HOLISTIC APPROACH TO WATER QUALITY: BIOTIC AND ABIOTIC CONTAMINATIONS IN WATER

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Abstract

Effectively addressing water contamination requires a comprehensive approach that considers both biological and chemical contaminants, highlighting their interconnected impact and presents an accurate picture of the real environmental conditions. Microplastics (MP), per- and polyfluoroalkyl substances (PFAS) and antibiotic-resistant bacteria (ARB) coexist in real-world environments. These stressors are considered emerging waterborne contaminants. The interaction between MP/PFAS and ARB may synergistically impact several endpoints, potentially exacerbating environmental risks and posing significant challenges to water management systems and human health. This thesis is structured into two primary sections. The first part focuses on chemical contamination (MP and PFAS) in water systems, investigating their toxicological impact on *Daphnia magna*, a sentinel species for assessing ecological health due to its sensitivity to water quality changes. The second part investigates drinking water contamination from a microbiological perspective with a focus on ARB, providing a comprehensive analysis of water quality concerns. Research on environmentally relevant concentrations of MP/PFAS, their combined effects, and their interactions within ecosystems remains limited. A chronic toxicity exposure test was conducted on two genotypes of *Daphnia magna* with different exposure histories to chemicals (naive and experienced). The experimental setup mimicked natural environmental conditions to investigate *Daphnia's* ecotoxicological response to the individual and combined effects of MP, PFOA and PFOS. This study enhances our understanding of the mechanisms of toxicity triggered by these environmental stressors and their impact on *Daphnia's* life history traits and overall fitness throughout its life cycle. It was discovered that the stressors negatively affected the plasticity in both genotypes. However, the naive genotype exhibited greater tolerance to chemical stressors than the experienced genotype. The interactions between these chemicals were also investigated. Prior to chronic exposure, a proof-of-concept study was conducted to optimise the experimental design using *Daphnia magna* to assess the ingestion, retention and egestion rates of four common MP both in the presence and absence of algae as a food source. This groundwork prepared the study for chronic exposure and helped validate key techniques, such as MP characterisation, their concentration, dispersal methods, refining the choice of one MP and ensuring that the study is well-designed and methodologically accurate. Despite the significance of ARB as emerging biological contaminants, comparatively little research has focused on their presence, diversity, and concentration within household drinking water systems compared to clinical or wastewater systems. Research into the isolation, characterisation and identification of ARB from household drinking systems addresses a critical gap in understanding how these bacteria per-

sist and proliferate in domestic environments. In the next phase of this thesis, the presence, prevalence, and diversity of ARB were investigated in three sites (showerheads, bathroom taps and kitchen taps) within 30 residences. This study provides valuable insight into the link between environmental ARB contamination in community settings and the quality of treated wastewater. This knowledge enhances our understanding of their environmental and public health implications, supports the development of effective mitigation strategies, and promotes increased public awareness of antimicrobial resistance. This thesis emphasises the need to incorporate realistic environmental conditions to accurately represent natural processes and better understand the impacts and interactions of these contaminants on the environment and humans, inspiring further research in this critical field.

Dedication

In memory of my late mother, whose dream was for me to become a doctor and whose love, influence and support have left an unforgettable mark on my life.

And to my dear father, whose dedication, love, and encouragement have been an enduring source of strength throughout my journey.

Acknowledgements

At the beginning of my PhD, the final COVID-19 lockdown caused delays in initiating my laboratory work. I initially started with a microbiology-focused project assessing biological contamination in the drinking water system. However, due to the unexpected departure of my initial supervisor, I had to adapt my PhD project while maintaining its environmental relevance. This led to a shift from biological contamination to chemical water pollution assessment in water systems. At the end of my second year, I approached Professor Luisa Orsini at the School of Biosciences, University of Birmingham, who kindly and professionally supervised me in refining my research focus. Under her supervision, I transitioned into the field of ecotoxicology, investigating the toxicological impact of emerging contaminants. I would like to express my deepest gratitude to Professor Luisa Orsini for welcoming me with open arms and supervising me with unwavering support, kindness, and professionalism throughout my PhD journey. From the very beginning, she warmly accepted me not just as a student but as a valued member of her research group, creating an environment where I felt encouraged, inspired, and empowered to grow. Her trust and belief in my potential have been truly remarkable, encouraging me to build confidence, overcome challenges and push the boundaries of my research. More than just an exceptional supervisor, she has taught me the importance of compassion, integrity, and caring for others, demonstrating that true leadership extends beyond academia. Her vast expertise and insightful mentorship have profoundly shaped my work, influencing every stage of my research. Her patience, thoughtful feedback, and constructive criticism have been invaluable, constantly motivating me to improve and strive for excellence. I am incredibly grateful for the many opportunities she has provided, helping me build confidence, present my work, expand my professional network, and gain invaluable experience. Working with Professor Orsini has been an enriching and transformative experience, one that I will always cherish. I deeply admire her dedication, work ethic, and commitment to her students, and I

feel truly privileged to have been mentored by such an inspiring scientist and human being.

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Abbreviations

ADC Albumin, Dextrose, and Catalase. 132

AMP Ampicillin. 130

AMR Antimicrobial Resistance. 66

AOP Adverse Outcome Pathway. 171

ARB Antibiotic-Resistant Bacteria. 11, 65, 70, 131, 145, 162, 176

ARG Antibiotic-resistant Gene. 5, 17, 65, 67, 69, 158, 161, 162

BDL Below Detection Limit. 144

CF Cystic Fibrosis. 161

CFU Colony Forming Unit. 132, 139, 141, 145, 151, 153, 155

CRB Chlorine-Resistant Bacteria. 66

DLS Dynamic Light Scattering. 84

DWTP Drinking Water Treatment Plant. 67

HGT Horizontal Gene Transfer. 64, 65

HWPS Home Water Purification System. 67

LOD Limit Of Detection. 144

MIE Molecular Initiating Events. 171

MP Microplastic. 65, 169

MPAC MPs Contain Additive Chemicals. 45

NTM Nontuberculous Mycobacteria. 158

OADC Oleic Acid-Albumin-Dextrose-Catalase. 130

OARB Opportunistic Antibiotic-resistant Bacteria. 69

OP Opportunistic Pathogen. 160, 161

PBP Penicillin-Binding Proteins. 68

PDI Polydispersity Index. 84

PE Polyethylene. 21, 168

PEG Polyethyleneglycol. 144

PET Polyethylene Terephthalate. 21, 168, 169

PFAS Per- And Polyfluoroalkyl Substances. 4, 65, 169

PFOA Perfluorooctanoic Acid. 27, 166

PFOS Perfluorooctane Sulfonate. 27, 166

PMC Persistent Mobile Chemicals. 3

PMMA Polymethyl Methacrylate. 21, 168

PP Poly Propylene. 21

PS Polystyrene. 21, 35, 168

PVC Polyvinyl Chloride. 21

QA/QC Quality Assurance/Quality Controls. 81

RE Renewable Energy. 173, 180

REACH Registration, Evaluation, Authorisation And Restriction Of Chemicals. 56

RSD Relative Standard Deviation. 81

SOP Operating Procedure. 84

TAE Tris-Acetate-EDTA. 137

VGT Vertical Gene Transfer. 64

WWTP Waste Water Treatment Plants. 64

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Chapter 1

Introduction

1.1 Introduction

1.1.1 Global Water Pollution

Water is vital for life, and access to safe water sources is crucial for human health and development in every society. Water pollution is a major global concern impacting both developed and developing countries. It poses a significant threat to the environment and public health. Globally, water pollution has significantly worsened in recent decades due to many factors, including rapid population growth, industrialisation (leading to increased industrial discharge), and intensified agricultural activities, contributing to the runoff of chemical fertilizers, pesticides, insecticides, and manure. In addition, untreated domestic and hospital sewage, improper waste disposal, and the impacts of climate change have exacerbated the situation (Shady, Siddique, and W. Yu 2023). Water contamination can be biological or chemical, ranging from pathogenic bacteria to chemicals such as anthropogenic and persistent

pollutants. All these contaminants co-occur and are considered a major global water concern in the natural environment, jeopardising aquatic environments and public health. Once these biological and chemical pollutants have entered the environment, they are challenging to remove because they are not fully biotransformed, degradable or eliminated by current effluent treatment. High contamination levels and long-term exposure to pollutants, such as persistent chemicals in water systems, create a cumulative health burden. Their presence has been linked to a range of chronic diseases, including respiratory disorders, cardiovascular diseases, anaemia, liver cirrhosis, kidney failure, developmental issues, and various types of cancer (Duan et al. 2011). Also, the potential for microbial contamination makes it crucial to properly manage and treat surface water before it is used for any human activity to ensure public safety and prevent waterborne illnesses (Some et al. 2021). Tap water serves as a direct route for human exposure to pollutants through ingestion, skin contact, or inhalation of waterborne aerosols (Jie Wang et al. 2018). Vulnerable populations, including children, the elderly, immunocompromised individuals, and low-income communities, are particularly susceptible to these compounded effects. Also, understanding the geographic distribution of these pollutants is vital for identifying high-risk areas and prioritising interventions. Heavy industrial zones, agricultural regions with pesticide use, metropolitan cities, coastal areas affected by runoff, and regions with poor water sanitation are among the most contaminated locations. Therefore, immediate action is required to identify the adverse effects of these contaminants and mitigate water pollution by implementing stricter environmental policies, investing in wastewater treatment, and promoting sustainable practices to ensure reliable access to sufficient and safe water.

1.1.2 Microplastics as Persistent Environmental Challenge

Plastics are the ideal materials for a wide range of applications due to their adaptability, stability, lightweight, low-cost production, resistance, and easy handling compared to other chemicals (Botterell et al. 2019). Global plastic production exceeds 320 million tons annually (S. L. Wright and Kelly 2017) and is anticipated to double within the next two decades (De Smet 2016). However, only 6-26% of this plastic is effectively recycled, leaving up to 94% to either be improperly managed or end up in landfills, where between 21-42% of plastics accumulate (Nizzetto, Futter, and Langaas 2016; Browne et al. 2011). The remaining is often released into the environment due to poor waste management practices, finding its way into ecosystems through various pathways. Plastic debris forms 60-80% of marine litter (Q. Qiu et al. 2016). Then, large plastic debris breaks down and forms microplastics (MP). MP are particles smaller than 5 mm in size. As a result of their extensive use across industries and daily life, MP has become a persistent pollutant and is ubiquitous in environments. Due to their small size, these tiny particles easily infiltrate water bodies, accumulate in aquatic species, and move into the food chain, leading to human exposure through contaminated water, seafood, and other food products. Their widespread presence raises significant concerns about potential health risks and long-term environmental impacts. Unfortunately, these effects could aggravate marine life in the near future.

1.1.3 PFAS as Forever Chemicals and Infamous for its Ubiquity

Persistent mobile chemicals (PMCs), often referred to as “forever chemicals”, are compounds extremely resistant to environmental degradation and persist in the environment for decades or longer. Their properties make them a significant concern

for environmental and public health. Among these chemicals, PFAS stand out due to their widespread use, persistence, mobility, and potential toxicity. PFAS are found in various products, including stain repellents, nonstick cookware, food packaging, textiles (oil and water-resistant clothing), firefighting foams, pesticides, pharmaceuticals, personal care products, cosmetics, paper and board, cleaning products, paint and coatings, chrome plating, as surfactants and additives in the manufacture of many materials (Kissa 2001; Qi Wang et al. 2018). Due to their wide range of applications in numerous sectors, PFAS are almost everywhere in the environment (Glüge et al. 2020). PFOA and PFOS are the most common PFAS that are considered long chains, having eight carbons in their carbon chain. Also, they are mobile in water; they travel from their original source and gradually spread and accumulate in groundwater at trace levels worldwide (A. O. D. Silva et al. 2021). Hence, there is a need to address the toxic effects of these chemicals on the environment and public health.

1.1.4 Antibiotic-Resistant Bacteria Another Emerging Contaminant in Water

Antibiotics were discovered several decades ago as a remarkable achievement in history. They are used to treat several incurable diseases and save many lives worldwide (Cook and G. D. Wright 2022). However, despite this success, antibiotic resistance is among the top 10 significant threats to human health today. Without proper action, we risk facing a future where effective antibiotics are no longer available (R. J. Melander and C. Melander 2017). Antibiotic resistance is increasing globally, as bacterial infections remain a major cause of illness and death and consequently lead to significant financial burdens on societies (R. J. Melander and C. Melander 2017; Y. Zhang et al. 2018). Antibiotic resistance occurs due to the natural evolution

of bacteria, mutation and gene transfer between bacteria via vertical (VGT) and horizontal gene transfer (HGT), resulting in protective mechanisms that make antibacterial agents ineffective (Chinemerem Nwobodo et al. 2022). VGT and HGT are the main factors contributing to the evolution and emergence of antibiotic-resistant bacteria (ABR), driven by the uncontrolled release of wastes containing antibiotics as a result of misuse or overuse of antibiotics in medical and veterinary (Shakibaie, Jalilzadeh, and Yamakanamardi 2009; He et al. 2016). Approximately 25%–75% of used antibiotics cannot be metabolised and enter the environment (Yulin Chen et al. 2023). Freshwater environments are likely exposed to antibiotic residuals produced by various sources, such as pharmaceutical waste, agricultural run-off, sewage discharges of hospitals, veterinary, and leaching of antibiotics from nearby farms (Chika F. Nnadozie and Odume 2019b). Consequently, freshwater environments could likely be used as reservoirs, where other bacteria can become resistant and cause a serious public health problem. Therefore, it is crucial to investigate the fate of antibiotics, ARB and ARGs in household water distribution systems.

1.1.5 Environmental Co-Existence of MP, PFAS and ARB (MP as Potential Vector for ABR and PFAS)

MP, PFAS, and ARB are present in the natural environment and are known for their adverse effects. However, their simultaneous occurrence exacerbates bioaccumulation and increases ecological toxicity. Wastewaters carry both biological and chemical contaminants, which ultimately enter water bodies, transforming water systems into vast reservoirs of these pollutions. These pollutants severely degrade the quality of aquatic ecosystems, endangering biodiversity and human health. MP tend to adsorb a vast number of environmental compounds (e.g., ARB, heavy metals, persistent organic compounds and pharmaceuticals) due to their small sizes and

large surface area. It occurs because MP can serve as a substrate for the colonisation of ABR by biofilm formation on MP, creating microhabitats for ARB that can facilitate the transfer of ARGs among bacterial communities. MP and PFAS can exacerbate the spread of ARB by creating stressful environments that drive microbial resistance. Moreover, PFAS are known for their hydrophobic and lipophobic properties. Plastics tend to sorb hydrophobic chemicals due to their non-polar nature and, hence, act as potential carriers of PFAS to different organisms upon exposure or facilitate the transport of these substances across various ecosystems, (C. Li and Tang 2023; Robert C Buck, Franklin, Berger, Conder, Cousins, Voogt, et al. 2011; Lagarde et al. 2016). The adsorption of PFAS onto MP typically shows a positive correlation with the length of the PFAS's carbon chains. In general, longer carbon chains tend to enhance the adsorption process. However, this interaction depends not only on chain length; additional factors also significantly influence the adsorption behaviour, like functional groups on the PFAS, specific characteristics of the MP, and environmental conditions (Llorca et al. 2018). The coexistence of these pollutants can disrupt natural processes and contribute to broader ecological imbalances, highlighting the need for a comprehensive understanding of their interactions and effects (Tang 2023). Addressing these challenges is essential for safeguarding environmental health, protecting biodiversity and integrated water management. (Álvarez-Ruiz, Picó, and Campo 2021).

1.1.6 Interaction of Multiple Stressors in Environment

In the natural environment, organisms are exposed to multiple stressors simultaneously that cause a quantifiable change, whether positive or negative response. Understanding the physicochemical dynamics of the contaminants is critical because how organisms respond to multiple stressors depends on the magnitude and

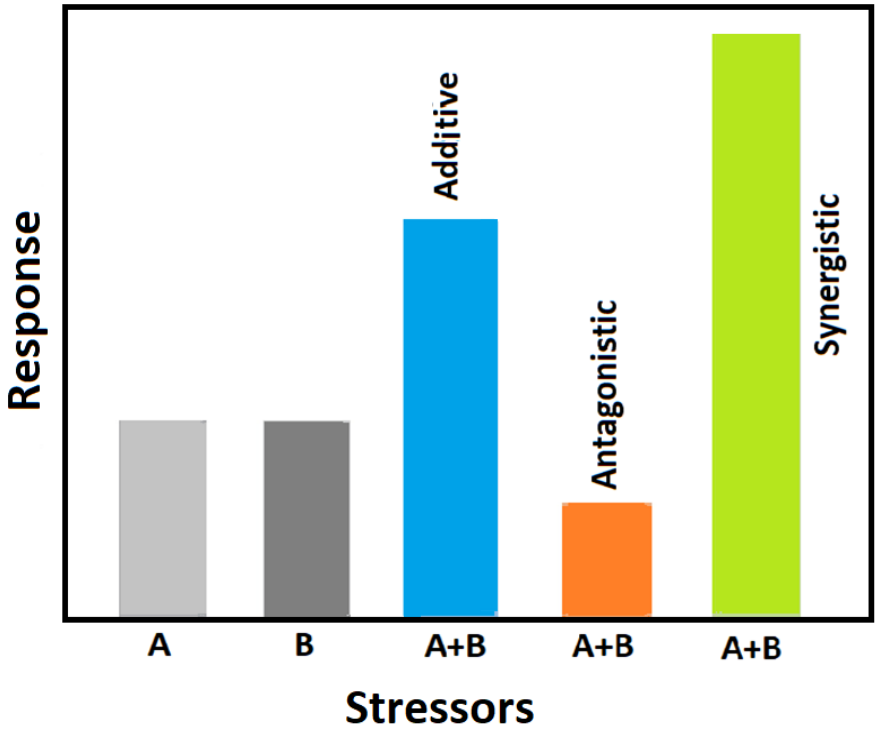


Figure 1.1: Depicts possible interactions of multiple stressors using the additive null model. It defines how multiple stressors interact and establishes a baseline for distinguishing these interactions and how they affect a biological response. In this model, the effects of two or more stressors are assumed to combine in a simple additive way, meaning the total impact is the sum of each individual stressor's effect when acting independently. The interaction of two stressors (A and B) can be additive, antagonistic, or synergistic.

relative timing of each stressor (Gunderson, Armstrong, and Jonathon H Stillman 2016). The interaction of combined stressors can jeopardise biological abundance, processes, functions and biodiversity in the ecosystem (Todgham and Jonathon H. Stillman 2013; Côté, Darling, and Brown 2016); (Côté, Darling, and Brown 2016).

Fig. 1.1 shows the interaction of multiple stressors according to the additive null model that provides a "null" or default expectation for how stressors should interact (Côté, Darling, and Brown 2016). Predicting the type of interactions and complex ecological responses to multiple stressors could be a helpful approach to managing or tackling their negative impact. However, identifying and understanding the nature of interactions occurring among stressors before managing them will be challenging at any scale. The vast number of stressors affect the aquatic and terrestrial ecosystems

simultaneously, making it nearly impossible to measure all potential interactions between every combination of stressors. The ecological interaction of multiple stressors remains a significant challenge in the environment, and distinguishing the impacts of multiple stressors from the response to a single stressor further complicates this issue. The interaction of combined stressors can jeopardise biological abundance, processes, functions and biodiversity in the ecosystem. Synergistic interaction is when their combined effect is greater than the sum of two individual stressors; antagonistic when their combined effect is smaller than the sum of two individual stressors; and additive is when their combined effect is the sum of the two individual stressors (Côté, Darling, and Brown 2016). The antagonistic interaction is the most common interaction of multiple stressors in freshwater organisms, with a value of 41% compared to other interactions (Jackson et al. 2016). Investigating the interactions between stressors uncovers the underlying mechanisms and the factors that influence them and provides deeper information on their associated risks and ecotoxicity in the environment (Tang 2023).

1.1.7 *Daphnia* as a Sentinel Species for Toxicity Tests

Daphnia (water flea) is a small planktonic crustacean found in diverse aquatic environments ranging from large freshwater lakes to smaller bodies of water such as shallow ponds, streams, rivers, and even temporary pools (Ebert 2005). *Daphnia* has been used as a model organism for many toxicological, ecological and evolutionary studies due to its fundamental characteristics in aquatic biodiversity, rapid parthenogenic reproductive cycle (sexual and asexual reproduction alternate), its easy culturing and maintenance in the laboratory and its fully sequenced genome (Ebert 2005; Stollewerk 2010). *Daphnia* shares many ancestral genes with humans, including those linked to diseases; this genetic similarity makes *Daphnia* particu-

larly useful for studying the genetic basis of diseases and understanding how these processes work across different species (Chaturvedi, J. Zhou, et al. 2021). *Daphnia* is a key species in freshwater ecosystems, as it consumes phytoplankton and is the prey of several types of invertebrates (Ebert 2005). *Daphnia* is widely used as a biomarker in toxicology research, an indicator of water quality due to its central role in the food web and its sensitivity to environmental changes (including xenobiotics, temperature fluctuations, and nutrient levels).

1.2 Research Key Elements

1.2.1 Problem Statement

Aquatic environments worldwide are increasingly contaminated with chemical and biological pollutants, posing significant risks to marine ecosystems and human health. Most research tends to focus on either biotic or abiotic contaminants separately, with relatively limited studies simultaneously addressing both in water systems. This is partly because these areas often require distinct methodologies and expertise. Although this interdisciplinary approach is gaining attention, it remains underexplored in the current literature. Contaminations from biological and chemical sources often co-occur in the environment, interact with each other and can influence each other's behaviour and impact. The potential environmental and public health implications of their bioavailability highlight a significant research gap that needs to be addressed. The key motivation for this study was to assess water contamination from both biotic and abiotic perspectives and explore potential correlations between these two in the environment. Contamination by MP and persistent chemicals like PFAS has emerged as a global environmental issue, impacting the world's aquatic life due to their ubiquity. These chemicals have gained significant attention due

to their adverse effects on the ecosystem and public health. Although their toxic effects have been extensively studied individually, their combined toxicological effect remains poorly understood, even though these contaminants often co-occur and interact in the natural environment (Ojo, Peng, and Ng 2021). In addition, conventional chemical toxicity tests often overlook the effects of long-term exposure and typically use chemical concentrations that do not reflect the levels organisms encounter in a real environment. This narrow approach fails to account for the potential negative cumulative impacts of chemical mixtures on both the environment and human health. Therefore, a multidisciplinary approach is needed to address the complexities of environmental pollution. Understanding these combined health risks is essential for a comprehensive view of environmental and public health challenges as it mirrors the real environment while focusing on single chemicals that often overlook their true impact. To address this research gap, the toxicological impact of these MP, PFOA and PFOS was investigated individually and in combination on *Daphnia magna* as a sentinel species through a chronic exposure. By investigating the chronic effects of these pollutants, this research seeks to uncover the broader ecological risks associated with MP and PFAS contamination and provide insights into their long-term environmental impact. *Daphnia* is an ideal model organism as it holds significant potential to advance effective water quality management strategies. As the first research to report the combined effects of PFAS and MP on *Daphnia magna*, this study aims to fill critical knowledge gaps and provides valuable insights into impacts, thresholds and the ecological risks posed by these pollutants that ultimately contribute to the safeguarding of aquatic ecosystems and the promotion of public health by ensuring clean and safe water resources. Moreover, current studies on the ecotoxicological impact of MP and PFAS often use uniform shapes of MP (commercially available) and concentrations that do not fully represent the complexity of the natural environment. In the natural ecosystems, MP comes in a variety of irregular shapes and sizes due to weathering, and the concentrations of both MP

and PFAS fluctuate based on local pollution levels. This lack of environmental relevance in laboratory testing limits the applicability of results to real-world scenarios, potentially underestimating or misrepresenting the actual ecological risks posed by these contaminants. There is a critical need for ecotoxicological research employing environmentally relevant shapes and concentrations of persistent chemicals to better understand their actual impacts on aquatic organisms and ecosystems. Without such realistic testing parameters, our understanding of how these pollutants behave and interact in natural environments remains incomplete, hindering the development of effective mitigation strategies. This study addresses this gap by using irregularly shaped MP, environmentally realistic shapes, and concentrations of MP and PFAS that reflect those found in polluted water bodies. Given that these chemicals coexist and are likely to interact in the environment, and with limited research addressing their combined effects and interactions, this study also investigated their potential interactions and provides valuable insights into their potential risks to environmental health, biodiversity and public health. Understanding these dynamics is essential for predicting the real-world implications of chemical mixtures.

From a microbiological perspective, ARB, a growing biotic threat, proliferates in water systems, accelerating the spread of antibiotic-resistant genes. The prevalence of antibiotic-resistant bacteria is steadily increasing, posing significant environmental and public health risks. Among these, β-lactam antibiotics are a major concern because of the increase in multidrug-resistant Gram-negative bacteria. Although the presence of ABR has been extensively studied in clinical settings and wastewater treatment plants, far less is known about the variation in bacterial species and levels of contamination in different households and water sources (community settings). Another key motivation was to investigate the presence, prevalence and diversity of ARB in household plumbing systems. Identifying contaminated sites within households that serve as reservoirs for antibiotic resistance is essential, as it

provides valuable insights into strategies to minimise their proliferation and mitigate the risks posed by these environmental reservoirs. Analysing the domestic environment as a potential reservoir for resistant bacteria is essential to determine whether households contribute to the spread of antibiotic resistance or serve as habitats for resistant bacteria. Mapping the distribution of resistant bacteria through this research provides valuable baseline data on the prevalence and resistance profiles of ARB in domestic environments.

To conclude, conducting in-depth research on the interplay between different pollutants and stressors helps scientists to accurately assess the cumulative and synergistic effects on ecosystems. The motivation for this research originated from the need to better understand the impact of multiple contaminants of different natures (MP, PFAS and ABR) that simultaneously exist in water systems. They can be linked as complementary aspects of water pollution that may contribute to a holistic assessment of waterborne hazards.

1.2.2 Primary Research Questions

1. What are the chronic toxicological impacts of MP, PFOA, and PFOS on the phenotypic plasticity and life history traits of *Daphnia magna*?

2. How do two-way and three-way interactions between PFOA, PFOS, and MP influence the life history traits of *Daphnia magna*, and what are the combined effects of these mixtures on the organism's fitness?

3. To what extent can ARB be isolated from different locations within various households, and what are the most common bacterial strains identified across all samples? How do microbial communities vary across different sections of household plumbing systems, and which areas serve as the most favourable

sites for the proliferation and dissemination of ABR and resistance genes?

1.2.3 Research Aim and Objectives

The primary aim of this thesis was to develop a comprehensive approach for designing studies to assess the impact of various types of emerging contaminants on water quality, aquatic species, and human health. Specifically, this research investigated how different stressors, such as MP and PFAS as abiotic factors and ARB as biotic factors, affect aquatic environments. This study aimed to investigate emerging waterborne hazards, including MP, PFOA, and PFOS, and their adverse effects on freshwater biota using freshwater invertebrate *Daphnia magna* as a model organism, as well as the ecological risks posed by these contaminants. Also, to investigate how naive and experienced genotypes of *Daphnia magna* respond to these chemicals, focusing on differences in their physiological and ecological responses to assess potential variations in tolerance and adaptive capacity. Lastly, this research aims to explore these contaminants' combined effects and interactions under environmental conditions, focusing on their potential synergistic, additive, or antagonistic effects.

In parallel, the second part of this thesis aimed to detect, quantify, and compare antibiotic-resistant bacteria (ARB) across three sites within household water distribution systems. The aim is to investigate their prevalence, diversity, and associated risks to environmental and public health, as well as to identify contaminated sites within households that act as a potential reservoir for resistant bacteria and facilitate the spread of antibiotic resistance. Overall, this research focuses on mimicking real-world scenarios to better understand how these stressors coexist and interact within natural ecosystems. This holistic approach allows us to investigate multiple forms of water contamination, offering a comprehensive perspective on the environmental and public health challenges posed by chemical and biological contaminants.

This research aims to improve environmental management, raise awareness, educate the public about the adverse effects of these contaminants on the environment, and support policies that protect aquatic ecosystems and public health. Three objectives break down the different stages of the study design to increase our understanding of how these chemical and biological threats potentially may affect water quality.

1. To assess the toxicological impact of MP and PFAS on the overall fitness response of *Daphnia magna* as a sentinel species by assessing key life history traits such as growth, reproduction, and survival (Chapter 3 and 4)
2. To investigate the two-way and three-way interactions of MP, PFOA, and PFOS on *Daphnia magna* to quantitatively assess the synergistic, antagonistic, and additive effects of these multiple stressors on the organism's fitness and life history traits (Chapter 4)
3. To assess the environmental occurrence, diversity, and potential public health implications of antibiotic-resistant bacteria (ARB) within household plumbing systems by identifying contamination hotspots that facilitate ARB persistence, proliferation, and dissemination (Chapter 5).

1.2.4 Publications Associated with this Work

The following research papers have been published or submitted for publication as part of my PhD work. This thesis serves as a comprehensive resource, offering in-depth discussions, conceptual frameworks, and the key contributions presented in these publications. Moreover, it integrates the theoretical and practical insights that underpin the findings reported in these papers.

1. Soltanighias, Tayebeh, Abubakar Umar, Muhammad Abdullahi, Mohamed

Abou-Elwafa Abdallah, and Luisa Orsini. "Combined toxicity of perfluoroalkyl substances and microplastics on the sentinel species Daphnia magna: Implications for freshwater ecosystems." Journal of Environmental Pollution 363 (2024): 125133. Doi: 10.1016/j.envpol.2024.125133.

Author's contribution: Tayebeh Soltanighias: Writing – original draft, Investigation, Formal analysis. Abubakar Umar: Investigation, Formal analysis. Muhammad Abdullahi: Formal analysis, Data curation. Mohamed Abou-Elwafa Abdallah: Writing – review & editing, Supervision, Funding acquisition, Conceptualization. Luisa Orsini: Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization.

2. Abubakar Umar; Tayebeh Soltanighias; Luisa Orsini and Mohamed Abdallah (2025), Toxicity of Microplastics and Perfluoroalkyl Substances in Sentinel Freshwater Models, Daphnia, Zebrafish and Unicellular Green Algae: A Systemic Review, Journal of Environmental Pollution and Management (under review)

   Author's contribution: Tayebeh Soltanighias: Writing – original draft, Investigation, Formal analysis. Abubakar Umar: Writing – original draft, Investigation, Formal analysis. Mohamed Abou-Elwafa Abdallah: Writing – review & editing, Supervision, Funding acquisition, Conceptualization. Luisa Orsini: Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization.

3. Tayebeh Soltanighias, Kiran Tota-Maharaj, Mujib Rahman, Tala Kasim (2025) Occurrence of antibiotic-resistant bacteria in the household plumbing system, AquaEcOmics International Conference, 17-20 March, Evian-Les-Bains, France, (accepted)

1.2.5 Thesis Roadmap

Fig. 1.2 depicts the structure of the thesis roadmap as outlines below.

Chapter 1: This chapter provides background knowledge and an introduction to the study. It presents overview of key elements to the research, covering the problem statement, research aims and objectives and research questions.

Chapter 2: This chapter is divided into three main sections. The first section provides a literature review on microplastics (MP) and per- and polyfluoroalkyl substances (PFAS) contamination in aquatic environments, with a particular focus on the use of *Daphnia* as a model organism in ecotoxicological studies. The second section presents a systematic review of relevant research conducted over the past 25 years, synthesising key findings and identifying major trends and knowledge gaps in the field. The third section expands the scope of the review to address antibiotic-resistant bacteria (ARB) as a form of biological contamination, with a specific focus on their occurrence in domestic water systems. This chapter aims to provide a deeper understanding of the critical challenges and knowledge gaps in research on MP and PFAS from an environmental perspective, using freshwater sentinel species such as *Daphnia*, zebrafish, and green algae to investigate their toxicological impacts and identifying persistent issues in the field, while also proposing new directions for future research to improve environmental realism and relevance in MP and PFAS studies. Also this chapter discusses potential environmental interactions among MP, PFAS, and ARB, and explores how these co-occurring pollutants may collectively contribute to environmental and public health risks.

