International Journal of Design & Nature and Ecodynamics
Vol. 21, No. 4, April, 2026, pp. 1015-1028
Journal homepage: http://iieta.org/journals/ijdne

Histopathological Damage and Mortality in Rainbow Trout Exposed to Microplastics from Disused Tires

Alexander Benjamín Paucar-Villanueva[1], Ana María Guzmán-Neyra[1], Francisco Carlos Prosopio-Carranza[1], Ingrid Marisol Roldán-Díaz[1], Nora R. Malca-Casavilca[2], Silvia Iglesias-León[2], Jorge L. Lopez-Bulnes[2], Gina Paola Cubillas-Terreros[2], Luis M. Soto-Juscamayta[2*]

[1] Faculty of Biological and Agricultural Sciences, Universidad Nacional San Agustín de Arequipa, Arequipa 04001, Peru
[2] Faculty of Geological, Mining, Metallurgical and Geographic Engineering, Universidad Nacional Mayor de San Marcos, Lima 07011, Peru

Corresponding Author Email: lsotoj@unmsm.edu.pe

https://doi.org/10.18280/ijdne.210409

Received: 8 August 2025
Revised: 14 November 2025
Accepted: 2 March 2026
Available online: 30 April 2026

Keywords:
acute toxicity, tires, rainbow trout, bioassays, histopathology, microplastics

ABSTRACT

This study evaluated the acute toxicity of end-of-life tire fragments (EDT) in juvenile *Oncorhynchus mykiss*, analyzing the relationship between digestive bioaccumulation, histopathological damage, and mortality. A completely randomized design was conducted following OECD Guideline 203, exposing 105 fish to five concentrations (0, 6.25, 12.5, 25, and 50 mg/L) for 96 h. Digestive density and particle count were used as indicators of bioaccumulation, reflecting the retention of tire-derived particles in the digestive tract and enabling the association between ingestion and toxic effects. The highest particle accumulation was recorded at 6.25 mg/L (1912 particles), which is attributed to stress-induced reductions in feeding activity at higher concentrations, limiting particle ingestion. Mortality showed a clear concentration–response pattern, reaching a maximum of 71.43% at 50 mg/L after 96 h, confirming acute toxicity. Histopathological analysis revealed sinusoidal congestion and cytoplasmic vacuolization, progressing to hepatic necrosis at concentrations ≥12.5 mg/L. These results demonstrate that exposure to EDT induces digestive bioaccumulation and dose-dependent liver damage, even at low concentrations, representing an underestimated ecotoxicological risk for freshwater fish species.

1. INTRODUCTION

Rubber pollution is one of the fastest-growing environmental concerns today, due to the proliferation of products made with this synthetic material, which is present not only in the automotive industry, but also in sectors such as footwear, construction, and the manufacturing of industrial products [1, 2]. Synthetic rubber, highly durable and resistant, presents a great advantage in terms of functionality, but, at the same time, poses a serious environmental challenge, since its decomposition is extremely slow [3-5]. Tires, as one of the most widely used rubber products, contribute significantly to the generation of solid waste, known as end-of-life tires (EDT), which have become a major source of pollution [6, 7].

Each year, global production of end-of-life tires exceeds 1.5 billion units, and more than 3.5 million tons of discarded tires are generated in Europe alone, reflecting the global extent of the problem [8-10]. Globally, end-of-life tires not only occupy vast spaces in landfills but also contribute to the release of microplastics into the environment, one of the most persistent and difficult-to-manage forms of pollution [11, 12]. The lack of efficient solutions for the proper recycling of these materials has increased pollution in both terrestrial and aquatic bodies [13, 14].

End-of-life tires, when exposed to environmental conditions such as friction wear, temperature variations, and rain, release small fragments that break down into microplastics [15]. These fragments are easily transported by water to rivers, lakes, and oceans, where they can be ingested by aquatic organisms, seriously altering the health of species and aquatic ecosystems [16]. Microplastics derived from tires contain not only synthetic rubber but also dangerous chemical compounds such as mineral oils, which, when released into water, aggravate the pollution of water bodies [17-19].

Synthetic rubber released from end-of-life tires not only physically affects aquatic organisms when ingested but also carries a series of harmful chemical compounds that increase the toxicity of water. These compounds include heavy metals, additives, and other substances derived from tire manufacturing processes. These contaminants affect aquatic species at the cellular level, causing negative effects such as digestive obstructions, endocrine disruptions, and, in more severe cases, the death of exposed organisms [20, 21].

The impact of microplastics on aquatic ecosystems has gained great relevance in the scientific community in recent years [22].

These pollutants, largely derived from the wear and tear of end-of-life tires, not only affect the aquatic species that ingest

them but also alter the physical and chemical properties of aquatic ecosystems. The accumulation of microplastics in sediments and aquatic organisms generates changes in key parameters such as pH and water quality, disrupting the homeostasis of aquatic ecosystems [23, 24]. In the long term, the presence of these fragments can contribute to the biomagnification of contaminants through the food chain, directly or indirectly affecting species that consume these microplastics. Although the full effects of this contamination are not yet fully understood, scientific evidence indicates that microplastics pose a significant threat to aquatic biodiversity, generating negative impacts on the health of organisms and the functionality of aquatic ecosystems [25-27]. This is shown by the study presented by the study [28] on the toxicity of tire wear-derived pollutants, particularly 6PPD-quinone and nanoplastics (PS-NPs), in fish such as zebrafish. This study showed that exposure to 6PPD-quinone, both alone and in combination with PS-NPs, significantly altered fish locomotor behavior, increasing traveled distance, speed, and time spent in specific areas of the tank, indicating a neurological impact. The results suggest that the effects of combined exposure to 6PPD-quinone and PS-NPs are more intense than when the pollutants are administered separately. However, no significant damage was observed in liver or intestinal tissues, indicating that while the effects are noticeable at the behavioral level, severe morphological damage is not present in the short term. Furthermore, transcriptomic analysis revealed alterations in genes related to lipid and cholesterol metabolism, which could affect cellular integrity and mitochondrial function. This gap in knowledge about the interactions between contaminants highlights the need for more in-depth research, such as that potentially conducted with rainbow trout, to determine the long-term impacts on the health of aquatic ecosystems.

The study [29], which investigated microplastic (MP) contamination in commercial tilapia feeds in Bangladesh, highlighted the presence of MPs in all samples analyzed, with grower feeds showing the highest contamination (2150 ± 70.71 MPs/kg). Fibers were the predominant morphotype (85%), and the most common polymers were polypropylene (38.74%) and polyethylene (33.61%). The polymer hazard index (PHI) was high, especially due to polyvinyl chloride (PVC), which poses a significant health risk to tilapia and human consumers. MPs mainly originate from feed ingredients, packaging materials, and wear and tear on processing machinery. Although the pollution loading index (PLI) values were low, indicating minor contamination, the results show that MP contamination remains a risk in aquaculture. The study suggests reducing the use of single-use plastics in animal feed production and conducting further studies to assess the toxicological effects of MPs, especially in different geographic regions. It also calls for greater regulation and oversight of commercial animal feed production to mitigate this problem.

Likewise, there is the study [30] analyze microplastic pollution in rivers and its interaction with freshwater fish, highlighting the spatial and temporal dynamics of these contaminants. The study describes how microplastic input sources affect their concentration and composition in rivers, influencing their distribution throughout the river system. Microplastics interact with fish primarily through the ingestion of particles that are mistaken for food. Furthermore, the article analyzes the physical and toxic effects of microplastics on fish species, with special attention to environmentally relevant concentrations. The results suggest that microplastics can accumulate in fish, affecting their physiology and behavior, and that benthic species are especially susceptible due to the high concentration of microplastics in riverbed sediments. However, it was observed that the behavior of microplastics varies according to their size, density, and polymer type, which affects their retention and translocation in aquatic organisms. Despite the impacts found, the authors suggest that pollutant management could be improved through better monitoring and control of microplastic sources in river ecosystems, which would allow for more accurate data on their influence on aquatic species and ecosystem health.

