Short thesis for the degree of doctor of philosophy (PhD)

Bioaccumulation and toxic effects of anthropogenic contaminants in freshwater organisms

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Abbreviations

- **ALP** – Alkaline phosphatase
- **ALT** – Alanine aminotransferase
- **AST** – Aspartate aminotransferase
- **CAT** – Catalase
- **ChE** – Cholinesterase
- **DNA** – Deoxyribonucleic acid
- **EFSA** - European Food Safety Authority
- **GIT** - Gastrointestinal tract
- **GPx** – Glutathione peroxidase
- **GR** – Glutathione reductase
- **Hb** – Haemoglobin
- **IBR** - Integrated Biomarker Response
- **LC_{50}** - Median lethal concentration (50% mortality)
- **LDH** – Lactate dehydrogenase
- **MP** – Microplastic
- **MPI** – Metal pollution index
- **NC** – Nitrocellulose
- **NC-PT** - Nitrocellulose-based paint thinner
- **PBDEs** – Polybrominated diphenyl ethers
- **PCV** – Packed cell volume
- **PET** - Polyethylene terephthalate
- **PFAS** - Per- and polyfluoroalkyl substances
- **RBC** – Red blood cell count
- **ROS** - Reactive oxygen species
- **SCCPs** – Short-chain chlorinated paraffins
- **SEM** – Scanning electron microscopy (if used for MP morphology)
- **SOD** – Superoxide dismutase (include only if measured)
- **TI%** – Tail intensity percentage (comet assay)
- **WBC** – White blood cell count
- **µm** – Micrometre

1. INTRODUCTION

1.1. Introduction

Freshwater ecosystems sustain global biodiversity, underpin food security, support human livelihoods, and regulate essential biogeochemical cycles. Yet they are increasingly compromised by anthropogenic contamination arising from rapid urbanisation, industrialisation, intensive agriculture, mining activities, and inadequate waste management. Persistent and emerging pollutants, such as potentially toxic elements- PTEs (heavy metals and metalloids), polybrominated diphenyl ethers (PBDEs), short-chain chlorinated paraffins (SCCPs), volatile organic solvents (including nitrocellulose-based paint thinners- NC-PT), and microplastics (MPs), accumulate in water, sediments, and biota, resulting in bioaccumulation, biomagnification, and toxicity across trophic levels (United Nations, 2017; Bashir *et al*., 2020; European Commission, 2020). These contaminants disrupt biochemical pathways, physiological processes, behaviour, reproduction, and immune function in aquatic organisms, threatening population viability, ecosystem stability, and the services they provide, while simultaneously creating direct pathways for human exposure through consumption of contaminated fish and shellfish (Bukola *et al*., 2015; Egan *et al*., 2023).

Evaluating these impacts is inherently complex. Contaminants differ widely in chemical composition, bioavailability, toxicity, and interaction potential, while emerging pollutants such as MPs introduce additional challenges due to their heterogeneous size, shape, polymer composition, and role as vectors for adsorbed chemicals and additives (Kong *et al*., 2016; Amoatey & Baawain, 2019; Saad *et al*., 2022). Robust assessment, therefore, requires integrative approaches that combine field-based biomonitoring with controlled laboratory toxicity testing and sensitive biomarkers. Sentinel species play a pivotal role: filter-feeding mussels integrate ambient contamination over time, while fish reflect trophic-level exposure through direct ingestion and dietary transfer. Despite substantial global research, region-specific, multi-method studies remain scarce in Central and Eastern Europe, particularly in Bulgarian reservoirs impacted by long-term industrial and mining legacies, and in the Hungarian section of the Tisza River affected by transboundary textile-derived microfibres and inadequate wastewater treatment. This short thesis addresses these regional gaps by examining bioaccumulation patterns and toxic effects of three major contaminant classes (PTEs/organics, organic solvents, MPs) in three representative freshwater organisms using complementary field and laboratory approaches.

1.2. Problem Statement

In Central and Eastern Europe, freshwater pollution is intensified by historical and ongoing anthropogenic pressures. Bulgarian reservoirs such as Kardzhali, Studen Kladenets, and Zhrebchevo have accumulated PTEs, PBDEs, and SCCPs over decades from mining, industrial discharges, and agricultural runoff, yet localised biomonitoring using integrated biomarker responses remains limited. Similarly, the Tisza River, one of Europe's major transboundary waterways, exhibits elevated MP loads, particularly textile-derived fibres, driven by inadequate wastewater treatment in upstream rural catchments (especially Ukraine) and Hungary's growing plastic waste generation, historically low plastic recycling rates and high landfilling (Balla *et al*., 2022; Plastics Europe, 2022; European Commission, 2023). Despite the ecological and commercial significance of species such as the Chinese pond mussel (*Sinanodonta woodiana,* Lea, 1834), African catfish (*Clarias gariepinus*), and burbot (*Lota lota*), comprehensive studies linking contaminant exposure to multi-level biological effects (biochemical, physiological, behavioural, and tissue-specific) are largely absent in these regions.

This knowledge deficit impedes the development of targeted monitoring programmes, early-warning systems, and evidence-based mitigation strategies aligned with frameworks such as the EU Water Framework Directive. There is a pressing need for localised, multi-method research that elucidates bioaccumulation mechanisms, toxicological responses, and potential human health risks to inform effective regional pollution control and freshwater conservation.

1.3. Objectives of the Study

The overarching aim of this dissertation was to advance understanding of bioaccumulation patterns and toxic effects of anthropogenic contaminants in freshwater organisms through integrated field and laboratory approaches in understudied Central and Eastern European systems. The specific objectives, addressed through three complementary experiments, were:

1. To identify and validate sensitive biochemical, metabolic, and genotoxic biomarkers for assessing freshwater pollution using caged *S. woodiana* in three anthropogenically impacted Bulgarian reservoirs (Experiment I).
2. To characterise the acute and chronic toxicological effects of sublethal NC-PT exposure on behavioural, haematological, and serum biochemical responses in juvenile *C. gariepinus*, including post-exposure recovery (Experiment II).
3. To provide the first comprehensive assessment of MP contamination in *L. lota* from the Hungarian section of the Tisza River, characterising abundance, tissue distribution, polymer composition, and potential sources, and evaluating ecological and human health implications (Experiment III).

These objectives were pursued through the following six central thesis questions, which guide the research and encapsulate its original scientific contributions:

Experiment I: Biomarker-Based Biomonitoring Using Caged Mussels in Bulgarian Reservoirs

1. Can caged *S. woodiana* effectively integrate site-specific contaminant exposure and elicit sensitive, correlated multi-biomarker responses (oxidative stress, metabolic suppression, genotoxicity) across pollution gradients in Bulgarian reservoirs?
2. To what extent do antioxidant enzyme activities (CAT, GPx, GR) reflect pollutant-driven oxidative stress and correlate quantitatively with contaminant burden (MPI, PTEs, SCCPs, PBDEs) in transplanted mussels?

Experiment II: Toxicological impacts of paint thinner exposure

1. What is the toxicity potential (96-hour LC_{50}) of NC-PT, and how does acute sublethal exposure affect the behavioural patterns of juvenile *C. gariepinus*?
2. How does chronic sublethal exposure influence haematological parameters, serum lipid profiles, and liver and kidney biochemical markers, and to what extent are these effects reversible following a depuration period?

Experiment III: Microplastic contamination in burbot

1. Is there evidence of MP uptake and tissue-specific distribution in the demersal, predatory fish *L. lota* from the Hungarian Tisza River, and what are the abundance and morphological characteristics of the identified particles?
2. Can polymer-level characterisation and morphological fingerprints of MPs in *L. lota* be used to identify potential upstream sources of contamination and establish a chemical signature for regional mitigation? And what is the estimated potential human exposure associated with consumption of contaminated edible tissues?

CHAPTER 2: MATERIALS AND METHODS

2.1. Experiment I: Biomarker-Based Biomonitoring Using Caged Mussels in Bulgarian Reservoirs

2.1.1. Test Organism

The Chinese Pond mussel *S. woodiana* was selected as the sentinel organism due to its wide distribution, large body size, filter-feeding behaviour, and well-documented capacity to bioaccumulate contaminants. Although invasive in Europe, *S. woodiana* is widely recognised for its sensitivity to environmental stressors and suitability for biomarker-based freshwater biomonitoring.

2.1.2. Study Sites

The field experiment was conducted in three Bulgarian reservoirs subjected to contrasting anthropogenic pressures: Kardzhali (41.638475 N, 25.304432 E), Studen Kladenets (41.622244 N, 25.441933 E), and Zhrebchevo (42.585571 N, 25.885592 E). Kardzhali and Studen Kladenets have a long history of PTE contamination linked to lead–zinc mining and ore processing. In contrast, Zhrebchevo is primarily influenced by intensive agriculture with documented PTE inputs. A reference site with no known anthropogenic impact was established at a fishpond within the Institute of Fisheries and Aquaculture, Plovdiv (42.143611 N, 24.816111 E).

Figure 1. Map of South-Eastern Europe and the localities of the study sites: Kardzhali (K), Studen Kladenets (SK) and Zhrebchevo (Z) reservoirs, and reference site in Plovdiv (P) in Bulgaria.

2.1.3 Field Experimental Design

Unsexed mussels of uniform size were collected from the reference site and acclimated under controlled laboratory conditions before deployment. Thirty individuals (mean body weight: 154 ± 5.5 g; shell length: 11 ± 3.5 cm) were placed in stainless-steel cages (30 × 15 × 10 cm) and deployed at ~2 m depth in the study reservoirs (n = 10 mussels per reservoir). The cages

allowed unrestricted water exchange while preventing organism loss. Mussels were exposed in situ for 30 days without supplementary feeding. Due to logistical constraints, the reference site served as the control, and no baseline (day-0) sampling was performed. Following exposure, soft tissues were excised and processed according to an adapted EMERGE mussel protocol (Rosseland *et al*., 2003).

2.1.4. Bioaccumulation Analyses

Bioaccumulation was assessed by quantifying 17 elements (Al, As, Ca, Cd, Co, Cr, Cu, Fe, Hg, K, Mg, Mn, Na, Ni, P, Pb, Zn), six PBDE congeners (28, 47, 99, 100, 153, 154), and short-chain chlorinated paraffins (SCCPs) in surface water and whole soft tissues. Analyses were conducted using ICP-AES, ICP-MS, and GC-MS (Thermo Scientific, USA) following established protocols. The Metal Pollution Index (MPI) was calculated to integrate trace metal burdens (excluding macro-elements: Al, As, Cd, Co, Cr, Cu, Fe, Hg, Mn, Ni, Pb, and Zn) and enable inter-site comparisons (Ju *et al*., 2017; Nyeste *et al*., 2019):

$$MPI = (C1 \times C2 \times C3 \times \ldots Cn) \wedge (1/n) \quad \textbf{(Eq. 1)}$$

where Cn represents the mean tissue concentration (mg kg^{-1} wet weight) of each element. Previously published bioaccumulation datasets (Gecheva *et al*., 2020; Gecheva *et al*., 2021) were used to contextualise biomarker responses.

2.1.5. Biomarker Analyses

A multi-biomarker approach was applied to evaluate biological responses in mussels:

Histochemical analysis: Gill sections (6 µm, cryostat) were stained with Periodic Acid–Schiff (PAS) reagent and graded semi-quantitatively (0: no staining reaction; 1: very weak positive reaction; 2: weak positive reaction; 3: moderate positive reaction; 4: strong positive reaction) for glycogen content under light microscopy in a blinded manner (McManus, 1948; Bernet *et al*., 1999).

