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GENOTOXICITY/CARCINOGENICITY OF BENZYL-PARABEN ON FISH (*SPARUS AURATA)* USING MICRONUCLEUS ASSAY

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ABSTRACT

Parabens are widely utilized as preservatives in an array of consumer products. During the lifecycle of these productsparabens such as benzylparaben (BeP) are released into the environment. In this study investigating the genotoxic effects of BeP on *Sparus aurata*, we are employed the micronucleus assay—a well established method for evaluating DNA damage at cellular levels. Fishs are exposed to varying concentrations of BeP (0.2 µg/L to 1 µg/L). The findings revealed significant increases in MN frequencies across different tissues; specifically, blood cells exhibited a frequency of 26.4 ‰ at the highest concentration tested, while gill cells showed an alarming frequency of 41‰ and liver cells recorded 33 ‰. These results underscore that exposure to benzylparaben induces genotoxic effects in S. *aurata*, highlighting its potential risk not only for marine life but also for ecosystems where such substances may accumulate through various pathways.

KEYWORDS: Micronucleus assay, Genotoxicity, *Sparus aurata*, Benzylparaben.

INTRODUCTİON

Cancer is a genomic disease associated with the accumulation of genetic damage. Micronuclei (MN) are a characteristic of human cancer (Di Bona et al., 2024). In recent years, MN has been evaluated as an early diagnostic marker in cancer patients. MN plays a fundamental role during cancer evolution and metastatic progression (Boveri, 2008). Water pollution, including contamination of groundwater and drinking water, signifies the pollution of these resources with harmful substances, resulting in serious health hazards. One of the greatest concerns associated with water pollution is its relationship to cancer. Numerous studies have demonstrated that exposure to pollutants in contaminated water sources can significantly increase the risk of developing various types of cancer. These carcinogens not only pollute the water but also accumulate in aquatic organisms consumed by humans, eventually entering our food chain. Prolonged exposure to these pollutants can weaken the immune system's ability to effectively combat cancer cells. Environmental exposures are known risk factors for cancer etiology. Biomarker-based approaches have been applied in the assessment of exposure to genotoxic/carcinogenic pollutants. Biomarkers enable early diagnosis of disease-related changes. The MN test is a well-known testing system commonly used in human and animal biomonitoring studies to assess DNA damage at the chromosomal level.We are conducting this study to determine the genotoxic effects of Benzylparaben (BeP), which is commonly used as a preservative in personal care products. The polluted water environment, waste pharmaceuticals, and their interactions with natural organisms have led to discussions on genotoxicity studies, as they continue to be an important approach for the environmental risk assessment of these substances. BeP has emerged as a vital preservative in various industries, including cosmetics, pharmaceuticals, food, and children's products (Petric et al., 2021; Oliveira et al., 2020). BeP does not have an effective degradation mechanism in wastewater treatment systems and is also found in plants and aquatic organisms (Bledzka et al., 2014, Soni et al., 2005; Andersen et al., 2005). Considering their use in products such as sunscreens, deodorants, hair gels, shampoos, creams, and toothpaste, the amount of parabens in the environment is expected to increase day by day (Bledzka et al., 2014).. The increasing prevalence of this compound in both treated and untreated wastewater effluents raises concerns regarding its persistence and bioaccumulation in aquatic ecosystems. (Wang and Kannan, 2016). BeP enter the environment via inefficient removal by sewage discharge from wastewater treatment plants, pharmaceuticals, and runoff from agricultural and industrial activities (Witorsch and Thomas, 2010). Studies have demonstrated that this compound can impair growth, reproductive success, and overall organismal health, particularly in sensitive species (Wang and Kannan,

