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ECO-TOXICOLOGICAL EFFECTS OF POLYETHYLENE MICROPLASTICS ON DNA DAMAGE IN ERYTHROCYTES OF BARILIUS VAGRA IN THE SARDARYAB STRETCH OF THE KABUL RIVER, PAKISTAN

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Abstract

In this study, genotoxic effects of polyethylene micro-plastics (PE-MPs) on a commercially and ecologically significant species of freshwater fish, Barilius vagra were evaluated in the stretch of the Kabul river, Pakistan. The fish were sampled from nine locations (S1 to S9) and held in controlled laboratory conditions for 7 and 14 days at different concentrations of PE-MPs (0, 5, 25 and 50 mg/L). The peripheral blood erythrocytes (PBE) were used for determination of DNA damage with the micronucleus (MN) assay. After 7 days of exposure, MN frequency increased from 4.8% in the control group to 24.0% at 50 mg/L. The genotoxic stress increased with concentration and time, with MN frequency increasing from 7.2% in the control to 27.4% at 50 mg/L after 14 days. Comparing data from the sampling stations revealed that the baseline MN frequencies of fish were significantly higher at downstream stations (S7–S9: 13.6–15.8%) than at upstream stations (S1–S2: 4.8–6.2%), indicating the overall increase in microplastic pollution in the river. The majority of the fibers, 80-92%, was the dominant type in all matrices, suggesting the presence of pollution from textiles and domestic wastewater. These results indicate ecological concerns due to micro-plastic pollution in the Kabul River basin and the need for more environmental regulations and monitoring approaches for particular species.

INTRODUCTION

The prevalence, persistence, and negative impacts of microplastics on aquatic environments have made it one of the most significant environmental issues of the twenty-first century. Microplastic (5 mm or less) and polyethylene (PE) are the most common types of plastics found in aquatic environments (Hamed et al., 2020). They are derived from the fragmentation of larger plastic debris and the addition of microplastics from the direct release of manufactured microplastics from industrial processes, personal care products and synthetic textiles (Cole et al., 2011). It is estimated that in 2018, 13 million tonnes of the 400 million tonnes of plastic produced became plastic waste and debris in aquatic ecosystems (Cunha et al., 2020).

First created in the early 1900s, plastics have grown to be essential to contemporary business and daily life. Since the 1950s, their manufacturing has increased dramatically (Worm et al., 2017). But due to their persistence and longevity, people have started worrying about plastic pollution in water bodies. Although records of plastic pollution in the marine environment were first reported in the 1970s, plastic pollution likely began a great deal earlier (Rochman, 2018). Since then, there has been an exponentially greater scientific interest, particularly with regard to microplastics (Borja and Elliott, 2019). Today, these particles are known to be present in a wide range of aquatic environments and organisms, ranging from freshwater to deep-sea habitats (Valente et al., 2019). The ecological and health issues linked to microplastics pollution are becoming a crucial priority in the context of sustainable fisheries and safe food, particularly with the current world trend of seafood consumption (FAO, 2020; Béné et al., 2015).

Rivers are significant conveyors of micro-plastic from land to ocean. Both Baldwin et al. (2016) and Vermaire et al. (2017) have shown that river systems are generally heavily contaminated with plastic litter. Likewise, Klein et al. (2015) reported that there was high spatial variability and accumulation of microplastics in river sediments, which showed freshwater systems are not just an avenue for transportation of plastic pollution, but also act as storage. Micro-plastics have been found in the sediment of many locations around the world. Some studies, for instance Peng et al. (2018) and Neama et al. (2020) have reported high concentrations in urban river sediments and similar contamination in the Euphrates River. Sediments are also important reservoirs as observed in North Africa by Toumi et al. (2019) who found microplastic accumulation in freshwater streams. The accumulation is affected by processes including aggregation, density-driven settling and biofouling (O'Connor et al., 2019).

Fish are extremely susceptible to microplastic pollution because of their feeding ecology and behavior. Microplastics can be ingested by fish directly by misidentifying them as food or indirectly through trophozooplankton that contain contaminated food (Worm et al., 2017; Watts et al., 2014). Bioaccumulation and biomagnification after ingestion raises concerns with exposure at higher trophic levels (Farrell and Nelson, 2013; Zhang et al., 2019). Microplastics have been found in the tissues, gills and guts of cultured fish as well as wild-caught fish (Rochman, 2018). Habitat type, feeding strategy, biogeography, and ecological niche are some of the factors that affect ingestion rates (Harris, 2020). The ingestion of microplastic by freshwater fish species has been confirmed from several studies. Microplastics have been found in the gastrointestinal system of fish, such as in Pazos et al. (2017) and Pegado et al. (2018), where the fish ingested the microplastics directly. Likewise, Roch et al. (2019) noted that there was species-specific variation in microplastic uptake, raising the question of the role of ecological characteristics such as feeding strategy.

Particles that are ingested can induce physical damage, oxidative stress, growth inhibition, and genetic effects (Banaei et al., 2022; Chae and An, 2017). Microplastics are especially dangerous for fish as they can accumulate in the digestive system, migrate into tissues, and even into the circulatory system (Jovanovic, 2017; Liu et al., 2021). The micronucleus assay is widely used in fish ecotoxicological studies (Thompson et al., 2004; Cavas and Ergene-Gozukara, 2005) and the DNA damage in erythrocytes of fish is a reliable indicator of genotoxicity. It has been previously reported that microplastics caused DNA damage, histopathological changes and biochemical alterations in fish (Hamed et al., 2020; Rochman et al., 2013).

Feed selection is an important factor in microplastic ingestion. Wang et al. (2020) and Sun et al. (2020) showed that bottom-feeding and omnivorous fish have higher MP concentrations than pelagic fish. This

is mainly attributed to the increased microplastics in sediments as mentioned by Zobkov and Esiukova (2017) and Scherer et al. (2020). The ingestion of microplastics can be by direct ingestion as well as from one trophoic level to the next via food chain interactions. Rochman et al. (2017) showed several direct and indirect effects of microplastics on aquatic organisms including predator/prey relationships. Moreover, Galloway et al. (2017) noted that microplastics can be transferred up trophozoos, which may lead to biomagnification and ecological risks.

Barilius vagra (Hamilton, 1822) is a wide-spread freshwater cyprinid fish in the hill streams and rivers of the Himalayan foothills in Pakistan, India, Nepal, Bangladesh and Afghanistan. It lives in medium to high gradient rivers; gravelly and rocky. B. vagra is an omnivorous benthopelagic which feeds on aquatic insects, insect larvae, micro-crustaceans, rotifers, and soft aquatic plants. It is especially susceptible to the ingestion of microplastics, particularly in areas of contaminated sediments, due to its feeding habits and habitat preferences. The common carp (Cyprinus carpio) and Barilius vagra are important aquatic species both ecologically and economically. The physiological and molecular effects of exposure to microplastics in these species can help to inform broader understanding of the impacts of the pollutant on aquatic systems. Physiological consequences include changes in food habits, metabolism, growth and reproductive success (Wang et al., 2021).

Although there is increasing concern over them, there is a lack of data regarding genotoxic effects of PE-MPs in freshwater fish species in Pakistan. Irfan et al. (2020a, 2020b) conducted the first study on the presence of microplastics in surface water and sediments of the country. Recently, Khan et al (2024) found microplastics in fish species collected from Panjkora River, which showed the widespread contamination of freshwater in K-P-K. Thus the present study was undertaken to assess the DNA damaging effects of polyethylene microplastic on Barilius vagra using micronucleus assay. The specific aims were to: (1) determine the baseline levels of micronuclei in B. vagra along the upstream-to-downstream pollution gradient in the part of the Kabul River studied; (2) quantify the concentration- and time-dependent genotoxic effects of PE-MPs under controlled laboratory conditions; and (3) assess the potential ecological and human health risks of microplastic pollution in this economically valuable fish species.

2. Materials and Methods

2.1 Study Area and Sampling Design

The study was conducted at the Sardaryab stretch of the Kabul River, located downstream of Peshawar in District Charsadda, Khyber Pakhtunkhwa, Pakistan. The Kabul River originates from the Hindu Kush region of Afghanistan and flows through densely populated and agriculturally active areas before joining the Indus River at Attock. Along its course in Pakistan, the river receives substantial anthropogenic inputs from urban settlements, agricultural lands, and small-scale industrial activities, making it increasingly vulnerable to pollution, including microplastics. Sardaryab is a key recreational and economic zone of the Kabul River where tourism, fishing, and boating generate high plastic waste input. Improper disposal of single-use plastics, along with sewage and agricultural runoff, strongly increases microplastic pollution, making the area a clear hotspot for microplastic accumulation.

A total of nine (n = 9) sampling sites were selected along the Sardaryab stretch of the Kabul River to represent a clear upstream-downstream pollution gradient and varying intensities of anthropogenic impact (Table 1; Figure1). These sites were strategically distributed from relatively less disturbed upstream areas to highly impacted downstream zones, including recreational, agricultural, and waste discharge points, to assess spatial variation in microplastic contamination.