To understand the specific toxicological effects of end-of-life tire fragments (EDT) in aquatic ecosystems, toxicological bioassays play a fundamental role. These experimental assays allow the toxicity of microplastics released into water to be assessed by observing parameters such as mortality, behavior, and histological changes in exposed organisms. Bioassays are essential for obtaining quantitative and qualitative data on the effects of microplastic pollution, facilitating the identification of toxicity levels and understanding the mechanisms of damage to exposed species [31-33]. In this context, the rainbow trout (*Oncorhynchus mykiss*) has been used as an experimental model in various investigations due to its high sensitivity to aquatic pollutants, its ability to bioaccumulate toxic substances and its relevance in both scientific research and aquaculture [34].

Rainbow trout is widely recognized as a model organism in ecotoxicological studies due to its biological characteristics, which make it especially sensitive to aquatic pollutants. This species has demonstrated a remarkable capacity to bioaccumulate contaminants, making it an ideal tool for assessing the toxicity of various substances, including microplastics derived from end-of-life tires [35, 36]. Furthermore, rainbow trout are of considerable economic importance to the aquaculture industry, reinforcing their relevance for studies seeking to assess the environmental impacts of pollutants. As a species commonly used in bioassays, rainbow trout allows for the simulation of the impact of microplastics on aquatic species in general, providing data that can be extrapolated to other species that may be exposed to similar pollutants [37].

To evaluate the acute toxic effects of EDT in juvenile *Oncorhynchus mykiss*, considering mortality, digestive bioaccumulation, and hepatic histopathological damage during 96 hours of exposure. The methodology will include fish mortality analysis and histological evaluation of internal organs to determine the median lethal dose (LD50), as well as other harmful effects caused by exposure. This approach will allow for an accurate measurement of the toxic effects of microplastics and provide relevant data for establishing safe exposure limits for aquatic organisms, in order to prevent negative impacts on the health of species and the stability of aquatic ecosystems.

2. MATERIALS AND METHODS

This study proposed an environmentally responsible methodology to assess the acute toxicity and histopathological effects of EDT on rainbow trout (*Oncorhynchus mykiss*) fingerlings, using a structured experimental protocol under controlled laboratory conditions. This methodology not only

allows for an understanding of the ecotoxicological impact of microplastic particles but also contributes to the development of biological risk assessment techniques for native aquatic organisms.

The methodology adopted was structured into four key experimental phases, as shown in Figure 1.

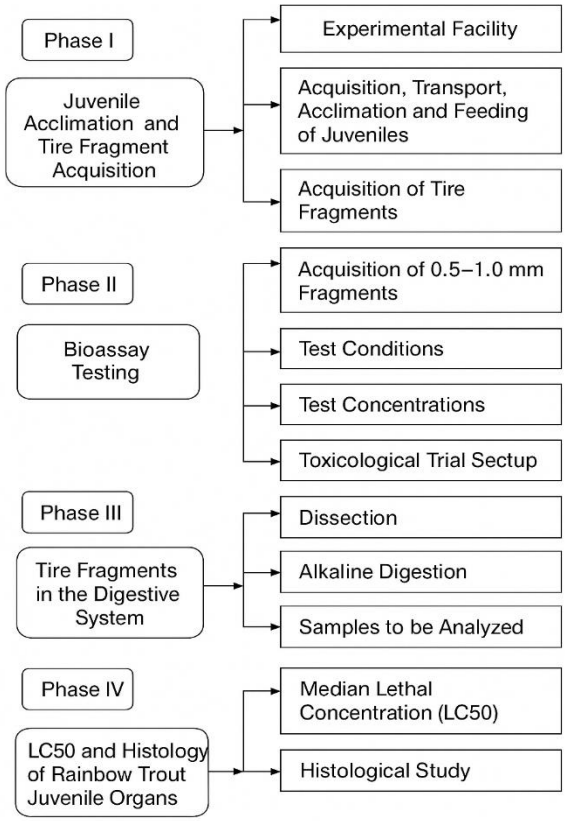

Figure 1. Phases of the investigation

2.1 Phase I: Experimental enclosure, acclimatization of organisms, and preparation of fragments

The study was conducted in the laboratory of the Professional School of Fisheries Engineering at the National University of San Agustín de Arequipa (UNSA), which is equipped with areas designated for fish maintenance, solution preparation, physicochemical measurements, biometry, sieving, microscopy, and cleaning.

For the experimental phase, fifteen 20 L plastic aquaria were arranged on shelving units as exposure tanks. Acclimatization was performed in a 500 L rectangular tank equipped with a submersible pump and continuous aeration using porous stone diffusers and silicone tubing, ensuring adequate dissolved oxygen levels and gentle water movement. The water used was dechlorinated and pre-aerated, following the CETESB L5.306 (2015) protocol.

Rainbow trout fingerlings (*Oncorhynchus mykiss*) were obtained from the Titire fish farm (Moquegua). Transportation was conducted in 200 L containers with oxygenated polyethylene bags for approximately 4 hours, ensuring the physiological stability of the organisms.

Acclimatization lasted 25 days, with weekly biometric monitoring (weight and length), maintaining the conditions recommended for the species according to OECD 203 (2019): temperature > 14 °C, dissolved oxygen > 6 mg/L, and pH between 6.5 and 7.5 (Table 1). The fish were fed commercial pellets (1–2 mm) twice daily, and feeding was suspended 48 hours prior to the start of the bioassays to minimize metabolic interference.

Table 1. Water control parameters during fry acclimatization

Parameter	Reference Value
Temperature	> 14 °C
Dissolved oxygen	> 6 mg/L
pH	6.5 – 7.5 units

The EDT was obtained from an authorized waste management company. The material was washed with distilled water, dried at 40 °C for 24 h, and subsequently sieved in cascade using 2.00 mm, 1.50 mm, and 1.00 mm metal mesh screens, obtaining an operational particle size fraction of 1.00–1.50 mm, homogeneous and suitable for toxicological testing. The selected fraction was stored in airtight containers, protected from light and moisture.

The EDT fragments used in the study were chemically characterized before the start of the bioassays, with the aim of identifying the main potentially toxic compounds associated with the solid material. As detailed in Table 2, the analysis included the detection of polycyclic aromatic hydrocarbons (PAHs), heavy metals, and organic additives using gas chromatography-mass spectrometry (GC-MS) and inductively coupled plasma mass spectrometry (ICP-MS). This characterization allowed for contextualizing the toxicological effects observed subsequently, without direct measurements of these compounds being performed in the aqueous exposure medium during the tests.

Table 2. Chemical characterization of end-of-life tire fragments (EDTs) and associated toxicological risks

Component	Analytical Method	Concentration Found	Potential Toxic Effects
Polycyclic Aromatic Hydrocarbons (PAHs)	GC-MS	5–10 µg/L	Carcinogenicity, mutagenesis, and neurotoxic effects in aquatic organisms
Benzothiazole	GC-MS	1–5 µg/L	Endocrine disruption, cellular damage
Lead (Pb)	ICP-MS	0.5–1 mg/kg	Hepatic toxicity, kidney damage, and neurotoxic effects
Cadmium (Cd)	ICP-MS	0.1–0.5 mg/kg	Renal damage, effects on the nervous system
Zinc (Zn)	ICP-MS	10–20 mg/kg	Hepatic damage, metabolic alterations
Phthalates (Plasticizers)	GC-MS	1–3 µg/L	Endocrine toxicity, reproductive alterations
Microplastics (Rubber Fragments)	Microscopic analysis	1000–2000 particles at 6.25 mg/L	Intestinal obstruction, stress, and alterations in digestion
Volatile Organic Compounds (VOCs)	GC-MS	2–4 µg/L	Cellular damage, alteration of the immune and digestive systems

Table 2 presents the chemical characterization of EDT and the associated toxicological risks. PAHs, heavy metals such as lead, cadmium, zinc, phthalates, and microplastics were detected, all known for their high toxicity to aquatic organisms. PAHs and heavy metals can cause liver, kidney, and neurological damage, while phthalates disrupt the endocrine system. Microplastics can cause intestinal blockages and disrupt digestion. These results suggest that tire fragments are a significant source of aquatic pollution, with harmful effects on exposed aquatic fauna.