2016)., experiments involving common aquatic invertebrates have shown that even low concentrations of benzylparaben can lead to reduced survival rates and altered behavioral patterns, ultimately impacting population dynamics and community structure (Wang and Kannan, 2016;). In fish, for example, exposure to benzylparaben has been linked to disruptions in gene expression related to reproductive health, resulting in skewed sex ratios and developmental anomalies (Osman ve diğ., 2018). Such disruptions can have cascading effects on population viability and the overall health of aquatic ecosystems. The broader ecological implications of benzylparaben pollution are considerable. As aquatic organisms serve critical roles in nutrient cycling and food webs, their decline could have far-reaching effects on ecosystem stability and function (Richardson and EL R. 1981). Additionally, the bioaccumulation of such pollutants in higher trophic levels may pose a risk to predators, including humans, who rely on these aquatic resources for sustenance. The presence of parabens in the environment is primarily due to wastewater discharges, which result in widespread contamination and their concentrations increased during the COVID-19 pandemic waves (Ramutshatsha-Makhwedzha and Munonde, 2024). According to previous studies, 90% of parabens in WWTP influents are removed through biological treatment (Haman et al., 2015).

Fish are the most preferred aquatic organisms in MN tests due to their dependence on aquatic environments with environmental pollutant discharges and their role as primary biomonitors affected by the genetic diversity of aquatic populations in changing environments. Additionally, they are frequently used to test the potential genotoxic properties of physical and chemical agents because they are exposed to a wide variety of chemical substances either directly through water or indirectly through the food chain in the ecosystem, and they respond to xenobiotics similarly to mammals. In particular, fish erythrocytes are used as a first step in assessing carcinogenic potential in MN tests (Hoftmank and Raat, 1982).

Aquaculture is one of the most common ways to produce fish species, and it has an increasing trend due to the high demand for animal protein. Sea bream is one of the most consumed species of sea fish grown in the geography of Turkey and the Mediterranean coasts (Rad and Köksal, 2000). In addition to these economic characteristics, it is an essential predator in the aquatic food chain in association with other aquatic organisms and biological food fish with high physiological and ecological significance. Sea bream is one of a group of species determined to be used effectively in toxicological research due to various anatomical biological traits, such as relatives with human pathology at the molecular level, organic solvents, and chemical metabolites that allow us to assess the number of compounds accumulated in the body and metabolite storage potentials (Rodriguez et al., 2018). It is considered a reliable indicator for specific maximum permissible levels in assessing the effects of environmental stressors or pollutants on aquatic community streams.

Based on these features, sea bream was selected as a model organism. Additionally, it aims to put forward the studies showing the damages that can be caused by the substances used in the field or in the aquatic ecosystems to the genotoxic molecular level and the effects on the associated production and health. One of the most used methods for determining genotoxic effects is the micronucleus test. This test, which is used to measure structural and numerical chromosome changes (Bolognesi and Hayashi, 2011, Arslan et al., 2015). Fish respond similarly to higher vertebrates to toxic agents, allowing the assessment of potentially hazardous substances to humans. The MN test has been successfully applied to evaluate the genotoxic activity of xenobiotic agents and complex environmental mixtures in fish, both in laboratory and field studies (Bolognesi and Hayashi, 2011). Despite the presence of BeP in the aquatic ecosystem, the genotoxic effects it may cause in aquatic organisms have not been focused on.For this purpose, in our study, it was investigated whether the *Sparus aurata* species, which is both an important trophic level in the aquatic ecosystem and an economic species, caused a genotoxic effect by exposure to BeP.

MATERİALS AND METHODS

Juvenile *Sparus aurata*, weigthing 0.7-1.5 g.which is commercially very important distributed in Turkey on the Aegean and Mediterranean coasts, was procured from the (Ege University, Faculty of Fisheries) Hatchery within the scope of our research and subjected to acclimatization for one week after it was brought to the laboratory. All the experiment was carried out with artificial sea water prepared in the laboratory. During acclimation, fish fry were fed according to their body weight and kept at the appropriate temperature (24°C).