Chapter 3: This chapter includes a preliminary analysis of the ingestion, retention, and egestion of the four (4) most common MP by *Daphnia magna* in the presence and absence of algae as a food source to assess the feeding behaviour and excretion

patterns when exposed to these MP. This pilot experiment is a critical step as it is a foundational study to optimise and establish the experimental setup/method by characterising MP and finalising one MP before proceeding to long-term exposure (chapter 4). The data generated in this chapter is used as baseline data for the subsequent study phase.

Chapter 4: This chapter investigates the chronic exposure of two genotypes of *Daphnia magna* (naive and experienced to the chemicals) to MP, PFOA, and PFOS, both individually and in various combinations. The exposure lasts throughout the life cycle of *Daphnia magna*, and its key life history traits such as survival, growth, and reproduction are investigated. Additionally, the chapter explores the interactions between these chemicals to understand their combined effects on *Daphnia's* fitness and their broader ecological impact on aquatic organisms and ecosystems. The findings of this chapter are reported for the first time and provide valuable insights that can inform the development of targeted strategies to enhance environmental protection and improve pollution management. This chapter represents a significant contribution to my thesis, addressing questions 1 and 2 of this thesis.

Chapter 5: This chapter explores the presence and prevalence of antibiotic-resistant bacteria (ARB), specifically ampicillin-resistant strains, in drinking water samples collected from household plumbing systems. It evaluates the diversity of bacteria found across three locations within households and identifies the most and least contaminated sites across the residences. ARB were isolated, characterised, and identified through 16S rRNA gene sequencing. Additionally, this chapter discusses the contributing factors to the dissemination of ARB and antibiotic resistance genes (ARGs) in the environment and their potential transmission to humans. Finally, it assesses strategies to reduce the occurrence of ARB in water supplies. The results of this chapter enhance public awareness, which could help minimise antibiotic resistance in drinking water and ultimately protect the environment and public health.

This chapter addresses the third research question in this thesis.

Chapter 6: This chapter discusses the overall results, key thesis findings, and future plans. It synthesises data from various experiments and analyses, interpreting them in the context of the research objectives. By comparing the outcomes with existing literature, the discussion highlights the contributions of the study to the current body of knowledge in the field. Although this chapter explores the broader implications of the results and identifies significant trends and patterns, it also addresses the strengths and limitations of the study. Additionally, the chapter offers insights into how the findings can inform future research directions, practical applications and development.

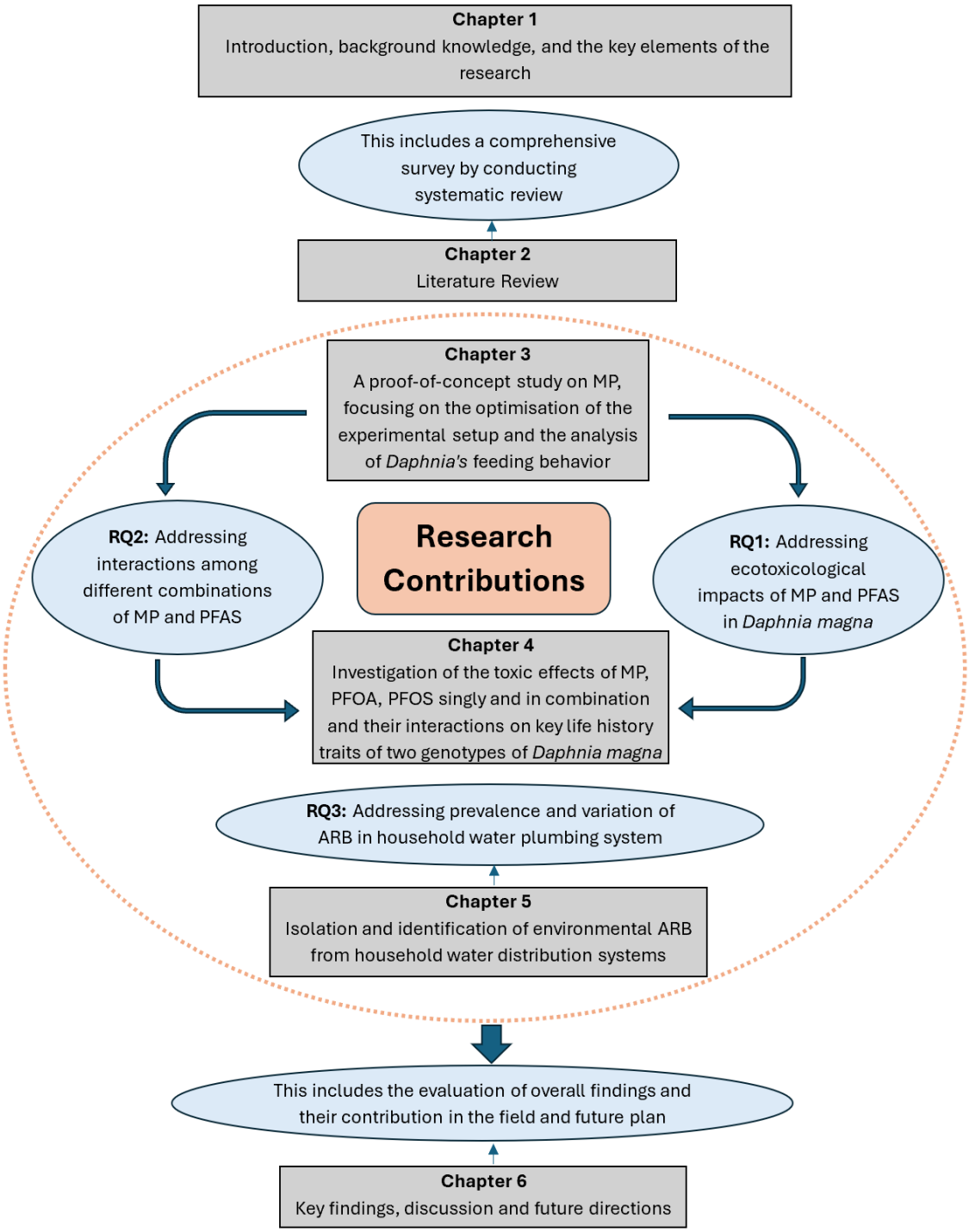


Figure 1.2: Research Roadmap

Chapter 2

Literature Review

2.1 Introduction

This chapter is organised into two major parts. The first section discusses *Daphnia* as a model organism in ecotoxicological studies and presents a literature review on the core concepts of microplastics (MP) and per- and poly-fluoroalkyl substances (PFAS), exploring their applications, environmental persistence, and ecological impacts on both ecosystems and public health. The second section presents an in-depth survey and applies a systematic workflow to identify and analyse 68 relevant studies from over 4,000 screened on the toxicity of MP and/or PFAS in three key freshwater ecotoxicology models: water fleas, zebrafish, and unicellular green algae. The review aims to provide a comprehensive overview of existing knowledge and identify research gaps, highlights key challenges in understanding the toxicological impacts of these contaminants, and key directions for future research in this evolving field. The review highlights differences between acute and chronic exposure effects, emphasising the need for long-term studies to capture life-cycle impacts. This review

further highlights the need for research on the toxicity of irregularly shaped MP with varying polymer types and sizes under realistic exposure conditions, as "Virgin" MP are rare in the natural environment. Also, this review identifies key knowledge gaps and research priorities to advance understanding of MP and PFAS mixtures, supporting efforts to protect freshwater ecosystems and public health. This knowledge is critical for informing policy and supporting efforts to protect environmental and human health from these pervasive emerging contaminants. Although evidence exists on the individual toxicity of MP and PFAS, no study was found addressing the combined effects of MP and PFAS on the mentioned model organisms despite their coexistence in freshwater ecosystems. To address these gaps, this thesis investigated the combined toxicity of PFAS and MP using irregularly shaped MP and environmentally relevant concentrations in Chapter 4. The current chapter does not cover a literature review on antibiotic-resistant bacteria (ARB) as a form of biological contamination in water. Instead, a dedicated literature review is presented within the empirical chapter (chapter 5) that specifically investigates ARB in domestic water systems.

2.1.1 Microplastics in the Environment

Plastics are extensively utilised across various industries, including manufacturing, medicine, engineering, construction, and household products Alomar, Estarellas, and Deudero 2016; Duis and Coors 2016. Their widespread adoption is primarily attributed to their advantageous properties, such as high adaptability, stability, lightweight nature, cost-effective production, durability, and ease of handling compared to other materials. (Botterell et al. 2019). PE, PS, PET, PMMA, PP and PVC are the most prevalent plastics released into the environment (Albert A. Koelmans et al. 2019). A significant proportion of these plastics degrades into microplastics

(MP) through the fragmentation of larger plastic items. MP are small particles of plastic with a size of smaller than 5 millimetres that can be found in various shapes and sizes X. Sun et al. 2017. MP are classified into primary or secondary MP based on their origin. Primary MP includes plastic fragments synthesised in microscopic sizes and used in various industrial and domestic applications. For instance, microfibers and microbeads can be found in synthetic textiles, personal care products like mouthwash, toothpaste shower gel and facial cleansers (Auta, Emenike, and Fauziah 2017, Fu and Jun Wang 2019). These primary MP commonly end up in either freshwater or seawater environments through various pathways: discharge from wastewater plant systems, road runoff and industrial or domestic drainage, landfill operations or wind transfer (Geyer, Jambeck, and Law 2017; Sherri A Mason et al. 2016). Secondary MP are the result of fragmentation and degradation of bigger plastic wastes due to weathering processes (e.g., UV photodegradation, hydrolysis, mechanical wearing, biodegradation) (Alimi et al. 2018; J. Yuan et al. 2020). Secondary MP sources include car tyres, microfibers from textiles, fishing nets, water bottles, disposable food containers, plastic sacks, boxes, agrarian plastic films, and marine paints and other discarded plastic debris (Ammala et al. 2011; McDevitt et al. 2017; E. Hernandez, Nowack, and Mitrano 2017). Fibers, fragments, film, foam and pellets are the most prevalent types of MP in the environment. It is important to use concentrations of MP for ecotoxicology studies that realistically reflect those found in the natural environment. Although using extreme concentrations of MP in studies can enhance our understanding, such results are unlikely to reliably assess their potential impact in a natural environment (Eerkes-Medrano, Thompson, and Aldridge 2015). MP are distributed in various environments heterogeneously. Hence, it is essential to identify ecosystems, hotspots, and species at high risk of interaction with MP to understand the risk of MP. Smaller MP can be found abundantly in the environment than larger particles (Cabernard et al. 2018). In natural aquatic systems, the concentrations of MP are ranged from tens to hundreds of particles per

litre (L. Ding et al. 2019; Scircle et al. 2020; Zhu et al. 2019; Watkins et al. 2019; Kreitsberg et al. 2021; Leslie et al. 2017). The concentration of MP in water bodies varies greatly depending on the location, proximity to urban or industrial areas, size/shape, sampling technique, analysis method, and the water body's usage, which can range from a few particles per litre to thousands of particles per cubic meter, depending on the water source (rivers, oceans, lakes, etc.) (Heidbreder et al. 2019). Analytical methods often complicate the comparison of MP concentrations because values are reported in different units.

2.1.2 PFAS as Forever Chemicals

per- and poly-fluoroalkyl substances (PFAS) are a class of synthetic aliphatic compounds (contain hydrophobic and oleophobic moieties) with strong carbon-fluorine bonds (Tang and Kristanti 2022; R. Buck et al. 2012). They were first discovered in the 1930s, and their large-scale production started during the 1940s due to their unique properties, such as resistance to water, stains, heat, and adhesion and oxidation (Babut et al. 2017). PFAS can be either a short or a long chain, dependent on the length of the perfluoroalkyl chain. Long-chain PFAS tend to be more bioaccumulative and toxic compared to short-chain counterparts. The length of the fluorinated carbon chain results in different physicochemical properties that affect the compound's behaviour, bioaccumulation and toxicity in organisms and the environment (Fenton et al. 2021). PFAS have been detected in numerous water sources worldwide, including groundwater, surface water, oceans, and even drinking water. Their concentrations in drinking water worldwide range from a few to several tens of ng/l (David Q. Andrews and Naidenko 2020). Hence, it is necessary to provide a scientific basis for risk assessment due to the significant toxic impact of PFAS on aquatic organisms, the environment and human health. However, until

the last few decades, there were no comprehensive risk assessments on the impact of anthropogenic chemicals on humans and wildlife (Dulio et al. 2018). Industrial discharge seems to be a major contributor to PFAS in aquatic ecosystems. Wastewater treatment Plants (WWTP) worldwide are another main source of detection and spreading of PFAS, as some types of PFAS are challenging to remove from the water using conventional wastewater treatment processes. They can even be found in the effluent after ozone or chlorine disinfection due to their resistance to degradation and low sorption to solids (Earnshaw et al. 2014). Concentrations of PFAS can be varied geographically, in urban vs rural environments and between the metropolitan and countryside (Simcik and Dorweiler 2005). These pollutants have been found in distant and isolated regions, such as the Arctic and Antarctic Oceans, demonstrating their global spread and persistence in the environment (Suja, Pramanik, and Zain 2009). As part of the food chain, humans are also vulnerable to bioaccumulation of these harmful substances through drinking water, food, vegetables, dairy items, refreshments, eggs, meat items, fish and shellfish, which has been already proven (Jian et al. 2017; Renzi, Guerranti, et al. 2013). PFAS are linked to a variety of health issues, including disrupting immunological systems, endocrine hormones (thyroid function), liver disorders, neurological problems, cancers and adverse impacts on reproduction and development (López-Arellano et al. 2019; Olsen et al. 2007; Y. Li et al. 2017; Tse et al. 2016; Y. Wang et al. 2019; Nicole 2013). Also, studies have shown that human blood samples collected from across the world consistently show the presence of PFAS, indicating widespread exposure and accumulation within the human population (J. Han et al. 2021).

2.1.3 *Daphnia* as a Model Organism for Toxicity Tests

Daphnia, typically found in ponds and slow-moving water bodies and is a keystone species because of its crucial role in the trophic food web (Ebert 2005). *Daphnia* reproduces many genetically identical clones during its life cycle (decreasing variation in experimental results), allowing a resurrected genotype to be maintained for many generations (Berg, Pálsson, and Lascoux 2001). In addition, their transparent body allows easy observation of internal changes under a microscope (F. Nasser and Iseult 2019). *Daphnia* assays offer crucial information on the acute and chronic toxicity of xenobiotics in aquatic environments (Colbourne et al. 2022). This information helps determine whether or not these changes will occur at higher tropical levels, highlighting how these changes affect the ecosystem as a whole. *Daphnia magna* and *Daphnia pulex* are the most common species that have been investigated in various research. *Daphnia* is often used as a model organism in ecotoxicology studies as a bioindicator to assess the impact of environmental contaminants due to their keystone characteristics in the environment, their rapid parthenogenic reproductive cycle (sexual and asexual reproduction alternate) (Fig. 2.1), their easy maintenance and their sensitivity to a range of contaminants (F. Nasser and Iseult 2019; F. Nasser, Constantinou, and Lynch 2020; Gaiser et al. 2012). However, comparing toxicity endpoints between laboratory-cultured daphnids with their counterparts in natural environments still remains unclear. The Organisation for Economic Co-operation and Development (OECD) has established standardised guidelines for evaluating chemical toxicity using *Daphnia* species. These guidelines, specifically the guideline for the testing of chemicals: Acute immobilisation test and chronic toxicity test on *Daphnia*, provide a framework for assessing the potential toxic effects of various chemical substances on aquatic invertebrates (Reilly 2022). The acute immobilization test measures the short-term impact of chemical exposure by determining the concentration at which *Daphnia* exhibit immobilisation, while the chronic toxicity test evaluates long-term

effects, including reproduction and growth inhibition. These standardised protocols ensure consistency and reliability in ecotoxicological studies, facilitating regulatory assessments and environmental risk evaluations.

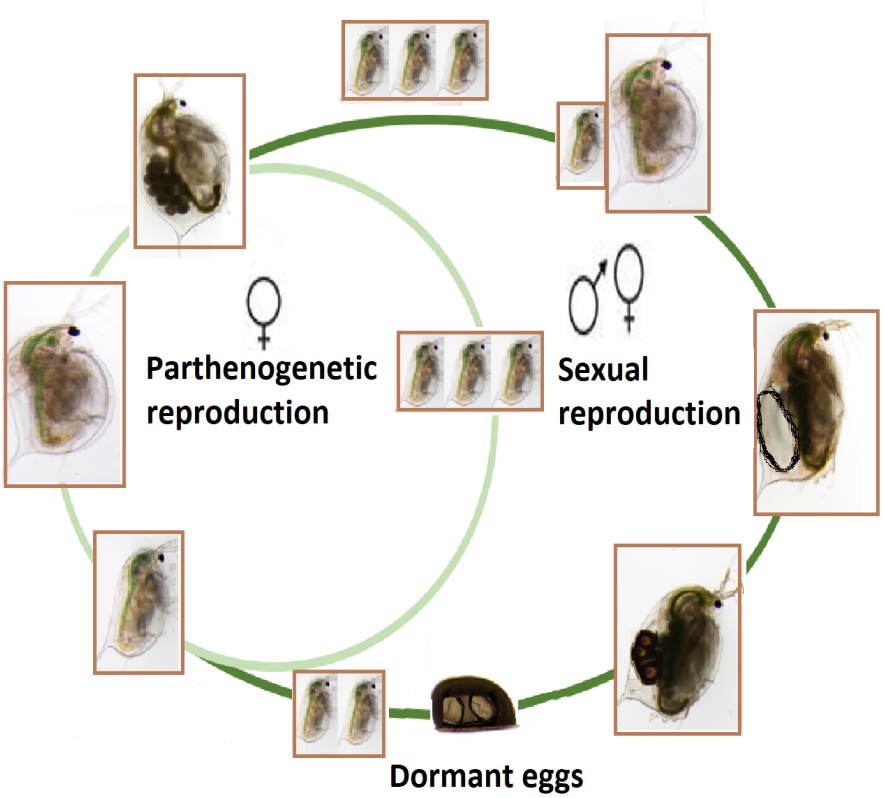


Figure 2.1: The life cycle of *Daphnia* involves both asexual and sexual reproduction. Adult females primarily produce asexual eggs that develop within their brood chamber (cycle: parthenogenetic reproduction). These eggs typically hatch into daughters, although in some instances, they develop into males. Under certain conditions, some females may switch to sexual reproduction, producing haploid eggs that require fertilization by males (sexual reproduction). The fertilized eggs are enclosed in a protective case formed from the female's carapace, known as an ephippium (brown structure at the bottom of the diagram), which contains dormant eggs. The ephippium sinks to the bottom of the water body, where the eggs enter a dormant state (diapause). After this period of dormancy, one or two sexual offspring hatch from the ephippium and develop into females.

2.2 Systematic Review of Literature

A large proportion of accessible freshwater designated for human consumption is polluted with persistent contaminants, which impact ecosystems and human health

(Landrigan et al. 2018). Among these contaminants, microplastics (MP) and per- and poly-fluoroalkyl substances (PFAS) are of high concern because of their ubiquitous presence in freshwater, high persistence and potential toxic impact on wildlife (Ackerman Grunfeld et al. 2024; Bhardwaj et al. 2024). Plastics degrade in the natural environment, producing particles of <5mm in size known as secondary MP. In addition to these secondary MP, primary MP enter the environment as microbeads washed out from cosmetics, textile microfibres, and industrial abrasives (Elizalde and Herrera 2023). Polyethylene (PE) and polypropylene (PP) are the most common MP in surface water (Dai, J. Zhao, C. Sun, Diying Li, et al. 2022). MP contains additive chemicals (MPACs) that are incorporated during production to enhance performance and physicochemical properties. These chemicals include plasticizers (Jasso-Gastinel and Kenny 2016), flame retardants (X. J. Luo et al. 2010a), biocides, and colourants, amongst others (Table 1, Appendix A) (Wee and Aris 2023). Additionally, MP may adsorb other environmental contaminants (e.g., heavy metals, persistent organic pollutants) by virtue of their large surface-to-area ratio (Dai, J. Zhao, C. Sun, Danyang Li, et al. 2022). PFAS are industrial chemicals widely used for their chemical and thermal resistance, combined with superior surface-active properties. This has led to their extensive production and application in a broad range of products, including some firefighting foams, nonstick cookware, water-repellent clothing, stain-resistant fabrics and carpets, cosmetics, and products that resist grease, water, and oil (Dirani et al. 2024). Currently, up to 15,000 PFAS have been identified in the environment (NIEHS n.d.). Among those, PFOA and PFOS are most commonly detected (Post, Cohn, and Cooper 2012; Dirani et al. 2024). In particular, PFAS is found in freshwater, groundwater, ice caps and rainwater (Dirani et al. 2024). Recent reviews have shown that MP, and PFAS co-occur in freshwater environments, facilitated by their common origin in consumer products (i.e., microfibres from water-repellent textiles or grease-resistant fabrics) (Dirani et al. 2024; Ehsan et al. 2024; Rekik et al. 2024; Y. Shi et al. 2024, Witczak et al. 2024;

B. Zhao, Richardson, and You 2024). MP and PFAS have been reported across geographic regions; River Thames, UK; (Rowley et al. 2020), River Rhine, Switzerland (Mani and Burkhardt-Holm 2020; Pan et al. 2018), Pearl River, China (Lin et al. 2018; Si et al. 2021), and the Great Lakes, North America (A. A. Koelmans et al. 2023; Lenaker, Corsi, and S. A. Mason 2021; McFarlan and Lemke 2024). Recent studies showed that adsorption via electrostatic interaction is the main mechanism of interaction between PFAS and MP. Other possible interaction mechanisms include surface complexation, $\pi-\pi$ interactions and micelle formation (Dai, J. Zhao, C. Sun, Diying Li, et al. 2022;F. Yu et al. 2024). Despite their abundance in freshwater, little is known about the mechanisms of toxicity on aquatic biota, and an in-depth assessment of their combined toxicity is lacking. To fill this knowledge gap, the current literature reviews the adverse effects of MP and PFAS, both as single chemicals and mixtures, on three premier freshwater ecotoxicology model species, waterfleas (*Daphnia*), zebrafish (*Danio*), and unicellular algae e.g. *Chlorella*, focusing on both acute and chronic toxicity. *Daphnia* serves as a sentinel species for water pollution (M. Abdullahi, J. Zhou, et al. 2022) and is well-suited for laboratory experiments due to its small size, ease of culturing, and well-defined ecological endpoints (Ebert 2022). Additionally, advanced 'omics' resources developed for *Daphnia* (Chaturvedi, X. Li, et al. 2023) facilitate the identification of biomolecular responses to chemical exposures and enable cross-species extrapolation of conserved biomolecular functions. This approach supports the early diagnosis of potential chemical hazards in ecosystems, based on the hypothesis that species similarities are shaped by shared evolutionary history, referred to as "toxicity by descent" (Colbourne et al. 2022). Sharing many advantages with *Daphnia*, such as ease of laboratory maintenance, low cost, small body size, high fecundity, and transparency at early developmental stages, *Danio* is a vertebrate surrogate species widely applied in ecotoxicology studies (Elizalde-Velázquez and Herrera-Vázquez 2023; Stegeman, Goldstone, and Hahn 2010; Choi et al. 2021). Beyond ecotoxicology, *Danio* is a prominent biomedical

model species used in genetics, developmental biology, and disease research. As primary producers in aquatic ecosystems, unicellular green algae, such as *Selenastrum capricornutum* (now reclassified as *Raphidocelis subcapitata*) and *Chlorella pyrenoidosa*, serve as bioindicators of water quality, helping to assess the effects of pollutants on ecosystem health. Algae-based toxicity assays are widely used to evaluate the impact of chemicals on photosynthesis, growth inhibition, and cellular stress responses, making them essential tools for assessing the ecological risks of chemical pollution in freshwater environments. In this review, an acute and chronic toxicity of MP and PFAS, both as single chemicals and in mixtures in the three model species are reported to quantify their short and long-term adverse effects. Acute exposure is defined based on standard regulatory tests, and it is used to define effect concentration or EC50, that is, the concentration of a given chemical that kills or immobilises 50% of a test population. The test substance should be administered to at least five concentrations in a geometric series with a factor preferably not exceeding 2.2. Mortality or immobilisation is observed at different time points depending on the test organism, as explained below. The cumulative percentage mortality for each exposure period is plotted against the concentration on logarithmic probability. Acute toxicity tests in *Daphnia* follow the *Daphnia* Acute Immobilisation Test (OECD, 2004). Juvenile *Daphnia magna* (<24 hours old) are exposed to a given chemical for 48 hours in either static (no renewal) or semi-static (media renewed at 24 hours) conditions. Immobilisation is recorded at 24 and 48 hours, with *Daphnia* considered immobilised if they cannot swim within 15 seconds from the beginning of the observation. EC50 (concentration immobilising 50% of Daphnia) can be calculated from these tests. Acute toxicity tests in fish follow the Fish Acute Toxicity Test (OECD, 2019). In these tests, the fish are exposed to the test substance, preferably for a period of 96 hours. Mortalities are recorded at 24, 48, 72 and 96 hours, and the concentrations which kill 50% of the fish (LC50) are determined. Acute toxicity tests in unicellular algae follow the Algal Growth Inhibition Test (OECD, 2011).

This test assesses the impact of a substance on the growth of freshwater microalgae or cyanobacteria. Exponentially growing cultures are exposed to the test substance for 72 hours under nutrient-sufficient conditions with continuous fluorescent illumination. Growth inhibition is measured by comparing biomass reduction across at least five test concentrations, with three replicates per concentration, against control cultures. Chronic toxicity exposures assess the sub-lethal effects of a test substance on ecological endpoints, such as growth inhibition, reproductive impairment, developmental effects, and biochemical or molecular changes. In regulatory ecotoxicology, chronic toxicity tests include:

- *Daphnia* Reproduction Test (OECD 211), in which *Daphnia magna* are exposed until they have released at least two broods. In these tests, survival, growth and reproductive output are typically measured.
- Fish Early Life-Stage Test (OECD 210), in which fish embryos and larvae (e.g., *Danio rerio*) are exposed over 28-60 days, and survival, growth, and development are measured.
- Algal Growth Inhibition Test (OECD 201), in which microalgae are exposed for 72 hours, and their growth inhibition is measured through biomass reduction over time. In addition to acute and chronic toxicity of MP and PFAS, we also review literature on short-term exposures. These refer to tests that extend beyond the typical acute toxicity phase but do not encompass the full life cycle of the organisms. Whereas the toxicity of MP and PFAS as individual stressors has been investigated on freshwater model organisms, the toxic effect of mixtures of these pollutants is poorly understood despite their ubiquitous presence in freshwater ecosystems worldwide (Islam et al. 2021; Rainieri et al. 2018; Parashar, Mahanty, and Hait 2023; F. Wang, Shih, and X. Y. Li 2015). Given the persistence and widespread presence of MP and PFAS, there is an urgent need for a comprehensive evaluation of their combined toxic effects on

aquatic organisms. Our systematic review provides a critical assessment of the past 25 years of research on the toxic effects of PFAS and MP on freshwater organisms. By synthesising existing findings, this review identifies key knowledge gaps in understanding the combined impacts of these pollutants and their potential ecological consequences. Furthermore, it outlines future research priorities needed to improve our understanding of the impact of chemical mixtures in freshwater ecosystems.

2.2.1 Methods

Research Strategy

The current review was conducted following a systematic framework (Pautasso 2013). The literature search was performed using the databases of Web of Knowledge and PubMed. The following keywords and keyword combinations were used: "PFOS AND PFOA AND Microplastic* AND (Daphnia OR Zebrafish OR Algae)", "PFOS AND PFOA AND Microplastic* AND toxic* AND (Daphnia OR Zebrafish OR Algae)", "PFAS AND Microplastic* AND toxic* AND (Daphnia OR Zebrafish OR Algae)", "PFCs AND Microplastic* AND toxic* AND (Daphnia OR Zebrafish OR Algae)", "PFOS AND Microplastic*AND (Daphnia OR Zebrafish OR Algae)", "PFOA AND Microplastic* AND (Daphnia OR Zebrafish OR Algae)", "PFOS AND PFOA AND Microplastic* AND (Daphnia OR Zebrafish OR Algae)", "PFOS AND PFOA AND (Daphnia OR Zebrafish OR Algae)", "PFOS AND PFOA AND Toxic* AND (Daphnia OR Zebrafish OR Algae)", "Microplastics* AND (Daphnia OR Zebrafish OR Algae)", "Microplastics AND toxic* AND (Daphnia OR Zebrafish OR Algae)", "PFC* AND toxic* AND (Daphnia OR Zebrafish OR Algae)", "PFAS AND toxic* AND (Daphnia OR Zebrafish OR Algae)", "PFOS AND PFOA AND Mi-

croplastics AND toxic*", "Microplastics AND toxic* AND (Daphnia OR Zebrafish OR Algae)".

The use of PFAS, PFCs, PFOS, and PFOA as alternative search terms is aimed at capturing as many relevant studies as possible because early studies may not have used the term PFAS. The search covered the period from 1st January 2000 to 6th June 2024 (last 25 years). A total of 4,221 studies were identified from both databases, 2,466 from Web of Knowledge and 1,755 from PubMed. After cross-referencing the two databases, a total of 2,920 studies were retained (Fig 2.2).

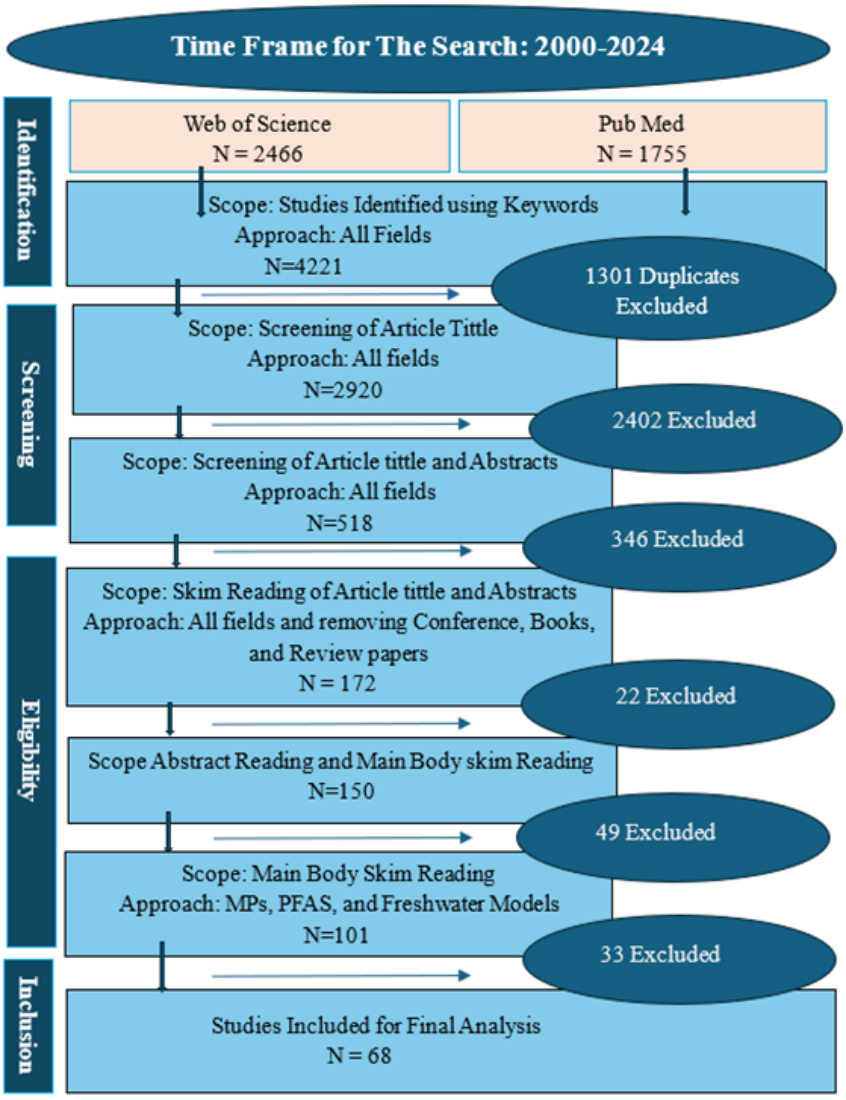


Figure 2.2: The workflow for the systematic search and selection process.