2.2 Phase II: Toxicological bioassays with NFU fragments

2.2.1 General test conditions

The bioassays were conducted following the OECD 203 guideline for acute toxicity testing in fish, maintaining the physicochemical parameters of the water within controlled ranges. Each treatment included a minimum of seven fish per concentration, with continuous monitoring of water conditions: dissolved oxygen > 60% saturation, stable temperature (variation ≤ ±1 °C), pH, and conductivity, under a 12 h light / 12 h dark photoperiod.

To ensure that the tire fragments remained suspended and that the concentrations of leached compounds stayed constant throughout the experiment, the exposure solution was renewed every 12 hours. Continuous aeration and gentle intermittent agitation were used to prevent sedimentation of the fragments and to distribute the leached compounds uniformly in the water. The total duration of the bioassay was 96 hours, with more frequent observations during the first 24 hours.

2.2.2 Selection of concentrations and preliminary tests

The final concentrations were established based on preliminary range-finding tests, considering previous reports on microplastic toxicity in fish as cited in the study [38, 39]. Five exposure levels were selected to represent a progressive environmental gradient, including a control:

- •C-T1 (0 mg/L – control).
- •C-T2 (6.25 mg/L).
- •C-T3 (12.5 mg/L).
- •C-T4 (25 mg/L).
- •C-T5 (50 mg/L).

The concentrations are expressed as the mass of suspended particles per volume (mg/L). To ensure a homogeneous suspension, the aquaria were maintained with continuous aeration and gentle intermittent agitation, preventing particle sedimentation.

During the bioassays, no direct chemical measurements of PAHs, heavy metals, or other additive concentrations were performed in the aqueous exposure medium. The experimental design focused on evaluating the toxicological effects resulting from the physical presence of EDT fragments and the potential release of leachable compounds under controlled laboratory conditions. However, a system of periodic renewal of the exposure medium was implemented to maintain stable physicochemical conditions and a consistent availability of any compounds released during the test period.

2.2.3 Experimental setup

The aquaria were arranged on a three-level metal rack, with random assignment of tanks to each treatment and replication in order to minimize positional bias. Three replicates were used per concentration, totaling 105 fish in the experiment. All tanks were labeled with internal identification codes and maintained under stable aeration conditions (Figure 2).

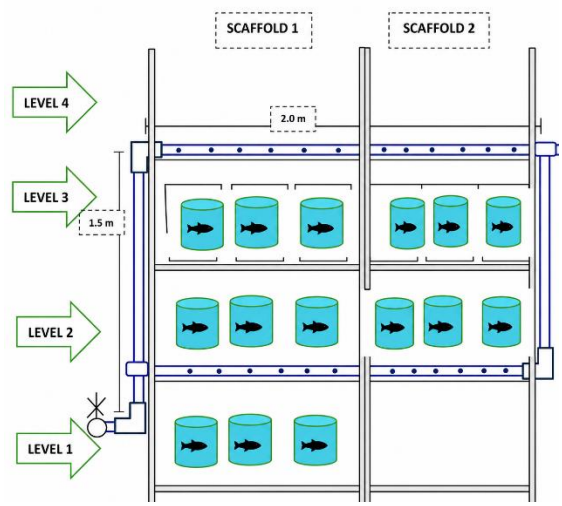

Figure 2. Setting up the fish tanks

2.2.4 Exposure and data recording

Fish are exposed to the defined concentrations for 96 hours. During this time, records are made every 12 hours, following the criteria established by OECD, which include sublethal and lethal parameters, such as:

- •Abnormal orientation (horizontal or vertical).
- •Loss of buoyancy control.
- •Changes in pigmentation.
- •Gulping behavior, isolation, or abnormal distribution in the water column.
- •Appearance of edema, hemorrhage, or aggressive behavior.

The observation process and arrangement of the experimental system are shown in Figure 3.

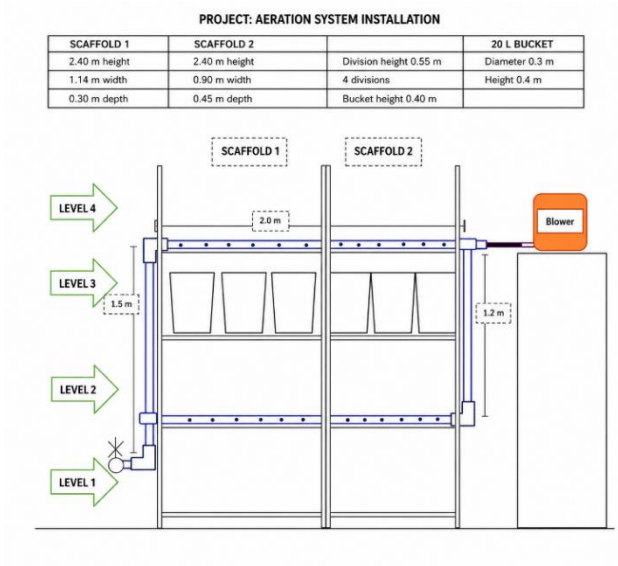

Figure 3. Installation schematic

2.3 Phase III: Analysis of the digestive tract and detection of NFU fragments

2.3.1 Dissection and removal of the digestive system

At the end of the exposure period, the fish were separated individually. Dissection was performed by making an incision from the mouth to the anus to extract the entire digestive

system (esophagus, stomach, and intestines). Each digestive system was weighed and then frozen in sealed plastic bags, following the standard laboratory procedure [38]. To determine the volume of the digestive system, a graduated cylinder filled with distilled water was used, measuring the liquid displacement generated by the introduction of the tissue and its contents. This procedure is illustrated in Figure 4.

