, Aminopielik, and MAC for PBDEs in biota (7 µg) and MAC for PBDEs in water (0.425 µg) (%).

This will contribute to a more detailed clarification of the mechanisms of action of the applied organic contaminants and the specificity of their mutagenic effects.

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