Bioaccumulation and toxic effects of anthropogenic contaminants in freshwater organisms

Thesis for the Degree of Doctor of Philosophy (PhD)

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Hereby, I declare that I prepared this thesis within the Doctoral Council of Natural Sciences and Engineering, Juhász-Nagy Pál Doctoral School, University of Debrecen in order to obtain a PhD Degree in Natural Sciences at Debrecen University.

The results published in the thesis are not reported in any other PhD theses.

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I support the acceptance of the thesis.

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Bioaccumulation and toxic effects of anthropogenic contaminants in freshwater organisms

Dissertation submitted in partial fulfilment of the requirements for the doctoral (PhD) degree in Environmental Sciences

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DEDICATION

This work is dedicated to my family, whose unwavering love, sacrifice, and encouragement have carried me through every stage of this journey.

To my parents, who taught me resilience; to my siblings, who kept me grounded; and to everyone who believed in me even when the path felt uncertain, this achievement is yours as much as it is mine.

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NOMENCLATURE AND ABBREVIATIONS

4-HNE: 4-Hydroxynonenal
Ag: Silver
Al: Aluminum
ALT: Alanine Aminotransferase
As: Arsenic
AST: Aspartate Aminotransferase
ATSDR: Agency for Toxic Substances and Disease Registry
BDE-47: Tetrabromodiphenyl ether
BEM: Biological Effect Monitoring
BFRs: Brominated Flame Retardants
BMPs: Best Management Practices
BPA: Bisphenol A
BTBPE: 1,2-Bis(2,4,6-tribromophenoxy)ethane
C. gariepinus: Clarias gariepinus
Ca: Calcium
CAT: Catalase
CAT: Catalase
Cd: Cadmium
ChE: Cholinesterase
Co: Cobalt
CPs: Chlorinated Paraffins
Cr: Chromium
Cu: Copper
DDC-CO: Dechlorane Plus
DDE: Dichlorodiphenyldichloroethylene
DDT: Dichlorodiphenyltrichloroethane
EDC: Endocrine-disrupting chemicals
EH-TBB: 2-Ethylhexyl-2,3,4,5-tetrabromobenzoate
EPA: Environmental Protection Agency
ERA: Environmental Risk Assessment
EROD: Ethoxyresorufin-O-deethylase
EtOH: Absolute Ethanol
FAO: Food and Agriculture Organisation
Fe: Iron
FR: Flame retardants
FTCA: Fluorotelomer Carboxylic Acids
FTOHs: Fluorotelomer Alcohols
FTS, FTSA: Fluorotelomer Sulfonates
GPx: Glutathione Peroxidase
GPx: Glutathione Peroxidase
GR: Glutathione Reductase
GSI: Gonadosomatic Index
GSTs: Glutathione S-transferases
H_2O_2: Hydrogen Peroxide
Hb: Hemoglobin Concentration

HBCD: Hexabromocyclododecane
HCB: Hexachlorobenzene
HCT: Hematocrit
HDL: High-Density Lipoproteins
HDPE: High-Density Polyethylene
HFRs: Brominated/Halogenated Flame Retardants
Hg: Mercury
HSI: Hepatosomatic Index
IBR: Integrated Biomarker Response
K: Potassium
KOH: Potassium Hydroxide
L. lota: Burbot (*Lota lota*)
LC-MS: Liquid Chromatography-Mass Spectrometry
LC: lethal concentration
LDH: Lactate Dehydrogenase
LDL: Low-Density Lipoproteins
LDPE: Low-Density Polyethylene
Li: Lithium
LPO: Lipid Peroxidation
MDA: Malondialdehyde
Mg: Magnesium
Mn: Manganese
MPI: Metal Pollution Index
MPs: Microplastics
Na: Sodium
NaCl: Sodium chloride
NaI: Sodium Iodide
NC: Nitrocellulose
Ni: Nickel
Ni: Nickel
NOAA: National Oceanic and Atmospheric Administration
NOS: National Ocean Service
O_2^-: Superoxide Anions
OCPs: Organochlorine Pesticides
OECD: Organisation for Economic Co-operation and Development
OH: Hydroxy group
OH^-: Hydroxyl Radicals
OPFRs: Organophosphate Flame Retardants
OS: Oxidative Stress
P: Phosphorus
PA: Polyamide
PAHs: Polycyclic Aromatic Hydrocarbons
Pb: Lead
PBBs: Polybrominated Biphenyls
PBDEs: Polybrominated Diphenyl Ethers
PCBs: Polychlorinated Biphenyls
PCDDs: Polychlorinated Dibenzo-p-dioxins

PCDFs: Polychlorinated Dibenzofurans
PCV: Packed Cell Volume
PE: Polyethylene
PET: Polyethylene Terephthalate
PEVA: Poly Ethylene-vinyl acetate
PFAAs: perfluoroalkyl acids
PFAO: Perfluorooctanoic Acid
PFAS: Per- and polyfluoroalkyl substances
PFBA: Perfluorobutanoic Acid
PFDA: perfluorodecanoic acid
PFHxA: Perfluorohexanoic Acid
PFHxS: Perfluorohexane Sulfonate
PFNA: Perfluorononanoic Acid
PFOS: Perfluorooctanesulfonic acid
POM: Polyformaldehyde
POPs: persistent organic pollutants
PP: Polypropylene
PPB: parts per billion
PPCPs: Pharmaceuticals and personal care products
PPM: parts per million
PS: Polystyrene
PT: Paint thinners
PU: Polyurethane
PVC: Polyvinyl Chloride
QA/QC: quality assurance and quality control
QCA: Quimica Clinica Aplicada
RBC: Red Blood Cell Count
ROS: Reactive Oxygen Species
SCCPs: short-chained Chlorinated Paraffins
Se: Selenium
SOD: Superoxide Dismutase
TAG: Triglycerides
TBBPA: Tetrabromobisphenol A
TBOEP: Tris(2-butoxyethyl)phosphate
TCEP: Tris(2-chloroethyl)phosphate
TCHOL: Total Cholesterol
TCIPP: Tris(2-chloroisopropyl)phosphate
TDCIPP: Tris(1,3-dichloro-2-propyl)phosphate
TI: Tail Intensity
TPHP: Triphenyl Phosphate
UNEP: United Nations Environmental Program
US EPA: United States Environmental Protection Agency
UV: Ultraviolet
VTG: Vitellogenin
WBC: White Blood Cell Count
WHO: World Health Organisation
Zn: Zinc

1. INTRODUCTION AND OBJECTIVES

1.1. Introduction

Freshwater ecosystems are indispensable for sustaining global biodiversity, supporting human livelihoods, and regulating biogeochemical cycles. However, these vital systems face escalating threats from anthropogenic contaminants, posing a pressing global challenge to the health and integrity of aquatic organisms and their habitats (Mendil & Uluozlu, 2007; Bashir *et al*., 2020). The surge in urbanisation, industrialisation, commercialisation, and intensive agriculture has intensified the discharge of agricultural runoff, industrial effluents (both untreated and partially treated), mining residues, and domestic waste into water bodies. This pollution introduces a complex array of contaminants, such as potentially toxic elements- PTEs (including trace metals and metalloids), agrochemicals, pharmaceuticals, microplastics (MPs), and antibiotics, that accumulate in sediments and biota, leading to ecological imbalances and potential toxicity (Ujah *et al*., 2017).

The toxicity of freshwater pollutants is a critical concern due to their persistence, non-biodegradability, and capacity for bioaccumulation and biomagnification through food webs (Adeaga *et al*., 2017). Elevated pollutant concentrations can induce a spectrum of adverse effects across species, ranging from molecular and cellular damage to physiological and behavioural disruptions. These effects may manifest as acute toxicity or chronic conditions, including immune suppression, impaired growth, developmental abnormalities, and reproductive disorders (Bukola *et al*., 2015; Egan *et al*., 2023). Moreover, the dietary reliance on fish and other aquatic organisms amplifies human health risks, as bioaccumulated contaminants transfer through the food chain, posing significant public health concerns (Adelana *et al*., 2006). Consequently, monitoring bioaccumulation and assessing toxicity are essential for ecological risk assessment and the development of effective management and remediation strategies (Schäfer *et al*., 2015).

Despite growing awareness of the ecological and public health implications of anthropogenic contaminants, the full scope of their impacts on freshwater ecosystems remains elusive. The variability in contaminant concentrations, toxicities, modes of action, and interactions with environmental factors complicates comprehensive assessments (Kong *et al*., 2016; Amoatey & Baawain, 2019). Understanding the intricate processes of bioaccumulation and toxicity, from legacy pollutants like PTEs and persistent organic compounds to emerging contaminants such as MPs, demands a multidisciplinary approach. Robust bioindicators and sensitive biomarkers are critical for evaluating ecological and health impacts and informing mitigation strategies (Ochoa-Esteso *et al*., 2024). There is an urgent need for integrative

research that synergistically combines field-based and laboratory-based approaches to comprehensively elucidate the mechanisms underlying contaminant exposure and its ecological and physiological consequences in representative freshwater species. Such studies are essential for advancing our understanding of anthropogenic impacts on aquatic ecosystems and informing effective conservation strategies.

Freshwater organisms across diverse trophic levels serve as valuable sentinels for monitoring aquatic pollution, owing to their capacity to bioaccumulate contaminants and exhibit quantifiable physiological and behavioural responses (Amoatey & Baawain, 2019). Fish, such as African catfish (*Clarias gariepinus*) and burbot (*Lota lota*), are widely used in ecotoxicological studies due to their diverse species, omnivorous feeding habits, broad geographic distribution, relative longevity, and constant aquatic exposure (Silva *et al*., 2016; Pouil *et al*., 2018). As integral components of freshwater food webs, these species are susceptible to direct and trophic contaminant transfer, making them ideal for bioaccumulation and toxicological research (Ray & Shaju, 2023). Similarly, mussels, with their filter-feeding behaviour, sedentary lifestyle, and high tolerance to abiotic changes, excel as bioindicators due to their ability to accumulate contaminants and their less efficient metabolic enzyme systems compared to fish (Gagné & Burgeot, 2013; Olenycz *et al*., 2015). Tissue-specific contaminant concentrations in these organisms provide critical insights into pollution patterns, enhancing water quality assessments (Azevedo *et al*., 2012).

Prior research has extensively documented the impacts of contaminants, including heavy metals (Javed and Usmani, 2016; Opasola *et al*., 2019), flame retardants (Oliveri *et al*., 2015; Georgieva *et al*., 2022), pesticides (Amaeze *et al*., 2020; Moreira *et al*., 2021), industrial effluents (Jabeen *et al*., 2022), pharmaceuticals (McCallum *et al*., 2017; Kayode-Afolayan *et al*., 2022), paint wastewater (Owolabi & Adewoye, 2017; Jekayinfa & Bawa-Allah, 2022), and antifouling paints (George *et al*., 2017), on aquatic ecosystems. However, region-specific studies in Central and Eastern Europe, particularly in Bulgarian reservoirs and the Tisza River, remain limited, emphasising the need for localised investigations to address knowledge gaps and inform mitigation policies.

This dissertation seeks to elucidate the bioaccumulation patterns and toxic effects of anthropogenic contaminants through three complementary experiments, each targeting distinct contaminants, organisms, and freshwater systems. By integrating biomarker identification, behavioural and physiological assessments, and MP quantification, this research aims to provide a multifaceted framework for assessing freshwater pollution and its ecological and human health implications. The findings are expected to deepen the understanding of

contaminant-organism interactions, unravel bioaccumulation mechanisms, and highlight the broader impacts on freshwater ecosystem health. Furthermore, this work aspires to contribute significantly to the scientific literature, offering valuable insights to guide pollution control strategies, conservation practices, and regulatory frameworks for protecting freshwater ecosystems and public health.

1.2. Aims and Objectives

This dissertation aims to advance the understanding of bioaccumulation and toxic effects of anthropogenic contaminants in freshwater organisms by:

1. Identifying and validating biomarkers for assessing freshwater pollution using caged mussels in Bulgarian reservoirs.
2. Investigating the behavioural, haematological, and serum biochemical responses of juvenile African catfish (*C. gariepinus*) to varying levels of paint thinner exposure.
3. Assessing the extent of microplastic pollution in the Hungarian section of the Tisza River through pioneer research on burbot (*L. lota*).

Specific objectives include quantifying contaminant concentrations (e.g., PTEs, MPs), characterising biological responses (e.g., biomarker expression, behavioural alterations, MP morphology), evaluating tissue-specific bioaccumulation patterns, and assessing ecological and human health implications. By combining field-based biomonitoring (mussels, burbot) with controlled laboratory experiments (catfish), this research seeks to deliver comprehensive insights into the uptake, accumulation, and biological impacts of diverse contaminants across multiple trophic levels and ecological contexts.

2. LITERATURE REVIEW

2.1. Sources and Pathways of Anthropogenic Contaminants in Freshwater Ecosystems

Freshwater ecosystems, characterized by low salinity (typically < 0.5‰), encompass diverse aquatic environments, including lentic systems (e.g., lakes, ponds, swamps), lotic systems (e.g., rivers, streams), wetlands (e.g., marshes, bogs), and groundwater reservoirs (e.g., aquifers). Covering approximately 15% of the Earth's surface and storing about 93,000 km³ of water, these ecosystems are critical for supporting biodiversity, human livelihoods, industrial processes, and ecological stability (Di Lorenzo *et al*., 2020). However, escalating anthropogenic pressures, such as industrial discharges, pharmaceutical effluents, inadequate urban waste management, agricultural intensification, and urban expansion, threaten these vital systems with contamination and degradation (Meijide *et al*., 2018; Amoatey & Baawain, 2019). To safeguard aquatic and human health and devise sustainable mitigation strategies, a comprehensive understanding of the major pollutants, their sources, transport mechanisms, and environmental fate is imperative (Zhao *et al*., 2018; Sumon *et al*., 2018; Amoatey & Baawain, 2019).

2.2. Major Classes of Anthropogenic Contaminants

Anthropogenic contaminants in freshwater systems encompass a broad spectrum of chemical, physical, and biological pollutants that disrupt aquatic ecosystems, human health, agricultural productivity, and ecological balance (Kumar *et al*., 2018). These contaminants are categorized based on their chemical composition, sources, modes of action, and environmental persistence.

2.2.1. POTENTIALLY TOXIC ELEMENTS (PTES)

PTEs comprise metallic, metalloid, and certain non-metallic elements of both natural and anthropogenic origin that can threaten human health and ecosystem stability. PTEs encompass heavy metals (sometimes used interchangeably), which are metallic elements and metalloids with atomic densities exceeding 4,000 kg/m³, including lead (Pb), arsenic (As), mercury (Hg), cadmium (Cd), chromium (Cr), zinc (Zn), copper (Cu), nickel (Ni), iron (Fe), aluminium (Al), silver (Ag), selenium (Se), manganese (Mn), cobalt (Co), and lanthanides (Edelstein & Ben-Hur, 2018; Amoatey & Baawain, 2019; Iordache *et al*., 2020). Generally, these elements are of particular concern because they persist in the environment, resist biodegradation, and can accumulate in biological systems, exerting harmful effects even at low concentrations. While certain elements (e.g., Zn, Fe, Cu, Ni) are essential for physiological functions in plants and animals at trace levels, excessive concentrations can be toxic (Vardhan

et al., 2019). Others, such as Pb, Hg, Cd, and As, are non-essential and inherently harmful (Akhtar *et al*., 2021a). Typically detected in freshwater at concentrations ranging from nanograms per litre (ng/L) to micrograms per litre (µg/L), PTEs originate from both natural processes (e.g., volcanic eruptions, soil erosion, metal corrosion) and anthropogenic activities, including industrial discharges, mining, agricultural runoff, and urban stormwater (Galindo-Miranda *et al*., 2019; Akhtar *et al*., 2021a).

The persistence, bioaccumulation, and toxicity of PTEs pose severe risks to aquatic organisms and human populations. These contaminants can induce neurological, reproductive, and developmental disorders, with cascading effects on ecosystem stability (Rhind, 2009; Chițescu *et al*., 2021). For instance, mercury bioaccumulates in fish, posing risks to predators and humans through dietary exposure. Given their widespread prevalence and grave health implications, PTEs remain a focal point of environmental research and regulatory efforts worldwide.

2.2.2. PHARMACEUTICALS AND PERSONAL CARE PRODUCTS (PPCPS)

Pharmaceuticals and personal care products (PPCPs) represent an emerging class of contaminants that have garnered significant attention due to their ubiquitous presence and ecological impacts. This diverse group includes pharmacologically active compounds (e.g., antibiotics, analgesics, anti-inflammatories, hormones) and chemicals from personal care products (e.g., fragrances, sunscreens, deodorants, insect repellents) (US EPA, 2013; Valdez-Carrillo *et al*., 2020). Advances in analytical technologies have recently enabled the detection of PPCPs in freshwater systems, revealing their introduction through wastewater treatment plants, hospital effluents, municipal waste, and agricultural runoff containing manure or biosolids (Patel *et al*., 2020; Sharma *et al*., 2024). A primary pathway is the excretion of unmetabolized or partially metabolized pharmaceuticals by humans and animals, which enter sewage systems and, subsequently, surface and groundwater (Wu *et al*., 2014; Kibuye, 2024). Conventional wastewater treatment processes often fail to remove PPCPs, exacerbating their persistence in aquatic environments (Hirsch *et al*., 1999; US EPA, 2013; Arumugam *et al*., 2025).

PPCPs have been detected globally in rivers, lakes, groundwater, and even treated drinking water at concentrations ranging from parts per billion (ppb) to parts per million (ppm) (Hughes *et al*., 2012; Paíga *et al*., 2016, 2024). A seminal study by Deo (2014) identified 93 pharmaceutical compounds in surface waters, including antibiotics, antidepressants, antihypertensives, and contraceptives, among others. Despite their low concentrations, PPCPs exert significant biological effects due to their pharmacological activity. They induce acute and

chronic toxicity in aquatic organisms, impairing algal growth, altering feeding behaviours, and disrupting endocrine, metabolic, and reproductive functions in invertebrates, fish, and higher vertebrates (Hagenbuch & Pinckney, 2012; Deo, 2014; Chakraborty *et al*., 2023; Sharma *et al*., 2024). Notably, antibiotics contribute to the proliferation of antibiotic-resistant genes, posing a public health crisis (Kümmerer, 2009). Endocrine-disrupting chemicals (EDCs) within PPCPs further exacerbate reproductive and developmental abnormalities in freshwater species (Thanigaivel *et al*., 2023).

Beyond ecological impacts, PPCPs have broader implications. Their accumulation may enhance methanogen activity, potentially contributing to climate change (Chakraborty *et al*., 2023). Transformation products of PPCPs, formed through environmental processes, may exhibit equal or greater toxicity than parent compounds, amplifying their threat (Shahid *et al*., 2020). Due to their persistence, bioaccumulation, and poorly understood long-term effects, PPCPs are classified as "contaminants of emerging concern" by the US Environmental Protection Agency and the European Union (Mariano *et al*., 2023). Their shared physiological pathways with target species emphasise the urgent need for further research and regulatory action.

2.2.3. PESTICIDES AND AGROCHEMICALS

Pesticides and agrochemicals, encompassing insecticides, herbicides, rodenticides, fungicides, and fertilizers, are integral to modern agriculture, enhancing crop yields and controlling pests and weeds. In 2020, global pesticide use reached an estimated 3.5 million tons, with significant contributions from countries such as China, the United States, and Argentina (Sharma *et al*., 2019). While these chemicals bolster agricultural productivity and profitability, their widespread and often indiscriminate application has raised critical concerns about their adverse effects on human health, biodiversity, and environmental integrity (Ahmad *et al*., 2024; Anjaria & Vaghela, 2024). In freshwater ecosystems, pesticides introduced through agricultural runoff and groundwater leaching pose significant risks to aquatic organisms, ecosystem stability, and human populations reliant on these water sources (Chițescu *et al*., 2021; Peluso *et al*., 2023).

Pesticides enter freshwater ecosystems primarily through diffuse sources, such as agricultural runoff and leaching into groundwater, and point sources, including improper disposal and accidental spills. Runoff from treated fields carries pesticide residues into rivers, lakes, and wetlands, while leaching introduces these chemicals into aquifers, contaminating groundwater reserves. The persistence and mobility of many pesticides exacerbate their environmental reach, allowing residues to accumulate in soils, sediments, and aquatic

organisms. Moreover, pesticide residues in crops and animal products enter the food chain, posing additional risks to human health through dietary exposure (Ergenler & Turan, 2023; Dureshahwar *et al*., 2024).

Pesticides are categorized based on their target organisms and chemical composition. Key subtypes, along with representative examples, include (Sharma *et al*., 2019; Ergenler & Turan, 2023):

- ***Insecticides:*** Aldrin, Azinphos-methyl, Chlordane, Chlorpyrifos, Diazinon, Dichlorodiphenyltrichloroethane (DDT), Dieldrin, Endrin, Heptachlor, Imidacloprid, Lufenuron (5% EC), Mirex, Pyriproxyfen, Quinalphos, Thiamethoxam.
- ***Herbicides:*** 2,4-Dichlorophenoxyacetic Acid (2,4-D), Atrazine, Glyphosate, Linuron, Pendimethalin, S-Metolachlor.
- ***Fungicides:*** Hexachlorobenzene (HCB), Penconazole, Pyraclostrobin, Triadimefon, Ziram.
- ***Biopesticides:*** Saponin-rich extracts.

Notably, several of these compounds, such as DDT and HCB, are classified as persistent organic pollutants (POPs) under the Stockholm Convention due to their environmental persistence and toxicity, amplifying their regulatory significance.

Pesticides exert profound effects on freshwater ecosystems, inducing oxidative stress, genotoxicity, impaired growth, altered behaviour, and disruptions in hormonal and reproductive functions in aquatic organisms (Vos *et al*., 2000; Ergenler & Turan, 2023). Studies have documented widespread pesticide contamination in freshwater systems, with toxicological impacts on algae, invertebrates, fish, and amphibians (Ccanccapa *et al*., 2016; Wang *et al*., 2018; Mondal *et al*., 2018; Demirci *et al*., 2018; Sumon *et al*., 2018; Fonseca *et al*., 2019). These effects cascade through ecosystems, reducing biodiversity and impairing ecosystem services such as nutrient cycling and water purification. For humans, exposure to pesticide-contaminated water and food products is linked to neurological disorders, endocrine disruption, and increased cancer risk (Chițescu *et al*., 2021).

The high toxicity, bioaccumulation, and biomagnification potential of pesticides stresses their status as significant environmental hazards. Their persistence in aquatic environments and food webs amplifies long-term risks, particularly for top predators and human consumers. Global regulatory frameworks, such as those established by the World Health Organisation and

the Food and Agriculture Organisation (2019), aim to control pesticide use and mitigate environmental impacts. However, inconsistent enforcement, particularly in low- and middle-income countries, contributes to the overuse and misuse of these chemicals. Addressing these disparities requires harmonised regulations, enhanced monitoring, and education on sustainable agricultural practices.

2.2.4. PER- AND POLYFLUOROALKYL SUBSTANCES (PFAS)

Per- and polyfluoroalkyl substances (PFAS) are a diverse group of synthetic fluorinated compounds, characterised by their strong carbon-fluorine (C-F) bonds, which confer exceptional thermal and chemical stability. Widely used in industrial and consumer products, such as electronics, textiles, firefighting foams, food packaging, and non-stick coatings (e.g., Teflon), PFAS are valued for their water- and oil-repellent properties (Brase *et al*., 2022; Parashar *et al*., 2023). However, their resistance to degradation, earning them the nickname "forever chemicals," combined with continuous emissions, has led to their pervasive presence in air, water, soil, sediments, and biota (Dai *et al*., 2022; Ehsan *et al*., 2025). In freshwater ecosystems, PFAS pose significant risks to aquatic organisms and human health due to their persistence, bioaccumulation, and toxicity.

PFAS enter freshwater ecosystems through multiple pathways, including industrial discharges, wastewater treatment plant effluents, domestic waste, biosolid applications, and direct releases from aqueous film-forming foams used in firefighting (Ehsan *et al*., 2025). Wastewater treatment processes often fail to remove PFAS, allowing these compounds to persist in surface and groundwater. Runoff from contaminated soils and atmospheric deposition further contributes to their environmental spread. The chemical stability of PFAS ensures their persistence and long-range transport, resulting in widespread contamination of rivers, lakes, and aquifers.

PFAS encompass fully fluorinated perfluoroalkyl substances, such as perfluoroalkyl acids (PFAAs), and partially fluorinated polyfluoroalkyl substances, which may degrade into PFAAs. Key examples include (Gonkowski *et al*., 2023; Ehsan *et al*., 2025):

- ***Perfluoroalkyl Acids (PFAAs)****:* Perfluorooctanoic Acid (PFOA), Perfluorooctane Sulfonate (PFOS), Perfluorohexane Sulfonate (PFHxS), Perfluorononanoic Acid (PFNA), Perfluorobutanoic Acid (PFBA), Perfluorohexanoic Acid (PFHxA).
- ***Polyfluoroalkyl Substances****:* Fluorotelomer alcohols (FTOHs), Fluorotelomer sulfonates (FTS, FTSA), Fluorotelomer carboxylic acids (FTCA), Polyfluoroalkyl ether acids, Fluorinated polymers.

The structural diversity of PFAS complicates their detection and regulation, as transformation products may exhibit similar or greater toxicity than parent compounds. PFAS have been detected globally in freshwater systems, often at concentrations exceeding regulatory thresholds. In China, rivers transport an estimated 4,000 kg of PFAS annually to the East China Sea, with PFOA and PFOS detected at levels up to 200 ng/L and 5.4 ng/L, respectively (Lu *et al*., 2015). In the United States, PFAS concentrations in some freshwater systems surpass the EPA's interim Health Advisory Levels for PFOA (0.004 ng/L) and PFOS (0.02 ng/L) (Ehsan *et al*., 2025). Elevated PFAS levels have also been reported in Singapore (Kurwadkar *et al*., 2022), South Africa (Groffen *et al*., 2018), Germany (Janousek *et al*., 2019), the United States of America (USA) (Brase *et al*., 2022; Barbo *et al*., 2023), and tropical South American French territories (Munoz *et al*., 2017), showcasing their global ubiquity.

PFAS exert profound effects on aquatic ecosystems and human health. In aquatic organisms, PFAS induce endocrine disruption, reproductive impairments (e.g., reduced fertility, hormone imbalances), and physiological damage, including oxidative stress, liver dysfunction, and immune suppression (Groffen *et al*., 2018; Nayak *et al*., 2023; Evich *et al*., 2022). Their bioaccumulation and biomagnification in food webs result in elevated concentrations in top predators, such as fish, which serve as a primary exposure route for humans. For example, individuals consuming fish from contaminated freshwater sources, such as Onondaga Lake, New York, exhibited PFOS and perfluorodecanoic acid (PFDA) blood levels 9.5 and 26.9 times, respectively, higher than median values (Wattigney *et al*., 2022). Human health risks include increased cancer risk, developmental disorders, and immune system dysfunction, highlighting the public health implications of PFAS contamination.

The persistence, bioaccumulation, and toxicity of PFAS necessitate urgent research to elucidate their environmental fate, long-term ecological impacts, and human health risks. Current regulatory frameworks, such as the EPA's Health Advisory Levels, provide some guidance, but global inconsistencies in monitoring and enforcement hinder effective management. Developing advanced detection methods, remediation technologies, and safer alternatives to PFAS is critical to mitigating their environmental and societal impacts.

2.2.5. MICROPLASTICS

Microplastics (MPs), defined as plastic particles smaller than 5 mm, have emerged as significant anthropogenic contaminants, posing profound risks to freshwater ecosystems and human health. Valued for their flexibility, durability, and affordability, plastics have driven global production from 1.5 million tonnes in the 1950s to 413.8 million tonnes by 2023 (Pellini *et al*., 2018; Statista, 2024). Approximately 41.4 million tonnes of this production enter aquatic

environments annually, with MPs infiltrating freshwater systems through diverse pathways (Rao, 2019; Iheanacho & Odo, 2020). MPs originate from primary sources, such as intentionally manufactured microbeads in cosmetics and coatings, and secondary sources, resulting from the degradation of larger plastics through physical, biological, and chemical processes. Key pathways into freshwater systems include sewage and wastewater discharges, urban and agricultural runoff, atmospheric deposition, industrial effluents, recreational activities, and tire and road wear. The mobility and persistence of MPs facilitate their widespread distribution, with freshwater systems and estuaries serving as critical conduits for terrestrial plastic waste entering marine environments (GESAMP, 2010; Horton *et al*., 2017; Karlsson *et al*., 2017).

Recent studies have documented alarming MP concentrations in freshwater systems globally, including surface waters, sediments, and biota (Kiss *et al*., 2021; Makhdoumi *et al*., 2021; Oh *et al*., 2023; Raza *et al*., 2023; Saad *et al*., 2024). Concentrations vary widely, ranging from 22.4 items/m^3 in the Tisza River, Hungary (Balla *et al*., 2022), to 7,571 items/m^3 in the Pearl River, China (Zhao *et al*., 2022), and up to 1.11 x 10^5 items/m^3 in the Sierra Gorda Biosphere Reserve, Mexico (Rafael & Elías, 2024). Sediments exhibit concentrations from 11 to 4,270 items/kg dry mass on beaches and 4 to 27,830 items/kg dry mass in benthic zones, while aquatic species may contain 0 to 1,242 MPs per individual (D'Avignon *et al*., 2022). Common polymers include polypropylene (PP), polyethylene (PE), polystyrene, low- and high-density polyethylene (LDPE, HDPE), polyurethane, polyethylene terephthalate (PET), and polyvinyl chloride (PVC), appearing as fibres, fragments, spheres, foams, sheets, and films (Rochman *et al*., 2019).

MPs exert significant physical and biological impacts on freshwater ecosystems. Physical effects include entanglements, blockages, and reduced feeding, while biological impacts encompass neurotoxicity, oxidative stress, endocrine disruption, behavioural abnormalities, and cancer (Issac & Kandasubramanian, 2021; D'Avignon *et al*., 2022). MPs act as vectors for toxic adsorbents, such as PTEs, ultra violet stabilisers, plasticisers, PFAS, and flame retardants, which bioaccumulate through trophic levels, amplifying risks to aquatic organisms and humans via the food chain (Zhao *et al*., 2022; Rafael & Elías, 2024). These effects disrupt ecological balance, impairing biodiversity and ecosystem services.

Despite growing research, the dynamics of MPs in freshwater systems remain underexplored compared to marine environments. Critical gaps include understanding MP prevalence in specific freshwater systems, their distribution mechanisms, and synergistic effects with co-occurring contaminants. Standardised sampling and analytical protocols are

needed to address variability in MP size, shape, and composition, which complicates monitoring and mitigation efforts.

2.2.6. PERSISTENT ORGANIC POLLUTANTS

Persistent Organic Pollutants (POPs) are a class of highly toxic, non-polar organic compounds containing chlorine or bromine, notorious for their environmental persistence, bioaccumulation, and widespread distribution. Recognised under the Stockholm Convention, POPs include organochlorinated pesticides (e.g., DDT, heptachlor, toxaphene), industrial by-products (e.g., dioxins, dibenzofurans), and compounds from industrial activities (e.g., polychlorinated biphenyls [PCBs], brominated flame retardants [BFRs], polycyclic aromatic hydrocarbons [PAHs]) (European Parliament and Council of the European Union, 2004; Chițescu *et al*., 2021; UNEP, 2025). POPs enter freshwater systems through improper disposal of agricultural and industrial chemicals, industrial by-products, agricultural runoff, oil spills, atmospheric deposition, and environmental processes like evaporation and temperature-driven volatilisation (UNEP, 2025). Their hydrophobic nature and low solubility enhance their persistence and mobility, allowing long-range transport and accumulation in water, sediments, and biota (Moradi *et al*., 2021; Vasseghian *et al*., 2021; Kumar *et al*., 2022).

POPs have been widely detected in freshwater systems globally, including the Danube River, Romania (Covaci *et al*., 2006), Mediterranean wetlands, Spain (Lorenzo *et al*., 2019), Sembrong Lake Basin, Malaysia (Sharip *et al*., 2017), Songhua River, China (Wang *et al*., 2018), and Buffalo River, South Africa (Yahaya *et al*., 2017). Mean concentrations rank highest in surface water (206.24 ± 2092.01 ng/L), followed by drinking water (61.73 ± 154.26 ng/L), seawater (48.81 ± 607.1 ng/L), and groundwater (42.26 ± 139.38 ng/L), with South Africa, India, and Turkey exhibiting the highest regional contamination (Vasseghian *et al*., 2021).

POPs pose severe risks due to their high toxicity, even at low concentrations. They induce carcinogenic, reproductive, endocrine, neurological, and immune system disruptions, as well as developmental abnormalities in aquatic organisms and humans (Yahaya *et al*., 2017). Their bioaccumulation and biomagnification in food webs result in elevated concentrations in top predators, amplifying exposure risks for humans through contaminated water and aquatic food products. The prolonged environmental presence of POPs exacerbates these hazards, necessitating urgent mitigation efforts.

Although many POPs, such as DDT and PCBs, have been phased out globally, their continued use in some developing countries sustains environmental contamination (Wang *et al*., 2018; UNEP, 2025). The Stockholm Convention provides a framework for regulation, but

inconsistent enforcement hinders progress. Enhanced monitoring and international cooperation are essential to reduce POP emissions and protect freshwater ecosystems.

2.2.7. FLAME RETARDANTS

Flame retardants (FRs) are anthropogenic chemicals incorporated into electronics, textiles, and plastics to reduce flammability, but their widespread use has led to significant contamination of freshwater ecosystems. Classified into brominated/halogenated, organophosphate, and inorganic/mineral types, FRs exhibit varying degrees of persistence, bioaccumulation, and toxicity (Vorkamp *et al*., 2019; Gupta *et al*., 2024). FRs are released into freshwater systems through product wear, improper disposal, industrial effluents, wastewater discharges, and atmospheric deposition. Their high mobility facilitates long-range transport, resulting in contamination of remote aquatic environments (Yang *et al*., 2019). Sediments and biota serve as sinks, accumulating FRs and amplifying their environmental persistence. FRs are categorised as follows (Vorkamp *et al*., 2019; Gupta *et al*., 2024):

- ***Brominated/Halogenated FRs (HFRs)****:* Polybrominated diphenyl ethers (PBDEs), polybrominated biphenyls (PBBs), tetrabromobisphenol A (TBBPA), hexabromocyclododecane (HBCD), dechlorane plus (DDC-CO), 1,2-bis(2,4,6-tribromophenoxy)ethane (BTBPE), 2-ethylhexyl-2,3,4,5-tetrabromobenzoate (EH-TBB), chlorinated paraffins.

- ***Organophosphate FRs (OPFRs)****:* Tris(2-chloroethyl)phosphate (TCEP), tris(2-chloroisopropyl)phosphate (TCIPP), tris(1,3-dichloro-2-propyl)phosphate (TDCIPP), triphenyl phosphate (TPHP), tris(2-butoxyethyl)phosphate (TBOEP).

- ***Inorganic/Mineral FRs****:* Zinc borate, aluminium hydroxide, magnesium hydroxide.

Several HFRs, such as PBDEs, PBBs, and HBCD, have been classified as POPs under the Stockholm Convention due to their environmental persistence and toxicity. FRs are widely detected in freshwater systems, with PBDEs and OPFRs reported in water, sediments, and biota. In China, aquatic toxicity thresholds for PBDEs range from 0.049 mg/L for pentabrominated diphenyl ethers (PeBDE) to 0.239 mg/L for decabrominated diphenyl ethers (DeBDE), with PeBDE exhibiting the highest toxicity for aquatic species (Lu *et al*., 2018). OPFRs show growth-dependent accumulation and maternal transfer in carp, indicating bioaccumulation risks (Choo *et al*., 2020).

FRs induce significant toxicity in freshwater organisms, including cancer, endocrine disruption, neurotoxicity, reproductive and developmental impairments, and immune dysfunction (Yang *et al*., 2019). Brominated FRs, particularly PBDEs, pose the greatest risk

due to their bioaccumulation and biomagnification in food webs. Inorganic FRs are less toxic but still contribute to environmental contamination. Human exposure through contaminated water and aquatic food products heightens health risks, necessitating further investigation.

Data on regional FR trends and effective remediation strategies remain limited, drawing attention to the need for expanded monitoring. Therefore, comprehensive risk assessments, advanced analytical techniques, and alternative FRs with lower toxicity are critical to addressing contamination challenges. Standardised protocols for monitoring FR distribution and impacts are also needed to inform regulatory frameworks.

2.3. Knowledge Gaps and Challenges in Monitoring and Regulating Emerging Contaminants

The proliferation of emerging contaminants, such as PFAS, novel PPCPs, nanomaterials, MPs, and FRs, presents significant challenges for monitoring and mitigation in freshwater ecosystems. These contaminants, characterised by complex chemical structures, low environmental concentrations, and synergistic interactions, present critical knowledge gaps that hinder effective management (Li *et al*., 2024).