2.2.2 Selection Process

Titles and abstracts in papers written in English language were assessed. Only peer-reviewed papers and scientific reports from International Organisations (e.g., US EPA, WHO) were selected for this review to minimise selection bias. Studies that explored the toxicity of MP, PFOS, PFOA, and other PFAS, both as individual chemicals or in mixtures on the model organisms identified above (waterflea, zebrafish, and green algae), were included in the review. All studies that explored the toxic effects of MP or "MP AND MPACs" on *Daphnia, Danio* or green algae were included. Finally, all studies that investigated the combined effects of MP, MPACs and/or PFAS (as individual chemicals or mixtures) on the ecotoxicology of these species were included. Conference papers, book chapters, and non-peer-reviewed preprints were excluded. Studies exploring the toxicity of MP or PFAS on animal models other than freshwater models (e.g., rats or mice) were also excluded. Applying the inclusion and exclusion criteria, a total of 68 articles were retained and critically analysed (Fig. 2.2).

2.2.3 Research Trends and Statistic

Seven studies were published on MP, PFAS or their mixture toxicity on the 3 model species between 2000 and 2014, whereas 61 studies were published between 2015 and 2024 (Fig 2.3). This suggests growing scientific interest and societal concern over emerging contaminants in the past decade, leading to an upward trend in studies of MP and PFAS in general and freshwater species in particular. *Daphnia magna* appeared to be the most studied model species (38%), followed by *Danio rerio* (35%), and unicellular green algae (e.g., *Pseudokirchneriella subcapitata*) (22%) (Fig. 2.4a). Studies including *D. magna* and green algae together represented 3% of the total

and studies including *D. magna* and *D. rerio* together were 2% of the total. In terms of contaminant studied, MP appeared in 46% of the reviewed studies, followed by the combined toxicity of PFOS+PFOA (22%) (Fig. 2.4b). Overall, the observed research trends indicate that while sufficient studies may explore the toxic effect of various MP on freshwater models, little is known about the combined toxicity of different PFAS and MP+PFAS mixtures. Interestingly, only one study looked at the combined effect of MP+PFOS+PFOA on *Daphnia*.

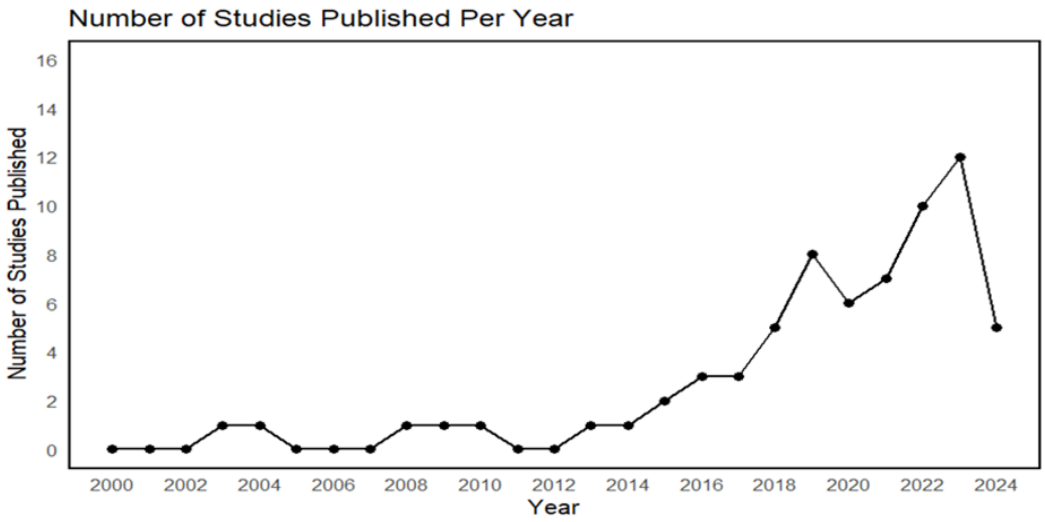


Figure 2.3: Number of studies published per year on MP, PFAS, and/or their mixture on *Daphnia, Danio*, and unicellular green algae.

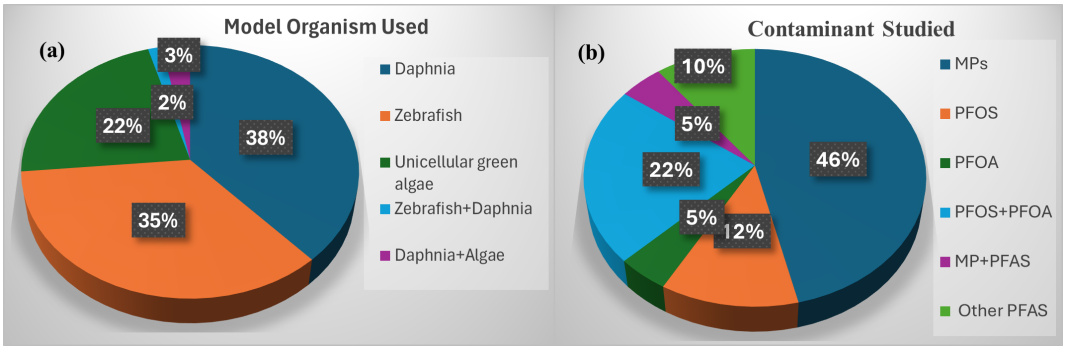


Figure 2.4: Percentage of total publications studied classified according to (a) model organism used, and (b) contaminant studied.

2.2.4 Toxicity of Microplastics Waterflea, Zebrafish and Green Algae

In the last 25 years, studies on the impact of MP on freshwater organisms were the most common (Malafaia et al. 2020; Medriano and Bae 2022; Pencik et al. 2023). Different MP polymers, concentrations, shapes, and sizes were explored for their potential short and long-term toxicological impacts. 12 acute and 5 short-term toxicity studies, 6 chronic studies were recovered and 4 studies in which both acute and chronic toxicity were investigated. These studies revealed various toxic effects (Table 2.1).

Acute Exposures to MP

Out of 12 acute toxicity studies, 11 investigated MP alone. One paper studied combined effects of MP and the biocide Triphenyltin chloride (TPTCl), (Yi et al. 2019). Even high concentrations of MP rarely caused immobilisation or mortality in the three species included in the review. For example, Kokalj et al. 2019) found no toxic effects of MP on *Daphnia* mobility when exposed to PE-MP (PE ≈30 μm and Aged-MP 4–12 μm at 100 mg/L concentration) and zebrafish embryos development at 96 h exposure. Also, Eltemsah and Bohn 2019 reported that PS beads of 6 μm size did not cause immobilisation or mortality on *Daphnia* following 48h exposure at a concentration range of 5-300 mg/L but caused mortality under short-term exposure (120h). This may show concentration-dependent effects (Eltemsah and Bohn 2019). Whereas immobilisation and/or mortality were rarely observed in *Daphnia* and *Danio* following acute exposures to MP, other metabolic and cellular responses have been reported. Medriano and Bae 2022 reported immune system disorders, gut microbiome dysbiosis, metabolic dysregulation, and altered fatty and

amino acids metabolism in zebrafish following acute exposure (96h) to PE fragments and Polyether sulfone (PES) fibres (<500 µm). Metabolic and altered cellular functions (altered antioxidant enzyme's function and photosynthesis disorder) were also reported in acute exposures (96h) of green algae exposed to 250 mg/L of MP particles <100 μm (polymer type not specified) (A. M. Nasser et al. 2022). Similar effects of altered cellular functions and structural damage (vacuolation, distortion of membrane structures, and plasmolysis in the cell) were confirmed in a study on acute green algae toxicity following exposure to regular-shaped PS beads (0.55 and 5.0 μm) at a concentration of 5 mg/L (Yi et al. 2019). The review revealed that acute toxic effects of MP were highly dependent on their size and shape. A study by Mizukami-Murata et al. 2021 compared the toxic effects of MP with different shapes and sizes on green algae. The researchers examined irregularly shaped MP, including nylon particles (Ny6: 15–20 μm; Ny12: 25–30 μm), and MP composed of various polymers such as low-density polyethylene (LDPE), polyethylene terephthalate (PET), polystyrene (PS), and ultra-high molecular weight polyethylene (UHPE), all smaller than 300 μm at a concentration of 750 mg/L. Additionally, they tested larger, regularly shaped granule MP, including nylon (Ny6-G) and polypropylene (PP), averaging 3000 μm in size and tested at a concentration of 7500 mg/L. The results demonstrated that large, regularly shaped MP (3000 μm) did not inhibit algal growth, even at high concentrations (7500 mg/L). Moreover, MP of different polymer types (PET, PS, and UHPE) with sizes smaller than 300 μm showed no acute toxicity to algae at concentrations up to 750 mg/L. However, the smallest irregular MP (Ny6, 15–20 μm) exhibited toxic effects, highlighting the size-dependent nature of MP toxicity. This size dependency is primarily attributed to differences in surface area, interaction mechanisms, and bioavailability. Small MP pose a greater risk because many algal species (e.g., *Chlorella, Scenedesmus*) absorb nutrients and particles through endocytosis or cell wall adhesion. In contrast, larger MP cannot penetrate the cell wall and are therefore less toxic. Additionally, small MP remain

suspended in the water column longer, increasing their exposure time to algae. Their greater surface area enhances their ability to attach to algal cell membranes, leading to oxidative stress and disruptions of key cellular processes such as macromolecule transport and photosynthesis. The shape of MP also plays a crucial role in their toxicity. Studies have shown that fibrous MP tend to be more harmful than granular MP. Y. Cheng et al. 2021 reported that PET fibres caused greater toxicity to zebrafish embryos compared to MP granules. Similarly, research by Jemec et al. 2016 found that PET fibres were 20–40% more toxic than granules in *Daphnia*. However, the observed mortality in *Daphnia* was likely due to starvation rather than direct MP toxicity, as the effect disappeared when the organisms were fed prior to MP exposure. While most studies focus on MP size and shape, only one study has explicitly examined the influence of MP concentration in combination with these factors. Miloloža et al. 2021 tested five different irregularly shaped MP (PP, PS, PET, PE, and PVC) at varying concentrations (10–1000 mg/L) and size ranges (100–700 μm) on unicellular green algae over a 3-day acute exposure period. The results showed that all tested MP inhibited algal growth, with the highest inhibition rates observed at the highest concentration (1000 mg/L). Furthermore, toxicity increased as MP particle size decreased, reinforcing the size-dependent nature of MP toxicity. The study also found that the rate of algal growth inhibition varied depending on MP polymer type, with toxicity following this order: PVC > PET > PS > PE > PP. This suggests that while size and shape are primary determinants of toxicity, polymer type may also play a role in influencing toxicity levels. The current review of the literature confirms that MP toxicity is primarily size- and shape-dependent, with smaller and irregularly shaped MP posing the greatest risk to algae. While polymer composition and concentration have some influence, their effects appear to be secondary to size and shape.

Short-Term Exposures to MP

Short-term studies highlight the potential cumulative effects of microplastics (MP). While acute toxicity is not associated with MP in *Daphnia*, short-term exposures could induce mortality, with PC, PET, and PBT exhibiting EC50 values of 2.6 mg/L, 4.7 mg/L, and >100 mg/L, respectively, suggesting that polymer chemical structures influence toxicity (Sönmez, Ercan, and Sivri 2022). In *Danio*, short-term toxic effects, including damage to intestinal mucosal cells, were observed following exposure to irregularly shaped PE (1–1000 μg/mL) for seven days (Y. Yuan et al. 2023). Another short-term exposure study (10 days) on zebrafish exposed to irregular MP of PE, PA, PVC, PP, and PS ($\approx$ 70 μm, 0.001-10.0 mg/L) reported significant mortality, with 59 fish dying in the following order: PP (n = 26), PVC (n = 25), PE (n = 5), and PA (n = 3) compared to the control. These MP caused intestinal damage, inhibited survival and reproduction rates, reduced body length, and induced oxidative stress (Lei et al. 2018). Zebrafish exposed to irregular PE (38.26 μm $\pm$15.64 μm) at concentrations of 6.2, 12.5, 25, 50, and 100 mg/L for 144h exhibited physiological and life-history effects, including low larval survival rates and significant changes in morphometric parameters (Malafaia et al. 2020). A study on green algae (*C. reinhardtii, C. vulgaris)* and cyanobacteria exposure to irregular PET (3 and 7 μm, 5–80 mg/L) for 120h resulted in reduced overall fitness, including 24% growth inhibition in *C. reinhardtii*, altered chlorophyll content, and cellular damage in all three organisms. Cryo-SEM analysis revealed cell wall disruption and shrivelling, though the cyanobacterium was the least affected (Pencik et al. 2023).

Chronic and Long-Term Exposures to MP

Chronic exposure to MP has been shown to impact the three species used in this review with effects dependent on particle size, polymer type, concentration, and ex-

posure length. Chronic exposure to small PS beads (6 µm, 5–300 mg/L) in *Daphnia* resulted in delayed maturity, impaired reproduction, reduced growth, and increased mortality, with effects intensifying at higher concentrations and longer exposures (Eltemsah and Bohn 2019). Similarly, PP (10–1000 mg/L) inhibited algal growth in a dose-dependent manner (Milošža et al. 2021). However, MP toxicity is not necessarily concentration-dependent, as mortality and reproductive defects in *Daphnia* exposed to PS beads (2 µm, 146 mg/L, 21 days) could be explained by food availability rather than MP exposure (Aljaibachi and Callaghan 2018). This is because MP can impair feeding, indirectly affecting long-term fitness. Extended exposure beyond the traditional chronic exposure time amplifies toxic effects. In green algae, PS (0.1–1 µm, 10–100 mg/L, 66 days) inhibited growth in a time-dependent manner (Leng et al. 2024). A 77-day exposure of *Daphnia* to PS (5–7 µm, 5–300 mg/L) resulted in an 80% reduction in neonate production after 45 days (Eltemsah and Bohn 2019). Polymer type and leachates also play a critical role in toxic responses. *Daphnia* exposed to Polylactic Acid (PLA) and PET (1–80 µm, 5 mg/L, 21 days) exhibited higher mortality in PLA (52.4%) than PET (85.7%) exposures, likely due to oxidative stress from PLA degradation (D. An et al. 2021). Similarly, green algae exposed to PP and PVC (5–500 mg/L, 11+ days) showed greater photosynthetic inhibition from PVC, potentially due to its high chlorine content (Y. Wu et al. 2019). MP mixtures in realistic exposure scenarios further highlight polymer-specific toxicity. Zebrafish exposed to a mixture of PET, PE, PS, and PP MP (5–28 days) exhibited neurotoxicity and behavioural changes at higher concentrations despite no acute effects at environmentally relevant doses (Yuling Chen et al. 2023). Several chronic exposure studies have assessed the effects of MP mixtures containing different polymer types to better reflect real-world exposure scenarios. Yuling Chen et al. 2023 examined both short-term (5 days) exposure in zebrafish embryos and long-term (28 days) exposure in larvae using a mixture of irregular PET, PE, PS, and PP MP. While acute exposure (102 particles/L) had no significant impact on embryo

development or behaviour, higher concentrations revealed polymer-specific effects: PET inhibited locomotion at 103 particles/L, whereas PP increased locomotion at 106 particles/L. Chronic exposure resulted in behavioural changes and neurotoxicity in larvae, with no significant differences between polymer types. Other studies report MP-induced metabolic disruption, mitochondrial dysfunction, and inflammation in zebrafish (Boopathi et al. 2023; Félix, Carreira, and Peixoto 2023). Chronic MP exposure in green algae has been linked to reduced chlorophyll content, photosynthetic inhibition, and growth suppression (H. Wang et al. 2023; Pencik et al. 2023). We identified a number of studies looking at the chronic toxicity of MP under prolonged exposure conditions. Malafaia et al. 2020 observed altered locomotor behaviour and reduced larval survival in zebrafish following 144h exposure to irregular PE (38 µm ± 16 µm, 6–100 mg/L). The study also highlighted the influence of exposure conditions (static vs. semi-static) on toxicity outcomes. Boopathi et al. 2023 reported severe toxic effects in zebrafish adults and larvae after 21-day exposure to irregular PE (<100 µm, 0.1 mg/L), including impaired locomotion, disrupted lipid metabolism, and inflammatory damage such as edema and necrosis. Similarly, Y. Yuan et al. 2023 exposed zebrafish to irregular MP (1–1000 mg/L, 7 days), and observed disruption in intestinal microbiota but no mortality, even at high concentrations. Félix, Carreira, and Peixoto 2023 examined cellular toxicity in zebrafish following 21-day exposure to irregular PP (<200 µm, 0.1–1 mg/L), identifying mitochondrial dysfunction, reduced membrane potential, and increased oxidative stress markers (superoxide dismutase and catalase). In green algae, chronic MP exposure led to photosynthetic inhibition, reduced chlorophyll content, and growth suppression, as reported by H. Wang et al. 2023 and Pencik et al. 2023.

These studies underscore the significant long-term impact of MP on aquatic organisms, with effects varying based on polymer type, particle size, and exposure conditions. Overall, our literature review revealed that chronic and long-term ex-

No.	Organism	Contaminant characteristics			Concentration	Mode (duration)	Endpoint	Observed effects	Reference
		Polymer*	Shape	Size					
1	Daphnia	PS	Beads	5-7 µm	5 – 300 mg/L	Acute (48 h), Chronic (5-77 days)	Physiologic/life-history traits and Metabolic	MP is not toxic to D. magna within 48 h but caused more mortality within 120 h and significant reduction of neonates after 45 days.	(Eltemsah and Bohn, 2019)
2	Daphnia	PLA and PET	Pellets	Each:1–80 µm	1 and 5 mg/L	Chronic (21 days)	Physiologic/life-history traits	At 5 mg/L PLA, 52.4 % of the Daphnia survived with deformed embryo and decreased offsprings. while 85.7% of the Daphnids survived the exposure to PET. Mean the PLA caused more mortality than PET under the same conditions.	(An et al., 2024)
3	Daphnia	PP	Irregular	11.86 to 44.62 µm	0 mg/L, 12.5 mg/L, 25 mg/L, 50 mg/L and 100 mg/L	Acute (48 h)	Physiologic/life-history traits and metabolic	Daphnia were Immobilized and caused metabolic dysregulation which caused generation of free radicals and decreased in the activity of neurotransmitter enzyme acetylcholinesterase (AChE).	(Jeyavani et al., 2023)
4	Daphnia (D. magna, Cladocera dubia, and Daphnia Pulex)	(Primary MP) =PMP and Secondary MP) =SMP	Irregular	PMP:1-5 µm SMP:1–10 µm)	103, 104, 105, 106, 107 particles/mL of either PMP or SMP	Short-term (72 or 96 h)	Physiologic/life-history traits and Metabolic	The time-dependent survival effects were recorded which became substantially more severe after the 48 h. SMP exerted more toxicity than the PMP	(Jaikumar et al., 2018)
5	Daphnia	PS	Regular	2 µm	1.46 × 102 mg/L	Chronic (21 days)	Physiologic/life-history traits	mortality after seven days of exposure in all treatments compared to the control	(Aljaibachi and Callaghan, 2018)
6	Daphnia	PC, PET, PBT	PC, fibre PET and PBT powder	PET=0.5 – 30µm, PBT=1 – 40µm, PC=2 –40µm	5, 10, 20, 50 and 100 mg/L	Short-term (72 h) and Chronic (21 days)	Physiologic/life-history traits and metabolic	The acute toxicity responses observed on Daphnia in terms of immobilization and death were at low level and increases over the chronic exposure. Also, slightly polymer type dependent (PC> PET> PBT)	(Sönmez et al., 2022)
7	Zebrafish	PE and PS	PE: fragments PES: fibre	<500 µm	1 mg/L each	Acute (96 h)	Metabolic	Both caused Immune disorder, biota dysbiosis, metabolomic dysregulation, altered fatty and amino acids metabolism.	(Medriano and Bae, 2022)
8	Zebrafish	PE	Irregular shape	Micro size (unspecified)	1-1000 ug/ml	Short-term (7 days)	Physiologic/life-history traits and Cellular	Damaged intestinal mucosal cells	(Yuan et al., 2023)
9	Zebrafish	PE	pellets	Size ranges (10–600 µm)	2 mg/L	Acute (96 h)	Metabolic and Physiologic/life-history traits	Seizures, significant upregulation, and tail bent downward.	(Mak et al., 2019)

10	Zebrafish	PE and Aged MPs	PE=regular Aged=Irregular	PE~ 30 µm Aged:4–12 µm	100 mg/L	Acute (96 h)	Behavioural (for Zebrafish)	In WWTP, the hatching of Zebrafish embryos after 96 h were significantly decreased. While in River, no acute toxicity for both Daphnia and Zebrafish	(Kokalj et al., 2019)
11	Zebrafish	PVC, PE, and PET	Fragments	~ 200 µm for each	0, 125, 250 and 500 mg/L each	Acute (72 h)	PVC: Metabolic. While HDPE and PET caused no effects	PVC increased expression of metallothionein 2 (mt2). While, HDPE and PET caused no changes in expression of any biomarkers	(Boyle et al., 2020)
12	Zebrafish	PE	Irregular	below 100 µm	0.1 mg/L MP + High fat Diet	Chronic (21 days)	Behavioural and cellular	Locomotor behaviour in adult Zebrafish and their larvae with inflammatory-associated damage such as hepatocytes, Edema, and necrosis. Also Impaired lipid metabolism.	(Boopathi et al., 2023)
13	Zebrafish	PE	Irregular	38.26 µm ± 1 5.64 µm	6.2, 12.5, 25, 50 and 100 mg/L	Short-term (144 h)	Behavioural, Physiologic/life history traits and metabolic	Caused low larval survival rate after egg hatching. Induced significant changes in morphometric parameters of Zebrafish.	(Malafaia et al., 2020)
14	Zebrafish	PP	Irregular	200 µm below	0.1 and 1 mg/L.	Chronic (21 days)	Behavioural, cellular, and metabolic	Anxiety-like behaviour. Deficiency in liver mitochondrial respiration and decreased mitochondrial membrane potential. Increase in hepatic superoxide dismutase and catalase	(Félix et al., 2023)
15	Zebrafish	PE, PA, PVC, PP and PS	Irregular	~ 70 µm	0.001–10.0 mg/L	Short-term (10days)	Cellular and metabolic	After 10 days exposure, 59 fishes died: PP (n = 26), PVC (n = 25), PA (n = 3), and PE (n = 5) Compared to the control. And they all caused. Damage of Zebrafish intestine, inhibited the rate of survival, reproduction, body length, and caused oxidative stress	(Lei et al., 2018)
16	Zebrafish	PE, PP, PS, PVC, PET, PMMA, PSAN	Mixed	2 mm max.	1-10 µg/mL	Acute (96 h)	Physiologic/Life-history traits	Zebrafish morphometric changes(cardiac)	(Moreno and Cooper, 2021)
17	Zebrafish	multiple polymers (PET, PE, PS, PP)	Irregular	52 to 74 µm	102,104 and 106 particles/L	Short-term (h (44 h) and Chronic (28-day)	Physiologic/life-history traits	Daily mortality of embryos occurred, sublethal and lethal effects of on Zebrafish embryo development at different exposure concentrations	(Chen et al., 2023)
18	Zebrafish	PET	Granules and Fibres	~3–5 mm	PET granules 20 mg/L and PET fibre 20 mg/L	Acute (96 h)	Physiologic/life-history traits	PET fibres or granules inhibited the embryonic development of Zebrafish.	(Cheng et al., 2021)
19	Daphnia	PET	Fibres	Length: 62–1400 µm, width: 31–528 µm	12.5-100 mg/L	Acute 48 h	Physiologic/life-history traits	Daphnia mortality was 20-40% range at exposure doses from 12.5 to 100 mg/L	(Jemec et al., 2016)
20	Green algae	MP particles from River	Regular	100 µm below	10, 20, 40, 60, 80, 100, 200, and 250 mg/L	Short-term (96 h)	Cellular and Metabolic	Caused photosynthesis disorder and affected antioxidant enzyme's function	(Nasser et al., 2022)
21	Green algae	PS particles and triphenyltin chloride (TPTCl)	Regular	0.55 and 5.0 µm	PS = 5 mg/L and TPTCl = 30.64 µg/L	Short-term (96 h)	Physiologic/life-history traits and Metabolic	96 h IC_{50} was observed at 9 mg/L for 0.55 µm PS. no toxicity observed for 5.0 µm.	(Yi et al., 2019)

								Damage to cell structure and affected the photosynthesis.	
22	Green Algae	PE, PP, PVC, PS, and PET	Irregular	100–700 µm	10–1000 mg/L	Acute (3 days)	Physiologic/life-history traits and metabolic	Growth inhibition mostly by PVC. PVC>PET>PS>PE>PP	(Miloloza et al., 2021)
23	Green algae	PP and PVC	Regular	PP: 64, 172, 236 µm PVC:111, 157, 216 µm	5–500 mg/L	Chronic (21 days)	Physiologic/life-history traits and Cellular	Reduce chlorophyl content of the algae and inhibited photosynthesis. PVC is more toxic than the PP in terms of the above effect	(Wu et al., 2019)
24	Green algae	LDPE, PET, PS Ny6-P, Ny12, UHPE, Ny6-G and PP	LDPE, PP, Ny6, PET, Ny12, PS, UHPE	Ny6 and Ny12=50 µm Other MPs <300 µm	6.25, 12.5, 25, and 50 mg/L for 72 h	Acute (72 h)	Physiologic/life-history traits	Ny6-P caused greater inhibitory effect on the algal growth, followed by Ny12, LDPE, PS, UHPE, and PET.	(Mizukami-Murata et al., 2021)
25	Green algae	PS and PE	Regular	30-38 µm below	PS = 1000 mg/L PE = 0, 1, 10, 100, 500, 1000, 5000 and 10,000 mg/L	Chronic (11 days)	Physiologic/life-history traits and metabolic	PE: (High concentration has decreased TSP content and the efficiency of photosynthesis PS: Decreased the chlorophyll pigment content in *Chlorella vulgaris* PS: Inhibited colony formation compared with PE The smaller sizes of PE and PS have inhibited green algae colony formation	(Wang et al., 2023b)
26	Green algae	PS	Regular	0.1, 0.5, and 1 µm)	10,50, and 100 mg/L	Acute and chronic (72 h to 66 days)	Physiologic/life-history traits and metabolic and Cellular	The inhibitory effect on algal growth increases with increasing size of MPs: 0.1 µm caused highest inhibitory effect on algal growth (68.42%), followed by 0.5 µm then least effect by 1 µm	(Leng et al., 2024)
27	Green algae (C. reinhardtii, C. vulgaris)	PET	Irregular	3 and 7 µm	5 to 80 mg/L	Short-term (120 h)	Physiologic/life-history traits and metabolic and Cellular	Growth inhibition in C. reinhardtii (− 24%), altered chlorophyll in C. vulgaris and C. reinhardtii. Caused damage to cell in all three organisms by CRYO-SEM (shriveling, cell wall disruption), but the cyanobacterium was the least damaged.	(Pencik et al., 2023)

Table 2.1: Toxicity of Microplastics and Associated Chemicals on *Daphnia*, *Danio* and Green Algae.

* PC = Polycarbonate, PET = polyethylene terephthalate, PBT = polybutylene terephthalate, PP = polypropylene, FPP = Flexible Polypropylene PS = polystyrene, HDPE = High-Density Polyethylene, LDPE = Low- Density Polyethylene, MDPE = Medium-Density Polyethylene, PVC = polyvinylchloride, PU = Polyurethane, PMMA = Polymethyl Methacrylate (Acrylic), PSAN: PSAN = Polystyrene Acrylonitrile, FPE = Flexible Polyethylene, SPA =Styrene polymers and elastomers, Ny6-P = Nylon 6 Polymer), Ny12 =(Nylon 12, UHPE = Ultra High Polyethylene, Ny6-G = Nylon 6 Cast, PLA = Polylactic acid.

posure to MP causes toxicity in aquatic organisms and it is influenced by exposure duration, polymer type, particle shape, and concentration. Only four studies examined both acute and chronic MP exposure: *Daphnia* (Eltemsah and Bohn 2019; Sönmez, Ercan, and Sivri 2022), zebrafish (Yuling Chen et al. 2023), and green algae (Leng et al. 2024). Acute exposure (48–72h) in *Daphnia* showed minimal toxicity, whereas chronic exposure (21–77 days) led to increased immobilisation, mortality, and reduced neonate production (Eltemsah and Bohn 2019). Similarly, zebrafish exhibited no short-term effects (5 days) at environmentally relevant MP concentrations, but chronic exposure (28 days) induced behavioural changes and neurotoxicity (Yuling Chen et al. 2023). In green algae, growth inhibition intensified with prolonged exposure, increasing from 72h to 66 days (Leng et al. 2024). These findings emphasise the importance of chronic MP exposure studies, ideally alongside acute assessments, to fully understand their long-term ecological risks.

2.2.5 Combined Toxicity of Microplastics and Microplastic-associated Chemicals (MPACs) on Waterflea, Zebrafish and Green Algae

The toxicological effects of MP on aquatic organisms are not solely linked to the polymeric composition of MP. They also result from the presence of microplastic-associated chemicals (MPACs), chemical additives that are incorporated during plastic manufacturing to enhance performance (Abihssira-Garcia et al. 2022). These additives, such as flame retardants, per- and polyfluoroalkyl substances (PFAS), and UV stabilisers, can leach from MP into surrounding water, posing additional toxicity risks (Wee and Aris 2023). Studies have suggested that MPACs, including phthalates, PFAS, and flame retardants, can be more hazardous than the MP themselves, contributing to greater toxicity when combined with MP (Thompson

and Pahl 2018; Rummel, Schäfer, et al. 2022). Furthermore, MP can act as vectors for toxic chemicals in freshwater environments, facilitating the transport and bioavailability of contaminants to aquatic organisms (J. Yuan et al. 2020). However, research on the combined effects of MP and MPAC is limited, despite its greater relevance to real-world exposure scenarios, as virgin plastic polymers are rarely detected in environmental samples (Amaneesh et al. 2022).