Biochemical analysis: Digestive gland tissues were homogenised in phosphate buffer (50 mM, pH 7.4, 300 mM NaCl) and centrifuged for 15 min at 9,000 rpm (4°C). Supernatants were used for enzyme assays. Antioxidant enzymes (CAT, GPx, GR), metabolic and neurotoxicity markers (Lactate dehydrogenase: LDH and Cholinesterase: ChE), and aminotransferases (Alanine aminotransferase: ALT and Aspartate aminotransferase: AST) were measured spectrophotometrically using standardised methods (Beutler, 1984; Wendel, 1980; Reitman & Frankel, 1957) and commercial kits where applicable. Total protein content was determined by the Bradford method (Bradford, 1976). All assays were performed in triplicate and normalised to protein content (U/mg protein).

Genotoxicity/Comet assay: DNA damage was assessed using the alkaline comet assay on haemolymph cells (Singh *et al*., 1988; Kolarević *et al*., 2016). Fifty nucleoids per individual were analysed using Comet Assay IV software, and DNA damage was expressed as tail intensity (%).

2.1.6. Integrated Biomarker Response (IBR)

To integrate individual biomarker responses, the Integrated Biomarker Response (IBR) index was calculated following Beliaeff and Burgeot (2002). Biomarker values were standardised, directionally adjusted, and transformed into positive scores. IBR values were derived from star-plot polygon areas, providing a single quantitative metric for comparing biological stress across sites.

2.1.7. Data Processing and Statistical Analysis

Data analysis was performed using PAST 3.03 and GraphPad Prism 7. Normality and homogeneity were assessed using Shapiro–Wilk and Levene's tests. Differences between reference and exposed groups were evaluated using one-way ANOVA followed by Tukey's post hoc test. Relationships between MPI values and biomarker responses were examined using Spearman's correlation. Statistical significance was accepted at $p < 0.05$, and results are presented as mean ± standard deviation.

2.2. Experiment II: Acute and Chronic Toxicity of NC-PT in Juvenile African Catfish

2.2.1. Ethical considerations and experimental animals

Procedures complied with institutional ethical guidelines (UNN/FBS/24/SS.12337) and international animal welfare standards (Borski & Hodson, 2003). A total of 220 healthy juvenile *C. gariepinus* (mean weight 67.73 ± 0.8 g; standard length 9.52 ± 0.2 cm) were obtained from a commercial hatchery and acclimated for two weeks in the laboratory (Fisheries and Hydrobiology Research Unit, Department of Zoology and Environmental Biology, University of Nigeria, Nsukka) with a commercial diet (45% crude protein) and regular water renewal.

2.2.2. Test compound and exposure design

A commercial NC-PT was used as the test toxicant. Although the precise formulation could not be determined, NC-based thinners typically contain mixtures of ketones, esters, alcohols, amides, nitroparaffins, and aliphatic and aromatic hydrocarbons, all of which possess varying toxicological properties. Test solutions were freshly prepared in dechlorinated tap water.

Acute toxicity: Acute toxicity followed OECD 203 guidelines (OECD, 2019): 80 fish were exposed to graded concentrations (11.0, 1.5, 2.0, 2.25, 3.7, and 5.0 mg L^{-1}) in 20 L tanks (10 fish/tank) for 96 h under semi-static conditions. Mortality was recorded every 24 h, while 96-hour median lethal concentration (96 h LC_{50}) was estimated via probit analysis (Finney, 1971).

Chronic sub-lethal exposure: Based on the LC_{50} value, fish were exposed to three sub-lethal NC-PT concentrations (0.1, 0.2, and 0.4 mg L^{-1}; Groups 1–3) for 21 days, followed by a 7-day depuration period in clean water. Treatments were arranged in a completely randomised design with triplicate tanks per concentration and an untreated control group. Fish were fed twice daily at 3% body weight. Water quality parameters (temperature, dissolved oxygen, and pH) were monitored regularly and remained within acceptable ranges throughout the experiment.

2.2.3. Blood Sampling and Analyses

Blood samples were collected on days 0, 7, 14, 21, and after depuration (day 28). Samples were divided into EDTA-treated tubes for haematological analyses and plain tubes for serum separation. Haematological parameters, including total red blood cell count (RBC), packed cell volume (PCV), haemoglobin concentration (Hb), total white blood cell count (WBC), and differential counts, were determined using an automated haematology analyser (Mindray BC2800) (Erkmen, 2022). Serum lipid profiles, including low-density lipoprotein (LDL), high-density lipoprotein (HDL), total cholesterol (TCHOL), and triglyceride (TAG), were analysed using a lipid-plus measuring system. Serum biochemical markers of hepatic and renal function (ALT, AST, and alkaline phosphatase: ALP) were quantified using commercial assay kits and a semi-automated clinical chemistry analyser.

2.2.4. Statistical Analysis

Acute toxicity data were analysed using probit analysis to estimate LC_{50} values with 95% confidence intervals. Haematological and biochemical data were analysed using mixed-model ANOVA with the rstatix package in R Studio (RStudio Team, 2023), wheras Visualisations used ggplot2 (R v3.3.3). The PT treatment was considered the between-subject factor, and the exposure time was taken as the within-subject factor. Post hoc comparisons were performed using Bonferroni correction. Statistical significance was set at $p < 0.05$, and results are presented as mean ± standard deviation.

2.3. Experiment III – Microplastic Contamination Assessment in Burbot from the Tisza River

2.3.1. Study area and sampling

Sampling was conducted at Tiszabecs (Hungarian–Ukrainian border), a key site for transboundary MP influx in the upper Tisza River (Fig. 2). Ten burbot (*L. lota*) were captured by electrofishing, transported alive in aerated containers, and stored at –20 °C. After thawing, total/standard/fork lengths and wet weight were recorded. Under clean laboratory conditions, the gastrointestinal tract (GIT, including contents), gills, liver, and dorsal muscle (mean 6.02 ± 0.81 g) tissues were dissected using stainless steel instruments and stored in pre-cleaned glass containers covered with aluminium foil to minimise contamination.

Figure 2. Map of the sampling site with the Tiszabecs point marked in red; HU: Hungary; UA: Ukraine.

2.3.2. Tissue digestion and MP extraction

Tissues were digested in filtered 10% KOH (1:3 sample: KOH) at 60 °C for 24 h with periodic agitation. Liver samples included absolute ethanol (1:4 ratio) to prevent saponification (Dawson *et al.*, 2020). Density separation used saturated NaI (1.8 g/cm³, 1:2 ratio). Supernatants were vacuum-filtered through glass-fibre filters (1.6 µm pore). Procedural blanks with Milli-Q water were prepared and processed in parallel to monitor environmental contamination throughout.

2.3.3. Microplastic Identification, Morphological and Chemical Analysis

Filters were examined under a stereomicroscope with imaging software for quantification and categorisation by shape, colour, and size (Bessa *et al.*, 2018). Ninety-eight representative particles underwent Raman spectroscopy (532 nm laser, 0–3,500 cm^{-1}, LabSpec 6) for polymer

and pigment identification using Spectragryph/OpenSpecy with a minimum match threshold of ≥70% (Cowger *et al*., 2021; Menges, 2022).

2.3.4. Quality Assurance and Quality Control

Rigorous quality assurance and quality control measures were employed throughout the duration of sample collection, preparation, and laboratory analysis to minimise environmental contaminations. Personnel wore nitrile gloves and cotton lab wear, while analysis utilised pre-filtered reagents, glass/stainless equipment, and laminar flow where possible, with blanks prepared and analysed concurrently with the various samples (Karami *et al*., 2017; O'Connor *et al*., 2020). Airborne controls and subtraction of blank-matched particles were carried out before analysis to ensure data integrity.

2.3.5. Estimation of Human Microplastic Intake

Muscle MP concentration (0.96 ± 0.56 items/g) was used to estimate weekly and annual intake based on EFSA guidelines (EFSA, 2014) and Hungarian per capita fish consumption (~129 g/week; Szűts *et al*., 2022) (Eq. 2).

$$\boldsymbol{Estimated\ weekly\ MP\ intake}\left(\boldsymbol{MP\ items}\frac{\boldsymbol{items}}{\boldsymbol{week}}\right) = Average\ MP\ item\ in\ the\ muscles\left(MPs\frac{items}{g}\right) \times Recommended\ fish\ food\ intake\ per\ week\ (g)$$

(Eq. 2)

2.3.6. Statistical Analysis

MP abundance (items/g tissue) was tested for normality using the Shapiro–Wilk test and for homogeneity using Levene's test. One-way ANOVA with Tukey's HSD or Kruskal–Wallis with Dunn's test was employed to compare tissue means in R (version 4.3.3; R Core Team, 2023). Correlations between tissue weights and MP abundance were analysed using Pearson's correlation. Statistical significance was set at $p < 0.05$. Pearson's correlations were used to examine organ weight–MP relationships (R v4.3.3, ggpubr package).

3. RESULTS AND EVALUATION

The results are presented and evaluated in three subsections, each aligned with a core objective of the thesis. Within each subsection, the corresponding research questions formulated in the Introduction are addressed explicitly and answered. These questions are presented in italics, followed by an integrated synthesis of key findings and their interpretation. Tables and figures are referenced narratively where essential to ensure clarity and conciseness.

3.1 Experiment I: Biomarker-Based Biomonitoring Using Caged Mussels in Bulgarian Reservoirs

This pilot study deployed caged *S. woodiana* for 30 days in three Bulgarian reservoirs (Zhrebchevo, Kardzhali, and Studen Kladenets), alongside a reference site. No mortality was recorded, enabling robust assessment of site-specific contaminant bioaccumulation and integrated multi-biomarker responses.

Can caged S. woodiana effectively integrate site-specific contaminant exposure and elicit sensitive, correlated multi-biomarker responses (oxidative stress, metabolic suppression, genotoxicity) across pollution gradients in Bulgarian reservoirs?

Caged *S. woodiana* proved highly effective as a sentinel species, integrating ambient contaminant exposure and generating coherent, site-specific biological responses. Analyses of water samples (Table 1) and mussel tissues (Table 2) revealed a clear pollution gradient, with Studen Kladenets exhibiting the highest contamination (elevated Pb, Zn, Cd, PBDEs, and SCCPs; MPI = 1.72), followed by Kardzhali (MPI = 1.65) and Zhrebchevo (MPI = 1.58), while the reference site showed the lowest burden (MPI = 0.95). These patterns are consistent with known reservoir-specific pressures, including mining and industrial activities in Studen Kladenets and Kardzhali (Gecheva *et al*., 2020), and predominantly agricultural inputs at Zhrebchevo (Kalchev *et al*., 2013).