Detecting emerging contaminants requires advanced analytical techniques, such as liquid chromatography-mass spectrometry (LC-MS), which can identify trace levels but are often cost-prohibitive, limiting their accessibility in resource-constrained regions (Richardson & Kimura, 2020). The non-degradability of PFAS complicates monitoring, as their persistence and bioaccumulation in aquatic organisms demand specialised detection methods (Ahrens & Bundschuh, 2014). MPs pose unique challenges due to their variable sizes, shapes, and ability to adsorb co-contaminants, necessitating standardised sampling and analytical protocols to ensure consistency and comparability (Li *et al*., 2018).

Also, the combined occurrence and synergistic effects of emerging contaminants remain poorly understood. Interactions between MPs, PFAS, and other pollutants may amplify toxicity, yet few studies address these cumulative impacts. Comprehensive assessments of ecological and public health risks require integrated approaches that account for contaminant mixtures and their long-term effects on aquatic ecosystems.

Inadequate regulatory frameworks exacerbate monitoring challenges. Many emerging contaminants lack established environmental standards, complicating risk assessments and enforcement. For instance, the structural diversity of PFAS and novel PPCPs hinders the development of universal regulatory thresholds. Strengthening global regulatory harmonisation and investing in affordable monitoring technologies are critical to addressing these gaps.

Bridging knowledge gaps requires interdisciplinary research to elucidate contaminant distribution, fate, and synergistic impacts. Developing cost-effective analytical tools, standardised methodologies, and innovative remediation strategies is essential for effective monitoring and mitigation. Policy efforts should also prioritise international cooperation, capacity building in low-resource regions, and the adoption of safer chemical alternatives to reduce contaminant emissions.

2.4. Point and Non-Point Sources of Anthropogenic Contaminants in Freshwater Ecosystems

Freshwater ecosystems, which are vital for ecological balance and human sustenance, face increasing threats from anthropogenic contaminants introduced through diverse pathways. These pathways are broadly classified as point and non-point sources, each characterised by distinct properties and associated contaminants (Environmental Protection Authority [EPA], Victoria, 2018; National Ocean Service [NOS], 2025). Understanding these sources is essential for developing effective pollution management and mitigation strategies, as their identification and regulation directly influence the health of aquatic systems and human populations.

2.4.1. POINT SOURCES

Point sources are distinct, identifiable origins of pollution, such as pipes, drains, smokestacks, or ships, that release contaminants directly into freshwater systems. Their localised nature makes them easier to monitor and regulate compared to non-point sources, yet their potential to introduce highly toxic substances and pathogens poses significant risks, including biomagnification in aquatic organisms and humans (Singh, 2024). Key examples of point sources include:

- ***Industrial Effluents:*** Discharges from industries, such as pulp and paper mills, oil refineries, and chemical, electronics, and automobile manufacturing plants, release PTEs, organic chemicals, and other pollutants into rivers and lakes (Ritter *et al*., 2002; NOS, 2025). These effluents can degrade water quality and harm aquatic life.

- ***Municipal Sewage:*** Sewage treatment plants and combined sewer overflows discharge nutrients, pathogens, and organic compounds into surface and groundwater. Inadequately treated sewage introduces contaminants that threaten ecosystem stability and public health (Malone, 2015).

- ***Landfills and Industrial Impoundments:*** Urban landfills and industrial waste storage sites can leach contaminants, including PTEs and organic pollutants, into groundwater systems, compromising drinking water quality and aquatic habitats.

- ***Mining and Extraction Activities:*** Mining operations and oil drilling sites release PTEs, sediments, and hazardous chemicals into freshwater ecosystems through spills and runoff, exacerbating contamination and toxicity.

The concentrated nature of point source pollution enables targeted regulatory interventions, such as effluent standards and treatment technologies, to mitigate their environmental impact. However, their high toxicity emphasises the need for stringent oversight and continuous monitoring. Figure 1 presents a schematic diagram of the various point sources and transport routes of environmental contaminants (adopted from FAO & UNEP, 2021).

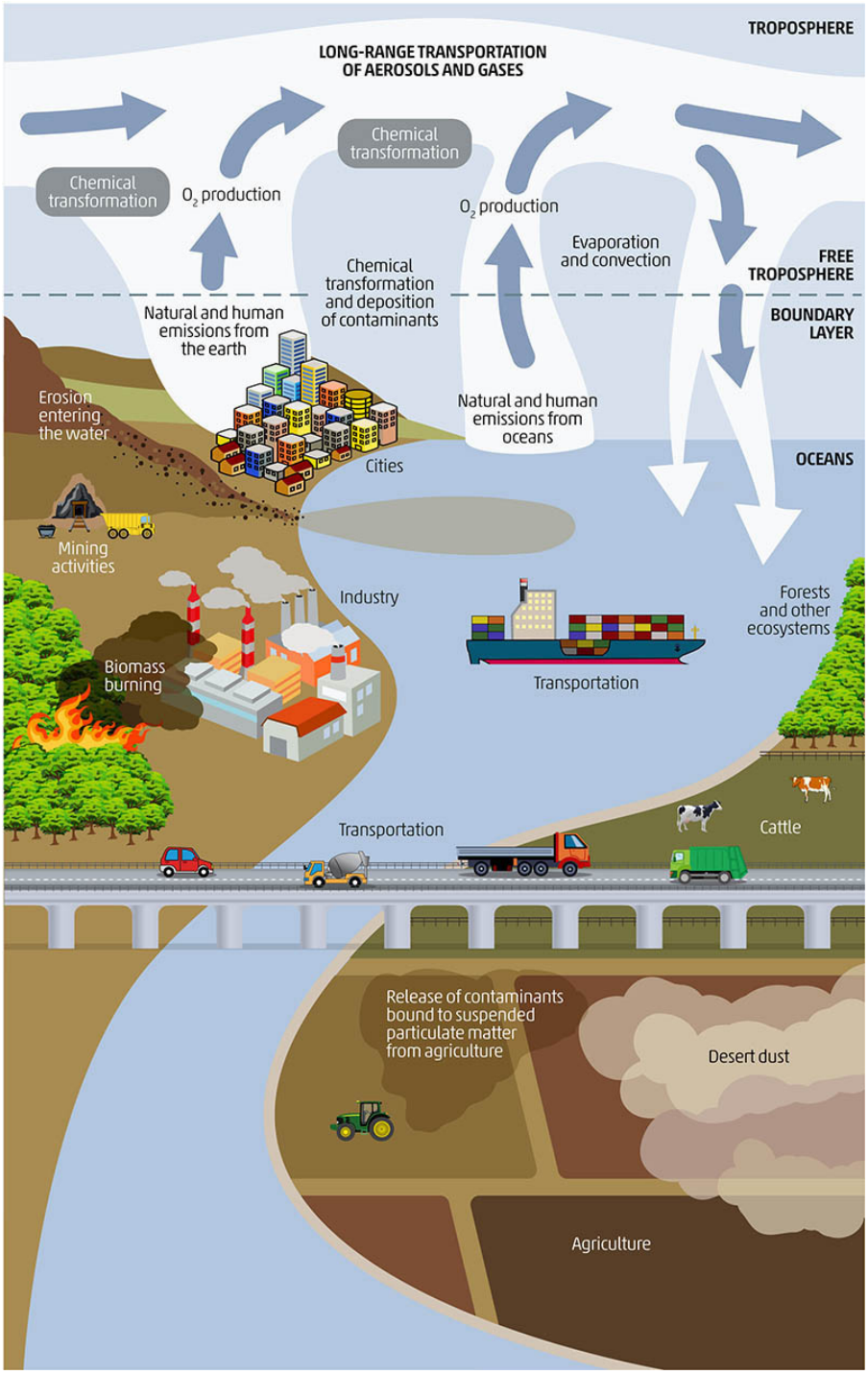

Figure 1. Point source and transport of Environmental contaminants (adopted from FAO & UNEP, 2021)

2.4.2. NON-POINT SOURCES

Non-point sources involve diffuse pollution from widespread areas, lacking a single identifiable origin, which complicates monitoring and regulation. These sources contribute to cumulative and transboundary contamination, transporting diverse pollutants across landscapes and borders (Horton *et al*., 2017; Singh, 2024). Key examples of non-point sources include:

- **Urban Stormwater Runoff:** Runoff from urban areas carries a complex mixture of contaminants, including oil, grease, PTEs, microplastics, and chemicals from roads,

buildings, and infrastructure. This runoff pollutes rivers, lakes, and wetlands, impairing water quality and ecosystem health.

- **Agricultural Runoff:** Runoff from agricultural fields introduces fertilisers, pesticides, herbicides, pathogens, and animal manure into freshwater systems. These contaminants can cause toxicity, eutrophication, and algal blooms, disrupting aquatic ecosystems and threatening human health (Ritter *et al*., 2002).

- **Soil Erosion:** Erosion from agricultural and deforested lands transports sediments and associated pollutants, such as pesticides and PTEs, into water bodies, increasing turbidity and smothering aquatic habitats.

- **Atmospheric Deposition:** Pollutants from vehicular and industrial emissions, such as volatile organic compounds and mercury, settle into freshwater systems through precipitation or dry deposition. This widespread source contributes to long-range contamination, affecting even remote ecosystems.

The diffuse and variable nature of non-point sources poses significant challenges for management, requiring integrated approaches like watershed management, sustainable agricultural practices, and urban planning to reduce their environmental footprint.

The distinct characteristics of point and non-point sources necessitate tailored strategies for pollution control. Point sources, with their identifiable origins, benefit from regulatory measures such as discharge permits, advanced wastewater treatment, and regular inspections. Non-point sources, however, demand broader interventions, including best management practices (BMPs) for agriculture, green infrastructure for stormwater management, and policies to reduce atmospheric emissions. Understanding the interplay between these sources and their associated contaminants, ranging from PTEs and microplastics to nutrients and pathogens, is critical for prioritising mitigation efforts and safeguarding freshwater ecosystems.

2.5. Environmental Fate and Transport Mechanisms of Anthropogenic Contaminants

2.5.1. Environmental Fate of Anthropogenic Contaminants

Understanding the environmental fate of anthropogenic contaminants requires a comprehensive analysis of their sources, emission patterns, and the biological, chemical, and physical processes governing their behaviour in aquatic ecosystems (Agency for Toxic Substances and Disease Registry [ATSDR], 2025). Upon entering aquatic environments, contaminants undergo transformations that influence their form, bioavailability, and distribution. Microbial activity plays a pivotal role in these processes, with certain

microorganisms degrading, transforming, or bioaccumulating contaminants such as hydrocarbons, polychlorinated biphenyls (PCBs), PAHs, PTEs, and pharmaceuticals (Fenner *et al*., 2013; Ayilara & Babalola, 2023). For instance, hydrocarbonoclastic bacteria and fungi, such as *Aspergillus* species, facilitate biodegradation and bioremediation in polluted aquatic systems. However, the efficacy of these microbial processes is modulated by environmental factors, including temperature, pH, and oxygen availability (Ayilara & Babalola, 2023).

Chemical reactions, such as oxidation, reduction, hydrolysis, and photolysis, further alter the structure, toxicity, and persistence of contaminants. Photochemical degradation and hydrolysis are particularly significant for transforming and detoxifying pharmaceutical compounds in aquatic environments (Boreen *et al*., 2003). Physical processes, including volatilisation, adsorption/desorption, diffusion, and dissolution, also contribute to contaminant dynamics. For example, adsorption to sediments, organic matter, or contaminants like MPs, can immobilise contaminants, while desorption may facilitate their remobilisation. Fine sediments, with their high surface area, exhibit greater adsorption capacity, acting as both sinks and vectors for contaminants like PTEs and microplastics (Kasperek *et al*., 2013). In mountainous river systems, sediments have been associated with elevated contaminant levels in upstream sections, limiting downstream transport (Dhivert *et al*., 2022). Sediments, soils, and biota often serve as long-term reservoirs for POPs and PTEs, which resist degradation and prolong ecological and human exposure risks. High-flow events, such as heavy precipitation or floods, can resuspend these contaminants, leading to further dispersion and the formation of pollution hotspots (Hurley *et al*., 2018).

2.5.2. Transport Mechanisms and Dynamics of Anthropogenic Contaminants

The transport of anthropogenic contaminants in freshwater systems is governed by their physicochemical properties and environmental conditions. Key properties, including water solubility, density, volatility, partition coefficients, bioconcentration factors, and half-life, dictate contaminant mobility and persistence (ATSDR, 2025). Environmental variables, such as temperature, precipitation, wind patterns, topography, and soil characteristics, further influence transport dynamics and exposure risks (FAO & UNEP, 2021). Contaminants are mobilised from emission sources to freshwater systems through several pathways, each with distinct mechanisms and implications:

- ***Atmospheric Transport:*** Volatile and semi-volatile contaminants, such as pesticides, PAHs, and PCBs, are primarily transported through the atmosphere via evaporation and deposition cycles, often referred to as the "grasshopper effect" (FAO & UNEP, 2021). This process enables long-range dispersal, with contaminants reaching remote regions, such as the Arctic and Antarctic, through wet (e.g., rain, snow) or dry (e.g., dust,

particulates) deposition. Wind patterns and atmospheric circulation significantly influence this transport.

- ***Waterborne Transport:*** Contaminants with varying solubility are conveyed through surface runoff, rivers, lakes, reservoirs, and groundwater. Hydrological features, such as glaciers, can store and release contaminants into aquatic systems upon melting, amplifying their distribution.

- ***Soil and Sediment Transport:*** Soils, sediments, and microplastics act as adsorbents and vectors, binding contaminants and facilitating their movement during erosion or surface runoff. Finer sediments, due to their larger surface area-to-volume ratio, accumulate higher contaminant loads (Kasperek *et al*., 2013).

- ***Biological Transport:*** Aquatic organisms, including microorganisms and higher trophic-level species, contribute to contaminant dispersal through uptake, bioaccumulation, and biomagnification within food webs. This process is particularly significant for persistent contaminants that accumulate in biota.

2.5.3. FACTORS INFLUENCING FATE AND TRANSPORT IN FRESHWATER SYSTEMS

Climatic and environmental variables profoundly influence the fate and transport of contaminants across terrestrial, aquatic, and atmospheric ecosystems (Bolan *et al*., 2024). Extreme weather events, such as heavy rainfall or droughts, exacerbate contaminant mobilisation. For instance, intense precipitation can trigger runoff and flooding, dispersing contaminants widely, while droughts may concentrate pollutants in reduced water volumes (Schaffer-Smith *et al*., 2023). Climate change further complicates these dynamics by altering rainfall patterns, intensifying storms, and raising temperatures, which affect the behaviour of contaminants like pesticides, pharmaceuticals, and PFAS (Bolan *et al*., 2024). Indirect climate change effects, such as water shortages or changes in land management, can also elevate contaminant levels in surface and groundwater (Lenka *et al*., 2022).

Hydrological factors, including topography, water flow velocity, precipitation, and geological composition, significantly modulate contaminant transport. In the Cape Fear River Basin, for example, hydrological conditions like droughts and floods, combined with landscape and point-source contributions, have been shown to influence contaminant loads, particularly during dry seasons (Schaffer-Smith *et al*., 2023). Runoff, a primary dispersal mechanism, is challenging to manage due to its dependence on slope, rainfall, and soil properties. Additionally, groundwater recharge and discharge play critical roles in the transport of contaminants like

PFAS, which can leach into aquifers and pose long-term risks to freshwater systems (Post *et al*., 2012).

2.6. Freshwater Organisms as Bioindicators of Water Pollution

Freshwater ecosystems are increasingly subjected to anthropogenic contaminants, necessitating robust methods for assessing water quality. Traditional physicochemical assays, often referred to as "performance-based standards," have historically been employed to evaluate soil and water quality, particularly in low- and middle-income countries (Garg *et al*., 2022). While these methods remain valuable for identifying immediate pollution sources and statuses, they are limited in addressing the complexities of emerging contaminants, contaminant mixtures, variable persistence, bioavailability, and long-term ecological and toxicological impacts (Garg *et al*., 2022). Consequently, biomonitoring using freshwater organisms has emerged as a sensitive, comprehensive, and cost-effective approach for detecting water pollution and evaluating its ecological consequences over time. These organisms, responsive to both natural and anthropogenic stressors, serve as bioindicators, providing insights into the health of freshwater systems and potential human health risks (Garg *et al*., 2022).

Bioindicator organisms reflect environmental conditions through their presence, absence, or physiological and behavioural responses, offering a historical perspective on pollution dynamics due to their lifelong exposure to chemical, physical, and biological changes in their habitats (Maine Department of Environmental Protection, 2019). The selection of appropriate bioindicators depends on the contaminant or pollution type under investigation. Hellawell (1986) outlined key criteria for selecting bioindicators, including:

- *Ease of Identification:* Organisms should be readily identifiable to minimise taxonomic complexities that could confound data interpretation.

- *Simple and Quantitative Sampling:* Species should be easily sampled with minimal equipment and personnel to ensure reliable quantitative assessments.

- *Wide Distribution:* Cosmopolitan species with broad ecological ranges are preferred to avoid misattributing the absence of narrowly distributed species to pollution.

- *Availability of Autecological Data:* Extensive ecological knowledge about the species facilitates data analysis and the development of pollution and biotic indices.

- *Economic Significance:* Species with economic value (e.g., fish) or nuisance potential (e.g., algae) provide additional justification for monitoring.

- *Pollutant Accumulation:* Bioindicators should effectively accumulate contaminants, enabling straightforward assessment of environmental contamination levels.

- *Ease of Laboratory Culture:* Species amenable to laboratory culture support experimental studies that complement field observations.

- *Low Variability:* Species with minimal genetic and ecological variability ensure consistent and reliable results across broader biological communities.

These criteria guide the selection of bioindicator species, which should ideally possess several of these attributes. The following section examines the role of fish as bioindicators, highlighting their responses, advantages, and limitations in freshwater pollution monitoring.

2.6.1. FISH AS BIOINDICATORS

Fish, comprising 41.24% of global fish species as freshwater inhabitants, are a diverse and ecologically significant group widely used in biomonitoring due to their nutritional, economic, and sociocultural importance. Their application as bioindicators likely stems from historical observations linking fish population declines to environmental stressors (Muñoz *et al*., 2022). Fish are ideal bioindicators due to their ecological roles, varying sensitivities to pollutants, presence across trophic levels, and diverse habitat requirements throughout their life stages (Fritsch, 1872; Ortmann, 1909; Forbes & Richardson, 1913; Brinley, 1942; Trautman, 1957; Grabarkiewicz & Davis, 2008). Their migratory behaviours and sensitivity to habitat fragmentation further enhance their utility in assessing ecological changes. Positioned near the top of aquatic food webs, fish bioaccumulate contaminants over time, reflecting long-term pollution impacts on both themselves and other aquatic organisms (Hellawell, 1986; Chovanec *et al*., 2003). Factors such as eutrophication, invasive species, pollution, and altered water flow dynamics can significantly affect fish communities (Wilson *et al*., 2010).

Despite their advantages, using fish as bioindicators presents challenges, including the need for multi-person sampling teams, potential misinterpretation of data due to complex life cycles and migratory behaviours, difficulties in sampling deep or fast-flowing waters, and biases inherent in sampling methods such as electroshocking or seining (Hellawell, 1986; Grabarkiewicz & Davis, 2008). Nonetheless, fish remain integral to biomonitoring, particularly through bioaccumulation monitoring (BAM).

2.6.2. BIOACCUMULATION MONITORING (BAM)

Fish are extensively utilised in BAM due to their capacity to accumulate contaminants across time and space, providing insights into water pollution status and exposure levels.

Contaminant uptake in fish occurs directly through waterborne exposure (e.g., via gills or skin) or indirectly through consumption of contaminated prey or food material (Okwuosa *et al*., 2019). **Bioconcentration** refers to direct uptake from water, while **biomagnification** describes the increased contaminant concentrations in higher trophic levels due to dietary intake. Recent studies have employed various fish species for BAM in freshwater systems, demonstrating their efficacy in detecting pollutants (Subotić *et al*., 2013a, 2013b; Ujah *et al*., 2017; Nyeste *et al*., 2019).

Lipophilic contaminants, such as POPs and pesticides, tend to biomagnify in fish tissues, accumulating at hazardous levels in top predators (Topić Popović & Strunjak-Perović, 1999). The bioaccumulation rate depends on the chemical properties of contaminants. Hydrophobic organic pollutants, including PCBs, organochlorine pesticides (OCPs), PAHs, polychlorinated dibenzofurans (PCDFs), and polychlorinated dibenzo-p-dioxins (PCDDs), are influenced by lipid and organic matter content in sediments, water, and biota (Van der Oost *et al*., 2003). Lipophilic substances with high octanol-water partition coefficients (log Kow values of 5–8) exhibit low water solubility and poor metabolism, leading to elevated uptake and storage in fish tissues, particularly in lipids and muscles (Topić Popović & Strunjak-Perović, 1999). This raises public health concerns, as fish with high lipid content can retain significant contaminant loads.

Contaminant distribution in fish varies by compound type and exposure duration. Lipophilic substances preferentially accumulate in muscles and fatty tissues, while PTEs concentrate in the liver and kidneys due to high metallothionein levels and active metabolic roles in detoxification (Emon *et al*., 2023). Fish bile is a reliable indicator of PAH exposure, with high concentrations of PAH metabolites facilitating sensitive assessments (Beyer *et al*., 2010). Bioaccumulation patterns also differ across fish species and families, influenced by metabolic rates, lipid content, feeding behaviours, and habitats (Nichols *et al*., 2004; Nyeste *et al*., 2019; Feng *et al*., 2020). For example, salmonids exhibit higher liver copper levels than other families (Okwuosa *et al*., 2019), while chubs are effective for monitoring trace metal pollution due to their tolerance of contaminated waters (Dragun *et al*., 2007; Nyeste *et al*., 2019).

2.6.3. Biological Effect Monitoring (BEM) and Environmental Risk Assessment (ERA)

Assessing the full spectrum of natural and anthropogenic contaminants in freshwater systems is impractical due to their diversity and complexity. An effective alternative involves evaluating biological responses, or biomarkers, which reflect biochemical and physiological changes in aquatic organisms exposed to contaminants. These biomarkers serve as sensitive

indicators of physiological impairments, enabling early detection of water pollution and facilitating timely mitigation to prevent long-term environmental damage (Van der Oost *et al*., 2003). By capturing immediate and chronic effects, biomarkers provide critical insights into the ecological and toxicological impacts of pollutants. Key biomarker categories for biomonitoring and ecotoxicological studies include biotransformation enzymes and products, oxidative stress markers, haematological indices, reproductive and endocrine parameters, stress and immunological proteins, and genotoxic indices (Van der Oost *et al*., 2003).

2.6.3.1. BIOTRANSFORMATION ENZYMES AND PRODUCTS

Biotransformation enzymes are critical biomarkers due to their specificity, sensitivity, and ability to link pollutant exposure to biochemical responses in fish (Van der Oost *et al*., 2003). Exposure to xenobiotics can induce or inhibit these enzymes, altering their activity levels. Phase I enzymes, such as cytochrome P450 1a (CYP1A), measured through ethoxyresorufin-O-deethylase (EROD) activity, are highly sensitive to organic pollutants like PAHS and PCBS. Phase II enzymes, including glutathione S-transferases (GST) and glucuronyltransferases, facilitate xenobiotic conjugation and detoxification (Iacopetta *et al*., 2023; Grădinariu *et al*., 2025). Biotransformation products, such as PAH metabolites in bile, provide direct evidence of pollutant metabolism and excretion (Van der Oost *et al*., 2003). While detoxification mitigates toxicity, it may produce metabolites more harmful than the parent compound, posing additional environmental risks.

2.6.3.2. OXIDATIVE STRESS MARKERS AND ANTIOXIDANT DEFENCES:

Pollutant exposure induces oxidative stress (OS) in fish by generating reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-), and superoxide anions (O_2^-), which can overwhelm antioxidant defences and damage cellular components, including lipids, proteins, and DNA (Van der Oost *et al*., 2003; Karina *et al*., 2022). Lipid peroxidation (LPO), a primary consequence of OS, compromises cell membrane integrity and produces toxic byproducts like malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) (Ayala *et al*., 2014). These byproducts, along with protein carbonyls, serve as reliable OS markers in fish (Grădinariu *et al*., 2025). Antioxidant defences, including enzymatic antioxidants like superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), and non-enzymatic antioxidants like glutathione, are also upregulated in response to ROS, acting as sensitive indicators of OS and aiding environmental risk assessment (Van der Oost *et al*., 2003).

2.6.3.3. HAEMATOLOGICAL PARAMETERS

Haematological indices provide a rapid, cost-effective method for assessing fish physiological health under toxicant exposure (Maksoud *et al*., 2018; Fazio, 2019). Parameters such as red blood cell (RBC) count, haemoglobin concentration (Hb), haematocrit, white blood cell (WBC) count, and differential leukocyte counts reflect stress responses and health status. Contaminants like PTEs, organochlorines, and PAHs can damage erythrocyte membranes, impair haemoglobin synthesis, induce OS, cause immunosuppression or inflammation, and disrupt hematopoietic tissues, leading to anaemia and other abnormalities (Srivastava & Reddy, 2020; Ahmed *et al*., 2022). Studies have reported reduced Hb, RBC, and packed cell volume (PCV) levels, alongside elevated WBC counts, in fish exposed to various toxicants (Avni & Jagruti, 2017; Deepika & Noorjahan, 2018; Alaguprathana & Poonkothai, 2021; Chris *et al*., 2022). These indices are valuable for ERA due to their sensitivity to sublethal stress, though their interpretation requires baseline data to account for natural variability influenced by age, diet, and environmental conditions (Van der Oost *et al*., 2003).

2.6.3.4. Reproductive and Endocrine Parameters

Reproductive and endocrine biomarkers, including plasma vitellogenin (VTG) levels, gonad development, sex steroid concentrations, and fertility, are essential for evaluating the impacts of EDCs such as bisphenol A, PFAS, phytoestrogens, phthalates, and pesticides (Van der Oost *et al*., 2003; Amoatey & Baawain, 2019; Guimaraes *et al*., 2023). EDCs disrupt endocrine signalling by mimicking or antagonising hormones, causing measurable changes even at low concentrations. Elevated VTG levels in male fish, a female-specific egg yolk precursor protein, indicate estrogenic EDC exposure (Celino-Brady *et al*., 2021). Altered plasma levels of sex steroids (e.g., 17β-oestradiol, testosterone, 11-ketotestosterone) reflect disruptions in the hypothalamic-pituitary-gonadal (HPG) axis, affecting gametogenesis and spawning.

Further, the gonadosomatic index (GSI) and hepatosomatic index (HSI) assess gonadal development and metabolic activity, respectively, with reduced GSI signalling impaired reproduction and elevated HSI indicating increased detoxification or pathology. Histological and cellular markers like the increased presence of degenerated oocytes in the ovarian tissues and the presence of both ovarian and testicular tissues in the same gonad are indicative of severe endocrine and immunological disruptions (Noleto *et al*., 2022). Similarly, histopathological changes, such as tissue alterations (e.g., necrosis, vacuolization, hyperplasia) in liver and gills, further correlate with pollutant exposure (Orso *et al*., 2023; Segura *et al*., 2024). Reduced reproductive behaviours (such as courtship or spawning) and fertility also highlight the reproductive and ecological consequences of endocrine disruption (Van der Oost *et al*., 2003). However, developmental plasticity and complex endocrine regulation necessitate integrating

these biomarkers with other physiological indices for robust ERA of pollutants in freshwater systems.

2.6.3.5. Other Physiological and Genotoxicity Indices

Stress and immunological proteins, along with genotoxic indices, provide additional insights into contaminants' physiological and molecular impacts. Heat shock proteins (HSPs), such as HSP60 and HSP70, are upregulated under environmental stress, protecting cells from damage. Immunological proteins, including cytokines like tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6), regulate immune responses and indicate pollution-induced inflammation or immune dysfunction (Yıldırım & Danabaş, 2014). The aryl hydrocarbon receptor (AhR) mediates xenobiotic effects, altering immune gene expression and increasing disease susceptibility in fish (Segner *et al*., 2021). Furthermore, genotoxic assays, such as the micronucleus test and comet assay, detect chromosomal damage and DNA strand breaks, serving as cost-effective tools for assessing carcinogenic and mutagenic risks (Braham *et al*., 2017; Žegura & Filipič, 2019). Collectively, these biomarkers reveal sublethal and chronic pollution effects. However, their non-specificity, technical complexities, and confounding factors (e.g., environmental variability) require careful study design and baseline data for accurate interpretation.

2.6.4. Ecosystem Indicators and Food Web Implications of Contaminant Bioaccumulation

The bioaccumulation of contaminants within aquatic ecosystems presents profound ecological challenges, compromising the integrity, stability, and functionality of freshwater environments. Ecosystem indicators, encompassing metrics such as species composition, abundance, and biodiversity, serve as indispensable tools for evaluating ecosystem health and the pervasive impacts of environmental pollutants (Van der Oost *et al*., 2003). This discourse elucidates the critical role of ecosystem indicators, explores the cascading effects of contaminant bioaccumulation and biomagnification, and evaluates their broader implications for ecosystem services and environmental risk assessment (ERA).

Effective monitoring of freshwater ecosystems relies on standardised metrics to discern the ecological consequences of contaminant exposure. Community-level assessments, such as the Fish-Based Multi-Metric Index of Biotic Integrity (mIBI-F) and the Fish Integrated Biological Index (F-IBI), integrate diverse fish characteristics—tolerance levels, trophic guilds, and reproductive behaviours—to provide a robust measure of ecosystem health (Adnan *et al*., 2024; Jargal *et al*., 2024). Ideal bioindicator species for ecological monitoring exhibit sensitivity to specific contaminants, quantifiable and concentration-dependent responses,

widespread distribution, ease of sampling, and the capacity to accumulate pollutants over time (Holt & Miller, 2010). Species that sequester highly toxic, persistent compounds with extensive spatial and temporal distribution function as early warning systems, signalling the onset of pollution and its ecological ramifications (Van der Oost *et al*., 2003).

2.6.5. BIOACCUMULATION AND BIOMAGNIFICATION IN AQUATIC FOOD WEBS

Bioaccumulation occurs when aquatic organisms absorb contaminants at a rate exceeding their metabolic or excretory capacities, resulting in elevated tissue concentrations. This phenomenon is particularly pronounced for persistent, lipophilic compounds such as DDT, polychlorinated biphenyls (PCBs), and methylmercury, which resist degradation (Chojnacka & Mikulewicz, 2014). As these contaminants traverse trophic levels, biomagnification amplifies their concentrations in apex predators, disrupting predator-prey dynamics, diminishing biodiversity, and precipitating population declines (Eero *et al*., 2021). Such disruptions destabilise food webs, alter energy flows, and undermine ecosystem resilience.

2.6.6. IMPLICATIONS FOR ECOSYSTEM SERVICES

Freshwater ecosystems provide indispensable services, including fisheries production, water quality regulation, and nutrient cycling, which underpin both ecological and human well-being. Contaminants exert direct and indirect effects that jeopardise these services, with significant ecological and societal consequences. The multifaceted impacts of contaminants on freshwater ecosystem functions and services include:

DIRECT IMPACTS

- ***Nutrient Pollution and Hypoxia:*** Excessive nutrient inputs drive eutrophication, elevating primary production and causing oxygen depletion (hypoxia) during decomposition. This creates “dead zones” that impair ecosystem vitality and reduce fish survival and yields.

- ***Toxicity to Key Species:*** Hazardous chemical pollutants diminish the survival and functionality of critical species, such as macroinvertebrates, adversely affecting essential processes like leaf litter decomposition.

- ***Human-Linked Ecosystem Services:*** Contaminants can degrade cultural and health-related services. For instance, diclofenac toxicity has been linked to declines in Gyps vulture populations in India, correlating with increased human rabies incidence.

INDIRECT IMPACTS

- ***Disruption of Keystone Species and Ecosystem Engineers:*** Contaminant accumulation disproportionately affects keystone species and ecosystem engineers (such as microbial communities and macroinvertebrates), which exert an outsized influence on ecosystem structure and function. Keystone species, such as bivalves and certain fish, enhance water quality, nutrient cycling, biodiversity, and habitat complexity. Their decline due to contaminant exposure triggers cascading effects, including shifts in community composition, reduced biodiversity, compromised ecosystem stability, food web disruptions, and habitat degradation (Peters *et al*., 2013; Rosa, 2022). Ecosystem engineers, such as mussels and macroinvertebrates, shape habitats by creating or maintaining physical structures. Contaminant-induced impairments to their populations or activities disrupt habitat integrity, nutrient cycling, and biodiversity, with far-reaching consequences for ecosystem dynamics (Mayer-Pinto *et al*., 2016; Fleeger, 2020).

- **Habitat Degradation and Alterations in Community Structure**

  Chronic exposure to contaminants undermines ecosystem engineers, leading to the deterioration of physical habitat structures and declines in species richness and evenness (Borja *et al*., 2011; Gusmao *et al*., 2024). This shift favors tolerant species, fostering biotic homogenization and reducing ecosystem resilience (Schiesari *et al*., 2018). The resultant loss of biodiversity heightens vulnerability to additional stressors, further impairing ecosystem functions and services (Mustafa *et al*., 2024).

- ***Reduced Fisheries and Resource Availability:*** Contaminant pollution diminishes primary production, limiting energy flow through food chains and reducing resource availability for consumers (Johnston *et al*., 2015). Bioaccumulation of toxic xenobiotics in fish necessitates fishery closures and public health advisories, threatening food security and livelihoods.

- ***Ecological Traps in Restoration Efforts:*** Contaminated soils in restored habitats, such as wetlands, may attract colonizing species but impair their survival and reproduction, hindering biodiversity recovery and ecosystem service restoration.

- ***Recreational and Cultural Values:*** Declines in biodiversity and ecosystem degradation diminish freshwater systems' recreational and cultural significance, adversely affecting community well-being.

2.6.7. ECOSYSTEM MONITORING AND ENVIRONMENTAL RISK ASSESSMENT

Ecosystem monitoring employs systematic, standardised surveys conducted across temporal and spatial scales, often integrated with chemical and biological assessments through methodologies like the Triad approach, enhancing the robustness of classical ERA (Grassi *et al*., 2022). These surveys provide comprehensive data on the effects of pollution on food webs and ecosystem functions (Van der Oost *et al*., 2003). While ecosystem indicators effectively capture broad ecological impacts, their sensitivity to early pollutant effects is limited, and their implementation demands extensive field-based data collection, rendering them resource-intensive compared to laboratory or mesocosm studies. Nevertheless, integrating these indicators with complementary monitoring approaches enhances the precision and reliability of environmental assessments.

3. EXPERIMENT I

IDENTIFICATION OF BIOMARKERS FOR THE ASSESSMENT OF FRESHWATER POLLUTION USING CAGED MUSSELS IN THREE RESERVOIRS IN BULGARIA: A PILOT STUDY

3.1. Background of Study

Anthropogenically driven freshwater pollution has emerged as a critical global challenge, threatening aquatic ecosystems and human health. Inorganic contaminants, such as heavy metals and metalloids (e.g., As, Al), and organic pollutants, including PBDEs, CPs, PAHs, and pesticides, are widely used in industrial and commercial applications. However, their persistence, toxicity, and bioaccumulation properties have elevated them to priority status for international concern and regulation (United Nations, 2017; European Commission, 2020; Kostka & Leśniak, 2020). These pollutants disrupt ecological balance, impair organismal health, and pose significant risks to human populations through dietary exposure, necessitating robust monitoring and assessment strategies.

Biomarkers serve as indispensable tools for evaluating aquatic pollution by quantifying contaminant exposure and its biological impacts on aquatic organisms. An integrated biomarker approach, encompassing biochemical, histological, genotoxic, and physiological indices, provides a comprehensive framework for assessing the type, extent, and severity of pollutant-induced alterations (Turja *et al*., 2015; Larsson *et al*., 2018). Such methodologies are vital for ecotoxicological research, enabling scientists to elucidate the mechanisms of contaminant toxicity and inform environmental management (Swaleh *et al*., 2020). This pilot study investigates the bioaccumulation of selected priority substances in water, transplanted moss, and mussels, focusing on three anthropogenically stressed reservoirs in Bulgaria: Kardzhali, Studen Kladenets, and Zhrebchevo. These reservoirs, subjected to decades of industrial, agricultural, and domestic pollution, offer a critical opportunity to examine contaminant impacts in a real-world setting.

The study hypothesises that long-term exposure to contaminated waters in these reservoirs will induce measurable changes in selected biomarkers in caged mussels after a 30-day exposure period. Its primary aim is to develop an integrated methodology for assessing PTEs (e.g., trace and macro-elements), PBDEs, and short-chain CPs using mussel transplants. Specifically, the research utilised the Chinese pond mussel (*Sinanodonta woodiana* [Lea, 1834]), a filter-feeding species known for its bioaccumulation capacity and sensitivity to pollutants. The methodology employs a multi-biomarker approach, incorporating biometric

measurements, histochemical, biochemical, and genotoxic assays to explore potential correlations between pollutant concentrations and biological responses. By characterising these relationships, the study seeks to establish a robust framework for monitoring freshwater pollution and evaluating its ecological implications in Bulgarian reservoirs, contributing to broader efforts to safeguard aquatic ecosystems and public health.