Studies on the combined toxicity of MP and MPACs in aquatic biota are scarce (Table 2.2). Five studies have examined these effects in *D. magna*, while only one study was on zebrafish. All studies consistently reported higher toxicity from MP + MPAC mixtures compared to MP alone. Renzi, Grazioli, and Blašković 2019 exposed *D. magna* to polyvinyl chloride (PVC), polypropylene (PP), and polyethylene (PE) MP, alone or in combination with the surfactant Triton X-100 (0.05 g/L) for 96h. The results showed that PVC + surfactant caused the highest immobilisation and mortality, followed by PE + surfactant, compared to either MP or surfactant alone. Similarly, G. An et al. 2024 investigated the effects of PE fragments and beads, either alone or in combination with Benzophenone-3 (BP-3, a UV stabiliser), on *Daphnia* for 48h. The study found that PE fragments were 80 times more toxic than PE beads, likely due to their larger surface area and irregular shape. Moreover, the EC50 for the PE + BP-3 mixture was lower than that of MP alone, and the combination caused a synergistic increase in oxidative stress markers, including total antioxidant capacity, reactive oxygen species (ROS), and lipid peroxidation, attributed to the bioaccumulation of BP-3 in *Daphnia*.

Rehse, Kloas, and C. Zarfl 2018 found a different toxicity pattern when *Daphnia* were exposed to a mixture of polyamide MP (PA-MP) and the plasticizer Bisphenol A (BPA). While the PA + BPA mixture caused reduced mobility, BPA alone induced the highest immobilisation, suggesting that PA-MP adsorbed BPA, reducing its bioavailability and subsequent toxicity under test conditions. Tarasco et al. 2022

No	Organism	MPs characteristics			MPACS**	Concentration	Mode	Endpoint	Observed effects	Reference
		Polymer*	Shape	size			(duration)			
1	*Daphnia magna*	PVC, PP, PE	Irregular	10-100 µm	Triton x-100 (surfactant)	0.05 g/L	Acute (96h)	Physiologic/life history traits	PVC+Triton caused the highest immobilization and mortality, followed by PE+Triton, then Triton alone then PE. PE and PP were more toxic than PVC when each is alone	(Renzi et al., 2019)
2	*Daphnia magna*	PUR, PVC, PLA	Irregular	≤59 µm)	Kaolin (filler)	500 mg/L, 100 mg/L, 50 mg/L and 10 mg/L	Chronic (21 days)	Physiologic/life history traits	All MPs affected daphnia life-history, but PVC caused the highest impact on reproduction (neonates from 117 to 25/Daphnids), PLA caused the highest effect on survival, kaolin alone was less toxic compared to MPs	(Zimmermann et al., 2020)
3	*Daphnia magna*	PE	fragments and beads	Fragments (25-50µm). Beads (40-48µm)	BP-3 (UV stabilizer)	Fragments 20 mg/L; Beads 500 mg/L; BP-3 20 mg/L	Acute (48h)	Behavioral	EC50: Beads= 323.05, Fragment=3.90, PE+BP-3=0.99, while BP-3 alone=2.29 mg/L. Mixture of BP-3 + MPs posed more toxicity than MPs alone	(Na et al., 2021)
4	*Daphnia magna*	PVC	Irregular PVC	10-60 µm	DiNP (plasticizer)	PVC containing 30% DiNP	Chronic (31days)	Physiologic/life history traits	PVC+DiNP reduced neonate population and caused an increase in body length. PVC alone caused primiparity shift.	(Schrank et al., 2019)
5	*Daphnia magna*	PA	Irregular	15-20 µm	Bisphenol A (BPA)	PA = 200 mg/L; BPA= 5-15 mg/L	Acute (48h)	behavioral	BPA alone caused high immobilization while PA+BPA always caused reduced immobilization	(Rehse et al., 2018)
6	Zebrafish	PE	Beads	20–27 µm	Benzo[a]pyrene (BaP)	250 mg PE+50 mL BaP (1% w/w)	Chronic (30 days)	Metabolic	PE alone negatively affects skeletal development. PE+BaP impaired reproductive performance, reduced the breeding efficiency, growth and bone development. Skeletal deformities increase with the mixture of PE+BaP.	(Tarasco et al., 2022)

Table 2.2: Combined toxicity of MP and MPACs on *Daphnia* and zebrafish.

* PVC = polyvinylchloride, PU = Polyurethane, PP = polypropylene, PA = polyamide, PE = polyethylene PLA = Polylactic acid. ** BP-3 = benzophenone-3, DiNP = Diisononylphthalate.

investigated the effects of regularly shaped PE beads (20–27 μm) alone and in combination with Benzo[a]pyrene (BaP, a polycyclic aromatic hydrocarbon) on zebrafish for 30 days. Chronic exposure to PE MP alone impaired skeletal development, while the PE + BaP mixture further reduced reproductive performance, growth, and bone development. Also, the combination altered oxidative stress markers and upregulated genes associated with the BaP response pathway, suggesting heightened toxicity due to chemical interactions between MP and MPACs. Schrank et al. 2019 conducted a 31-day chronic exposure study on *D. magna* using PVC MP with diisononyl phthalate (DiNP). The result showed that PVC + DiNP reduced reproduction and increased body length, while PVC alone only affected primiparity. This suggests that DiNP caused metabolic and reproductive disruption.

2.2.6 Toxicity of PFAS on Waterfleas, Zebrafish, and Green Algae

The current review identified 35 studies on the toxicity of PFAS on the three model species studied (Table 2.3). Most studies focused on PFOS, PFOA and/or a combination of both (Figure 3b), which may be explained by the ubiquitous co-occurrence of these two PFAS in freshwater environments (Jeong, Yuk, et al. 2016; Liang et al., 2017; M. Abdullahi, J. Zhou, et al. 2022; Jeong and Simpson 2020). Different PFAS concentrations were explored for their potential short and long-term toxicological impacts. 8 acute, 11 short-term, and 11 chronic toxicity studies were recorded; acute and chronic toxicity were investigated together in 5 studies (Table 2.3).

Acute Exposures to PFOS

PFOS toxicity in aquatic organisms varied significantly between acute and chronic exposures, with short-term exposure primarily affecting metabolism and physiology,

while prolonged exposure led to mortality, reproductive impairment, and oxidative stress. Acute exposures to PFOS generally did not cause mortality in *Daphnia* but induced metabolic and physiological adverse effects. For instance, *Daphnia* exposed to 0–150 mg/L for 48h exhibited an EC50 for immobilisation at 79.35 mg/L, along with increased heart rate and disrupted antioxidant and neurological responses (R. Y. Liang et al. 2017). Acute (24h) exposure to 2 and 20 mg/L resulted in dysregulation of energy metabolism pathways, including adenosine, arginine, tyrosine, and monophosphate (Jeong and Simpson 2020). Similarly, acute exposure of zebrafish to PFOS exhibited physiological and behavioural alterations. Observed effects included ventroflexion of the tail, edema, failed swim bladder inflation (Gaballah et al. 2020), inhibited liver cell growth (Cui et al. 2015), and locomotion impairments (Hawkey et al. 2023). On a molecular level, exposure of zebrafish embryos and larvae (0.04–1 μM, 166h) resulted in motor inhibition, altered transcription of muscle and nervous system genes, and increased neurotransmitter levels (AChE and DA) (Xin Wang et al. 2022). Acute PFOS exposure affected unicellular green algae, leading to growth inhibition at 20 μg/L over 12 days, particularly under simulated heatwave conditions (Liao et al. 2024). Another study found up to 50% growth inhibition after 48h exposure to 6–100 mg/L (Boudreau et al. 2003). At the molecular level, green algae exposed to 40–200 mg/L PFOS (96h) exhibited a dose-dependent increase in reactive oxygen species (ROS), potentially leading to oxidative stress and cell damage (Xu, X. S. Chen, and Shao 2017).

Short-Term Toxicity of PFOS

Several studies have investigated the short-term toxicity of PFOS on the model species included in this review. In a study by Jeong, Yuk, et al. 2016, *D. magna* was exposed to PFOS concentrations ranging from 0.001 to 10 mg/L over a 10-day period. The results indicated significant adverse effects on reproduction and

growth at concentrations as low as 0.1 mg/L. Specifically, there was a reduction in the number of offspring and a decrease in body size, suggesting that even relatively low levels of PFOS can impair the fitness of *Daphnia* populations over short-term exposures. Xin Wang et al. 2022 conducted a study exposing zebrafish embryos and larvae to PFOS concentrations between 0.04 and 1 μM for 166 hours (approximately 7 days). The exposure led to inhibited motor behaviour in both larvae and embryos. Additionally, there was altered transcription of genes associated with the muscular and nervous systems, along with increased levels of neurotransmitters such as acetylcholinesterase (AChE) and dopamine (DA). These findings suggest that short-term PFOS exposure can disrupt neurological development and function in zebrafish. Xu, X. S. Chen, and Shao 2017 investigated the effects of PFOS on green algae over a 96-hour exposure period, with concentrations ranging from 40 to 200 mg/L. The study observed a concentration-dependent increase in reactive oxygen species (ROS) production, indicating oxidative stress. Elevated ROS levels can lead to cellular damage, impairing growth and photosynthetic efficiency. This suggests that short-term exposure to PFOS can have detrimental effects on algal health and productivity.

Chronic Toxicity of PFOS

Chronic PFOS exposure in *Daphnia* cause fitness-related adverse effects, including mortality, reduced body size, impaired reproduction, and metabolic disruption (Jeong, Yuk, et al. 2016; Seyoum et al. 2020; M. H. Li 2009; Logeshwaran et al. 2021; M. H. Li 2010; Boudreau et al. 2003; Sanderson et al. 2004. Importantly, whereas acute toxicity of PFOS is uncommon, chronic exposures to environmentally relevant concentrations of this PFAS in *Daphnia* showed a genotype-dependent reduction in fecundity, smaller size at maturity and mortality. In zebrafish, chronic exposure can lead to behavioural and neurotoxic effects. Larvae exposed to PFOS for 28 days dis-

played altered locomotion and neurotoxicity, with no significant short-term toxicity at environmentally relevant concentrations (Yuling Chen et al. 2023). Chronic exposure studies for unicellular algae suggest that longer exposures exacerbate PFOS toxicity. Growth inhibition was observed at 20 μg/L (12 days) and was more severe under thermal stress, which likely increases the bioavailability of PFOS (Liao et al. 2024). Additionally, prolonged PFOS exposure resulted in higher oxidative stress, disrupting cellular function and overall growth (Xu, X. S. Chen, and Shao 2017).

2.2.7 Toxicity of PFOA on Waterfleas, Zebrafish, and Green Algae

Studies on PFOA toxicity are less exhaustive than studies on PFOS. Overall, this review of the existing literature revealed that toxicity of PFOA varies across different species and exposure durations, with zebrafish showing developmental and transgenerational effects, while green algae exhibit species-specific growth inhibition and photosynthetic impairment.

Acute Toxicity of PFOA

Existing studies on the toxicity of PFOA in *Daphnia* have primarily been investigated in combination with PFOS. These studies are summarised in Table 2.3. Due to the combined exposure to PFOS, the specific acute, long-term, and chronic effects of PFOA alone in *Daphnia* remain unclear. Acute exposure of zebrafish to PFOA has been linked to larval mortality and developmental abnormalities. D. L. Wu et al. 2022 exposed zebrafish to 400 μM of PFOA for 96 hours, reporting significant inhibition of liver cell viability and transcriptional alterations in 1,055 genes, with 977 upregulated and 78 downregulated genes. Hawkey et al. 2023 also observed increased larval motility after exposing zebrafish larvae to PFOA concentrations ranging from

0.1 to 100 μM for 5 to 122 hours post-fertilization (hpf).

Short-Term Toxicity of PFOA

Short-term exposures to PFOA have been associated with developmental delays and reduced reproductive success. Zebrafish exposed to PFOA during early developmental stages exhibited delayed growth, reduced egg production, and decreased egg viability (Hawkey et al. 2023; D. L. Wu et al. 2022; Jantzen et al. 2017). Short-term exposures to PFOA have been shown to affect algal growth rates in a species-dependent manner. C. Hu, Q. Luo, and Q. Huang 2014 conducted an 8-day study on two algal strains exposed to PFOA concentrations between 10 and 40 mg/L. They observed significant growth inhibition in *Chlamydomonas reinhardtii*, whereas the effects on growth were mild in *Scenedesmus obliquus*. These findings suggest that different algal species exhibit varying levels of sensitivity to PFOA contamination.

Chronic Toxicity of PFOA

The developmental toxicity of PFOA in zebrafish has been observed across generations. Jantzen et al. 2017 demonstrated that exposure to PFOA during early development led to transgenerational effects, including delayed growth in subsequent generations, highlighting potential long-term ecological risks. Chronic effects of PFOA on algae are less well studied. However, the observed growth inhibition, photosynthetic impairment, and biomass reduction in short-term studies suggest long-term risks to algal populations under prolonged exposure. Given the foundational role of algae in aquatic ecosystems, such chronic impacts could have cascading effects on higher trophic levels.

2.3 Mixture toxicity of PFOA + PFOA on Waterfleas, Zebrafish, and Green Algae

The combined toxicity of PFOS and PFOA is not as commonly studied as the toxicity of the respective individual compounds. However, our systematic review revealed that the toxicity of PFOS + PFOA varies with exposure duration and species. Acute exposure affects mobility, cell viability, and metabolism, while short-term exposure leads to neurological and metabolic impairment. Chronic exposure significantly disrupts reproduction, genetic integrity, and neurobehavioral functions.

Acute Toxicity of PFOA+PFOS

Acute exposure to PFOS and PFOA has been shown to induce immobilisation in *Daphnia*. K. Ji et al. 2008 determined 48h EC50 values of 37 mg/L for PFOS and 477 mg/L for PFOA, confirming that PFOS is ≈10 times more toxic than PFOA. H. B. Yang et al. 2019 reported synergistic toxicity of PFOS + PFOA, with increased mortality under 48h exposure, attributed to their combined disruption of the antioxidant defence system. Acute exposure primarily affects zebrafish cell viability and metabolic function. Cui et al. 2015 reported that a PFOS (27.9 μg/mL) + PFOA (84.8 μg/mL) mixture inhibited liver cell growth within 48h, with PFOS being more toxic than PFOA.

Short-Term Toxicity of PFOA+PFOS

Short-term studies on the combined effects of PFOS and PFOA on *Daphnia* indicate non-lethal effects but significant metabolic and reproductive impacts.Seyoum

et al. 2020 exposed *Daphnia* to PFOS + PFOA mixtures (1, 10, and 25 μM) for 7 days, observing reduced growth, reproduction, and lipid metabolism. PFOS reduced *Daphnia's* body length, whereas PFOA did not, reinforcing the greater toxicity of PFOS. Short-term exposure to PFOS + PFOA in *Danio* impacts neurological function and lipid metabolism. Z. Y. Yang et al. 2023 reported dysregulated lipid metabolism in zebrafish embryos (25 μg/mL, 250 μg/mL, 2.5 mg/mL, 72 hpf), associated with oxidative stress and energy metabolism dysfunction. Whereas both PFOS and PFOA impacted embryonic survival, PFOA exhibited slightly greater toxicity, but the difference was not statistically significant.

Chronic Toxicity of PFOA+PFOS

Chronic exposure to PFOS and PFOA in *Daphnia* is larger than the toxic effects of PFOS and PFOA as individual compounds, leading to genetic damage, delayed reproduction, and reduced fecundity. Only one study in *Daphnia* has reported the combined effects of PFOS and PFOA, showing synergistic and additive effects of the two compounds on ecological endpoints. Logeshwaran et al. 2021 found that PFOS (1 and 10 mg/L) caused 23% mortality, whereas PFOA (10 mg/L) caused 12% mortality. Both chemicals delayed the first brood production and reduced fecundity. A study by Sanderson et al. 2004 further showed variability in toxicity across different experimental setups, highlighting the need for field validation of laboratory findings. Chronic exposure to PFOS and PFOA in *Danio* results in transgenerational and neurobehavioral effects. Haimbaugh et al. 2022 observed persistent behavioural changes in zebrafish across two generations, including altered responses to dark and light stimuli. Similarly, Hawkey et al. 2023 linked long-term PFOS + PFOA exposure to neurotoxicity and locomotion defects. There is limited research on PFOS + PFOA mixture toxicity in algae. Mojiri, Nazari Vishkaei, et al. 2023 exposed green algae to PFOS + PFOA ($\leq$ 10 mg/L) for 7 days, reporting significant reductions in

cell viability, protein content, and chlorophyll levels.

Mixture Toxicity of PFOA+PFOA+MP on Waterfleas, Zebrafish and Unicellular Algae

Y. Y. Li et al. 2021) provided an overview of the interactions and combined toxicity of microplastics and PFAS in aquatic environments, concluding that the co-occurrence of these pollutants could lead to synergistic toxic effects, posing a significant threat to aquatic organisms (Dai, J. Zhao, C. Sun, Diying Li, et al. 2022). Yet, no study has investigated the combined toxic effects of complex mixtures such as PFOS, PFOA, and MP on aquatic organisms. This research gap was addressed in Chapter 4 of this thesis for the first time. The chronic toxicity of PFOS, PFOA, and polyethylene terephthalate (PET) microplastics, both individually and in two-way and three-way mixtures, was investigated using *Daphnia manga* as a model organism. The combined exposure led to more severe effects on ecological endpoints than individual compounds and two-way mixtures of PFOS and PFOA, including reduced reproduction and delayed development. The study highlighted that *Daphnia* genotypes with different histories of chemical exposure exhibited varying sensitivities to these contaminants. This study was also the first to show that the combined effect of the three compounds analysed was 59% additive and 41% synergistic, whereas no antagonistic interactions were observed.

2.3.1 Toxicity of other PFAS

While most research has focused on the toxicity of PFOS and PFOA, a few studies have investigated the effects of other PFAS on *Daphnia*, *Danio*, and unicellular green algae (Table 2.3). These studies suggest that newer PFAS compounds exhibit similar

toxic effects, which can be exacerbated when combined with PFOS, PFOA, or other PFAS mixtures. Short-chain PFAS can influence the bioconcentration and toxicity of longer-chain PFAS. Q. R. Zhang et al. 2022 examined the effects of a mixture of PFOA, PFDA (perfluorodecanoic acid), and PFDoA (perfluorododecanoic acid) on *Daphnia* at 60 and 120 μg/L for 48h. The study found that the longer-chain PFDoA inhibited the bioconcentration of the shorter-chain PFOA and PFDA. Immobilisation effects were more severe at higher temperatures. Lu et al. 2015 investigated the combined effects of PFNA (perfluorononanoic acid) and PFOA at concentrations ranging from 0.008 to 5 mg/L under both acute (48h) and chronic (21-day) exposure conditions. The PFAS mixture inhibited *Daphnia* growth and reproduction in a concentration-dependent manner, with significant reductions in ingestion rates and enzymatic activity, including acetylcholinesterase (AChE), superoxide dismutase (SOD), and catalase (CAT). PFOA was found to be more toxic than PFNA. Labine et al. 2022 reported a similar metabolic disorder following 48h exposure to a mixture of traditional (PFOA, PFOA) and the short-chain PFAS GenX. The study found anabolic disruption in protein and energy metabolism, suggesting that newer PFAS can interfere with fundamental biological pathways in *Daphnia*. Gaballah et al. 2020 examined the toxicity of multiple PFAS (PFOA, PFHxS, PFHxA, PFOA, ADONA, and PFESA1) on zebrafish for 6 days. The exposure resulted in developmental defects, including failed swim bladder inflation, as well as neurotoxicity, with severity increasing with PFAS carbon chain length. Similarly, Albers et al. 2024 exposed zebrafish embryos to a PFAS mixture containing PFOA (528.6 ppm), PFHxS (14.3 ppm), and PFOA (2.1 ppm) for 120 hours post-fertilization (hpf). The study reported morphological deformities, altered embryonic activity, delayed startle responses, and decreased lipid levels, highlighting PFAS-induced disruptions in development and metabolism. The effects of PFAS on unicellular algae include growth inhibition, oxidative stress, and damage to photosynthetic machinery. W. Liu et al. 2018 examined the toxicity of chlorinated polyfluorinated ether sulfonate

(Cl-PFESA) on green algae at concentrations of 5–60 mg/L over 72 hours. The study determined an IC50 value of 40.3 mg/L, indicating significant growth inhibition. L. L. Zhang et al. 2023 investigated the toxicity of three PFAS mixtures: (PFBS + 6:2 FTS), (PFOA + 6:2 FTS), and (PFOA + PFBS), each at 100 mg/L, following 12-day exposure in *C. vulgaris*. The results showed chlorophyll reduction rates of 87.9%, 55.0%, and 89.7%, respectively, demonstrating severe impairment of photosynthetic capacity. Y. Y. Li et al. 2021 studied the toxicity of GenX (a PFOA substitute) under chronic exposure (12 days) and found that it physically damaged algal cells and downregulated photosynthetic genes, reinforcing concerns about the ecological impact of newer PFAS compounds.

2.3.2 Policies and Legislations to Control, or Mitigate these Problems

Countries worldwide are increasingly recognising the environmental and health hazards posed by MP and PFAS. They are implementing policies that focus on reducing or banning these harmful substances' production, use and disposal to limit their release into the environment and, ultimately, their impact on ecosystems and public health (Carlini and Kleine 2018). In the mid-2000s, after half a century of production and commercialisation of over 3,000 PFAS, some of these chemicals (including PFOA and PFOS) have been restricted or phased out at both European and global levels under the REACH Regulation and the United Nations Stockholm Convention on Persistent Organic Pollutants (POPs) list due to their persistency, bioaccumulation and carcinogenicity in the environment as non-biodegradable substances (Lim 2019). The European Commission, the US Ecological Insurance Organisation and the United States Environmental Protection Agency (US EPA) and numerous other public and global organisations have restricted the use of PFAS and MP and also

No.	Organism	Contaminant	Concentration	Mode (duration)	Endpoint	Observed effects	Reference
1	Daphnia	PFOS	1 - 10,000 µg/L	Chronic (25 days)	Physiologic/life-history traits and metabolic	GST and AChE as well as body weight, were significantly affected in subsequent generations. Individual fitness-related adverse effects were observed from F1 not in F0 and deteriorated as the generation number increased. (Reproduction was inhibited in F0 and was recovered in subsequent generations)	(Jeong et al., 2016)
2	Daphnia	PFOS	30 - 150 mg/L	Acute (48 h)	Physiologic/life-history traits	EC50 Immobilization occurred at 79.35 mg/L, Heartbeat was significantly stimulated, and reproductive parameters were affected significantly	(Liang et al., 2017)
3	Daphnia	PFOS	70 ng/L	Chronic (min 21 days)	Physiologic/life-history traits and metabolic	Negative effect on detoxification, catabolism, and endocrine pathways of Daphnia	(Abdullahi et al., 2022)
4	Daphnia	PFOS	2 and 20 mg/L	Acute (24 h) and Chronic (16 days)	Metabolic	Acute: Dysregulation of adenosine, arginine, tyrosine, and monophosphate (energy status). Chronic: Bioaccumulations of PFOS are more in younger D. magna and remained consistent from 72 h-16 days	(Jeong and Simpson, 2020)
5	Daphnia and green algae	PFOS	6-100 mg/L	Acute (48 h) and Chronic (21 days)	Physiologic/life-history traits	Acute: PFOS 48-h immobility NOEC was observed. Chronic: Daphnia mortality at 100 mg/L PFOS and 50% inhibition of growth of algae	(Boudreau et al., 2003)
6	Green algae	PFOS	20 µg/L	Chronic (12 days)	Physiologic/life-history traits	20 µg/L inhibited algal growth under heat wave	(Liao et al., 2024)
7	Green algae	PFOS	40 - 200 mg/L	Acute (96 h)	Physiologic/life-history traits, metabolic, cellular	Caused proportional increase of ROS to concentrations of PFOS. This ROS increase may cause Algal cell damage peroxidation	(Xu et al., 2017)
8	Zebrafish	PFOA	2.0 nM and 8.0 ppm	Chronic (3 to 120 hpf)**	Physiologic/life-history traits and Behavioural	decrease of transporters slco2b1, slco4a1, slco3a1 and tgfb1a, and a significant increase of slco1d1 expression. And caused fewer eggs production with reduced viability and developmental stage delay in F1.	(Jantzen et al., 2017)
9	Zebrafish	PFOA	400 µM	Acute (96 h)	Physiologic/life-history traits and Metabolic	Inhibition of cell viability of Zebrafish liver cells and changes in transcription levels of 1055 (977 up- and 78 down-regulated genes) and 520 (446 up- and 74 down-regulated genes)	(Wu et al., 2022)
10	Daphnia	PFOA or PFOS separate each	PFOA; 1 -250 mg/L. PFOS; 0.5 - 50 mg/L	Acute (96 h) and Chronic (21 days)	Behavioural and Physiologic/life-history traits	Acute: PFOS significantly damaged daphnid's genetic makeup. Chronic: Both delayed time for 1st brood and impaired fecundity. PFOS at 10 mg/L caused 23% mortality. PFOA at10 mg/L caused 12% mortality.	(Logeshwaran et al., 2021)
11	Daphnia	PFOS+PFOA	1, 10 and 25 µM	chronic (7 days)	Physiologic/life-history traits and Metabolic	No acute lethality. Impeded growth, reproduction, lipid metabolism and lifespan in Daphnia magna. PFOS reduced the length of Daphnia.	(Seyoum et al., 2020)
12	Daphnia	PFOS, PFOA and mixture	PFOA: (1.6 – 4.0 $\times 10^{-4}$ M); PFOS: (1.6 – 11.8 $\times 10^{-5}$ M).	Chronic (21-days)	Physiologic/life-history traits and Metabolic	Combined toxicities showed strong synergistic effect on mortality with the EC50 of the mixture at 4.44 × 10−5 mol/L	(Yang et al., 2019)
13.	Daphnia	PFOS and PFOA	PFOS; 10–400 mg/L PFOA; 31–250 mg/L	Acute (96 h)	Physiologic/life-history traits	PFOA LC50 at 24h was 298 mg/L and at 48h was 181 mg/L. PFOS LC50 at 24h was 34 mg/L and at 48 h was 27 mg/L	(Li, 2009)

14	Daphnia	PFOS and PFOA	PFOS: 0.0, 15.5, 25, 62.5, 125, 250, and 450 mg/LPFOA: 0.0, 26.3, 52.6, 105, 210, and 420 mg/L	chronic (21 days)	Physiologic/life-history traits	LOEC of PFOS and PFOA were tested under 3 exp designs (Laboratory, indoor, and outdoor microcosm) using 2 species of Daphnia. LOEC conservatively set to 1 and 10 mg/L. PFOS outdoor microcosm showed a temporal reduction in the total abundance >10 mg/L. The richness of species was significantly reduced from day 7 at 30 mg L^{-1}	(Sanderson et al., 2004)
15	Daphnia	PFOS and PFOA	PFOA; 1-100 mg/L and PFOS; 0.5-20 mg/L	chronic (21 day)	Behavioural	The LC50 values of PFOS after 21 days was 9.1 mg/L while for PFOA the LC50 was >100 mg/L. Also, 1 mg/L PFOA and 10 mg/L PFOS were the no observed effect concentrations after 21-day exposure. Zero survival at 100 mg/L PFOA. Only 1 Daphnid out of 30 survived 20 mg/L PFOS	(Li, 2010)
16	D.magna	PFOS and PFOA	PFOS: 0.3 - 5 mg/L. PFOA: 3 - 50 mg/L	Acute 48 h and Chronic (21 days)	Physiologic and Metabolic	ACUTE: For PFOS 48-h EC50(immobility) = 37 mg/L. PFOA = 477 mg/L. CHRONIC: Both PFOS and PFOA caused delay in reproduction.	(Ji et al., 2008)
17	Zebrafish	PFOS+PFOA	PFOS: 54.9 mg/L at 72h and 54.4 mg/L at 96h. PFOA: 1.5 mM at 72h and 0.9 mM at 96h	Acute (96 h)	Physiologic/life-history traits	Observed synergistic effect with the increasing ratio of PFOS. For the single exposures, the PFOS was more toxic. PFOS+PFOA showed an additive effect after 72 h of exposure.	(Ding et al., 2013)
18	Zebrafish	PFOA+PFOS	PFOA: 84.8 µg/mL. PFOS:27.9 µg/mL	Acute (48 h)	Physiologic, Metabolic and Cellular	Both PFOA and PFOS inhibited the growth of zebrafish liver cells (PFOS>PFOA). Bcl-2 expression has increased compared to control group.	(Cui et al., 2015)
19	Zebrafish	PFOA, PFOS, and mixture	PFOA= 7-700 ng/L. PFOS = 24- 2400 ng/L	Chronic (5days)	Behavioural and Physiologic.	PFOA, PFOS, and their mixture affected Zebrafish behaviour and caused untargeted gene expression.	(Haimbaugh et al., 2022)
20	Zebrafish	PFOA+ PFOS	PFOA+PFOS = 25-2500 µg/mL	Acute (72 h)	Behavioural and metabolic	Caused dysregulations of lipid metabolism in Zebrafish rerio embryos exposed to PFOA or PFOS. PFOA has greater effect than PFOS	(Yang et al., 2023)
21	Zebrafish	PFOS, PFOA, PFNA	PFOS, PFOA, or PFNA: 0.02 – 2 uM	Chronic (3-120 h)	Physiologic/life-history traits and Metabolic	Caused a decrease in total body length, increased tfc3a (muscle development) upon post fertilization exposure.	(Jantzen et al., 2016)
22	Zebrafish	PFOA, PFOS, PFHxS, PFHx, ADONA, or PFESA1	0:04–80:0 uM	(Chronic 6 days)	Cellular, and Metabolic	PFOS caused failed swim bladder inflation and ventroflexion of the tail. developmental neurotoxicity increases with increasing C-chain length but not developmental toxicity	(Gaballah et al., 2020)
23	Daphnia	PFOA, PFDA, PFDoA, and mixture	60 and 120 µg/L	Acute (48 h)	Physiologic	The toxicity increases with increase in C-chain length of Perfluoroalkyl acids (PFAAs) PFAs on Daphnia. Eventually caused Antagonistic effects	(Zhang et al., 2022)
24	Daphnia	PFNA, PFOS and mixture	0.008 to 5 mg/L alone and in combine	Acute (48 h) and Chronic (21 days)	Physiologic/life-history traits and metabolic	The activities of Acetylcholinesterase (AChE), superoxide dismutase (SOD), and catalase (CAT) and the growth were all inhibited. The mixture caused antagonistic effects related to the reproduction and growth.	(Lu et al., 2015)
25	Daphnia	PFOS, PFOA, GenX, and mixture	PFOS = 1 - 45 mg/L. PFOA = 5 - 65 mg/L. GenX = 1 -60 mg/L.	Acute (48 h)	Metabolic	Caused anabolic disruption in protein and energy pathways.	(Labine et al., 2022)
26	Zebrafish	PFOA or PFOS	(0.1-100 uM PFOA) or (0.01–1.0 uM PFOS)	Chronic (5 - 122 hpf)	Metabolic and Behavioural	PFOA was associated with increased larval motility, but PFOS caused time-dependent changes in locomotor activity. Overall, both PFOA and PFOS caused neurobehavioral toxicity.	(Hawkey et al., 2023)
27	Daphnia	22 PFAS (mixture)	0.03 to 0.58 µM	Acute (24 h)	transcriptomic	Determined the transcriptomic Point of Departures (tPODs)# to be from 0.03 to 0.58 µM.	(Villeneuve et al.) 2024

28	Zebrafish	PFOA, PFHxS, and PFOS	PFOA: 528.6 ppm, PFHxS: 14.3 ppm, PFOS: 2.1 ppm	Chronic (120 hpf)	Cellular and Physiologic/life-history traits	morphological deformities, embryo activity, and startle response time, as well as decreased lipid levels in 120 hpf Zebrafish embryos.	(Albers et al., 2024)
29	Daphnia, Zebrafish, and green algae	13 PFAS (mixture)	219 to 375 ng/g dry weight at (16, 20, and 24 °C)	Chronic (28 days)	Metabolic	Bioconcentration in zebrafish increases with increasing temperature	(Wang et al., 2023a)
30	Zebrafish	PFOS	0.04 - 1 µM PFOS	Chronic (166 h)	Physiologic/Life-history traits, and metabolic	Interference with gene transcription in the muscular and nervous systems. Accelerated motor behaviours in larvae and embryos. Increased the levels of neurotransmitters (ACh and DA).	(Wang et al., 2022)
31	Green algae	PFOA	10 - 40 mg/L	Chronic (8 days)	Physiologic/Life-history traits.	Slowed the growth of S. obliquus slightly. While inhibited the growth of C. reinhardtii significantly.	(Hu et al., 2014)
32	Green algae	PFOS+PFOA Mixture	0 to 10 mg/L	Chronic (7 days)	Physiologic/life-history traits and metabolic	There is significant decrease in cell viability, protein and chlorophyll content.	(Mojiri et al., 2023)
33	Green Algae	Cl-PFESA	From 5 mg/L to 60 mg/L	Acute (72 h)	Metabolic	The IC50 of Cl-PFESA to Algal growth was ~ 40.3 mg/L. no sign effect from 5 mg/L	(Liu et al., 2018)
34	Green algae	PFBS, PFOS, 6:2-FTS and mixture	0.01 - 500 mg/L	Chronic (12 days)	Physiologic/life-history traits	Inhibited algal growth for up to 95%	(Zhang et al., 2023)
35	Green algae	PFOA or GenX	100 ng/L, 100 µg/L	Chronic (12 days)	Physiologic/life-history traits and Metabolic	physical damage and metabolic disorders. downregulated the photosynthetic genes	(Li et al., 2021)

Table 2.3: Toxicity of PFAS* on *Daphnia*, zebrafish and green algae.