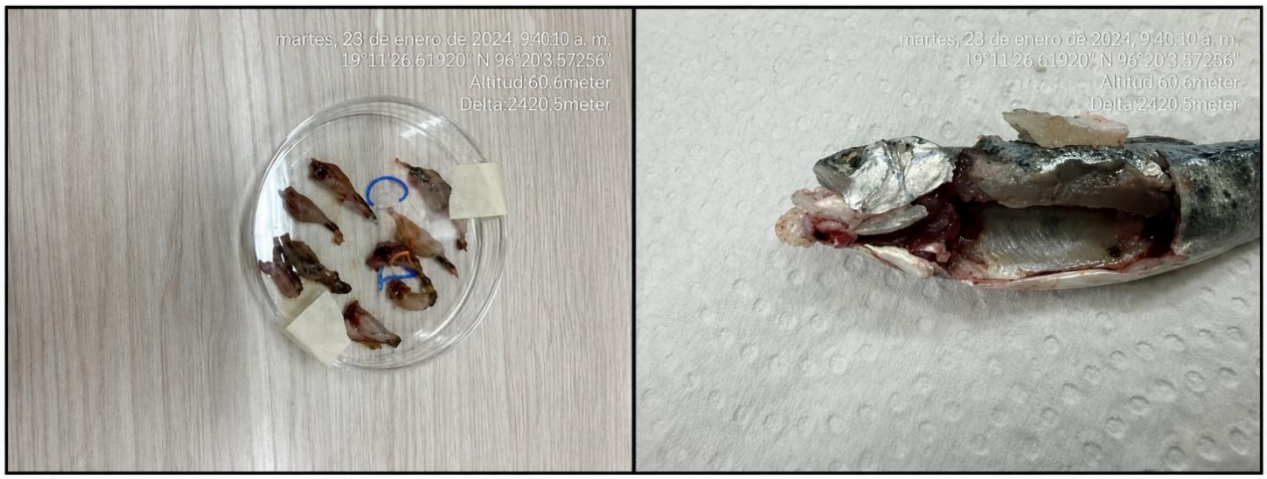

Figure 4. Illustration of trout dissection

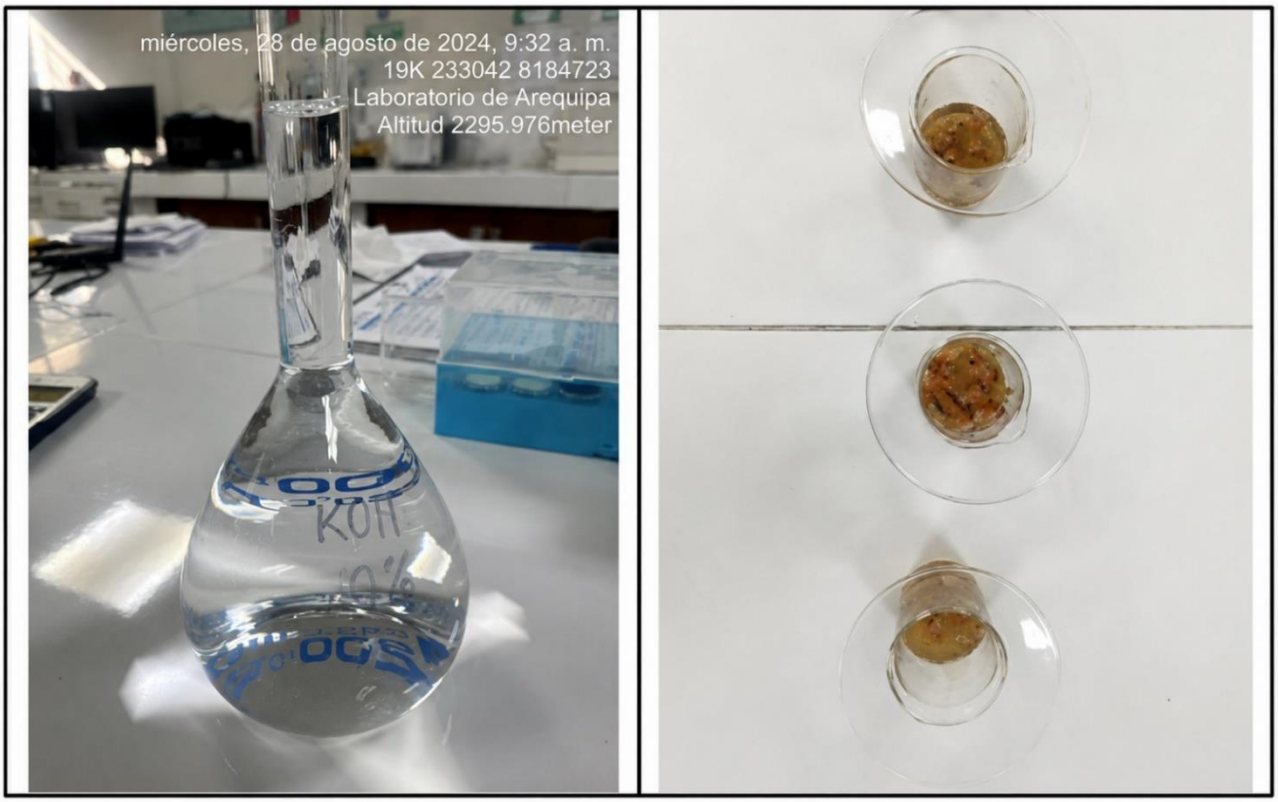

Figure 5. Alkaline digestion of the digestive systems

2.3.2 Alkaline digestion of tissues

Each digestive system was transferred to a 50 to 100 mL beaker, depending on its size, to which a 10% potassium hydroxide (KOH) solution, previously filtered through a 0.45 µm membrane, was added. The amount of reagent used was equivalent to up to three times the volume of the tissue, following the laboratory recommendations [40].

The beakers were covered with aluminum foil to prevent contamination and were left to stand for 3 to 4 days, allowing complete tissue digestion. This process is depicted in Figure 5.

2.3.3 Sample preparation and sieving

For microplastic retention analysis, the fish were previously sacrificed by overdosing with eugenol [41]. A subsample of 12 individuals (3 to 4 per replicate) from each treatment was selected. The resulting digestion solution was filtered using 1.5 mm and 0.5 mm metal sieves, sizes defined based on the range of concentration of number of tire fragments at the end of their useful life (NFU) used in this study. The material retained on the 0.5 mm sieve was collected with fine-tip forceps and transferred to Petri dishes, which were dried at 40 ℃ for 24 hours to facilitate observation.

2.3.4 Identification of NFU fragments

The fragments are examined under a stereoscope, following the criteria described by the study [40]. Only those fragments that do not have cellular or organic structures are considered microplastics. In cases where there is doubt about the nature of the fragment, the hot needle test described by the study [42], confirming the plastic composition when the particle deforms or melts upon contact with the hot metal. Finally, the identified fragments are counted for subsequent quantitative analysis.

2.4 Phase IV: statistical analysis and calculation of LC50-96h

To ensure the reliability of the acute bioassay, the acceptance criteria established by the OECD were applied. These criteria state that the test is valid if mortality in the control group is less than 10% during the 96 hours of exposure. This threshold was strictly monitored and met throughout the experimental process.

2.4.1 Data logging and variable control

During the trial, lethal and sublethal effects on exposed fish are systematically recorded, such as loss of balance, changes in swimming pattern, pigmentation, irregular ventilation, and other clinical signs detailed in OECD observations, which are made every 12 hours throughout the exposure period, ensuring traceability and consistency in data collection.

Records include:

•Number of live and dead fish per replicate and treatment.

•Clinical signs observed (according to OECD protocol).

•Physical and chemical conditions of the water: temperature, pH, dissolved oxygen, and conductivity.

2.4.2 Calculation of the median lethal concentration (LC50-96 h)

The median lethal concentration (LC50-96 h) is calculated using a probit-type statistical analysis, according to the model of the study [43]. This analysis is recommended by the OECD and widely used in toxicological studies. This analysis allows for estimating the concentration of NFU fragments that causes death in 50% of individuals exposed for 96 hours.

The following steps are used to achieve this:

Data coding: The responses (number of deaths per concentration) are organized into a matrix by treatment.

Probit transformation: The cumulative mortality proportion is converted into probit units.

Regression fitting: The relationship between concentrations (in logarithms) and probit responses is graphed, fitting a linear regression model.

LC50 estimation: The concentration value corresponding to probit point 5 (50% mortality) is determined, along with the 95% confidence interval.

This analysis is performed using IBM SPSS Statistics software, ensuring the accuracy of the results and compliance with international standards in aquatic toxicology.

3. RESULTS

3.1 Results, acclimatization, and preparation of fragments

Phase I constituted the methodological starting point for the study, ensuring controlled conditions for both the model organisms (*Oncorhynchus mykiss*) and the contaminants (disused tire fragments – DUT). A simulated aquatic environment was established on an experimental scale with homogeneous and monitored physicochemical conditions, which was essential to guarantee the reliability of the experiment. In parallel, the DUT fragments were processed and characterized morphologically and compositionally. These parameters are essential to ensure that subsequent toxicological tests are performed with homogeneous and comparable inputs, as shown in Table 3, Figure 6, Table 4, and Figure 7, respectively.

Furthermore, to understand the nature of the observed toxicity, a detailed chemical characterization of the tire fragments used in this study was performed.