3.2. Materials and Methods

3.2.1. TEST ORGANISM

This study was carried out using the Chinese pond mussel, *S. woodiana* (Lea, 1834), commonly referred to as the Eastern Asiatic freshwater clam or swan-mussel, as the test organism. This mussel species was selected due to its role as an effective biofilter, its widespread presence across Asia, Europe, and the Americas, and its relatively large size, which allowed for various analytical procedures (Kim *et al*., 2011; Douda *et al*., 2012). Although classified as an invasive species in Europe, with recognized negative impacts on the native Unionid species, the Chinese pond mussel also has some known important nutritional and economic values, particularly in its native ranges, including China and Indonesia (Chen *et al*., 2015; Bolotov *et al*., 2016)

3.2.2. STUDY SITES

Three major dam reservoirs in Bulgaria were chosen for this investigation: Kardzhali (41.638475 N, 25.304432 E), Studen Kladenets (41.622244 N, 25.441933 E), and Zhrebchevo (42.585571 N, 25.885592 E). These reservoirs experience varying degrees and types of long-term anthropogenic pressures (Figure 2).

The Kardzhali Reservoir, created by damming the Arda River in the Rhodope Mountains, supports extensive power generation and cage aquaculture, including one of Europe's largest sturgeon farms (Dochin and Iliev, 2019). The Arda River, flowing through Bulgaria, Greece, and Turkey before reaching the Aegean Sea, has historically been impacted by PTE pollution from a now-closed lead-zinc processing facility and ore mines, resulting in persistent water quality degradation (Velcheva, 2006). However, recent data on contamination levels and their effects on bioindicator species in this reservoir remain limited (Zhelev *et al*., 2015).

Figure 2. 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. Geocoordinates of the localities: K – 41.638475 N, 25.304432 E; SK – 41.622244 N, 25.441933 E; Z – 42.585571 N, 25.885592 E; P – 42.164785 N, 24.756515 E.

Studen Kladenets Reservoir, the third biggest in Bulgaria, has been heavily affected by a former lead-zinc ore processing plant. Previous studies, though sparse and dated, have primarily focused on PTE accumulation in freshwater fish (Velcheva, 1995; Velcheva, 1997). Research on PTE content in fish organs has been reported (Bachvarov and Velcheva, 1995), but there is a notable lack of information on the effects of human-induced pollutants on biomarkers in vital aquatic species, including fish or mussels (Arnaudova *et al*., 2008; Velcheva *et al*., 2010).

Zhrebchevo Reservoir, situated alongside the Tundzha River within the Maritsa River's drainage basin (Aegean Sea), is primarily contaminated by intensive agricultural activities, with some evidence of PTE pollution (Kalchev *et al*., 2013; Zhelyazkov *et al*., 2014). The reservoir serves multiple purposes, including generating energy, irrigation, fish farming, leisure, and angling (Ognjanova-Rumenova *et al*., 2013). Fluctuations in zooplankton composition due to water pollution and hydro-technical structures were documented earlier in 1981 (Naidenow *et al*., 1981).

3.2.3. FIELD EXPERIMENT

Mussels were manually collected from a fishpond at the Institute of Fisheries and Aquaculture (42.143611 N, 24.816111 E) in Plovdiv, Bulgaria, which represents a reference site with no recorded anthropogenic stress (www.ira-plovdiv.bg). The mussels, all from a uniform size class (weight: 154 ± 5.5 g; shell length: 11 ± 3.5 cm), were gathered a few days before deployment. Plovdiv, Bulgaria's second-largest city and cultural hub, lies along the Maritsa River. Though the city is a fast-developing one, the Institute is located in a rural area surrounded by fields, far from Plovdiv's urban centre, which provides optimal conditions for aquaculture.

No mussels were transplanted to the reference site from other locations, nor were external mussels introduced to the three study reservoirs. On the day of collection, the mussels were transported to Plovdiv University's laboratory and maintained in a 100 L tank with a 50:50 mix of dechlorinated water and water from the reference location, equipped with air pumps. Parameters such as conductivity, dissolved oxygen, pH, and temperature were monitored and found to be stable, ensuring no effect on the biomarkers of interest. Subsequently, 30 mussels were transported in oxygenated water containers to the reservoirs and placed in stainless-steel cages (30 × 15 × 10 cm) at a 2 m depth, with 10 mussels per cage in each reservoir (Gecheva *et al*., 2020). The cages, with a 25 mm mesh size, allowed water flow while securing the mussels (Kazour and Amara, 2020). The mussels were not supplemented with food and were retrieved after 30 days of exposure. Due to funding constraints, the reference site served as the control, and no initial (day 0) sampling was conducted, as biomarker changes were assumed to require longer exposure to manifest. Soft tissues were extracted at the study sites for biomarker analysis, following the EMERGE protocol adapted for mussels (Rosseland *et al*., 2003). Mussel sex was not considered, as it was not a variable of interest.

3.2.4. BIOMARKER DETERMINATIONS

3.2.4.1. BIOACCUMULATION ANALYSES

Concentrations of 17 elements (Al, As, Ca, Cd, Co, Cr, Cu, Fe, Hg, K, Mg, Mn, Na, Ni, P, Pb, Zn) and organic pollutants, including PBDE congeners (28, 47, 99, 100, 153, 154) and short-chain chlorinated paraffins (SCCPs), were measured in surface water and whole soft tissues of transplanted mussels at day 30. For element analysis of mussel tissues, samples were subjected to microwave-assisted acid digestion in closed vessels to prevent loss of volatile elements, such as Hg (U.S. EPA, 1996). Water samples were analysed directly. Determinations were performed using inductively coupled plasma atomic emission spectrometry (ICP-AES), inductively coupled plasma mass spectrometry (ICP-MS), and gas chromatography-mass

spectrometry (GC-MS) (Thermo Scientific, USA) following established protocols (Gecheva *et al*., 2020; Gecheva *et al*., 2021). The Metal Pollution Index (MPI) was calculated to assess the total metallic and trace elemental contents (excluding macro-elements: Al, As, Cd, Co, Cr, Cu, Fe, Hg, Mn, Ni, Pb, Zn) in mussels from the reference and study sites, using the formula:

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

where Cn represents the mean concentration of element n in the tissue (mg kg^{-1} wet weight) (Ju *et al*., 2017; Nyeste *et al*., 2019). The bioaccumulation results and factors for mussels in the three reservoirs were previously reported (Gecheva *et al*., 2020; Gecheva *et al*., 2021). This study primarily examines biomarker responses in *S. woodiana* relative to toxicant levels in the water.

3.2.4.2. HISTOCHEMICAL TECHNIQUE

Histochemical analysis was performed using a cryostat (Leica, CM 1520, Germany) to section samples. Gill sections (6 µm) from each mussel were prepared following the standard PAS method (McManus, 1948) and examined under a light microscope (Leica DM 2000 LED, Germany) with an attached camera in a blinded manner. Gill lesions were evaluated semi-quantitatively using Bernet's grading system, adapted for this study (Bernet *et al*., 1999). A positive PAS reaction appeared as purple-magenta staining. Histochemical changes were averaged and graded as follows: (0) no staining reaction; (1) very weak positive reaction; (2) weak positive reaction; (3) moderate positive reaction; (4) strong positive reaction.

3.2.4.3. BIOCHEMICAL ANALYSES

All chemicals used were of analytical grade, sourced from Sigma Aldrich-Merck (Germany). Biochemical assays were conducted at 25°C using a spectrophotometer (Beckman Coulter, DU 800, USA). Digestive glands were rapidly thawed on ice, homogenised in chilled phosphate buffer (pH 7.4, 50 mM, 300 mM NaCl) using a Potter Elvehjem homogeniser with a Teflon pestle (Thomas Scientific, USA), and centrifuged at 9000 rpm for 15 minutes at 4°C (MPW 351 R, Poland). Supernatants were aliquoted, preserved at -80°C, and later used for antioxidant and metabolic enzyme analyses.

Catalase (CAT, EC 1.11.1.6) activity was determined by the reduction in absorbance at 240 nm due to H_2O_2 decomposition (Beutler, 1984; Aebi, 1984). Glutathione reductase (GR, EC 1.8.1.7) activity was assessed by observing NADPH oxidation at 340 nm (Zhou *et al*., 2019). Glutathione peroxidase (GPx, EC 1.11.1.9) activity was determined following the methodological descriptions of Wendel (1980). Cholinesterase (ChE, EC 3.1.1.8) activity was measured by absorbance reduction at 405 nm (Burtis and Ashwood, 1994). Lactate

dehydrogenase (LDH, EC 1.1.1.27) activity was quantified by pyruvate consumption via NADH oxidation at 340 nm (Vassault, 1983). Alanine aminotransferase (ALT, EC 2.6.1.2) and aspartate aminotransferase (AST, EC 2.6.1.1) activities were measured using commercial kits (Merck, Germany) per Reitman and Frankel (1957) and Bergmeyer *et al*. (1985). Total protein content was determined using the protocol of Bradford (1976) with Coomassie Brilliant Blue G-250, standardized against bovine serum albumin, and measured at 595 nm (mg protein/mL homogenate). All assays were carried out in triplicate, with enzyme activities expressed as international units per milligram of protein (U/mg protein).

3.2.4.4. COMET ASSAY

Haemolymph was extracted from the adductor muscle of individual mussels, and the samples were subjected to centrifugation at 1000 rpm for 10 minutes using a centrifuge (MPW 351 R, Poland) prior to comet assay analysis. The alkaline comet assay was performed, adapted from the protocol described by Singh *et al*. (1988) with minor modifications. Agarose layer preparation on microscope slides followed the method outlined by Kolarević *et al*. (2016). After staining with SYBR Green I (diluted 1:10,000), nucleoids exhibiting comet-like structures were visualised at 400× magnification using a Leica DM1000 LED epifluorescence microscope equipped with an I3 filter (Leica, Germany) and an attached camera. For each mussel, 50 nucleoids were analysed using the Comet Assay IV software (Perceptive Instruments, UK). DNA damage was quantified using the Tail Intensity (TI%) parameter, which represents the proportion of DNA present in the comet tail.

3.2.4.5. INTEGRATED BIOMARKER RESPONSE (IBR) CALCULATION

Integrative indices were employed to consolidate biomarker responses for biomonitoring purposes (d'Errico *et al*., 2021; Manfra *et al*., 2021). The Integrated Biomarker Response (IBR) was computed following the procedures of Beliaeff and Burgeot (2002) for a structured approach for mussel-based biomonitoring. The IBR calculation process is outlined as follows:

1. *Calculation of Descriptive Statistics*: For each biomarker, compute the mean and standard deviation across all sampling sites.
2. *Data Standardisation:* Standardise the biomarker data for each site using the formula [Eq. (2)]:

$$Y = \frac{X - m}{s} \qquad \text{(Eq. 2)}$$

where (Y) is the standardized biomarker value, (X) is the mean biomarker value at a specific site, (m) is the overall mean of the biomarker across all sites, and (s) is the standard deviation of (m).

3. *Determination of Z Value*: Calculate the (Z) value based on the biological effect, using [Eq. (3)]:

$$Z = -Y \text{ or } Z = Y \quad \text{(Eq. 3)}$$

Depending on whether the biomarker indicates inhibition (negative effect) or activation (positive effect), respectively.

4. *Score Calculation*: Compute the score (S) for each biomarker by adding the absolute value of the minimum standardised value (min) across all sites to the Z value, as per [Eq. (4)]:

$$S = Z + |\min| \quad \text{(Eq. 4)}$$

Where S ³ 0.

This process is applied to all biomarkers. To derive the IBR, star plot areas are calculated by multiplying the score of each biomarker (Si) by the score of the subsequent biomarker in a circular arrangement, dividing each product by 2, and summing the results [86]. The IBR is expressed as [Eq. (10)]:

$$IBR = \left(\left(\frac{S1 \times S2}{2}\right) + \left(\frac{S2 \times S3}{2}\right) + \cdots \left(\frac{Sn-1 \times Sn}{2}\right) + \left(\frac{Sn \times S1}{2}\right)\right) \quad \text{(Eq. 5)}$$

This formula yields the final IBR value, integrating the responses of all biomarkers into a single metric for comparative analysis across sampling sites.

3.2.5. DATA PROCESSING

Statistical analyses were conducted using Past 3.03 (Hammer *et al*., 2001) and GraphPad Prism 7 (USA). Data normality was assessed with the Shapiro-Wilk test, and variance homogeneity was evaluated using Levene's test. Differences between reference and exposed mussel groups were analysed using one-way ANOVA followed by Tukey's post-hoc test. Spearman's non-parametric correlation test examined relationships between MPI and biomarker responses from the reference and sampling sites based on the gill's PAS-reactions, the biochemical indices from the digestive gland, and the haemocytes' DNA damage. Significance was set at $p < 0.05$, and biomarker results were reported as mean ± standard deviation (SD).

3.3. RESULTS

3.3.1. BIOACCUMULATION ANALYSES

This research constitutes a component of a pilot study aimed at evaluating contamination levels in three Bulgarian reservoirs, Zhrebchevo, Kardzhali, and Studen Kladenets, while examining the bioaccumulation and physiological effects of pollutants on mussels through biomarker assessments. Concentrations of elements and organic contaminants

in water and mussel samples from the reservoirs and reference site are detailed in Tables 1 and 2. Findings indicate that Studen Kladenets exhibited the highest pollution levels among the reservoirs, with Kardzhali and Zhrebchevo showing lesser but notable contamination (Table 1). The elevated pollution in Studen Kladenets likely stems from its role as a terminal sink for pollutants along the Arda River, including inputs from an operational alloy factory and discharges from four decommissioned mining sites.

The MPI values for mussels were 0.95 (reference site), 1.58 (Zhrebchevo), 1.65 (Kardzhali), and 1.72 (Studen Kladenets). These values reflect the lowest PTE bioaccumulation at the reference site and the highest in Studen Kladenets mussels. Additionally, mean concentrations of brominated diphenyl ethers (BDEs) peaked in Studen Kladenets mussels, while short-chain chlorinated paraffins (SCCPs) were most elevated in Zhrebchevo mussels (Table 2).

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

		Sampling sites		
	Reference site	**Zhrebchevo**	**Kardzhali**	**Studen Kladenets**
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

3.3.2. BIOMARKER RESPONSES

Biomarkers serve as critical indicators of biological responses, offering insights into freshwater contamination levels, organism health, and potential human health risks (Blaise *et al*., 2017). No mortality was observed in mussels across any reservoir during the exposure

period. Results of the PAS reaction in gills, activities of oxidative stress and metabolic enzymes, and the IBR for mussels from all sites are presented in Table 3.

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.

3.3.3. HISTOCHEMICAL OBSERVATIONS

Histochemical analyses revealed a moderately positive PAS reaction in the gills of reference site mussels, indicative of glycogen presence. In contrast, mussels from the tested reservoirs displayed a general reduction in gill glycogen content. Mussels from Kardzhali and Studen Kladenets reservoirs exhibited a weak positive PAS reaction, characterised by faint pink-violet staining, while those from Zhrebchevo showed a moderately positive reaction, comparable to the reference group, suggesting similar glycogen levels in gill cells (Table **3**; Figure 3).

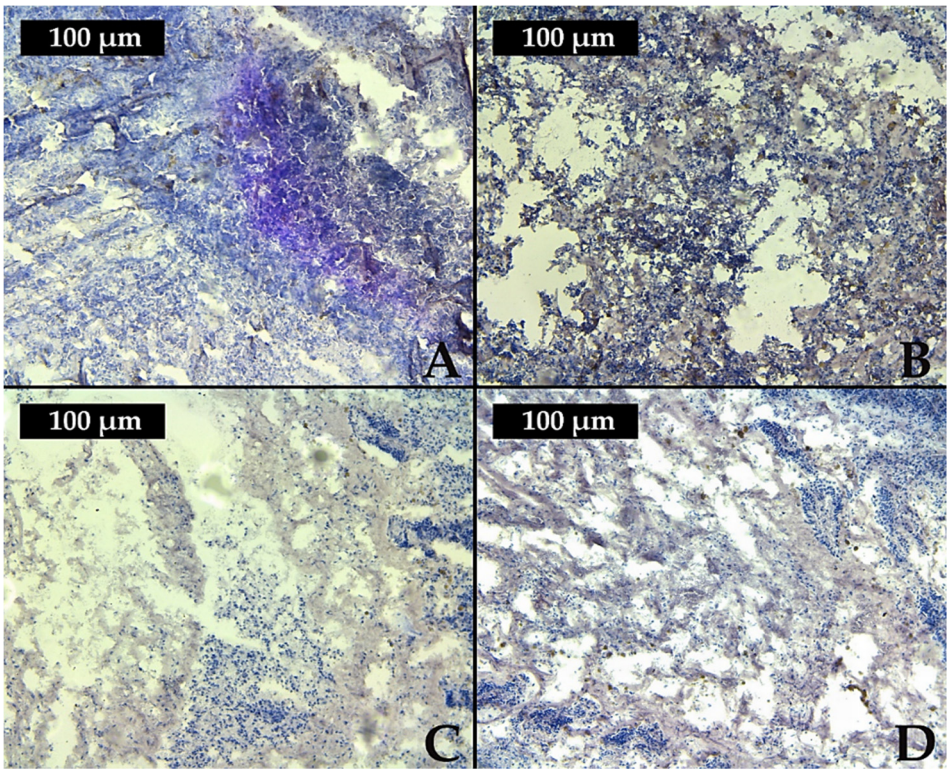

Figure 3. Intensity of PAS-reaction in the gills of mussels: A – reference site (400× magnification), B – Zhrebchevo (400× magnification); C – Kardzhali (400× magnification); and D – Studen Kladenets reservoirs (400× magnification).

3.3.4. Biochemical Responses

3.3.4.1. Oxidative Stress-Related Enzymes

Activities of oxidative stress-related enzymes, CAT, GPx, and GR, in mussel digestive glands are summarised in Table 3. CAT activity was significantly elevated in mussels from Studen Kladenets and Kardzhali compared to the reference group (ANOVA, $F = 324.3$, $p < 0.001$). No significant differences were observed in oxidative stress enzyme activities between Zhrebchevo and reference site mussels ($p > 0.05$). GPx activity was significantly higher in Studen Kladenets mussels compared to those from the reference site and Zhrebchevo (ANOVA, $F = 7.066$, $p < 0.01$). Similarly, GR activity was significantly increased in mussels from Studen Kladenets and Kardzhali relative to the reference and Zhrebchevo sites (ANOVA, $F = 15.680$, $p < 0.001$).

3.3.4.2. Metabolic-Related Enzymes

The present study revealed significant differences ($p < 0.05$) in the activities of metabolic enzymes in *S. woodiana* mussels across the sampled reservoirs (Table 3). LDH activity was markedly lower in mussels from Zhrebchevo and Studen Kladenets reservoirs compared to the reference site (ANOVA, $F = 81.69$, $p < 0.001$), while LDH levels in Kardzhali mussels were comparable to the reference. Similarly, AST activity varied significantly among sites (ANOVA, $F = 412.80$, $p < 0.001$), with all tested reservoirs showing reduced AST levels

relative to the reference. ALT activity was also significantly lower in mussels from Studen Kladenets and Kardzhali (ANOVA, $F = 29.20$, $p < 0.001$). Additionally, ChE activity exhibited significant differences (ANOVA, $F = 83.57$, $p < 0.001$), with the lowest levels observed in mussels from Kardzhali and Studen Kladenets compared to the reference site.

3.3.5. DNA DAMAGE

Significant variations were observed in the DNA tail intensity percentage within haemocytes of mussels collected from different sampling locations (ANOVA, $F = 277.1$, $p < 0.001$) (Table 2). Additionally, a notable increase in DNA migration was detected in the nucleoids of haemocytes from caged mussels across the three studied reservoirs, with mussels from Studen Kladenets Reservoir exhibiting the highest degree of migration (Figure 4).

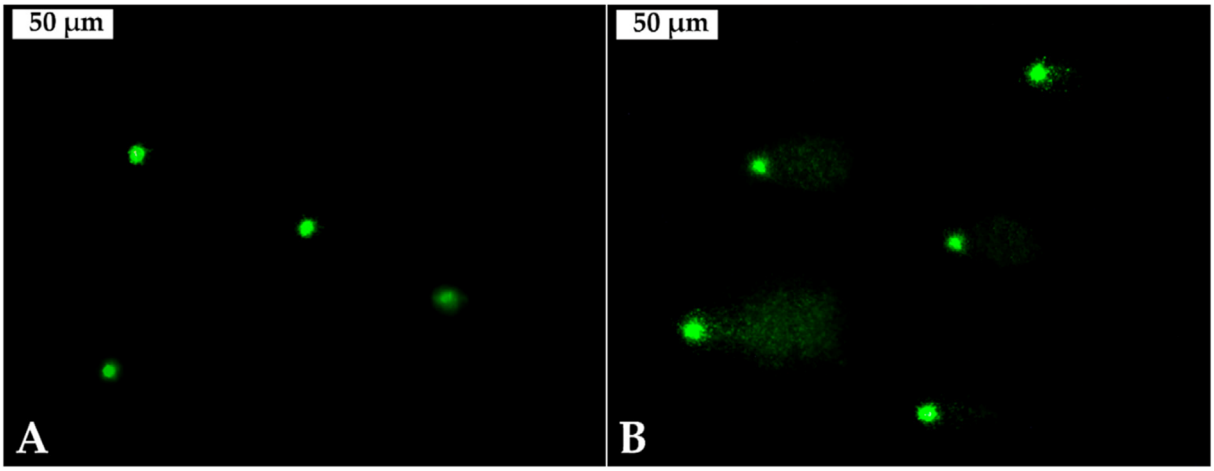

Figure 4. Comet assay images of haemocytes from Sinanodonta woodiana mussels: (A) Reference site; (B) Studen Kladenets Reservoir (400× magnification).

3.3.6. RELATIONSHIPS BETWEEN POLLUTANT LEVELS AND BIOMARKER RESPONSES

Correlation analyses revealed significant relationships ($p < 0.05$) between the MPI and biomarker responses in *S. woodiana* mussels (Table 4). Oxidative stress enzyme activities (CAT, GPx, GR) showed positive correlations with MPI, while metabolic enzyme activities (AST, ALT, ChE) exhibited negative correlations, except for LDH. DNA damage, measured as TI%, was positively correlated with MPI ($p < 0.05$). The Integrated Biomarker Response (IBR) index indicated the lowest pollution stress at the reference site, followed by Zhrebchevo Reservoir, with higher values in Kardzhali and Studen Kladenets reservoirs (Table 3).

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).

3.4. DISCUSSION

3.4.1. BIOACCUMULATION AND ENVIRONMENTAL CONTAMINATION

The elevated contamination in Studen Kladenets, as evidenced by higher MPI and BDE concentrations, emphasises its role as a cumulative repository for pollutants along the Arda River. The presence of an alloy factory and legacy mining discharges likely exacerbates this condition, aligning with prior studies on PTE impacts in the region (Gecheva *et al*., 2020; Gecheva *et al*., 2021). Zhrebchevo's higher SCCP levels may reflect agricultural runoff, given its location in an intensively farmed region (Kalchev *et al*., 2013). These findings highlight the reservoirs' varying contamination profiles, with Studen Kladenets being the most impacted, consistent with bioaccumulation data (Tables 1 and 2).

3.4.2. HISTOCHEMICAL INSIGHTS

Histochemical and histological biomarkers are pivotal for assessing environmental pollution effects, as they directly reflect organism health (Au, 2004). The observed glycogen depletion in the gills of mussels from Kardzhali and Studen Kladenets suggests chemical stress induced by PTE and organic pollutants, which aligns with reports of glycogen metabolism disruption under contaminant exposure (Javed and Usmani, 2015; Ngo *et al*., 2022). Glycogen is metabolised via gluconeogenesis to meet energy demands during stress, a process requiring substantial energy reserves (Köhler *et al*., 1996; Ansaldo *et al*., 2006). The weaker PAS reaction in these reservoirs compared to Zhrebchevo and the reference site indicates varying degrees of energy reserve depletion, potentially linked to glycolysis under stress conditions (Da Silva Souza *et al*., 2011). These results corroborate findings of glycogen and lipid depletion in molluscs exposed to contaminants (Triebskorn and Köhler, 1996; Ansaldo *et al*., 2006), with no lethal outcomes observed, suggesting mussels' resilience to short-term exposure.

3.4.3. BIOCHEMICAL RESPONSES

3.4.3.1. OXIDATIVE STRESS ENZYMES

ROS are highly reactive molecules generally generated by various cellular processes and are much higher during exposure to environmental stress. Oxidative stress occurs when there is an imbalance between the generation of ROS and the body's antioxidant capacity to neutralise them, which may lead to various pathological conditions, including disruption of normal cellular functions, impairment of DNA repair mechanisms, inflammation, cellular dysfunctions, and even death. PTEs and organic (PAHs, PCBs) contamination in waters are widely known to promote the generation of ROS in aquatic organisms (Ferreira *et al*., 2019; Ferreira *et al*., 2020; Ferreira *et al*., 2021), which may trigger defence mechanisms or cause oxidative cellular damage to macromolecules, such as lipid peroxidation (Wang *et al*., 2019). Overall, our findings reveal that the oxidative stress enzyme activities of mussels from the Zhrebchevo Reservoir did not significantly differ from those of the reference location. The observed increase in the oxidative stress-related enzymatic activities in mussels from Studen Kladenets when compared to those from Zhrebchevo Reservoir and the reference site indicates that Studen Kladenets Reservoir was the most polluted water basin, whereas Zhrebchevo Reservoir was the least polluted. These findings are in line with earlier reports of higher bioaccumulation of contaminants in mussels from these sites, and as presented in Tables 1 and 2 (Gecheva *et al*., 2020; 2021).

Elevated CAT activity in *S. woodiana* mussels from the Studen Kladenets and Kardzhali reservoirs, relative to the reference group, likely reflects heightened pollutant exposure at these sites. This observation aligns with findings by Regoli *et al*. (2014), who noted that under environmental stress, organisms upregulate antioxidant enzyme activities, including CAT, to neutralise excessive ROS, particularly hydrogen peroxide (H_2O_2), thereby mitigating oxidative damage to cellular components. The increased CAT activity observed in this study corroborates reports by Duarte *et al*. (2011), Freitas *et al*. (2014), and Velez *et al*. (2015), which documented enhanced CAT levels in various mussel species exposed to PTEs. These findings emphasise the critical role of the antioxidant enzymatic system in mussel cells, particularly in detoxifying superoxide (O_2^-), a primary precursor of other ROS, to maintain cellular homeostasis under polluted conditions. This adaptive response highlights the sensitivity of CAT as a biomarker for oxidative stress induced by anthropogenic contaminants in freshwater ecosystems.

The GPx enzyme plays a pivotal role in mitigating oxidative stress by catalysing the reduction of H_2O_2, a byproduct of superoxide radical dismutation mediated by SOD. Additionally, GPx facilitates the conversion of lipid hydroperoxides into less reactive alcohols,

thereby protecting cellular membranes from oxidative damage (Lesser, 2006). The elevated GPx activity observed in *S. woodiana* mussels from the Studen Kladenets reservoirs aligns with findings by Orbea and Cajaraville (2006), who reported increased GPx levels in invertebrates from heavily contaminated sites compared to reference locations. Similarly, Cong *et al*. (2012) documented enhanced GPx activity in bivalves exposed to PTEs under controlled laboratory settings. These consistent patterns point out GPx's critical role in counteracting oxidative stress induced by environmental pollutants, particularly in freshwater ecosystems subject to anthropogenic pressures.

Complementing GPx, GR is an NADPH-dependent enzyme essential for regenerating GSH from its oxidised form (GSSG). GSH is a vital cofactor for antioxidant enzymes like GPx and biotransformation enzymes such as glutathione S-transferase (GST), which are integral to ROS neutralisation and xenobiotic detoxification (Regoli *et al*., 2014; Ferreira *et al*., 2019). The increased GR activity observed in the Studen Kladenets and Kardzhali reservoirs when compared to the reference and Zhrebchevo sites corroborates findings by Lacroix *et al*. (2015), who suggested that enhanced GSH production supports ROS scavenging and phase-II biotransformation processes in polluted environments. Similarly, Ferreira *et al*. (2019) observed elevated GR activity in transplanted oysters from a Brazilian estuary, attributing it to an elevated cellular GSH demand to sustain detoxification processes. These results highlight the coordinated upregulation of GR and GPx as part of an adaptive antioxidant response to maintain redox homeostasis under pollutant stress.

The biochemical adaptations observed in caged mussels, including heightened GPx and GR activities, are likely energetically demanding, leading to increased metabolic costs. Sokolova *et al*. (2012) noted that such responses divert energy from basal physiological functions, such as growth and reproduction, toward detoxification and stress mitigation. This hypothesis is supported by the present study's histochemical analyses, which revealed reduced glycogen levels (via PAS staining), indicative of energy reallocation to cope with pollutant exposure. Drawing on Lacroix *et al*. (2015), the study proposes that the mussels may have activated aerobic metabolism to meet these toxicant-induced elevated energy demands, potentially increasing ROS production and lipid peroxidation as byproducts of enhanced mitochondrial activity. ROS generation may occur directly from toxicants, such as PTEs or organic pollutants (e.g., polycyclic aromatic hydrocarbons [PAHs], polychlorinated biphenyls [PCBs]), or indirectly through the electron transport chain, a primary site of ROS production during heightened aerobic respiration (Sokolova *et al*., 2012). The observed enzymatic responses in caged mussels reflect a dynamic interplay between pollutant-induced stress and adaptive defence mechanisms. The significant upregulation of GPx and GR in mussels from

polluted reservoirs, particularly Studen Kladenets, suggests a robust antioxidant response to counteract ROS production driven by PTEs and organic pollutants. These findings align with broader ecotoxicological research, which emphasises the role of antioxidant enzymes in mitigating oxidative stress in aquatic organisms (Murphy, 2009; Sokolova *et al*., 2012). However, the energetic trade-offs associated with these responses, as evidenced by glycogen depletion, highlight the physiological costs of chronic pollutant exposure.

The interplay between pollution and oxidative stress is well-documented, with PTEs and organic contaminants known to promote ROS formation, triggering either protective antioxidant defences or oxidative damage to macromolecules, such as lipids, proteins, and DNA (Wang *et al*., 2019; Regoli & Giuliani, 2014). In this study, the focus on antioxidant enzymes (CAT, GPx, GR) as biomarkers of oxidative stress in transplanted mussels provides valuable insights into their response to environmental contaminants in Bulgarian reservoirs. However, due to funding constraints, key complementary markers, such as GST, SOD, and lipid peroxidation (e.g., MDA levels), were not assessed. This limitation highlights the need for future research to comprehensively evaluate the balance between ROS generation and antioxidant defences. As noted by Lacroix *et al*. (2015), when antioxidant mechanisms fail to neutralise excessive ROS, oxidative damage, including lipid peroxidation, may occur, compromising cellular integrity and organismal health.

3.4.3.2. METABOLIC ENZYMES

Metabolic enzymes are critical for cellular processes, including energy production, nutrient metabolism, and environmental stress responses, facilitating survival and homeostasis (Dong *et al*., 2017). These enzymes, encompassing dehydrogenases, transferases, and others, mediate functions such as cellular respiration, proteolysis, and detoxification. The observed reduction in LDH, AST, ALT, and ChE activities in mussels from contaminated reservoirs suggests metabolic disruptions linked to pollutant exposure, reflecting compromised physiological functions in polluted environments. LDH, a key enzyme in anaerobic metabolism, supports energy production under oxidative stress, particularly following exposure to PTEs (Das *et al*., 2004). It catalyses the conversion of pyruvate to lactate, generating ATP during cellular respiration. Pollutants, such as PTEs, can impair gill function, reducing oxygen availability and promoting anaerobic respiration, typically marked by elevated LDH activity **(**Parveen *et al*., 2017). However, the decreased LDH activity observed in Zhrebchevo and Studen Kladenets may indicate tissue damage or metabolic suppression, potentially due to chronic pollutant exposure, as lower enzyme activity can also signify cellular impairment (Parveen *et al*., 2017). LDH activity thus serves as a reliable indicator of anaerobic capacity

and oxidative stress-induced damage, including necrotic lesions and increased cell permeability (Gagnon & Holdway, 1999).

AST and ALT, transaminases central to amino acid metabolism, are sensitive markers of hepatotoxicity and tissue damage in aquatic organisms (Williams & Hoofnagle, 1988; Narvia & Rantamaki, 1997; Giannini *et al*., 2005). The reduced AST and ALT activities in mussels from contaminated sites likely reflect degenerative processes in tissues, such as the hepatopancreas, caused by pollutant-induced lesions. These findings are consistent with Almeida *et al*. (2002), who proposed that alterations in LDH, AST, and ALT activities serve as robust biomarkers for histological damage, enabling rapid detection of toxicological impacts.

ChE, a neurotoxic biomarker, is widely used to assess exposure to inorganic and organic pollutants, including PTEs, organophosphorus pesticides, and carbamates (Almeida *et al*., 2011; Rouane-Hacene *et al*., 2015). The significant inhibition of ChE activity in mussels from Kardzhali and Studen Kladenets aligns with studies by Bocquené *et al*. (1990; 1995) and Galgani and Bocquené (2000), which demonstrated ChE suppression as an indicator of environmental toxicant stress. This inhibition likely results from ChE's high affinity for neurotoxic compounds, including PTEs and organic pollutants, leading to neurotoxic effects in bivalves (Maisano *et al*., 2017; Pirone *et al*., 2019). Reduced ChE activity also reflects broader physiological stress, impacting mussel health (Sandoval-Herrera *et al*., 2019).

The consistent reduction in metabolic enzyme activities across contaminated reservoirs suggests that pollutants, including PTEs and organic compounds (e.g., PAHs, PCBs), disrupt critical biochemical pathways in caged mussels. These findings align with Sevgiler *et al*. (2011), who identified enzymatic alterations as sensitive biomarkers of anthropogenic toxicant effects. The observed metabolic suppression likely stems from tissue damage and energy reallocation toward detoxification, compromising cellular homeostasis. This emphasises the utility of metabolic enzymes as early-warning indicators of pollution-induced stress in freshwater ecosystems.

3.4.4. DNA DAMAGE

The comet assay, or single-cell gel electrophoresis, is a well-established method in ecotoxicology for detecting DNA damage in aquatic organisms, offering rapid and sensitive evaluation of genotoxicity induced by environmental pollutants (Jha, 2008; Frenzilli *et al*., 2009; Bolognesi and Cirillo, 2014; Collins, 2015; Lapuente *et al*., 2015**)**. This technique identifies primary DNA lesions, including single- and double-strand breaks, alkali-labile sites, and crosslinks, enabling timely detection of exposure to genotoxic agents (Jha, 2008; Collins, 2015;

Lapuente *et al*., 2015; Glei *et al*., 2016). In this study, the comet assay applied to haemocytes of caged *S. woodiana* mussels demonstrated high sensitivity for monitoring genotoxicity in freshwater ecosystems, consistent with prior research on both native and transplanted bivalves (Kolarević *et al*., 2016; Marić *et al*., 2020). Our findings affirm the Chinese pond mussel as an effective bioindicator for in situ genotoxicity assessments, as previously validated in polluted aquatic systems (Vuković-Gačić *et al*., 2014). Significant variations in TI% among caged mussels from Studen Kladenets, Kardzhali, and Zhrebchevo reservoirs (ANOVA, $p < 0.001$) underscore the mussels' responsiveness to waterborne genotoxicants, including PTEs, polybrominated diphenyl ethers (PBDEs), and short-chain chlorinated paraffins (SCCPs). Mussels from Studen Kladenets exhibited the highest DNA damage (TI% = 25.79 ± 1.58), likely due to the historical lead-zinc ore processing plant, "Kardzhali", which elevated tissue concentrations of cadmium (Cd), lead (Pb), zinc (Zn), PBDEs, and SCCPs (Gecheva *et al*., 2020). These pollutants are known to induce DNA strand breaks and chromosomal fragmentation in freshwater organisms (Vincent-Hubert *et al*., 2011; Ali *et al*., 2012; Mahaye *et al*., 2017). Cd, for instance, indirectly causes genotoxicity through oxidative stress and inhibition of DNA repair enzymes, while Pb's genotoxic mechanisms involve free radical production and repair suppression (García-Lestón *et al*., 2010; Mahaye *et al*., 2017). PBDEs, particularly BDE-209, and SCCPs further exacerbate DNA damage via ROS-mediated pathways, as observed in bivalves (zebra mussel, *Dreissena polymorpha*) and human cell lines (Riva *et al*., 2007; Ji *et al* 2011; Pereira *et al*., 2016; Wang *et al*., 2019).

Mussels from Kardzhali and Zhrebchevo reservoirs also displayed elevated DNA damage (TI% = 21.84 ± 2.60 and 13.23 ± 1.31, respectively), primarily linked to PTEs such as Cr, Cu, and Ni in Kardzhali, and Al, As, Co, Fe and Mn in Zhrebchevo reservoirs (Gecheva *et al*., 2020). These results align with those of Khan *et al*. (2019), who documented increased DNA damage in mussels with higher metal (Cd, Cr, Cu, Fe, Mn, Ni, Pb, Zn) bioaccumulation from polluted sites compared to reference locations. The synergistic effects of multiple toxicants, including PTEs and pesticides from agricultural runoff in Zhrebchevo, likely amplify genotoxicity, resulting in more severe DNA damage than that caused by single pollutants (Singh *et al*., 2017). This synergism explains the significant DNA damage in mussels from the Zhrebchevo reservoir, which experienced pollution from both severe agricultural activities, in addition to earlier reports of metal pollutant profiles (Kalchev *et al*., 2013; Zhelyazkov *et al*., 2014).