* PFAS = Perfluoroalkyl Substances; PFOS = Perfluorooctanesulfonic acid; PFOA = Perfluorooctanoic acid; PFHxS = perfluoro hexane sulfonate; GenX (Trade name) = hexafluoropropylene oxide dimer acid (HFPO-DA); PFNA=Perfluoro nonanoic acid; PFESA1 = Perfluoroethylcyclohexane sulfonate; ADONA = 4,8-dioxa-3H-perfluorononanoate; Cl-PFESA = Chlorinated polyfluorinated ether sulfonate; PFBS = Perfluoro butane sulfonic acid. ** hpf = hours post fertilization. tPOD values is the maximum dose level which cause no adverse effects.

proposed health advisory rules and suggested new studies to understand why these substances need to be limited. Despite the phase-out, they were replaced by shorter-chain PFAS compounds as alternatives, which were initially thought to pose fewer risks to human health and the environment. European Union (EU), United Kingdom (UK), United States (US), Canada, and Australia have taken strong actions to ban PFAS applications in different sectors by introducing alternatives. The UK was the first European country to propose the restriction on PFAS following a national assessment of the environmental risks of using PFOS and its derivatives (Agency 2004). Also, several policies have been introduced by OECD and non-OECD countries regarding the negative impact of MP to mitigate MP leakage into environments (UNEP 2018). Countries like the UK, the US, Canada and the EU have banned the use of MP in cosmetic and personal care products to prevent the release of more MP into the environment, and they are working on tighter regulations on plastic waste and single-use plastics (California Code of Regulations. Microplastics Materials. Sect. 1 2018, https: //leginfo.legislature.ca.gov/faces/billTextClient.xhtml?bill_id=201720180SB1263). Comprehensive risk assessments and prioritisation of the most common PFAS compounds and their sources are still under investigation to develop future UK policy on PFAS to ensure they are used safely and to identify critical evidence gaps for further work (Agency 2021). All companies that supply, formulate, distribute or use mixtures that contain PFAS quantities above the REACH registration threshold in the UK (higher than one tonne per year) need to register under UK REACH environmental legislation, which refers to the laws and rules designed to protect the natural environment and human well-being. It is a web of guidelines, strategies, and resolutions pointed toward resolving issues of air and water quality, waste administration and control of pollution. Environmental legislation plays a crucial role in the UK in preventing water-related problems and ensuring the sustainable management of water resources. The Water Framework Directive (WFD, 2000/60/EC) in Europe is considered the best legislation used for

water quality management (Wuijts et al. 2023). WFD aims to achieve a good ecological and chemical level for all the EU surface water and a decent substance status with enough water by 2027. Every sector that uses PFAS and MP follows specific legal requirements, which are evaluated by different organisations. To improve our knowledge of the dissemination and risks of PFAS and MP in the environment, further information from industrial sectors needs to be shared about the range and amounts of PFAS and plastics used and potential pathways for release to the environment. Global collaboration is essential to regulate MP and PFAS effectively, especially considering their widespread use in international supply chains. Hence, international agreements could be crucial in setting global standards. Also, public awareness about the dangers of MP and PFAS can drive consumer demand for safer products, while holding companies accountable for environmental practices will ensure better compliance with regulations. Last but not least, Governments should invest in research to develop safer and biodegradable alternatives to MP and PFAS that industries can widely adopt.

2.3.3 Research Gaps and Recommendations for Future Research

This systematic literature review aimed to assess the current state of knowledge on the toxic effects of MP and PFAS on key ecotoxicology species as proxies for freshwater ecosystems. While existing research provides insight into the toxic effects of individual contaminants (e.g., PE beads, PFOS, PFOA) on model freshwater species, several significant knowledge gaps remain:

1. **Limited studies on combined MP and PFAS toxicity**

   Despite the widespread coexistence of MP and PFAS in freshwater ecosystems, only one study has explored their combined toxic effects on *D. magna*.

This is a critical knowledge gap, as co-exposure to MP and PFAS is more environmentally relevant than exposure to individual contaminants alone.

2. **MP Toxicity Needs Refinement**

   MPs' toxicity is primarily size- and shape-dependent, with polymer type playing a secondary role. However, research is limited on the toxicity of different MP shapes and their interactions with persistent pollutants. Furthermore, many studies use unrealistically high MP concentrations, limiting their relevance to regulatory toxicology and environmental risk assessments.

3. **Higher toxicity of MP with MPACs**

   The limited available evidence suggests that MP associated with chemical additives induces greater toxicity than MP alone, particularly at the molecular level. Given the vast diversity of MPACs—including flame retardants, plasticizers, PFAS, and UV stabilisers—further research is needed to assess the real-world impact of MP as complex chemical carriers. Studies focusing only on MP or plastic leachates do not accurately reflect environmental exposure conditions and may underestimate actual toxicity risks.

4. **Increased Toxicity of Complex Mixtures**

   Studies show that the toxicity of complex mixtures—including multiple PFAS or MP + PFAS combinations—is greater than that of individual contaminants, often revealing synergistic or additive effects. This suggests that the global phase-out of PFOS and PFOA may not significantly reduce environmental risks. This is particularly worrying as emerging evidence indicates that "newer" short-chain PFAS alternatives (e.g., GenX, PFBS, ADONA) exhibit similar toxic effects than long-chain PFAS on aquatic organisms.

5. **Shortcomings of Acute Toxicity Studies in Regulatory Toxicology**

   Acute toxicity studies, widely applied in regulatory toxicology, often conclude that MP and PFAS are non-toxic due to a lack of immediate mortality or im-

mobilisation. However, chronic exposure studies reveal significant long-term effects, even at trace concentrations, demonstrating these contaminants' cumulative and sublethal impacts on growth, reproduction, metabolism, and genetic integrity. This result calls for an urgent reassessment of toxicity tests that define the safe use of chemicals in regulatory toxicology.

In conclusion, this review demonstrates the need for further research that i) evaluates the entire life cycle of organisms under chronic exposure conditions and ii) examines realistic environmental mixtures rather than single contaminants. Investigates the long-term ecological consequences of MP and PFAS co-exposure. Such knowledge is essential to inform policy decisions and environmental regulations aimed at protecting freshwater ecosystems and human health from these persistent contaminants.

2.4 Environmental Antibiotic-Resistant Bacteria

2.4.1 Antibiotic and Antibiotic-Resistant Bacteria Communities in Water Sources

Since the discovery of penicillin in the 20th century, antibiotics have been used to treat diseases caused by pathogenic microorganisms (Wenhui Li et al. 2012). However, misuse and overuse of antibiotics have led to antimicrobial resistance (AMR), posing a significant public health and environmental issue (Chika F Nnadozie and Odume 2019a). Antimicrobial resistance is considered one of the most serious threats to global health (Aljeldah 2022). Currently, there is limited availability of effective therapies, insufficient prevention strategies, and only a small number of new antibiotics in development, highlighting the urgent need for innovative treatment options

and alternative antimicrobial approaches. It is projected that 1.27 million global deaths annually are associated with antimicrobial resistance (AMR) (Murray et al. 2022), and this figure will rise to 10 million people by 2050 (WHO 13 April 2022). Antibiotic-resistant bacteria (ARB) and antibiotic-resistant genes (ARGs) have been detected across various ecosystems (X. Bai et al. 2015; Célia M Manaia et al. 2016). Infections caused by ARB and the dissemination of ARGs in the environment have become a serious global threat (Tao et al. 2022) and are now recognised as emerging environmental contaminants. The costs of antibiotic contamination remain a major concern in the world (Hutchings, Truman, and Wilkinson 2019). The growing human population and increasing anthropogenic activities, including the extensive use of antibiotics in medicine, agriculture, and veterinary practices, have exacerbated this issue. For example, China uses around 50% of the total antibiotics in the world, of which approximately 25%–75% are released to the environment unmetabolised (Yulin Chen et al. 2023). These antibiotics enter freshwater ecosystems directly or indirectly through multiple sources, such as wastewater treatment plants (WWTP), hospital and domestic sewage discharges, agricultural runoff, and farm leaching. These activities lead to contamination, persistence, and transmission of ARB in freshwater (S. Faria, Joao, and Jordao 2015).

ARB serve as carriers of ARGs and play a critical role in their dissemination in the environment. In aquatic environments, ARB transfer their ARGs to other indigenous bacteria primarily via horizontal gene transfer (HGT) and vertical gene transfer (VGT), which are the key mechanisms driving the spread of antibiotic resistance both in vitro and in vivo (Baquero, J.-L. Martínez, and Cantón 2008; J. L. Martínez, Baquero, and Andersson 2007). Antibiotic residuals in water will not only negatively affect aquatic organisms but also induce the emergency of resistome (genes involved in antibiotic resistance), which could pose adverse effects to marine life and human health through drinking water or the food chain that cause illness and even death,

especially in immunocompromised people (Ramay et al. 2020; Chika F Nnadozie and Odume 2019a). Understanding resistome is important for predicting and mitigating the emergence of antibiotic resistance, as it helps identify the genetic reservoirs and pathways that enable bacteria to become antibiotic-resistant. Resistome refers to a group of genes, often found in pathogenic and non-pathogenic bacterial populations, that have the potential to develop or be modified into antibiotic-resistant genes under certain conditions. Resistomes are capable of being transferred to other organisms, including dormant or latent ones. These genes may not initially confer resistance but can acquire mutations, be transferred between bacteria, or be activated in response to selective pressures, such as exposure to antibiotics, heavy metals, or other environmental stressors (D'Costa et al. 2006). Once converted, these genes can provide bacteria with mechanisms to survive and thrive even in the presence of antibiotics, contributing to the broader problem of antimicrobial resistance. The resistome serves as a critical reservoir of genetic material, facilitating the spread of antibiotic resistance through HGT. This process can contribute to the emergence of multidrug-resistant pathogens. Understanding the resistome is vital for tackling the global challenge of antimicrobial resistance (Shakibaie, Mansouri, and Hakak 2002; J. L. Martínez, Coque, and Baquero 2015; R. L. Finley et al. 2013). Despite advanced filtration and disinfection technologies in water treatment, ARB contamination remains a persistent issue, often leading to microbial infections and related illnesses (Craun et al. 2010).

Wastewater treatment plants (WWTPs) and hospital effluents constantly release antibiotic residuals into the environment and are globally recognised as major sources of ARB and ARGs that enter rivers, lakes, domestic water systems, and drinking water reservoirs (Baquero, J.-L. Martínez, and Cantón 2008). In addition to the gradual accumulation of antibiotics, also detergents, disinfectants, and industrial pollutants (including heavy metals, MPs and PFAS) in the environment play a

significant role in promoting the evolution and dissemination of resistant bacteria in aquatic ecosystems (Baquero, J.-L. Martínez, and Cantón 2008). Some bacteria may survive in chlorinated water after inadequate treatment of water and chlorination (Zhaojun Chen et al. 2017) as chlorination is a common disinfection method used worldwide to eliminate bacteria from drinking water (Mazhar et al. 2020). These bacteria are known as chlorine-resistant bacteria (CRB). When CRB acquire ARGs, they become ARB, carry resistance to both chlorine and antibiotics and ultimately enter the water distribution system (Z. Zhou et al. 2023). Studies have shown that chlorination can make CRB more resistant to antibiotics than common bacteria (C. Faria et al. 2009; Khan, Knapp, and Beattie 2016; S.-S. Liu et al. 2018).

Studies have identified ARGs such as *vanA* (vancomycin resistance), *mecA* (methicillin resistance), and *ampC* (Beta-lactamase-mediated resistance) in wastewater, surface water, and drinking water biofilms (Schwartz et al. 2003). The persistence of ARB and ARGs in freshwater environments increases the risk of infections caused by resistant pathogens, reducing the effectiveness of antibiotic treatments (Chika F Nnadozie and Odume 2019a). Protecting public health, therefore, requires minimising microbial contamination in water intended for consumption. The prevalence and distribution of ARB in drinking water vary geographically and are influenced by several factors: urban and rural areas (Asaduzzaman et al. 2022); developed and developing countries (Collignon et al. 2018), and seasonal variations (Wolf-Baca and Siedlecka 2023). Therefore, strategies to tackle ABR differ across countries based on economic status and development levels. High-income and low-income countries exhibit differences in ABR patterns, antibiotic use, healthcare access, sanitation, and regulatory infrastructures (Collignon et al. 2018). In high-income countries, antimicrobial use and better stewardship reductions have been linked to lower AMR prevalence, especially in the healthcare and agriculture sectors (Ramay et al. 2020). Ke et al. 2023 studied seasonal variation and abundance of microbial communities

and ARGs in tap water and confirmed that ARGs were more abundant at higher temperatures. However, other factors, such as ammonia level, also had an important role in the variation of ARGs.

The prevalence of ARB and ARGs has been widely reported in environmental settings. However, research on their dynamics, geographic variations in contamination levels, and their potential impact remains less developed compared to studies in clinical contexts, despite evidence indicating that the environment is a significant reservoir of resistance genes for pathogens. Regulatory systems have not implemented adequate measures to monitor, reduce, or prevent resistance to antibiotics within ecosystems (Ashbolt et al. 2013). One particular area of focus is the presence of beta-lactam-resistant bacteria in household water systems. Recently, the presence of antibiotics and ARGs in tap water, drinking water sources, drinking water treatment plants (DWTPs), and distribution systems (pipelines) has become a severe threat (K. Zhang et al. 2020; Kinsey et al. 2017; Kizny Gordon et al. 2017; Marcela F Dias et al. 2018; L. Ma, B. Li, and T. Zhang 2019; Marcela França Dias et al. 2020). Many people worldwide use home water purification systems (HWPSs) to obtain clean drinking water. However, the reliability of HWPSs in providing safe water is unknown or not well-proven (Gu, Zhai, and S. Cheng 2021). Antibiotic resistance speeds up when antibiotics are misused or overused. However, antibiotic resistance has decreased in developed countries due to stewardship programs and a reduction in the use of antimicrobials, particularly in the healthcare and agriculture sectors (Chantziaras et al. 2013). The high levels of resistance observed in ARGs for cephalothin, penicillin, and tetracycline up to a milligram in rivers and lakes indicate the presence of these antibiotics in freshwater environments (Ana, Madriaga, and Espino 2021). Beta-lactam antibiotics, commonly used as frontline antibiotics and fundamental treatments for a wide range of bacterial infections, are likely among the most prevalent in aquatic ecosystems, contributing to the spread of resistance.

2.4.2 Ampicillin: A β-Lactam Antibiotic

β-lactam antibiotics are one of the most widely used drugs that serve a broad spectrum of clinical applications and represent 65% of the total antibiotics market (Thakuria and Lahon 2013). β-lactam antibiotics are a broad class of antibiotics that share a common chemical structure known as the beta-lactam ring, which is essential for their antibacterial activity. Resistance to beta-lactams is an alarming and growing concern and poses a public health challenge. Bacterial resistance to β-lactam antibiotics is primarily expressed by the production of beta-lactamase enzymes. However, other mechanisms also contribute to resistance, including decreased penetration to the target site, alteration of the target site (penicillin-binding protein), and Efflux mechanisms that ultimately inhibit antibiotic effectiveness (Ibrahim et al. 2019). Ampicillin, a β-lactam antibiotic, is effective against both gram-positive and gram-negative bacteria. It works by inhibiting penicillin-binding proteins (PBPs) involved in bacterial cell wall synthesis. Ampicillin is widely used to treat bacterial infections such as pneumonia, urinary tract infections, and systemic respiratory illnesses (Karlowsky et al. 2019). It accounts for approximately 50% of global antibiotic usage (Lima et al. 2020; World health organization 2019). Resistance to ampicillin in domestic water sources poses significant health risks, particularly for immunocompromised individuals who may develop severe infections from ARB.

2.4.3 Biofilm Formation

Biofilm formation initiates with water droplets adhering to the pipeline walls, followed by the accumulation of bacteria and other loose particles, including antibiotics, MPs and PFAS, that adhere to the inner surfaces of the pipeline. These bacteria secrete a slimy, protective matrix composed of polysaccharides, proteins, and DNA,

which helps them stick together and to the surface (Kalu et al. 2024). Biofilms play a significant role in the survival and spread of ARB. Biofilms provide a microenvironment that supports the growth of ARB and facilitates the transmission of ARGs and other contaminants like MPs and PFAS (J. Chen, Weiying Li, J. Zhang, et al. 2020; Z. Zhou et al. 2023). Biofilms in pipelines are common in domestic, industrial and municipal water systems and can have significant consequences. Pathogenic/opportunistic microbial biofilms are recognised as a global challenge and pose a significant risk due to their unique structure and way of living, which naturally make them resistant to antibiotics (Mah 2012). Interestingly, low doses of antibiotics can induce biofilm formation (Kaplan 2011). However, it remains unclear whether there is a quantitative correlation between biofilm formation and the level of antibiotic resistance.

2.4.4 Opportunistic Antibiotic Resistant Bacteria

Opportunistic antibiotic-resistant bacteria (OARB) in water systems are a significant public health concern (Bartell 2015). While these bacteria typically do not pose a threat to healthy individuals, they can act as opportunistic pathogens in immunocompromised individuals, where antibiotic resistance makes infections particularly difficult to treat, increasing morbidity and healthcare costs (Curran et al. 2021). Opportunistic bacteria are highly adaptable and can survive in diverse environments, including water systems, where they form biofilms that protect them from antibiotics and environmental stresses (J. Chen, Weiying Li, Tan, et al. 2022). Additionally, their resistance to disinfection and heat enables them to thrive in low-nutrient and stressed conditions (J. Huang et al. 2021). These pathogens can cause severe and potentially life-threatening infections in individuals with weakened immune systems. This bacterial species can exhibit antibiotic resistance through

intrinsic or acquired mechanisms that either block antibiotics from reaching their targets or inactivate the antibiotics themselves (Blair et al. 2015; Nadeem et al. 2020). Kalu et al. 2024 summarised the studies performed on the occurrence of ARB in drinking water treatment plants and distribution systems between 2013 and 2023, and they showed that ABR are present throughout drinking water treatment plants and distribution systems, after filtration (3%), disinfection (23%), and distribution systems (24%). These risks highlight the importance of monitoring and managing microbial contamination in water systems to protect vulnerable populations. Furthermore, exploring bacterial communities in drinking water is crucial for understanding the overall dynamics and contamination of water systems.

Chapter 3

Ingestion, Retention and Egestion of Microplastics by *Daphnia*

3.1 Introduction

3.1.1 Microplastics Ingestion, Retention and Egestion by *Daphnia*

This chapter serves as a proof-of-concept study and provides the foundation for the next chapter (Chapter 4, Toxicological Impacts of Perfluoroalkyl Substances and Microplastics on the Sentinel Species *Daphnia magna*). In the current chapter, the ability of *Daphnia magna* to ingest, retain, and egest four common microplastics (MP): polyethylene (PE), polystyrene (PS), and polymethyl methacrylate (PMMA) and polyethylene terephthalate (PET) is investigated and compared. These MP are frequently found in the environment due to their wide range of applications. The experiment was conducted under two conditions: in the presence and absence of algae as a food source, to assess the feeding dynamic of *Daphnia*. Additionally, it

was crucial to verify whether *Daphnia magna* could tolerate the concentration used in this experiment to ensure its suitability for the next phase of the study. Dynamic light scattering (DLS) and microscopy were employed to characterise MP, determining their particle size, shape, and surface charge. By the end of this chapter, the MP are characterised, the experimental design is optimised, and the most suitable MP candidate is identified for use in ecotoxicology experiments in Chapter 4 and the following questions are addressed:

1. Is *Daphnia* able to ingest different MP?
2. Do ingestion and egestion patterns vary under two experimental conditions (presence and absence of algae) and with different types of MP?
3. Does the used concentration of MP (100 mg L-1) cause any mortality?
4. Does stirring or mixing help keep MP in suspension and evenly distribute it in the test jars?

3.1.2 Occurrence of Microplastics in Aquatic Environments

The selection of four different types of microplastics (MP) was made intentionally to represent a diverse range of polymer types commonly found in aquatic environments. The aim was to assess their general physical and biological effects, particularly focusing on *Daphnia magna* as a sentinel species. While detailed chemical characterisation was not conducted, this study prioritised the investigation of physical properties such as particle size, shape, and surface area which are well-established factors influencing ingestion, gut retention, and physical toxicity in aquatic organisms, independently of chemical composition. All MP used in this study were obtained from well-characterised commercial sources, reducing uncertainty regarding their polymer

identity and ensuring consistency across experimental treatments. Although chemical analysis techniques such as FTIR, Raman spectroscopy, or GC-MS would provide further insight into potential leachates or additives contributing to chemical toxicity, such analysis was beyond the scope of this initial investigation. Nonetheless, the current study offers a foundational understanding of the physical interactions between MP and aquatic organisms. The use of four representative MP types is therefore justified as an appropriate first step toward evaluating baseline biological responses. These findings will inform and support more targeted chemical characterisation and mechanistic toxicity studies in future research. Cryogrinding is a process that involves pulverizing plastics at low temperatures. It significantly impacts the shape, size, surface properties, and morphology of the MP it produces, which can affect their behaviour and interactions and, consequently, their impact on the environment by generating irregularly shaped fragments and fibres. Also, these variations in the shape and size of MP are critical because they influence their aggregation potential, bioavailability, and uptake by organisms. Understanding the diversity of microplastic particles produced through cryogrinding is essential for replicating real-world conditions and accurately evaluating their ecological and toxicological effects. Cryogrinding leads to more efficient fragmentation of plastic particles due to the brittle state of polymers at extremely low temperatures (Molina-Boisseau and Le Bolay 1999). Cryogrinding was employed as a controlled and reproducible method to fragment foure types of plastic into smaller particles with irregular shapes and heterogeneous sizes, closely resembling the physical degradation observed in natural environments. While cryogenic grinding itself does not occur in nature, environmental processes such as mechanical abrasion, weathering, and fragmentation from UV exposure result in irregularly shaped microplastics. Real environmental microplastic samples is not used in this study in order to maintain experimental control, consistency, and reproducibility. Environmental MP are highly heterogeneous in terms of polymer type, size, shape, and chemical contaminants (e.g., adsorbed pollutants or

biofilms), which can introduce variability and make it difficult to isolate the specific factors responsible for observed biological effects. By using laboratory-prepared MP with known composition, size range, and surface characteristics, we ensured that the biological responses observed in exposed animals could be clearly attributed to defined physical properties of the microplastics, rather than to unknown or variable environmental contaminants. This approach provides a necessary baseline for understanding MP toxicity under controlled conditions. Future studies will incorporate real-world MP samples to build on this baseline and assess combined effects of environmental complexity. However, the current use of defined synthetic MP is essential for generating reliable, interpretable data to understand fundamental biological interactions.

3.1.3 Ingestion of MP by *Daphnia magna*

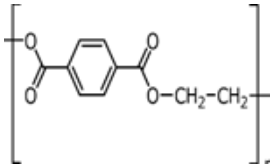
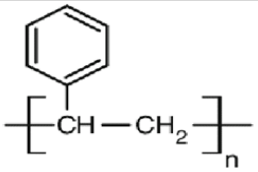
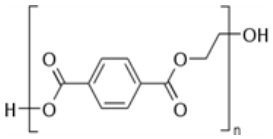
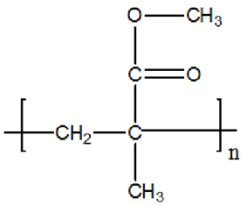
Daphnia ingests MP through a filter-feeding process by which these tiny organisms draw in water and filter out particles as they feed on algae and other suspended particles. Green algae are the main diet for *Daphnia*, which naturally can grow and colonise on the surface of plastic particles P. S. Kumar, Pavithra, and Naushad 2019. *Daphnia* unintentionally ingests MP while feeding on particles in the water as it is a non-selective zooplankton that cannot differentiate between algae as food and MP particles. *Daphnia* ingests particles with sizes from <1-70 µm approximately Rosenkranz et al. 2009; Nørgaard and Roslev 2016. The MP are almost similar in size to the algae and other suspended particles that *Daphnia* normally ingests. However, plastic materials can obstruct food intake in *Daphnia* due to the accumulation of MP in their gut. The interference of MP on food intake by *D. magna* is not fully understood yet. The ingestion of MP by *Daphnia* can cause several potential effects, such as impacting digestion and nutrient absorption, phys-

ical blockage of the gut, and leaching of the chemicals associated with the MP into the *Daphnia*, eventually affecting their overall fitness such as survival, growth, and reproduction. Additionally, MP are transferred into the food web and have become widespread in aquatic ecosystems. Many marine and freshwater zooplankton species can ingest MP. However, several factors affect MP ingestion, such as type of plastic, concentration, shape, weight, and frequency of ingestible MP particles S. L. Wright, Thompson, and Galloway 2013. *Daphnia's* behavioural and morphological changes in response to environmental stressors have been identified (M. Abdullahi, J. Zhou, et al. 2022.

3.1.4 Dynamic Light Scattering and Zeta Potential for Characterisation of MP

It is important to understand the physio-chemical characteristics of the MP due to their physical nature and high surface area. Several MP characterisation techniques have been established, such as Transmission Electron Microscopy (TEM), Scanning Electron Microscopy (SEM) and Dynamic Light Scattering (DLS) (F. Nasser and Lynch 2016). SEM, TEM, and DLS are widely used and complementary techniques for microplastic (MP) characterisation, each providing unique and valuable information. SEM generates detailed three-dimensional images by scanning the sample surface with a focused electron beam. This technique is particularly effective for studying the texture, shape, and surface degradation of microplastics. TEM, on the other hand, transmits electrons through an ultra-thin sample to produce high-resolution two-dimensional images. It is ideal for examining internal structures and fine details such as nanoplastic morphology and crystallinity, features that SEM cannot resolve due to its focus on surface imaging. Dynamic Light Scattering (DLS) complements these electron microscopy methods by offering a rapid, non-destructive

Table 3.1: Outlines specifications of four (4) used MP in this study and provides information about their chemical structures, density, CAS number, and common applications.

Microplastics	**Chemical Formula**	**Chemical Structure**	**Density (g/cm^3)**	**CAS number**	**Application**
Polyethylene (PE)	$(C_2H_4)_n$		0.93	9002-88-4	Packaging materials, bottles, plastic bags, Piping, Plumbing and Construction
Polystyrene (PS)	$(C_8H_8)_n$		0.895	9003-53-6	Packaging, containers, medical, toys, and construction
Polyethylene Terephthalate (PET)	$(C_{10}H_8O_4)_n$		1.68	25038-59-9	Single-use plastic items, beverage bottle, and textile
Poly (methyl methacrylate) (PMMA)	$(C_5H_8O_2)_n$		1.18	9011-14-7	Optics and Eyewear, Electronics and Lighting, Medical and Dental Applications, etc

way to measure the hydrodynamic diameter of particles suspended in liquid. DLS is especially useful for detecting and analysing submicron and nanoscale particles that may be difficult to observe through SEM or TEM. DLS is a common technique to determine the size, potential agglomeration, and hydrodynamic behaviour of the particles in suspension by measuring the scattered light source through a solution as the particles move under Brownian motion (Berne and Pecora 2000; Reilly 2022). DLS helps to understand more about the characteristics of the MP particles in solution (L. M. Hernandez, Yousefi, and Tufenkji 2017). In light-scattering, the sample is typically exposed to a monochromatic light wave, the signal is detected using a suitable detector, and the sample size is then interpreted mathematically (Berne and Pecora 2000; Stetefeld, McKenna, and Patel 2016). DLS has many advantages over other techniques, such as working with various buffers, temperatures and concentrations, reliability, and non-invasive and easy application (Stetefeld, McKenna, and Patel 2016). It is commonly employed in different fields of chemistry to characterise particles, aggregates, and macromolecules in solutions. It allows rapid measurements of particle size distribution without the need for extensive sample preparation. A laser is directed into a solution, and the light is scattered off the particles. A detector then measures the light intensity. Variations in particle size result in differences in light scattering, which can be analysed by DLS to calculate the average particle size in the solution. The DLS method is based on the principle that larger particles move more slowly in a solution, allowing their size to be correlated with the intensity of light detected. When particles agglomerate, their movement becomes slower, leading to a shift in the backscattered light detected by the system (Reilly 2022). This variation in light scattering provides valuable information about the extent of particle aggregation, allowing for an accurate assessment of particle size and dynamic behaviour within the solution. It enables researchers to monitor the formation of clusters and gain a deeper understanding of particle interactions and stability. (P. S. Kumar, Pavithra, and Naushad 2019; El Hadri et al. 2020). Zeta

potential shows the particle's net charge in the solution. Zeta potential measures the electrophoretic mobility of particles in an ionic suspension; particles' surface charge attracts a surrounding layer of oppositely charged ions, consisting of a strong inner layer and a weaker outer layer (Reilly 2022). The movement between these layers, known as the slipping plane, indicates the particle's surface charge and electrostatic stability. This measurement provides valuable insights into the particle's interaction with its environment and helps assess its potential for aggregation or dispersion within a solution (P. S. Kumar, Pavithra, and Naushad 2019; El Hadri et al. 2020; Reilly 2022).

3.1.5 Hydrophobic Behaviour of MP in Water

The hydrophobic nature of MP significantly influences their behaviour in the environment and aquatic life, and understanding this concept when conducting toxicity tests is essential. Hydrophobic MP tend to clump together (agglomerate) to minimise their exposure to water (Julapong et al. 2022). This aggregation can affect their environmental distribution and interaction with organisms. Agglomerated particles often form larger, irregularly shaped clusters. It can impact how organisms ingest them and how they move through aquatic systems. Agglomeration reduces the surface area of individual MP, potentially decreasing the surface available for pollutant adsorption and influencing their sedimentation rates and distribution. Hence, it is important to have realistic exposure scenarios and consider the hydrophobic nature of MP in laboratory settings. Proper test suspensions and dispersion methods must be used to avoid unrealistic clumping of MP in toxicity tests. It might involve using surfactants or mechanical agitation to ensure a uniform distribution of the particles. Artificial surfactants like Sodium Dodecyl Sulphate (SDS), ethanol and Tween are widely used to reduce surface tension between liquids or between a liquid and a

solid. However, many of these surfactants pose toxicity risks to organisms, potentially causing cellular damage through several mechanisms. These include disruption of lipid membranes, interference with endocrine signalling pathways, and direct cytotoxic effects that can compromise organismal health. Given these concerns, it is essential to identify safer alternatives for managing MP agglomeration in solution to ensure the integrity of experimental conditions. Effective dispersion of MP is critical for laboratory studies, as it allows for a homogenous suspension in MP stock solutions and uniform particle distribution across experimental setups. Consistent particle dispersion in working solutions is particularly crucial for obtaining accurate, reproducible results, as uneven MP distribution could lead to variable exposures and confound the findings of the study. Therefore, developing or identifying non-toxic surfactants or alternative methods to achieve stable and evenly distributed MP suspensions is a priority for maintaining ecological relevance and safety in experimental environments.