Table 1: Description of Sampling Sites along the Sardaryab Stretch

Site Code	Location Description	Latitude (N)	Longitude (E)	Characteristics
S1	Upstream (reference site)	34.15°	71.68°	Low pollution
S2	Entry point near agriculture	34.16°	71.69°	Runoff influence
S3	Midstream (low disturbance)	34.17°	71.70°	Moderate flow
S4	Near fishing activity zone	34.18°	71.71°	Net/plastic waste
S5	Main Sardaryab picnic point	34.19°	71.72°	High tourism
S6	Food street discharge area	34.20°	71.73°	High plastic input
S7	Downstream mixing zone	34.21°	71.74°	Accumulation zone
S8	Far downstream	34.22°	71.75°	Sediment deposition
S9	Extreme downstream	34.23°	71.76°	Max accumulation

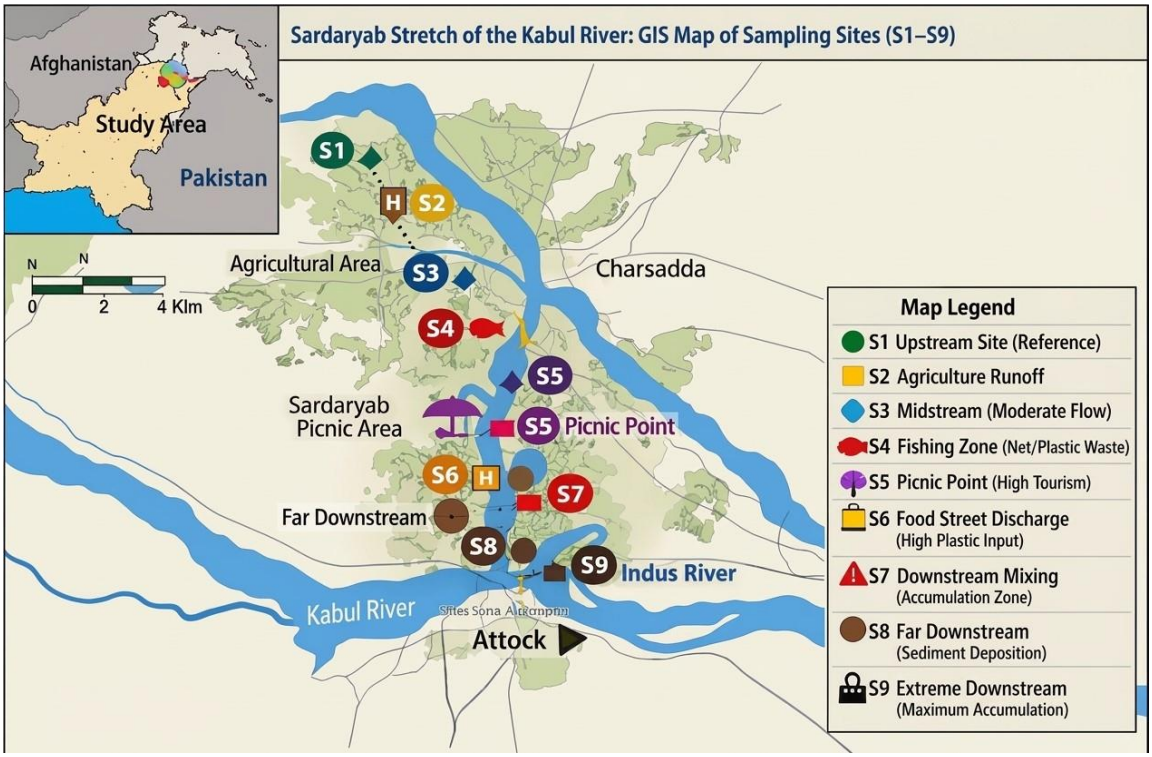


Figure 1: X. GIS map of sampling sites (S1–S9) along the Sardaryab stretch, Kabul River (Charsadda, Pakistan)

2.2 Sample Collection

All selected stations of the Sardaryab stretch were sampled for water, sediment and fish samples using standard field procedure and to ensure reliable data and avoid contamination. The water sample was collected at surface level (up to 0-30 cm depth) in pre-sterilised glass bottles to represent floating and suspended microplastics. Sediment samples were collected by grab samplers, from the upper 5cm of the riverbed sediments that is known to be the primary area where micro-plastics tend to accumulate.

The conventional fishing nets were used to catch fish specimens of Barilius vagra at all sampling sites with average weight of 45 ± 8 g and length of 14 ± 2 cm. The species were selected for their broad ecological niche, feeding behaviour and habitat preferences to evaluate species-specific susceptibility to ingestion of micro plastics. Fish were transported to the laboratory in an aerated tank filled with river

water and acclimatized for two weeks in glass aquaria with aerated, dechlorinated water at 25 ± 2°C with a light-dark cycle of 12:12 h and a pH of 7.5 ± 0.5. During acclimatization and experimentation, fish were fed the commercial diet (PCG Company, ~25% protein) twice a day.

2.3 Experimental Chemicals and Organism

The polyethylene micro plastics (PE-MPs) with ~500 µm particle size were extracted as raw powder from Alfa Aesar (Thermo Fisher Scientific). To have a constant physical and chemical property, low-density polyethylene (LDPE) with typical particle size of 500 µm and high purity was used. Polyethylene was selected because it is widely used in consumer goods, is buoyant, and is inert in water (Catalog No. A10239, CAS No. 9002-88-4). The high purity reduced the number of contaminants, enabling the controlled and repeatable evaluation of the behavior of microplastics under different conditions.

2.4 Experimental Design

Fish from each site were acclimatised, and then exposed to four concentrations of polyethylene microplastics; 0 (control), 5, 25 and 50 mg/L for 7 and 14 days. Five fish were used in each treatment and experiments were replicated 3 times. Control groups were provided with identical conditions with the exception of exposure to PE-MP. The concentrations were chosen from previous studies to find the dose-response relationship and threshold effects (Hamed et al., 2020; Banaee et al., 2019).

2.5 Microplastic Extraction from Environmental Samples

A multi-step extraction protocol, based on a protocol applied to freshwater sampling for microplastics, was used for the extraction of microplastics from water, sediment and fish samples.

Wet peroxide digestion was employed to remove organic matter from water samples, by adding 20 mL of hydrogen peroxide (35%) and 20 mL of Fenton's reagent (acidified ferrous sulfate solution) to 200 mL of each water sample. The mixture was boiled in a water bath until the organic matter was completely digested at 75°C. The microplastics were separated after digestion by density separation in saturated sodium chloride (NaCl) solution (density 1.18 g/cm^3). Around 600 mL of NaCl solution was added, and left to settle for 6-8 hours. The collected supernatant, which contained the microplastic particles, was carefully filtered through a series of sieves (300 µm, 150 µm, 50 µm) to create various particle size fractions. Both sieves were rinsed, and the particles retained were collected on membrane filter papers by using a vacuum filtration assembly.

A similar wet peroxide digestion method was used for sediment samples, where 25 mL of hydrogen peroxide (35%) and 25 mL of Fe(II) catalyst solution was poured into the beaker containing the sediment. The mixture was heated to 75°C until all the organic matter was digested. The digested material was then sieved with 50 µm sieves followed by sequential sieving using sequential sieves of 300 µm, 150 µm and 50 µm. Microplastic isolation was performed by transferring the filtered material from each fraction to filter papers by vacuum filtration. In fish, gastrointestinal tracts were removed following the unfreezing of the specimens for 1-2 hours prior to dissection. Each gut sample was placed in a 250 mL glass bottle with 10% potassium hydroxide (KOH) solution at a 5:1 ratio (solution to sample) and was subsequently digested. The mixture was incubated at 55°C for 36 hrs, to complete the digestion of tissues. Following digestion, a solution of sodium chloride (3:1 v/v) was added, stirred for 20 minutes and allowed to settle for 2 hours. The supernatant was then passed through a series of filters of different pore sizes (300 µm, 150 µm, 50 µm). The particles retained in each fraction was back washed and collected on membrane filter papers (0.45 µm pore size, 47 mm diameter) by vacuum filtration. The filter paper was then dried in covered Petri dish for 24 hours before analyzed under the microscope.

2.6 Identification and Classification of Microplastics

After filtration, the retained material on filter papers was air-dried and analyzed under a stereomicroscope equipped with a digital camera. Both coarse and fine fractions were examined to ensure comprehensive detection of microplastics in all samples. The coarse fraction (retained on 300 µm sieves) was first observed under the microscope, followed by detailed examination of finer fractions

collected from 150 µm and 50 µm sieves using higher magnification to detect smaller particles, in line with the method described by Irfan et al. (2020a).

To confirm the plastic nature of suspected particles, a hot needle test was applied to all observed items. Particles that melted or deformed upon contact were verified as microplastics and included in further analysis. Only confirmed plastic particles were counted to ensure data reliability. Microplastics were then classified based on their physical characteristics, including shape, color, and surface texture. Identified particles were categorized into fibers, fragments, films/sheets, foams, and beads. For quantification, standardized reporting units were used: microplastics were expressed as MPs per individual fish, MPs per cubic meter of water, and MPs per kilogram of sediment, following internationally recognized guidelines reported by Hidalgo-Ruz et al. (2012) and Bilal et al. (2022).

2.7 Micronucleus Assay

Fish were placed in anesthetic solution, and blood was drawn from caudal veins of the fish through sterile syringes and transferred into EDTA tubes. Thin blood smears were made on clean glass slides, air dried then fixed in 100% methanol for 10 minutes and stained with 6% Giemsa solution for 15 minutes. The number of micronuclei in each blood sample was counted in 500 erythrocytes of each blood sample using a light microscope at 1000× magnification. The small chromatin bodies, which are separated from the main nucleus, were termed micronuclei and were less than 1/3 of the diameter of the main nucleus (Cavas and Ergene-Gozukara, 2005).To Calculate: MN Frequency (%), the number of MN observed in each of the 100 cells counted is divided by the total number of cells counted and multiplied by 100.