Benzylparaben (CAS No: 380709) was obtained from Sigma Aldrich. 1000 ppm stock solutions were obtained by dissolving 10 mg of each paraben derivative in 10 ml of DMSO (CAS No: 67-68-5). Paraben concentrations used in the experiment were calculated according to the EC50 value determined according to the results of sea urchin embryotoxicity (Karaaslan et al. 2019). Exposure concentrations were determined as 0.2, 0.3, 0.5 and 1 mg BeP/L.

Glass aquariums (57x39x28 and 45 lt volume) were used in our experimental setup, which was designed according to the semi-static test method. Experiments were carried out in 2 repetitions. After the end of acclimation, 10 fish in each aquarium were tested at five concentrations in addition to the control group for 72 hours. Sampling was done every day at 24, 48 and 72 hours. Blood, gill and liver tissues were taken for micronucleus test.

Cell preparation in different tissues of fish (Sparus aurata)

Blood

A small incision was made from the caudal fin and spread on slides (10 µl). After being kept in pure ethanol, it was stained with 5% Giemsa solution. Slides stained with 5% Giemsa were closed with the help of entellan and coverslip to prevent air leakage. In the preparations prepared for microscopic observations, a minimum of 1000 cells were examined microscopically for each living thing. The occurrence and frequency of MN/BN were counted as 2000 cells from each living organism and the frequency of MN/BN was calculated as ‰ (Arslan et al., 2015, Nalbantlar and Arslan, 2017; Bolognesi and Fenech, 2012).

Gill

After the gills were removed, they were placed in tubes containing acetic acid. Shedding of epithelial tissue from the gills was achieved with the help of a pipette and then centrifuged at 2000 rpm for 10 min. After spreading on the slide, it was kept in ethanol and left to dry. Slides stained with 5% Giemsa were closed with the help of entellan and coverslip to prevent air leakage In the preparations prepared for microscopic observations, a minimum of 1000 cells for each living thing were examined microscopically. A total of 2000 cells were counted from each living thing and the MN/BN frequency was calculated as ‰ (Arslan et al., 2015, Nalbantlar and Arslan, 2017; Bolognesi and Fenech, 2012).

Liver

The liver tissue taken was transferred into 45% acetic acid and the tissue was fragmented with the help of a pipette. After spreading, it was kept in ethanol and left to dry. Slides stained with 5% Giemsa were closed with the help of entellan and coverslip to prevent air leakage. In the preparations prepared for microscopic observations, a minimum of 1000 cells were examined microscopically for each living thing. A total of 2000 cells were counted from each living creature and the MN/BN frequency was calculated as ‰ ‰ (Arslan et al., 2015, Nalbantlar and Arslan, 2017; Bolognesi and Fenech, 2012).

Statistical Analyses

Data from treatments and controls were compared using one-way analysis of variance (ANOVA), followed by Dunnett's comparison post-hoc test whenever applicable ($p < 0.05$) to compare the mean differences in MN frequency between groups and exposure durations. Results with $P < 0.05$ were considered statistically significant. MN frequencies are reported as mean ± standard error for each experimental group.