3.2 Methodology

3.2.1 Culturing of *D. magna*

Model organism *Daphnia magna* was previously resurrected (revived) from a biological archive of Lake Ring, a shallow mixed lake in Denmark (55°57'51.83" N9°35'46.87" E) with a well-recorded history of anthropogenic impact (Cuenca Cambronero et al. 2018. In the late 1950s, sewage discharge from a nearby town caused severe eutrophication symptoms in the lake. Although sewage inflow was diverted by the late 1970s, the intensification of agricultural land began in 1975 simultaneously, which caused substantial pesticide and herbicide runoff into the lake. Between 1989 and 1990, the lake was stocked with white fish to investigate the effects of predation on

the invertebrate community. From the late 1990s onwards, the lake has shown signs of partial recovery from hyper-eutrophication. Dormant embryos of *Daphnia magna* that were used in this experiment were collected during the lake's recovery phase (post-1999) and identified as LRV0_1 genotype. Then, the embryos were transferred to the laboratory and maintained for several years in the *Daphnia* facility laboratory, school of Biosciences, University of Birmingham. The *D. magna* clone was obtained from cultures that were maintained in the laboratory at 10°C for over a year as isoclonal lines in the following standard laboratory conditions: 16:8-h light-dark photoperiod; 0.8 mg L-1 *Chlorella vulgaris* (CCAP 211/11B) was refrigerated at 4°C and fed weekly; ambient temperature. *Daphnia* was cultured continuously in the laboratory at 20 °C and a 16 8-h (light-dark) photoperiod, feeding 0.8 mg L-1 *Chlorella vulgaris* daily and with the light intensity of 500-1000 lux for at least three generations to acclimate and reduce interference from maternal effects following the guidelines of Test No. 211 of the Organization for Economic Co-operation and Development (OECD 2012). After at least three generations in these conditions, 24h old juveniles from the third brood were randomly isolated and assigned to experimental conditions. Borehole water (pH 8-8.2) was used as growth media for culturing *Daphnia*. Physio-chemical characteristics of borehole water are shown in Table 2 in Appendix B.

3.2.2 Microplastics Preparation by Cryogrinding and Sieving

Four types of microplastic granules were purchased from Sigma Aldrich, UK, and their specification is shown in Table 3.1. Cryogrinding was used as a simulation technique to replicate these natural degradation effects in a time-efficient and controlled laboratory setting, without introducing thermal or chemical changes that could alter the plastic's composition. The operation was indeed designed with the

intent to produce irregularly shaped and non-uniformly sized particles, aligning with environmental microplastic profiles. By avoiding uniform spherical particles (which are often used in studies for convenience), our approach improves the environmental relevance of the test materials. Therefore, cryogrinding is justified as a practical and valid technique for producing microplastic samples that simulate the physical characteristics of those found in nature. MP were cryo-milled in liquid nitrogen using SPEX samplePreP 6775 freezer/mill cryogenic grinder (6 cycle with the rate of 15 CPS) to generate the ingestible size of MP by *Daphnia* (*Daphnia* are usually able to ingest MP less than 50-60 μm due to their size). Steel sieves with pore sizes of 50 µm and 38 µm were used to screen out the particles between 38 and 50 µm to achieve precise sizing. Following the filtration process, the MP were collected and stored in clean glass vials with screw caps to prevent contamination for further analysis.

3.2.3 Preparation of Dosing Solutions and Dispersion of MP

As MP particles naturally agglomerate in water, a mechanical stirring setup was employed to minimise agglomeration and ensure even distribution of MPs when dosing into each test jar. After the 1 g L-1 MP stock solution was prepared using filtered borehole water in a clean glass beaker containing a stir bar, the beaker was placed on a magnetic stirrer set at 500 rpm to achieve a homogenised solution before dosing. Before the experiment, the mass of used MP was quantified, and their recovery percentage was calculated to validate the accuracy of the solutions for dosing MP by following QA/QC protocols. By calculating recovery and relative standard deviation (RSD), the accuracy and precision of the analytical methods for MP can be evaluated. 10 ml of each MP stock solution was pipetted into ten (10) jars containing 90 ml of borehole water. Then, each jar was filtered through

the Whatman GF/D filter with a diameter of 47 mm membrane glass microfiber, using a vacuum pump, placed in a clean glass petri dish and dried at 60°C overnight and weighed to calculate the standard deviation (if all 10 jars contain almost the same quantity of MP after each pipetting). All processes were performed in the laminar flow cabinet except when using an incubator. The recovery percentage of MP particles was determined by comparing the mass of MP particles initially added to the borehole water (nominal total mass) with the mass recovered on the filter papers after filtration. The recovery percentage was calculated using the formula:

$$\text{Recovery Percentage} = \frac{\text{Recovered MP after filtration (mg/L)}}{\text{Spiked concentration (mg/L)}} \times 100 \tag{3.1}$$

$$RSD(\%) = \frac{\text{Standard Deviation (SD)}}{\text{Mean Value}} \times 100 \tag{3.2}$$

Where the mass of recovered MP was measured after drying and weighing the filter papers, added MP is the actual (spiked) total mass of 100 mg L-1.

3.2.4 Characterisation of Four MP

To conduct microscopic observations, a concentrated MP solution at a concentration of 1 mg/ml was prepared. Then, 3-4 ml of this solution was pipetted onto a clean glass petri dish, ensuring an even spread across the surface. The petri dish was then divided into four equal quadrants to facilitate a systematic counting process and detailed observation of particle distribution. The individual MP particles in each

quadrant were characterised, counted and examined using a Digital OPTIKA™ IM-3LD4D PROVIEW, Italy microscope with multiple lenses Plan Fluor (4X, 10X, 20X, 40X). MP were visualised and measured using the bespoke "Optika™ ProView Image Analysis" software for the built-in 6.3-megapixel USB 3.0 CMOS digital camera. The average size and shape of the particles used in the experiment were determined based on the analysis of up to 50 randomly selected particles for each plastic (except PET, up to 300 particles were selected).

3.2.5 Dynamic Light Scattering (DLS) and Zeta Potential

To validate the size and surface charge of the particles of each MP, 5 ml solutions of 2 mg/ml of all 4 MP were prepared and analysed using Dynamic Light Scattering (Malvern 5000 Nano Series Zetasizer). Subsequently, four 1 ml aliquot replicates of each solution were tested in the DLS to determine average particle size, zeta potential, and polydispersity index. The experiment was conducted at room temperature (24–25 °C), with distilled water as the dispersant. The water had a refractive index of 1.330, a viscosity of 0.8872cP, and a dielectric constant of 78.5. The following steps were carried out separately for the four MP samples:

- 2 ml of concentrated solution 2 mg/ml of MP was pipetted into low-volume cuvettes.
- The cuvette was placed into the DLS sample holder, with the clear panel positioned to face the laser beam.
- The appropriate Standard Operating Procedure (SOP), previously established based on the refractive index and absorption values specific to each MP sample, was selected and pre-saved in the SOP files.

- A new file was generated and assigned for the sample run for MP.
- The DLS analysis was conducted after selecting the respective file for each sample. Each sample was analysed in four replicates during a single run using the nominated SOP.
- After all the sample runs were finished, an average of the four (4) runs was calculated.
- Then, the sample was removed, and a fresh aliquot from the same original stock solution was introduced. Steps 3-6 were repeated to ensure that a total of four distinct aliquots of the stock solution were analysed for each MP sample.
- Following the DLS analysis, a 1ml sample aliquot was carefully injected into a zeta potential capillary cell, ensuring no air bubbles were introduced. Then, the caps were securely replaced.
- The capillary was then placed into the sample holder, and the corresponding Zeta SOP was selected using the same parameters as those applied in the DLS SOP.
- A new measurement file was also created for the zeta readings.
- The zeta potential of the sample was analysed after selecting the measurement file. This was also done for four replicates (as shown in steps 6 and 7)

The DLS and zeta potential data can be processed and presented by exporting them as CSV files or analysing the raw files directly using the Zetasizer software. The particle size distribution can be plotted, and the mean particle size can also be calculated. Additionally, the instrument output for DLS measurements reports the polydispersity index (PDI), which quantifies particle distribution and uniformity of particle sizes within the sample. The Z-average score, which represents the average

zeta potential of the sample, can also be recorded to provide valuable information about the particle surface charge properties. It indicates how the particles may interact or react with charged ions in the medium, offering insights into their stability, aggregation behaviour, and overall colloidal behaviour in various environments.

3.2.6 Exposure of *Daphnia magna* to MP

MP were incorporated into the environment of *Daphnia magna*. The procedure for the experiment is briefly presented as follows:

- 24 h old neonates of *Daphnia* were filtered from running cultures and pooled from different culture jars. The test used broods 3-4 from well-maintained, healthy cultures
- Neonates were selected from the pooled stock and allocated to a 100 ml test jar to eliminate any potential bias from the different culture jars that could affect the results.
- *Daphnia* were grouped, typically with n=10 neonates per vessel for short-term testing, with each treatment having three replicates, and were kept in a fresh medium.
- MP were then introduced to the test vessel at a nominal concentration of 100 mg L-1 (for each MP).
- All the labelled test tubes were then incubated in a standard laboratory condition for 96h at 22 °C.
- The experiment was conducted under two conditions: in the presence and absence of algae as a food source in parallel experiments.

- Under conditions with algae, feeding was performed daily, with each animal receiving 20 μm of algae solution.

- This experiment was carried out in the presence of a control in which *Daphnia* was fed algae. Microplastic control samples, consisting of water and MP, were also prepared to monitor the agglomeration of plastic particles.

- After 24 hours of exposure, *Daphnia* were carefully collected using a clean glass pipette to avoid contamination or damage. The collected animals were then gently transferred onto a clean glass slide, and the surrounding water was reduced to minimise their movement and prepare them for microscopic examination.

- Their gut contents were observed under the Nikon Optical stereomicroscope to assess the ingestion of MP, and high-resolution images were captured with the attached camera and J software and then saved for further analysis and documentation.

- All the exposure and control conditions were in triplicate. The experimental design is shown in Fig 3.1.

3.2.7 Retention and Egestion of MP from *Daphnia* Gut

After exposing *Daphnia* to MP for 24 hours and confirming that their guts were nearly filled with MP particles, the animals were then removed, rinsed three times to remove MP bound to the exoskeleton and then transferred to fresh media without MP and the media was renewed daily to ensure that any MP excreted into the media did not get re-ingested by the *Daphnia*. *Daphnia* and their gut content were observed microscopically every 24 h to monitor the progress of MP excretion until *Daphnia*

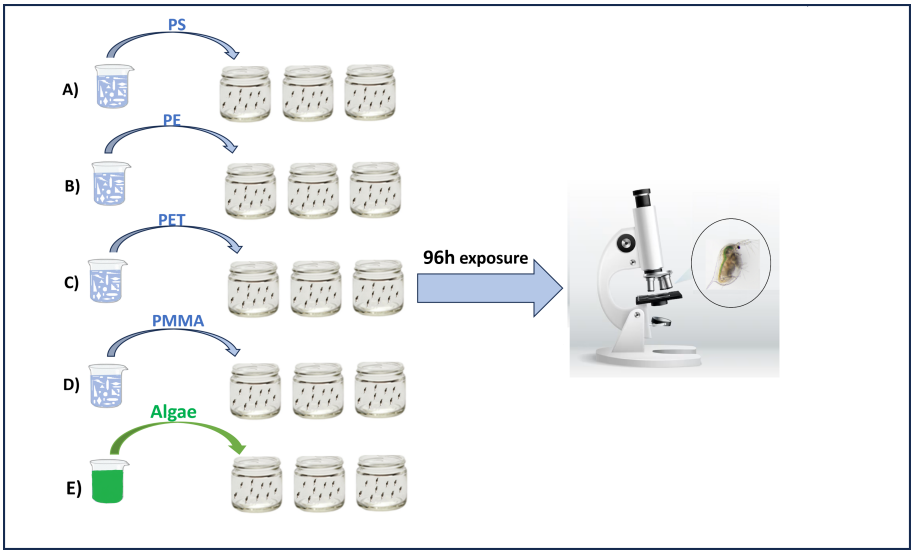


Figure 3.1: Schematic representation illustrating the experimental setup for exposing *Daphnia magna* to four different types of MP: A) exposure to PS (polystyrene), B) exposure to PE (polyethylene), C) exposure to PMMA (polymethyl methacrylate), and D) exposure to PET (polyethylene terephthalate). Panel E depicts the control condition, where *Daphnia* was fed algae alongside the other microplastic exposures. The same experimental design was used for two scenarios: with and without algae as a food source. All microplastics were administered at a concentration of 100 mg L-1. The exposure period lasted 96 hours, including ingestion, retention, and egestion of MP. The gut content of the *Daphnia* was examined microscopically every 24 hours to monitor and document changes over time until animals emptied their gut.

gradually emptied their gut and to estimate the gut retention time. The pictures were captured and saved.

3.2.8 Quality Assurance/ Quality Control for MP Contamination

Environmental pollution studies rely on rigorous quality assurance and quality control (QA/QC) standards to ensure that reported findings accurately reflect pollutant concentrations in various ecosystems. These standards involve implementing strict measures to validate the collected data, thus providing a reliable assessment of environmental contamination levels. However, widely adopted QA/QC guidelines for MP studies are still lacking. A comprehensive QA/QC protocol contains several critical aspects, including contamination prevention, standardisation of sampling and analytical methods, replication, and extensive data quality assessment. To minimise

the risk of cross-contamination in our experiment, Ten key control measures, as described by Prata et al. 2021, were followed in order to ensure the integrity of the results as follows: (1) Wearing 100% cotton lab coats and nitrile gloves,to reduce potential contamination from synthetic fibres and MP; (2) Exclusively using glass or metal laboratory materials, avoiding plastic equipment to prevent the introduction of MP; (3) Properly washing all laboratory materials and equipment with destiled water 3-4 times before use to remove any residual contaminants; (4) Pre-filtering all working solutions to eliminate any MP particles present in the reagents; (5) Covering samples whenever possible, using materials like aluminium foil caps, to prevent contamination from airborne particles; (6) Conducting all laboratory procedures in a laminar flow hood or a clean room (cleaning the workspace with 70% alcohol) with controlled access and ventilation to maintain a contaminant-free environment; (7) Cleaning the surface of filtration system before use to remove any potential contaminants; (8) Utilising blanks as negative controls throughout the procedure to detect any background contamination; (9) Using air-exposed filters to monitor and control for airborne contamination; (10) Carefully washing all materials between sample processing to avoid cross-contamination (Prata et al. 2021; Ziajahromi and Leusch 2022). By thoroughly adhering to these steps, the highest level of data integrity and reliability was ensured, and this experimental procedure was aligned with best practices in MP research to minimise contamination and enhance the credibility of our findings throughout this chapter and the next chapter.

3.2.9 Statistical Analysis

Microsoft Excel 2007 was used to calculate the mean and standard deviation (sd) of MP particle sizes. Excel was also used to analyse DLS data, including calculating the mean particle size from the DLS readings and visualising the data as plots.

3.3 Result and Discussion

3.3.1 Characterisation of MP by Microscope and DLS

All four types of MP were assessed under the microscope to evaluate their physical characteristics. Microscopic images of the irregularly shaped MP used in this study are shown in Fig 3.2. For each type of MP, up to 50 particles were randomly selected and counted, and their size was measured to ensure a statistically representative sample (except for PET, up to 300 particles were selected and counted). For every type of MP, the shape, average size, standard deviation (sd), maximum size, and minimum size of the particles were calculated. The result of microscopic characterisation is shown in Table 3.2. Microscopic analysis revealed that the majority of observed particles across all four types of MP were classified as fragments and fibres, which varied in size and shape, with average proportions of 59.5% and 40.25%, respectively. Fragments were identified as irregularly shaped pieces, likely resulting from the breakdown of larger plastic items, while fibres were long, thin strands (thread-like shapes). Particle diversities also highlight the complex nature of MP and the significant influence of cryogrinding on their physical characteristics. Similar fragmentation processes could occur in the environment through natural weathering and mechanical forces, such as wave action, sediment transport, or anthropogenic activities (Born and Brüll 2022).

The zeta potential is an important parameter in understanding the stability of colloidal systems, as it reflects the surface charge of particles and their ability to resist aggregation under given environmental conditions. A higher zeta potential, whether it is positively or negatively charged (e.g., above +30 mV or below -30 mV), indicates that dispersion is electrostatically stabilised; the particles in a solution experience greater electrostatic repulsion that prevents the particles from aggregating

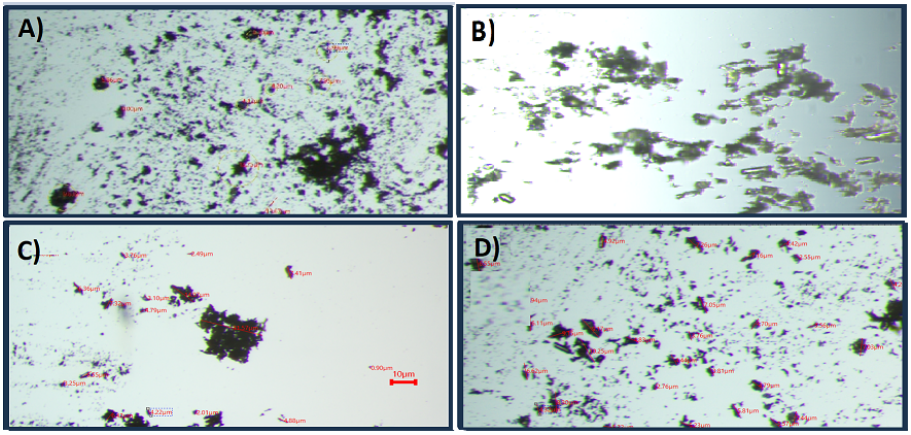


Figure 3.2: During microscopic analysis of four MP particles, it was confirmed that MP are irregular shapes with diverse sizes and shapes. A, B, C and D show PE, PS, PMMA and PET plastic particles, respectively. MP particles were randomly selected and examined under the microscope to assess their sizes and shapes. The size of each MP particle was measured using calibrated microscopic imaging software, which provides precise dimensions for each particle observed. The recorded sizes ranged from small MP to larger particles (ranging from 1 to 60 µm), covering a broad spectrum of potential environmental MP. The red line shows the scale bar.

and minimises flocculation. As a result, the solution becomes more stable because the particles remain dispersed and less likely to clump together. Hence, maintaining a well-mixed and uniform distribution of particles within the solution (Reilly 2022). Due to the lack of homogeneity in the sample, it is expected to have above-average polydispersity (PDI) in our experiment compared to the studies using homogenous particle sizes. Due to the heterogeneous nature of MP in the environment, it is unlikely that using water as a dispersant instead of synthetic surfactants will reduce the environmental accuracy of the exposure. This is especially true when considering that the potential confounding effects of using a surfactant are unlikely to be significant factors in toxicity during environmental exposures. Naturally, MP found in the environment might be more toxic than the plastic used in the laboratory, either due to the additional chemicals the original plastic products contain or the chemicals that the particles can acquire from the environment (Andrady 2017). Zeta potential indicates how reactive the surface layer may be with charged ions in the medium, which, in our case, four used MP were negatively charged. The zeta potential values were -90.63 mV for PE, -33.0 mV for PS, -31.0 mV for PMMA, and -46.4 mV for

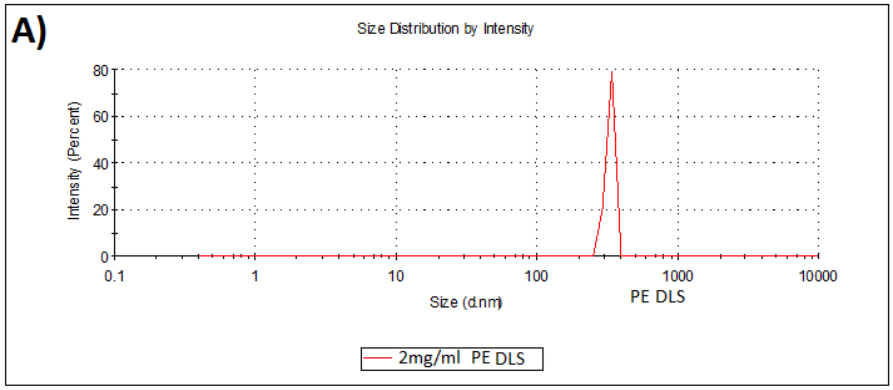
Table 3.2: Presents detailed data on the microscopic characterisation of four different MP, providing important information about their shape, size range, and the prevalence of each shape within each sample.

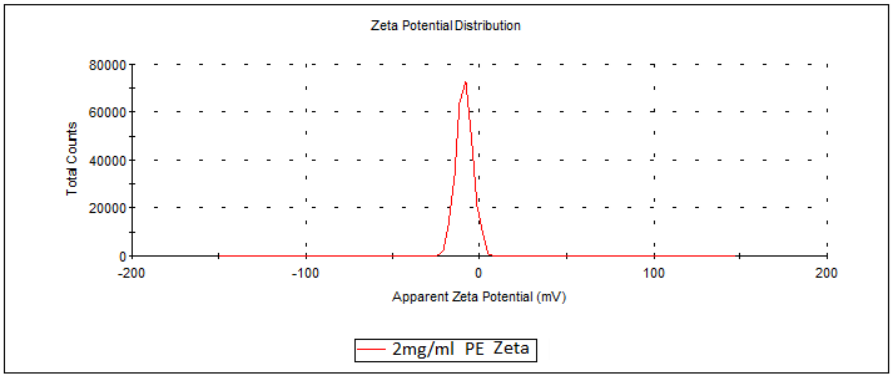
Microscopic Characterisation of MPs								
Shape	**Fiber**				**Fragment**			
MPs	PE	PS	PMMA	PET	PE	PS	PMMA	PET
Average Particles size	17.98	31.12	12.56	20.03	16.21	18.99	23.11	14.93
Standard Deviation (sd)	3.7	2.1	1.68	5.8	3.1	4.7	5.54	1.27
Minimum Size	4.16	5.75	2.56	7.23	5.82	4.76	3.64	8.93
Maximum Size	42.33	50.10	47.77	54.72	45.77	48.90	53.88	45.91
Percentage	32%	47%	49%	33%	68%	53%	51%	66%

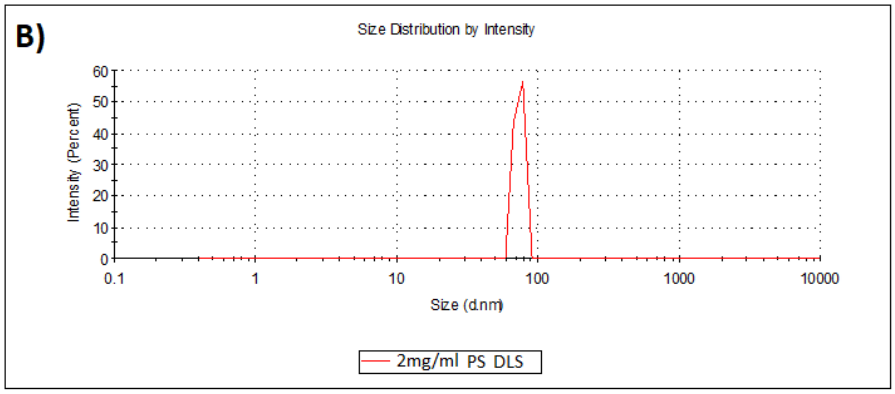
PET. The size intensities were 445.4 d.nm, 255 d.nm, 2295 d.nm, and 132.1 d.nm, respectively. More details of DLS and Zeta potential analysis are provided in Fig 3.3

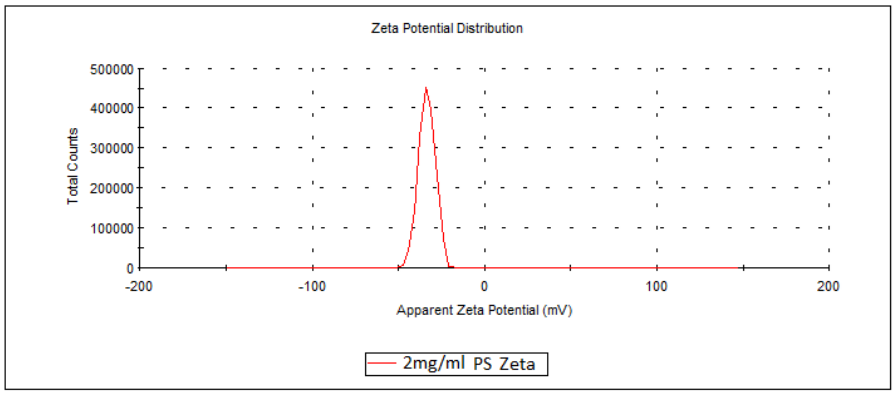
3.3.2 QA/QC for Accuracy and Validity of Dosing MP

Since MP are everywhere, including indoor air, they can easily contaminate samples if no contamination control measures are seriously adopted. Due to the complexity of detecting and quantifying MP in various environmental matrices and biological samples, proper QA/QC measures help prevent contamination, standardise methods, and ensure accurate data interpretation, which is essential for advancing the field and supporting environmental policy decisions. To assess the MP dosing and dispersion technique, the relative standard deviation (RSD) and recovery percentage of MP were calculated after filtering the samples and weighing the filter papers. The recovery of MP particles demonstrated a reasonable efficiency of 88%, 90%, 95% and 92% for PS, PE, PMMA and PET, respectively, across multiple replicates but also showed moderate variability, as reflected by an RSD of 19%. 18%, 15% and 19% receptively. The ideal RSD (precision) should be <10%. However, 10-20%


A)
Size Distribution by Intensity
Intensity (Percent)
80
60
40
20
0
0.1
1
10
100
1000
10000
Size (d.nm)
PE DLS
2mg/ml PE DLS


Zeta Potential Distribution
Total Counts
80000
60000
40000
20000
0
-200
-100
0
100
200
Apparent Zeta Potential (mV)
2mg/ml PE Zeta


B)
Size Distribution by Intensity
Intensity (Percent)
60
50
40
30
20
10
0
0.1
1
10
100
1000
10000
Size (d.nm)
2mg/ml PS DLS


Zeta Potential Distribution
Total Counts
500000
400000
300000
200000
100000
0
-200
-100
0
100
200
Apparent Zeta Potential (mV)
2mg/ml PS Zeta

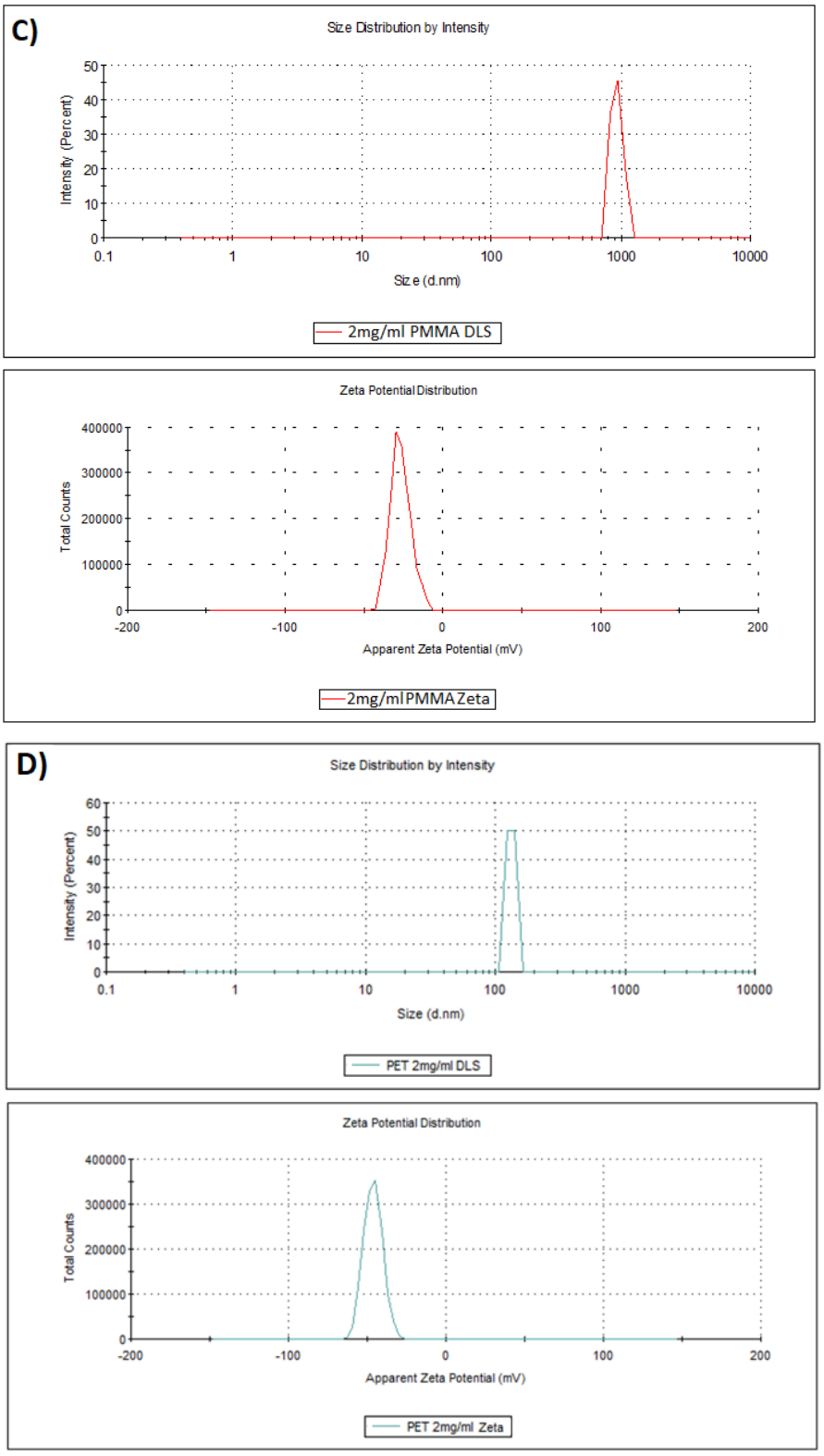


Figure 3.3: Presents DLS and Zeta potential results for PE (A), PS (B), PMMA (C) and PET (E)

is acceptable but can be improved. It may be necessary to further standardise the filtration and weighing procedures, minimise sample handling errors, or refine the recovery protocol to improve RSD.

3.3.3 MP Uptake (Ingestion) by *Daphnia magna*

Daphnia is an ideal test organism for toxicity tests involving nanomaterials, micromaterials, and chemicals because of their keystone role in the environment and their proven sensitivity, which makes them a reliable indicator species. Transparent bodies of *Daphnia* enabled the visualisation of plastic particles within their guts. This transparency allows researchers to observe and track the uptake and retention of plastics in *Daphnia* (F. Nasser and Lynch 2016). In this experiment, the uptake of irregularly shaped MP by *Daphnia magna* was confirmed through microscopic observation. The study confirmed that *Daphnia magna* could ingest all four types of MP (PE, PS, PMMA and PET) within 24 hours, with clear evidence of a full gut observed in both the presence and absence of algae as a food source alongside the control condition (Fig 3.4 and 3.5).