The TI% values in Studen Kladenets and Kardzhali reservoirs are comparable to those reported for *Unio* sp. (28.8 ± 1.6) and *S. woodiana* (21.9 ± 1.5) in polluted Danube River sites, indicating similar genotoxic pressures (Kolarević *et al*., 2016). Lower TI% values (10.95–18.42)

reported in other Danube River studies suggest site-specific differences in pollutant loads (Marić *et al*., 2020). The comet assay's sensitivity as a biomarker is further evidenced by its correlation with oxidative stress markers (e.g., CAT, GPx, GR) in all the studied reservoirs, particularly in Studen Kladenets mussels, where high SCCP levels (6.12 μg/kg, 27 times higher than Zhrebchevo) coincided with elevated antioxidant enzyme activities, reflecting ROS-driven DNA damage (Gecheva *et al*., 2020). The pronounced DNA damage in caged mussels confirms the genotoxic potential of pollutants in the studied reservoirs, driven by PTEs, PBDEs, and SCCPs. The comet assay's efficacy in detecting these effects positions it as a critical tool for biomonitoring, complementing chemical analyses and other biomarkers. These findings highlight the need for targeted pollution control in Studen Kladenets, where industrial legacies pose significant risks, and underscore the broader ecological and public health implications of genotoxic contaminants in freshwater systems.

3.4.5. Relationships between pollutant levels and biomarker responses in Caged Mussels

The positive correlation between MPI and oxidative stress enzymes (CAT, GPx, GR) suggests an adaptive antioxidant response to counteract ROS induced by pollutants like PTEs and SCCPs in Studen Kladenets and Kardzhali reservoirs (Regoli & Giuliani, 2014). Conversely, the suppression of metabolic enzymes (AST, ALT, ChE) indicates pollutant-induced tissue damage or neurotoxicity, consistent with their roles as hepatotoxicity and neurotoxicity biomarkers (Almeida *et al*., 2002; Lastumaki *et al*., 2020). The lack of correlation with LDH may reflect its specific role in anaerobic metabolism. The positive MPI-TI% correlation underscores the genotoxic impact of PTEs (e.g., Cd, Pb) and SCCPs, which cause DNA strand breaks via ROS (Bolognesi and Cirillo, 2014). These findings corroborate the findings of Ferreira *et al*. (2019), who reported correlations between PTE levels and biochemical indices in *Crassostrea gasar* oysters, and between organic toxicants (PAHs, PCBs) and molecular responses. Similarly, Capolupo *et al*. (2016) noted a dose-dependent link between CAT activity, DNA damage, and caffeine in *Mytilus galloprovincialis*, paralleling our observations with SCCPs and PTEs. Overall, the observed associations between MPI and biomarkers reveal adaptive (oxidative stress enzymes) and adverse (metabolic suppression, DNA damage) responses to pollution.

3.5. Conclusions

In conclusion, the mussel caging method for biomonitoring freshwater ecosystems is relatively new in Bulgaria. The waters of Studen Kladenets and Kardzhali reservoirs were observed to be heavily polluted and, generally, affected different biomarker responses in the

caged mussels more significantly compared to the waters of Zhrebchevo Reservoir due to a different anthropogenic load. The results from this pilot study are essential for improving the assessment and monitoring programs of water pollution effects. Further, the results of this study suggest that transplanted mussels can and should be applied regularly in ecotoxicological studies in freshwater studies, along with classical bioaccumulation analyses of water/sediments and biota for the effective representation of toxicant impacts on aquatic systems.

4. EXPERIMENT 2

BEHAVIOURAL, HAEMATOLOGICAL, AND SERUM BIOCHEMICAL RESPONSES OF JUVENILE AFRICAN CATFISH (*CLARIAS GARIEPINUS* [BURCHELL, 1822]) SUBJECTED TO VARYING LEVELS OF PAINT THINNER EXPOSURE.

4.1. Background

The global paint industry has experienced substantial growth, driven by initiatives from both governmental and private sectors, leading to heightened paint production and a consequent increase in the release of paint-related effluents into aquatic ecosystems (Owolabi and Adewoye, 2017; Rafay and Singh, 2020). Paint thinners (PTs) comprise a range of chemical solvents employed in the paint and decorative sectors to dilute oil-based paints, varnishes, and other coatings. Among these, nitrocellulose (NC) lacquer thinners, which are volatile organic solvent blends, are specifically formulated to dissolve and thin NC lacquers (Xu *et al*., 2020; Liang *et al*., 2022).

Exposure to PTs in animals is known to elevate the generation of ROS, inducing oxidative stress (Agin *et al*., 2016). In humans, contact with these compounds, through inhalation, ingestion, or dermal absorption, may lead to elevated methaemoglobin levels in the blood, causing symptoms such as dizziness, respiratory distress, nausea, hallucinations, and chronic damage to the cardiovascular, respiratory, hepatic, renal, and nervous systems (Singh *et al*., 2012; Patrick-Iwuanyanwu *et al*., 2013). Despite their critical role in industrial and painting applications, PTs present considerable environmental hazards due to their toxicity and potential for bioaccumulation, raising significant concerns regarding their ecological and health implications (Patrick-Iwuanyanwu *et al*., 2013; Agin *et al*., 2016; Pandey and Kiran, 2020; John *et al*., 2024). These solvents can infiltrate aquatic environments through multiple routes, including storage and transport leaks, improper disposal, stormwater runoff, and industrial discharges, thereby posing risks to aquatic organisms and ecosystem safety (Nair *et al*., 2021; Viktoryová *et al*., 2021). With the ongoing rise in urbanization and construction, assessing the toxicological effects of PTs on aquatic species is essential for devising effective environmental protection measures.

Toxicological research is vital for elucidating the health risks posed by chemical exposures (Djordjevic *et al*., 2023). In fish, exposure to toxicants triggers a spectrum of physiological, biochemical, morphological, and histological responses, which may impair normal physiological functions and compromise health, survival, and product quality (Van der

Oost *et al*., 2003; Jabeen *et al*., 2022; Shah and Parveen, 2022). Blood parameters serve as sensitive indicators of environmental stressors, reflecting a broad range of physiological changes in fish (Witeska *et al*., 2023). Haematological assessments offer a cost-effective and reliable approach to evaluating physiological health in fish exposed to toxicants across diverse environmental conditions (Maksoud *et al*., 2018; Fazio, 2019; Ali *et al*., 2021).

Lipids play a critical role in fish physiology, serving as energy reserves, structural components of cellular membranes, and precursors for hormones and signalling molecules (Bailey *et al*., 2022; Higashi, 2022). Alterations in lipid profiles can disrupt membrane integrity, release intracellular contents, and initiate cascading physiological effects, providing insights into liver function and overall health in toxicant-exposed fish (Sayed *et al*., 2011; Hook *et al*., 2018; Cocci *et al*., 2019). Additionally, biochemical markers such as ALT, AST, alkaline phosphatase (ALP), creatinine, urea, bilirubin, and albumin are valuable for assessing liver and kidney function, serving as indicators of the physiological impacts of toxicants and environmental stressors (Mohamed *et al*., 2019; Hamed *et al*., 2021; Said *et al*., 2021; Farrag *et al*., 2022; Hamed *et al*., 2023). Consequently, blood profile metrics, encompassing haematological parameters, serum lipid profiles, biochemical markers, and behavioural changes, have been widely utilised to evaluate the effects of sublethal pollutant concentrations on aquatic organisms (Nikalje *et al*., 2012; Jarema *et al*., 2015; Jacquin *et al*., 2020).

Prior studies have extensively explored the impacts of various contaminants, including heavy metals (Javed *et al*., 2015; Opasola *et al*., 2019), flame retardants (Oliveri *et al*., 2015; Georgieva *et al*., 2022), pesticides (Amaeze *et al*., 2020; Moreira *et al*., 2021), industrial effluents (Jabeen *et al*., 2022), pharmaceutical wastewater (McCallum *et al*., 2017; Kayode-Afolayan *et al*., 2022), paint wastewater (Owolabi and Adewoye, 2017; Jekayinfa and Bawa-Allah, 2022), and antifouling paints (George *et al*., 2017), on aquatic ecosystems. Furthermore, research has investigated the toxicological effects of PTs and other organic solvents on humans (Agin *et al*., 2016; Liu and Zheng, 2020; Akhter *et al*., 2024) and laboratory animals such as albino rats (Uboh *et al*., 2012; Fifel *et al*., 2013; Patrick-Iwuanyanwu *et al*., 2013; Hussein, 2019). However, the toxicological impacts of PTs on aquatic systems and the health of aquatic organisms remain underexplored. This study seeks to address this gap by investigating the acute and chronic toxicities of sublethal PT concentrations on African catfish (*Et al* Burchell, 1822), with a focus on behavioural changes, haematological parameters, serum lipid profiles, and biochemical markers. Additionally, the study evaluates the recovery potential of exposed fish following a 7-day depuration period. The results will enhance understanding of the risks posed by PT contamination in aquatic environments and support the development of strategies to mitigate its effects on freshwater ecosystems.

4.2. Materials and Methods

4.2.1. Ethical Approval

All experimental procedures involving fish adhered to the institutional animal welfare guidelines outlined in the UNN Research Policy Document (2013). Measures were implemented to minimise stress and suffering, in accordance with the Animal Welfare Act (AWA) and Public Health Service (PHS) standards (Borski and Hodson, 2003). Ethical clearance (Reference No.: UNN/FBS/24/SS.12337) was granted by the Faculty of Biological Sciences Research Ethics Committee (FBSREC) at the University of Nigeria, Nsukka.

4.2.2. Experimental Fish and Study Location

Juvenile African catfish (*C. gariepinus*; n = 220; mean body weight = 67.73 ± 0.8 g; mean standard length = 9.52 ± 0.2 cm) were sourced from Freedom Fisheries, Nsukka, Enugu State, Nigeria. To reduce transport-related stress, the fish were conveyed in 50-liter plastic containers. The study was conducted at the wet laboratory of the Fisheries and Hydrobiology Research Unit, Department of Zoology and Environmental Biology, University of Nigeria, Nsukka. During a two-week acclimation period, fish were housed in 60 L plastic containers and fed twice daily (9:00–10:00 A.M. and 3:00–4:00 P.M.) with a commercial feed (Aler aqua®, Aller Aqua Group, Denmark) comprising of 42% crude protein. Water was replaced at intervals of 72 hours to ensure optimal water quality by removing uneaten feed and metabolic waste.

4.2.3. Procurement and Preparation of Test Compound

The study utilised HITRO Nitrocellulose-based paint thinner® (PT), a widely used nitrocellulose (NC)-based paint thinner in Enugu State, Southeast Nigeria. Although the exact formulation is proprietary, NC-based thinners typically comprise “true solvents” (e.g., ketones like acetone or methyl ethyl ketone, esters such as methyl or ethyl acetate, amides, and nitroparaffins), “cosolvents/latent solvents” (e.g., alcohols like ethanol or isopropanol), and “non-solvents” (e.g., aliphatic and aromatic hydrocarbons such as benzene, toluene, or xylene), which pose varying degrees of toxicity and environmental risk. Test concentrations were prepared by accurately measuring PT volumes with calibrated syringes and thoroughly mixing them into bioassay containers to create the test media.

4.2.4. Acute Toxicity Testing

Preliminary range-finding experiments were conducted to identify appropriate concentrations for acute toxicity evaluations of nitrocellulose-based paint thinner (NC-PT) on juvenile African catfish. A 96-hour semi-static renewal bioassay, following Organisation for Economic Co-operation and Development (OECD) guidelines (OECD, 2019), was performed

in glass aquaria to determine the 96-hour median lethal concentration (LC50). Initial tests showed 0% mortality at 1.50 mg/L and 100% mortality at 5.0 mg/L. Eighty acclimated juvenile catfish were randomly assigned to eight 20 L glass tanks (10 fish per tank). Seven groups were exposed to PT concentrations of 1.0, 1.5, 2.0, 2.25, 2.5, 3.7, and 5.0 mg/L, while a control group was maintained in tap water under identical conditions. Feeding was discontinued during the test. Mortality and behavioural responses were recorded every 24 hours over 96 hours, and the LC50 was estimated using probit analysis (Finney, 1971).

Table 5 presents the outcomes of the Probit analysis conducted to evaluate the acute toxicity of paint thinner (PT) on the mortality of *C. gariepinus* juveniles following a 96-hour exposure period. The determined 96-hour LC50 value for paint thinner was 2.0 mg/L. No mortalities were recorded in the control group or at PT concentrations of 1.00 mg/L and 1.50 mg/L throughout the 96-hour exposure (Table 5). At concentrations of 2.00 mg/L and 2.25 mg/L, mortality rates reached 50% and 60%, respectively, while concentrations of 2.50 mg/L and higher resulted in complete (100%) mortality. The results indicate a clear concentration-dependent toxic effect of paint thinner on *C. gariepinus* juveniles during the 96-hour acute exposure.

Table 5. Logarithmic concentration and mortality response of *Et al* juveniles to paint thinner exposure

Conc. (mg/L**)**	**24 h**	**%**	**48 h**	**%**	**72 h**	**%**	**96 h**	**%**
Control	0	0	0	0	0	0	0	0
1.00	0	0	0	0	0	0	0	0
1.50	0	0	0	0	0	0	0	0
2.0	2	20	4	40	5	50	5	50
2.25	3	30	5	50	6	60	6	60
2.50	8	80	10	100	10	100	10	100
3.70	10	100	10	100	10	100	10	100
5.0	10	100	10	100	10	100	10	100

***Conc.: Concentration**

Table 6 summarises the estimated lethal concentration and mortality response of *C. gariepinus* catfish to paint thinner (PT) during a 96-hour exposure period. The estimated 96-h median lethal concentration (LC_{50}) of paint thinner to juvenile *C. gariepinus* was shown to be 1.874 mg/l (95% CI = 1.698 – 2.044). Approximately 90% mortality of juvenile *C. gariepinus* is estimated to occur at 2.493 mg/l (95% CI = 2.270 – 2.851).

Table 6. 96 h acute toxicity of paint thinner on juveniles of the *Et al* catfish

Mortality Response (%)	Lethal Concentration (CL) (mg/L)
20	1.554 (1.345 – 1.714)
30	1.668 (1.473 – 1.826)
40	1.772 (1.588 – 1.933)
50	1.874 (1.698 – 2.044)
60	1.983 (1.810 – 2.168)
70	2.106 (1.931 – 2.319)
80	2.260 (2.072 – 2.521)
90	2.493 (2.270 – 2.851)
99	3.146 (2.769 – 3.887)

***LC: lethal concentration (measured at a confidence interval of 95%); CL: Confidence Limit.**

4.2.5. CHRONIC SUB-LETHAL TOXICITY TEST

For the chronic toxicity testing, three sub-lethal PT concentrations - 0.1 mg/L (1/20th of 96-h LC50), 0.2 mg/L (1/10th of 96-h LC50), and 0.4 mg/L (1/5th of 96-h LC50), derived from the estimated 96-h LC50 of 2.0 mg/L, were considered. Juvenile *C. gariepinus* were randomly distributed to four groups (A, B, C, and D) with three replicates per group (10 fish per replicate) in a completely randomized design. Groups A, B, and C were exposed to 0.1, 0.2, and 0.4 mg/L PT, respectively, while Group D (control) were given no PT. Fish were fed a commercial diet (Aler aqua®, Aller Aqua Group, Denmark) twice daily at 3% body weight. Water quality indices, determined per APHA *et al*. (2005), remained steady throughout the study: dissolved oxygen (6.47 ± 0.12 mg/L), temperature (27.5 ± 0.1°C), pH (6.77 ± 0.06), and alkalinity (0.0 ± 0.0 mg/L). The experiment followed a natural 12 h light/12 h dark photoperiod. Water was renewed every 48 hours to maintain quality and dissolved oxygen levels, with PT concentrations replenished during water changes to ensure consistent exposure. The exposure lasted 21 days, followed by a 7-day depuration period to assess recovery from PT exposure.

4.2.6. BLOOD SAMPLING AND ANALYSIS

Blood samples were collected on days 0, 7, 14, and 21 of exposure and day 28 (after 7 post-depuration). Four fish per group were randomly taken for sampling. Blood was drawn from the dorsal arteries using a 3 ml syringe inserted along the lateral line, following a 24-hour fasting period to minimise stress. Collections were performed swiftly to reduce handling stress.

4.2.6.1. HAEMATOLOGY

Blood for haematological analysis was collected in ethylenediaminetetraacetic acid (EDTA)-containing bottles, labelled, and analysed using a Mindray automated haemato-

analyser (Model BC2800) (Erkmen, 2022). Parameters assessed included total red blood cell count (RBC), packed cell volume (PCV), haemoglobin (HB), total white blood cell count (WBC), and WBC differentials.

4.2.6.2. SERUM BIOCHEMISTRY

Blood aliquots for serum biochemistry were collected in EDTA-free bottles, tilted to separate serum, and transferred to capped test tubes using a Pasteur pipette. Lipid profile analysis (low-density lipoprotein [LDL], high-density lipoprotein [HDL], total cholesterol [TC], and triglycerides) was conducted using a Lipid-Plus measuring system (Jant Pharmacal Corporation, Encino, CA). Serum proteins were quantified using Randox® bilirubin and albumin test kits (Randox Laboratories Ltd., County Antrim, UK). Serum albumin was measured via the bromocresol green method (Doumas and Peters, 1997; Johnson, 2008), and total bilirubin was assessed using the Jendrassik and Grof method (Doumas *et al*., 1973; Higgins *et al*., 2008). Creatinine was determined using Jaffe's reaction (Toora and Rajagopal, 2002), and urea was quantified as described by Machado and Horizonte (1958). Serum ALT, AST, and ALP activities were measured using assay kits from Quimica Clinica Aplicada (QCA), Spain. ALT and AST were determined via the Reitman-Frankel colourimetric method (Reitman and Frankel, 1957; Colville, 2002), and ALP activity was assessed using a QCA assay kit based on phenolphthalein monophosphate hydrolysis (Klein *et al*., 1960; Colville, 2002). All biochemical assays were performed using a CHEM5V3® Semi-automated Clinical Chemistry Analyser (Erba Diagnostics, Manheim, Germany), with analytical-grade reagents.

4.2.7. STATISTICAL ANALYSIS

The 96-h lethal concentrations (LC) of PT were estimated via probit analysis using the lognormal distribution method (Finney, 1971) in StatPlus Pro 5.9.8 (AnalystSoft Inc., Walnut, Canada). Mortality percentages were calculated as (number of deaths/initial sample size) × 100. Haematological and serum biochemistry data were analysed using mixed-model ANOVA with the rstatix package (Kassambara, 2021) in R Studio (RStudio Team, 2023). PT treatment was the between-subject factor, and sampling time was the within-subject factor. Mean differences were evaluated using Bonferroni corrections for multiple comparisons, with significance set at $p < 0.05$. Visualisations of PT treatment effects over time were generated using the ggplot2 package in R Studio (v3.3.3; Wickham, 2016).

4.3. Results

4.3.1. BEHAVIOURAL AND MORTALITY RESPONSES TO ACUTE TOXICITY

Table 7 outlines the behavioural responses of *C. gariepinus* juveniles exposed to varying concentrations of paint thinner over a 96-hour period. The exposure elicited a spectrum of aberrant behavioural alterations in the fish. No unusual behaviours were noted in the control group or fish exposed to 1.00 mg/L of paint thinner across all assessed behavioural parameters. As paint thinner concentrations increased, behavioural anomalies became more pronounced. At 1.50 mg/L, fish displayed slight erratic swimming, surface air gulping, attempts to leap from the water, and a moderate decline in feeding response. At 2.00 mg/L, more pronounced behavioural aberrations were observed, including intense efforts to escape the water, moderate air gulping, and delayed feeding responses. At higher concentrations of 2.50 mg/L, 3.70 mg/L, and 5.00 mg/L, fish exhibited severe air gulping, frequent escape attempts, sluggish feeding, protruding eyes, agitation, and reduced fin movement.

Table 7. Morphological, behavioural and mortality responses in juvenile *Et al* on 96-h exposure to paint thinner

Behavioural responses	**Concentrations (mg/L)**							
	0.0	**1.00**	**1.50**	**2.00**	**2.25**	**2.50**	**3.70**	**5.00**
Erratic swimming	-	-	+	+	++	++	+++	+++
Gulping of air	-	-	+	++	++	+++	+++	+++
Attempts to jump out of water	-	-	+	+++	+++	+++	+++	+++
Slow response to feeding	-	-	++	++	+++	+++	+++	+++
Bulging of eyes	-	-	-	+	++	+++	+++	+++
Restlessness	-	-	-	+	++	++	+++	+++
Fin movement	+++	+++	++	++	+	-	-	-

***Key: - absent; + mild; ++ moderate; +++ severe.**

4.3.2. CHRONIC TOXICITY TEST

4.3.2.1. HAEMATOLOGY

Main Effects of PT Treatment on Haematological Parameters

The haematological parameters of juvenile *C. gariepinus* exposed to sub-lethal concentrations of paint thinner are presented in Table 8. Mixed-model ANOVA indicated significant main effects of PT treatment on PCV ($F_{3,12} = 41.9$, $p < 0.001$, $\eta^2_p = 0.91$), RBC ($F_{3,12} = 182.97$, $p < 0.001$, $\eta^2_p = 0.98$), WBC ($F_{3,12} = 96.8$, $p < 0.001$, $\eta^2_p = 0.96$), HB ($F_{3,12} = 390.01$, $p < 0.001$, $\eta^2_p = 0.99$), neutrophils ($F_{3,12} = 34.07$, $p < 0.001$, $\eta^2_p = 0.90$), and lymphocytes ($F_{3,12} = 24.95$, $p < 0.001$, $\eta^2_p = 0.86$). No significant effects were observed for monocytes ($F_{3,12} = 3.13$,

$p = 0.066$, $\eta^2_p = 0.44$), basophils ($F_{3,12} = 1.90$, $p = 0.184$, $\eta^2_p = 0.32$), or eosinophils ($F_{3,12} = 1.0$, $p = 0.426$, $\eta^2_p = 0.20$). Compared to the control, WBC and neutrophil levels were significantly elevated in all PT-treated groups (Groups 1, 2, and 3) with no significant differences among them ($p \geq 0.05$). Conversely, PCV, RBC, HB, and lymphocyte levels were lower in treated groups ($p < 0.05$), with Group 1 showing the lowest RBC and HB values, differing significantly from Groups 2 and 3 ($p < 0.05$).

Table 8. Effect of chronic exposure to sub-lethal doses of paint thinner on the haematological indices of African catfish (*Et al*) juveniles

Source	PCV (%)	RBC ($\times 10^{12}$/L)	WBC ($\times 10^9$/L)	HB (g/dL)	NEUT	Lymph	MONO	BASO	EOSIN
Sampling Time									
Day 0 (Pre-Treat.)	31.69	10.6	8.44	10.34	20.75	76.56	1.69	0.38	0.13
	(0.48)^a	(0.07)^a	(0.06)^d	(0.09)^a	(0.64)^d	(0.88)^a	(0.26)^a	(0.14)^a	(0.13)^a
Day 7	26.38	9.31	11.10	8.16	29.25	68.38	1.75	0.50	0.13
	(0.46)^c	(0.08)^b	(0.12)^b	(0.06)^c	(0.66)^b	(0.48)^b	(0.14)^a	(0.1)^a	(0.07)^a
Day 21	24.63	8.47	11.55	7.54	35.38	63.75	1.13	0.38	0.0
	(0.39)^d	(0.10)^c	(0.07)^a	(0.05)^d	(0.73)^a	(0.61)^c	(0.22)^a	(0.13)^a	(0.0)^a
Day 28 (7-Days Post-Treat.)	29.5 (0.31)^b	10.44 (0.03)^a	10.0 (0.07)^c	9.79 (0.07)^b	24.38 (0.65)^c	73.25 (0.87)^a	1.63 (0.16)^a	0.75 (0.14)^a	0.0 (0.0)^a
PT - Treatment									
Group1	25.75	9.27	10.8	8.07	29.0	69.13	1.50	0.38	0.13
	(0.53)^b	(0.05)^c	(0.11)^a	(0.06)^c	(0.83)^a	(0.97)^b	(0.26)^a	(0.11)^a	(0.07)^a
Group2	26.13	9.54	10.74	8.53	30.19	67.0	2.19	0.69	0.0
	(0.53)^b	(0.05)^b	(0.11)^a	(0.06)^b	(0.83)^a	(0.97)^b	(0.26)^a	(0.11)^a	(0.07)^a
Group3	27.19	9.26	10.88	8.62	30.31	68.19	1.38	0.38	0.0
	(0.53)^b	(0.05)^c	(0.11)^a	(0.06)^b	(0.83)^a	(0.97)^b	(0.26)^a	(0.11)^a	(0.07)^a
Control	33.13	10.75	8.67	10.6	20.25	77.63	1.13	0.56	0.13
	(0.53)^a	(0.05)^a	(0.11)^b	(0.06)^a	(0.83)^b	(0.97)^a	(0.26)^a	(0.11)^a	(0.07)^a
Interactions									
Group1 X Day 0	31.0	10.53	8.47	10.11	20.5	74.5	2.0 (0.52)^a	0.5	0.5
	(0.97)^a	(0.14)^a	(0.12)^a	(0.17)^a	(1.29)^a	(1.75)^a		(0.27)^a	(0.25)^a
Group2 X Day 0	31.5	10.55	8.36	10.23	21.25	77.0	1.75	0.25	0.0
	(0.97)^a	(0.14)^a	(0.12)^a	(0.17)^a	(1.29)^a	(1.75)^a	(0.52)^{ab}	(0.27)^a	(0.25)^a
Group3 X Day 0	31.8	10.5	8.47	10.58	21.75	76.75	2.0 (0.52)^a	0.5	0.0
	(0.97)^a	(0.14)^a	(0.12)^a	(0.17)^a	(1.29)^a	(1.75)^a		(0.27)^a	(0.25)^a
Control X Day 0	32.5	10.79	8.47	10.46	19.5	78.0	1.0 (0.52)^a	0.25	0.0
	(0.97)^a	(14)^a	(0.12)^a	(0.17)^a	(1.29)^a	(1.75)^a		(0.27)^a	(0.25)^a
Group1 X Day 7	24.5	8.53	11.95	7.18	29.5	69.0	1.5 (0.29)^a	0.0	0.0
	(0.92)^b	(0.16)^b	(0.25)^c	(0.12)^b	(1.32)^b	(0.97)^b		(0.20)^a	(0.14)^a
Group2 X Day 7	23.5	9.28	11.7	7.60	32.5	62.5	3.5 (0.29)^c	1.5	0.0
	(0.92)^c	(0.16)^b	(0.25)^c	(0.12)^b	(1.32)^c	(0.97)^c		(0.20)^b	(0.14)^a
Group3 X Day 7	26.0	8.96	12.05	7.35	36.5	62.5	1.0 (0.29)^a	0.0	0.0
	(0.92)^b	(0.16)^b	(0.25)^c	(0.12)^b	(1.32)^b	(0.97)^b		(0.20)^a	(0.14)^a
Control X Day 7	31.5	10.48	8.7	10.50	18.5	79.5	1.0 (0.29)^a	0.5	0.5
	(0.92)^a	(0.16)^a	(0.25)^{ab}	(0.12)^a	(1.32)^a	(0.97)^a		(0.20)^a	(0.14)^a
Group1 X Day 21	21.0	7.89	12.15	6.35	44.5	57.0	1.0 (0.43)^a	0.0	0.0
	(0.78)^c	(0.20)^b	(0.13)^c	(0.10)^c	(1.45)^c	(1.23)^c		(0.25)^a	(0.0)^a
Group2 X Day 21	21.5	7.83	12.55	6.70	39.5	59.5	1.0 (0.43)^a	0.0	0.0
	(0.78)^c	(0.20)^c	(0.13)^c	(0.10)^c	(1.45)^d	(1.23)^c		(0.25)^a	(0.0)^a

Group3 X Day 21	21.5 (0.78)^{c}	7.40 (0.20)^{c}	13.15 (0.13)^{d}	6.50 (0.10)^{c}	36.5 (1.45)^{b}	62.0 (1.23)^{b}	1.0 (0.43)^{a}	0.5 (0.25)^{a}	0.0 (0.0)^{a}
Control X Day 21	34.5 (0.78)^{a}	10.75 (0.20)^{a}	8.35 (0.13)^{a}	10.60 (0.10)^{a}	21.0 (1.45)^{a}	76.5 (1.23)^{a}	1.5 (0.43)^{a}	1.0 (0.25)^{a}	0.0 (0.0)^{a}
Group1 X Day 28	26.5 (0.61)^{b}	10.14 (0.07)^{a}	10.65 (0.14)^{b}	8.65 (0.14)^{d}	21.5 (1.30)^{a}	76.0 (1.73)^{a}	1.5 (0.32)^{a}	1.0 (0.29)^{a}	0.0 (0.0)^{a}
Group2 X Day 28	28.0 (0.61)^{b}	10.5 (0.07)^{a}	10.35 (0.14)^{b}	9.60 (0.14)^{a}	27.5 (1.30)^{b}	69.0 (1.73)^{b}	2.5 (0.32)^{b}	1.0 (0.29)^{ab}	0.0 (0.0)^{a}
Group3 X Day 28	29.5 (0.61)^{a}	10.13 (0.07)^{a}	9.85 (0.14)^{b}	10.05 (0.14)^{a}	26.5 (1.30)^{a}	71.5 (1.73)^{a}	1.5 (0.32)^{a}	0.5 (0.29)^{a}	0.0 (0.0)^{a}
Control X Day 28	34.0 (0.61)^{a}	10.99 (0.07)^{a}	9.15 (0.14)^{b}	10.85 (0.14)^{a}	22.0 (1.30)^{a}	76.5 (1.73)^{a}	1.0 (0.32)^{a}	0.5 (0.29)^{a}	0.0 (0.0)^{a}
P-Value									
Sampling Time	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.1	0.169	0.37
PT-Treatment	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.066	0.184	0.426
Interaction	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.018	0.003	0.197

[a,b,c,d] 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; PCV: Packed cell volume; RBC: Red Blood Cells; WBC: White Blood Cells; HB: Haemoglobin; NEUT.: Neutrophil; Lymph: Lymphocytes; Mono: Monocytes; Baso: Basophil; Eosin: Eosinophils.

Main Effects of Sampling Time on Haematological Parameters

Sampling time significantly affected PCV ($F_{3,36} = 73.0$, $p < 0.001$, $\eta^2_p = 0.86$), RBC ($F_{3,36} = 155.57$, $p < 0.001$, $\eta^2_p = 0.92$), WBC ($F_{3,36} = 352.57$, $p < 0.001$, $\eta^2_p = 0.97$), HB ($F_{3,36} = 346.66$, $p < 0.001$, $\eta^2_p = 0.97$), neutrophils ($F_{2.03,24.35} = 107.95$, $p < 0.001$, $\eta^2_p = 0.90$), and lymphocytes ($F_{1.8,21.6} = 79.29$, $p < 0.001$, $\eta^2_p = 0.87$). No significant effects were observed for monocytes ($F_{1.85,22.21} = 107$, $p = 0.1$, $\eta^2_p = 0.18$), basophils ($F_{3,36} = 1.78$, $p = 0$□□, $p = 0.17$, $\eta^2_p = 0.13$), or eosinophils ($F_{1.5,18.0} = 1.0$, $p = 0.37$, $\eta^2_p = 0.08$). PCV, HB, RBC, and lymphocyte levels declined over the 21-day exposure period. By day 28 (post-depuration), PCV and HB partially recovered, while RBC and lymphocytes fully recovered. WBC and neutrophil levels increased until day 21 and decreased by day 28, remaining above baseline.

Interaction Effect of PT Treatment and Sampling Time on Haematological Indices

Significant interaction effects between PT treatment and sampling time were observed for PCV ($F_{9,36} = 12.03$, $p < 0.001$, $\eta^2_p = 0.75$), RBC ($F_{9,36} = 15.77$, $p < 0.001$, $\eta^2_p = 0.80$), WBC ($F_{9,36} = 48.10$, $p < 0.001$, $\eta^2_p = 0.92$), HB ($F_{9,36} = 41.20$, $p < 0.001$, $\eta^2_p = 0.91$), neutrophils ($F_{6.09,24.35} = 17.81$, $p < 0.001$, $\eta^2_p = 0.82$), lymphocytes ($F_{5.4,21.6} = 13.12$, $p < 0.001$, $\eta^2_p = 0.77$), monocytes ($F_{5.6,22.21} = 3.38$, $p = 0.018$, $\eta^2_p = 0.46$), and basophils ($F_{9,36} = 3.51$, $p = 0.003$, $\eta^2_p = 0.47$). No interaction effect was found for eosinophils ($F_{4.5,18} = 1.67$, $p = 0.197$, $\eta^2_p = 0.29$). In the control group, no significant changes occurred in PCV, RBC, HB, neutrophils, lymphocytes, monocytes, or basophils, though WBC increased by day 28, remaining lower than in PT-treated groups (Figure 5).

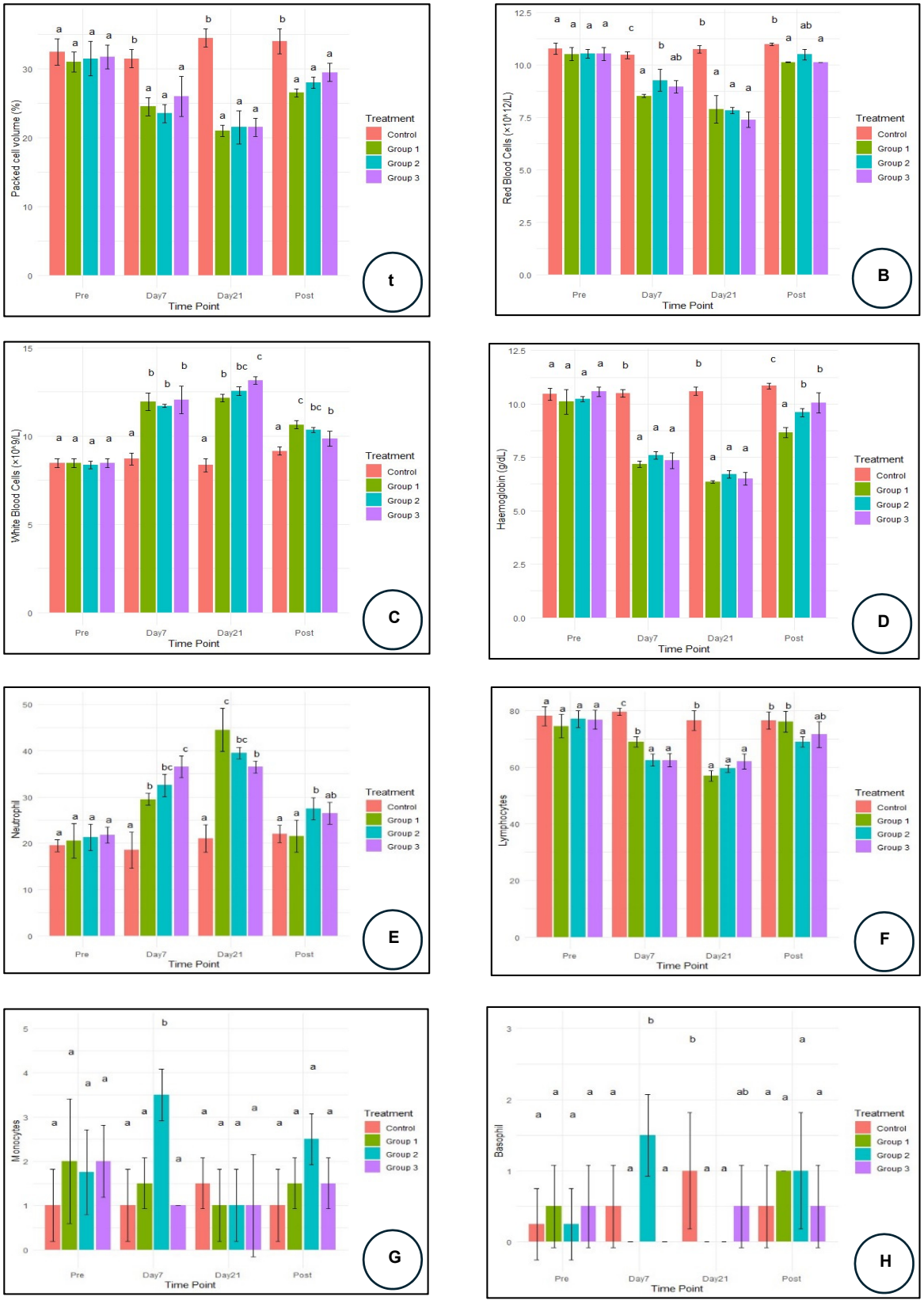

Figure 5: A within-sampling time comparison of the haematological indices of African catfish juveniles exposed to sub-lethal doses of paint thinner

[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; **A** - Packed cell volume (%); **B** - RBC: Red Blood Cells (**$\times 10^{12}$/L**); **C** - White Blood Cells ($\times 10^9$/L); **D** - Haemoglobin (g/dL); **E** - Neutrophil; **F** – Lymphocytes; **G** – Monocytes**; H –** Basophil.