Table 3 shows that the conditions of the experimental enclosure remained within the limits recommended by the OECD (2019), which validates the correct acclimatization process of the organisms. The temperature (12.3 °C) and dissolved oxygen (8.1 mg/L) guarantee an optimal environment for the model species, minimizing possible stress effects unrelated to exposure to the contaminant. Likewise, the distribution of the 180 fingerlings in six aquariums made it possible to maintain an adequate population density to avoid aggressive behavior or stress due to overcrowding, which strengthens the reproducibility and reliability of the bioassays.

Table 4 shows that the NFU fragments used in the study exhibit homogeneous physical and chemical characteristics. Their average size of 2 mm and angular shape ensure adequate contact surface area with the fish's digestive tract, maximizing physical interaction and potential toxicity. Negative buoyancy ensures that the fragments remain submerged, increasing the likelihood of ingestion by fish during exposure. Finally, the use of techniques such as standardized sieving and FTIR spectroscopy for characterization ensures that the material used in subsequent experimental phases is replicable and scientifically validated.

3.2 Results of toxicological bioassays with NFU fragments

During the 96-hour acute toxicological bioassays, juvenile trout (*Oncorhynchus mykiss*) were exposed to five concentration levels of EDT. The objective was to determine the evolution of cumulative mortality as a function of exposure time, following the OECD 203 protocol. Mortality observations were performed every 12 hours. As shown in Table 5, mortality was progressive in the treatments with the highest concentrations, while no deaths were recorded in the control group. The results are complemented by those illustrated in Figure 8, corresponding to the experimental system installation model.

In Table 5, C-T5 (50 mg/L) shows a mortality rate of 71.43% at 96 hours, which is consistent with the data and allows for the calculation of CL_{50}.

C-T4 (25 mg/L) shows a mortality rate of 28.57% at 96 hours, confirming that higher concentrations result in higher mortality.

C-T1 (0 mg/L) shows a mortality rate of 0%, confirming that there was no external interference.

In Table 6, the highest density was observed for C-T3 (1.0633 g/mL), while the lowest was for C-T5 (0.8189 g/mL). This suggests that treatments with intermediate concentrations could be associated with greater accumulation of biomass or digestive residues before filtration.

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intermediate concentrations could be associated with a greater accumulation of biomass or digestive waste before filtration.

Table 3. Conditioning of organisms and conditions of the experimental enclosure

Parameter	Average Value	Allowable Range (OECD)	Observation
Water temperature (°C)	12.3	12–15	Within range
Dissolved oxygen (mg/L)	8.1	>7	Adequate
pH	7.6	6.5–8.5	Optimal
Electrical conductivity (µS/cm)	238	<300	Within limits
Acclimatization time (days)	14	≥7	OECD recommendation met
Total number of fingerlings	180	—	Distributed across 6 aquariums

Table 4. Conditioning of organisms and conditions of the experimental enclosure

Property	Registered Value	Measurement Method
Average size (mm)	2	Standardized sieving
Dominant shape	Irregular angle	Visual microscopic inspection
Dominant composition	Rubber + fillers (carbon black)	FTIR
Buoyancy in water	Negative	Direct immersion test
Cleaning before use	Washed with distilled H_2O	Standardized procedure

Table 5. Cumulative mortality (%) by treatment

Concentration (mg/L)	Mortality at 12 h (%)	Mortality at 24 h (%)	Mortality at 36h (%)	Mortality at 48 h (%)	Mortality at 72 h (%)	Mortality at 96 h (%)
C-T1 (0 mg/L)	0%	0%	0%	0%	0%	0%
C-T2 (6.25 mg/L)	0%	0%	0%	0%	4.76%	4.76%
C-T3 (12.5 mg/L)	0%	0%	9.52%	9.52%	14.29%	23.81%
C-T4 (25 mg/L)	0%	0%	9.52%	14.29%	23.81%	28.57%
C-T5 (50 mg/L)	0%	4.76%	9.52%	19.05%	47.62%	71.43%

Table 6. Sample mass, volume, and density after digestion with 10% KOH

Sample	Weight (g)	Volume (mL)	Density (g/mL)
C-T1	4.4214	5	0.8843
C-T2	4.1115	4	1.0279
C-T3	4.253	4	1.0633
C-T4	5.0754	6	0.8459
C-T5	3.2757	4	0.8189

Table 7. Particle counts after digestion with 10% KOH

Sample	Particle Count
C-T2	1912
C-T3	1842
C-T4	765
C-T5	630

3.3 Results of digestive tract analysis and detection of NFU fragments

Following exposure of aquatic organisms to fragments of end-of-life tires (EDTs), a physiological and anatomical evaluation was performed to verify the presence, accumulation, and histological impact of these contaminants. This phase was structured in two key parts: (a) digestive analysis with alkaline digestion to quantify retained particles, and (b) histological analysis of the liver to assess the internal damage caused by the treatments.

As shown in Table 6, the digestion process allowed the samples to be reduced to an optimal state for sieving and microscopic analysis. Table 7 and Figure 9 then quantify the particles retained in the fish's digestive system, which is complemented by the series of Figures 10 to 14, which progressively demonstrate liver damage.

The results of the bioassay shown in Figure 15 demonstrate a clear progression of liver damage in rainbow trout as NFU increases. In the control group (0 mg/L), no liver damage was observed, with a score of 0, indicating healthy tissue. At 6.25 mg/L, the liver showed mild damage, with early signs of stress such as mild vascular congestion, resulting in a score of 1. At 12.5 mg/L, the damage became more pronounced, with the appearance of fat vacuoles and cell swelling, yielding a score of 2, indicating moderate liver damage. At 25 mg/L, severe liver dysfunction was observed, with greater congestion and altered hepatocytes, resulting in a score of 3. Finally, at 50 mg/L, the most severe damage occurred, with necrosis, loss of liver architecture, and significantly altered hepatocytes, reaching a score of 4. This dose-dependent increase in liver damage clearly reflects the toxicity of NFU fragments, with higher concentrations causing progressively more severe damage to liver tissue.

3.4 Results of statistical analysis and calculation of LC50-96 h

The toxicological evaluation of *Oncorhynchus mykiss* exposed to synthetic rubber fragments for 96 hours was based on statistical analysis of cumulative mortality by concentration. A completely randomized design was used with five treatments (0, 6.25, 12.5, 25, and 50 mg/L) and three replicates per concentration, recording data at 24, 48, 72, and 96 hours. Normality tests (Shapiro-Wilk), homogeneity of variance (Levene), and statistical significance (ANOVA or Kruskal-Wallis) were applied with an $\alpha = 0.05$ level.

As shown in Table 8, at 96 hours, the 25 mg/L and 50 mg/L treatments showed the highest mortality rates, at 28.57% and 71.43%, respectively. The 50 mg/L treatment exceeded the 50% threshold, allowing the LC50 to be calculated at 96 hours. The lowest lethal concentration observed (LOEC) was identified at 6.25 mg/L, the lowest concentration with an observable lethal effect, while the NOEC corresponds to the control group.