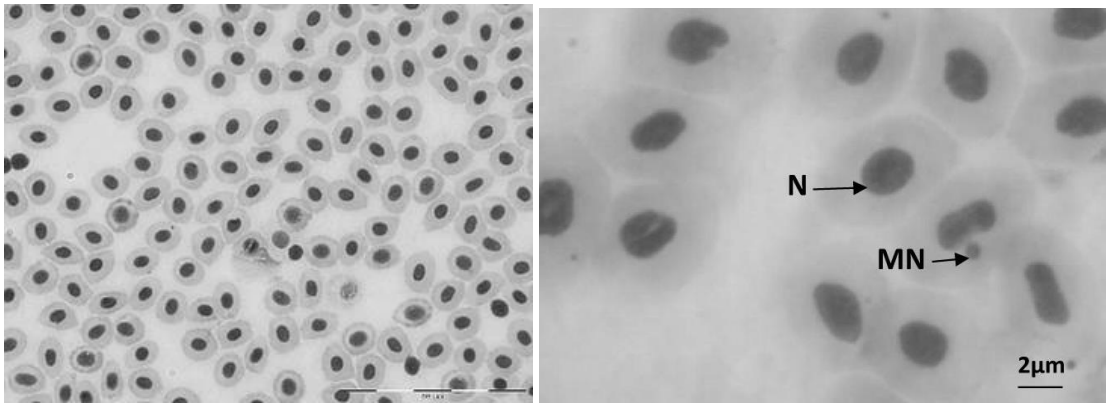
RESULTS

This study focused on genotoxic effects of benzlyparaben (BeP) on the test organisms *Sparus aurata* as an economically important species. The study investigated erytrocythes, gill and liver cells of *Sparus aurata* exposed to increasing concentrations of BeP for a period of three days under semi-static laboratory conditions. The present study attempted to determine the genotoxic potential of BeP for the test organism *Sparus aurata* using a micronucleus test (Figure 1). Our microscopic investigations also revealed other nuclear aberrations such as nuclear buds. However, they were not included into counts as their numbers were statistically insignificant.

The frequency of micronuclei in investigated cells of test organisms (fish) is presented in Table 1,2,3. Table 1 shows the micronucleus frequencies determined as a result of the samplings made for every 24 hours of fish blood cells exposed to increasing concentrations. Compared to the control, the micronucleus frequency was found to be 26.4‰ at the highest concentration (1 ppm). The micronucleus frequency determined at benzylparaben concentrations increases for 24, 48 and 72 hours (Figure 2).

Table 2 shows the micronucleus frequencies determined as a result of the samplings made for every 24 hours of fish gill cells exposed to increasing concentrations. Compared to the control, the micronucleus frequency was found to be 41‰ at the highest concentration (1 ppm). Micronucleus frequency in gill cells is higher than that determined in blood cells. An increase in micronucleus frequency was detected depending on the concentration in the gill cells for 3 days (Figure 3).

Table 3 shows the micronucleus frequencies determined as a result of the samplings made for every 24 hours of fish liver cells exposed to increasing concentrations. Compared to the control, the micronucleus frequency was found to be 33,75‰ at the highest concentration (1 ppm). Based on increasing concentrations, the micronucleus frequency determined in liver cells is lower than that determined in gill cells. The highest micronucleus frequency was detected in gill cells, followed by blood and liver cells, respectively (Figure 4).



(a) (b)

Figure 1: a: Erythrocytes with normal nuclei (magnifying power: 1000×), b: Erythrocytes with micronucleus (*MN*)