In Group 1, monocytes and basophils were stable, while PCV, RBC, HB, and lymphocytes decreased during exposure, with partial recovery for PCV and HB and full recovery for RBC and lymphocytes post-depuration. WBC and neutrophils increased until day 21, with WBC partially and neutrophils fully returning to baseline. Groups 2 and 3 showed similar trends, with partial recovery in PCV and lymphocytes (Group 2) and full recovery in PCV, RBC, HB, and lymphocytes (Group 3). WBC and neutrophils partially recovered in both groups, while monocytes and basophils remained stable.

4.3.2.2. LIPID PROFILE

Main Effects of PT Treatment on Lipid Profile

Mean lipid profile values are presented in Table 9. Significant main effects of PT treatment were observed for HDL ($F_{3,12} = 8.48$, $p < 0.01$, $\eta^2_p = 0.68$), LDL ($F_{3,12} = 5.76$, $p < 0.01$, $\eta^2_p = 0.59$), TAG ($F_{3,12} = 4.65$, $p < 0.05$, $\eta^2_p = 0.54$), and TCHOL ($F_{3,12} = 7.33$, $p < 0.01$, $\eta^2_p = 0.65$). The control group had the lowest HDL, similar to Group 2 but significantly lower than Groups 1 and 3 ($p < 0.05$). The control group had the highest LDL, TAG, and TCHOL, comparable to Group 1. Group 3 had similar LDL and TAG to the control, while Group 2 had the lowest LDL, TAG, and TCHOL, similar to Group 3.

Table 9. 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.

Main Effects of Sampling Time on Lipid Profile

Sampling time significantly affected LDL ($F_{3,36} = 7.87$, $p < 0.001$, $\eta^2_p = 0.40$) and TAG ($F_{2.08,24.99} = 32.31$, $p < 0.001$, $\eta^2_p = 0.73$), but not HDL ($F_{3,36} = 0.04$, $p = 0.988$, $\eta^2_p = 0.003$) or TCHOL ($F_{1.91,22.87} = 3.0$, $p = 0.052$, $\eta^2_p = 0.22$). LDL decreased from day 0 to day 7, returning to baseline by days 21 and 28. TAG remained stable until day 7, then decreased significantly by day 21, remaining low post-depuration.

Interaction Effects of PT Treatment and Sampling Time on Lipid Profile

Significant interaction effects were found for HDL ($F_{9,36} = 7.06$, $p < 0.001$, $\eta^2_p = 0.64$), LDL ($F_{9,36} = 7.37$, $p < 0.001$, $\eta^2_p = 0.65$), and TCHOL ($F_{5.72,22.87} = 6.86$, $p < 0.001$, $\eta^2_p = 0.63$), but not TAG ($F_{6.24,24.99} = 1.06$, $p = 0.415$, $\eta^2_p = 0.21$). The control group showed stable HDL and TCHOL, with a transient LDL increase on day 21, returning to baseline by day 28. Groups 1 and 3 had minimal changes in LDL and TCHOL, but HDL increased significantly by day 21, returning to baseline by day 28. In Group 2, HDL was stable, while LDL and TCHOL increased from day 7 to day 21, returning to baseline post-depuration (Figure 6).

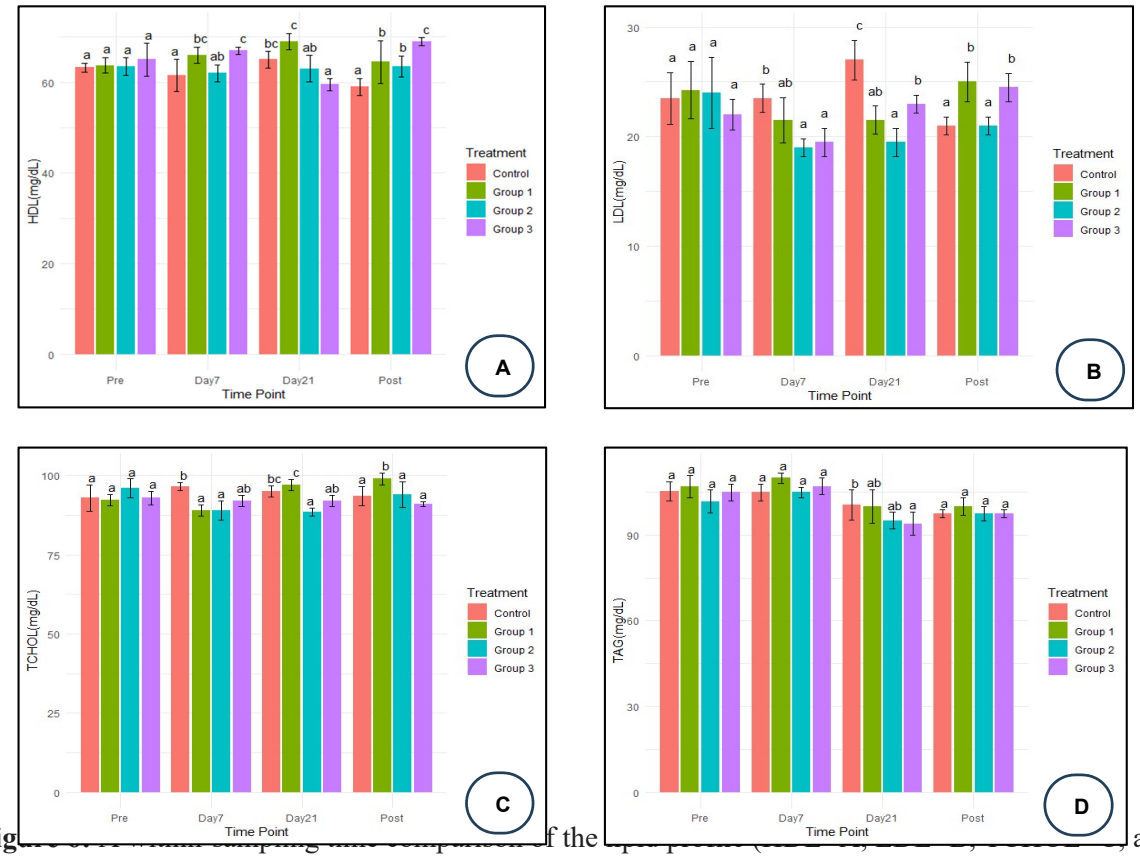

Fig [illegible] of the [illegible] and TAG- D) of African Catfish (C. gariepinus) Juveniles exposed to sub-lethal doses of Paint Thinner [a,b,c,] Bars with different letters for each sampling time are significantly different ($p < 0.05$); Control – 0 mg/L paint thinner; Group 1 – 0.1 mg/L paint thinner; Group 2 – 0.2 mg/L paint thinner; Group 3 – 0.4 mg/L paint thinner; Pre.: Pre-treatment period; HDL: High-Density Lipoproteins; LDL: Low-Density Lipoproteins; TCHOL: Total Cholesterol.

4.3.2.3. SERUM BIOCHEMISTRY

Main Effects of PT Treatment on Serum Biochemistry

Table 10 details the serum biochemistry of PT-exposed juveniles. Significant main effects were observed for ALT ($F_{3,12} = 131.57$, $p < 0.01$, $\eta^2_p = 0.97$), AST ($F_{3,12} = 166.18$, $p < 0.01$, $\eta^2_p = 0.98$), ALP ($F_{3,12} = 201.61$, $p < 0.01$, $\eta^2_p = 0.98$), urea ($F_{3,12} = 147.87$, $p < 0.01$, $\eta^2_p = 0.97$), creatinine ($F_{3,12} = 278.66$, $p < 0.01$, $\eta^2_p = 0.99$), bilirubin ($F_{3,12} = 155.53$, $p < 0.01$, $\eta^2_p = 0.98$), and albumin ($F_{3,12} = 177.35$, $p < 0.01$, $\eta^2_p = 0.98$). The control group had the lowest values for all parameters. PT-treated groups (1, 2, and 3) showed elevated AST, ALP, creatinine, bilirubin, and albumin, with no significant differences among them ($p \geq 0.05$). Groups 2 and 3 had the highest ALT, and Group 3 had the highest urea, comparable to Group 1, indicating a dose-dependent effect on ALT and urea.

Main Effects of Sampling Time on Serum Biochemistry

Sampling time significantly affected ALT ($F_{3,36} = 260.98$, $p < 0.001$, $\eta^2_p = 0.96$), AST ($F_{1.98,23.74} = 452.44$, $p < 0.001$, $\eta^2_p = 0.97$), ALP ($F_{2.09,25.12} = 480.59$, $p < 0.001$, $\eta^2_p = 0.98$), urea

($F_{3,36} = 149.02$, $p < 0.001$, $\eta^2_p = 0.93$), creatinine ($F_{3,36} = 316.53$, $p < 0.001$, $\eta^2_p = 0.96$), bilirubin ($F_{3,36} = 182.79$, $p < 0.001$, $\eta^2_p = 0.94$), and albumin ($F_{3,36} = 593.51$, $p < 0.001$, $\eta^2_p = 0.98$). All parameters increased from day 0 to day 21, then decreased by day 28. ALT, AST, and urea returned to day 7 levels, ALP and albumin dropped below day 7 but remained above baseline, creatinine returned to baseline, and bilirubin fell below baseline.

Table 10. **Effect of chronic exposure to sub-lethal doses of paint thinner on the serum biochemistry of African catfish juveniles**

Source	ALT (U/L)	AST (U/L)	ALP (U/L)	Urea (mg/dL)	Creatinine (mg/dL)	Bilirubin (µmol/L)	Albumin (g/dL)
Sampling time							
Day 0 (Pre-Treat.)	31.25 (0.43)^{c}	18.63 (0.25)^{c}	41.75 (0.40)^{d}	62.44 (0.54)^{c}	4.23 (0.08)^{c}	84.5 (0.75)^{c}	3.4 (0.04)^{d}
Day 7	40.38 (0.39)^{b}	30.25 (0.4)^{b}	58.63 (0.56)^{b}	73.13 (0.42)^{b}	6.01 (0.05)^{b}	94.38 (0.66)^{b}	5.36 (0.06)^{b}
Day 21	47.63 (0.59)^{a}	38.38 (0.5)^{a}	66.5 (0.57)^{a}	80.38 (0.58)^{a}	7.15 (0.09)^{a}	99.75 (0.55)^{a}	7.08 (0.07)^{a}
Day 28 (Post-Treat.)	39.0 (0.35)^{b}	31.25 (0.47)^{b}	50.5 (0.40)^{c}	71.63 (0.77)^{b}	4.3 (0.09)^{c}	80.13 (0.67)^{d}	4.9 (0.09)^{c}
TP-Treatment							
Group1	40.19 (0.54)^{b}	33.31 (0.49)^{a}	57.31 (0.5)^{a}	75.44 (0.54)^{ab}	6.09 (0.08)^{a}	94.06 (0.64)^{a}	5.69 (0.08)^{a}
Group2	43.19 (0.54)^{a}	32.19 (0.49)^{a}	57.56 (0.5)^{a}	73.81 (0.54)^{b}	5.95 (0.08)^{a}	93.94 (0.64)^{a}	5.83 (0.08)^{a}
Group3	44.25 (0.54)^{a}	32.75 (0.49)^{a}	58.75 (0.5)^{a}	76.19 (0.54)^{a}	6.10 (0.08)^{a}	93.06 (0.64)^{a}	5.61 (0.08)^{a}
Control	30.63 (0.54)^{c}	20.25 (0.49)^{b}	43.75 (0.5)^{b}	62.13 (0.54)^{c}	3.55 (0.08)^{b}	77.69 (0.64)^{b}	3.63 (0.08)^{b}
Interactions							
Group1 X Day 0	31.25 (0.86)^{a}	18.25 (0.49)^{a}	41.75 (0.80)^{a}	63.75 (1.07)^{a}	4.38 (0.16)^{a}	84.75 (1.49)^{a}	3.43 (0.08)^{a}
Group2 X Day 0	30.75 (0.86)^{a}	18.25 (0.49)^{a}	41.75 (0.80)^{a}	61.75 (1.07)^{a}	3.97 (0.16)^{a}	85.25 (1.49)^{a}	3.31 (0.08)^{a}
Group3 X Day 0	31.5 (0.86)^{a}	19.0 (0.49)^{a}	41.5 (0.80)^{a}	62.75 (1.07)^{a}	4.41 (0.16)^{a}	83.25 (1.49)^{a}	3.31 (0.08)^{a}
Control X Day 0	31.5 (0.86)^{a}	19.0 (0.49)^{a}	42.0 (0.80)^{ab}	61.5 (1.07)^{a}	4.16 (0.16)^{a}	84.75 (1.49)^{a}	3.56 (0.08)^{a}
Group1 X Day 7	41.0 (0.78)^{b}	36.0 (0.79)^{b}	64.0 (1.13)^{b}	76.0 (0.83)^{b}	6.68 (0.09)^{b}	98.0 (1.32)^{b}	5.84 (0.12)^{b}
Group2 X Day 7	46.0 (0.78)^{b}	35.5 (0.79)^{b}	59.5 (1.13)^{b}	73.5 (0.83)^{b}	7.07 (0.09)^{b}	101.0 (1.32)^{b}	6.32 (0.12)^{b}
Group3 X Day 7	46.0 (0.78)^{b}	32.0 (0.79)^{b}	66.5 (1.13)^{b}	82.5 (0.83)^{b}	6.42 (0.09)^{b}	99.0 (1.32)^{b}	6.01 (0.12)^{b}
Control X Day 7	28.5 (0.78)^{a}	17.5 (0.79)^{a}	44.5 (1.13)^{ab}	60.5 (0.83)^{a}	3.89 (0.09)^{a}	79.5 (1.32)^{ab}	3.28 (0.12)^{a}

Group1 X Day 21	49.0 (1.18)^{c}	43.0 (0.99)^{c}	71.5 (1.14)^{c}	86.0 (1.16)^{c}	8.37 (0.19)^{c}	111.5 (1.1)^{c}	7.67 (0.13)^{c}
Group2 X Day 21	53.0 (1.18)^{c}	42.5 (0.99)^{c}	76.5 (1.14)^{c}	87.5 (1.16)^{c}	8.14 (0.19)^{c}	106.0 (1.1)^{b}	8.37 (0.13)^{c}
Group3 X Day 21	57.0 (1.18)^{c}	47.0 (0.99)^{c}	72.0 (1.14)^{c}	85.5 (1.16)^{b}	8.78 (0.19)^{c}	105.5 (1.1)^{c}	8.49 (0.13)^{c}
Control X Day 21	31.5 (1.18)^{a}	21.0 (0.99)^{ab}	46.0 (1.14)^{a}	62.5 (1.16)^{a}	3.31 (0.19)^{b}	76.0 (1.1)^{b}	3.79 (0.13)^{a}
Group1 X Day 28	39.5 (0.71)^{b}	36.0 (0.94)^{b}	52.0 (0.79)^{d}	76.0 (1.53)^{b}	4.94 (0.18)^{a}	82.0 (1.33)^{a}	5.81 (0.18)^{b}
Group2 X Day 28	43.0 (0.71)^{b}	32.5 (0.94)^{b}	52.5 (0.79)^{d}	72.5 (1.53)^{b}	4.63 (0.18)^{a}	83.5 (1.33)^{a}	5.31 (0.18)^{d}
Group3 X Day 28	42.5 (0.71)^{d}	33.0 (0.94)^{b}	55.0 (0.79)^{d}	74.0 (1.53)^{c}	4.8 (0.18)^{a}	84.5 (1.33)^{a}	4.62 (0.18)^{d}
Control X Day 28	31.0 (0.71)^{a}	23.5 (0.94)^{b}	42.5 (0.79)^{d}	64.0 (1.53)^{a}	2.83 (0.18)^{b}	70.5 (1.33)^{c}	3.89 (0.18)^{a}
P-Value							
Sampling time	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
TP-Treatment	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Interactions	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

^{a,b,c,d} 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; ALT: alanine aminotransferase; AST: aspartate aminotransferase; ALP: alkaline phosphate.

Interaction Effects of PT and Sampling Time on Serum Biochemistry

Significant interaction effects were observed for ALT ($F_{9,36} = 34.57$, $p < 0.001$, $\eta^2_p = 0.90$), AST ($F_{5.93,23.74} = 48.39$, $p < 0.001$, $\eta^2_p = 0.92$), ALP ($F_{6.28,25.12} = 41.77$, $p < 0.001$, $\eta^2_p = 0.91$), urea ($F_{9,36} = 19.08$, $p < 0.001$, $\eta^2_p = 0.83$), creatinine ($F_{9,36} = 45.17$, $p < 0.001$, $\eta^2_p = 0.92$), bilirubin ($F_{9,36} = 27.27$, $p < 0.001$, $\eta^2_p = 0.87$), and albumin ($F_{9,36} = 70.43$, $p < 0.001$, $\eta^2_p = 0.95$). The control group showed no significant changes in ALT, ALP, urea, or albumin with sampling time, but AST increased, and creatinine and bilirubin decreased by day 28. PT-treated groups showed increased indices until day 21, reversing by day 28. Across all treated groups, AST returned to day 7 levels, ALP dropped below day 7 but remained above baseline, and creatinine and bilirubin reverted to baseline. ALT in Groups 1 and 2 returned to day 7, while Group 3 dropped below day 7 but remained above baseline. Urea in Groups 1 and 2 returned to day 7, while Group 3 reverted to baseline. Albumin in Group 1 returned to day 7, while Groups 2 and 3 dropped below day 7 but remained above baseline. Group 3 exhibited the greatest recovery, particularly for ALT, urea, and albumin (Figure 7).

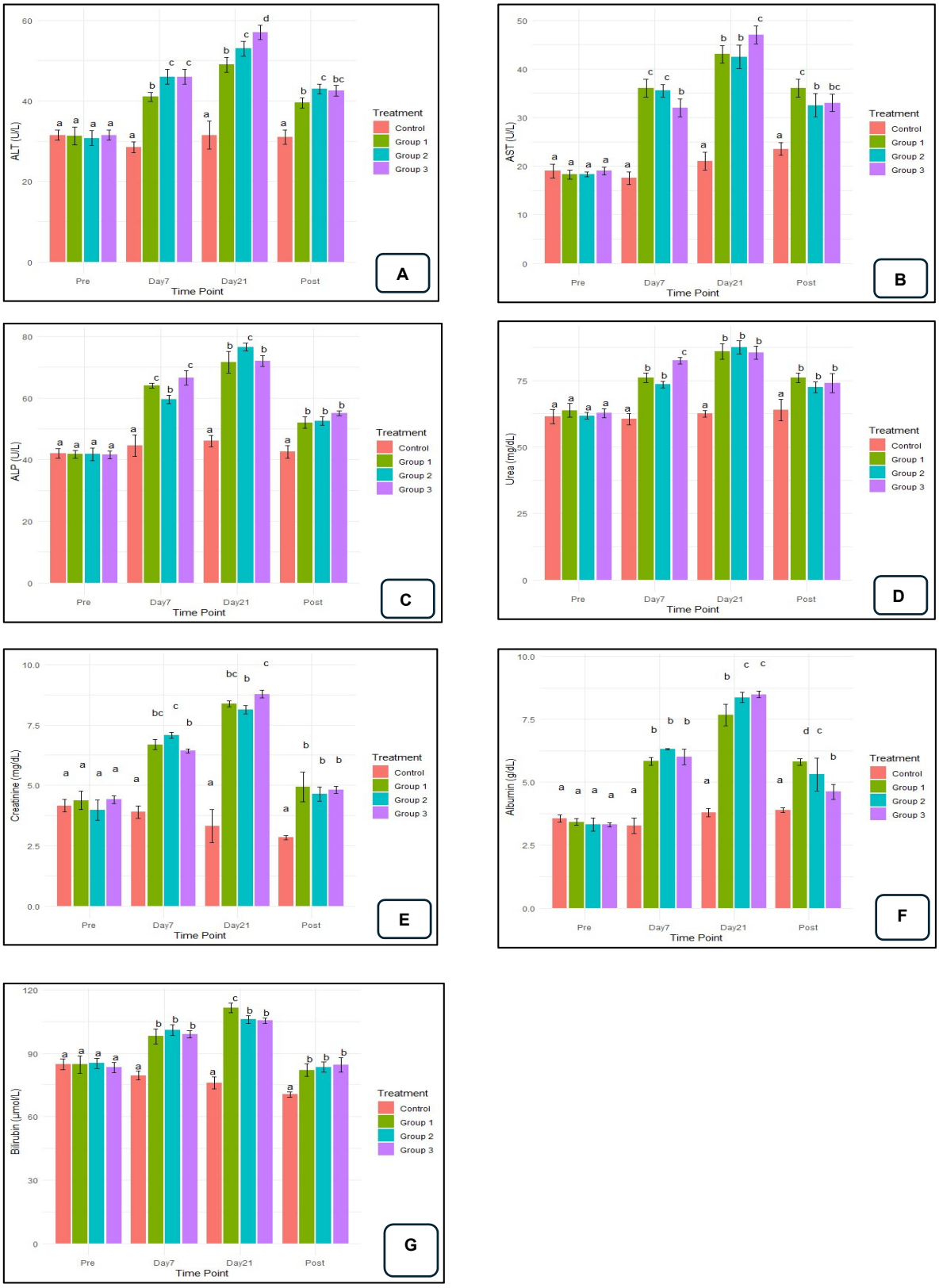

Figure 7: A within-sampling time comparison of the serum biochemical indices of African catfish juveniles exposed to sub-lethal doses of Paint Thinner

[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).

4.4. DISCUSSION

The contamination of aquatic ecosystems by hazardous industrial residues and by-products represents a critical global environmental challenge (Uboh *et al*., 2012; Owolabi & Adewoye, 2017). PT, comprising a mixture of toxic organic solvents such as toluene, xylene, acetone, turpentine, N-hexane, and various aromatic and halogenated hydrocarbons, enters water bodies through industrial effluents, storage or transportation leaks, and surface runoff (Liang *et al*., 2022). These pollutants threaten aquatic biodiversity, including species like *C. gariepinus*, a vital food and economic resource in many regions. Given the increasing urbanisation, infrastructure development, and widespread use of paint-related products, particularly in developing nations, this study evaluated the toxicological effects of a commercially available NC-based lacquer thinner (Hitro Universale Lacquer Thinner) on juvenile *C. gariepinus*. The investigation focused on the acute and chronic sub-lethal exposures over 21 days, analysing behavioural and physiological responses, biomarkers of organ health, alongside the potential for recovery during a 7-day depuration phase.

4.4.1. BEHAVIOURAL AND MORTALITY RESPONSES TO ACUTE TOXICITY

Behavioural responses are integral to the fitness of organisms, influencing their ability to reproduce, defend territories, evade predators, and forage effectively (Söffker and Tyler, 2012; Brodin *et al*., 2014). This study investigated the effect of acute exposure to PT on the behaviour and survival of *C. gariepinus* juveniles, revealing a pronounced dose-dependent relationship between PT concentrations and aberrant behavioural patterns. At elevated concentrations (≥ 1.50 mg/l), fish exhibited heightened erratic swimming, surface air gulping, and attempts to escape the water, indicative of stress responses similar to the "general adaptation syndrome" outlined by Wedemeyer *et al*. (1990). Such toxicant-induced hyperactivity is likely driven by the release of stress hormones, which precipitate physiological adjustments that may lead to exhaustion, neurological impairments, and gill dysfunction, culminating in mortality at higher concentrations (Erondu and Chindah, 1991; Chris *et al*., 2022). Prolonged hyperactivity and hyperventilation may further exacerbate nervous system dysfunction and toxicity, contributing to the observed mortality at higher PT levels (Omoregie, 1995; Chris *et al*., 2022). These factors, combined with potential reductions in CO2 levels due to hyperventilation, likely account for the dose-dependent mortality observed in this study. Increased fin movements, suggestive of escape behaviour, align with responses to other environmental contaminants (Ufodike and Omoregie, 1991; Okwuosa and Omoregie, 1995; Chris *et al*., 2022). Diminished feeding activity may reflect appetite suppression or impaired feeding capacity, possibly linked to disruptions in serotonin signalling pathways (Fent *et al*., 2006; Nassef *et al*., 2010).

The precise physiological mechanisms underlying pollutant-induced behavioural changes in fish remain an active area of research. Potential mechanisms include disruptions in cholinesterase activity, neurotransmitter function, or hormonal signalling (Brodin *et al*., 2014; Vindas *et al*., 2017). Dopamine, a key catecholaminergic neurotransmitter, plays a critical role in regulating locomotion, cognition, emotion, motivation, and decision-making, and its disruption may contribute to the observed behavioural alterations (Messias *et al*., 2016; Van der Zouwen and Ryczko, 2020). Previous studies have reported decreased dopamine and serotonin levels in larval zebrafish exposed to toxicants such as chlorpyrifos and tris(1,3-dichloro-2-propyl) phosphate, with dopamine deficiencies persisting post-exposure (Wang *et al*., 2015). This suggests a potential neurotoxic effect of PT, mediated by dopamine dysregulation. Two primary mechanisms have been proposed to explain pollutant-induced behavioural disruptions. First, pollutants may directly interact with neural receptors, such as gamma-aminobutyric acid (GABA) receptors, causing neuronal overstimulation and potentially leading to mortality (Huang *et al*., 2019). Second, pollutants may interfere with inhibitory neurotransmission in the central nervous system, triggering a cascade of effects that manifest as behavioural changes (Grillner, 2003; López-Corcuera *et al*., 2001; Wang *et al*., 2016).

Furthermore, the energetic costs associated with detoxification and stress responses can deplete energy reserves, further influencing fish behaviour (Sokolova *et al*., 2012; Sokolova, 2013; Montiglio and Royauté, 2014). Prior research has also documented reduced ketosteroid levels, linked to agonistic behaviours and reproductive fitness, in male fish exposed to environmental contaminants (Martinović *et al*., 2007). The complex chemical composition of PT likely affects fish behaviour and survival through intricate physiological and metabolic pathways involving the endocrine and nervous systems. Consequently, additional studies are warranted to clarify the specific biochemical and physiological processes mediating these effects. The findings of this study are consistent with prior research demonstrating altered behaviour and locomotion in fish exposed to sublethal concentrations of insecticides (Chaulet *et al*., 2019; Moreira *et al*., 2020), MS-222 anaesthetics (Kane *et al*., 2004), flame retardants (Jarema *et al*., 2015), and pharmaceuticals, which have been shown to impair feeding rates and swimming behaviours across various fish species (Nassef *et al*., 2010).

4.4.2. CHRONIC TOXICITY

4.4.2.1. HAEMATOLOGICAL RESPONSES

Blood parameters serve as sensitive indicators of environmental stress, providing insights into physiological changes in fish (Witeska *et al*., 2023). Haematological assessments are a reliable and cost-effective approach to evaluate the impact of toxicants on fish health

across diverse aquatic environments (Maksoud *et al*., 2018; Fazio, 2019; Ali *et al*., 2021). Statistical analysis using a mixed-model ANOVA revealed a significant interaction between PT exposure and sampling intervals for most Haematological parameters, except eosinophils. Post-hoc Bonferroni tests indicated that, excluding WBC counts, all Haematological indices in the control group remained consistent across sampling points, reflecting a stable physiological state in the absence of stressors.

RBCs and Hb are critical for oxygen transport, nutrient delivery, and inter-organ signalling, influencing nitric oxide metabolism, redox equilibrium, and blood viscosity (Kuhn *et al*., 2017). Hb levels are widely used to detect adverse health conditions in fish (Kopp *et al*., 2010; Casanovas *et al*., 2021), while PCV reflects RBC volume and is affected by RBC count and size (Vali *et al*., 2020). Alterations in RBC count, Hb concentration, or PCV can signal anaemic conditions in aquatic species (Palanisamy *et al*., 2011). This study observed significant, dose-dependent reductions in Hb, RBC, and PCV levels in PT-exposed groups (Groups 1, 2, and 3) with prolonged exposure, indicating PT's haematotoxic effects on erythropoiesis. These changes suggest either accelerated RBC degradation or suppressed RBC production, as previously noted by Sayed *et al*. (2023). The volatile organic compounds (VOCs) and hydrocarbons in PT, known to induce oxidative stress and inflammation, likely disrupted haematopoiesis by interacting with blood-forming tissues (Uboh *et al*., 2012; Kuang *et al*., 2021). Excessive ROS accumulation may further impair hematopoietic stem cells, leading to DNA damage, apoptosis, or anaemic states (Ghaffari, 2008; Porto *et al*., 2015). The high polyunsaturated fatty acid content in RBC membranes and haemoglobin's susceptibility to auto-oxidation render RBCs vulnerable to oxidative damage, compromising membrane integrity and enzyme function (Jewell *et al*., 2013; Orrico *et al*., 2023). PT's biochemical components likely triggered oxidative stress, impairing hematopoietic cell function, degrading haemoglobin, and disrupting ionic homeostasis, resulting in the observed declines in Hb, RBC, and PCV (Kuhn *et al*., 2017). Comparable haematotoxic effects have been reported for solvents like benzene, carbon disulfide, and hexane, where metabolites suppress hematopoietic activity (Amarnath *et al*., 1991; Valentine *et al*., 1993; Synder & Hedli, 1996). Additionally, increased swimming activity due to higher PT concentrations may have contributed to altered PCV levels, as suggested by Chris *et al*. (2022). These findings align with prior research documenting reduced Hb, RBC, and PCV in *Oreochromis niloticus* exposed to benzene, toluene, and xylene (Sayed *et al*., 2023) and in *C. gariepinus* exposed to xylene (Chris *et al*., 2022), as well as in other fish species exposed to dyes (Srivastav & Roy, 2015; Avni & Jagruti, 2017; Deepika & Noorjahan, 2018; Alaguprathana & Poonkothai, 2021).

WBCs are integral to the fish immune system, with distinct subtypes responding to stressors, pathogens, or toxins (Vali *et al*., 2020). This study found dose-dependent increases in WBC and neutrophil counts in PT-exposed groups, contrasted by reductions in lymphocyte levels with prolonged exposure. Elevated WBC counts may reflect enhanced antibody production or spleen-mediated WBC release to counteract PT-induced stress (Begg & Pankhurst, 2004; Hori *et al*., 2008; Amaeze *et al*., 2020). Tissue damage from PT exposure could also stimulate leucocytosis (Uboh *et al*., 2012). These results are consistent with studies reporting increased WBC counts in fish exposed to VOCs (Chris *et al*., 2022; Sayed *et al*., 2023), dyes (Oluah *et al*., 2018; Alaguprathana & Poonkothai, 2021; Singh and Pandey, 2023), pesticides (Maurya *et al*., 2019; Kanu *et al*., 2023), and aniline (Sharma & Chadha, 2023). Similar patterns were observed in rats exposed to nitrocellulose and polyurethane paint thinners (Uboh *et al*., 2012; Patrick-Iwuanyanwu *et al*., 2013; Hussein, 2019). The decline in lymphocyte levels likely indicates immune suppression due to PT's overwhelming toxicity, contrasting with reports of increased lymphocytes in mice exposed to similar thinners (Savithri *et al*., 2010; Uboh *et al*., 2012; Hussein, 2019). These discrepancies emphasise the need for further research to reveal species-specific immune responses.

4.4.2.2. LIPID METABOLISM

Lipids play essential roles in fish physiology as energy reserves, membrane components, and signalling molecule precursors. Lipid profile analysis is a valuable tool for assessing liver function and overall health under toxicant exposure (Sayed *et al*., 2011; Hook *et al*., 2018; Cocci *et al*., 2019). This study identified significant interactions between PT exposure and sampling time on serum HDL, LDL, and TChol levels. Fluctuations in TChol and LDL suggest disruptions in lipid metabolism, likely driven by the mobilisation of stored lipids to meet energy demands under PT-induced stress. Chronic stress depletes glycogen, shifting energy reliance to lipids and proteins (Malik *et al*., 2023). These changes may result from membrane damage, increased hepatic cholesterol production, or thyroid dysfunction affecting TAG metabolism (Hossain *et al*., 2000; Javed & Usmani, 2016; Singh & Pandey, 2023). Liver damage could also impair cholesterol conversion to bile acids, elevating TChol and TAG levels (Singh & Pandey, 2023).

Elevated TAG levels, indicative of metabolic or liver dysfunction, were observed in PT-treated fish, potentially due to reduced lipoprotein lipase activity (Hamdy *et al*., 2018; Singh & Pandey, 2023). Similar lipid profile alterations have been reported in fish from contaminated waters, such as *Tor putitora* (Yousafzai & Shakoori, 2011), *Channa punctatus* (Javed & Usmani, 2016), and Nile tilapia (Osman *et al*., 2018), as well as in fish exposed to copper sulphate (Mutlu *et al*., 2015), pesticides, heavy metals (Fırat *et al*., 2011; Kanu *et al*., 2023) and detergents

(Singh & Pandey, 2023). However, some studies, such as those on Atlantic cod exposed to oil pollutants, reported no lipid profile changes (Bratberg *et al*., 2013), highlighting variability in species-specific responses. The partial recovery of lipid profiles post-depuration suggests a capacity for restoring lipid homeostasis, though the extent of recovery varies with exposure duration and intensity.

4.4.2.3. LIVER AND KIDNEY FUNCTION

This study evaluated serum levels of AST, ALT, ALP, creatinine, urea, bilirubin, and albumin to assess hepatic and renal function, as well as overall physiological health, in *C. gariepinus* across multiple sampling intervals. The liver, due to its central role in the portal circulatory system, is particularly susceptible to damage from xenobiotics, making it a primary target for toxicant-induced harm (Jyothi and Narayan, 1999). Alterations in fish blood biochemistry are reliable indicators of the effects of toxicants and environmental stressors on critical organs, including the liver and kidneys (Mohamed *et al*., 2019; Hamed *et al*., 2021; Said *et al*., 2021; Farrag *et al*., 2022; Hamed *et al*., 2023). AST and ALT, enzymes integral to protein and amino acid metabolism, are released into the bloodstream following structural or functional damage to the liver, heart, gills, kidneys, or muscle tissues (Atli *et al*., 2015; Morcos *et al*., 2015). Elevated serum ALP levels are often associated with hepatopathology, particularly conditions such as obstructive jaundice or impaired bile flow, and may indicate chronic liver dysfunction (Hayat *et al*., 2005; Okechukwu and Auta, 2007). The observed increases in serum AST, ALT, and ALP in PT-exposed fish suggest that these enzymes may have been released into the bloodstream due to the toxicant-induced damage and dysfunction in the liver, kidneys, heart, and other organs (Osman *et al*., 2018; Reddy *et al*., 2023).

Although limited research has explored the hepatotoxic effects of PT on fish, studies on human occupational exposure to paint solvents have reported elevated liver enzymes and hepatopathology, including hepatitis and hepatic necrosis, among painters and industrial workers (Døssing, 1986; Lundberg *et al*., 1994; Malaguarnera *et al*., 2012; Aziz, 2015; Agrawal *et al*., 2020). Organic solvents, such as those found in PT, may disrupt liver structure and function through mechanisms such as inflammation, cytochrome P450 dysregulation, mitochondrial impairment, and oxidative stress (Malaguarnera *et al*., 2012; Agrawal *et al*., 2020). Comparable elevations in serum ALT, AST, and ALP have been documented in various fish species exposed to diverse chemical contaminants, including pesticides (Agrahari *et al*., 2007; Kanu *et al*., 2023), herbicides (Akhtar *et al*., 2021b), heavy metals (Oner *et al*., 2008; Mekkawy *et al*., 2011), and polluted aquatic environments (Osman *et al*., 2018; Hamdy *et al*., 2018). Furthermore, exposure to NC-PT fumes has been shown to increase serum ALT, AST, and ALP levels in rats (Patrick-Iwuanyanwu *et al*., 2013), indicating similar toxicological

effects across species. These findings collectively stress the potential of PT to induce significant hepatic and systemic toxicity in *C. gariepinus*, highlighting the need for further investigation into the mechanisms underlying these effects.

Bilirubin, a byproduct of heme metabolism resulting from red blood cell degradation, serves as an indicator of haemolytic anaemia or hepatic dysfunction (Adams and Fortner, 2012; Rezaei-Moghadam *et al*., 2012). Albumin, a key protein responsible for maintaining osmotic pressure and transporting biomolecules, is also a marker of liver function (Rezaei-Moghadam *et al*., 2012; Pastorino *et al*., 2022). In this study, the progressive increase in serum bilirubin and albumin levels in *C. gariepinus* exposed to PT over time suggests hepatic impairment. Elevated bilirubin levels may reflect liver damage, bile duct obstruction, or an overburdened liver unable to process excessive pigment breakdown, as seen in certain haemolytic anaemias (Jyothi and Narayan, 1999). Previous studies have reported increased bilirubin in fish exposed to contaminants such as crude oil derivatives (Nwamba *et al*., 2006), pesticides (Jyothi and Narayan, 1999), and PTEs in polluted rivers (Ullah *et al*., 2021). Similarly, elevated albumin levels have been observed in *Oreochromis niloticus* exposed to benzene (Sayed *et al*., 2023) and in fish from contaminated aquatic systems (Hamdy *et al*., 2018).