Figure 6. Acclimatization of fingerlings in the laboratory 1

Figure 7. Acclimatization of fingerlings in the laboratory 2

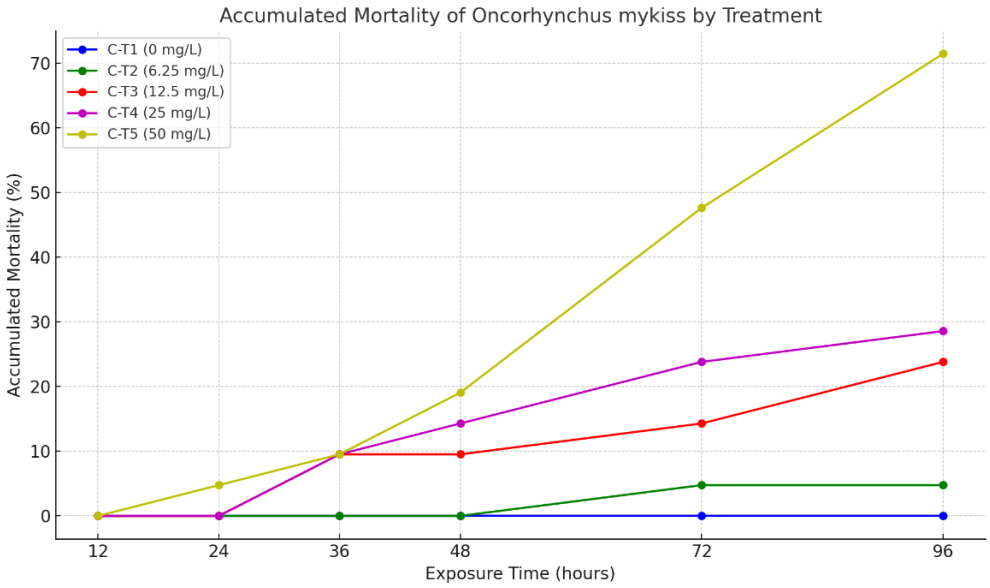

Figure 8. Percentage of cumulative mortality in juvenile trout by treatment

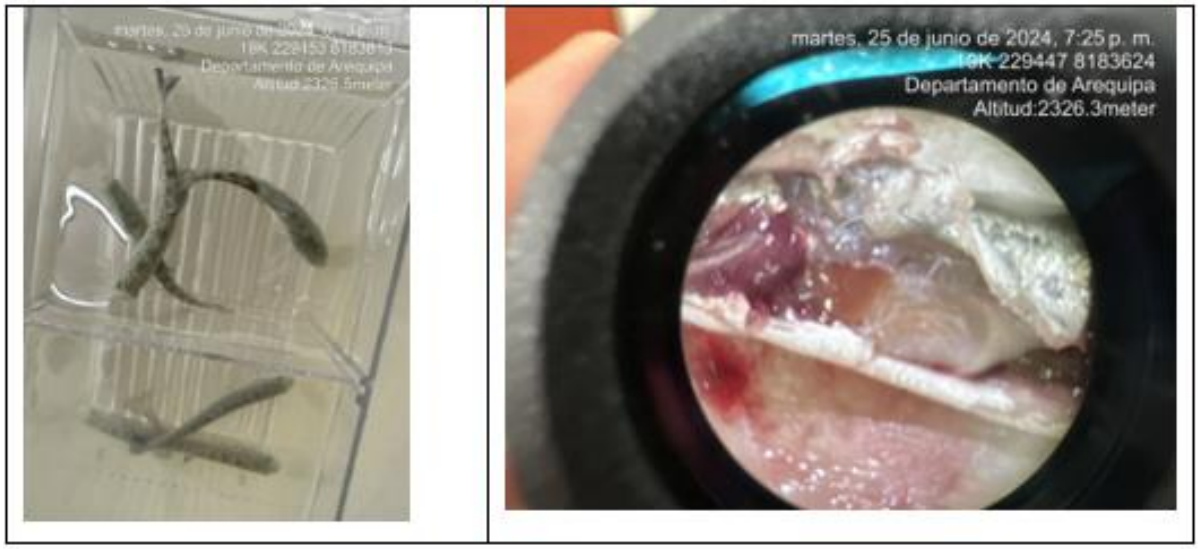

Figure 9. Fish digestion

Note: Post-digestion microscopic observation confirms the effectiveness of the chemical digestion method applied, achieving the dissolution of biological matter without degrading potential NFU particles. This ensures the validity of the subsequent analysis (sieving, identification, and counting), thus facilitating the accurate detection of pneumatic microfragments under controlled conditions.

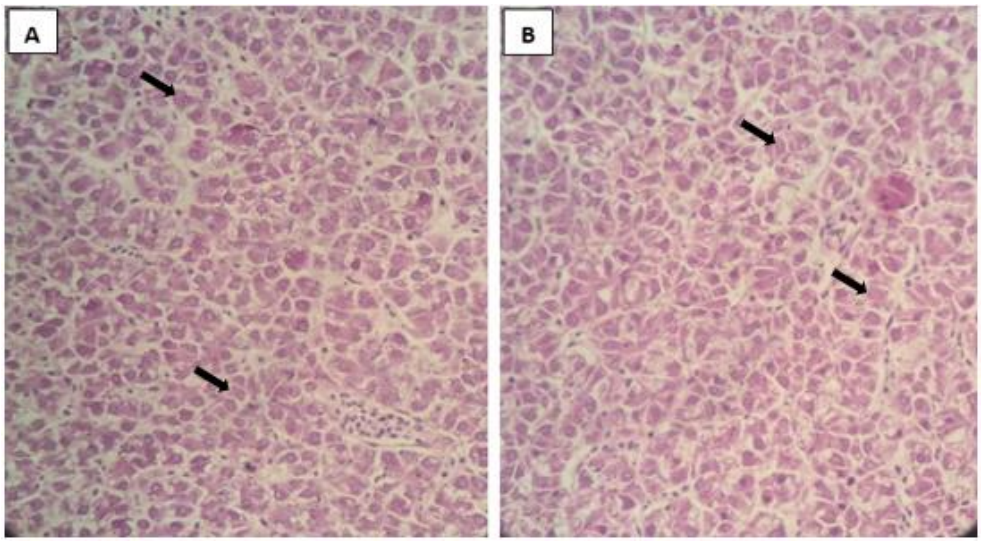

Figure 10. Histology of the control group (0 mg/L) at 400× magnification

The black arrows indicate well-preserved hepatocytes and normal hepatic tissue architecture.

Note: Healthy liver tissue with normal architecture is observed. Hepatocytes are well defined, and there are no signs of cellular degeneration, necrosis, hemorrhage, or vascular damage.

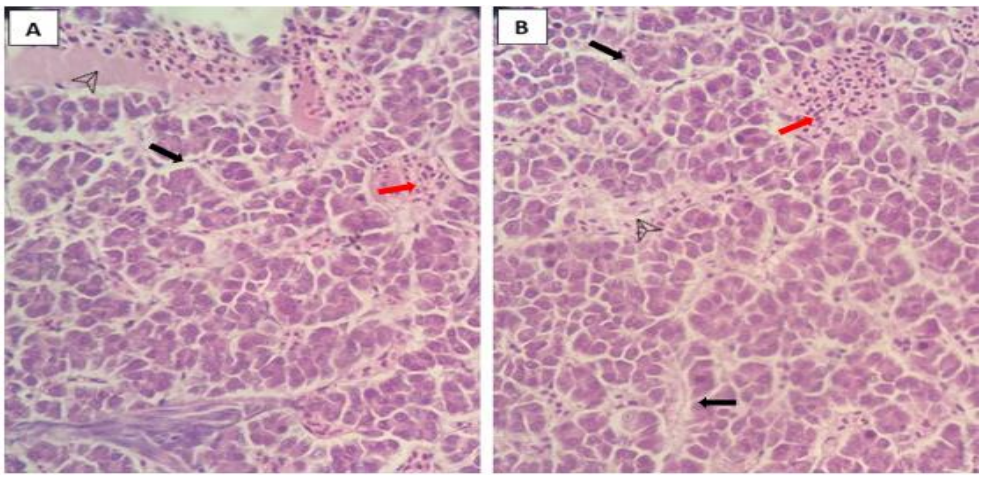

Figure 11. Light micrographs of liver tissue from the 6.25 mg/L treatment group at 400× magnification

Black arrows indicate sinusoidal dilation, red arrows indicate early vascular congestion, and open arrowheads indicate vacuolar alteration of hepatocytes.

Note: Sinusoidal dilation and early vascular congestion are evident, indicating an early liver response to toxic stress.