Table 1. Concentrations (mean mg L^{-1} and their relative standard deviation (RSD%)) of studied elements and organic pollutants in water samples from different sampling sites in Bulgaria

		Sampling sites		
	Reference site	**Zhrebchevo**	**Kardzhali**	**Studen Kladenets**
		Water		
Macro elements (mg L^{-1})				
Ca	n.a.	n.a.	n.a.	n.a.
K	n.a.	n.a.	n.a.	n.a.
Mg	10.7 (3.3%)	12.1 (2.6%)	2.6 (5.9%)	3.6 (6.4%)
Na	14.9 (2.6%)	11.1 (2.7%)	4.8 (3.6%)	7.6 (2.1%)
P	0.13 (3.5%)	<0.01	0.11 (7.6%)	0.04 (8.2%)
Trace elements (µg L^{-1})				
Al	130 (3.5%)	50 (11.4%)	110 (7.6%)	30 (13.3%)
As	1.7 (8.2%)	<1	1.2 (9.4%)	5.2 (4.9%)
Cd	<0.1	<0.1	<0.1	0.32 (7.9%)
Co	0.28 (5.1%)	<0.01	<0.01	0.26 (5.4%)
Cr	0.38 (5.3%)	0.13 (4.2%)	0.18 (4.1%)	0.06 (6.9%)
Cu	6.4 (4.2%)	0.3 (7.3%)	1.7 (6.2%)	1.8 (5.7%)
Fe	0.27 (9.8%)	<0.01	<0.01	<0.01
Hg	<0.05	<0.05	<0.05	<0.05
Mn	0.049 (4.3%)	0.005 (5.3%)	0.008 (5.2%)	0.039 (3.3%)
Ni	1.0 (3.1%)	0.4 (2.5%)	0.4 (7.5%)	0.6 (3.4%)
Pb	2.1 (4.5%)	0.3 (5.9%)	0.6 (5.0%)	17.7 (4.6%)
Zn	<1	<1	<1	19.9 (4.8%)

Organic compounds (μg L^{-1})				
BDE 28	<0.004	<0.004	0.023 (26.0%)	0.032 (28.1%)
BDE 47	0.005 (0.1%)	0.005 (0.1%)	0.012 (0.1%)	0.005 (0.1%)
BDE 99	0.012 (0.1%)	<0.004	0.017 (0.1%)	0.018 (0.1%)
BDE 100	0.009 (0.1%)	<0.004	<0.004	<0.004
BDE 153	0.014 (0.1%)	0.014 (0.1%)	0.012 (0.1%)	0.018 (0.1%)
BDE 154	<0.004	<0.004	0.010 (0.1%)	<0.004
SCCPs	0.58 (20.7%)	3.9 (20.5%)	0.86 (19.8%)	1.2 (20.0%)

BDE: brominate diphenyl ethers; SCCPs: short-chain chlorinated paraffins

Table 2. Concentrations (mean mg kg^{-1} and their relative standard deviation (RSD%)) of studied elements and organic pollutants in mussel samples from different sampling sites in Bulgaria

		Sampling sites		
	Reference site	**Zhrebchevo**	**Kardzhali**	**Studen Kladenets**
		Mussels		
Macro elements (mg kg^{-1})				
Ca	89 (3.1%)	196 (2.3%)	145 (2.1%)	185 (2.4%)
K	211 (4.2%)	201 (4.7%)	229 (4.0%)	234 (5.5%)
Mg	268 (3.8%)	659 (2.6%)	454 (2.8%)	450 (3.1%)
Na	331 (3.6%)	514 (3.0%)	780 (3.0%)	436 (3.1%)
P	0.43 (6.4%)	1.20 (5.7%)	0.93 (6.0%)	1.07 (5.1%)
Trace elements (mg kg^{-1})				
Al	14.4 (11.1%)	36.9 (6.9%)	36.6 (6.2%)	35.7 (5.7%)
As	0.37 (6.4%)	1.15 (8.8%)	0.80 (7.5%)	0.63 (9.3%)
Cd	0.09 (10%)	0.19 (13%)	0.17 (6.0%)	0.38 (5.6%)
Co	0.14 (6.0%)	0.27 (7.4%)	0.20 (7.1%)	0.21 (5.8%)
Cr	0.15 (3.4%)	0.19 (8.6%)	0.22 (3.2%)	0.14 (11.3%)
Cu	5.32 (4.9%)	25.35 (4.3%)	63.03 (3.4%)	31.43 (3.9%)
Fe	170 (6.4%)	294 (3.4%)	212 (3.4%)	196 (5.0%)
Hg	0.004 (16%)	0.009 (12%)	0.007 (12%)	0.009 (11%)
Mn	1.34 (7.5%)	1.88 (5.0%)	2.54 (4.0%)	1.98 (5.6%)
Ni	0.14 (5.0%)	0.4 (2.5%)	0.37 (6.5%)	0.6 (3.4%)
Pb	1.6 (6.8%)	1.9 (3.6%)	1.7 (5.7%)	4.0 (7.9%)
Zn	55.1 (3.3%)	15.70 (3.8%)	11.47 (3.6%)	32.59 (4.7%)
Organic compounds (mg kg^{-1})				
BDE 28	0.005 (0.1%)	<0.003	<0.003	0.005 (0.1%)
BDE 47	0.005 (0.1%)	0.005 (0.1%)	0.012 (0.1%)	0.005 (0.1%)
BDE 99	0.013 (0.1%)	<0.003	0.015 (0.1%)	0.010 (0.1%)
BDE 100	0.007 (0.1%)	<0.003	<0.003	<0.003
BDE 153	0.014 (0.1%)	<0.003	0.016 (0.1%)	0.014 (0.1%)
BDE 154	<0.003	<0.003	<0.003	0.010 (0.1%)
SCCPs	7.4 (29.7%)	0.22 (31.8%)	0.56 (30.4%)	6.1 (29.5%)

BDE: brominate diphenyl ethers; SCCPs: short-chain chlorinated paraffins

Differential contaminant exposure was mirrored by coordinated, multi-level biomarker responses (Table 3). Pronounced glycogen depletion in gill tissues (weak PAS reaction) was observed in Kardzhali and Studen Kladenets, indicating stress-induced energy reallocation (Javed and Usmani, 2015; Ngo *et al*., 2022). Antioxidant enzymes (Catalase: CAT; Glutathione peroxidase: GPx, and Glutathione reductase: GR) were significantly upregulated in Studen Kladenets and Kardzhali ($p < 0.01–0.001$), whereas metabolic enzymes (ALT, AST, LDH, ChE) were consistently suppressed across impacted sites ($p < 0.001$), reflecting hepatopancreatic and neuromuscular impairment (Almeida *et al*., 2011; Parveen *et al*., 2017; Pirone *et al*., 2019). Genotoxic damage followed the same spatial trend, with the highest DNA

tail intensity recorded in Studen Kladenets (25.79 ± 1.58%), followed by Kardzhali (21.84 ± 2.60%) and Zhrebchevo (13.23 ± 1.31%) ($p < 0.001$) (Fig. 3).

The IBR index ranked overall stress as reference = 0 < Zhrebchevo = 0.50 < Studen Kladenets = 4.38 < Kardzhali = 7.51, confirming strong coherence among biochemical, cellular, and genetic endpoints. Collectively, these results demonstrate that caged *S. woodiana* effectively integrates cumulative contaminant exposure over time and translates it into biologically meaningful, site-discriminatory responses, validating its suitability for detecting pollution gradients rather than transient contamination events.

Table 3. Average results (± standard deviation) PAS-reaction in the gills; oxidative stress-related enzymes' activities and metabolic-related enzymes' activities in the digestive glands and the integrated biomarker response (IBR) values of mussels from different sampling sites in Bulgaria.

		Sampling sites		
	Reference site	**Zhrebchevo**	**Kardzhali**	**Studen Kladenets**
		Histochemical alterations in gills		
Intensity of PAS-reaction	++	+	+/-	+/-
		Oxidative stress-related enzymes in the digestive gland (U/mg protein)		
CAT	$25.79 \pm 2.99^{a,c}$	17.74 ± 2.52^{a}	34.23 ± 7.59^{c}	104.75 ± 5.06^{b}
GPx	0.68 ± 0.19^{a}	0.74 ± 0.18^{a}	$0.93 \pm 0.23^{a,b}$	1.27 ± 0.29^{b}
GR	0.31 ± 0.11^{a}	0.49 ± 0.09^{a}	0.86 ± 0.28^{b}	1.10 ± 0.25^{b}
		Metabolic-related enzymes in the digestive gland (U/mg protein)		
ChE	31.82 ± 3.73^{a}	30.39 ± 2.46^{b}	5.98 ± 2.76^{d}	14.52 ± 3.18^{c}
AST	84.35 ± 2.97^{a}	72.19 ± 3.00^{b}	24.98 ± 2.54^{d}	65.18 ± 2.79^{c}
ALT	47.07 ± 3.64^{a}	46.12 ± 2.70^{a}	34.41 ± 1.72^{b}	35.32 ± 2.85^{b}
LDH	147.28 ± 16.22^{a}	52.28 ± 6.70^{b}	$140.81 \pm 8.76^{a,c}$	125.43 ± 9.16^{c}
		DNA damage in haemocytes		
Tail intensity, TI (%)	5.54 ± 0.92^{a}	13.23 ± 1.31^{b}	21.84 ± 2.60^{c}	25.79 ± 1.58^{d}
		Integrated biomarker response (IBR)		
IBR	0	0.50	7.51	4.38

[a,b,c] The values with different letters in the same row are significantly different (Tukey's test, $p < 0.05$); CAT: catalase; GPx: glutathione peroxidase; GR: glutathione reductase; ChE: cholinesterase; AST: aspartate aminotransferase; ALT: alanine aminotransferase; LDH: lactate; IBR: integrated biomarker response.

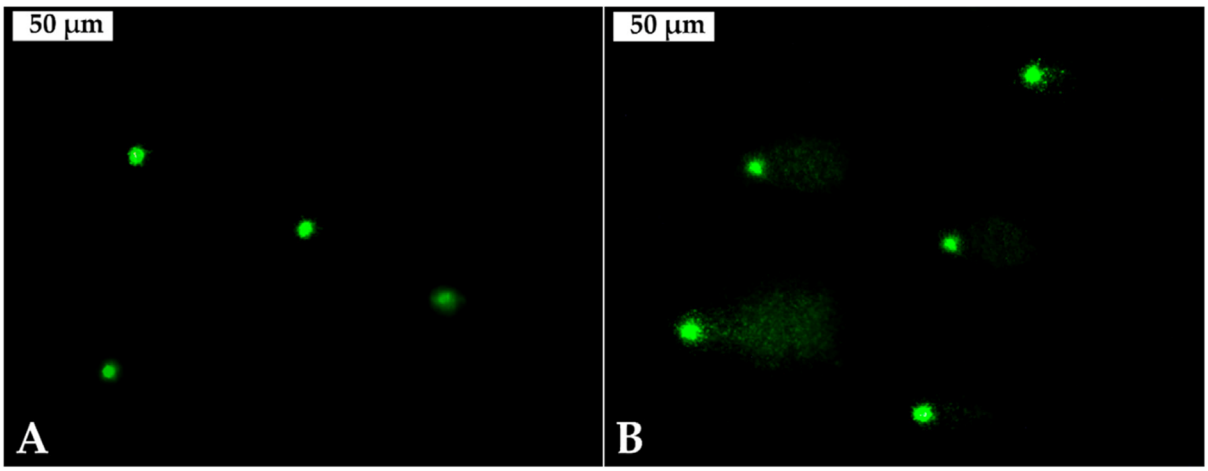

Figure 3. Comet assay images of haemocytes from Sinanodonta woodiana mussels: (A) Reference site; (B) Studen Kladenets Reservoir (400× magnification).

To what extent do antioxidant enzyme activities (CAT, GPx, GR) reflect pollutant-driven oxidative stress and correlate quantitatively with contaminant burden (MPI, PTEs, SCCPs, PBDEs) in transplanted mussels?