Urea and creatinine, end products of protein metabolism, are critical indicators of organ function in fish (Salazar, 2014). Urea, derived from the breakdown of dietary and tissue proteins, is synthesised in the liver and primarily excreted via the gills in fish, unlike mammals, where renal excretion predominates (Ip and Chew, 2010; Hamdy *et al*., 2018). Consequently, elevated serum urea levels are widely used as biomarkers for gill and liver dysfunction or impaired nitrogen excretion (Nelson *et al*., 1999; Adams and Fortner, 2012; Alkaladi *et al*., 2015). The increased urea levels observed in this study may result from compromised gill and liver functions or altered nitrogen metabolism in PT-exposed fish (Hamdy *et al*., 2018). These findings are consistent with prior reports of gill and liver damage, including hyperplasia, tissue degeneration, and necrosis, in *C. gariepinus* exposed to antifouling paints (George *et al*., 2017) and paint wastewater (Owolabi and Adewoye, 2017) under acute and chronic conditions.

Elevated creatinine levels, indicative of renal dysfunction such as tubular necrosis, were also observed in PT-treated fish, with increases correlating with exposure duration (Murray *et al*., 1990; Toffaletti and McDonnell, 2008; Deka and Mahanta, 2015). This suggests progressive kidney impairment with prolonged PT exposure. Additionally, proteins are essential for cellular homeostasis and energy provision in fish (Aragão *et al*., 2022), with increased protein catabolism reported under stress (Herrera *et al*., 2019). Amino acids such as glutamate, glutamine, and aspartate serve as primary metabolic substrates in fish tissues, including the liver, kidney, and skeletal muscle (Falco *et al*., 2020). The elevated serum urea and creatinine

levels in PT-treated fish may therefore reflect heightened protein metabolism to meet increased energy demands induced by toxicant stress. These results align with studies reporting elevated urea and creatinine in *C. lazera* exposed to dyestuff and chemical wastewater (Abdel-Moneim *et al*., 2008), as well as in *C. gariepinus*, *O. niloticus* (Kanu *et al*., 2023), *Channa punctatus* (Bharti and Rasool, 2021), and *C. lazera* (Aziz *et al*., 2009) exposed to pesticides or sampled from polluted waters (Osman *et al*., 2018; Hamdy *et al*., 2018).

Collectively, these findings indicate that PT exposure induces dysfunction and structural damage to the liver, gills, and kidneys of *C. gariepinus*, underscoring the toxicant's potential to impair internal organ function (Stoskoph, 1993; Abdel-Moneim *et al*., 2008). These observations are consistent with reports of significant liver and kidney damage, increased inflammation, reduced blood flow, and widespread cell death in catfish exposed to benzene, toluene, and xylene (BTX) mixtures (Sayed *et al*., 2023). Earlier studies have also documented renal damage, including tubular necrosis and vacuolization, in guinea pigs exposed to toluene (Kronevi *et al*., 1979).

Notably, some biochemical indices showed signs of recovery following PT withdrawal, suggesting that the observed liver and kidney dysfunction may be reversible. Fish exposed to higher PT concentrations exhibited faster recovery, possibly due to enhanced homeostatic mechanisms compensating for greater physiological stress. However, the limited duration of the depuration period necessitates further studies to assess long-term recovery and organ histopathology. These findings are consistent with reports of reversible Haematological and neurological effects in humans exposed to paint solvents (Atkinson *et al*., 1989), indicating potential cross-species recovery mechanisms.

4.5. Conclusion

This investigation provides the inaugural assessment of the toxicity and physiological impacts of NC-PT on the economically significant *C. gariepinus*. The results highlights the considerable toxicological effects of PT on this species. Acute exposure elicited substantial mortality and behavioural abnormalities, while prolonged exposure led to physiological impairments, as evidenced by alterations in haematological, serum lipid, and biochemical profiles. The observed changes in biomarkers of liver and kidney function highlight the hepatotoxic and nephrotoxic potential of PT. Although partial recovery in certain blood parameters was noted after a seven-day depuration period, the broader adverse implications for aquatic ecosystems are evident. To address these environmental risks, there is an urgent need for stringent regulations to govern the production, application, and disposal of PT, thereby safeguarding aquatic biodiversity and human health.

5. EXPERIMENT 3

ASSESSMENT OF MICROPLASTIC POLLUTION IN THE HUNGARIAN SECTION OF TISZA RIVER: A PIONEERING STUDY ON BURBOT (*LOTA LOTA*)

5.1. BACKGROUND OF STUDY

Microplastic (MP) pollution, defined as plastic particles smaller than 5 mm, has emerged as a pressing global environmental issue, threatening both ecosystems and human health. Originating from primary sources (e.g., microbeads in personal care products) or secondary sources (e.g., fragmentation of larger plastics), MPs pervade aquatic environments, causing physical harm, chemical toxicity, and trophic transfer in organisms, with implications for biodiversity and food safety (Sarijan *et al*., 2021; Xi *et al*., 2022). While marine MP pollution has been extensively studied, freshwater systems, which serve as critical pathways for terrestrial plastic waste to reach oceans, remain underexplored, particularly in Central and Eastern Europe (Horton *et al*., 2017; Li *et al*., 2018; Balla *et al*., 2022). Rivers are key conduits, with 122 heavily polluted rivers contributing over 90% of the 1.15–2.41 million tons of plastic waste entering oceans annually (Lebreton *et al*., 2017). In Europe, MP concentrations in rivers such as the Rhine, Danube, and Seine vary widely, ranging from 0 to 187,000 particles/m³ in water and 18 to 72,400 particles/kg in sediments, highlighting the persistent and ubiquitous nature of this pollutant, even in regions with advanced waste management (Sarijan *et al*., 2021; European Environmental Agency, 2023).

Eastern Europe, including Hungary, faces elevated levels of mismanaged plastic waste, compounded by inadequate wastewater treatment infrastructure (Lebreton & Andrady, 2019). In the Upper Tisza catchment in Ukraine, only 1.5% of rural households are connected to wastewater systems, leading to untreated effluents rich in textile-derived microfibres from washing machines, which are increasingly used with affordable synthetic fabrics (Lebreton & Andrady, 2019; Kiss *et al*., 2021; Rebelein *et al*., 2021). Consequently, the Hungarian section of the Tisza River exhibits significant MP contamination, with surface water containing 4–63 items/m³ and sediments holding 528–8,067 items/kg, driven by wastewater discharges, agricultural runoff, and urban activities (Kiss *et al*., 2021; Balla *et al*., 2022). Further, Hungary's elevated per capita plastic packaging waste generation (from 21 kg/cap in 2010 to 35 kg/capkg in 2019), post consumer plastic land fills (57%) and low recycling rate (22%) and waste treatment (26%) (Plastics Europe, 2022; European Commission, 2023) may also exacerbate MP pollution in the Tisza River, necessitating urgent investigations to elucidate the impacts of inadequate waste management on riverine ecosystems. Despite these findings,

comprehensive biomonitoring of MP ingestion by fish in the Tisza River remains limited, hindering efforts to assess ecological impacts and pollution sources.

Fish are effective bioindicators for MP pollution due to their ecological roles and susceptibility to particle ingestion (Koutsikos *et al*., 2023). The burbot (*Lota lota,* Linnaeus, 1758), a benthic predator native to the Tisza River, presents an ideal option for a sentinel species. Its diverse diet, including macroinvertebrates and small fish, and its high trophic position predispose it to MP accumulation via direct ingestion and trophic transfer (Lehtonen, 1998; Wetjen *et al*., 2019). Burbot's longevity, rapid growth, and ecological significance have earned it a critical and strategic place for the European water quality assessments under the EU Water Framework Directive, further enhancing its suitability for pollution studies (Olin *et al*., 2014; Bosveld *et al*., 2015; Walther *et al*., 2022). Additionally, burbot's commercial value as a nutritious fishery resource, rich in protein and omega-3 fatty acids, further buttresses the need to evaluate MP contamination for both ecological and human health considerations (Bruce *et al*., 2020; Matuha *et al*., 2024). To date, no studies have investigated MP ingestion in burbot, representing a critical gap in understanding species-specific MP impacts in freshwater systems.

Prior research has documented MPs pollution across global freshwater systems and various fish species (Yuan *et al*., 2019; Hamed *et al*., 2022; Saad *et al*., 2022; Erdoğan, 2025). In the Tisza River, the only biomonitoring study to date, conducted by Almeshal *et al*. (2022), utilised two freshwater mussel species, *Unio tumidus* and *Unio crassus*, reporting MP abundances of 2.7–4.9 and 5.2–8.3 items per individual, respectively. Building on this, the present study provides the first assessment of MP pollution in a fish species, the burbot (*L. lota*), from the Hungarian section of the Tisza River. Employing advanced Raman spectral analysis, this investigation characterises the abundance, diversity, and chemical composition of ingested MPs, including polymer types and associated pigments. The study aims to: (1) quantify MP prevalence in burbot, (2) identify dominant polymers and pigments, (3) infer potential pollution sources, and (4) evaluate the ecological implications of MP ingestion in this sentinel species. As a pioneering effort, this research addresses critical gaps in understanding riverine MP dynamics in Central Europe, establishing a baseline for future monitoring and informing policy interventions to protect the Tisza River and comparable freshwater ecosystems.

5.2. MATERIAL AND METHODS

5.2.1. STUDY AREA

The Tisza River, a major waterway in Central and Eastern Europe, is the largest tributary of the Danube, with a catchment area of 157,200 km² spanning Ukraine, Romania, Slovakia, Hungary, and Serbia (Kiss *et al*., 2021). Originating in the Carpathian Mountains, where its sub-catchments in Ukraine, Romania, and Slovakia receive 1,000–1,400 mm of annual precipitation, the river transitions to the Carpathian Plains in Hungary and Serbia, with the Hungarian section forming the longest segment (Vizi *et al*., 2018; Balla *et al*., 2024). The Ukrainian section is heavily polluted due to inadequate waste management, untreated sewage, and runoff from mining and agriculture, contributing significant plastic and inorganic waste to the river system (Bocskor & Tóth, 2021). These pollutants, accumulating along riverbanks, are transported downstream during floods, exacerbating MPs contamination in the upper Hungarian section of the Tisza (Balla *et al*., 2024).

Plastic pollution in the Tisza has escalated in recent years. A 2017 cleanup effort removed approximately 90 tons (3,084 m³) of plastic waste, predominantly from the upper Hungarian section near the Ukrainian border (Siklós, 2017). By 2019, plastic debris in this region surged to an estimated 10,000 tons, reflecting worsening pollution inputs (index.hu, 2019). Between 2021 and 2022, MP transport in the Tisza increased by 17%, with the highest concentrations observed in the upper Hungarian section (near the Ukrainian border) and the lower Serbian section, both characterised by deficient waste management systems (Balla *et al*., 2022). To comprehensively assess MP pollution in the upper Hungarian Tisza, fish sampling was conducted at Tiszabecs, located at the Hungarian-Ukrainian border (Figure 8). Tiszabecs, where the Tisza enters Hungary's Northern Great Plain, is a critical site for evaluating MP influx from the heavily polluted Ukrainian section.

Figure 8. Map of the sampling site with the Tiszabecs point marked in red
HU: Hungary; UA: Ukraine

5.2.2. FISH SAMPLING AND PREPARATION

The burbot, a piscivorous freshwater species, was selected for this study due to its ecological significance as a bioindicator, its growing role in aquaculture, and its nutritional value in human diets. As a demersal, benthivorous predator, burbot inhabits riverbeds, which act as sinks for MPs and other pollutants, making it an ideal candidate for assessing MP contamination and associated chemical pollutants (Parvin *et al*., 2021). Ten burbot specimens were collected from the Tisza River at Tiszabecs, near the Hungarian-Ukrainian border, using an electrofishing device and transported alive to the laboratory in aerated containers. Upon arrival, specimens were rinsed with deionised water to remove external contaminants and immediately frozen at -20°C to preserve tissue integrity until analysis. For processing, frozen fish were thawed at room temperature, and residual blood and debris were removed with deionised water. Total, standard, and fork lengths were measured to the nearest 0.1 cm using a calibrated vernier calliper or meter rule, and wet weight was recorded to the nearest 0.01 g using a digital balance. Each fish was dissected in a sterile, controlled environment using stainless steel dissection tools to prevent contamination. Organs, including the gastrointestinal tract (GIT) and its contents, gills, and liver, were excised and placed in separate, pre-cleaned glass beakers covered with aluminium foil to minimise airborne contamination (Hossain *et al*., 2023). Additionally, samples of dorsal muscle tissue (mean weight: 6.02 ± 0.81 g) were collected from each specimen, transferred to sealed glass containers, and prepared for chemical digestion and subsequent MP analysis.

5.2.3. *Tissue Digestion and Vacuum Filtration of Microplastics*

Tissue digestion of burbot organ samples, including the GIT, gills, liver, and dorsal muscle, was performed following established protocols (Saad *et al*., 2022). Each sample, contained in pre-cleaned glass beakers, was fully submerged in a filtered 10% (w/v) potassium hydroxide (KOH) solution at a 1:3 volumetric ratio (sample: KOH) and sealed with aluminium foil to prevent airborne contamination. Beakers were incubated at 60°C for 24 hours in a laboratory oven, with samples gently stirred at 12-hour intervals to facilitate tissue breakdown. For liver samples, which are rich in fatty tissues, absolute ethanol (EtOH, 97% purity) was added at a 1:4 ratio (sample & KOH:EtOH) to prevent saponification, following Dawson *et al*. (2020) and Mancuso *et al*. (2023). Procedural blanks, prepared with filtered Milli-Q water and processed concurrently, were included to monitor and correct for environmental contamination. Following digestion, saturated sodium iodide (NaI) solution (density: 1.8 g/cm³) was added to the digestate at a 1:2 volumetric ratio (digestate:NaI), and the mixture was vigorously shaken, sealed with aluminium foil, and allowed to settle overnight. The supernatant was vacuum-filtered through Whatman GF/A filter paper (47 mm diameter, 1.6 µm pore size). The filtration funnel was thoroughly rinsed with filtered Milli-Q water between samples to prevent cross-contamination, ensuring all adhering particles were captured on the filter. Filter papers were placed in clean, labelled Petri dishes, sealed with aluminium foil to minimise contamination, and air-dried at room temperature for 24 hours. Dried filters were stored in sealed Petri dishes for subsequent microplastic analysis.

5.2.4. *Microplastic Identification and Morphological Analysis*

MPs extracted from the fish tissues were identified and quantified using a stereoscopic microscope equipped with an integrated camera and image analysis software. MPs on each filter paper were visually inspected, counted, and categorized by shape (fibres, fragments, films, microspheres, foams) and colour (black, red, blue, green, yellow, grey, white, transparent), following established protocols (Bessa *et al*., 2018; Rodrigues *et al*., 2018). Particle sizes were measured at their largest cross-section in situ using imaging software and classified into size ranges: ≤0.5 mm, 0.5–1 mm, and 1–5 mm. To facilitate subsequent analysis, individual MPs were transferred to compartmentalised filter papers for labelling and organisation, ensuring accurate tracking for Raman spectroscopy.

5.2.5. *Chemical Characterisation of Microplastics*

To confirm polymer composition, 98 MP particles, representing all observed shapes, colours, and sizes, were analysed using a Raman spectrometer coupled with an Olympus BX41 microscope and a 532 nm (green) Lexel Model 95-SHG argon laser. The laser wavelength was

set to 1–2 μm, and spectra were acquired over a range of 0–3,500 cm^{-1} using LabSpec v5 software (Horiba Scientific). Spectral data were processed with polynomial baseline correction in LabSpec 6 software to reduce noise and enhance quality. Polymer identification was performed using Spectragryph v1.2.16 (Menges, 2022) with reference databases (SLoPP, minerals, and organic compounds) or OpenSpecy software (Cowger *et al*., 2021) with preloaded libraries and matching algorithms. A hit quality index of ≥70% or a probability match of ≥0.7 was required for positive identification of polymer types or composite mixtures.

5.2.6. Quality Assurance and Quality Control

Rigorous quality assurance and quality control (QA/QC) measures were implemented during sample collection, preparation, and laboratory analysis to minimise environmental contamination, following established protocols (Karami *et al*., 2017; O'Connor *et al*., 2020; Kiss *et al*., 2021). Personnel wore nitrile gloves and cotton (non-synthetic) laboratory coats throughout sampling and experimental procedures to prevent fibre contamination (Liu *et al*., 2020). All solvents, including KOH, (EtOH), and NaI, were pre-filtered through Whatman filter paper (8 μm pore size). Working surfaces were cleaned with 70% ethanol before and between analyses, and only glass or stainless steel equipment and containers were used, each pre-washed with Milli-Q water, rinsed with 70% ethanol, and oven-dried to eliminate plastic residues. To mitigate airborne contamination, critical procedures, including tissue dissection and filtration, were conducted in a horizontal laminar flow cabinet when feasible. Procedural blanks, prepared with filtered Milli-Q water, were processed concurrently with fish samples to monitor background contamination throughout the experiment. Stereomicroscopy and Raman spectroscopy were performed in dedicated laboratories with restricted access to minimise human traffic and airborne particles. Additionally, clean Petri dishes containing fresh filter paper were placed adjacent to the analysis setup during microscopy and Raman analysis to detect airborne contaminants, following Conger *et al*. (2024). Suspected MPs in samples matching the shape and colour of particles found in blanks were subtracted from the total count before data analysis to ensure accuracy. A schematic of the processes and procedures followed for the MPs analysis is presented in Figure 9.

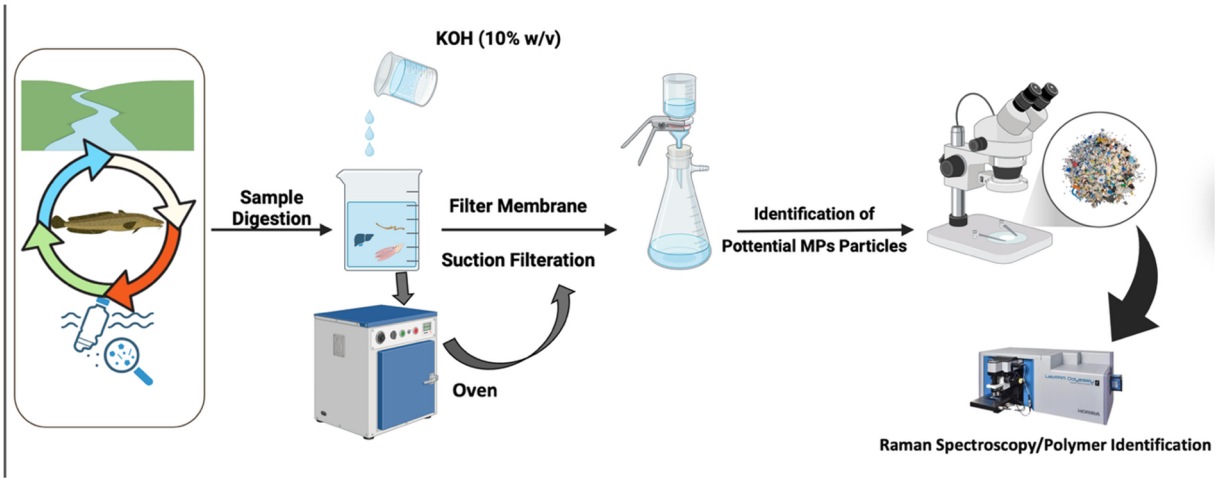

Figure 9. Workflow for Microplastic Extraction and Identification in Fish Tissues

5.2.7. ESTIMATED INTAKE OF MICROPLASTICS FROM HUMAN CONSUMPTION

To assess potential human exposure to MPs via burbot consumption, the methodology of Barboza *et al*. (2020) was adapted. The mean MP concentration in burbot dorsal muscle tissue was determined to be 0.96 ± 0.56 MPs/g, calculated by dividing the total number of MPs identified in muscle samples by the total muscle weight (6.02 ± 0.81 g per fish) across 10 specimens. Human MP intake was estimated using the European Food Safety Authority's (EFSA, 2014) recommended weekly fish consumption rates: 40 g for 1-year-olds, 50 g for children aged 2–6 years, 200 g for individuals over 6 years, and 300 g for adults. These were contextualised with Hungary's estimated annual fish consumption of 6.7 kg per capita (Szűts *et al*., 2022), equivalent to approximately 129 g/week. Weekly and annual MP intake (items/week and items/year) were calculated by multiplying the mean MP concentration (MPs/g) by the respective consumption rates (g/week or g/year) for each age group. Results provide insights into potential human health risks associated with MP ingestion through burbot consumption in the Tisza River region. The estimates of the human MP intakes were made using the formulas (Eqs. 6 and 7).

$$\textbf{\textit{Human MP intake per week}}\ (\textbf{\textit{MP items/week}}) = \textit{Average MP item in the muscles}\ (\textit{MPs items/g}) \times \text{Recommended fish food intake per week (g)} \quad \text{(Eq. 6)}$$

$$\textbf{\textit{Human MP intake per year}}\ (\textbf{\textit{MP items/year}}) = \textit{Average MP item in the muscles}\ (\textit{MPs items/g}) \times \text{Recommended fish food intake per year (g)} \times \text{Number of week per year (52)} \quad \text{(Eq. 7)}$$

5.2.8. STATISTICAL ANALYSIS

Morphometric data for burbot specimens and MPs morphology in fish tissues were summarised using descriptive statistics and visualised with appropriate libraries in Python (Python Software Foundation, 2023). MP counts in tissues (GIT, gills, liver, and dorsal muscle) were categorized by shape (fibres, fragments, films, microspheres, foams), colour (black, red, blue, green, yellow, grey, white, transparent), and size (≤0.5 mm, 0.5–1 mm, 1–5 mm), and also expressed as particles per gram of tissue (MPs/g). Data on MP prevalence across tissues were tested for normality and homogeneity of variance using the Shapiro-Wilk and Levene's tests, respectively. Normally distributed data were analysed using one-way analysis of variance (ANOVA), followed by Tukey's Honestly Significant Difference (HSD) test for pairwise comparisons. Results were visualised using the *ggpubr* package in R (version 4.3.3; R Core Team, 2023). For non-normal data, the Kruskal-Wallis test was applied, with Dunn's test for post hoc multiple comparisons. Pearson's correlation analysis was conducted to evaluate relationships between organ weights and MP counts, with significance set at $p < 0.05$ for all tests.

5.3. RESULTS

5.3.1. MORPHOMETRIC CHARACTERISTICS AND MICROPLASTIC ABUNDANCE IN BURBOT

Ten burbot specimens were sampled from the Tisza River at Tiszabecs, near the Hungarian-Ukrainian border, to assess microplastic (MP) contamination. Morphometric measurements, including total length, body weight, and organ weights (GIT, gills, liver, and dorsal muscle), were recorded, with means ± standard deviations and ranges presented in Table 11. The average body weight was 132.49 ± 20.77 g, and the total length was 265.0 ± 15.32 mm. Organ weights were as follows: GIT, 4.08 ± 0.42 g; gills, 2.41 ± 0.23 g; liver, 9.43 ± 0.80 g; and dorsal muscle, 6.02 ± 0.81 g (Table 12).

Table 11. Morphometric Measurements of Fish Samples and Their Tissues Analyzed for Microplastic Contamination

Species	ATL (Range) mm	ASL(Range) mm	GIT_W (Range) g	GILL_W (Range) g	LIV_W (Range) g	MUS_W (Range) g
Burbot	265 ± 15.52 (250 - 294)	237 ± 15.29 (220 - 266)	4.08 ± 0.42 (3.39 – 4.92)	2.41 ± 0.23 (2.09 – 2.89)	9.43 ± 0.80 (6.4 – 10.1)	6.02 ± 0.81 (4.50 – 7.13)

ATL: Average Total Length; ASL: Average Standard Length; MBW: Mean Body Weight; GIT_W: Mean Gastrointestinal Tract (GIT) Weight; GILL_W: Mean Gill Weight; LIV_W: Mean Liver (LIV) Weight; MUS_W: Mean Muscle (MUS) Weight.

Table 12. Quantification of microplastic particles in different fish 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.

5.3.2. MICROPLASTIC ABUNDANCE AND TISSUE-DEPENDENT DIFFERENCES

Microplastics were detected in 100% of sampled burbot, with a total of 246 MP particles identified across the GIT, gills, liver, and dorsal muscle. The mean intensity was 24.6 ± 7.46 particles per individual, and the mean abundance was 188.48 ± 58.44 particles/kg body weight (Table 12). Particle distribution by tissue was: liver, 76 particles (30.89%); GIT, 68 particles (27.64%); dorsal muscle, 57 particles (23.17%); and gills, 45 particles (18.29%). Per individual, the liver exhibited the highest mean MP count (7.6 ± 2.3 particles), followed by dorsal muscle (5.7 ± 1.8 particles), gills (4.5 ± 1.5 particles), and GIT (2.6 ± 0.9 particles). One-way ANOVA revealed significant differences in MP counts among tissues ($p < 0.05$). When normalized by tissue weight, gills (1.88 ± 1.28 MPs/g) and GIT (1.68 ± 0.69 MPs/g) showed significantly higher MP concentrations than dorsal muscle (0.96 ± 0.56 MPs/g) and liver (0.81 ± 0.41 MPs/g) ($p < 0.05$), with no significant differences between muscle and liver or between gills and GIT ($p \geq 0.05$) (Figure 10). The overall mean MP concentration was 0.19 ± 0.06 MPs/g body weight.

Pearson's correlation analysis was conducted to explore relationships between MP counts and organ weights. Results indicated a moderate positive correlation with GIT weight ($r = 0.41$, $p = 0.245$), strong positive correlations with liver weight ($r = 0.58$, $p = 0.080$) and muscle weight ($r = 0.63$, $p = 0.052$), and a strong negative correlation with gill weight ($r = -0.56$, $p = 0.089$) (Figure 11). However, none of these correlations were statistically significant ($p \geq 0.05$),

suggesting inconclusive evidence for associations between MP abundance and organ weights in this sample.

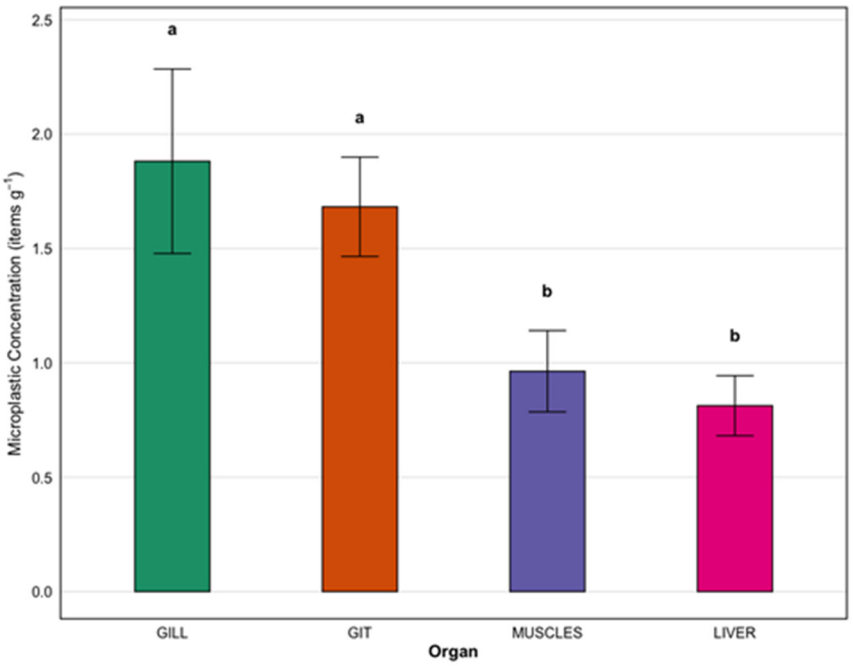

Figure 10. One-way ANOVA of suspected microplastic particle abundance across fish tissues *Bars with different alphabets differ significantly (P < 0.05)

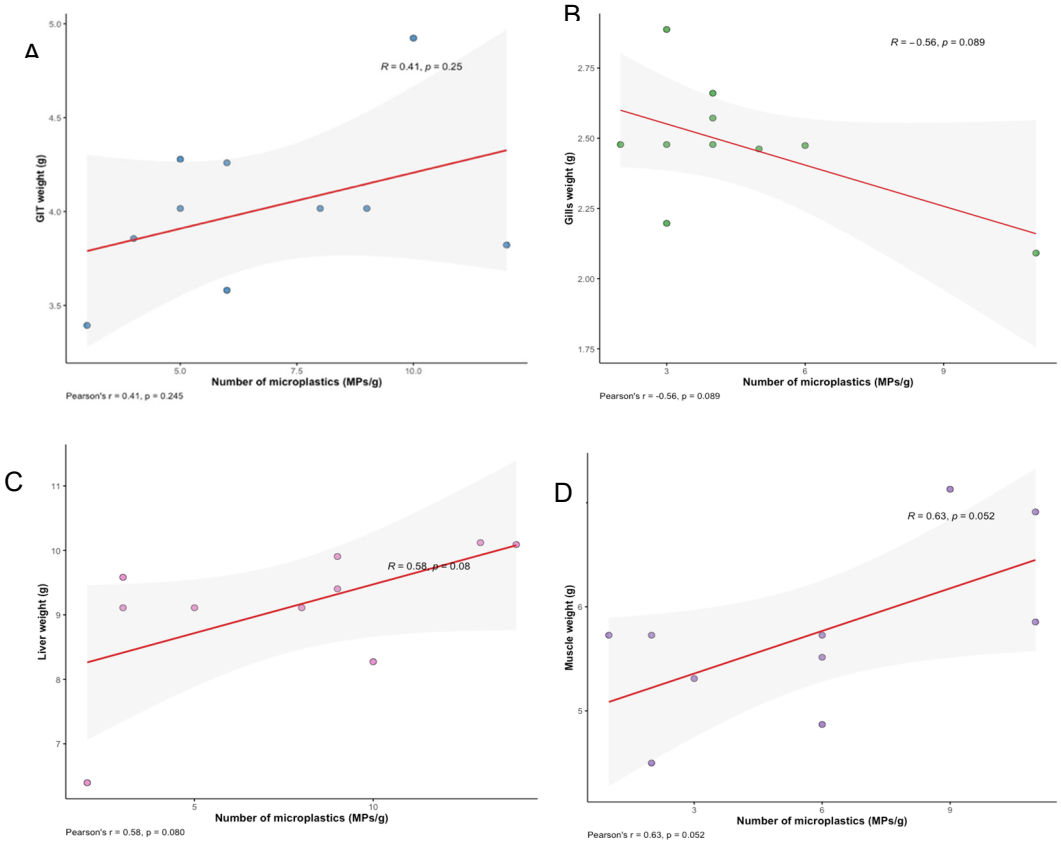

Figure 11 Bivariate plots of the identified suspected MPs per gram (MPs/g) (A) GIT weights, (B) Gill weights, (C) Liver weights, and (D) Muscle weights. The solid lines represent predicted associations, while the shaded areas are the 95% confidence intervals

5.3.3. MORPHOLOGICAL CHARACTERISTICS OF MICROPLASTICS IN BURBOT TISSUES

The morphological characteristics (shape, colour, and size) of MPs detected in burbot tissues, including the GIT, gills, liver, and dorsal muscle, are summarised in Figures 12–17. Fibres were the dominant shape, comprising 83.3% (n=205) of the 246 total MP particles identified, followed by fragments at 11.0% (n=27). Films, sheets, and beads were less common, representing 4.1% (n=10), 1.2% (n=3), and 0.4% (n=1), respectively (Figures 12 and 13). Across tissues, fibres remained predominant, with the highest prevalence in dorsal muscle (92.98%, n=53) and the lowest in gills (68.89%, n=31). Fragments were the second most common shape, peaking in the GIT (16.18%, n=11). Sheets were absent in liver samples, and films were not detected in the GIT. Beads were exclusive to the dorsal muscle, though rare (1.75%, n=1).

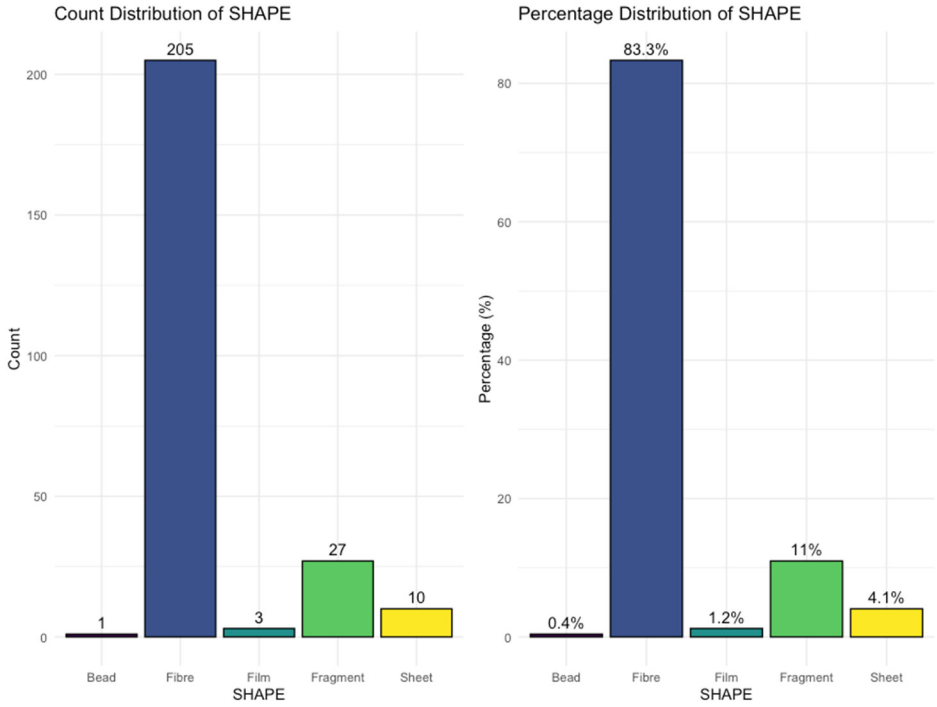

Figure 12. Suspected Particles' Shape Distributions (count and percentage) in Fish Samples

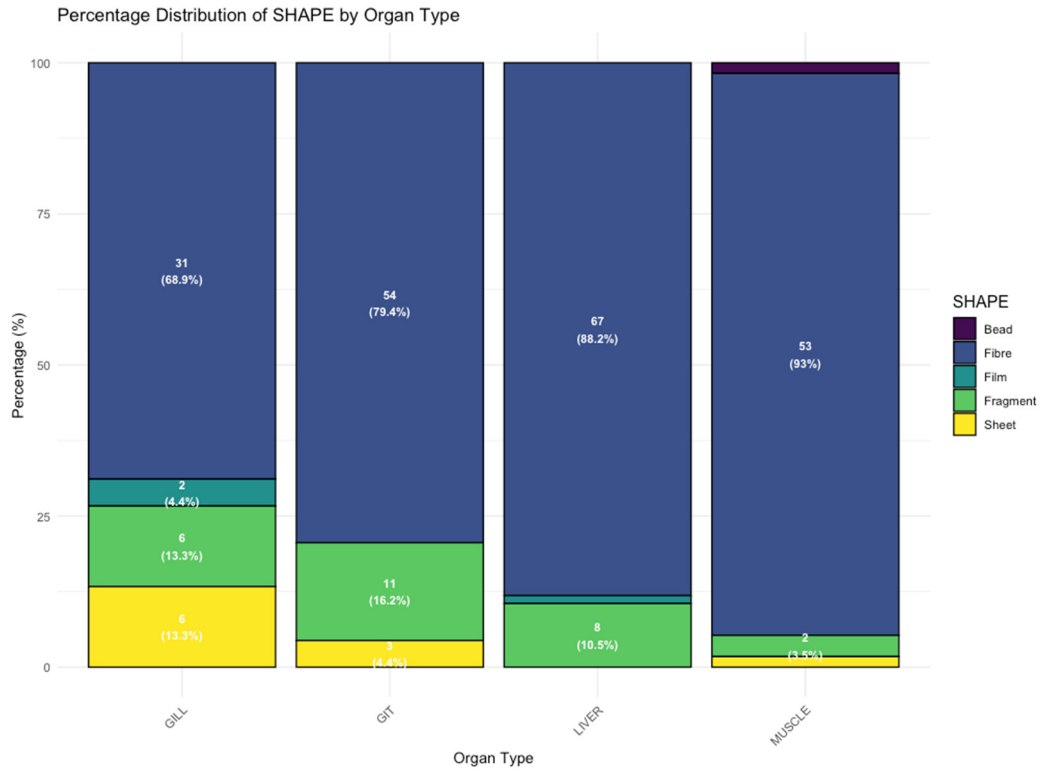

Figure 13. Suspected Particles' Shape Distributions (count and percentage) in various Fish Tissues.