MN Frequency (%) = (Number of Micronuclei Observed / Total Cells Counted) × 100

2.8 Data Analysis

The number of microplastics in each individual fish and in each water and sediment sample was used to quantify microplastics depending on the matrix type. The concentration of microplastics in the various sampling locations and fish species was statistically compared to examine the difference in concentration of microplastics in different locations and in different fish species. Data are presented as mean ± SD. To evaluate significant difference in MN frequency between different concentrations and exposure time, one way analysis of variance (ANOVA) was used. Tukey's test was used for post-hoc comparisons. All statistical analysis were done on Statistic software (version 8.1) and the results were considered statistically significant when $p<0.05$.

3. Results

3.1. Microplastic concentration in water samples

There was a clear gradient from S1 to S9 with increasing concentration of microplastics (MPs/m³) in the water samples collected from the Sardaryab stretch, from 150 ± 40 MPs/m³ at S1 to 510 ± 130 MPs/m³ at S9, and overall mean of 196.4 ± 88 MPs/m³ (Figure 2). Relatively low MPs levels were found upstream (S1-S2) where agricultural runoff was the dominant factor. Relative increases were moderate across midstream sites (S3-S4) corresponding to fishing activity and plastic waste found locally. The tourism hotspots (S5-S6) showed significant increase (310-360 MPs/m³) due to the improper disposal of single-use plastics at picnic/discharge points of the food street. The highest concentrations were detected at the downstream stations (S7-S9) which indicate cumulative pollution and depositional conditions.

3.2 Microplastic Concentrations in Sediments Samples

The concentration of micro-plastics in the sediment samples varied from 210 ± 45 MPs/kg at S1 to 610 ± 140 MPs/kg at S9 with an average of 281.8 ± 11 MPs/kg (Table 2). The data indicate a consistent increasing trend from upstream to downstream, which is likely due to the cumulative effect of anthropogenic inputs, as well as depositional processes. The MP levels were lower at the upstream locations (S1-S2) where agricultural run-off was the dominant source and human impact was minimal. Moderate increases (295-340 MPs/kg) were observed at midstream sites, potentially associated with

fishing operations and plastic waste from fishing nets and packaging. The highest concentration was found at S5-S6 (410-465 MPs/kg), showing that the main picnic point (Sardaryab) and the area of food street discharge of the waste are the areas with high level of tourism and presence of single use plastics (SUP) which are not disposed of properly (Figure 2.). The highest MPs accumulation (510-610 MPs/kg) was found in downstream sites (S7-S9), which is the result of the cumulative pollution load and the lower velocity of flow, favoring deposition of MPs.

3.3 Microplastics Concentrations in Fish Samples

The number of MPs per fish ranged from 6 ± 2 MPs/fish (S1) to 18 ± 9 MPs/fish (S9), with an overall mean of 12.5 ± 8.02 MPs/fish (Table 2). The data show a clear upstream to downstream increase suggesting that cumulative exposure of MPs via feeding and habitat contamination was present. Lower MPs ingestion was observed in upstream species, perhaps because of anthropogenic effects and lower water quality. The MPs level for midstream species (10-12 MPs/individual) was the same as the fishing activity and plastic waste. MPs ingestion was the highest in downstream species (14-18 MPs/individual) indicating bioaccumulation and trophic transfer from contaminated sediments and water (Figure 2).

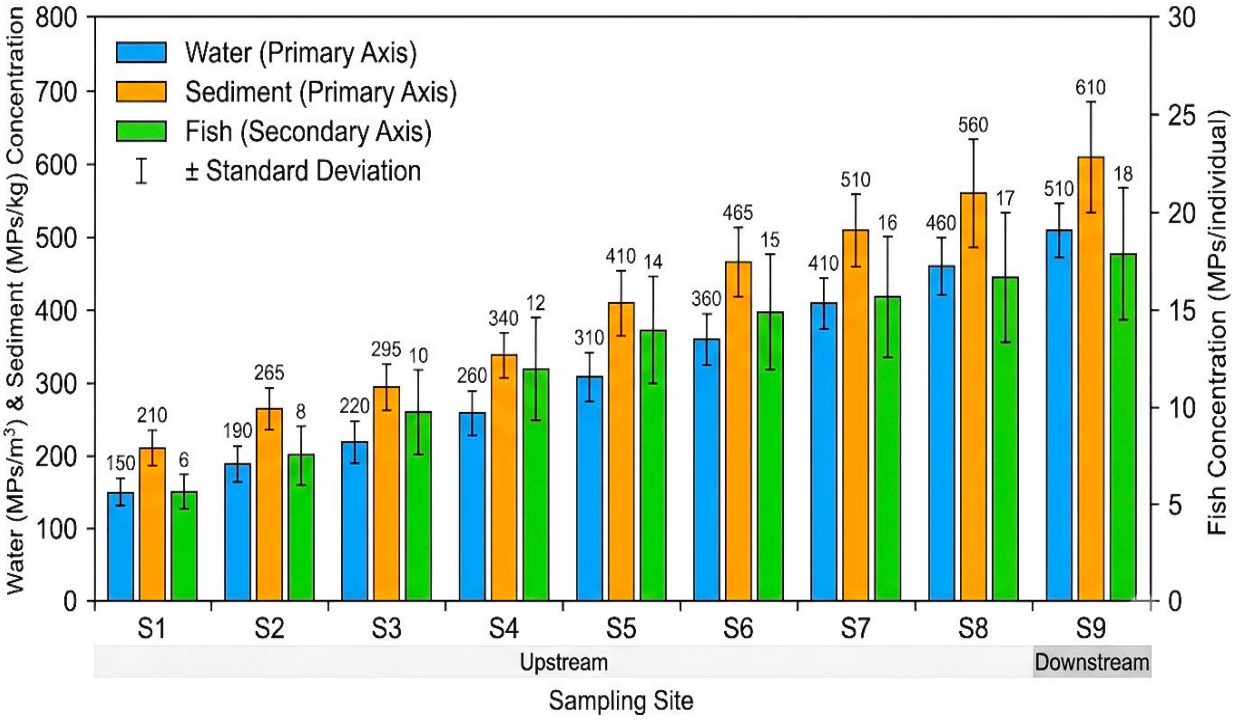


Figure 2. A comparative bar graph representing the microplastic (MPs) concentrations in water (MPs/m³), sediment (MPs/kg) and fish (MPs/individual) at nine sampling sites (S1–S9) along the Sardaryab section of the Kabul River, Pakistan. Standard deviation (± SD) is shown as error bars. The figure shows a steady increase in concentration of MPs downstream in all matrices, which suggests an increasing cumulative anthropogenic input and potential for trophic transfer.

3.4 Baseline Micronucleus Frequency in Barilius vagra

The frequencies of the baseline micronuclei (MN) of Barilius vagra examined in the nine sampling sites are provided without laboratory exposure to polyethylene microplastic (PE-MP). The results showed that the frequency of the MN increased in an upstream to downstream direction, which highlighted the upstream to downstream accumulation of microplastic pollution along the stretch of the Kabul River from Sardaryab (Figure 3a & 3b).

3.4.1 The spatial distribution of the contamination by microplastics

Microplastic concentrations were determined in each of three environmental matrices (water, sediment and fish tissue) at nine sites (S1–S9) assessed across the study area (Figure 3a). The microplastic concentration increased towards upstream in all matrices. The lowest water concentrations were recorded at S1 (150 ± 40 MPs/m³) and the highest at S9 (510 ± 130 MPs/m³) which corresponds to an increase of 240% from the reference location. The same was observed in sediment samples, where the concentration increased from 210 ± 45 MPs/kg at S1 to 610 ± 140 MPs/kg at S9, indicating the progressive depositional processes and the lower velocities of flows in the downstream sections of the river. The absolute concentration of MPs in fish tissue ranged between 6 ± 20.23 MPs/individual in S1 and 18 ± 92.23 MPs/individual in S9, suggesting bioaccumulation via dietary exposure and trophic transfer.

The observed gradient was very similar to the strength of the anthropogenic activities along the river corridor. The upstream sites (S1-S2) were relatively low in contamination, mainly due to agricultural runoff and low human population. Moderate increases (220–260 MPs/m³ for water, 295–340 MPs/kg for sediment) were observed at the midstream sites, similar to localized inputs from fishing activities and plastic waste from fishing nets and packaging, as well as small-scale domestic discharge. The highest level of contamination was found at the S5–S6 (310–410 MPs/m³ in water; 410–465 MPs/kg in sediment) the main picnic site of Sardaryab and area of food street disposal, where cases of tourism and improper disposal of single use plastics are common. The highest number of microplastics accumulated was at the downstream sites (S7-S9) with values of 410-510 MPs/m3 in water and 510-610 MPs/kg in sediment, likely resulting from combined effects of cumulative pollution inputs, depositional conditions and reduction in turbulence, which aids settling of microplastics.