Antioxidant enzyme activities in transplanted *S. woodiana* strongly reflected pollutant-driven oxidative stress and showed significant quantitative relationships with contaminant burden (Table 4). CAT, GPx, and GR activities were strongly and positively correlated with MPI values ($r = 0.745–0.904$, $p < 0.05$), confirming adaptive enzymatic upregulation in response to reactive oxygen species generated by combined PTE and organic pollutant exposure. This response was most pronounced in Studen Kladenets, where elevated contaminant loads likely exceeded detoxification capacity, contributing to downstream metabolic suppression (negative correlations with AST, ALT, and ChE) and enhanced genotoxicity (positive correlation between TI% and MPI; $r = 0.953$, $p < 0.05$).

Correlation analyses further demonstrated strong associations between antioxidant responses and specific contaminant classes, including PTEs, SCCPs, and PBDEs, reinforcing a mechanistic link between chemical exposure and oxidative stress pathways. In contrast, mussels at the reference site exhibited baseline enzyme activities consistent with minimal contaminant pressure. The parallel modulation of multiple antioxidant enzymes, rather than isolated changes, emphasises their diagnostic robustness and supports their use as early-warning biomarkers (Almeida *et al*., 2002). Overall, these findings confirm *S. woodiana* caging as a practical and sensitive biomonitoring approach for Bulgarian freshwater ecosystems.

Table 4. Correlation coefficients between metal pollution index (MPI) and biomarker responses of mussels from different sampling sites in Bulgaria, significant at $p < 0.05$ (N=20).

Biometric indices/ biomarker responses	Spearman's rang correlation coefficient.
CAT	0.745
GPx	0.718
GR	0.904
ChE	-0.710
AST	-0.760
ALT	-0.772
LDH	n.s.
Tail intensity	0.953

n.s.: Non-significant; AST – aspartate aminotransferase, ALT – alanine aminotransferase, CAT – catalase, ChE – cholinesterase, GPx – glutathione peroxidase, GR – glutathione reductase, and LDH – lactate dehydrogenase in the digestive glands of caged mussels (U/mg protein).

3.2 Experiment II – Toxicological Impacts of NC-PT in *C. gariepinus*

This experiment evaluated the acute and chronic sublethal effects of NC-PT on juvenile *C. gariepinus* over a 21-day exposure period, followed by a 7-day depuration phase.

What is the toxicity potential (96-hour LC_{50}) of NC-based paint thinner, and how does acute sublethal exposure alter behavioural patterns in juvenile C. gariepinus?

NC-PT exhibited moderate acute toxicity (GESAMP, 2019) to juvenile *C. gariepinus*, with a 96-hour LC_{50} of 1.87 mg L^{-1} (95% CI: 1.698–2.044 mg L^{-1}) (Fig. 4). The narrow margin between sublethal and lethal concentrations was evident, with no mortality at ≤1.5 mg L^{-1} and complete mortality at ≥2.5 mg L^{-1}, indicating steep concentration-dependent lethality consistent with solvent mixture toxicodynamics.

Behavioural responses occurred at concentrations well below those inducing mortality, highlighting their high sensitivity as toxicological endpoints. Behavioural alterations (Table 5)

were dose-dependent and progressive, ranging from mild distress (erratic swimming, air gulping, reduced feeding) at 1.5 mg L^{-1} to severe manifestations (restlessness, eye bulging, fin immobility) at ≥2.0 mg L^{-1}. These responses are indicative of neurotoxicity, respiratory distress, osmoregulatory disruption, and metabolic exhaustion, likely mediated through interference with neurotransmitter pathways and elevated detoxification demands (Nassef *et al*., 2020). Such early behavioural impairment has clear ecological relevance, as it would compromise predator avoidance, foraging efficiency, and overall fitness under natural conditions.

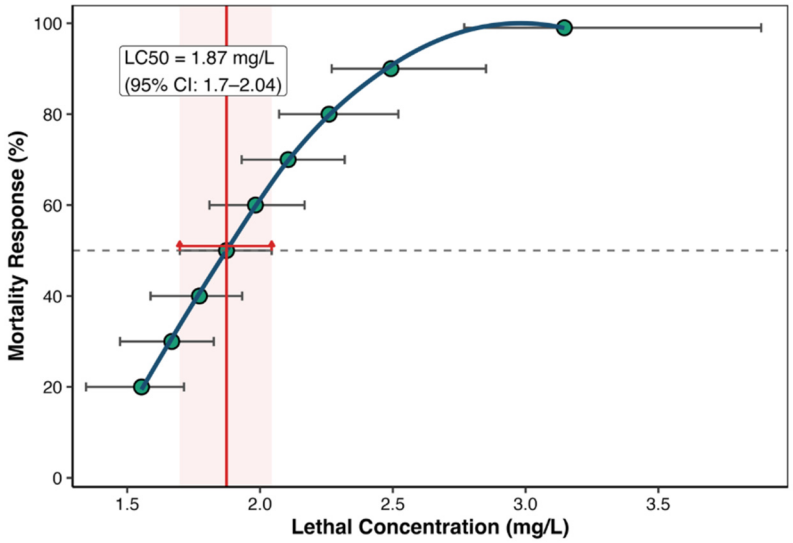

Figure 4. Probit-Based 96-Hour LCx Estimates with 95% Confidence Intervals for Clarias gariepinus Juveniles Exposed to Paint Thinner

Points show LCx (Lethal Concentration) with 95% Cls (Confidence Interval); the curve is a LOESS fit. The shaded band indicates LC50 (95% CI).

Table 5. Morphological and behavioural abnormalities, and mortality response of juvenile *Clarias gariepinus* during 96-h exposure to paint thinner

Conc. (mg/L)	Erratic Swim.	Gulping of air	Attempts to jump	Slow Response to Feeding	Bulging eyes	Restless.	Fin Movt.	Mort. (%)
0.00 (Control)	–	–	–	–	–	–	+++	0
1.00	–	–	–	–	–	–	+++	0
1.50	+	+	+	++	–	–	++	0
2.00	+	++	+++	++	+	+	++	50
2.25	++	++	+++	+++	++	++	+	60
2.50	++	+++	+++	+++	+++	++	–	100
3.70	+++	+++	+++	+++	+++	+++	–	100
5.00	+++	+++	+++	+++	+++	+++	–	100

***Key:** – absent; + mild; ++ moderate; +++ severe; Swim: Swimming; Restless: Restlessness; Movt.: Movement; Mort.: Mortality

How does chronic sublethal exposure influence haematological parameters, serum lipid profiles, and liver and kidney biochemical markers, and to what extent are these effects reversible following a depuration period?

Chronic sublethal exposure to NC-PT (0.1–0.4 mg L^{-1}) induced pronounced systemic physiological disruption in juvenile *C. gariepinus*. Haematological analysis revealed dose-dependent anaemia, characterised by reductions in Hb, PCV, and erythrocyte counts, alongside leukocytosis with neutrophilia and lymphopenia ($p < 0.001$) (Fig. 5). These changes reflect oxidative damage to erythrocytes and stress-mediated immune modulation (Kuhn *et al*., 2017; Sayed *et al*., 2023).

Serum lipid responses were moderate and variable, with increased HDL and decreased LDL and total cholesterol levels ($p < 0.01–0.05$) following PT exposure over time (Table 6), suggesting secondary metabolic adjustment rather than primary toxicity. In contrast, liver and kidney biomarkers exhibited marked dose- and time-dependent elevations, including ALT, AST, ALP, urea, and creatinine ($p < 0.001$), indicating hepatocellular damage and impaired renal filtration (Mohamed *et al*., 2019; Hamed *et al*., 2023).

Following the 7-day depuration period, most parameters showed substantial recovery. Full normalisation was observed for erythrocyte indices, lipid profiles, and renal markers (creatinine and bilirubin), while hepatic enzymes (ALT, ALP, and AST) and leukocyte profiles showed partial recovery. Persistently elevated WBC and neutrophil counts (Fig. 5) suggest residual immune activation. Interestingly, recovery patterns were dose-dependent, with Group 3 exhibiting the greatest recovery potential across several blood indices, possibly reflecting enhanced adaptive physiological responses triggered at higher exposure levels. Overall, *C. gariepinus* demonstrated notable resilience, although the findings highlight the risk of lingering effects under prolonged or repeated solvent exposure, with implications for aquaculture and wild fish populations in contaminated waters.

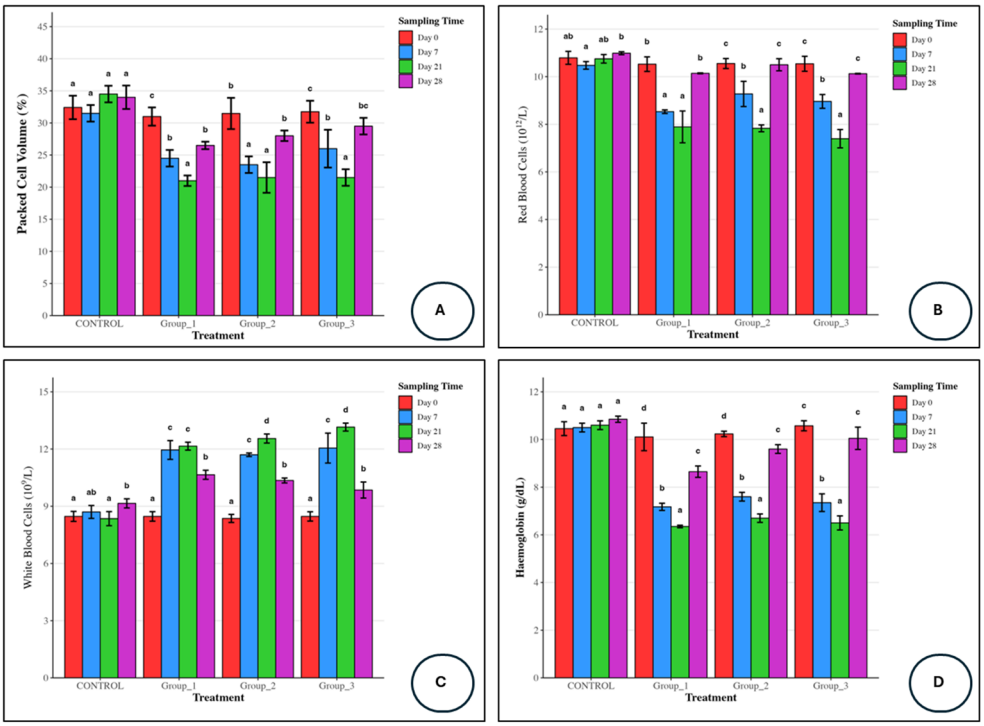

Figure 5: A within-Treatment comparison of the main haematological indices of African catfish juveniles exposed to sub-lethal doses of paint thinner

[a,b,c,d,] Bars with different letters for each PT-Treatment x sampling time interaction are significantly different ($p < 0.05$); Control – 0 mg/L paint thinner; Group 1 – 0.1 mg/L paint thinner; Group 2 – 0.2 mg/L paint thinner; Group 3 – 0.4 mg/L paint thinner; Day 0: Pre-treatment period; Day 28: Post-Treatment; **A** - Packed cell volume (%); **B** - RBC: Red Blood Cells (**×10^{12}/L**); **C** - White Blood Cells (× 10^9/L); **D** - Haemoglobin (g/dL)