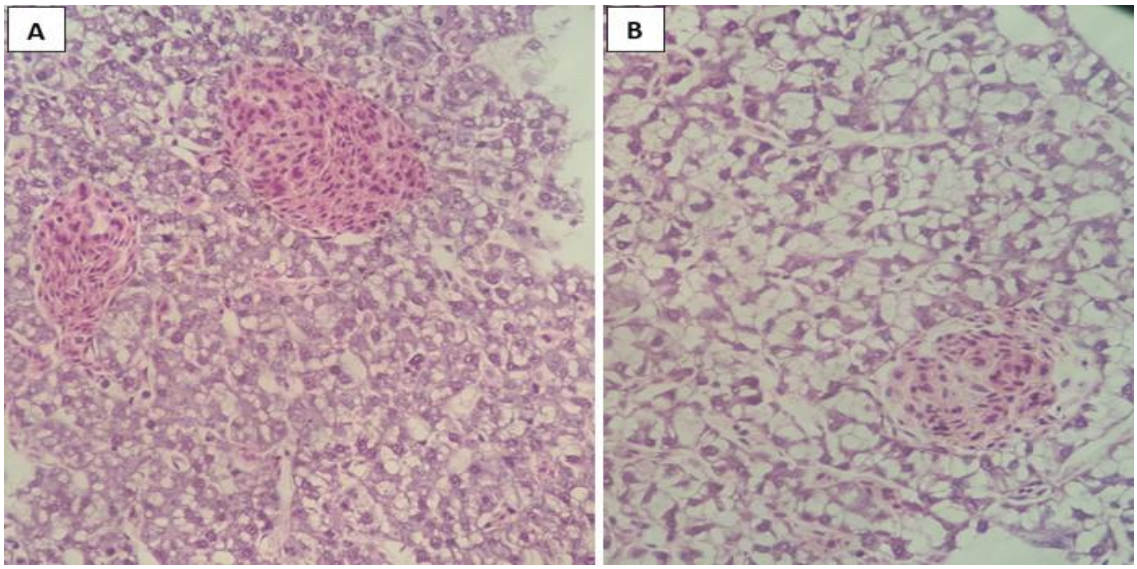

Figure 12. Light micrographs of liver tissue from the 12.5 mg/L treatment group at 400× magnification
Histological alterations include fat vacuoles, cell ballooning, and karyopyknosis, indicating sublethal liver damage after exposure to NFU.
Note: Fat vacuoles, cell ballooning, and karyopyknosis are observed, representing clear signs of sublethal hepatic damage induced by exposure to NFU.

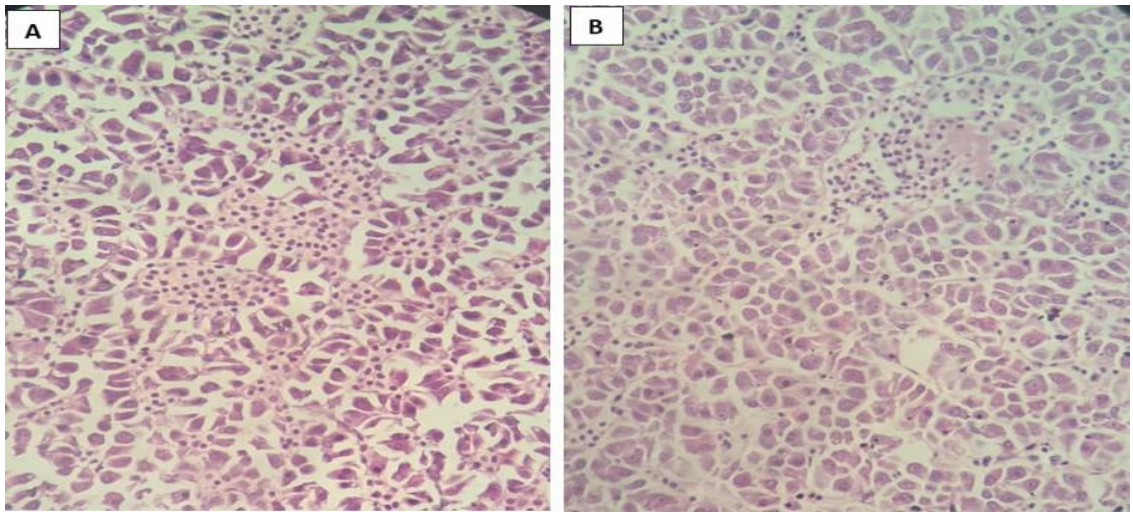

Figure 13. Light micrographs of liver tissue from the 25 mg/L treatment group at 400× magnification.
Note: More advanced structural damage is seen: greater sinusoidal dilation, intense congestion, and degenerating nuclei, suggesting progressive liver dysfunction.

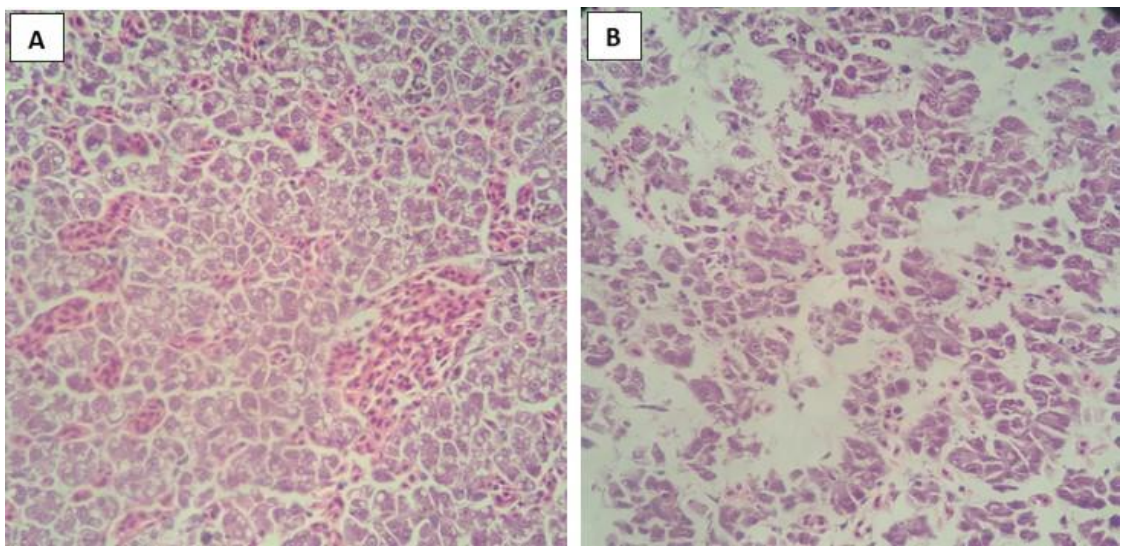

Figure 14. Light micrographs of liver tissue from the 50 mg/L treatment group at 400× magnification
Note: The tissue shows massive necrosis, loss of hepatic architecture, and severely altered hepatocytes. This is the most severe manifestation of toxicity observed.