3.4.2 Baseline Micronucleus Frequency

Micronucleus frequency, determined as absolute number of micronuclei in 500 erythrocytes examined, and as percentage frequency (MN%) in Barilius vagra erythrocytes, was found to be the same as in the case of P. pulcher. The frequency of MN was found to increase from upstream to downstream, corresponding to the spatial pattern of microplastic contamination. The baseline frequency of MN (24MN/500 cells) was the lowest in the fish collected from the reference site (S1), and the highest in the fish collected from the most contaminated downstream site (S9) (4.8% vs. 15.8%) (Figure 3b). The results show three different contamination zones along the river gradient, with relatively low baseline MN frequencies found in the Fish S1 (4.8%, 24 counts) and Fish S2 (6.2%, 31 counts) zones, indicative of relatively clean water quality and limited microplastic pollution. The sites are used as reference points to evaluate the genotoxic effect of anthropogenic pollution.

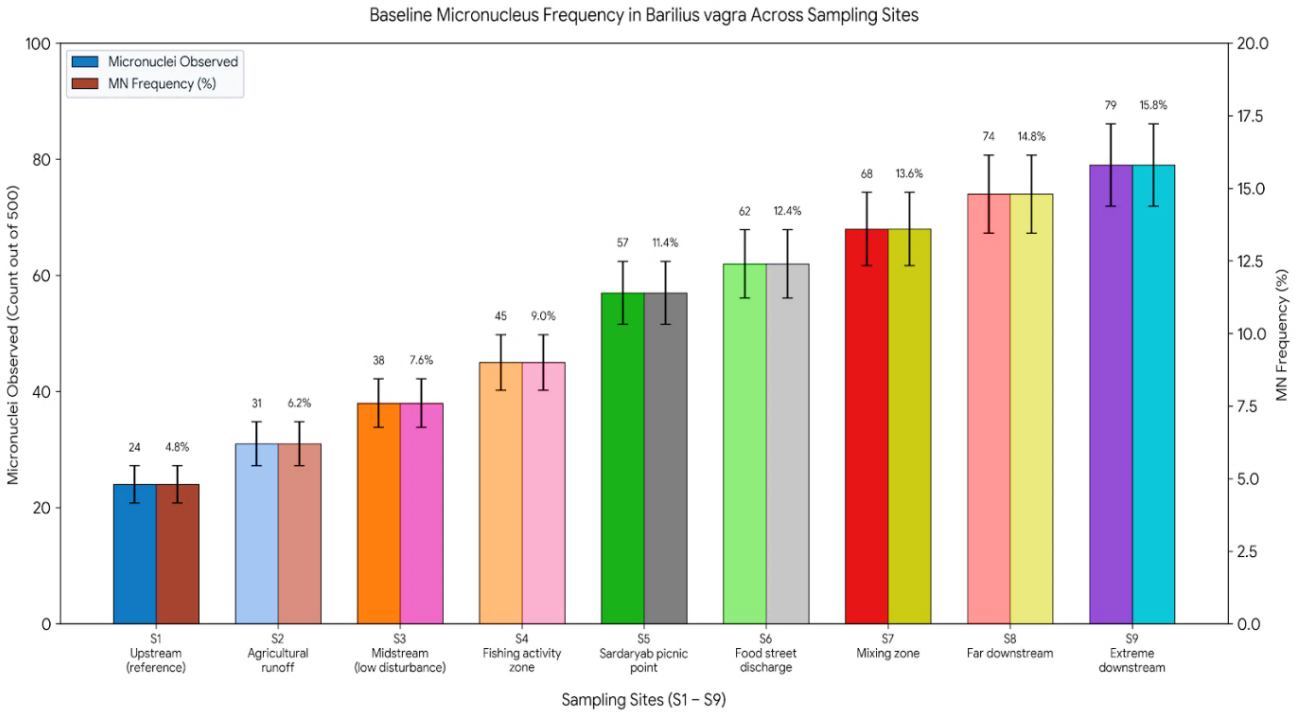
As anthropogenic pressure (agricultural runoff, fishing, tourism and domestic waste discharge) increased, the prevalence of MN also increased in a progressive manner from S3 (7.6%; 38 counts) to S6 (12.4%; 62 counts). A significant increase was observed in the transition from S5 (11.4%; 57 counts) to S6 (12.4%; 62 counts) which was correlated to food street discharge and plastic waste disposal.

At downstream sites, most populations had high baseline MN frequencies with S7 (13.6%, 68 counts), S8 (14.8%, 74 counts), and S9 (15.8%, 79 counts) having significantly higher frequencies than upstream populations. The MN frequency of fish sampled from S9 was almost 3.3 times higher than of fish sampled from S1, suggesting high genotoxic stress from the cumulative effect of microplastic pollution and the synergistic effects of multiple pollution sources.The positive correlation between environmental microplastics loads (particularly the concentration in the sediments) and the baseline MN frequencies indicates that B. vagra is a suitable bioindicator species for freshwater monitoring of microplastic pollution. The high level of baseline DNA damage in fish from downstream sites, exposed to microplastics for long periods, suggests that chronic exposure to the microplastics could predispose individuals to further genotoxic stress, like a decrease in DNA repair capacities or still accumulating oxidative damage.

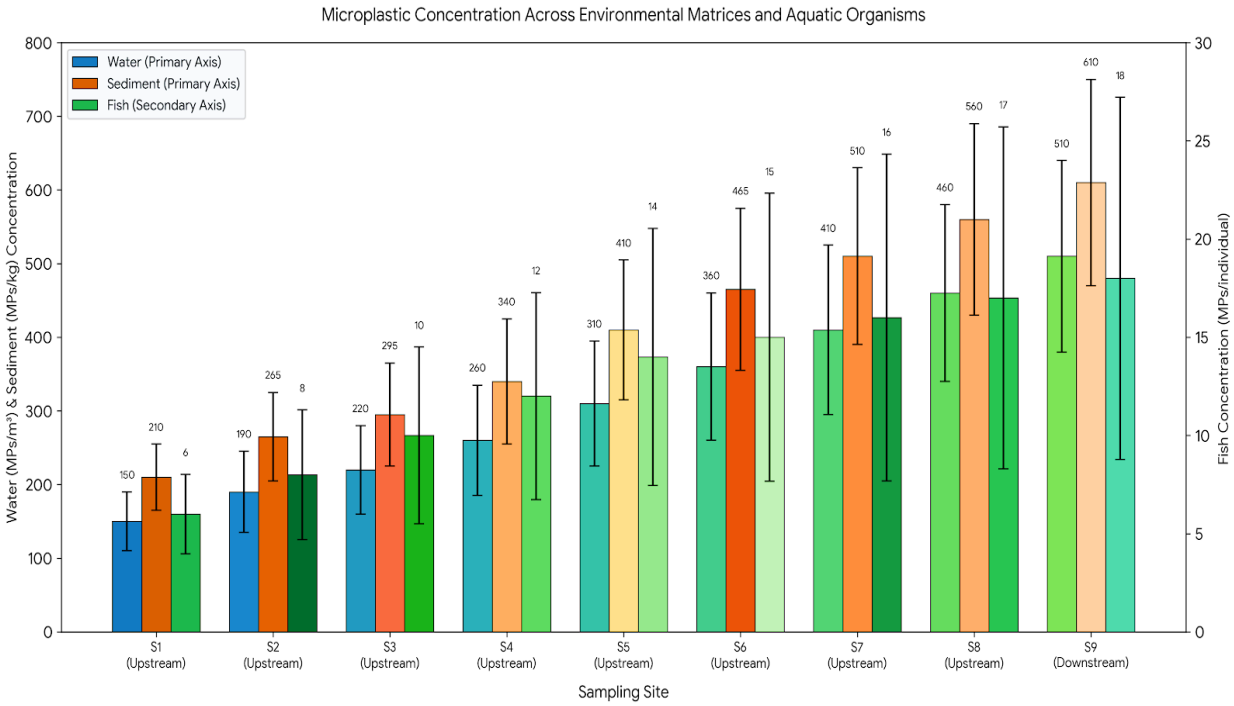
3.4.3 Observed at the Site Level and its Ecological Implications

The spatial gradient of MN frequency is similar to that of hydrological and landuse characteristics of Sardaryab region. The mixing zone (S7) and the far downstream sites (S8-S9) can be depositional areas where microplastics settle from the water as the turbulence and interaction with the sediment decreases, thereby increasing the exposure duration for benthic and benthopelagic species. The feeding habits and habitat preferences of Barilius vagra result in moderate to high vulnerability to ingestion of microplastics. Generally, bottom and omnivorous fish have higher levels of microplastic contamination than pelagic fish, and the higher concentration of microplastics in sediments than in water column is the main reason.

This site-specific variation emphasizes the combined effect of urban development, agricultural drainage, recreational use of waterway, and household effluent into the river along the river corridor. The rapid rise of MN at S5-S6 has been attributed to high tourism and food street discharge rates, thus underlining single-use plastics as a contributor to microplastic pollution. The highest measurements at S9 are due to all the upstream pollution sources; therefore it is important that integrated watershed management strategies are implemented. Spatial distribution of MP in water (MPs/m³), sediment (MPs/kg) and Barilius vagra tissue (MPs/individual) across the nine sampling sites (S1–S9) as shown in figure 3a. Means values are represented by bars and error bars are standard deviation (±SD). Microplastic bioaccumulation levels in fish tissue are measured in secondary y-axis.