Table 6. Effect of chronic exposure to sub-lethal doses of paint thinner on the lipid profiles of African catfish juveniles

Source	HDL(mg/dL)	LDL(mg/dL)	TAG(mg/dL)	TCHOL(mg/dL)
Sampling time				
Day 0 (Pre-Treat)	63.88 (0.56)^{a}	23.44 (0.52)^{a}	104.75 (0.89)^{a}	93.56 (0.72^{ab}
Day 7	64.13 (0.55)^{a}	20.88 (0.52)^{b}	106.75 (0.61)^{a}	91.63 (0.52)^{a}
Day 21	64.13 (0.52)^{a}	22.75 (0.52)^{a}	97.38 (1.18)^{b}	93.13 (0.43)^{ab}
Day 28 (7 Day Post-Treatment/ Depuration)	64.0 (0.7)^{a}	22.88 (0.52)^{a}	98.13 (0.53)^{b}	94.38 (0.69)^{b}
PT-Treatment				
Group1	65.81 (0.59)^{a}	23.06 (0.63)^{a}	104.25 (0.88)^{a}	94.31 (0.53)^{a}
Group2	63.0 (0.59)^{bc}	20.88 (0.36)^{b}	99.81 (0.88)^{b}	91.88 (0.53)^{b}
Group3	65.13 (0.59)^{ab}	22.25 (0.34)^{ab}	100.88 (0.88)^{ab}	92.0 (0.53)^{b}
Control	62.19 (0.59)^{c}	23.75 (0.32)^{a}	102.06 (0.88)^{ab}	94.5 (0.53)^{a}
Interactions				
Group1 X Day 0	63.75 (1.12)^{a}	24.25 (1.26)^{ab}	107.0 (1.79)^{a}	92.25 (1.45)^{ab}
Group2 X Day 0	63.5 (1.12)^{a}	24.0 (1.26)^{a}	101.75 (1.79)^{a}	96.0 (1.45)^{a}
Group3 X Day 0	65.0 (1.12)^{a}	22.0 (1.26)^{ab}	105.0 (1.79)^{a}	93.0 (1.45)^{a}
Control X Day 0	63.25 (1.12)^{ab}	23.5 (1.26)^{ac}	105.25 (1.79)^{a}	93.0 (1.45)^{a}
Group1 X Day 7	66.0 (1.11)^{ab}	21.5 (0.72)^{a}	110.0 (1.23)^{a}	89.0 (1.03)^{a}
Group2 X Day 7	62.0 (1.11)^{a}	19.0 (0.72)^{b}	105.0 (1.23)^{a}	89.0 (1.03)^{b}
Group3 X Day 7	67.0 (1.11)^{a}	19.5 (0.72)^{b}	107.0 (1.23)^{a}	92.0 (1.03)^{a}
Control X Day 7	61.5 (1.11)^{ab}	23.5 (0.72)^{a}	105.0 (1.23)^{a}	96.5 (1.03)^{a}
Group1 X Day 21	69.0 (1.03)^{b}	21.5 (0.68)^{a}	100.0 (2.35)^{a}	97.0 (0.85)^{b}
Group2 X Day 21	63.0 (1.03)^{a}	19.5 (0.68)^{b}	95.0 (2.35)^{a}	88.5 (0.85)^{b}
Group3 X Day 21	59.5 (1.03)^{b}	23.0 (0.68)^{a}	94.0 (2.35)^{a}	92.0 (0.85)^{a}
Control X Day 21	65.0 (1.03)^{a}	27.0 (0.68)^{b}	100.5 (2.35)^{a}	95.0 (0.85)^{a}
Group1 X Day 28	64.5 (1.40)^{a}	25.0 (0.63)^{b}	100.0 (1.05)^{a}	99.0 (1.38)^{b}
Group2 X Day 28	63.5 (1.40)^{a}	21.0 (0.63)^{ab}	97.5 (1.05)^{a}	94.0 (1.38)^{a}
Group3 X Day 28	69.0 (1.40)^{a}	24.5 (0.63)^{a}	97.5 (1.05)^{a}	91.0 (1.38)^{a}
Control X Day 28	59.0 (1.40)^{b}	21.0 (0.63)^{c}	97.5 (1.05)^{a}	93.5 (1.38)^{a}
P-Value				
Sampling time	0.988	<0.001	<0.001	0.052
PT-Treatment	0.003	0.011	0.022	0.005
Interaction	<0.001	<0.001	0.415	<0.001

^{a,b,c} Row means with different letters for each factor and the interaction are significantly different ($p < 0.05$); SEM: standard error of mean; Control – 0 mg/L paint thinner; Group 1 – 0.1 mg/L paint thinner; Group 2 – 0.2 mg/L paint thinner; Group 3 – 0.4 mg/L paint thinner; Treat.: Treatment; PT: Paint thinner; HDL: High-Density Lipoproteins; LDL: Low-Density Lipoproteins; TAG: Triglycerides; TCHOL: Total Cholesterol.

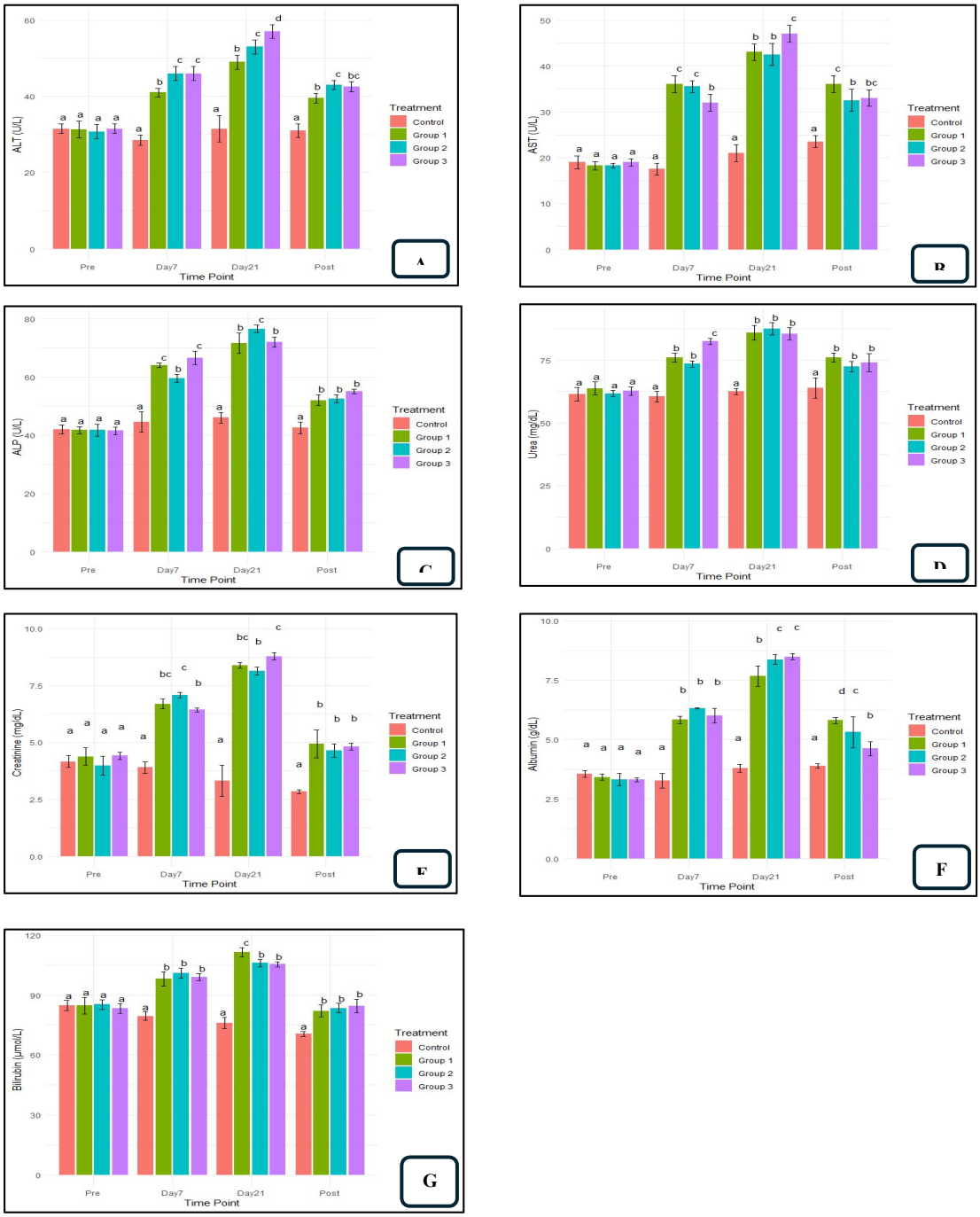

Figure 6: A within-sampling time comparison of the serum biochemical indices of African catfish juveniles exposed to sub-lethal doses of Paint Thinner

[a,b,c,] Bars with different letters for each sampling time are significantly different ($p < 0.05$); Control – 0 mg/L paint thinner; Group 1 – 0.1 mg/L paint thinner; Group 2 – 0.2 mg/L paint thinner; Group 3 – 0.4 mg/L paint thinner; Pre.: Pre-treatment period; ALT: alanine aminotransferase (**U/L**); AST: aspartate aminotransferase (**U/L**); ALP: alkaline phosphate (**U/L**); urea (**mg/dL**); creatinine (**mg/dL**); bilirubin (**mg/dL**); albumin (g/dL).

3.3. Experiment III – Microplastic Contamination in Burbot (*L. lota*) from the Hungarian section of the Tisza River

Ten burbot specimens collected from Tiszabecs were analysed, revealing 100% MP (MP) prevalence (246 particles total; mean 24.6 ± 7.46 particles per fish; 188.48 ± 58.44 items kg^{-1}; 0.19 ± 0.06 items g^{-1}).

Is there evidence of microplastic uptake and tissue-specific distribution in the demersal, predatory fish L. lota from the Hungarian Tisza River, and what are the abundance and morphological characteristics of the identified particles?

MPs were detected in all examined *L. lota* specimens, providing unequivocal evidence of uptake in this demersal predatory species. Absolute particle counts were highest in the liver (30.89%) and GIT (27.64%). However, when normalised to tissue mass (Fig. 7), MP concentrations were significantly higher in gills (1.88 ± 1.28 items g^{-1}) and GIT (1.68 ± 0.69 items g^{-1}) than in liver (0.81 ± 0.41 items g^{-1}) and muscle (0.96 ± 0.56 items g^{-1}) ($p < 0.05$), indicating combined waterborne and dietary exposure routes, with subsequent translocation to internal tissues.

Fibres dominated the MP assemblage (83.3%) (Fig. 8), particularly in muscle and liver tissues (Fig. 9). Blue (37.4%) and green (19.5%) particles were most prevalent (Fig. 10–11), while small particles (<500 µm) accounted for 41.9% of all MPs (Fig. 12–13), a size range associated with enhanced tissue penetration and retention (Zhao *et al*., 2021). Weak and non-significant correlations between organ weight and MP abundance (r = –0.56 to 0.63) suggest largely size-independent retention patterns (Fig. 14). Representative stereomicroscope images of suspected MPs are shown in Fig. 15. The detection of MPs in edible muscle tissue raises concerns regarding trophic transfer and potential human exposure, confirming *L. lota* as a suitable sentinel species for freshwater MP monitoring.