Colour distribution analysis (Figures 14 and 15) revealed blue MPs as the most prevalent across all fish, constituting 37.4% (n=92) of particles, followed by green (19.5%, n=48) and transparent (15.4%, n=38). Brown (1.2%, n=3) and yellow (3.7%, n=9) particles were least frequent. Blue MPs dominated in all tissues, ranging from 32.89% in the liver (n=25) to 42.11% in dorsal muscle (n=24). Green MPs varied, with the highest proportion in the liver (27.63%, n=21) and the lowest in gills (13.33%, n=6). Transparent MPs were most common in the GIT (22.06%, n=15) but least prevalent in the liver (7.89%, n=6). Black MPs were notable in the GIT (13.24%, n=9), liver (10.53%, n=8), and gills (8.89%, n=4). Brown, grey, red, and yellow MPs occurred in lower proportions across tissues, with brown absent in the dorsal muscle.

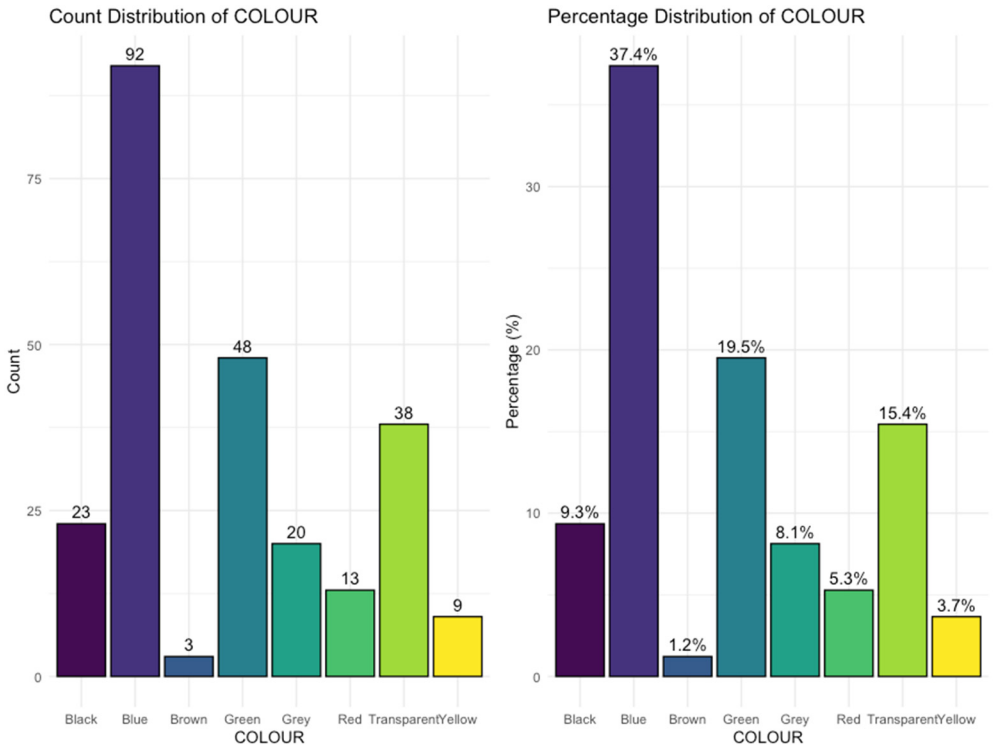

Figure 14. Suspected Particles' Colour Distributions (count and percentage) in Fish Samples

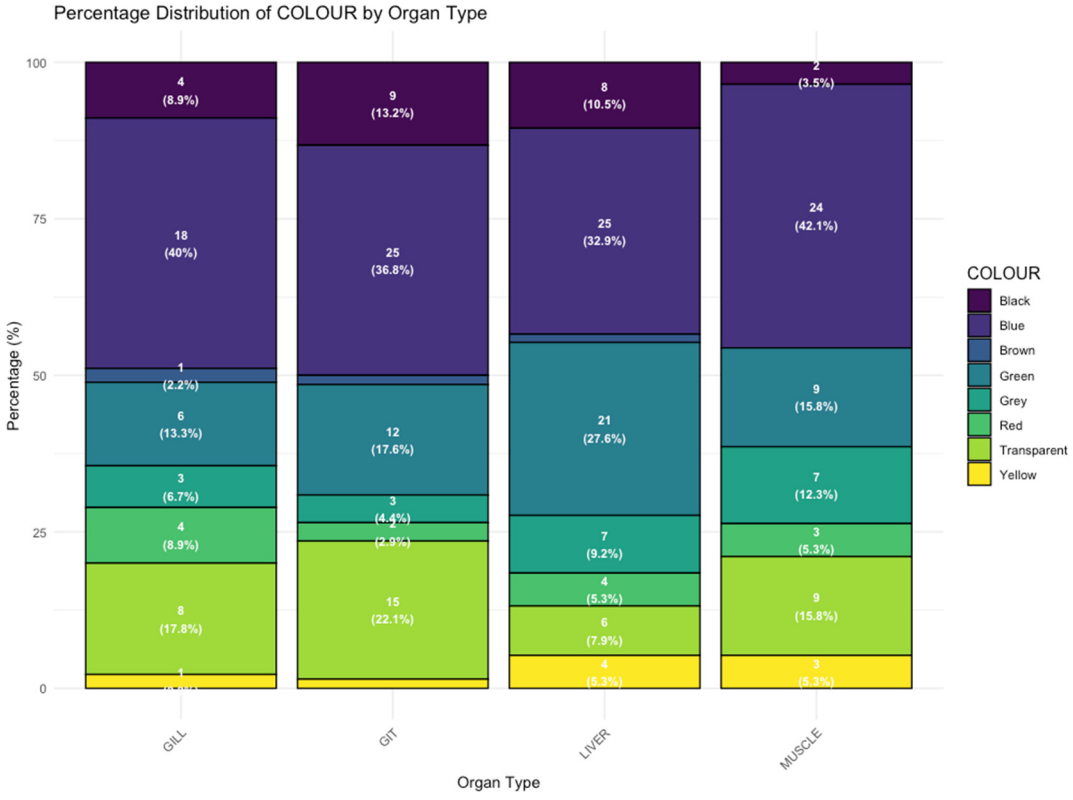

Figure 15. Suspected Particles' colour Distributions (count and percentage) in various Fish tissues

Small particles dominated the size distribution, comprising 41.9% (n=103) of the 246 total MPs identified, followed by large particles at 35.4% (n=87) and medium particles at 22.8% (n=56) (Figure 16). Across tissues (GIT, gills, liver, and dorsal muscle), small particles were the most abundant, ranging from 39.71% in the GIT (n=27) to 46.67% in the gills (n=21). Large

particles were the second most prevalent, with the highest proportion in the gills (37.78%, n=17) and the lowest in the dorsal muscle (31.58%, n=18). Medium particles were least frequent, varying from 15.56% in the gills (n=7) to 28.07% in the dorsal muscle (n=16) (Figure 17). The stereomicroscope images of identified suspected MPs in various tissue parts of the Burbot are presented in Figures 1 – 13 (appendix).

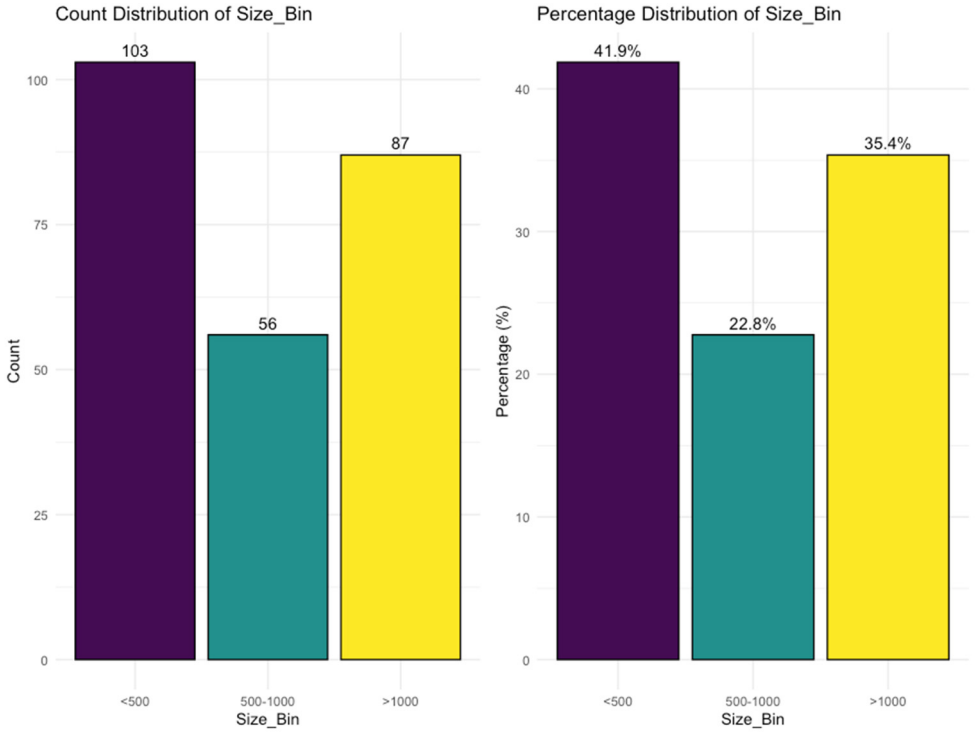

Figure 16. Suspected Particles' Size Distributions (count and percentage) in Fish Samples

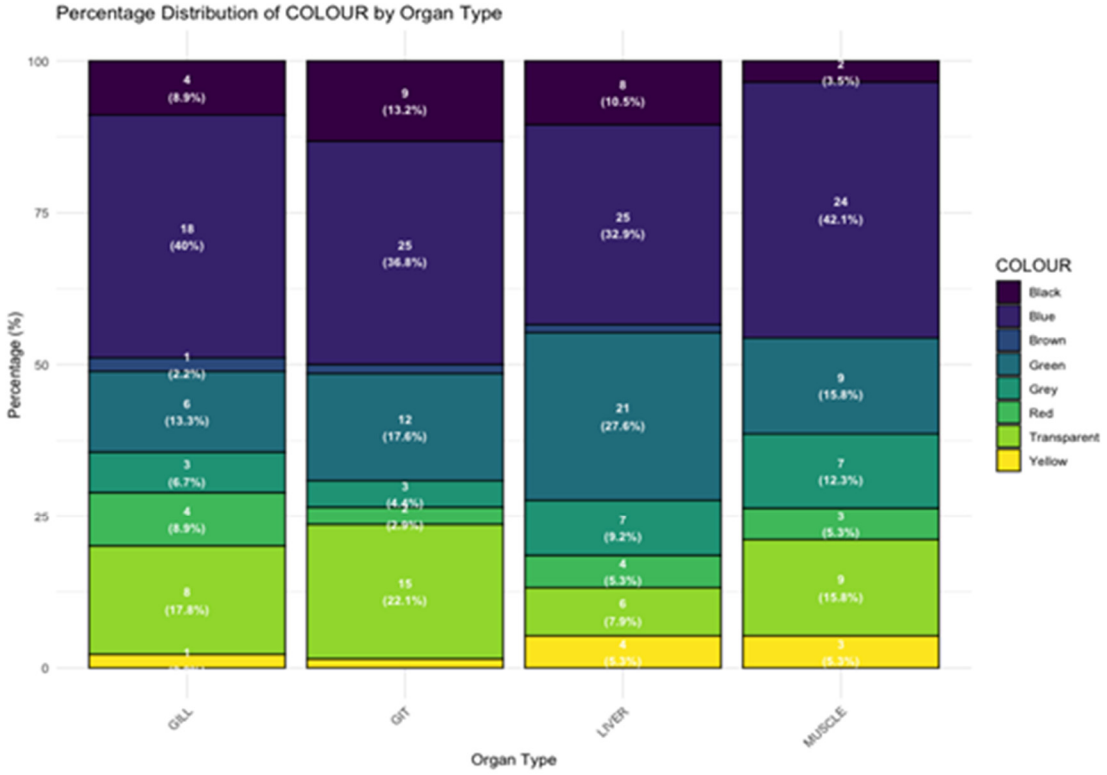

Figure 17. Suspected Particles' Size Distributions (count and percentage) in various Fish tissues

5.3.4. CHEMICAL PROPERTIES OF MPS

The spectral analysis and identification of MPs particles (Figure 18) revealed a diverse array of 20 distinct polymer types among the 98 particles examined. These polymers included 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-pigmented fibre (IBF), polyamide (PA), polycarbonate and polyphenylene oxide (PC-PPO), polyethylene (PE), polyethylene terephthalate (PET), polyethylene terephthalate-cotton blend (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), and polyvinyl chloride (PVC) (See Figures 14-20 in appendix). The PET-cotton blend, encompassing both PET-CO and PET-CO-PU, was the most prevalent MP type, constituting 34.7% of the total particles (PET-CO: 24.5%, n=24; PET-CO-PU: 10.2%, n=10). Cotton alone accounted for 20.4% (n=20) of the particles, followed by polyurethane at 12.7% (n=12). Polypropylene contributed 6.1% (n=6), and dyed cellulose represented 4.1% (n=4). The remaining polymers and copolymers were detected at lower frequencies (Figure 18).

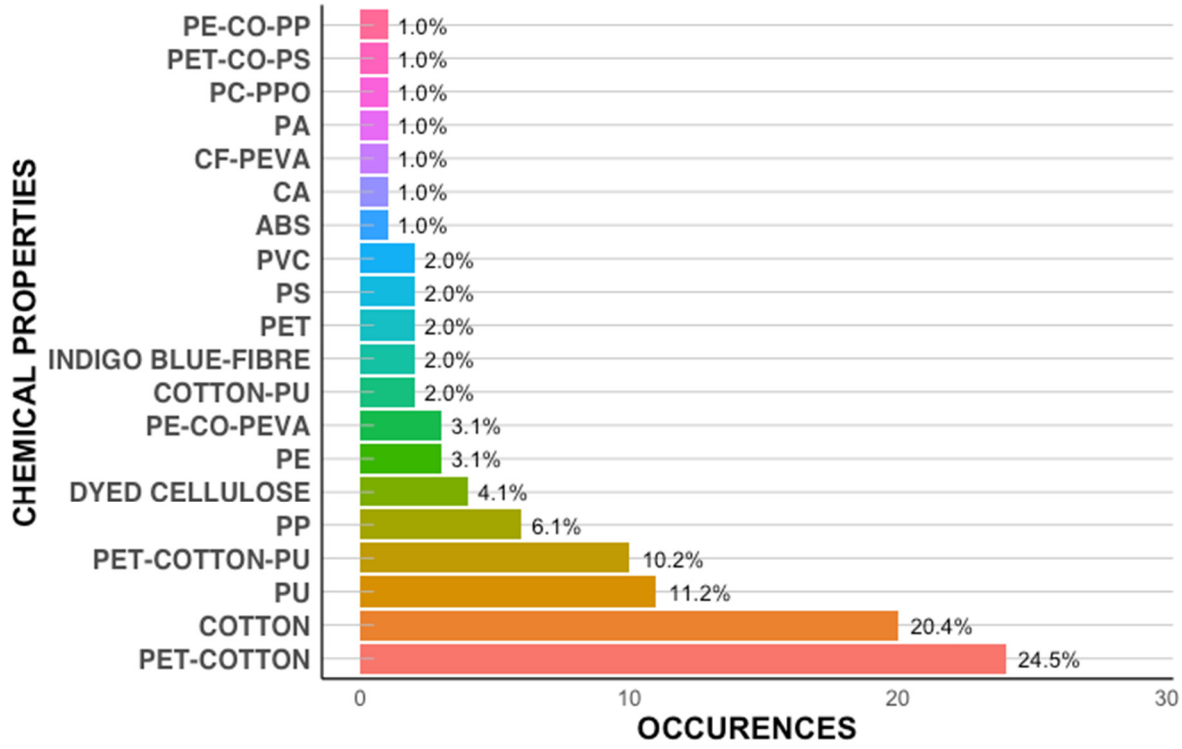

Figure 18. Percentage Distribution of Chemical Properties of Suspected Microplastics (MPs) Identified in Burbot Fish

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).

5.3.5. ESTIMATED MPS INTAKE FROM HUMAN CONSUMPTION OF BURBOT

Human MP intake through burbot consumption was estimated using the mean MP concentration in dorsal muscle tissue (0.96 ± 0.56 MPs/g) and the European Food Safety Authority's (EFSA, 2014) recommended weekly fish consumption rates: 40 g for 1-year-olds, 50 g for children aged 2–6 years, 200 g for individuals over 6 years, and 300 g for adults. The results of the estimated human MP intake (Table 13) show that weekly MP intake ranged from 38.4 MPs (1,996.8 MPs/year) for 1-year-olds to 288 MPs (14,976 MPs/year) for adults. Based on Hungary's per capita fish consumption of 6,700 g/year (approximately 128.8 g/week; Szűts *et al*., 2022), the estimated MP intake was 123.6 MPs/week (6,427.2 MPs/year) per capita. These estimates highlight potential human health risks associated with MP exposure through fish consumption in the Tisza River region.

Table 13. 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

5.4. DISCUSSION

5.4.1. ABUNDANCE AND DISTRIBUTION OF MICROPLASTICS

Microplastic (MP) pollution is a pervasive global challenge, with extensive evidence documenting its presence across diverse aquatic species, ecological niches, and feeding strategies (Parker *et al*., 2021). While marine environments have been the primary focus of MP research, freshwater systems, such as the Tisza River, are increasingly recognised as critical areas of concern due to their emerging contamination profiles (Raza *et al*., 2022). The Hungarian section of the Tisza River, particularly its upstream (Hungarian-Ukrainian border)

and downstream (Serbian border) regions, exhibits elevated MPs levels, driven by inadequate wastewater management and transboundary pollution from upstream sources (Siklós, 2017; Kiss *et al*., 2021; Balla *et al*., 2022). Previous studies have confirmed significant MPs contamination in the river's surface water (4–63 items/m³) and sediments (528–8067 items/kg), with mussels serving as effective biomonitors (Almeshal *et al*., 2022). Building on this foundation, the present study pioneers the use of the benthivorous, demersal *L. lota* as a bioindicator to assess MP pollution in the upper Hungarian Tisza River, leveraging its ecological and commercial significance in freshwater ecosystems.

Our findings reveal a mean MP intensity of 24.6 ± 7.46 particles per individual and 188.48 ± 58.44 particles/kg (0.19 ± 0.06 particles/g) body weight across 10 burbot specimens, highlighting the heightened pollution and bioavailability of microplastic particles to aquatic biota in the upper section of the Hungarian Tisza. These results align with reports of increasing MP prevalence in the Tisza River, particularly in the upper section, where rural communities in Ukraine lack adequate wastewater treatment infrastructure (Kiss *et al*., 2021; Balla *et al*., 2022). The high-water flow velocity and steep gradients in these areas facilitate the rapid transport of MPs into the Hungarian section, exacerbating contamination levels (Kiss *et al*., 2021). Wastewater effluent may be the primary source of MPs in rivers, and the presence of MPs in freshwater systems has been linked to their detection in aquatic organisms inhabiting these environments (Su *et al*., 2018; Saad *et al*., 2022). The burbot's benthivorous feeding habits, which include consuming invertebrates and small fish, likely contribute to MP ingestion through direct uptake or trophic transfer (Romeo *et al*., 2015; Barboza *et al*., 2020). The burbot serves as a valuable bioindicator for evaluating MP pollution in freshwater systems, complementing prior research on MP detection in mussels (2.7–8.3 items/individual) from the lower Tisza River (Almeshal *et al*., 2022) and in fish species across various aquatic environments (Roch *et al*., 2019; Tien *et al*., 2020). Notably, high MP levels have been documented in demersal carp from the Han River, China (59.4 ± 45.5 items/individual) (Oh *et al*., 2023), and the Vaal River, South Africa (26.23 ± 12.57 items/individual) (Saad *et al*., 2022). In contrast, lower MP abundances (0.07–0.53 items/individual) have been reported in pelagic coastal fish from Kerala, India (Daniel *et al*., 2020), highlighting the influence of habitat and feeding behaviour on MP accumulation (Lusher *et al*., 2016; Abbasi *et al*., 2018; Lopes *et al*., 2020).

5.4.2. TISSUE-SPECIFIC DISTRIBUTION OF MICROPLASTICS

A key finding of this study is the tissue-dependent distribution of MPs in burbot, with significant differences ($P < 0.05$) in abundance and concentration across organs. The liver exhibited the highest absolute MP count (7.6 ± 2.3 particles/individual), while the

gastrointestinal tract (GIT) had the lowest (2.6 ± 0.9 particles/individual). However, when normalized by tissue weight, the gills (1.88 ± 1.28 MPs/g) and GIT (1.68 ± 0.69 MPs/g) showed significantly higher concentrations than the liver (0.81 ± 0.41 MPs/g) and muscle (0.96 ± 0.56 MPs/g). These patterns likely reflect distinct accumulation mechanisms, with gills and GIT directly exposed to waterborne MPs and ingested prey, respectively (Hossain *et al*., 2023; 2024). The elevated MP presence in the liver suggests translocation from the GIT or gills via the circulatory system, a process potentially facilitated by the liver's role in detoxification and metabolism (Hossain *et al*., 2024). Similarly, MPs detected in muscle tissue may result from translocation mechanisms, such as endocytosis or persorption, as reported in previous studies (Karami *et al*., 2017; Abbasi *et al*., 2018; Barboza *et al*., 2019). These findings are consistent with reports of higher MP concentrations in inedible tissues (gills and GIT) compared to edible tissues (muscle and skin) in various fish species (Daniel *et al*., 2020; Hossain *et al*., 2023). However, contrasting patterns, such as lower muscle MP concentrations in *Silondia silondia* (Hossain *et al*., 2024), underscore the role of species-specific physiology and environmental factors in MP distribution. The presence of MPs in burbot muscle and liver raises significant public health concerns, given their importance as consumable tissues in this commercially valuable species.

The variability in MP abundance across tissues may be influenced by particle characteristics (e.g., size, type) and physiological factors, including membrane permeability, tissue vascularity, and translocation efficiency (Akhbarizadeh *et al*., 2018; Hossain *et al*., 2023). Although not statistically significant ($P > 0.05$), positive correlations between MP abundance and liver ($r = 0.58$) and muscle ($r = 0.63$) weights suggest that larger organs may have greater MP retention capacity. Conversely, a negative correlation with gill weight ($r = -0.56$) may indicate that smaller gills, with higher surface area-to-volume ratios, are more prone to MP adhesion. These trends, while inconclusive due to the limited sample size, warrant further investigation with larger cohorts to elucidate the mechanisms governing MP distribution. Previous studies have reported mixed results, with no significant correlations between tissue weight and MP accumulation in carp (Oh *et al*., 2023), but significant associations in other species (Hossain *et al*., 2023, 2024).

5.4.3. Morphological Characteristics of Microplastics in Burbot Tissues

The morphological diversity of ingested MPs, shape, size, and colour, provides valuable clues to their environmental sources and ecological interactions (Rochman et al., 2019). In this study, five morphologies were identified in *L. lota*: fibres dominated overwhelmingly (83.3%, n = 205), followed by fragments (11.0%, n = 27), sheets (4.1%, n = 10), films (1.2%, n = 3), and beads (0.4%, n = 1). This fibre predominance mirrors global patterns in freshwater and

marine systems, where textile-derived microfibres from laundry wastewater and effluents consistently prevail (Makhdoumi et al., 2021; Hossain et al., 2024).

In the Tisza River, microfibres account for 84–98% of suspended and sediment-bound MPs, largely reflecting discharges from inadequately treated municipal and industrial wastewater in the upper Hungarian–Ukrainian section (Kiss et al., 2021; Balla et al., 2022). These fibres, which can comprise up to 90% of wastewater-derived MPs, originate predominantly from textile laundering and are only partially retained by conventional treatment technologies (Mason et al., 2016; Prata, 2018). Against this environmental backdrop, the overwhelming dominance of fibres in burbot tissues (83.3% of all detected MPs) is best explained by their exceptionally high environmental abundance combined with physical and ecological processes that favour incidental uptake during benthic foraging, rather than selective ingestion based on visual resemblance to prey as proposed for some piscivorous and benthivorous fishes (Makhdoumi et al., 2021).

Burbot is a demersal and largely nocturnal predator that inhabits turbid, low-light riverbed environments, where foraging relies primarily on tactile input from barbels, chemosensory cues, and mechanosensory signals rather than fine visual discrimination of micrometre-scale particles (Ryder & Pesendorfer, 1992; Fischer et al., 2001). In such habitats, fibres exhibit low settling velocities, remain suspended for prolonged periods, and become loosely associated with fine sediments and organic detritus, precisely the substrates and food matrices that burbot probes while feeding on macroinvertebrates, small fish, and particulate organic matter (Nguyen et al., 2022; Serra & Colomer, 2023; Guo et al., 2024). Moreover, fibres rapidly accumulate biofilms and organic coatings, rendering them chemically and texturally similar to natural detrital filaments, algal strands, or invertebrate appendages, which further facilitates their co-ingestion with genuine food items (Guo et al., 2024).

The disproportionate accumulation of fibres in internal tissues (92.98% in muscle and 88.2% in liver) suggests that their small size, flexibility, and filamentous morphology enhance translocation from the gastrointestinal tract or gills into systemic circulation and internal organs (Zhao et al., 2021). This pattern raises important concerns regarding trophic transfer and bioaccumulation, particularly in edible tissues, with direct implications for human dietary exposure. In contrast, fragments, the second most common morphology (11%), were most prevalent in the GIT (16.18%), likely reflecting ingestion of degraded plastic particles alongside prey and sediment. The relative scarcity of beads and films probably reflects their lower environmental abundance and reduced bioavailability in benthic feeding zones.

Overall, the dominance of fibres in Tisza burbot is consistent with observations in other benthic and freshwater fishes (Barboza et al., 2020; Saad et al., 2022; Hossain et al., 2024), although some marine systems report higher fragment prevalence, highlighting the strong influence of habitat, hydrodynamics, and pollution sources on MP profiles (Karami et al., 2017; Karbalaei et al., 2019; James et al., 2020). These findings reinforce the role of upstream laundry wastewater as a major vector and emphasizes the need for improved textile effluent management in the Tisza basin.

The colour characteristics of MPs in aquatic environments can serve as indicators of their potential sources (Saad *et al*., 2022). Coloured fibres are likely derived from textiles, while transparent and white fragments often reflect the widespread use of packaging materials, such as plastic carrier bags; conversely, black fragments are typically associated with vehicle tires. Transparent and white fibres, meanwhile, are frequently linked to fishing gear, highlighting their prevalent origins in aquatic settings. In this study, colour analysis of MPs in burbot tissues revealed blue (37.4%), green (19.5%), and transparent (15.4%) particles as the most dominant, aligning with trends observed in other aquatic ecosystems (Neves *et al*., 2015; Bessa *et al*., 2018; Barboza *et al*., 2020). Coloured microfibres, particularly blue particles often originating from textiles like denim, are abundant in the Tisza River, likely due to upstream wastewater discharges (Balla *et al*., 2022; McQueen *et al*., 2016). These coloured particles may be mistaken for prey, thereby elevating ingestion rates among aquatic organisms (McNeish *et al*., 2018; Ferreira *et al*., 2020; Barboza *et al*., 2020). Transparent MPs, potentially resembling phytoplankton and thus mimicking natural food sources, were also prevalent (Atamanalp *et al*., 2021). Although MP colours may shift due to environmental weathering (Zhang *et al*., 2021), the preference of aquatic organisms for blue particles is well-established (Ory *et al*., 2018). Significantly, coloured fibres pose a notable health risk due to the potential release of embedded colourants and additives following fish ingestion and subsequent human consumption (Saad *et al*., 2022).

In this study, small-sized MPs (<500 μm) accounted for 41.9% of the particles identified, showing a higher prevalence in gills (46.7%), liver (42.1%), and muscle (40.4%) compared to the gastrointestinal tract (GIT). Particle size appears to be a critical physical factor influencing MP mobility, long-range transport, and distribution, which in turn affects their behaviour in the water column, sediment accumulation, and ingestion by aquatic organisms (Saad *et al*., 2022). Smaller MPs demonstrate enhanced mobility, facilitating their transport and increasing their bioavailability for uptake by a wide range of aquatic species, including fish (Besseling *et al*., 2017; Alfonso *et al*., 2021; Du *et al*., 2021; Saad *et al*., 2022). The elevated prevalence of small MPs in this study suggests that these particles, which are abundant in the Tisza River, are more

readily ingested and translocated due to their high surface area and bioavailability (Wright *et al*., 2013; Hossain *et al*., 2024). This trend may be exacerbated by their frequent degradation in natural environments and inherent persistence, contributing to their overall abundance (Yuan *et al*., 2019; Egessa *et al*., 2020). Notably, smaller fish, such as juvenile burbot, may be more prone to accumulating these particles due to their feeding habits and physiological limitations (Hossain *et al*., 2024). These findings align with previous research reporting a higher prevalence of small-sized MPs in common carp from South Africa's Vaal River (Saad *et al*., 2022). The significant presence of small MPs in liver and muscle tissues highlights their ability to bypass gut barriers and enter the circulatory system, consistent with patterns observed in prior studies (Barboza *et al*., 2020; Zitouni *et al*., 2020; Hossain *et al*., 2024). Furthermore, these small, fibrous MPs pose increased health risks due to their potential for cellular penetration and their capacity to adsorb environmental contaminants, such as PFAS, PTEs, and bacteria, owing to their large surface area-to-volume ratio (Corcoran *et al*., 2020; Bhuyan, 2022; Hamed *et al*., 2022). The heightened toxicity of smaller MPs is also linked to their ability to infiltrate cells and tissues, amplifying molecular damage and elevating associated risks. These observations underscore the urgent need for further research into the mechanisms of MP uptake, translocation, and toxicity in aquatic organisms, as well as their broader implications for human health.

5.4.4. CHEMICAL PROPERTIES OF MPS

Spectral analysis and polymer identification of suspected MP particles in burbot fish from the Tisza River, Ukraine, revealed a diverse chemical composition, indicative of varied pollution sources. Among the 98 particles analyzed, polyethylene terephthalate-cotton blends (PET-CO: 24.5%, n=24; PET-CO-PU: 10.2%, n=10), cotton (CO: 20.4%, n=20; CO-PU: 2.0%, n=2), polyurethane-indigo blue (PU-IB: 12.2%, n=12), polypropylene (PP: 6.1%, n=6), and dyed cellulose (DC: 4.1%, n=4) constituted the majority of the samples (Fig. 18). These findings emphasise the prominence of synthetic textiles and packaging materials as major contributors to MP pollution, with PET-CO, cotton, and dyed cellulose likely originating from textile processing, clothing, and household waste (Zou *et al*., 2011).

The identified polymers have widespread industrial applications. PE is prevalent in packaging (e.g., bags, films, bottles); PP is used in containers and piping for its chemical resistance; PVC is common in construction and medical devices; CA is found in films, textiles, and eyeglass frames; PEVA is used in textiles and packaging due to its flexibility; ABS is valued in electronics and automotive parts for its rigidity; PA is employed in textiles (e.g., nylon) and engineering plastics; PS is used in packaging and insulation; and PU is integral to foams, coatings, and textiles (Govender *et al*., 2020; De Vos *et al*., 2021; Ghazal *et al*., 2024). The prevalence of PET-CO and cotton-based materials highlights laundry effluents as a significant

MP source, with PET, a dominant thermoplastic in the textile industry, often blended with cotton to enhance versatility (De Vos *et al*., 2021). Polyurethane's role in polymer-coated textiles, which improve fabric properties like water repellency and flame retardancy, further highlights its contribution to MP pollution (Eyssa & Hassan, 2014; Ghazal *et al*., 2024).

Results also showed that blue pigments, predominantly indigo blue, comprised the majority of the identified pigment-based particles in the MPs polymer and cellulosic particle types (Figure 19). Samples Indigo blue, a key pigment in denim production, was notably prevalent (66.7%, n=48), reflecting the global popularity of denim jeans, with the market projected to reach US$85 billion by 2025 (Athey *et al*., 2020). Laundry-generated microfibres, particularly denim microfibres, likely contribute to the high presence of blue-pigmented MPs, exacerbated by inadequate wastewater treatment in the region (Kiss *et al*., 2021; Balla *et al*., 2022). An estimated 640,000 to 1,500,000 microfibre particles per kilogram of clothing are released during washing, with smaller fibres evading wastewater treatment and accumulating in aquatic ecosystems (De Falco *et al*., 2019; Berglund *et al*., 2019; Almeshal *et al*., 2022). Previous studies on the Tisza River have reported elevated levels of blue microfibres in surface water, sediments, and freshwater mussels, consistent with the current findings (McQueen *et al*., 2016; Balla *et al*., 2022; Almeshal *et al*., 2022). This study's findings corroborate those of Almeshal *et al*. (2022), who used Raman spectroscopy to identify indigo-dyed PET, cellulose-based fibres, and PA fragments in freshwater mussels from the same section of the Tisza River. The prevalence of these polymer types in burbot tissues reinforces the persistence of textile-derived microplastics in this riverine ecosystem. Additionally, 21–51% of microfibres in sediments are anthropogenically modified cellulose, with 40–57% comprising indigo denim microfibres (Athey *et al*., 2020). The dominance of smaller, mobile, blue-pigmented MPs in burbot fish aligns with their reported accumulation in aquatic organisms across various systems (Barboza *et al*., 2020; Saad *et al*., 2022; Erdoğan, 2025). These results emphasise the urgent need to investigate the ecological and health implications of MP pollution, particularly from textile-related sources, and to improve waste management strategies to mitigate microfibre release into aquatic environments.

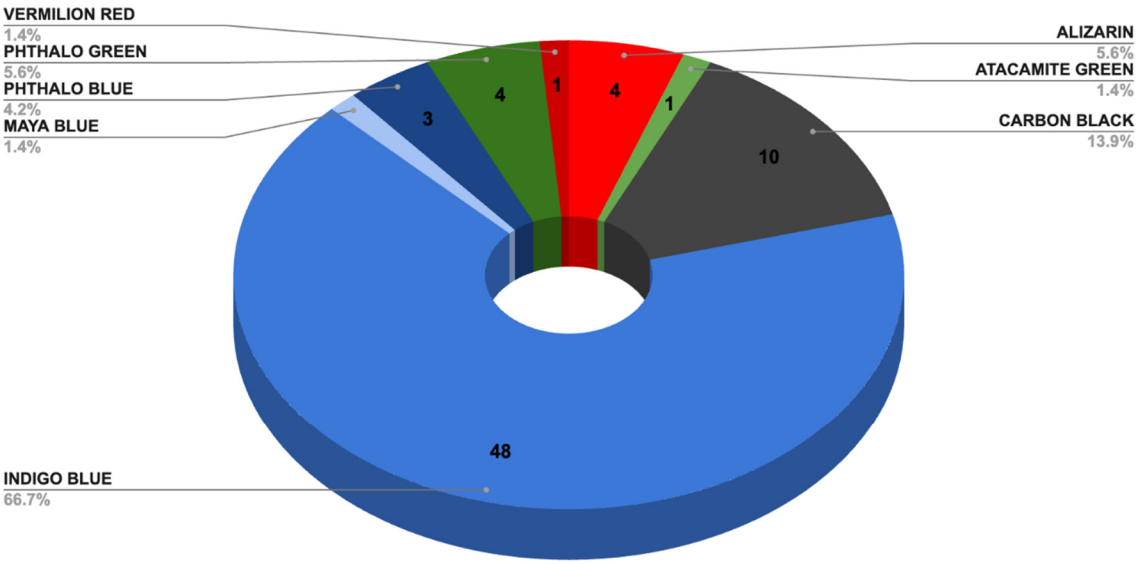

Figure 19. Distribution of Identified Pigments in Microplastic Polymer Samples

Composite polymer materials were excluded from the analysis, and only pigment components were considered.

5.5. CONCLUSION

This study represents the first comprehensive assessment of MPs pollution in *L. lota* from the Hungarian section of the Tisza River, revealing ubiquitous MP contamination with a mean intensity of 24.6 ± 7.46 particles per fish, predominantly small (<500 µm), fibre-shaped, and blue particles, particularly concentrated in gills (1.88 ± 1.28 MPs/g) and GIT (1.68 ± 0.69 MPs/g), and to a lesser extent in liver (0.81 ± 0.41 MPs/g) and dorsal muscle (0.96 ± 0.56 MPs/g). The chemical analysis shows a preponderance of indigo-dyed PET, cotton, and cellulose-based polymers in the analysed samples. The findings of this study confirm burbot's role as a sentinel species for monitoring riverine pollution and raising concerns about human exposure through consumption, with estimated weekly MP intake ranging from 38.4 particles for infants to 123.6 particles per capita in Hungary. The prevalence of textile-derived MP pollution likely stemming from inadequate upstream waste management and wastewater treatment, establish a critical baseline for Central European freshwater systems, advocating for robust waste management policies, enhanced wastewater treatment, and further research into MP bioaccumulation, polymer-specific toxicity, and regional mitigation strategies to protect aquatic ecosystems and public health, while affirming burbot's significance in ongoing environmental monitoring and conservation efforts within the Tisza River basin.