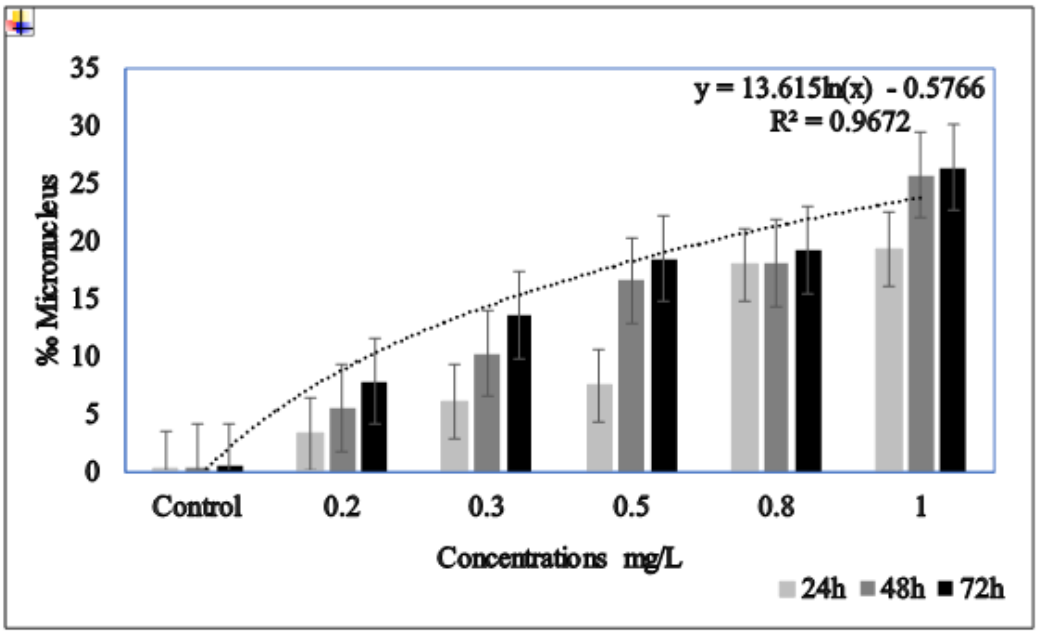


Figure 2. Micronucleus frequencies in erytrocytes depending on exposure time (24h, 48, 72h).

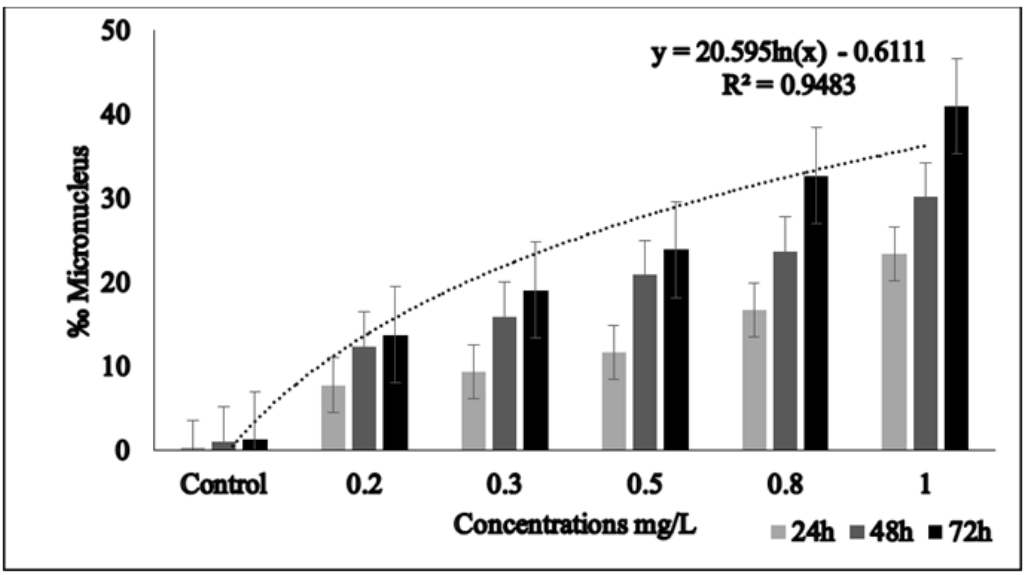


Figure 3: Micronucleus frequencies in gill cells depending on exposure time (24h, 48, 72h).