Table 7. Quantification of microplastic particles in different fish and tissue samples

FISH ID	Body Weight (g)	Tissue MPs No				MPs/Ind.	MPs/BW(kg)
		GIT	Gill	Muscle	Liver		
Burbot_1	132.49	5	4	6	3	18	135.86
Burbot_2	173.14	8	3	1	8	20	115.51
Burbot_3	139.96	9	2	2	5	18	128.61
Burbot_4	150.49	10	3	11	10	34	225.93
Burbot_5	116.56	5	3	11	14	33	283.12
Burbot_6	103.32	6	5	9	2	22	212.93
Burbot_7	119.2	6	6	2	3	17	142.62
Burbot_8	123	4	11	6	9	30	243.90
Burbot_9	148.82	12	4	6	13	35	235.18
Burbot_10	117.94	3	4	3	9	19	161.10
Total		**68 (27.64%)**	**45 (18.29%)**	**57 (23.17%)**	**76 (30.89%)**	**246**	
Mean	**132.49**	**6.80**	**4.50**	**5.70**	**7.60**	**24.60**	**188.48**
SD	**20.77**	**2.86**	**2.55**	**3.71**	**4.22**	**7.46**	**58.44**

*Gastrointestinal Tract: GIT; Ind.: Individual.

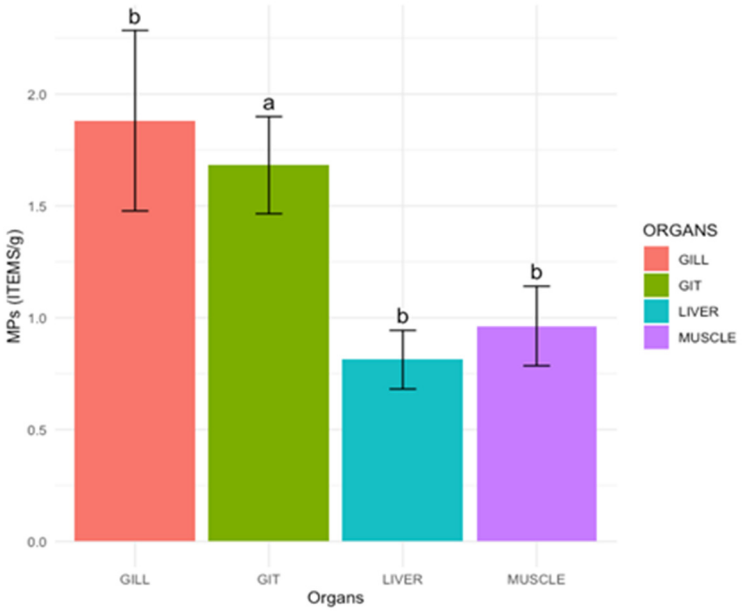

Figure 7. One-way ANOVA of suspected microplastic particle abundance across fish tissues
*Bars with different alphabets differ significantly ($P < 0.05$)

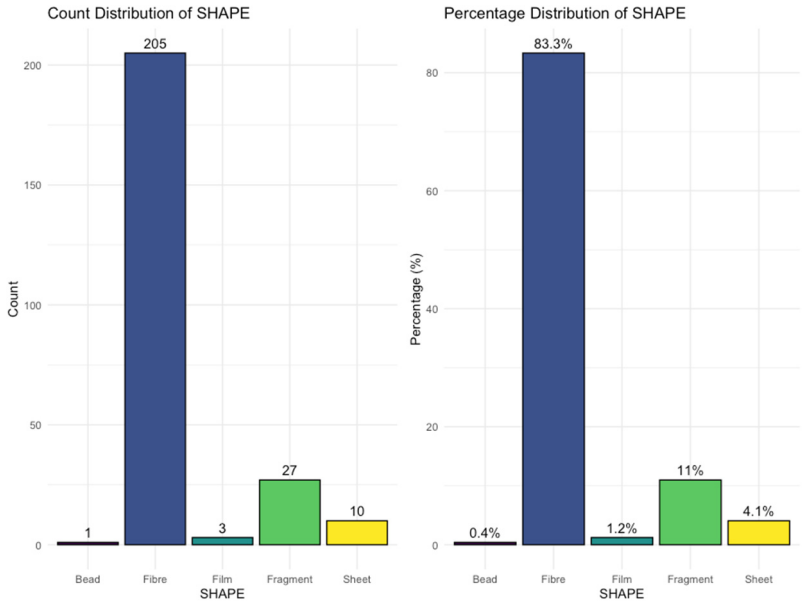

Figure 8. Suspected Particles' Shape Distributions (count and percentage) in Fish Samples

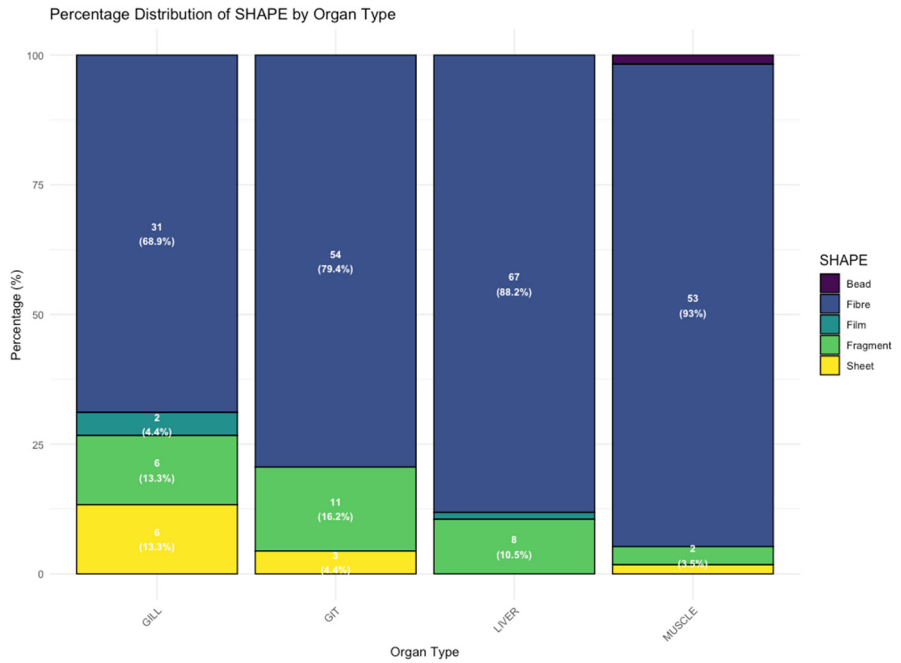

Figure 9. Suspected Particles' shape distributions (count and percentage) in various Fish tissues.

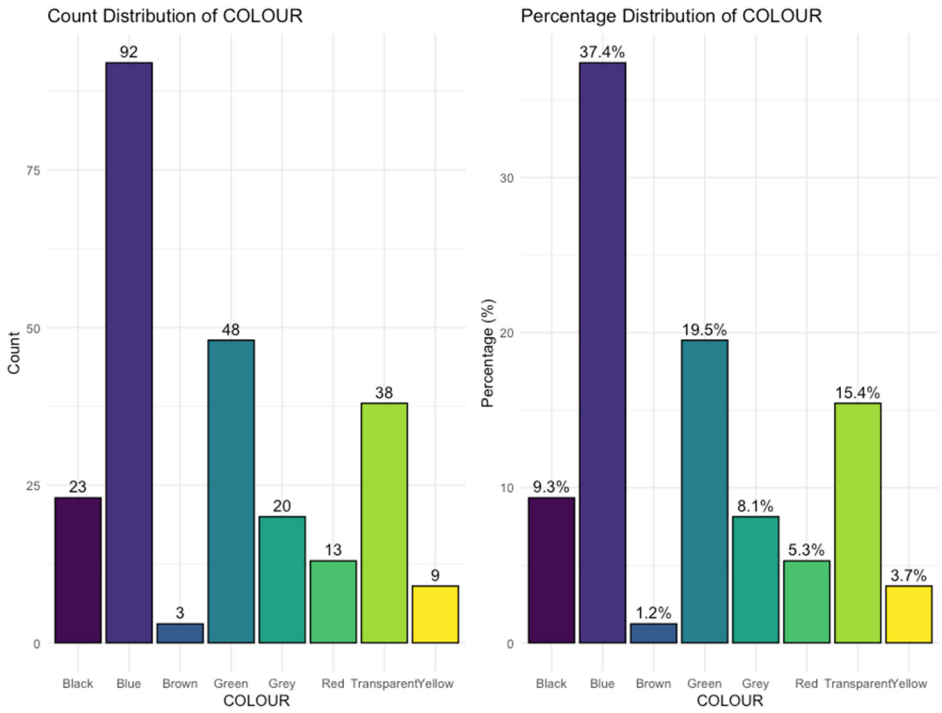

Figure 10. Suspected Particles' Colour Distributions (count and percentage) in Fish Samples

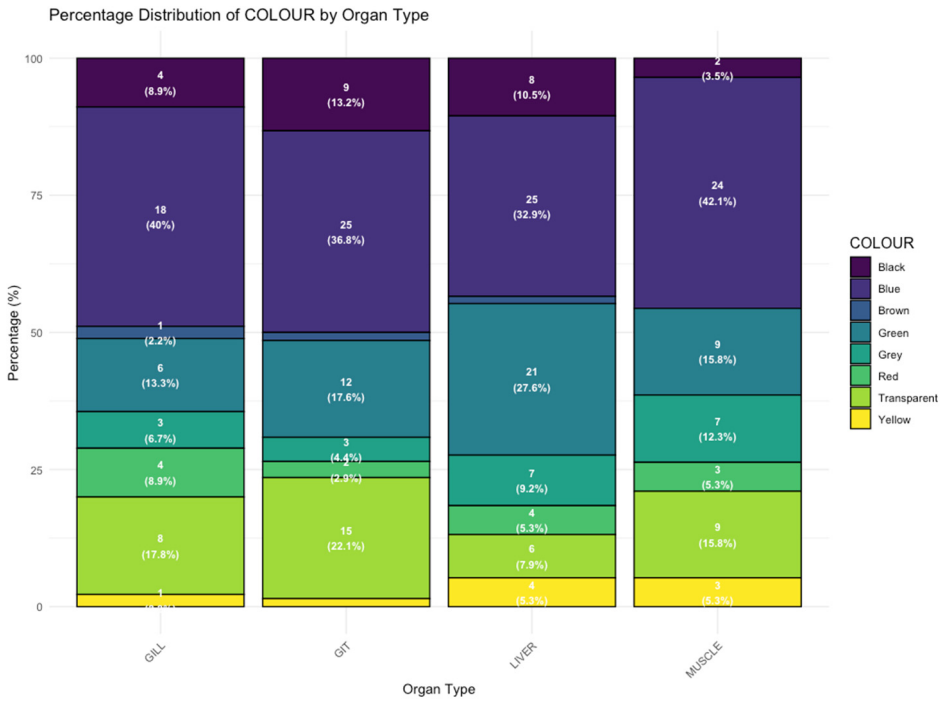

Figure 11. Suspected Particles' colour Distributions (count and percentage) in various Fish tissues