Daphnia ingested four types of MP at almost comparable levels in both feeding conditions, and no significant difference was noted in the ingestion of MP in these two conditions, confirming that *Daphnia magna* is a non-selective feeder regardless of whether algae were present or absent (F. Nasser and Lynch 2016). This finding suggests that the presence of algae does not significantly alter the uptake of MP by *Daphnia*, highlighting their potential vulnerability to MP pollution in various environmental contexts where natural food sources may or may not be available. The observation that *Daphnia* ingested all four types of microplastics at comparable levels under both feeding and non-feeding conditions supports the conclusion that *Daphnia* is a non-selective feeder. This non-selective feeding behaviour is well-documented in

the literature and explains the lack of significant differences in MP ingestion across different polymer types or feeding conditions. The similar ingestion levels observed in this study indicate that *Daphnia* does not actively discriminate between particle types based on polymer identity, and instead ingests MP based primarily on physical characteristics such as size and availability in the water column. This justifies the use of multiple MP types in the experiment and supports the ecological relevance of the findings, as environmental MP also occur in mixed and non-uniform forms.These results underscore MP's ecological risk in aquatic ecosystems, even in environments rich in natural food sources. These findings align with earlier studies indicating that zooplankton readily ingests MP, likely due to the similarity in size between MP particles and their natural food sources. The overlap in size range between MP and typical prey items, such as phytoplankton and debris, suggests that these animals cannot easily distinguish between these synthetic particles and their usual diet (Galloway and Lewis 2016). Consequently, this misidentification leads to the incidental ingestion of MP, which can affect their feeding behaviour, energy intake, and overall health. In interpreting the results, it is important to acknowledge the fundamental biological and functional differences between algae and microplastics (MP). Algae are living organisms with active metabolic processes, capable of growth, photosynthesis, and interactions with their environment, whereas MP are inert, non-living particles that do not possess metabolic activity. Despite these differences, both can influence aquatic organisms through physical presence, ingestion, and surface interactions. In this study, care was taken to consider these distinctions when evaluating biological responses, ensuring that interpretations did not conflate the active biological effects of algae with the passive, potentially disruptive effects of MP. Recognising these differences is crucial for accurately assessing toxicity and ecological risk. Furthermore, the results have broader implications for understanding how both natural and anthropogenic particulate matter can interact with aquatic biota, informing environmental monitoring strategies and risk assessment frameworks.

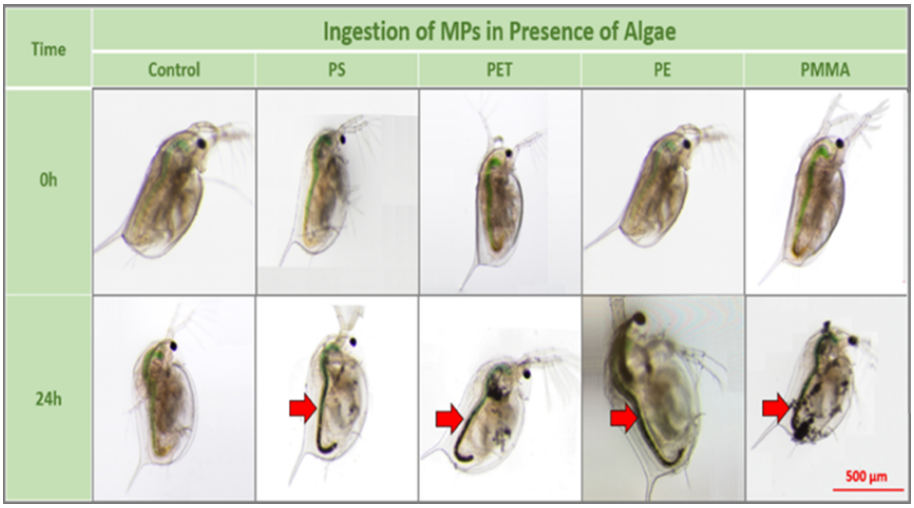


Figure 3.4: Depicts the ingestion of MP in the presence of algae within 24h of exposure. Red arrows pointed to the animals' guts filled with MP. The red bar shows the scale.

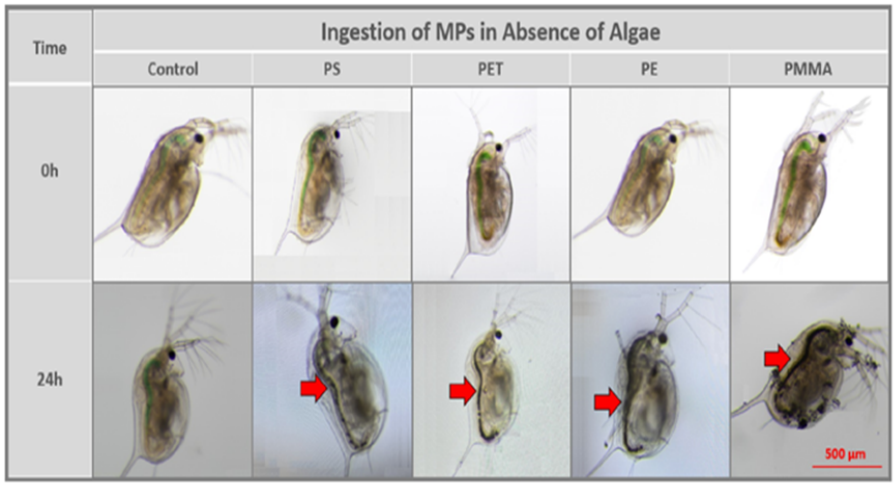


Figure 3.5: Depicts ingestion of MP in the absence of algae within 24h of exposure. Red arrows pointing the animals' guts. The red bar shows the scale.

3.3.4 Retention and Egestion of MP by *Daphnia magna*

Fig 3.6 and Fig 3.7 show the retention and egestion of MP in the presence and absence of algae.

Although *Daphnia magna* was able to rapidly excrete a large amount of ingested MP, a small proportion of MP may become temporarily lodged or retained in specific

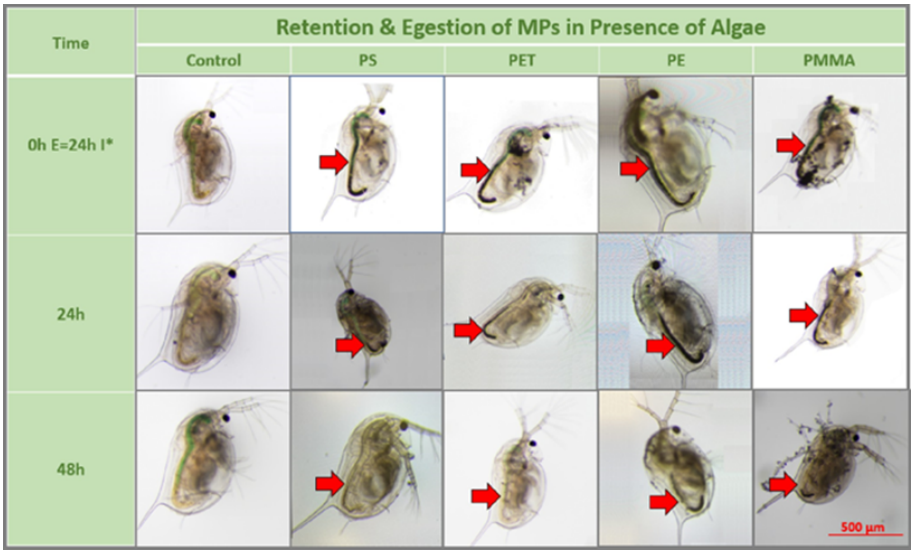


Figure 3.6: Shows the retention and egestion of four MP in the presence of algae as a food source. "I" presents injestion and "E" presents egestion.

sections of the gut, especially lower gut segments, that potentially could be due to their size, shape, and particle composition, or surface properties that interact with gut structures and influence their passage through the gut. This observation was particularly evident in the case of PMMA and PE, where *Daphnia magna* were unable to completely empty their guts. These observations emphasise the complexity of MP egestion dynamics in aquatic organisms and highlight the need for further investigation into the factors influencing retention and excretion rates, especially considering the potential for chronic exposure to affect gut health and overall organismal fitness, as well as for understanding how MP move through aquatic food webs. Frydkjær, Iversen, and Roslev 2017 confirmed that *D. magna* could rapidly ingest both regular and irregular-shaped MP (PE); however, the egestion of regular-shaped MP was faster than that of irregular ones. Gut clearance was longer for irregularly shaped MP particles. Frydkjær, Iversen, and Roslev 2017 found that ingestion of irregular MP fragments significantly declined at 10 g/L compared to 1.0 and 0.1g/l. This difference might be due to notable agglomeration of the MP at high concentrations. Based on their observation, Egestion of irregular fragments was slower than that of MP beads. They also reported that 83% of *Daphnia magna*

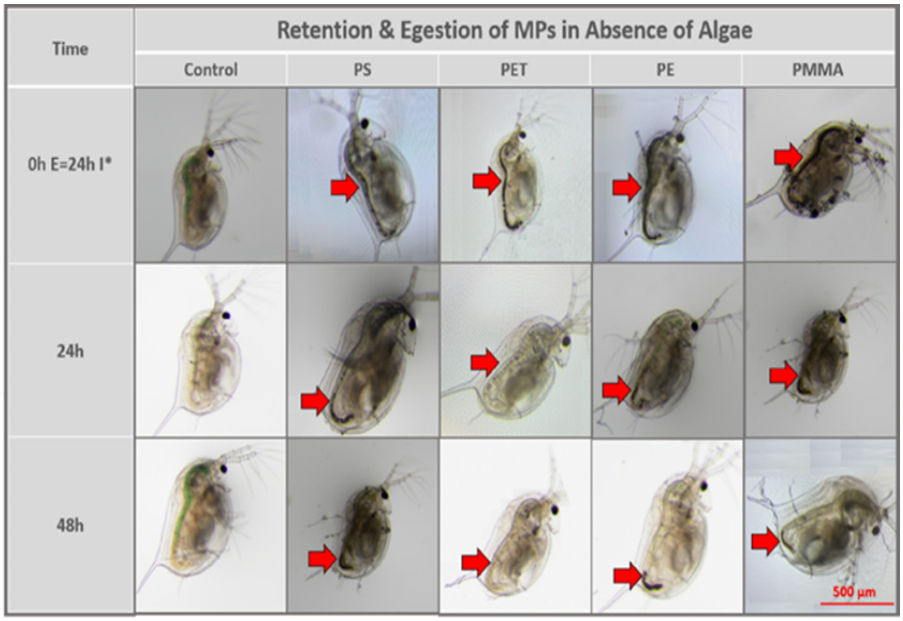


Figure 3.7: Depicts the retention and egestion of four different MP in *Daphnia's* gut in the absence of algae. "I" presents injestion and "E" presents egestion.

fed regular-shaped microplastic particles successfully emptied their guts during the initial depuration period. In contrast, none of the *Daphnia* fed irregularly-shaped MP could clear their guts within 90 minutes, which almost aligns with our findings. It appeared that regularly shaped plastic particles are quickly expelled from animal gastrointestinal tracts, indicating that microbeads are less likely to accumulate within these tracts (Rosenkranz et al. 2009). The significant difference in egestion rates between regular and irregularly shaped MP is likely due to the smoother surface of the spherical beads, which makes it easier for animals to pass through their gut, compared to the spikier, more complex structure of irregular MP particles. In addition, Rosenkranz et al. 2009 showed that the uptake of smaller MP was lower as compared to larger particles in terms of mass. However, in terms of surface area or the number of particles, the uptake of smaller particles was equal to or even greater than that of larger particles. Furthermore, removal of larger beads (1,000 nm) from the *Daphnia* gut was relatively quick, with their concentration dropping by more than 90%.

In this experiment, MP residues were observed to attach to the *Daphnia's* carapace and antennae, particularly in the case of PMMA. It could be due to several physical and chemical factors associated with both the MP and the *Daphnia's* external morphology and environment that are listed as follows: 1) surface properties of MP; some MP have a hydrophobic nature, which makes them prone to sticking to biological surfaces that also exhibit hydrophobic properties. Studies have shown that MP, particularly those with irregular or rough surfaces, can more easily attach to organisms due to large surface area and the presence of microscopic pores and grooves that enhance adhesion (Besseling et al. 2014; Lenz, Enders, and Nielsen 2016); 2) electrostatic interaction also plays a critical role in the attachment process. The surface charge of MP can interact with the charged biological surfaces of *Daphnia*. For example, many plastics are negatively charged in aquatic environments, which can lead to electrostatic attraction with positively charged areas on the exoskeleton or antennae of the *Daphnia* (C. B. Crawford and Quinn 2016); 3) MP are often coated with a layer of biofilm consisting of bacteria or algae. This biofilm can enhance the attachment of MP to the *Daphnia* by creating a sticky matrix that binds the particles to the carapace and antennae (Rummel, Jahnke, et al. 2017). In addition, other factors could be considered in future studies, such as assessing the morphology of the MP particles due to the potential difference in retention and mechanistic toxicity. For example, it has been confirmed that fibres can be retained longer in organisms due to potential tangling (Kukkola et al. 2021).

3.4 Conclusion

In this study, *Daphnia magna* was exposed to PE, PS, PMMA and PET, which are the four most common types of MP in the environment due to their wide application in different sectors. It was confirmed that the *Daphnia* was capable of

ingesting, retaining, and eventually egesting the irregular shaped and diverse size of MP that was produced through cryogrinding of plastic granules. The presence and absence of algae as a food source didn't affect the ingestion pattern of four MP significantly. However, our observations revealed that *Daphnia magna* exhibited varying behaviours in the retention of MP in their gut as MP were egested at different time intervals, highlighting the complexity of their interactions with different plastic particles. This chapter represents a preliminary analysis of MP exposure, providing a foundational understanding of the ingestion and egestion patterns of MP in *Daphnia magna*. This preliminary study was essential to establish before advancing to the next phase of our research, which focuses on the chronic exposure of *Daphnia* to MP and PFAS, investigating the longer-term effects and ecological implications (Chapter 4).

Chapter 4

Toxicological Impacts of Perfluoroalkyl Substances and Microplastics on the Sentinel Species *Daphnia magna*

4.1 Introduction

This chapter builds on the foundational research and findings presented in our published paper, "Combined Toxicity of Perfluoroalkyl Substances and Microplastics on the Sentinel Species *Daphnia magna*: Implications for Freshwater Ecosystems" (Soltanighias et al. 2024. The current chapter aims to address the second and third research questions as a continuation of the previous chapter. The primary objective of this chapter is to investigate the toxicological impacts of polyethylene terephthalate (PET) microplastics, perfluorooctanoic acid (PFOA), and perfluorooctane sulfonate (PFOS), both individually and in combination, on *Daphnia* ecological endpoints. The co-occurrence of PFAS and MP may exacerbate their impact by

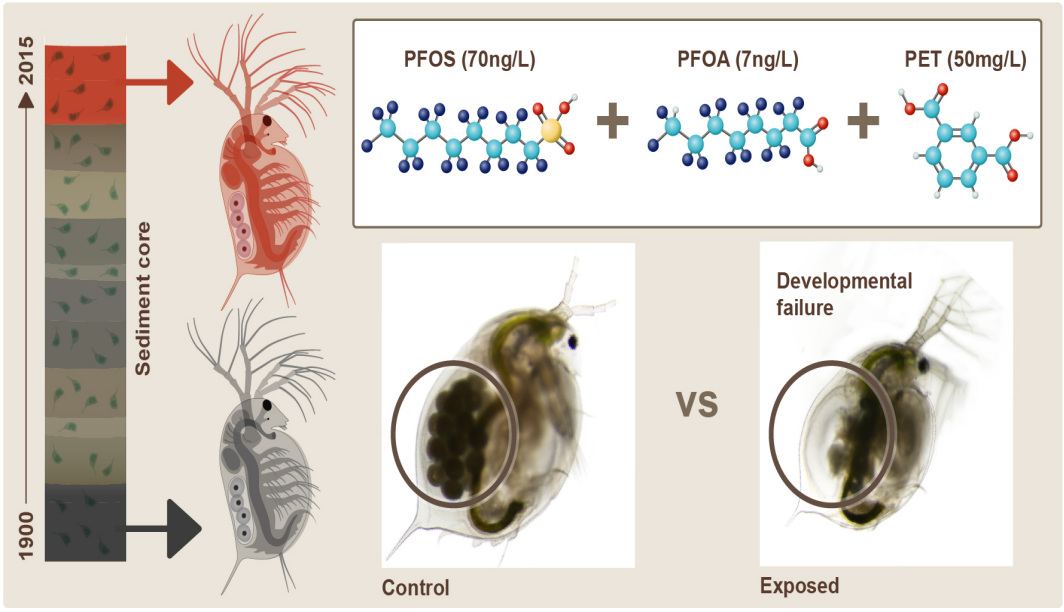


Figure 4.1: Illustrates graphical summary of chronic toxicological effects of MP and PFAS on *Daphnia magna* under environmentally relevant exposure conditions, assessing their chronic effects on key life history traits such as growth, reproduction, and survival

facilitating sorption and/or altering their transport and transformation pathways. Although the occurrence and fate of PFAS and MP in aquatic ecosystems have been individually studied, the toxicity of PFAS mixtures and their co-occurrence with MP remains poorly understood, particularly regarding their long-term effects on organism's life cycles. This study addresses these gaps by reporting the combined toxicity of PFAS and MP on *Daphnia magna*, a sentinel species central to aquatic food webs and a well-established model in ecotoxicology, for the first time. Unlike conventional studies, this research used two *Daphnia* genotypes with distinct histories of chemical exposure. Fig. 4.1 provides a graphical summary of the toxicological impact of MP and PFAS, both individually and in combination, on *Daphnia*, as discussed in this chapter.

Persistent chemicals from domestic and industrial processes have become ubiquitous in the environment due to their long half-lives, recalcitrant nature, and bioaccumulative properties (Robert C Buck, Franklin, Berger, Conder, Cousins, De Voogt, et al. 2011). Among these, perfluoroalkyl substances (PFAS) are increasingly de-

tected in soil, sediment, and water at concentrations ranging from picograms to nanograms per litre (Mojiri, J. L. Zhou, et al. 2023). PFAS are synthetic chemicals used globally in industrial applications such as metal plating, aqueous film-forming foams, and paper and textiles. Their thermal resistance and repellence to water and oil make them valuable for industrial and consumer products, including food packaging, cookware, upholstery, water-resistant clothing, and personal care products (Jane L Espartero et al. 2022). The economic value of PFAS in 2023 exceeds $28 billion, reflecting their extensive use in both commercial and residential applications and a significant increase from $ 1 billion market size in 2006 (Chemsec 2023; Prevedouros et al. 2006).

Due to their widespread use, PFAS are released into the environment during manufacturing, distribution, use, and disposal. Their high persistence and bioaccumulative properties enable global transport through air, water, and land, leading to their detection even in remote locations such as the Arctic and Antarctic regions (Pozo et al. 2017; Vecchiato et al. 2015). Since the discovery of PFAS in Arctic wildlife tissue at the turn of the 21st century (K. S. Kumar et al. 2002) and later in human blood (Sunderland et al. 2019), these chemicals have become substances of very high concern. Recent regulations on PFAS have tightened significantly. In 2024, the US Environmental Protection Agency (EPA) banned PFAS from new production processes, with 13 states adopting the regulation (EPA 2024). New limits for PFOS and PFOA in drinking water are now set at 4 ng/L (USEPA 2024). In the EU, the European Commission's 2020 Chemicals Strategy aimed to phase out PFAS, while the European Parliament set a drinking water limit of 0.5 μg/L in 2019 (OECD, 2019), classifying PFAS as compound of very high concern ((OECD) Esther 2023). The European Food Safety Authority (EFSA) recommends a total weekly intake of no more than 4.4 ng/kg of body weight (Contaminants in the Food Chain (EFSA CONTAM Panel) et al. 2020; COMMISSION 2020; EPA 2024; OECD. 2019; USEPA

2024). Despite these measures, the decades-long use of PFAS and their persistence in the environment mean that a significant proportion of the global population is currently and will continue to be exposed to levels exceeding recommended limits. The Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) has classified PFAS as compounds of very high concern ((OECD) Esther 2023). In 2020, the European Commission's Chemicals Strategy for Sustainability included plans to phase out the use of PFAS in the EU (COMMISSION 2020). Humans are exposed to PFAS through food, water, occupation, and the environment (L. Crawford et al. 2023). Known adverse effects of PFAS include cancer, immune system dysfunction, cardiovascular issues, and developmental problems such as lower birth weight (L. Crawford et al. 2023; Gerwen et al. 2023). Drinking water accounts for up to 14% of exposure to PFOA and 10% to PFOS, while diet accounts for up to 40% of exposure in adults (X. C. Hu et al. 2016; Sunderland et al. 2019). The aquatic environment is the compartment more susceptible to PFAS because it serves as a major transport and transformation medium, particularly for PFAS with high water solubility, such as ionic PFASs (Ahrens and Bundschuh 2014). Despite regulatory restrictions and the mandatory phase-out of long-chain PFAS, these compounds continue to enter aquatic systems from the degradation of PFAS precursors and from historical products still in use. These sources can remobilize PFAS into the water from soil, sediment, and ice (Ahrens 2011). Additionally, emissions of other PFAS, particularly short-chain PFAS, are on the rise. Short-chain PFAS are more mobile in aquatic environments, while long-chain PFAS, which are more hydrophobic, tend to bind to particles. PFOS and PFOA are the most frequently detected PFAS in freshwater ecosystems and have been shown to exert toxic effects on freshwater organisms (Ahrens 2011; Ahrens and Bundschuh 2014; N. Wang et al. 2023). Typically, PFOS is found in higher concentrations and is more toxic than PFOA to aquatic species, as evidenced by acute toxicity tests (K. Ji et al. 2008; Mojiri, J. L. Zhou, et al. 2023).

The impact of PFAS is exacerbated by the presence of other persistent compounds, such as microplastics (MP), which can facilitate their sorption (Bhagwat et al. 2021). The enhanced toxicity of MP for other persistent contaminants, including PFAs, can be mediated by biofilm formation, which intensifies the vector role of MP (Qi et al. 2021). Biofilms can also bioconcentrate PFAS in freshwater environments (Munoz et al. 2018), and alter their transport and transformation (Dai, J. Zhao, C. Sun, Diying Li, et al. 2022). Additionally, MP and PFAS are often released simultaneously from consumer products, such as waterproof textiles, further exacerbating their environmental impact (Kang et al. 2023). Despite significant progress in understanding the occurrence and fate of PAFS and MP in aquatic ecosystems, the toxicity of PFAS mixtures and their co-occurrence with other high-concern pollutants remains poorly understood, especially over organisms' life cycles (Mojiri, J. L. Zhou, et al. 2023). Few in vitro studies have investigated mixture effects, primarily focusing on PFAS mixtures (e.g., G. Ding et al. 2013). These studies revealed that mixtures can exert higher toxic effects than individual compounds, showing additive or synergistic effects depending on mixture components, target species, and dose ratios (Garvey et al. 2023; Ojo, Peng, and Ng 2021). While the combined effects of PFOS and PFOA have been empirically studied in human cell lines (X.-Z. Hu and D.-C. Hu 2009), fish, birds, and mammals (Suja, Pramanik, and Zain 2009), most research relies on models such as concentration addition and independent action (Escher, Braun, and Christiane Zarfl 2020). These models, which assume no interactions in the analysed mixtures, may overestimate or underestimate the combined toxic effects of interacting chemicals. The scarcity of empirical studies on these combined effects can lead to inaccurate risk assessments. This study aims to address this gap by using the sentinel and keystone species *Daphnia*, a well-established ecotoxicology model, to define safe concentrations of chemicals in the environment (M. Abdullahi, X. Li, et al. 2022).

Daphnia is central to freshwater food webs, making it an ecologically relevant species (M. Abdullahi, X. Li, et al. 2022; Altshuler et al. 2011). With an exceptionally long dormancy, *Daphnia* allows us to access past populations with different exposure histories to environmental pollutants (Cuenca Cambronero et al. 2018). Capitalising on these properties, the chronic toxicity of perfluorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA), and polyethylene terephthalate microplastics (PET), were assessed both as single chemicals and in mixtures, on two genotypes of *Daphnia* resurrected from the sedimentary archives of Lake Ring. This lake has a well-documented history of anthropogenic impact over the past century (Cuenca Cambronero et al. 2018). One genotype originated from a semi-pristine environment and is naive to chemical pollution. At the same time, the other had been exposed to chemical pollution for multiple sexual generations, referred to as 'experienced' hereunder (M. Abdullahi, J. Zhou, et al. 2022). Irregular microplastics, including fibres and fragments, were used, which closely represent MP in the natural environment and environmentally relevant concentrations of PFOS and PFOA, two commonly co-occurring PFAS in water (G. Ding et al. 2013). While single PFAS have previously been shown to impair growth in *Daphnia*, this study is the first to investigate the combined effects of these chemicals, providing a comprehensive understanding of the chronic toxicity of two-way and three-way mixtures of high-concern chemicals that co-occur in the natural environment. Given *Daphnia's* central role in supporting freshwater food webs, findings of this study have potential implications for the entire freshwater community, including primary producers and consumers of *Daphnia*.

4.2 Materials and Methods

4.2.1 Study System

The *Daphnia magna* genotypes used in this study were previously revived from a biological archive of Lake Ring, Denmark (550 57´ 51.83´´ N, 9035´46.87´´E), a shallow mixed lake with a well-documented history of anthropogenic impact over the past century (Cuenca Cambronero et al. 2018). The lake was semi pristine between the 1900s and 1950s (semi-pristine phase; SP); it experienced high levels of eutrophication from the 1950s to the 1970s (eutrophic phase; EP). From the 1980s to the 1990s the lake suffered from pesticides inflow (1980-1990; pesticide phase; PP). In modern times, the lake experienced a partial recovery from eutrophication but still received some agricultural run-off (Recovery Phase; RP) (Cuenca Cambronero et al. 2018; Eastwood et al. 2023). Two *D. magna* genotypes, one from the pristine and one from the recovery phases were used in this study. These were chosen to assess the compounded effect of prior exposure to chemical pollutants on the tolerance to modern chemical pollutants, namely PFOS, PFOA and PET. This was done by comparing the impact of each of these chemicals and their two-ways and three-ways mixtures on the fitness of the naïve (LRII36_1) and the experienced genotype (LRV0_1) in common garden experiments following (M. Abdullahi, J. Zhou, et al. 2022). The concentrations of chemicals tested were within the range of environmentally relevant concentrations, especially for PFOS and PFOA (Ahrens 2011; Mojiri, J. L. Zhou, et al. 2023).

4.2.2 Characterisation of Contaminants

PET granules (Sigma-Aldrich, USA; density = 1.68×10^{12} ng/L ; CAS No 25038-59-9) were cryogenically grounded in liquid nitrogen in a Freezer/Mill 6775 (United States), SPEX SamplePrep to obtain irregularly shaped particles. These were size selected with stainless steel filters to retain particles between 38 μm and 50 μm, easily ingestible by *Daphnia*. Following the grounding process, the PET particles were characterised using both microscopy and Dynamic Light Scattering (DLS; Malvern Nano Series Zetasizer). A concentrated solution of 1mg/ml of PET was examined using a Digital OPTIKA™ IM-3LD4D PROVIEW, Italy) microscope with multiple lenses Plan Fluor (4X, 10X, 20X, 40X). Visualisation and measurements of MP were conducted using the bespoke "Optika™ ProView Image Analysis" software for the built-in 6.3 megapixel USB 3.0 CMOS digital camera. The average size and shape of the particles used in the experiment were determined based on the analysis of up to 300 randomly selected particles. A concentrated solution of PET particles 1 mg/ml was transferred to three replicated glass petri dishes for microscopic observations. Up to 100 fragments and fibres were randomly selected from each plate, equally split among the four quadrants of each plate. The selected MP were sized using the Optika™ ProView Image Analysis software. To confirm the size and surface charge of the MP, 5 mL solutions of 2×10^9 ng/L of PET were prepared and subjected to analysis using Dynamic Light Scattering. Subsequently, four replicates of 1 mL aliquots of the solution were tested on the DLS to determine average particle size, zeta potential, and polydispersity index (more details in Chapter 3).

The nominal concentration of PFOS and PFOA in single chemical and mixture exposures were, respectively, 70 ng/l and 7 ng/l. A concentrations that reflect the ones observed in lakes, where PFOS occurs at typically ten times higher concentration than PFOA in Europe (PFOS average concentration: <100 ng/l; PFOA

average concentrations: <10 ng/l) (Ahrens 2011) was used. Before exposing *Daphnia* to these chemicals, the medium spiked with these concentrations to confirm the nominal concentrations were quantified. Media spiked with PFOS and PFOA was subjected to Solid Phase Extraction followed by LC-MS/MS (Shimadzu LC-20AB prominence high-pressure liquid Chromatography: Shimadzu Kyoto, Japan), coupled with a Sciex API 2000mass Spectrometer (Applied biosystem Foster City, CA, USA) analysis according to a previously published method (M. Abdullahi, Stead, et al. 2023; Harrad et al. 2019). Briefly, the SPE cartridges were first conditioned with 6 mL of methanol (0.1% NH4OH) and rinsed with 6 mL of MilliQ water. The internal (surrogate) standards used were M8PFOA+M8PFOS 1 ng/ul, and the recovery determination (syringe) standard were M4PFOS 1 ng/ul (Wellington Laboratories). Duplicate samples per single chemical and mixtures were analysed and quantified.

All experiments were conducted in glass jars, following strict QA/QC protocols. To minimise background contamination by MP and PFAS in the laboratory, pure cotton lab coats and nitrile gloves were worn. Furthermore, all sample processing took place on a laminar flow bench, which was regularly wiped with 90% ethanol. Laboratory equipment and consumables were rinsed with Milli-Q water prior to use. Samples were processed in batches of five, with a 'method blank' (Milli-Q water in a clean glass bottle) included in each batch to monitor potential contamination during sample preparation. Additionally, a 'recovery sample' (Milli-Q water spiked with a known quantity of MP/PFAS) was analysed for each 20 samples to assess recovery rates and background interference. A 'field blank', consisting of experimental medium without MP/PFAS, was also included with every 20 samples to check for contamination from the medium or laboratory.