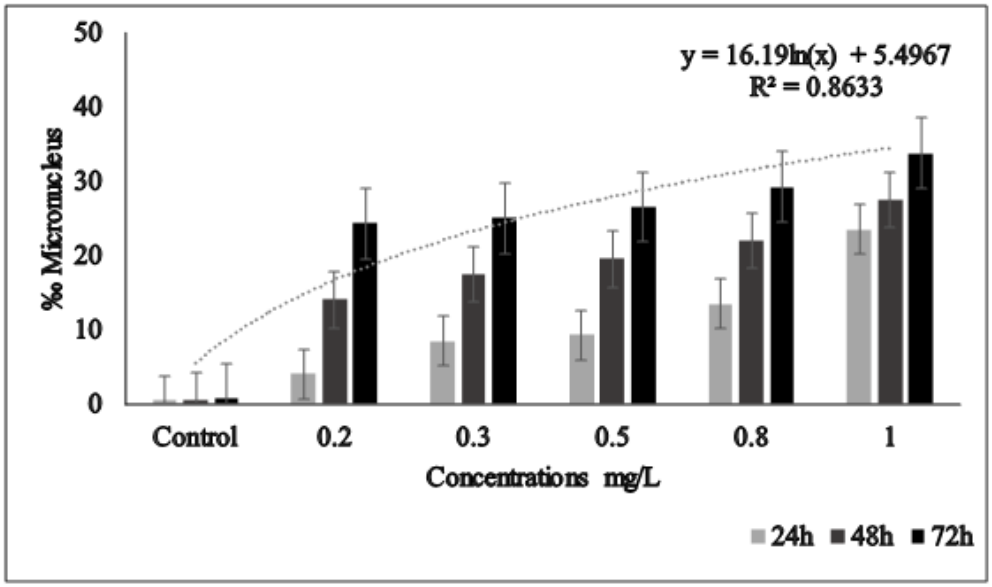


Figure 4: Micronucleus frequencies in liver cells depending on exposure time (24h, 48, 72h).

Tables

Table 1: Nukeus and Micronucleus score of erythrocytes (‰).

Concentrations mg BeP/L	Blood cells					
	N (n=10)			MN (n=10)		
	24h	48h	72h	24h	48h	72h
Control	999,6±0,16	999,6±0,16	999,5±0,18	0,4±0,16	0,4±0,16	0,5±0,18
0.2	997,3±0,42	990,4±3,98	992,1±0,89	3,33±0,42	5,6±0,67	7,87±0,89
0.3	993,8±0,58	989,7±0,74	986,4±1,72	6,12±0,58	10,3±0,74	13,6±1,72
0.5	992,3±0,46	983,4±1,17	981,2±2,70	7,5±0,42	16,6±1,17	18,5±2,67
0.8	981,8±0,83	981,8±1,51	980,7±2,95	18,17±0,83	18,17±1,51	19,25±2,95
1	980,4±1,73	974,2±1,10	973,6±0,71	19,3±1,85	25,75±1,10	26,4±0,71

Table 2: Nukeus and Micronucleus score of gill cells (‰).

Concentrations mg BeP/L	Gill cells					
	N (n=10)			MN (n=10)		
	24h	48h	72h	24h	48h	72h
Control	999,6±0,15	998,9±0,27	998,6±0,61	0,36±0,51	1,1±0,27	1,33±0,61
0.2	992,2±0,38	987,6±0,58	986,2±1,31	7,8±0,38	12,4±0,58	13,8±1,31
0.3	990,5±0,47	984,1±0,40	980,8±1,47	9,4±0,49	15,9±0,40	19,1±1,47
0.5	988,3±0,51	979,1±0,71	976,1±0,65	11,7±0,51	20,9±0,71	23,9±0,66
0.8	983,3±0,55	976,3±0,41	967,2±1,54	16,7±0,55	23,7±0,44	32,7±1,54
1	976,5±0,86	969,8±1,31	959±1,14	23,4±0,86	30,2±1,31	41±1,14

Table 3: Nukeus and Micronucleus score of Liver cells (‰).

Concentrations mg BeP/L	Liver cells					
	N (n=10)			MN (n=10)		
	24h	48h	72h	24h	48h	72h
Control	999,5±0,28	999,5±0,28	999,2±0,47	0,5±0,28	0,5±0,28	0,75±0,47
0.2	996±0,40	986±0,91	975,8±1,65	4±0,40	14±0,91	24,3±1,65
0.3	991,5±0,64	982,5±0,28	975±1,08	8,5±0,64	17,5±0,28	25±1,08
0.5	990,7±0,47	980,5±0,28	973,5±1,90	9,25±0,47	19,5±0,28	26,5±1,90
0.8	986,5±0,95	978±1,15	970,8±1,25	13,5±0,95	22±1,15	29,2±1,25
1	976,5±0,28	972,5±0,28	971,3±2,75	23,5±0,28	27,5±0,28	33,75±2,75