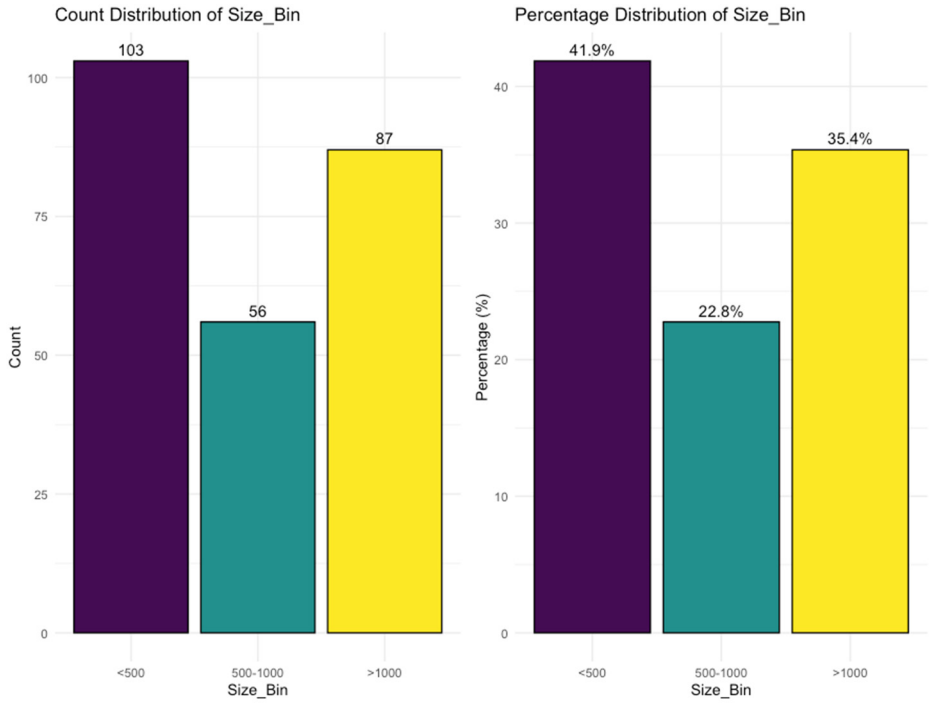

Figure 12. Suspected Particles' Size Distributions (count and percentage) in Fish Samples

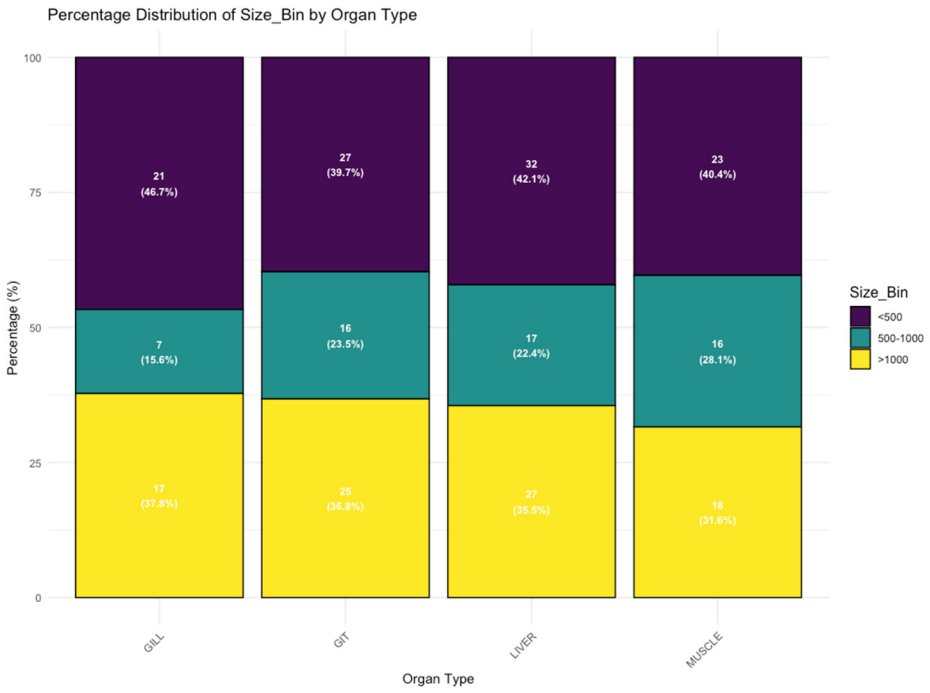

Figure 13. Suspected Particles' Size Distributions (count and percentage) in various Fish tissues

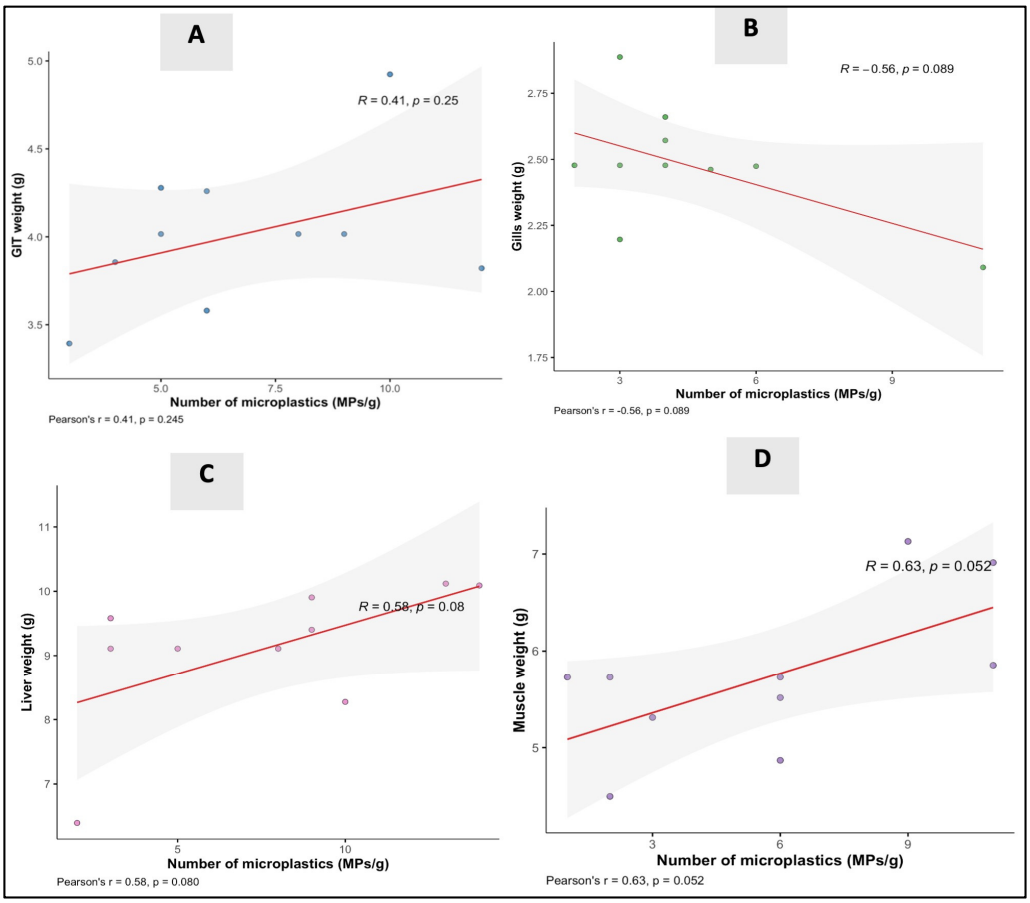

Figure 14. Bivariate plots of the identified suspected MPs per gram (MPs/g) (A) GIT weights, (B) Gill weights, (C) Liver weights, and (D) Muscle weights. The solid lines represent predicted associations, while the shaded areas are the 95% confidence intervals

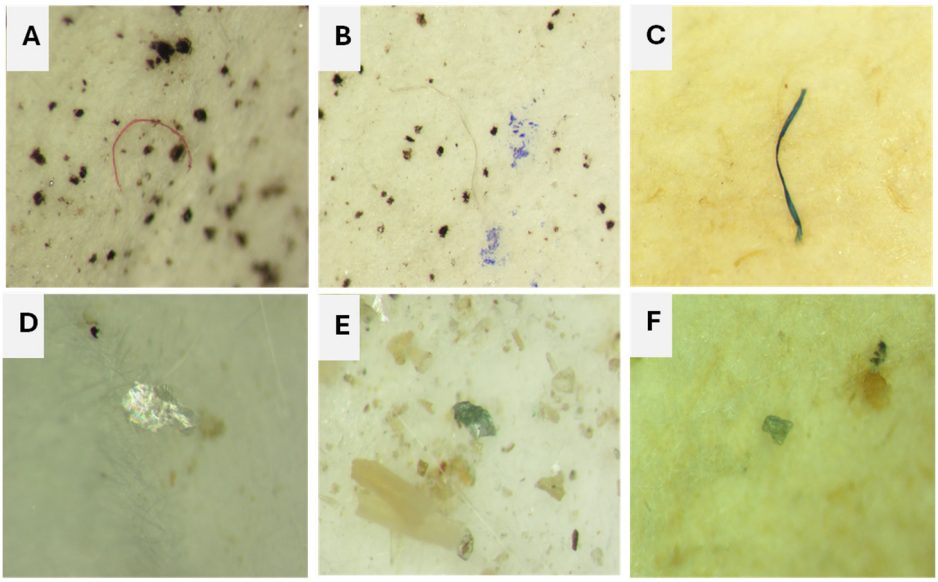

Figure 15. Stereomicroscope images of identified suspected microplastics in various burbot tissues: (A) Red fibre, (B) Transparent fibre, (C) Blue fibre, (D) Transparent sheet, (E) Green sheet, and (F) Green fragment (40× magnification).

Can polymer-level characterisation and morphological fingerprints of microplastics in L. lota be used to identify potential upstream sources of contamination and establish a chemical signature for regional mitigation? And what is the estimated potential human exposure associated with consumption of contaminated edible tissues?

Polymer-level characterisation identified 20 distinct polymer types (Fig. 16), providing strong evidence linking MP contamination in *L. lota* to upstream textile-related sources. PET–cotton blends (~34.7%), cotton (20.4%), and polyurethane (11.2%) dominated, with indigo-dyed fibres particularly prevalent (66.7%). This composition is consistent with emissions from laundry effluents and inadequate wastewater treatment upstream of the sampling area (De Vos *et al*., 2021; Balla *et al*., 2022; Ghazal *et al*., 2024). Representative Raman spectra of selected polymers are presented in Fig. 17.

The dominance of blue and indigo fibres, combined with polymer compositions characteristic of synthetic–natural blends, constitutes a distinct chemical and morphological fingerprint. This profile aligns with known wastewater inputs and demonstrates how polymer-level identification moves MP monitoring beyond particle counts toward source attribution.

Human exposure estimates based on muscle MP concentrations (0.96 ± 0.56 items g^{-1}) and EFSA consumption guidelines ranged from 38.4 items $week^{-1}$ (1,997 $year^{-1}$) in 1-year-olds to 288 items $week^{-1}$ (14,976 $year^{-1}$) in adults (Table 8). Using Hungary's per capita fish consumption (~129 g $week^{-1}$), the estimated intake averaged 124 items $week^{-1}$ (~6,427 $year^{-1}$). While muscle-only consumption poses a lower risk than whole-fish ingestion, these findings highlight the potential for chronic exposure in communities reliant on riverine fisheries.