(a) Frequency of micronucleated erythrocytes and (b) total micronuclei count in Barilius vagra from sampling sites (S1- S9) per 500 examined cells. A color coded scale indicates variation within the site (error caps are standard deviation ±SD). The total micronuclei count is plotted on the primary y-axis, the overall percentage frequency (MN%) is plotted on the secondary y-axis.



(a) Frequency of micronucleated erythrocytes and (b) total micronuclei count in Barilius vagra from sampling sites (S1- S9) per 500 examined cells. A color coded scale indicates variation within the site (error caps are standard deviation ±SD). The total micronuclei count is plotted on the primary y-axis, the overall percentage frequency (MN%) is plotted on the secondary y-axis.

3.5 Micronucleus Frequency after 7 Days of PE-MP Exposure

The frequencies of micronucleus (MN) in erythrocytes of Barilius vagra after 7 days exposure to different concentrations of polyethylene microplastics (PE-MPs) are shown in Table 4 and illustrated in Fig. 4. The results indicate that genotoxic stress increased as a function of the concentration of exposure with the highest genotoxic stress occurring at the highest concentration of exposure (50 mg/L).

3.5.1 Concentration-Dependent Effects

The overall genotoxic damage due to environmental microplastic contamination was assessed with MN frequency, which varied between 4.8% (at the upstream reference site, S1) and 15.8% (at the most contaminated downstream site, S9) in the control group (0 mg/L PE-MPs). The exposure to 5 mg/L PE-MPs led to a small but understandable rise in the percentage of MN at all the sites (6.0% - S1; 17.2% - S9). This is an average of 1.2–1.4 percentage points higher than controls, showing microplastic exposure at these low concentrations has measurable genotoxic effects.

The frequency of MN increased significantly for all sites at 25 mg/L PE-MP exposure, from 13.2% (S1) to 21.0% (S9). A dose-dependent response was observed in this concentration, with an increase in DNA damage of ~2.5-3.0 fold at most sites compared to control, indicating that at higher concentrations of microplastics, DNA damage may become significantly higher. The greatest rises were seen at 50 mg/L PE-MP exposure with MN frequency at 20.4% at S1 and 24.0% at S9. This is four times higher than the control population at S1 (4.8% to 20.4%) and 1.5 times higher at S9 (15.8% to 24.0%), proving that the fish in the upstream populations are more sensitive to acute exposure to PE-MP than are the fish at downstream sites where the environment is already chronically contaminated.

3.5.2 Site-Specific Response Patterns

The site specific investigation found that a gradient of upstream to downstream produced different reaction patterns. At 50 mg/L, fish exposed to PE-MP at upstream reference sites (S1-S3) had the highest relative increase in MN frequency when compared to controls: 4.8% to 20.4% (+15.6 percentage points) at S1, 13.0% to 25.0% (+12.0 percentage points) at S2, and 12.5% to 26.0% (+13.5

percentage points) at S3. This is a 325% rise and suggests that human populations that had not been previously exposed are very vulnerable to genotoxicity caused by acute exposure to micro-plastics. The relative increase was significantly lower (8.4–8.6 percentage points or 52–54%) at the downstream sites (S7–S9), perhaps because some DNA damage had occurred prior to sampling and the species may have adapted to this long-term exposure. The largest absolute frequencies of the MN were always detected at downstream sites for all treatment groups. At 50 mg/L exposure, S9 exhibited the maximum MN frequency of 24.0%, followed by S8 (23.6%) and S7 (23.2%). This trend provides further evidence of the synergism between environmental contamination and lab exposure, with the most severe overall genotoxic stress seen in the fish that are already stressed by baseline DNA damage who are exposed to PE-MP.

3.5.3 Dose-Response Relationships

The dose-response relationship was observed to have three phases at all the sampling sites. The low-dose phase (0-5 mg/L) gave very little genotoxic response, with an average difference of +1.3 percentage points compared to the control. The moderate dose range (5–25 mg/L) showed a rapid linear increase: MN frequency was increased by a median of 8.1 percentage points at all sites corresponding to 6.2-fold increase in MN frequency per unit concentration at moderate doses as compared to low doses. The high dose range (25–50 mg/l) showed a continuing but slightly reduced increase, with a further 5.3 percentage point average increase; this range might be approaching maximum genotoxic capacity.

The concentration dependent pattern was most pronounced at upstream sites where the dose response curve slope was greater compared downstream populations. It suggests that fish originating from relatively "pure" environments are more susceptible to genotoxic effects under increasing PE-MP exposure, thus it is of fundamental importance that REs are safeguarded from PE-MP pollution.

3.5.4 The mechanism underlying the genotoxicity of PE-MP

Several mechanisms of genotoxicity are suggested for the observed MN formation in Barilius vagra erythrocytes as a result of PE-MP. Polyethylene microplastics can produce reactive oxygen species (ROS) as a result of surface-mediated reactions and release of additives during leaching, which can cause DNA damage and strand breaks and chromosomal aberrations. Secondly, mechanical damage to erythrocyte membranes from the presence of microplastics in the blood stream and disruption of the normal functions of cells can occur. Third, PE-MPs can be vectors for adhesion of environmental pollutants, such as heavy metals and persistent organic pollutants, that can, through several mechanisms, synergistically increase the genotoxic effects. The progressive increase in MN frequency from baseline (Figure 3) to 7 days exposure (Figure 4) also suggests a progressive increase in DNA damage with exposure to microplastics. The uniform dose-dependent effect at all sampling sites indicates strong evidence for the direct genotoxic activity of PE-MPs in freshwater fish, highlighting the importance of implementing strict environmental regulations and implementing proactive measures to control pollution.

3.5.5 The ecological and risk assessment implications

This 7 day exposure experiment has many implications on ecological risk assessment and sustainable management of freshwater fisheries. This dose-response effect on the prevalence of MN suggests that genetic integrity of wild fish populations is at risk from PE-MP contamination. The absolute genotoxic stress was highest in fish exposed to the experimental pollutants, from downstream sites (S7-S9) where the baseline MN frequencies are already higher than the limits of environmental pollution. The observed response patterns indicate that species of great commercial and ecological importance, such as Barilius vagra, are at risk from microplastic pollution. This species is a benthopelagic omnivore and is more vulnerable to ingestion of microplastics because of the consumption of contaminated sediment and prey. The vulnerability of the species and the fact that they have shown genotoxic effects further highlight the need for the implementation of species-specific monitoring strategies in the Kabul River basin.

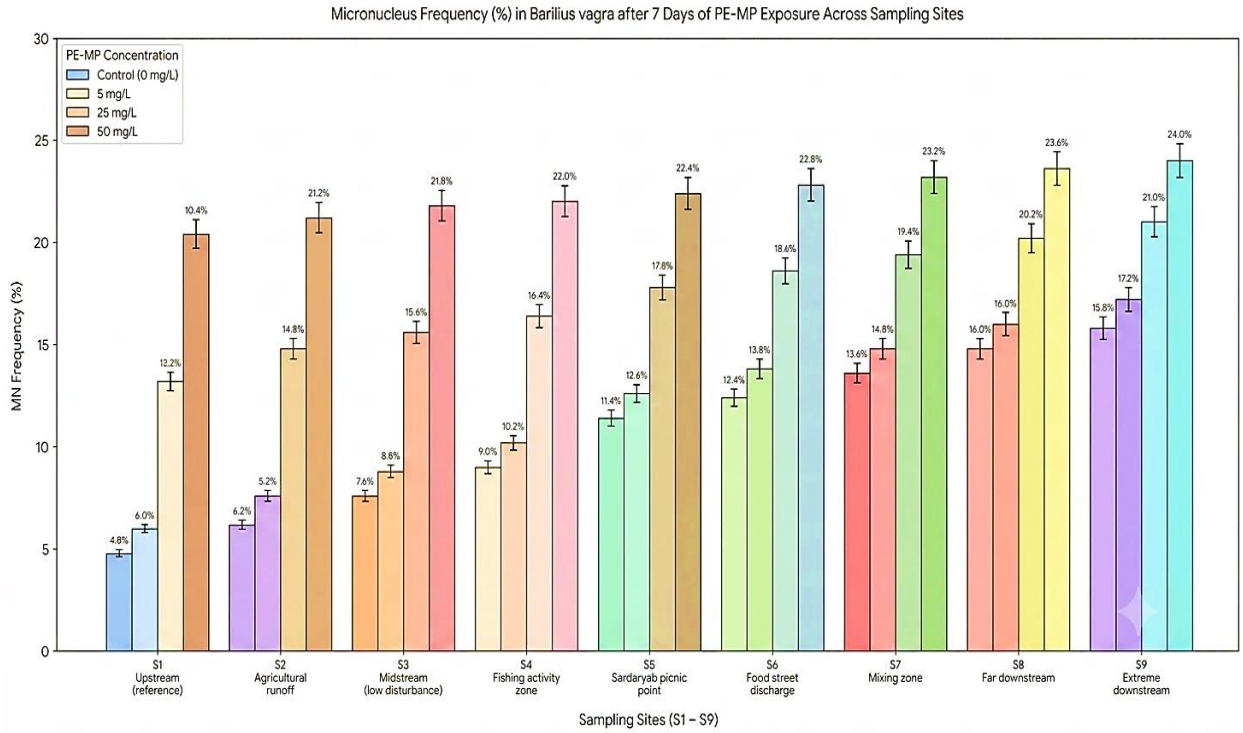


Figure 4: Mean micronucleus frequency (MN FR, %) in *B. vagra* exposed to different PE-MP concentrations (5, 25, and 50 mg/L) over 7 days. All sampling sites showed significantly higher MN frequencies compared to the control (0 mg/L), with downstream sites (S8 and S9) exhibiting the highest levels, particularly at 50 mg/L exposure. S1 to S9 denote specific sampling locations.