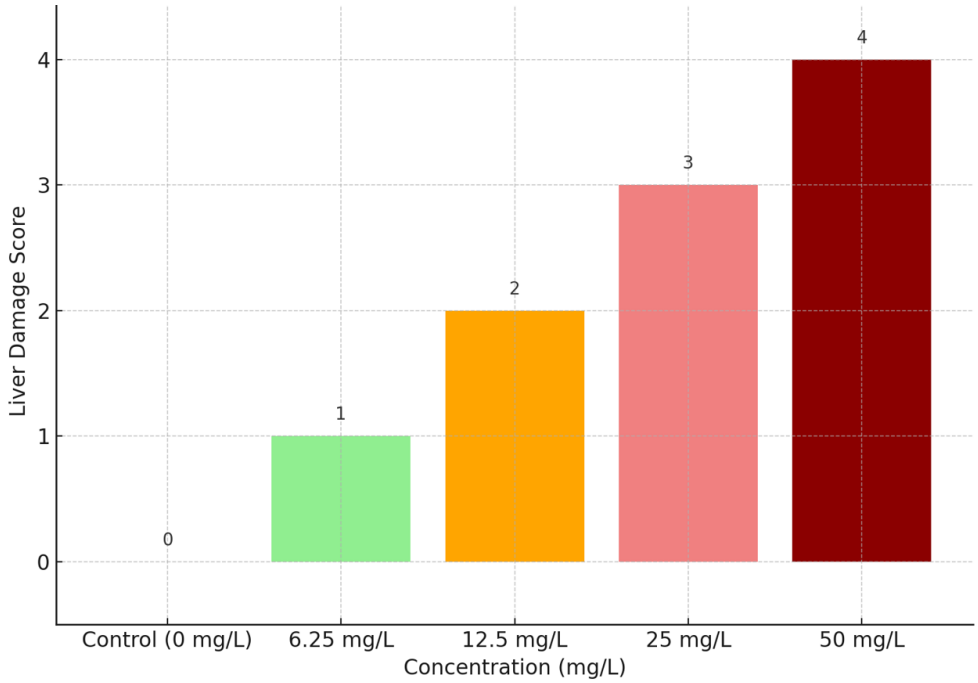

Figure 15. Semi-quantitative liver damage scores in rainbow trout

Table 8. Mortality of *Oncorhynchus mykiss* at different times of exposure, because of four treatments (mg/L) and a synthetic rubber control

Treatment (mg/L)	24 h Average ± SD (%)	Sig.	48 h Average ± SD (%))	Sig.	72 h Average ± SD (%)	Sig.	96 h Average ± SD (%)	Sig.
Control	00.00 ± 00.00	a	00.00 ± 00.00	a	00.00 ± 00.00	a	00.00 ± 00.00	a
6.25	00.00 ± 00.00	a	04.76 ± 8.24	a	04.76 ± 8.24	a	04.76 ± 8.24	ab
12.5	00.00 ± 00.00	a	04.76 ± 8.24	a	09.52 ± 8.24	a	23.81 ± 8.24	ab
25	00.00 ± 00.00	a	09.52 ± 16.49	a	14.28 ± 8.24	a	28.57 ± 8.24	b
50	00.00 ± 00.00	a	09.52 ± 16.49	a	19.04 ± 8.24	a	71.43 ± 14.29	b
No Observed Effect Concentration (NOEC)					50			
Lowest Observed Effect Concentration (LOEC)					<6.75			
DL50					ND			
S-W (Shapiro-Wilk)					0.51 (48 h), 0.78 (72 h), 0.82 (96 h)			
Levene					8.89 (48 h), 2.00 (72 h), 0.02 (96 h)			
p (Levene)					0.02, 0.17, 0.96, respectively			
K-W (Kruskal-Wallis)					2.35 (48 h), 7.62 (72 h), 10.52 (96 h)			
p (K-W)					0.67, 0.10, 0.04 respectively			

Note: Table 8 constitutes a fundamental matrix for the acute toxicological analysis of *Oncorhynchus mykiss* against progressive concentrations of synthetic rubber in suspension. From an ecotoxicological perspective, the data show a dose-dependent response with a cumulative temporal pattern. ND: Not Determined.

At 24 h, there was a complete absence of mortality in all treatments (0%), suggesting a lag phase in the compound's toxic action. No immediate lethal stress was observed, which may be attributed to a delay in hepatic biotransformation or interaction with target organs.

At 48 h, a transient sublethal response began in treatments ≥12.5 mg/L, with a mean mortality rate of up to 9.52 ± 16.49% (C-T4). However, the differences were not statistically significant ($p = 0.67$), which is consistent with interindividual heterogeneity and the initial physiological tolerance threshold.

At 72 h, an increasing pattern was detected, with 14.28% at 25 mg/L and 19.04% at 50 mg/L, but without exceeding the critical lethal threshold of 50%. Therefore, the LC50-96h value remains ND (Not Determined). Here, $p = 0.10$ indicates a significant developing trend, suggesting imminent cumulative cellular damage, especially in the liver, as confirmed by histological observations in later phases.

At 96 h, the C-T4 and C-T5 treatments recorded mortality rates of 28.57 ± 8.24% and 71.43 ± 14.29%, respectively, with confirmed statistical significance ($p = 0.04$, Kruskal-Wallis test). This confirms chronic sublethal toxicity associated with inflammatory processes, oxidative stress, and disruption of osmotic homeostasis.

LOEC (<6.75 mg/L): This implies that even concentrations close to the lowest threshold induce observable toxic effects. This is of great concern in environmental contexts where there is prolonged exposure to NFU microfragments.

NOEC (50 mg/L): This value, as it corresponds to the highest treatment, should be interpreted with caution, as the statistical significance at 96 h calls into question its actual validity in long-term studies. The use of the term "NOEC" here is only applicable to the first 72 h.

4. CONCLUSIONS

Acute exposure to synthetic rubber fragments caused Acute Acute exposure to synthetic rubber fragments induced clear toxic effects in *Oncorhynchus mykiss*, with a progressive concentration–response relationship observed from 6.25 mg/L

onwards. The highest exposure level (50 mg/L) resulted in a mortality rate of 71.43% at 96 hours, clearly exceeding the 50% threshold and allowing the estimation of the LC50-96h. This result unequivocally confirms the acute toxicity of EDTs in freshwater fish. Treatments at 25 mg/L and 50 mg/L produced pronounced lethal and sublethal effects, highlighting the ecotoxicological relevance of synthetic rubber microfragments in aquatic environments.

The lower number of particles recovered in the highest concentration group (50 mg/L), despite the elevated mortality, does not contradict the dose–response pattern but reflects alterations in fish behavior and particle dynamics within the aquatic medium. At higher concentrations, fish exhibited reduced swimming activity and feeding response, consistent with stress-induced feeding avoidance reported in acute microplastic exposure studies. In addition, particle aggregation and sedimentation at elevated concentrations are likely to reduce particle availability in the water column. Consequently, mortality at 50 mg/L appears to be driven not solely by ingestion, but by the combined effects of physiological stress, impaired respiration, and the increased release of leachable toxic compounds such as zinc and 6PPD derivatives, which intensify with concentration and exposure time. This mechanism explains why the 6.25 mg/L treatment showed the highest digestive accumulation (1912 particles) but substantially lower mortality.

Histopathological analysis of liver tissue revealed a clear dose-dependent progression of damage. While control organisms displayed preserved hepatic architecture, exposure to concentrations ≥12.5 mg/L resulted in cytoplasmic vacuolization, vascular congestion, and extensive necrosis at the highest exposure level, indicating acute hepatotoxicity. These findings confirm the liver as a primary target organ affected by exposure to synthetic rubber fragments.

Alkaline digestion and microscopic analysis confirmed the ingestion and digestive retention of tire-derived particles in treatments ranging from 6.25 to 50 mg/L, with particularly high accumulation at 6.25 and 12.5 mg/L. This demonstrates the bioavailability of these particles and their capacity to compromise gastrointestinal integrity, facilitating internal exposure to associated chemical additives.

The lowest observed effect concentration (LOEC) was identified at 6.25 mg/L, underscoring the ecological relevance of low-level exposure scenarios that may occur in freshwater systems affected by urban runoff and vehicular traffic. Overall, the evidence indicates that EDT represents a silent but tangible threat to freshwater and high-Andean aquatic ecosystems. *Oncorhynchus mykiss*, as a species of ecological and economic importance, proved to be a sensitive bioindicator, reinforcing the need for regulatory frameworks focused on the monitoring and management of tire-derived microplastic pollution.

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NOMENCLATURE

NFU	Concentration of number of tire fragments at the end of their useful life
C-T1, C-T2	Codes for experimental treatments with different concentrations of NFU
CL_{50}-96h	Median Lethal Concentration at 96 hours Concentration with no observable effect
KOH 10%	10% potassium hydroxide used for alkaline digestion