DİSCUSSİON

In environmental mutagenesis, MN tests provide quite practical results in monitoring the clastogenic and genotoxic effects of pollutants. Fish and shellfish are primary indicators of the health of aquatic environments. To obtain these results, aquatic organisms such as

bivalves *Mytilus galloprovincialis*, *Crassostrea gigas*, and *Chamelea gallina*, as well as fish like rainbow trout *Oncorhynchus mykiss* and *Oreochromis niloticus* (Tilapia) are commonly used (Cavaş and Ergene 2003, Arslan et al. 2010, Tsarpali and Dailianis 2012). Previous studies have examined aquatic organisms exposed to polluted waters to determine the effects of genotoxins in natural environments (Barśiene and Barśyte 2000; Dixon et al. 2002). The use of the micronucleus test to determine the genotoxic status of aquatic environments is rapidly increasing (Arslan et al. 2015, Dailianis et al. 2003, Tsarpali and Dailianis 2012).

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The MN test were used as a biomarker because it gave dependable results and also its easy applicability. When the results obtained from this study examined samples with different concentrations to determine genotoxic effects were found to be one of the pollutants that cause genotoxic damage in the light of BeP.

Despite the presence of parabens in the aquatic environment, the genotoxic effects they may cause in aquatic organisms have not been focused on. In previous studies, the effects of different paraben derivatives on the antioxidant defense mechanism were investigated(; Li et al., 2023 Silva et al. (2018) conducted research on antioxidant and oxidative damage in the gill and liver of *Oreochromis niloticus* species of parabens. They reported that SOD, GPx and GR activity increased significantly in the liver and gills after exposure.

The model organism *Sparus aurata*, which was also used in our study, is preferred to examine the effects of different chemicals. Banni et al. (2009) investigated the effects of the chemical with the accumulation, enzyme activity and comet method in the liver of *Sparus aurata* species exposed to benzo[a]pyrene. As a result of their study, reported that there was a change in enzyme activity and an increase of 12.17% in tail length. In our study, the Mn frequency increased depending on the concentration in the liver cells of *Sparus aurata* species exposed to benzylparaben, and it was found to be 33.75 ‰ at the end of the 72nd hour. Barreto et al. (2017) investigated the effects of gemfibrozil drug, which is widely available in the environment, on *Sparus aurata*. They reported an increase in Mn frequency and erythrocytic nuclear abnormalities depending on the concentration (1.5-15-150-1500-15.000 µg/L) in peripheral blood cells after 96 hours of exposure.

MN test, which can be applied to different species in ecotoxicological studies, is a suitable test method for evaluating the effects of various chemicals. Hussain et al. (2018) applied both the comet and micronucleus test in their study by collecting *Labeo rohita*, an economic species in the Chenab river. As a result of the study, they found the MN 6.30 ‰, the binucleus value as 2.56 ‰ and reported that it was parallel to each other with the tail length. The results of the study support the presence of pollutants in the region. In another study, *Cyprinus carpio* was exposed to mercury concentrations of 2.0, 20.0, 200.0 mg/L. The frequency of MN in blood cells has been reported to vary between 0.82 ‰ and 21.07 ‰ (Nepomuceno et al. 1997). Fish species *Channa punctatus* were exposed to pentachlorophenol (PCP) and 2,4-dichlorophenoxyacetic acid (2,4-D). While the MN frequency ranged between 2.89 and 8.08 for PCP, it was found 1.96 for 2.4 D after 48 hours of exposure (Farah et al. 2003). According to Cavas et al. (2005) used *Cyprinus carpio, Carassius gibelio and Corydoras paleatus* species. They exposed these three species to different concentrations of cadmium (0.005–0.1 mg/L) and copper (0.01–0.25 mg/L) for 21 days. As a result of the experiment, they reported that the MN values found for the liver and gills were higher than the values found for the blood. In our study, in which we demonstrated the genotoxic effect of benzylparaben, the highest frequency of MN was detected in gill cells. In another study, Arslan et al. (2015) applied the MN test with different fish species caught from clean and polluted areas in İzmir Aliağa Bay. As a result of the study, it was reported that high MN frequency was detected in fish caught from the polluted area. It is seen that the MN test can be used as a standard method for monitoring pollution in coastal