3.6 Micronucleus Frequency After 14 Days of PE-MP Exposure

The longer time of exposure caused even more DNA damage in all concentrations and locations. The baseline MN frequencies for the control group ranged from 7.2% (S1) to 19.6% (S9) and showed an increasing background DNA damage over time. The frequency of MN was 27.4% in the fish obtained from S9 at 50 mg/L, indicating a high level of genotoxicity that varies over time. The 14-day exposure period resulted in a significant increase in genotoxic effects as compared with the 7-day exposure period, with frequencies of MN being significantly higher in all treatment groups and sampling sites. An increase of 3.3 percentage points, on average across all sites, in MN frequency was observed at the highest concentration (50 mg/L) when compared to 7-day exposure, with an increase from 20.4% to 23.6% at upstream site S1 and from 24.0% to 27.4% at downstream site S9. The dose response relation was similar to 7 days, in which the more DNA damage that occurred, the higher the PE-MP concentration at all sites. In contrast, the same trend was observed in the control groups, with an increase in MN frequencies after 14 days compared to 7 days, ranging from 7.2% at S1 to 19.6% at S9; this increase in MN frequency may reflect ongoing physiological stress and/or continued genotoxic processes that have occurred in the background of the animals, even in the absence of PE-MP exposure. Therefore, the downstream populations were also found to have significantly higher Mn frequencies than the upstream ones at all the treatment concentrations for 14 days, which further confirms the genotoxic sensitivity of Barilius vagra under cumulative effect of exposure to microplastics in the environment. The highest genotoxic response was recorded in the fish at the most contaminated downstream site (S9) at the highest concentration (50 mg/L) with the MN frequency of 27.4%, showing that there is high damage to fish DNA with high concentrations of PE-MP for long periods of time.

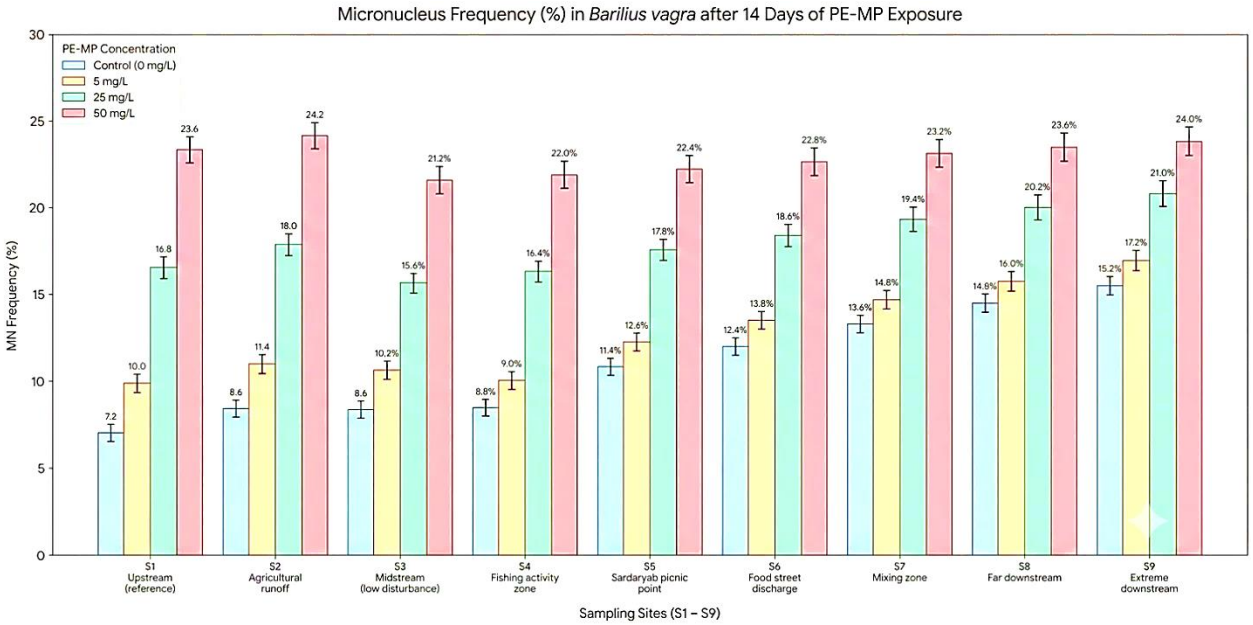


Figure 5: Nine sampling sites (S1–S9) of Barilius vagra showed significant difference in MN frequency (%) after PE-MP exposure at different concentrations (0, 5, 25, and 50 mg/l) in 14 days of exposure. Mean MN frequency (500 cells counted/treatment) is shown with bars, and increases with concentration and duration with the highest frequency observed at S9 at 50 mg/L.

3.7 Comparative Analysis of MN Frequency

All the sampling sites were then evaluated for MN frequencies after 7 and 14 days of exposure to PE-MPs at 50 mg/L. The results show that the genotoxicity significantly increases with time, and longer exposures result in higher numbers of MNs in all populations. The frequencies of the MN were generally higher in fish from downstream than in fish from upstream, suggesting that pre-existing environmental contamination worsens the genotoxic effects of PE-MP exposure (Figure 6).

The relative time-dependent increases were also different along the upstream-to-downstream gradient, being higher at upstream sites. The relative change in MN frequency at the reference site (S1) was 15.7% between 7 days and 14 days, with a change from 20.4% to 23.6% respectively. At the most contaminated downstream site (S9), however, relative increases were observed between the two sites with relative increases of 14.2% in MN frequency from 24.0% to 27.4%. This trend implies that some fish from relatively pristine upstream environments could be more sensitive to extended exposure to PE-MP, whereas some fish in downstream environments already have chronic DNA damage leading to a possible saturation point, where further genotoxic stress has less of a relative effect. However, absolute MN frequencies were always elevated at downstream locations for both exposure periods with the highest values at S9 (24.0% at 7 days; 27.4% at 14 days). At all sites, this increase was on average 3.3 percentage points (between 3.0% (S2-S3) and 3.6% (S5-S7)), indicating a progressive increase in genotoxic damage with the duration of exposure. These results confirm that DNA damage is an accumulative process when induced by PE-MP and indicate the vulnerable nature of fish downstream from contaminated water bodies where chronic environmental exposure and acute laboratory exposure add to the genotoxic stress.

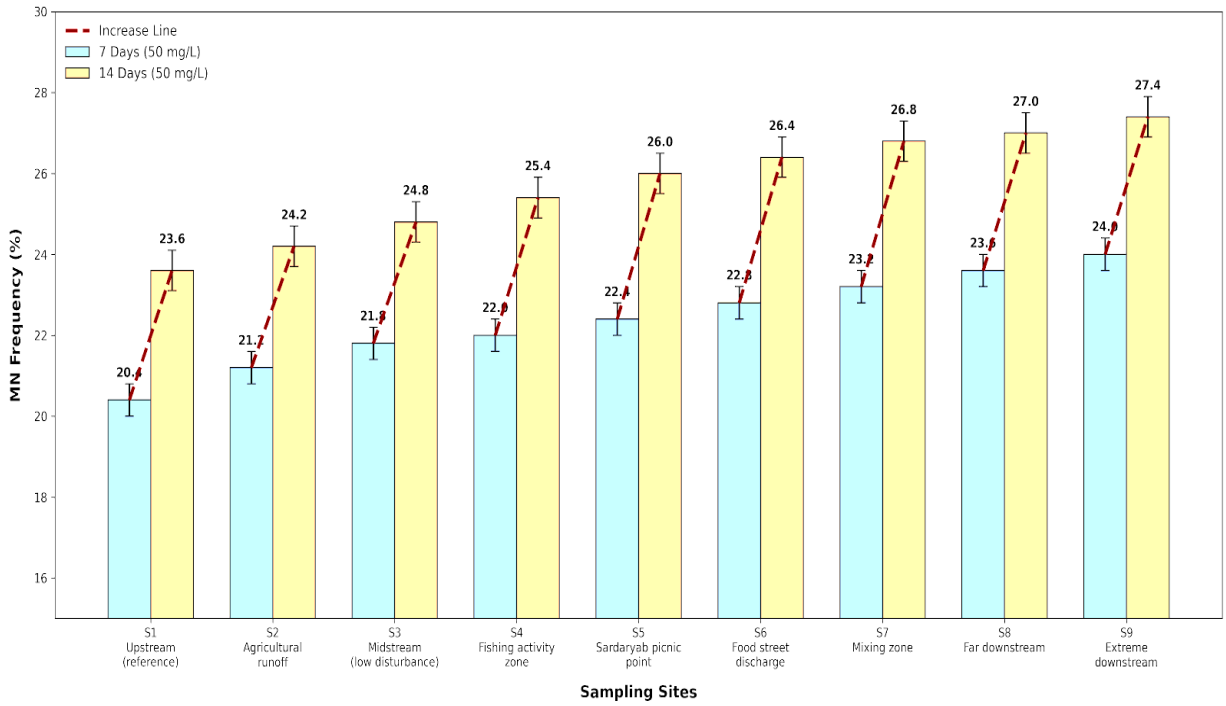


Figure 6: The comparative results of the frequency of the micronucleus (MN) in Barilius vagra after 7 and 14 days of exposure to polyethylene microplastics (PE-MPs) at nine sampling locations (S1–S9) are shown in Figure 6. The frequency of MN was significantly elevated for all sites with a mean increase of 3.3 percentage points at sites with exposure lasting >20 years. S9 had the highest frequency of MN after 14 days (27.4%). Means are represented by bars and are based on 500 cells treated.