CHAPTER 6: GENERAL CONCLUSION

This dissertation provides an integrated evaluation of the bioaccumulation patterns and toxicological effects of anthropogenic contaminants in freshwater organisms through three complementary experimental approaches. By combining field-based biomonitoring with controlled laboratory exposures, the research offers a holistic understanding of how complex pollutant mixtures, ranging from PTEs and short-chain chlorinated paraffins to solvent-based industrial chemicals and synthetic microplastics, affect freshwater biota across multiple biological levels.

The caged-mussel experiment established *S. woodiana* as a reliable sentinel species for freshwater biomonitoring in Bulgaria. Marked site-specific variations in bioaccumulation profiles and biomarker responses were observed, with mussels from the Studen Kladenets and Kardzhali reservoirs exhibiting heightened oxidative stress, metabolic impairment, and DNA damage. These biomarker patterns strongly aligned with elevated contaminant loads, demonstrating that the integrated biomarker approach is an effective early-warning system for ecosystem health assessment. This study also provides the first reservoir-scale comparison of genotoxic and biochemical pollution effects in this region, offering a valuable foundation for long-term monitoring programmes.

The toxicological assessment of nitrocellulose-based paint thinner in juvenile African catfish revealed substantial behavioural, haematological, biochemical, and organ-level disruptions under both acute and chronic exposure. The pollutant induced dose-dependent behavioural anomalies, anaemia, leukocytic alterations, dysregulated lipid metabolism, and significant hepatic and renal dysfunction. Although partial recovery occurred following depuration, the persistence of several impaired physiological parameters indicates that solvent pollution poses serious risks to fish health, with potential consequences for fisheries, aquaculture, and food security.

The microplastic assessment in burbot from the Hungarian section of the Tisza River demonstrated that microplastic contamination is widespread and dominated by small, fibre-shaped, blue particles derived primarily from textile waste. The detection of microplastics across all examined tissues, including edible muscle, raises critical concerns for trophic transfer and potential human exposure. Polymer analysis confirmed the prevalence of indigo-dyed PET and cellulose-based fibres, directly linking the observed contamination to textile effluent and inadequate wastewater treatment upstream. This represents the first polymer-resolved investigation of microplastics in burbot and provides essential baseline data for Central European riverine systems.

Overall, the findings of this dissertation reveal that anthropogenic contaminants exert significant adverse effects on freshwater organisms, impacting biochemical pathways, cellular integrity, organ function, and ecological behaviour. The research highlights the need for integrated biomonitoring frameworks, stricter regulation of industrial discharges, improved wastewater management, and sustained ecological surveillance. By advancing scientific understanding of contaminant exposure and biological response mechanisms, this work contributes important evidence to guide environmental protection policies, freshwater conservation strategies, and public health risk assessments.

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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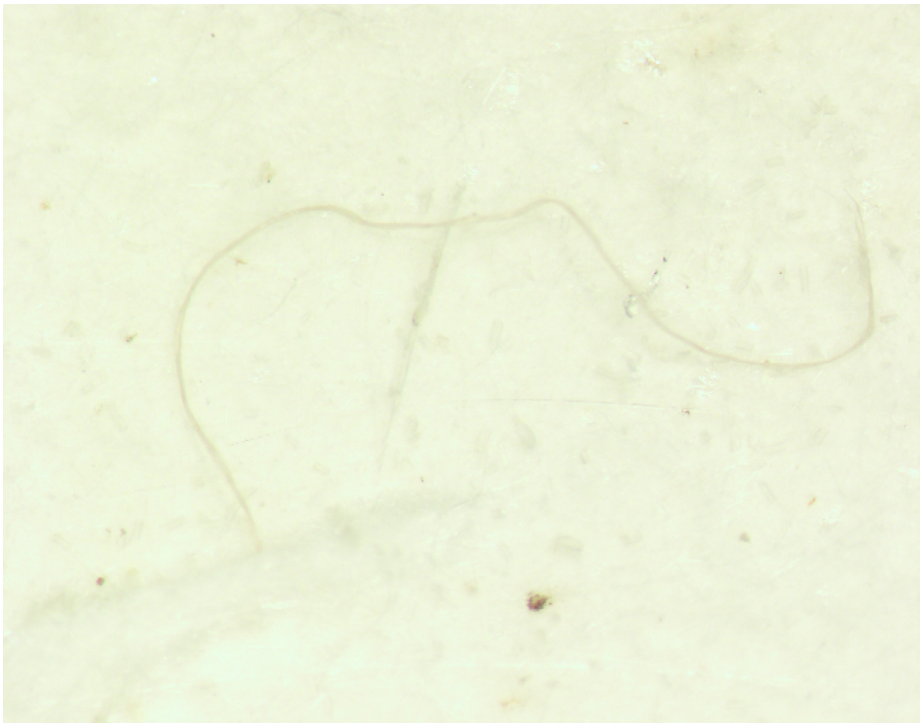
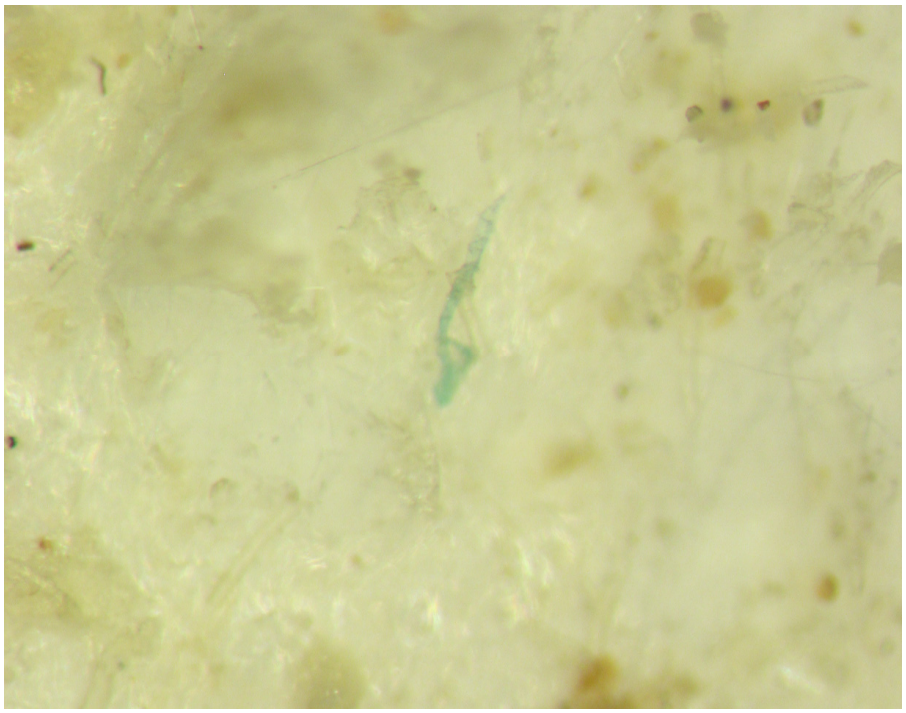
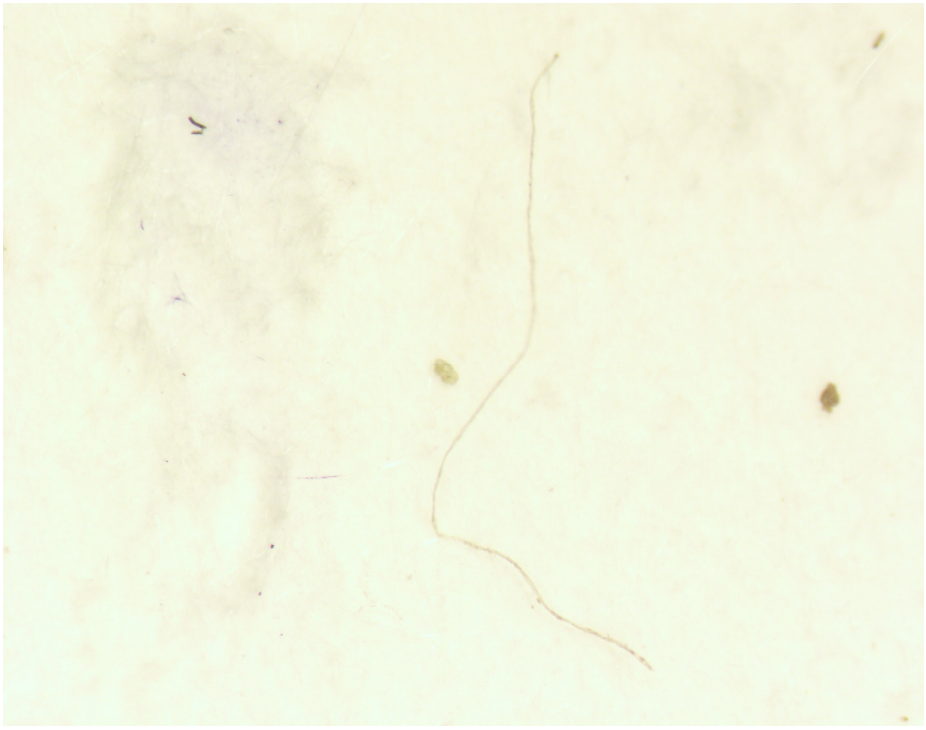
Appendix



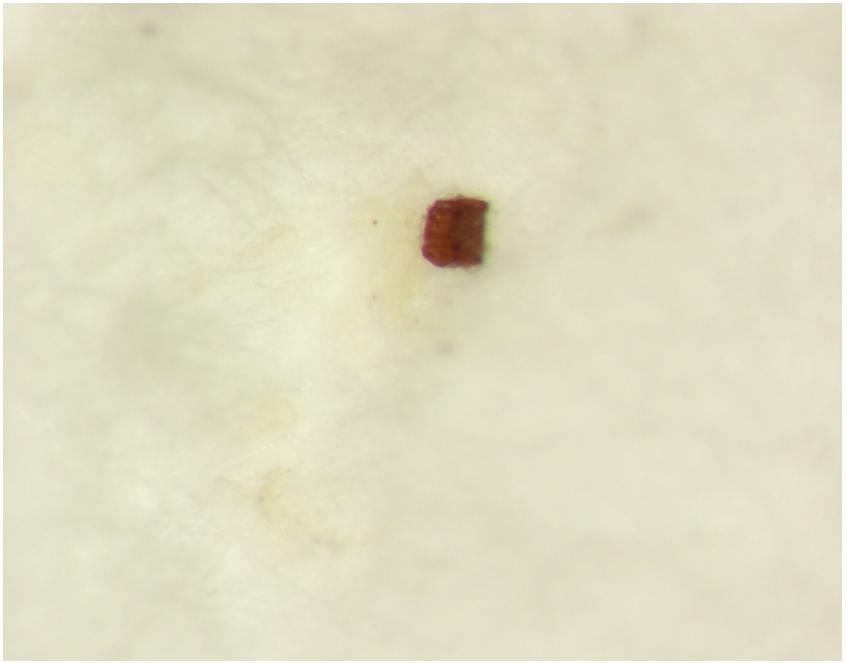
Appendix 1. Stereomicroscope image of white Cotton fibre found in the *Burbot (Lota lota) fish* collected from the upper Hungarian section of the Tisza River (40× magnification).



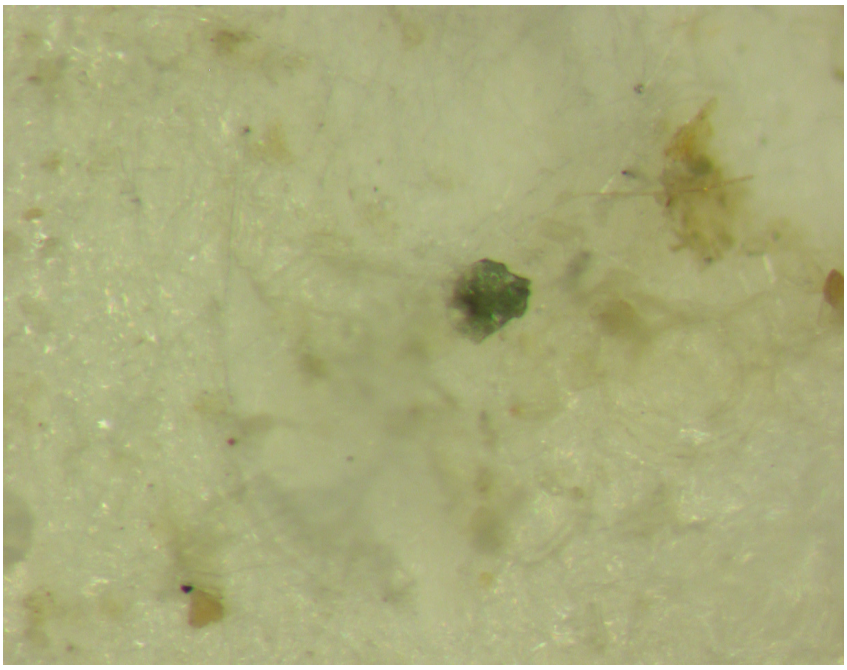
Appendix 2. Stereomicroscope image of Dyed Cellulose Fibre found in the *Burbot (Lota lota) fish* collected from the upper Hungarian section of the Tisza River (40× magnification).



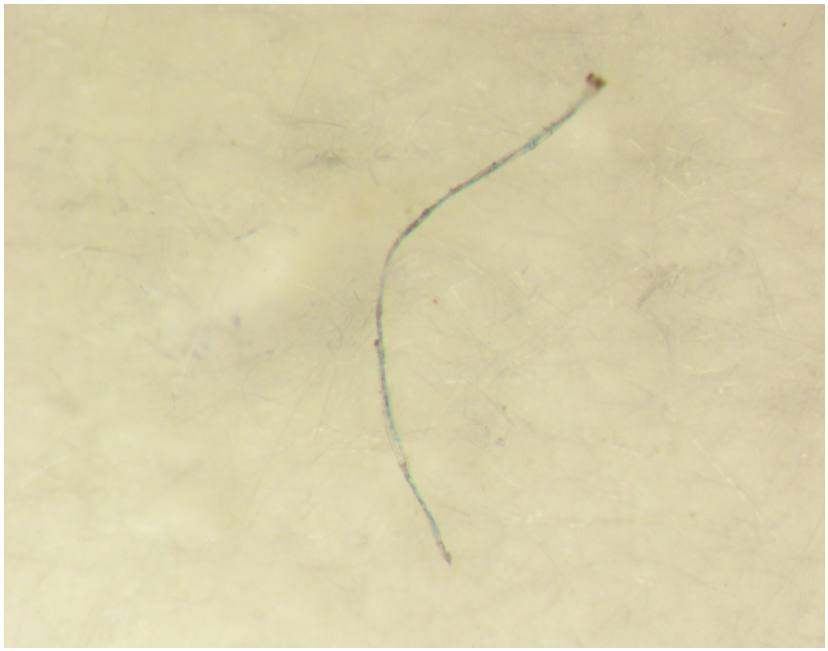
Appendix 3. Stereomicroscope image of white Cellulose acetate fibre found in the *Burbot (Lota lota) fish* collected from the upper Hungarian section of the Tisza River (40× magnification).



Appendix 4. Stereomicroscope image of Red PET Fragment found in the *Burbot (Lota lota) fish* collected from the upper Hungarian section of the Tisza River (40× magnification).



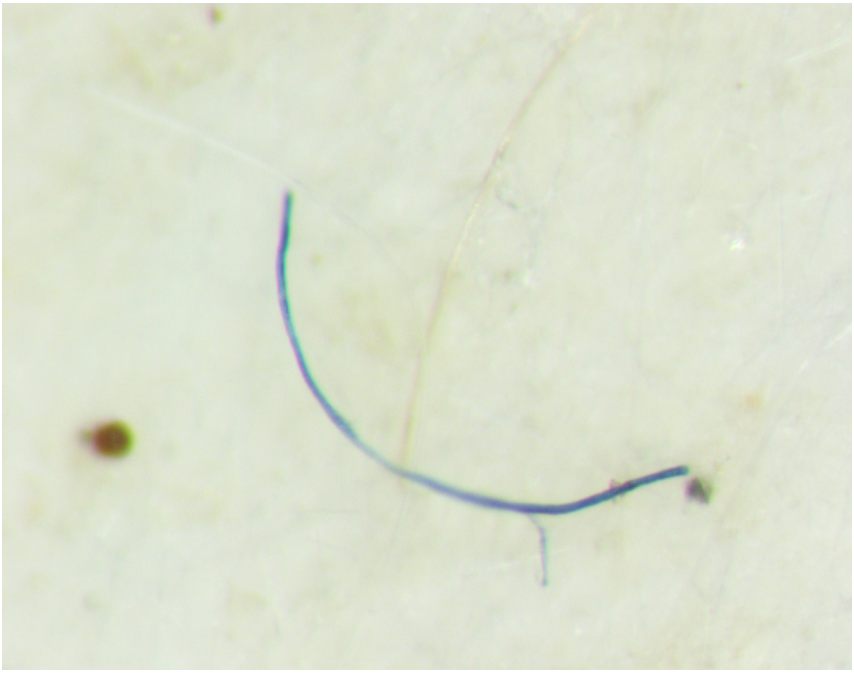
Appendix 5. Stereomicroscope image of a Green PU Fragment found in the *Burbot (Lota lota) fish* collected from the upper Hungarian section of the Tisza River (40× magnification).



Appendix 6. Stereomicroscope image of a Blue PVC Fibre found in the *Burbot (Lota lota) fish* collected from the upper Hungarian section of the Tisza River (40× magnification).

Appendix 7. Stereomicroscope image of green PE-CO-PP Fibre found in the *Burbot (Lota lota) fish* collected from the upper Hungarian section of the Tisza River (40× magnification).

Appendix 8. Stereomicroscope image of a Indigo blue PET-Cotton fibre found in the *Burbot (Lota lota) fish* collected from the upper Hungarian section of the Tisza River (40× magnification).

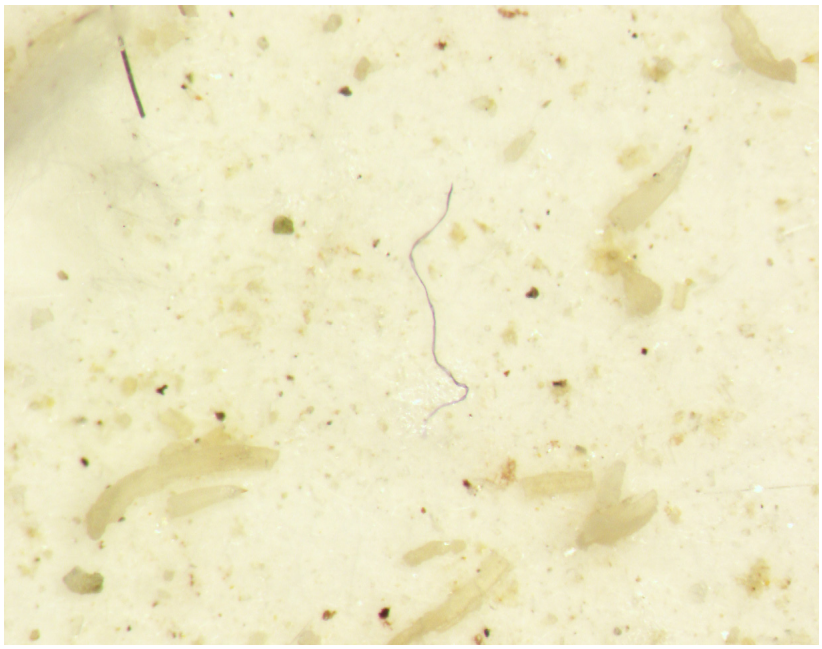


Appendix 9. Stereomicroscope image of an Indigo blue pigmented cotton-PU fibre found in the *Burbot (Lota lota) fish* collected from the upper Hungarian section of the Tisza River (40× magnification).

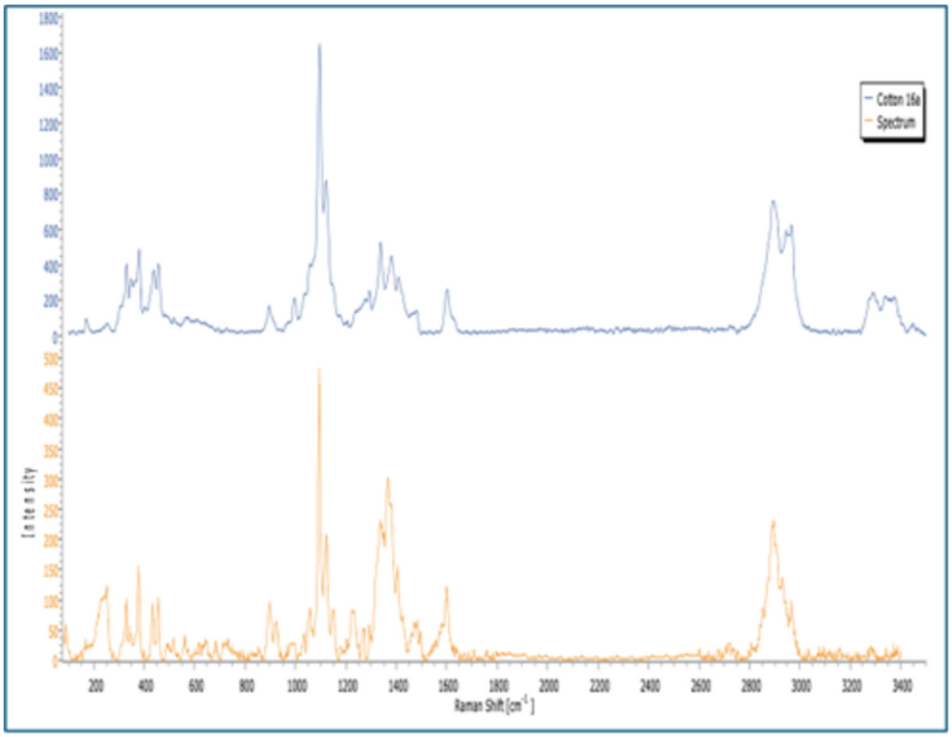
Appendix 10. Stereomicroscope image of a Phthalo green pigmented cellulose fibre found in the *Burbot (Lota lota) fish* collected from the upper Hungarian section of the Tisza River (40× magnification).

Appendix 11. Stereomicroscope image of an Alizarin pigmented polypropylene fibre found in the *Burbot (Lota lota) fish* collected from the upper Hungarian section of the Tisza River (40× magnification).

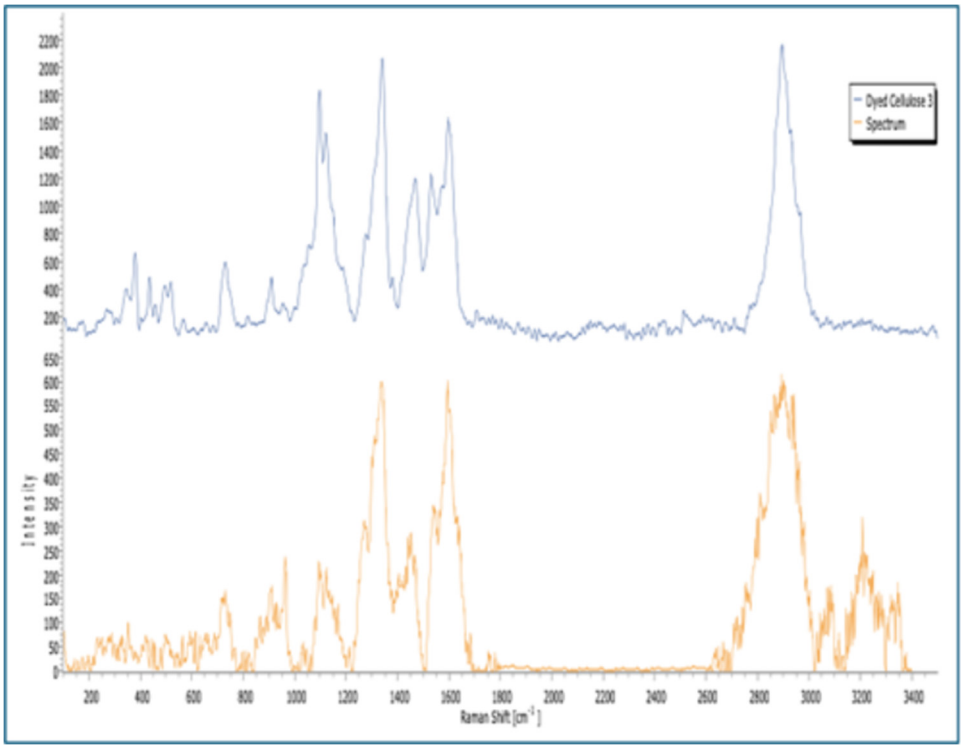
Appendix 12. Stereomicroscope image of Cotton fibre with carbon black pigment found in the *Burbot (Lota lota) fish* collected from the upper Hungarian section of the Tisza River (40× magnification).



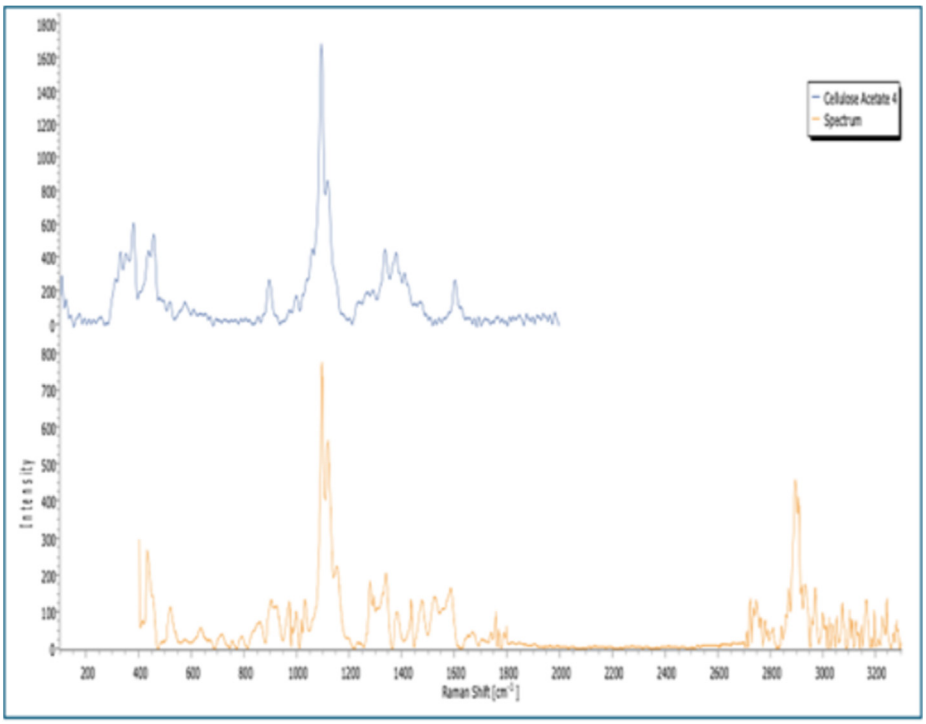
Appendix 13. Stereomicroscope image of Maya blue pigmented polyethylene terephthalate (PET) fibre found in the *Burbot (Lota lota) fish* collected from the upper Hungarian section of the Tisza River (40× magnification).



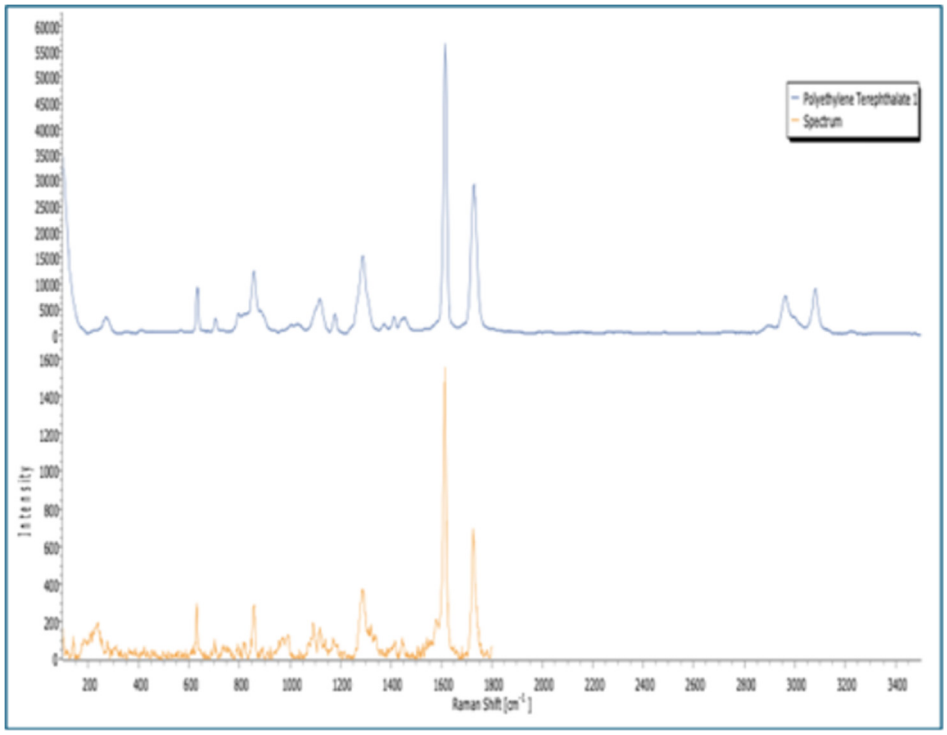
Appendix 14. Raman spectrum of Cotton fibre found in the *Burbot (Lota lota) fish* collected from the upper Hungarian section of the Tisza River.



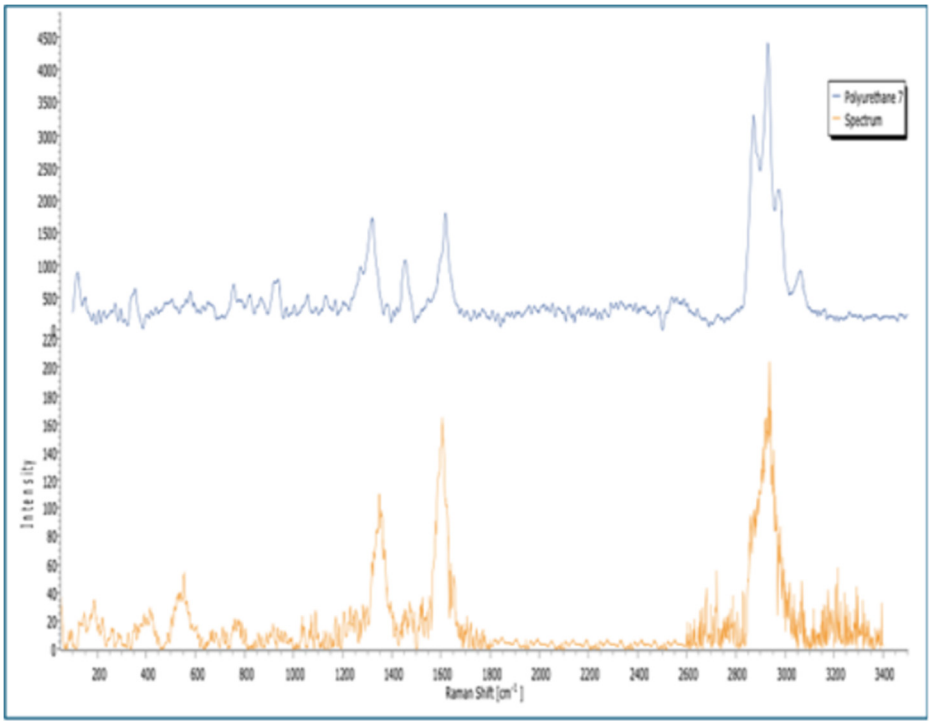
Appendix 15. Raman spectrum of Dyed cellulose fibre found in the *Burbot (Lota lota) fish* collected from the upper Hungarian section of the Tisza River.



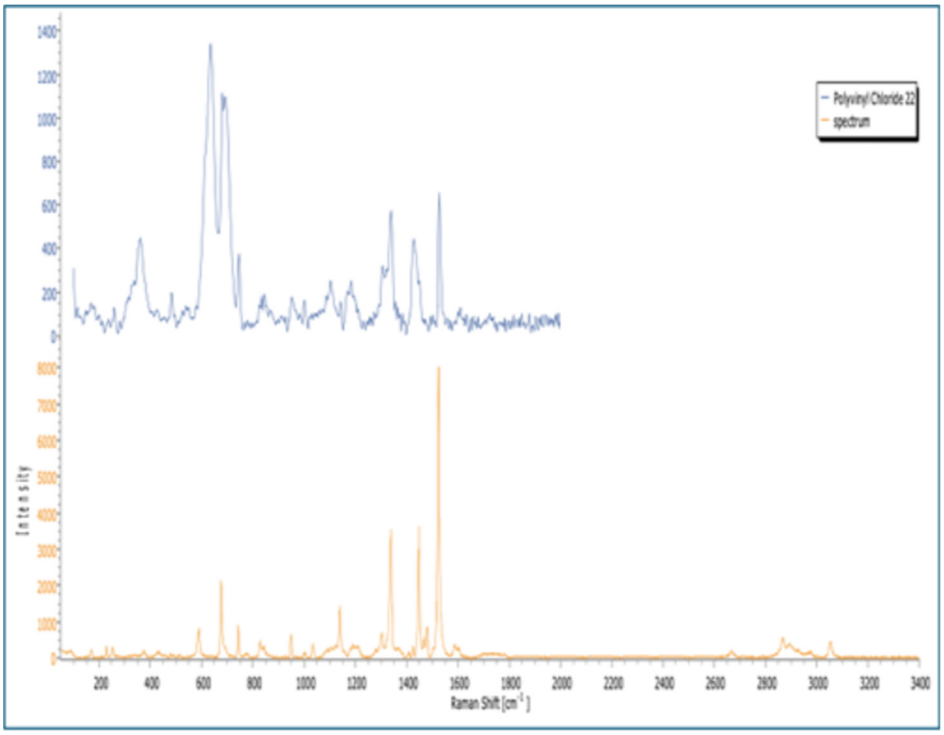
Appendix 16. Raman spectrum of Cellulose acetate particle found in the *Burbot (Lota lota) fish* collected from the upper Hungarian section of the Tisza River.



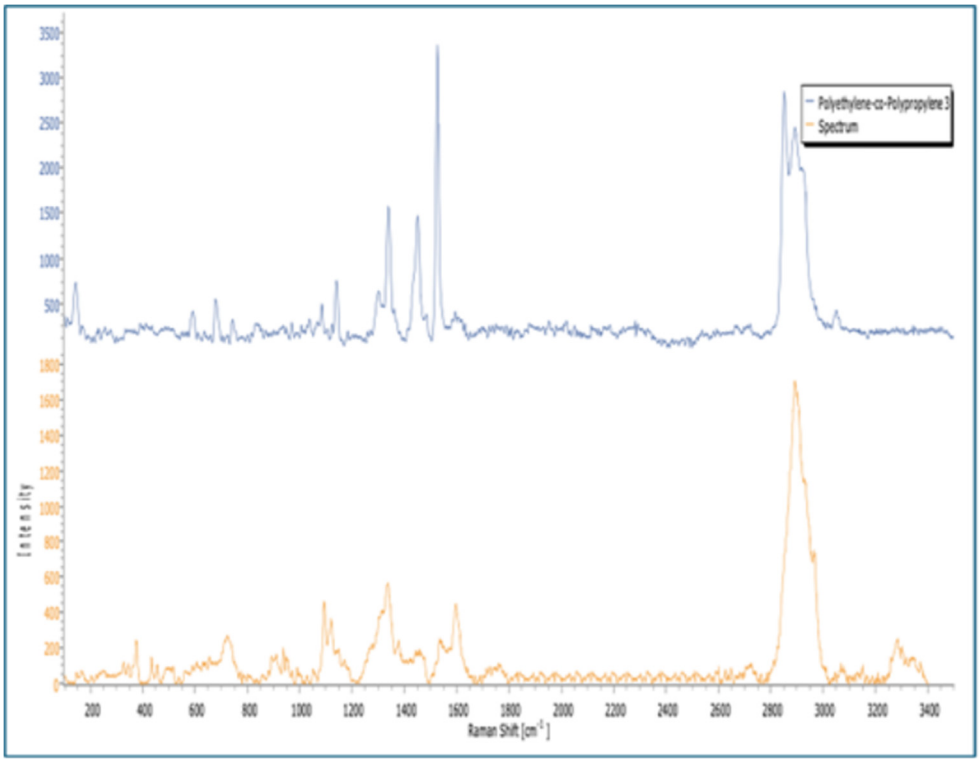
Appendix 17. Raman spectrum of Polyethylene terephthalate fibre found in the *Burbot (Lota lota) fish* collected from the upper Hungarian section of the Tisza River.



Appendix 18. Raman spectrum of Polyurethane based particle found in the *Burbot (Lota lota) fish* collected from the upper Hungarian section of the Tisza River.



Appendix 19. Raman spectrum of PVC fibre found in the *Burbot (Lota lota) fish* collected from the upper Hungarian section of the Tisza River.



Appendix 20. Raman spectrum of PE-CO-PP fibre found in the *Burbot (Lota lota) fish* collected from the upper Hungarian section of the Tisza River.