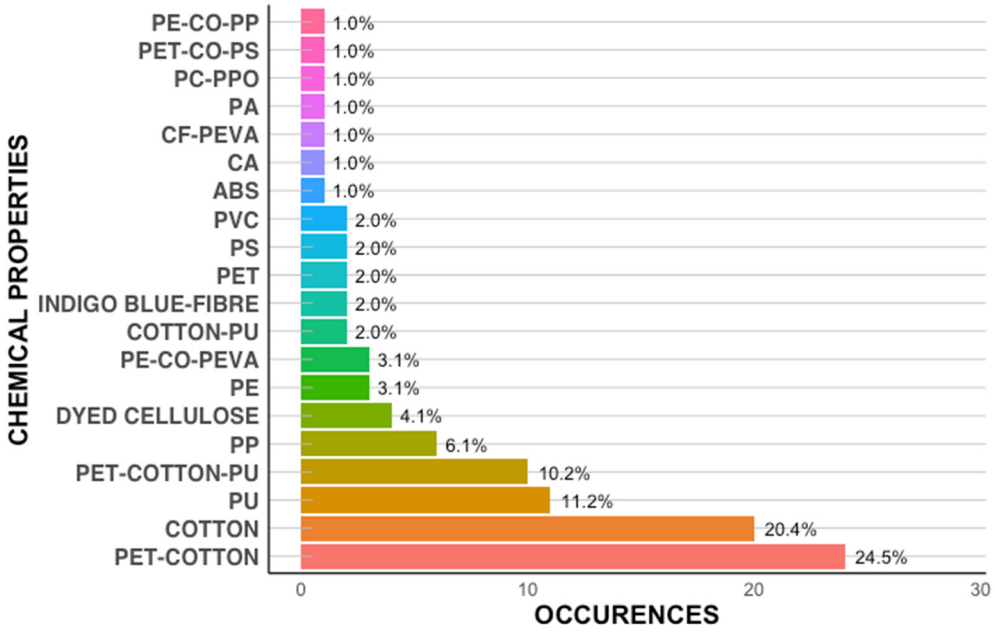

Figure 16. Percentage Distribution of Chemical Properties of Suspected Microplastics (MPs) Identified in Burbot Fish

Acrylonitrile Butadiene Styrene (ABS), Cellulose Acetate (CA), Cellulose Fibre-Polyethylene Vinyl Acetate (CF-PEVA), Cotton (CO), Cotton-Polyurethane (CO-PU), Dyed Cellulose (DC), Indigo Blue-Fibre (IBF), Polyamide (PA), Polycarbonate and Polyphenylene Oxide (PC-PPO), Polyethylene (PE), Polyethylene Terephthalate (PET), Polyethylene Terephthalate-Cotton (PET-CO), Polyethylene Terephthalate-Cotton-Polyurethane (PET-CO-PU), Polyethylene Terephthalate-Co-Polystyrene (PET-PS), Polyethylene-Co-Polyethylene Vinyl Acetate (PE-PEVA), Polyethylene-Co-Polypropylene (PE-PP), Polypropylene (PP), Polystyrene (PS), Polyurethane (PU), Polyvinyl Chloride (PVC).

Table 8. Estimated human exposure to microplastics through fish consumption, calculated using microplastic concentrations in fish and aligned with EFSA weekly fish consumption guidelines for children across various age groups and the adult population

	Children			**Adults or the general population (18 y)**	**Per capita fish consumption in Hungary**
	(1 y)	**(2-6 y)**	**(>6 y)**		
Recomm. fish muscle (g/week)	40 g	50 g	200 g	300 g	129 g
Estimated MP exposure (items/week)	38.4	48	192	288	123.84
Recomm. fish muscle (g/year)	2080 g	2600 g	10,400 g	15,600 g	6,700 g
Estimated MP exposure (items/year)	1,996.8	2496	9,984	14,976	6,432

***Recomm.: Recommendation**

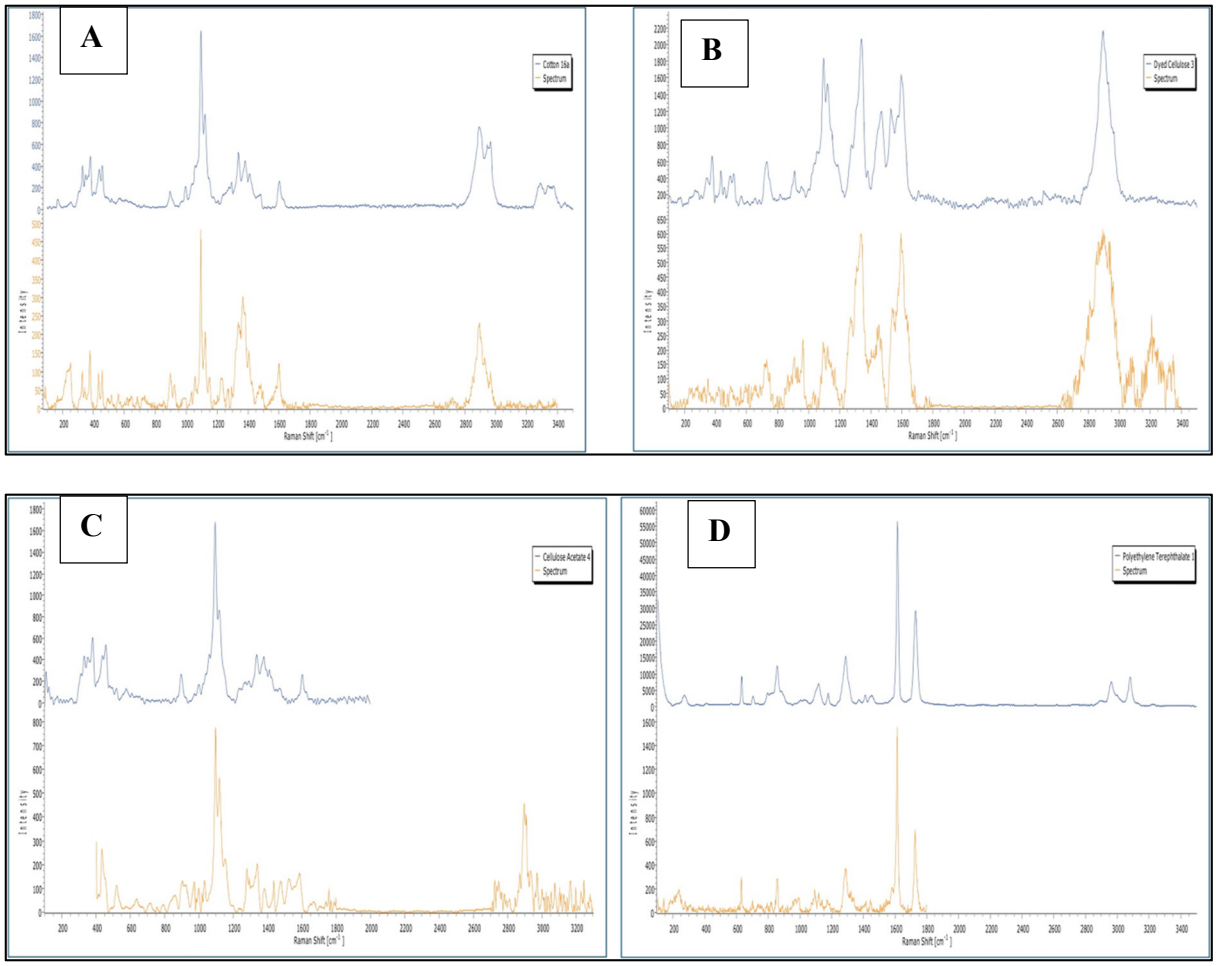

Fig 17. Raman spectrum of Cotton fibre (A), Dyed cellulose (B), Cellulose acetate (C) and Polyethylene terephthalate (D) found in the *Burbot (Lota lota) fish* collected from the upper Hungarian section of the Tisza River

New Scientific Results

- This study presents the first multi-reservoir biomonitoring assessment in Bulgaria using caged *S. woodiana*, demonstrating that transplanted mussels reliably integrate site-specific contaminant exposure and elicit sensitive biochemical, metabolic, and genotoxic responses across contrasting pollution gradients.
- A strong quantitative relationship was established between contaminant burden (MPI, PTEs, SCCPs, PBDEs) and antioxidant enzyme activity (CAT, GPx, GR), providing strong evidence for pollutant-driven oxidative stress mechanisms in mussels from industrially impacted reservoirs.
- The research provided the first evidence of significant metabolic suppression (ALT, AST, LDH, ChE) in caged mussels from Bulgarian reservoirs, indicating pollutant-induced impairment of hepatopancreatic and neuromuscular function and validating these enzymes as effective early-warning biomarkers.
- Reservoir-specific patterns of DNA damage were detected in mussel haemocytes, with the highest tail intensity observed at Studen Kladenets Reservoir, thereby confirming the high sensitivity of the comet assay for genotoxicity-based freshwater biomonitoring in Bulgaria.
- The study delivers the first comprehensive toxicological characterisation of NC-PT in juvenile *C. gariepinus*, demonstrating clear concentration-dependent impairments in locomotion, feeding behaviour, and stress responses, consistent with solvent-induced neurophysiological disruption.
- Systemic haematological and biochemical disturbances were documented in paint thinner–exposed fish, including dose-dependent anaemia, leukocytic alterations, and immune modulation, revealing previously underreported haematotoxic and immunotoxic effects associated with solvent mixtures.
- Pronounced hepatotoxic and nephrotoxic effects were observed in exposed fish, as evidenced by elevated AST, ALT, ALP, bilirubin, urea, and creatinine, highlighting the particular vulnerability of liver and kidney tissues to sublethal mixed-solvent exposure.
- This study reports the first documented occurrence of MP contamination in *L. lota* from the Hungarian section of the Tisza River, with substantial particle loads (mean 24.6 ± 7.46 particles per fish; 188.48 ± 58.44 items kg^{-1}), confirming the species as a valuable sentinel for freshwater MP monitoring.
- Small (<500 μm), fibre-shaped, blue MPs were identified as the dominant MP type in burbot tissues, with distribution patterns indicating translocation into liver and muscle, raising critical concerns for trophic transfer and potential human dietary exposure.
- Polymer-level analysis provided the first confirmation of indigo-dyed PET, cotton, and cellulose-based fibres in fish from the Hungarian Tisza River, directly implicating textile-related effluents as a dominant source and establishing a chemical fingerprint relevant for targeted regional mitigation strategies.

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Candidate: Ifeanyi Emmanuel Uzochukwu
Doctoral School: Pál Juhász-Nagy Doctoral School of Biology and Environmental Sciences
MTMT ID: 10083838

List of publications related to the dissertation

Foreign language scientific articles in international journals (3)

1. **Uzochukwu, I. E.**, Nagy, L., Somogyi, D., Pásztor, A., Ossai, N. I., Antal, L., Yancheva, V., Csarnovics, I., Nyeste, K. J.: Burbot (Lota lota) as a bioindicator of microplastic pollution in the Tisza River: Multi-tissue contamination, polymer characterisation, and implications for ecological and human-health risks.
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4. Machebe, N. S., Ikeh, N. E., **Uzochukwu, I. E.**, Baiyeri, P. K.: Livestock-crop interaction for sustainability of agriculture and environment (Chapter 13).
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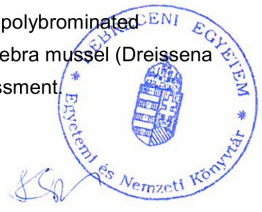

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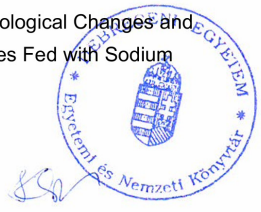

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