3.8 Shapes and Sizes of Microplastics

The morphology of microplastics identified revealed that fibers were the most abundant type in all matrices, fragments, sheets and foams, and not detected in beads. Of all the water samples, fibres made up about 80% of MPs, followed by fragments (6%), sheets (12%), and foams (2%). The fibers also had a greater dominance over the other types of MPs, with 92% of the total MPs in sediment samples, followed by fragments (6%) and sheets (2%). The same pattern was observed for fish samples: fibers (88% of the total MPs), sheets (7%), fragments (5%), and foams (0%). (Figure 7 and 8). In terms of particle size, large sized MPs (≈300 µm) were predominant (62%) followed by medium sized (20%) and small sized (18%) MPs in water samples. Additionally, large sized MPs dominated sediments (44%) as did medium size and small MPs. The size distribution of fish samples was also similar to that of mussels with large MPs (43%) most abundant followed by small (29%) and medium-sized particles (28%) (Figure 9).

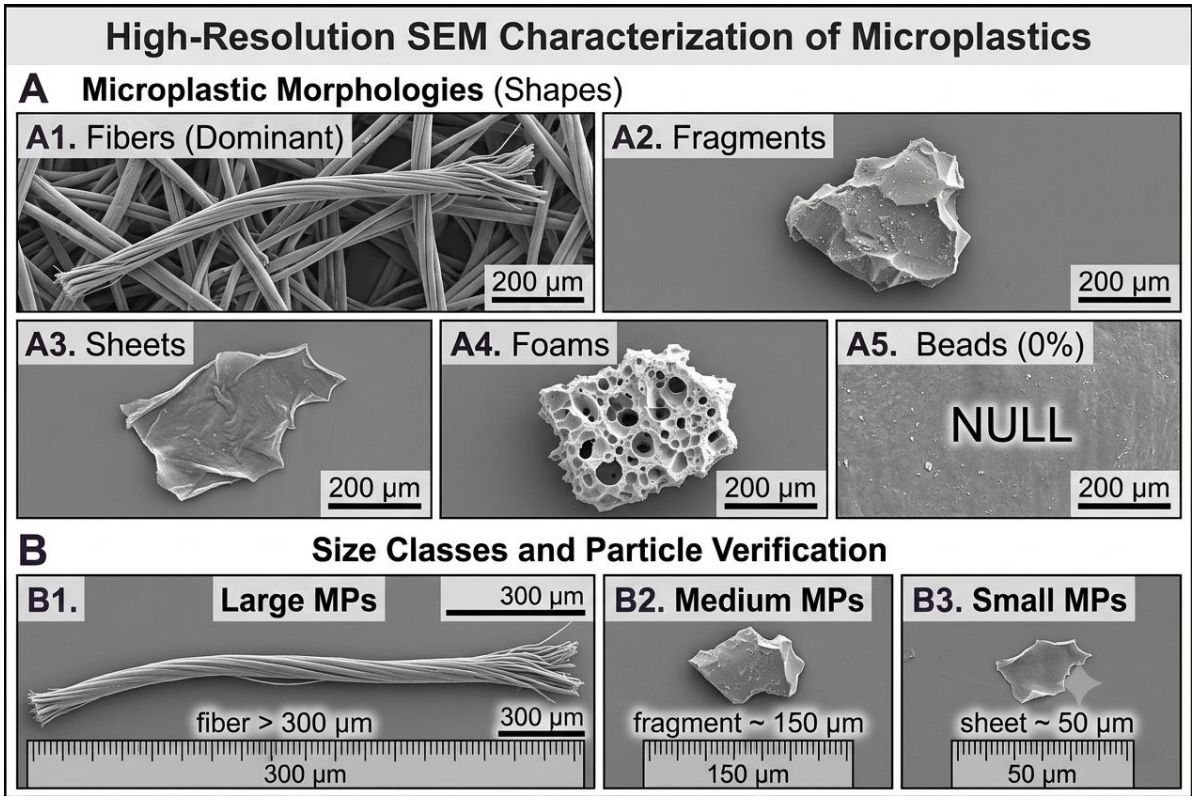


Figure 7: Morphology and size-class characterisation of microplastic (MP) particles extracted from sample matrices by scanning electron microscopy (SEM). (A) Morphological shapes: (A1) Fibers (dominant; 200 µm); (A2) Fragments (200 µm); (A3) Sheets (200 µm); (A4) Foams (200 µm); (A5) Beads (absent; 0%). (B) Size classes: (B1) Large MPs (~300 µm); (B2) Medium MPs (~150 µm); (B3) Small MPs (~50 µm). Scale bars are shown in each of the panes.

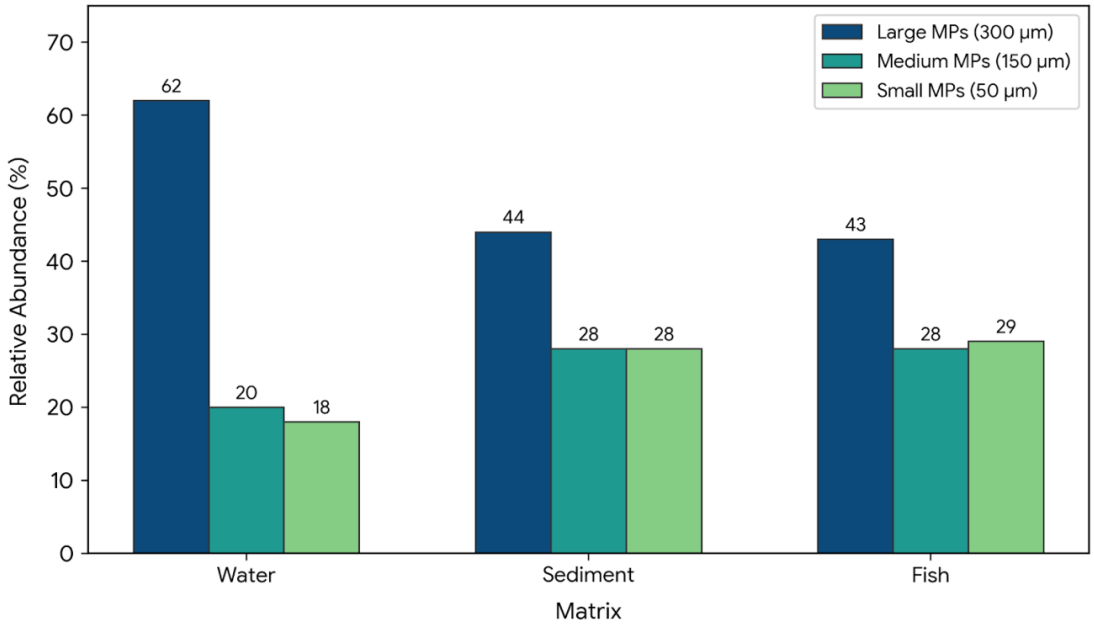


Figure 8: Relative abundance of microplastic size classes across environmental matrices.

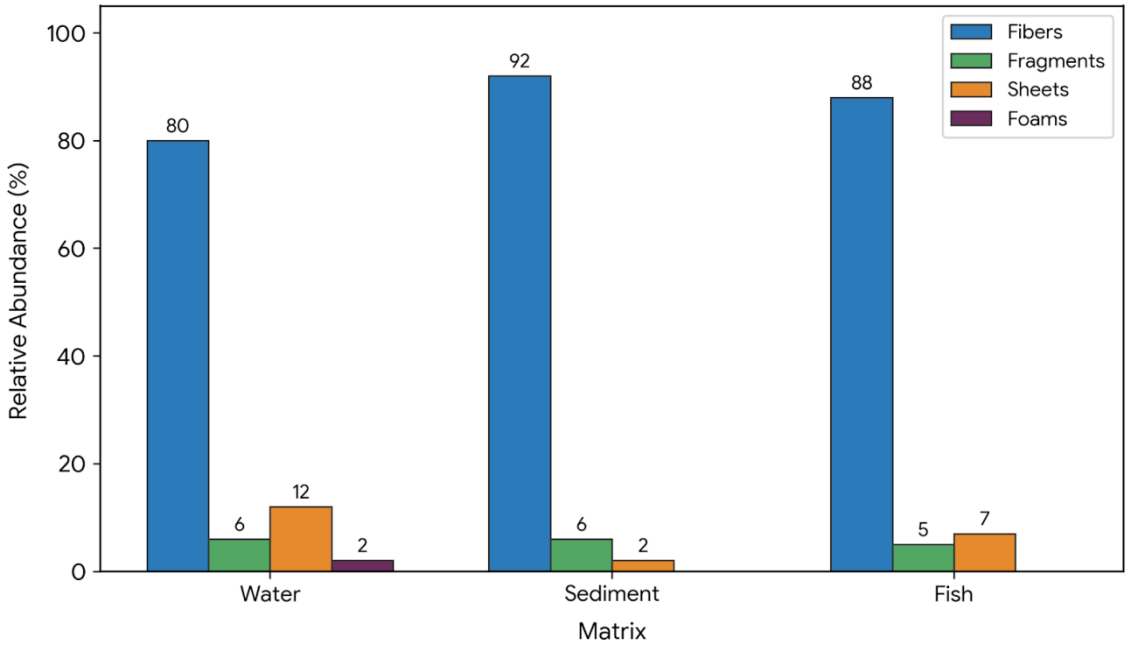


Figure 9: Relative abundance of microplastic morphological shapes across environmental matrices (water, sediment, and fish sample tissue).