ecosystems. In another study, Arslan et al (2016) determined the genotoxic effect of Bisphenol A, which is used in various applications, on *Mytilus galloprovincialis*. They reported that all concentrations of BPA increased genotoxic damage in *Mytilus galloprovincialis* and caused the formation of 11.33 ‰ to 37.35 ‰ micronuclei. Nalbantlar and Arslan (2017) investigated the genotoxic effect of PFOS, one of the persistent organic pollutants, on *M. galloprovincialis* species. As a result of the study, they reported that MN values increased depending on the concentration. The current study is consistent with previous research indicating that BeP is genotoxic/carcinogenic, primarily responsible for these nuclear changes. The relationship between chemical exposure and its detrimental effects on DNA synthesis within living organisms has been well-documented. According to Ventura et al. (2008), a myriad of chemicals can interfere with the DNA synthesis of exposed organisms, leading to nuclear abnormalities. This phenomenon has significant implications for both environmental health and the potential risks posed to higher vertebrates, including humans. Various studies have demonstrated a dose-dependent increase in micronucleus (MN) frequencies in erythrocytes, a crucial indicator of genotoxic damage. Das and Nanda (1986) noted a marked increase in the induction of micronuclei in the peripheral blood of *Heteropneustes fossilis*, correlating with the concentration of pollutants. Similarly, Yadav and Trivedi (2009) reported an increase in micronucleus frequencies in *Channa punctata* after 96 hours of heavy metal exposure, though a gradual decrease was observed with continued exposure. This underscores the complex dynamics of cellular response to toxic environments. Moreover, Nepomuceno et al. (1997) suggested that higher concentrations of pollutants could hinder normal cell division and damage erythrocyte chromosomes, ultimately disrupting DNA replication and influencing MN frequencies variably. In conclusion, these findings collectively indicate that exposure to microplastics significantly heightens genotoxic damage, as evidenced by elevated MN and nuclear abnormalities in fish. As bioindicators, fish are increasingly recognized for their role in detecting the effects of pollution, enabling early identification of aquatic environmental contamination. Notably, these organisms exhibit responses to toxic agents that are analogous to those observed in higher vertebrates, thereby facilitating the assessment of potentially hazardous substances for human health. Consequently, it becomes imperative to elucidate the genotoxic effects of pollutants such as microplastics, in light of their pervasive presence and implications for public health, particularly concerning cancer risks. The emerging threat posed by microplastics, especially as a potential carcinogen, has generated considerable concern within the public health domain. Experimental evidence suggests a plausible link between these small plastic particles and cancer development. Furthermore, microplastics have been linked to oxidative stress, inflammation, and cellular damage, all of which are recognized precursors to cancer. Addressing this pressing threat necessitates heightened public awareness and sustained scientific advancement to ensure a safer future for forthcoming generations.

It is seen that the MN test, which is used to determine the genotoxic effect of various chemicals with different species, is a safe test method. As a result, it has been determined that benzylparaben has a genotoxic effect on *Sparus aurata* species. The limited number of studies on Benzylparaben, which has been determined to cause a genotoxic effect, raises concerns. Therefore, more and different studies are needed in terms of the safety and sustainability of the aquatic ecosystem.

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Compliance with ethical standards

Conflict of interest

Author Özlem ÇAKAL ARSLAN, Author Beyza NALBANTLAR, Author Gizem GÜLSEVER and Author Koray BENAS declares that they have no conflicts of interest.

Ethical statement

This study under taken with" the certificate has been issued in accordance with the European Union Directive on the Protection of Animals Used for Scientific Purposes No. 2010/63/EU and the Ministry of Agriculture and Forestry dated 02.04.2019 and numbered 2019/3.

Data access statement

The data related to my article is not found on any data storage device.

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