4. Discussion

Based on the findings of this study, it is clear that polyethylene microplastics have a dose- and time-dependent genotoxic effect on erythrocytes of Barilius vagra. As exposure concentration increased from 0 to 50 mg/L, the frequency of micronuclei formation escalated from 4.8–15.8% to 20.4–24.0% at 7 days, and from 7.2–19.6% to 23.6–27.4% at 14 days. The results are consistent with the previous study that showed exposure to microplastics can cause DNA damage and chromosomal abnormalities in aquatic organisms; Menezes et al., 2024; Tang et al., 2025). The baseline frequencies of MN observed in fish samples from different sampling sites in Barilius vagra showed clear increase in upstream to downstream direction and fish sampled at S9 had MN frequency more than three times higher (15.8%) than S1 (4.8%). This spatial gradient is similar to the one observed in the Sardaryab stretch where the concentration of microplastics in the sediment ranged from 210 ± 45 MPs/kg in S1 to 610 ± 140 MPs/kg in S9. As baseline DNA damage was highly correlated with environmental microplastic load, B. vagra is a suitable bioindicator of microplastic pollution in freshwater bodies. The results agree with Menezes et al. (2024), which indicated that there is a high correlation between environmental contamination and genotoxic effects of microplastics in freshwater fish.

Several mechanisms may be responsible for the Genotoxicity seen. Microplastics induce the production of reactive oxygen species (ROS) and cause oxidative stress, which may result in DNA damage (Liu et al., 2025a). Besides, the direct contact with microplastics can lead to mechanical damage of cell membranes and disruption of cellular functions, which also plays a role in genotoxic effects (de Souza Freire et al., 2023). Atamanalp et al (2023) showed that PE exposure in rainbow trout affected growth and possibly as a promoting agent in tissue-based oxidative response, DNA damage and apoptosis. Likewise, da Costa Araújo et al. (2022) found the mixture of emerging pollutants and polyethylene microplastics caused genotoxicity, mutagenicity and redox unbalance in the zebrafish.

Intriguingly, the baseline MN frequencies in fish from the downstream sites (S7–S9) were higher than in those from the upstream sites and the response to PE-MP exposure was greater. This indicates that chronic exposure to microplastics in the environment could sensitise fish to further genotoxic stress, for example by lowering their capacity to repair DNA or by building up in the fish body, causing an overall increase in oxidative damage. The results emphasize the need for a holistic approach to evaluating fish population vulnerability to microplastic genotoxicity by taking into account their environmental history. Earlier, Islamy et al. (2025) found that the wild herbivorous fishes collected from polluted river water had high levels of MP ingestion, genotoxicity, and histopathological changes in their gills.

The fibers were the main microplastic type in all the matrices (80-92%) indicating that the main source of micro-plastics in Sardaryab region may be pollution from textiles and domestic wastewater discharge. Fibers that are shed during the washing process can be released into wastewater treatment systems, and then into natural water bodies, where they become trapped in sediments and eaten by aquatic animals. This finding coincides with the worldwide trend of fibers being the most commonly reported microplastic type associated with their widespread use and persistence in the environment (Jian et al., 2020; Zhang et al., 2018). Liu et al. (2025a) discussed the impact of micro(nano)plastics on the cell and the necessity of comprehending the mechanism of microplastic-induced damage.

Liu et al. (2025b) also reported that the polyethylene microplastics causes stress response in aquatic organisms by its oxidative and genotoxic pathways.

In the present study, MN frequency increased with the dose of exposure, in line with the results of Sampsonidis et al. (2024) who found that aged PE-MPs caused more severe DNA damage than virgin PE-MPs, indicating that aging may increase the toxicity of microplastics. This serves as a reminder of the need to take into account the environmental history of microplastics when evaluating their risks. Moreover, Liu et al. (2025c) proved the stress response of polyethylene microplastics in organisms, thus expanding the evidence base of the ecotoxicological effects of these contaminants. Based on species-specific analysis, Barilius vagra is found to be moderately to highly vulnerable to microplastic ingestion because of its feeding habits and environment preference as benthopelagic omnivore.

Generally, bottom-feeding and omnivorous fish have higher concentrations of microplastics than pelagic fish species, which is attributed to the concentration of micro-plastics in sediments (Wang et al., 2020; Sun et al., 2020). The high rates of increase in the frequency of the MNs moving downstream reflect the cumulative effects of the urban development, agricultural runoff and recreational uses of the river corridor. The study by Zhang et al. (2025) showed that polystyrene MPs and nanoplastics (nps) caused chronic reproductive toxicity in male mice, underscoring the potential cross-taxonomic effects of MPs exposure.

The results of this study have important implications for ecological risk assessment and public health. Microplastics in fish species of commercial importance are of concern for exposure to humans due to consumption of fish. Microplastics can provide a surface for the buildup of pollutants such as heavy metals and persistent organic pollutants that can increase the toxicity of the microplastics (Barboza et al., 2018). In addition, the movement of microplastics from the gut to muscle tissues, which also are eaten by humans, is a possible pathway for human exposure. Al-Ghanim et al. (2023) evaluated the biochemical, genotoxic, and histological effects of freshwater fish exposed to polypropylene microplastics and urged for thorough ecotoxicological evaluation. Spatial gradient of MPs concentration is similar to the hydrological and land-use attributes of the Sardaryab region. Upstream areas are relatively pristine, while downstream areas are constantly filled with urban effluents, agricultural canals and recreational wastes. The mixing zone (S7) and the far downstream sites (S8–S9) are depositional environments where lower turbulence and sediment interaction cause MPs to settle as described by Besseling et al. (2017) in their modelling of MPs fate in freshwater. The abundance of fibers and fragments found in the sediments of similar river (Peng et al., 2018; Zhang et al., 2019) indicates that textiles, packaging and fishing gear are likely the main sources. Similar to the Citarum River in Indonesia (Sembiring et al., 2020), seasonal low-flows further contribute to the retention of MPs in bottom sediments. The mean concentration (281.8 ± 11 MPs/kg) in the Sardaryab is lower than in Poyang Lake, China (1936 ± 121 MPs/kg; Jian et al., 2020) which is a heavily urbanized system, but similar to Veeranam Lake, India (309 MPs/kg; Srinivasalu et al., 2020) and the Ottawa River, Canada (Vermaire et al., 2017). These ranges are similar to those found in freshwater sediments in Australia (Nan et al., 2020) and coastal rivers in South Africa (Nel and Froneman, 2015) at the global level. The gradual increase downstream re-emphasises the importance of urban effluents, tourism and agricultural run-off as sources of MPs contamination.

5. Conclusion

This study offers all the evidence that polyethylene microplastic causes DNA damage in the erythrocytes of Barilius vagra in a dose and time dependent fashion. After 14 days of exposure, 50 mg/L showed the

highest genotoxic effects, including a frequency of MN of 27.4%. The baseline MN frequencies in the upstream and downstream populations of the wild Kabul River revealed a clear trend with an increase in the downstream population, indicating the cumulative effect of microplastic pollution along the upstream to downstream stretch of the river. The mean levels of microplastic in water, sediments and fish were 196.4 ± 88 MPs/m³, 281.8 ± 11 MPs/kg and 12.5 ± 8.02 MPs/individual, respectively, with the highest concentration found in the sediments.

The textile-derived pollution, domestic wastewater and fishing gear are among the major sources of contamination in the region as the fibers were found to be the dominant morphological type (80-92% of all matrices). Large-sized microplastics (≈300 μm) dominated the results, with especially high fractions of these particles in the water samples (62%), pointing to recent sources of input and low fragmentation levels. Analysis of the species showed that Barilius vagra has moderate to high vulnerability to ingesting microplastic, with fish from downstream sites of contamination having elevated baseline DNA damage requiring species-specific monitoring strategies.

The present results highlight the ecological threats from the presence of microplastics in freshwater environments and the need for public awareness, enhanced waste management, ongoing monitoring of aquatic systems, and source-specific mitigation measures to protect the integrity of aquatic ecosystems and public health in the region. Based on the results of the detection of microplastics in commercially important species, concerns should be raised regarding the possible human dietary exposure through fish consumption, and thus need for the development of dietary exposure assessment and implementation of pollution control measures in the Kabul River basin.

6. Authors' Contributions

Designed the study, performed experiments, analyzed data, and drafted the manuscript: **ZK**. Assisted in data collection, laboratory work, and manuscript revision: **SF, SN, ST, MA, MM, AM, AAK**. Supervised the research, reviewed the manuscript, and provided critical feedback: **AAK**. All authors read and approved the final manuscript..

7. Acknowledgment

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