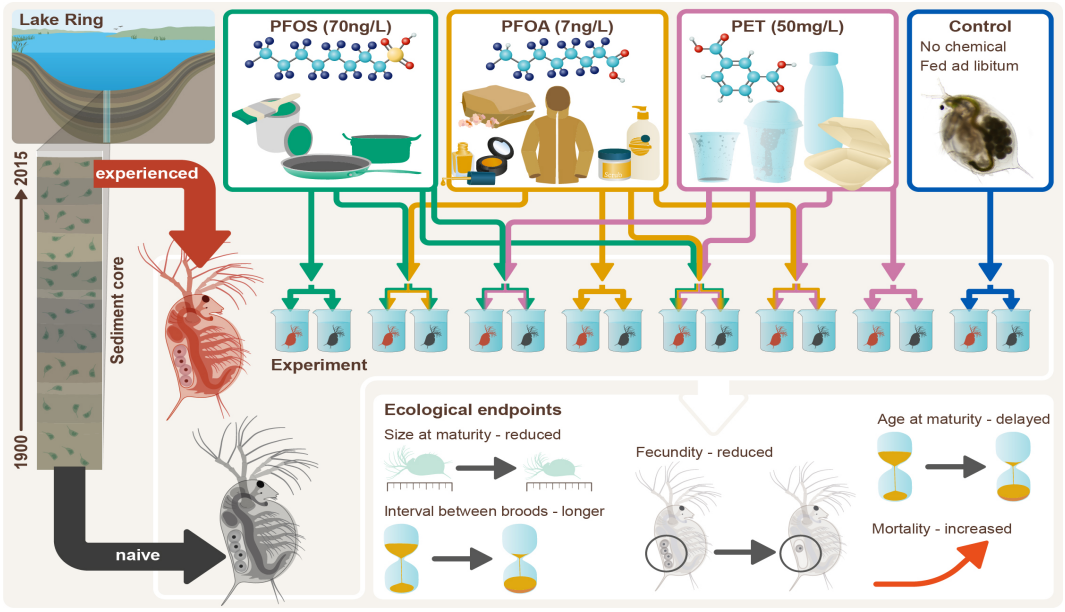


Figure 4.2: Experimental design. Populations separated in the time of *Daphnia magna* were previously resurrected from a sedimentary archive of Lake Ring (Denmark). The lake experienced four phases due to environmental impact. A genotype from the pristine (naive, black) and one from the recovery phase (experinced, red) of the lake were used in this study to assess the impact of PFOS (70 ng/L); PFOA (7 ng/L); PET (50 mg/L) and two-way and tree-way mixtures on *Daphnia's* ecological endpoints

4.2.3 Assessing the Chronic Toxicity of Single Chemicals and Mixtures

The toxicity of PFOS (70 ng/l), PFOA (7 ng/l), PET (50 mg/l) and their two-way and three-way mixtures were tested on the two *Daphnia* genotypes for the duration of their life cycles (until they released their second brood) Fig. 4.2. To ensure a homogeneous PET solution, a stock solution of 5 g/l was prepared and gently stirred on a magnetic stirrer before dosing the experimental vials at 50 mg/l (more details in Chapter 3).

Before the chronic toxicity test, a qualitative assessment of *Daphnia's* ingestion and egestion of microplastic over 72h was performed. We exposed three clonal replicates of the two genotypes used subsequently in the chronic toxicity tests to 50

mg/L of PET over 72h. Ingestion of MP was monitored every 24h in absence of feed. This experiment was run alongside a control in which *Daphnia* were fed algae. After 24h, *Daphnia* was transferred to clean medium every 24h, to visualise egestion of PET by microscope imaging. All images were captured using J software (Rueden et al. 2017). The medium was renewed every 24 h and up to 72h after the last PET ingestion to estimate the gut retention time of PET and the time to full egestion.

Clonal replicates of the two genotypes were obtained from the stock collection of the University of Birmingham, where they are maintained in standard laboratory conditions: 16:8-hr light-dark photoperiod; 0.8 mg L-1 *Chlorella vulgaris* fed weekly; ambient temperature: 10°C (Cuenca Cambronero et al. 2018). The growth medium used was borehole water, collected from a deep aquifer well and showing stable physico-chemical properties (borehole water chemistry analysis is provided in Appendix B, 2). Before commencing the exposures, clonal replicates of the two genotypes were acclimated in common garden conditions for three generations to the following conditions to reduce interference from maternal effects and synchronize reproduction: 16:8-hr light-dark photoperiod; 0.8 mg L-1 *C. vulgaris* fed daily; ambient temperature: 20± 2 °C. After three generations in these conditions, 24h old randomly selected juveniles from the second or following broods were assigned to experimental conditions.

A total of 64 exposures were completed, including seven treatments and one control group for both genotypes and four clonal replicates per genotype and condition Fig. 4.2. During the experiment, the growth medium (borehole water) was replenished every other day and spiked with the same concentration of chemicals at each medium change to ensure constant exposure throughout the experiment. Fitness-linked life history traits were measured during the experiment covering the *Daphnia's* life cycle (until the release of the second brood): age at maturity (the first time the parthenogenetic eggs are released in the brood pouch); size at maturity

(distance from the head to the base of the tail spine); fecundity (number of juveniles across the first two broods); the interval between broods (days between the first and second brood) and mortality. Size was measured after the release of the second brood (end of the test) using ImageJ software (Rueden et al. 2017).

4.2.4 Statistical Analyses

Univariate reaction norms were used to assess the effect of genotype (G), treatment (T-PFOS; PFOA; PET; PTE+PFOA; PTE+PFOS; PFOA+PFOS; PTE+PFOA+ PFOS) and their interaction terms (G x T) on the five fitness-linked life history traits (age at maturity, size at maturity, fecundity, interval between broods and mortality) using a two-way ANOVA. A linear mixed effect model (LMMs) was used with clonal replicates nested within genotype using the "lmerTest" package in R version 4.3.1 (Kuznetsova, Brockhoff, and Christensen 2017). Before applying the LMMs model, the data were checked for normality using the Q-Q plots (residual vs. fitted value) (Zuur, Ieno, and Elphick 2010).

The main effects of treatment, genotype and their interactions were visualised using 'reaction norm' plots. The mortality rate per treatment and genotype was estimated with the survival model fit using the psm function in the "rms" package in R version 3.6.0 (Harrell Jr 2023). The day of mortality and mortality events were combined as a response variable, while the term "genotype" was treated as a fixed effect. The mortality curves per generation were plotted with the survplot function from the rms package in R v.6.7.1 (Harrell Jr 2023). A separate model was fitted to each treatment.

Effects on the overall fitness were calculated using multivariate statistics (MANOVA) by combining the life history traits (age at maturity, size at maturity, fecundity, and

interval between broods) as response variable (y) and treatment and genotype as fixed terms (y treatment * genotype). As per the ANOVA analysis of individual fitness-linked life history traits, a nested linear mixed effect model (LMMs) was used with clonal replicates as random effects nested within genotype using the "lmerTest" package in R version 4.3.1 (Kuznetsova, Brockhoff, and Christensen 2017). The multivariate reaction norms were visualised using phenotypic trajectory analysis (PTA) plots to describe the difference between control and treatments in terms of magnitude and direction of change following (Adams and Collyer 2009).

4.2.5 Mixture Effects

In the common garden experiments, chemical mixtures (two-way and three-way combinations) were investigated to confirm their synergistic, antagonistic, or additive effects on *Daphnia* ecological endpoints (fitness-linked life history traits). To assess this, a null model of additivity (Côté, Darling, and Brown 2016) was used, which contrasts the expected additive effect of two or more compounds (i.e., the sum of each compound individual effects) on fitness linked life history traits with the empirical observed effect of mixtures. These inferences were possible because the same set of genotypes were exposed to the single chemicals and mixtures. The null addictive prediction of the joint effect of stressors combinations was calculated as:

$$Emix = EA + EB + EC \tag{4.1}$$

where EA, EB and EC are the effects of single compounds and Emix is the mixtures effect. For each trait 'y' (age at maturity, size at maturity, fecundity, and interval between broods) standardised effect sizes were used corresponding to

(ytreatment - ycontrol)/σ, where σ is the shared standard deviation of the pooled control and treatment trait values per treatment following (Côté, Darling, and Brown 2016). This analysis could not be done on mortality because individuals killed by one chemical cannot be killed by another simultaneously, and hence, the additive model cannot apply to this trait. The effect of mixtures was considered additive if the predicted joint effect 'Emix' was within the 95% confidence intervals of the observed effect from the multiple stressors and not significantly different from the null model. The effect of multiple stressors was antagonistic if the predicted joint effect was significantly smaller than the null model and synergistic if the predicted joint effect was significantly larger than the null model. Welch Two sample t-test was used to assess significant departure from the null model. This test compares the means of the predicted null model and the empirical observed values for each fitness-linked life-history trait.

4.3 Results

4.3.1 Characterisation of Chemicals

Results from blanks and recovery samples for both PET and PFAS are in Table 3 in Appendix C. None of the blank concentrations exceeded 5% of the average PET count or PFAS concentration in their respective sample batches, so no blank correction was necessary. Recovery of spiked MP and PFAS ranged from 79% to 117%, demonstrating good analytical performance (Appendix C, Table 3). The microscopic observation and DLS were used to confirm the physicochemical properties, size and surface charge of the PTE particles, revealed that 33% of the particles were fibres with an average size of 20.03 μm, and 67% were fragments with an average size of 14.93 μm (Table 4 in Appendix C). The DLS analysis showed that the PET particles

were negatively charged and that the zeta potential (electrophoretic mobility of particles in an ionic solution) was -46.4 mV. The size intensity was 132.1 (d.nm). The fibres size ranged between 7.23 μm and 54.72 μm, whereas the fragment size ranged between 8.93 μm and 45.91 (Table 4, Appendix C). Microscopic visualisation of MP particles is shown in Chapter 3 with more details. Bioaccessible concentrations of PFOS and PFOA in the media were confirmed with LC-MS/MS to not depart from the nominal concentrations (Appendix C, Table 5).

4.3.2 Assessing the Chronic Toxicity of Single Chemicals Mixtures

The preliminary experiment assessing the ingestion and egestion capacity of PET particles in *Daphnia* showed that the organisms could ingest and retain the particles for up to 72 h. Once transferred to a microplastic-free medium, their guts were emptied within 24 h (Fig 1, Appendix C). The effects of single, two-way and three-way mixtures of PFOS, PFOA and PTE Were studied on *Daphnia* fitness-linked life-history traits. Exposures to PET, PFOS and PFOA as single chemicals, all had a significant treatment effect (Table 4.1; MANOVA; Fig 4.3). This overall response for PET was driven by a significantly smaller size at maturity, a lower fecundity, a longer interval between broods and higher mortality in the exposed *Daphnia* (Table 4.1; ANOVA; Fig 4.4, PET). The response to PFOA was driven by a delay in sexual maturation, smaller size at maturity, reduced fecundity and longer interval between asexual reproduction (interval between broods) in the exposed *Daphnia* (Table 4.1;, ANOVA; Fig 4.3, PFOA). No mortality was observed in exposures to PFOA. In addition to a treatment effect, PFOS also induced a significant different response between genotypes (Table 4.1; MANOVA; Fig 4.3, PFOA). For this treatment, a significantly smaller size at maturity, lower fecundity, longer interval between broods and higher mortality were observed; in addition, age at maturity, interval between

broods and mortality significantly differed between genotypes (Table 4.1; MANOVA; Fig 4.3, PFOS). There were no significant interaction effects (GxT) in the single chemical exposures (Table 4.1; MANOVA).

Whereas all chemical mixtures showed a significant treatment effect, genotypes responded differently to PET+PFOA and PFOA+PFOS; in these treatments, a significant interaction term (G x T) was found, indicating a genotype dependent response to treatment (Table 4.1; MANOVA; Fig 4.3). The individual life-history traits contributing to these patterns differed among treatments. In the PET+PFOA, we observed a genotype dependent response in age and size at maturity and a response to treatment of the individual life history traits largely reflecting the patterns observed in the respective single stress exposures (Table 4.1; ANOVA; Fig 4.4; PET+PFOA). *Daphnia* exposed to PET+PFOS has significantly smaller size at maturity, lower fecundity, and longer interval between broods, but did not experience mortality, with a significant genotype dependent response for age at maturity (Table 4.1; ANOVA; Fig 4.4; PET+PFOS). The combination of PFOS and PFOA caused a smaller size at maturity, lower fecundity and longer interval between broods (Table 4.1; ANOVA, Fig 4.4; PFOS+PFOA). This treatment also showed a genotype dependent response in size at maturity and interval between broods (Table 4.1; ANOVA, Fig 4.4; PFOS+PFOA). A reduced number of offspring was paired with developmental failure, resulting in a high number of aborted broods in the PFOS treatment (Appendix C, Fig 2). Finally, the overall fitness response in the three-way chemical mixture was driven by a delayed maturation, smaller size and reduced fecundity; a genotype-dependent response was observed for size at maturity (Table 4.1; ANOVA, Fig 4.4; PFOS+PFOA+PET). Mortality did not significantly increase in the two-way and three-way chemical mixture treatments (Fig 3, Appendix C).

	MANOVA			ANOVA														
				Age at maturity (Day)			Size at maturity (mm)			Fecundity			Brood interval (Day)			Mortality		
	df	F	*p*-Value	df	F	*p*-Value	df	F	*p*-Value	df	F	*p*-Value	df	F	*p*-Value	df	F	*p*-Value
PET																		
Treatment (T)	1	38.67	**0.00**	1	1.79	0.21	1	122.08	**0.0002**	1	155.64	**0.0009**	1	9.56	**0.01**	1	**4.98**	**0.03**
Genotype (G)	1	1.29	0.37	1	0.52	0.49	1	1.29	0.30	1	0.04	0.85	1	0.03	0.86	1	0.54	0.46
T × G	1	2.67	0.14	1	5.61	**0.04**	1	0.03	0.86	1	0.78	0.44	1	9.56	**0.01**	1	0.00	1.00
PFOA																		
Treatment (T)	1	126.95	**2.86E-07**	1	5.41	**0.04**	1	244.67	**7.33E-09**	1	194.36	**2.46E-08**	1	35.54	**9.44E-05**		na	
Genotype (G)	1	7.17	0.01	1	2.76	0.12	1	2.30	0.16	1	3.17	0.10	1	11.60	**0.006**		na	
T × G	1	1.17	0.39	1	2.76	0.12	1	0.26	0.62	1	1.66	0.22	1	0.73	0.41		na	
PFOS																		
Treatment (T)	1	124.55	**6.68E-06**	1	7.12	**0.03**	1	171.31	**3.66E-07**	1	165.48	**4.25E-07**	1	117.34	**1.83E-06**	1	4.56	**0.03**
Genotype (G)	1	4.00	**6.45E-02**	1	4.95	**0.05**	1	0.82	0.39	1	1.29	0.29	1	6.94	**0.03**	1	4.56	**0.03**
T × G	1	2.21	0.18	1	0.00	1.00	1	0.12	0.74	1	2.09	0.18	1	0.77	0.40	1	0.00	1.00
PET+PFOA																		
Treatment (T)	1	110.46	**1.21E-07**	1	6.40	**0.03**	1	579.63	**3.37E-07**	1	98.58	**6.03E-05**	1	6.51	**0.04**		na	
Genotype (G)	1	1.56	**2.66E-01**	1	0.40	0.54	1	3.21	0.12	1	0.22	0.65	1	0.00	1.00		na	
T × G	1	8.02	**0.005**	1	6.40	**0.03**	1	22.33	**0.003**	1	0.89	0.38	1	0.92	0.38		na	

PET+PFOS																	
Treatment (T)	1	29.05	**8.17E-05**	1	10.82	**0.01**	1	132.23	**3.71E-05**	1	69.56	**0.0001**	1	13.91	**0.009**	1	1.53	0.22
Genotype (G)	1	2.69	0.11	1	1.20	0.30	1	0.43	0.54	1	0.00	0.26	1	0.12	0.74	1	1.53	0.22
T × G	1	3.35	0.07	1	6.55	**0.03**	1	5.16	0.07	1	2.70	0.15	1	1.33	0.29	1	0.00	1.00
PFOA+PFOS																	
Treatment (T)	1	47.57	**4.68E-06**	1	2.45	0.17	1	95.90	**4.49E-07**	1	124.16	**1.10E-07**	1	157.64	**2.92E-08**		na	
Genotype (G)	1	16.46	**0.0004**	1	4.74	0.07	1	7.75	**0.02**	1	0.03	0.868743	1	106.91	**2.49E-07**		na	
T × G	1	4.31	**0.032136**	1	2.45	0.17	1	1.98	0.18	1	0.03	0.868743	1	66.00	**3.21E-06**		na	
PET+PFOA+PFOS																	
Treatment (T)	1	54.46	**7.55E-05**	1	17.54	**0.00**	1	14.27	**0.009**	1	99.46	**0.0001**	1	0.11	0.75	1	3.45	0.06
Genotype (G)	1	1.69	0.27	1	0.88	0.37	1	2.82	0.14	1	5.58	0.06	1	2.10	0.18	1	0.06	0.81
T × G	1	2.25	0.18	1	0.88	0.14	1	1.36	**2.43E+26**	1	4.07	0.10	1	1.37	0.27	1	0.00	1.00

Table 4.1: Analysis of variance. Multivariate (MANOVA) and univariate (ANOVA) analysis of variance, testing response of five fitness-linked life history traits to single chemicals and mixtures. The effect of treatment (T), genotype (G) and their interaction term (T x G) on the life history traits is shown. The life history traits assessed were age at maturity (days), size at maturity (mm), fecundity (number of offspring produced in the first two broods), interval between broods (days) and mortality. The chemicals tested are PET 50 mg/l, PFOA 70 ng/l, PFOS 7 ng/l in isolation and combination. Significant p-values are shown in bold. na - no mortality occurred in the specific treatment.

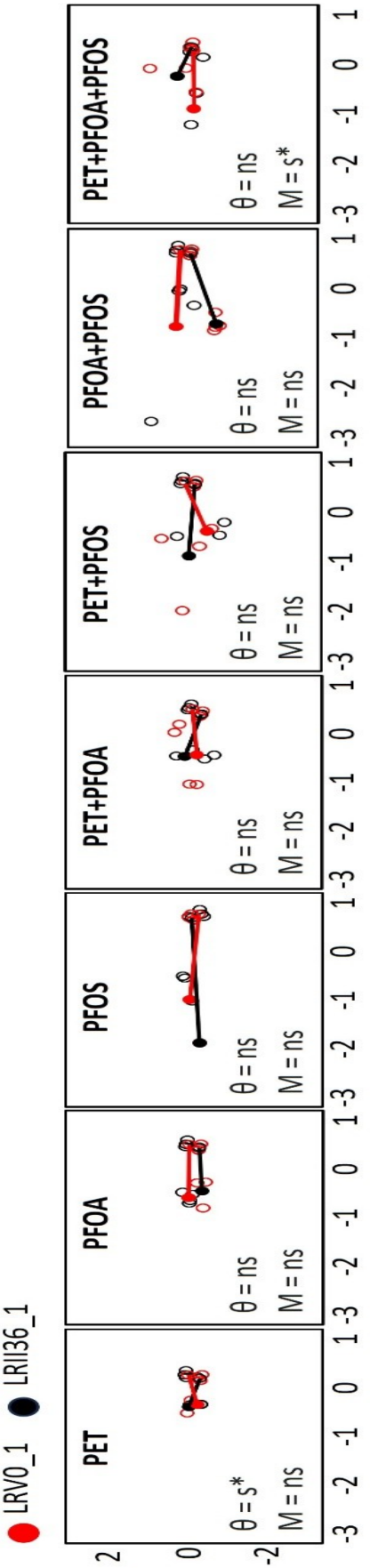


Figure 4.3: Phenotypic trajectory analysis (PTA) on the *Daphnia magna* genotypes following chronic toxicity tests to three single chemicals (PET [50 mg/L], PFOA [7ng/L], PFOS [70 ng/L] and their two-way and three-way mixtures. The multivariate response of five fitness-linked life history traits is shown. Open circles represent the control (nonexposed clonal replicates), and full circles represent the exposed clonal replicates. Genotype centroids are connected by reaction norms (solid lines), showing phenotypic change in direction and length. Differences among genotypes in terms of magnitude (M) and direction (θ) $of response are shown (s : significant, ns : non - significant). Genotypes are recolor - coded.$

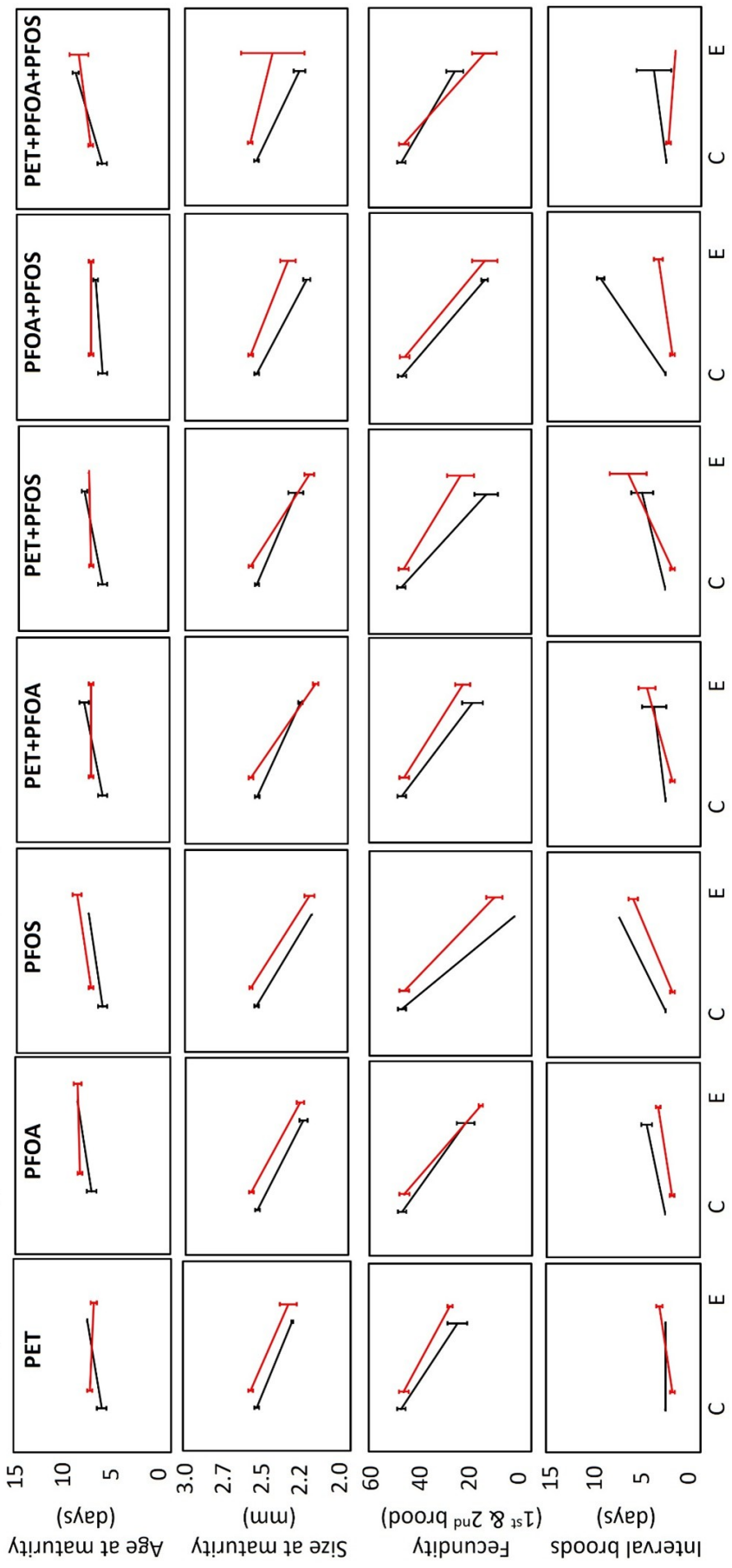


Figure 4.4: Univariate reaction norms. Univariate response of four fitness-linked life history traits between control and exposed Daphnia genotypes to single chemicals [PFOS (70 ng/L); PFOA (7 ng/L); PET (50 mg/L) and two-way and three-way mixtures. Size at maturity (mm), age at maturity (days), fecundity, and the interval between broods (time elapsed between the first two broods) are shown. Average and SD per genotype across four biological replicates are shown. Genotypes are color-coded.

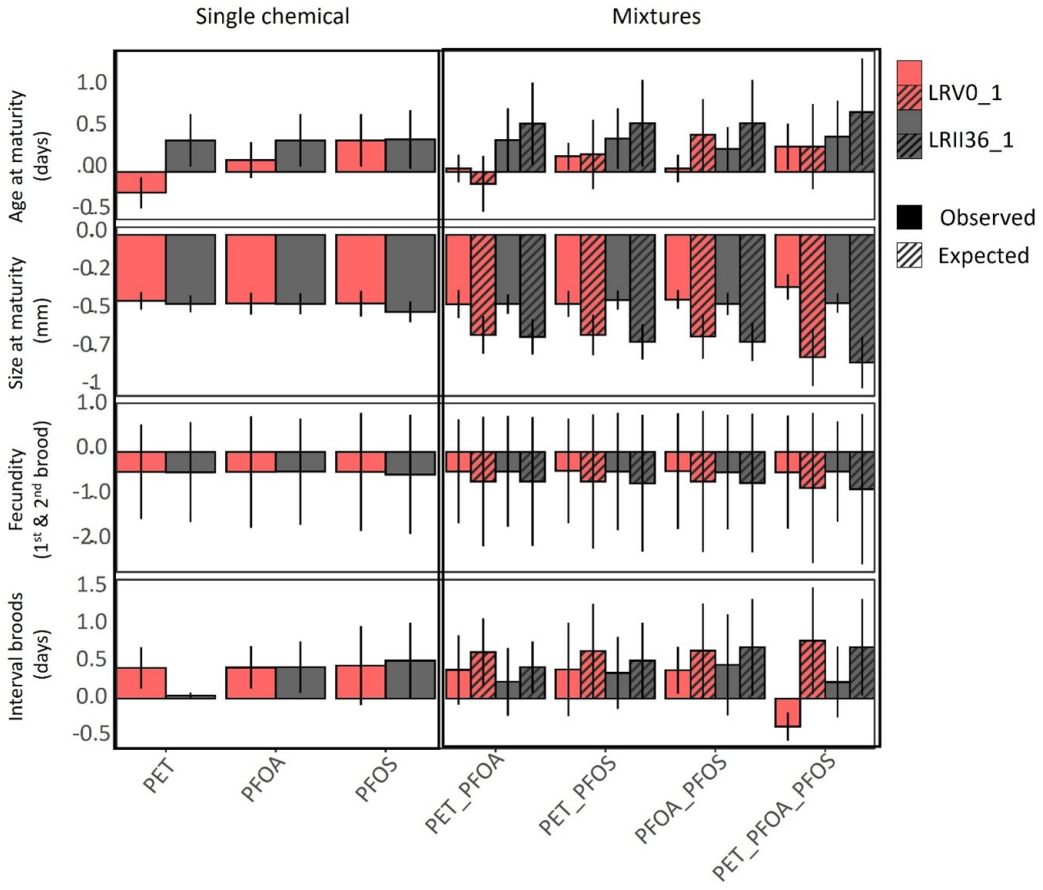


Figure 4.5: Mixture chemicals effects. Empirical observations from single chemicals (a) and chemical mixtures (b) are shown per genotype for four life history traits (age at maturity, size of maturity, fecundity and the interval between broods). The predicted additive effects are shown as patterned bars and the observed effect as solid bars. The effects are synergistic when the effect of chemical mixtures is greater than the sum of the effects of individual chemicals; antagonistic when the effect of mixtures is smaller than the sum of the effects of individual chemicals; and additive when the effects of mixtures is equivalent to the sum of the individual effects. The statistics used to assess significant departure from the null model of additivity are in Table 6 in Appendix C.

4.3.3 Mixture Effects

The effect of two-way and three-way chemical mixtures was quantified on the life-history traits using an additive null model. Across the life history traits and treatments, interactions were 59% additive and 41% synergistic, with no recorded antagonistic interactions (Fig 4.5, Table 6 in Appendix C). The effect of combined stressors on age at maturity was 50% synergistic and 50% additive (Fig 4.5, Table 6 in Appendix C). The combined effects on size at maturity was 100% synergistic

(Fig 4.5, Table 6 in Appendix C). The effect of multiple stressors on fecundity was 100% additive. The combined-stressors effects on the interval between broods were 87.5% additive and 12.5% synergistic (Fig 4.5, and Table 6 in Appendix C).

4.4 Discussion

4.4.1 Single Chemical Effects

In our study, both the reaction norms, supported by ANOVA analysis and the effect size analysis comparing exposures between single chemicals and mixtures corroborate impacts of PFOS and PFOA on *Daphnia's* fitness-related life history traits. However, a significant increase in mortality was observed only in individual exposures to PFOS. Previous studies indicating higher toxicity of PFOS compared to PFOA were predominantly acute toxicity studies or employed concentrations of the two compounds that were above environmental relevance >6 mg/l (Logeshwaran et al. 2021). Notably, the fitness effects previously documented for PFOA and PFOS align well with our findings for exposures involving concentrations below 3 mg/l (Logeshwaran et al. 2021). Our study, in alignment with existing results, revealed that part of the ingested MP is egested regularly (Frydkjær, Iversen, and Roslev 2017). However, the adverse effect on ecological endpoints suggests that MP can be internalised (Guilhermino et al. 2021). Consequently, the adverse effects of MP on *Daphnia* manifest either directly by impeding food intake or indirectly by affecting fitness (Scherer et al. 2017). Prolonged exposure of *D. magna* to microplastics has been linked to juvenile mortality, smaller somatic growth, and delayed/reduced reproduction (Guilhermino et al. 2021; Scherer et al. 2017; Ogonowski et al. 2016; Schür et al. 2023). Our findings validate prior research, showcasing a significant overall fitness impact of PET, evident through reduced fecundity and adult size,

alongside increased mortality. In this research, we opted for polyethylene terephthalate (PET) due to its widespread utilisation in textiles, packaging, water, and air filters, leading to its discharge into surface water (Dhaka et al. 2022). Additionally, we incorporated irregularly shaped PET to better mirror conditions encountered by freshwater species in the natural environment. Irregularly shaped and weathered MP, particularly fibres, have been shown to exhibit higher toxicity compared to their regularly shaped counterparts (Funke, Webb, and Wolinska 2024; C. Ma, H. Shi, and Slaveykova 2024; Rebelein et al. 2021). This heightened toxicity may be attributed to potential toxic or endocrine-disrupting additives, such as Bisphenol A, released as microplastics degrade into various shapes and sizes (D. An et al. 2021). Leachate from irregularly shaped MP was not quantified in our study, limiting our ability to conclude that additives may have contributed to the toxic response observed. Irregular MP are expelled at a slower rate than regularly shaped microplastics, potentially prolonging exposure within the digestive tract. Furthermore, irregular microplastics are expelled at a slower rate than regularly shaped microplastics, potentially prolonging exposure within the digestive tract (Frydkjær, Iversen, and Roslev 2017). In fact, irregularly shaped MP are the most found (up to 44%) in the tissue and guts of exposed organisms (De Sá et al. 2018). Smaller MP can be more toxic than larger ones due to their easier ingestion and prolonged retention in the digestive tract. In addition, smaller particles have a higher specific surface area, enabling them to adsorb greater amounts of persistent pollutants, such as perfluorinated compounds, compared to larger MP (De Sá et al. 2018; Dai, J. Zhao, C. Sun, Diying Li, et al. 2022; (L. Yin et al. 2023). Previous studies have demonstrated that fragments and fibers are eliminated more slowly than regularly shaped MP. While spherical MP are typically egested within 24 h (D. An et al. 2021). Our ingestion/egestion experiments show that nearly complete egestion of irregular particles and fibres requires at least 72 h. This longer residence time in the digestive tract, along with the increased potential for bioconcentration of irregularly

shaped MP, can contribute to higher toxicity. Smaller particles can penetrate epithelial and cellular barriers, further contributing to their harmful effects (Frydkjær, Iversen, and Roslev 2017).

4.4.2 Chemical Mixtures Effects

To date, the combined effects of PFOS and PFOA have predominantly been examined in human cell lines and vertebrates (e.g., (Dai, J. Zhao, C. Sun, Diying Li, et al. 2022; X.-Z. Hu and D.-C. Hu 2009; Watanabe et al. 2009)). The reported combined effects of these PFAS range from additivity in human cell lines (X.-Z. Hu and D.-C. Hu 2009) to antagonistic interactions in prokaryotes Rodea-Palomares et al. 2012, and a mix of synergistic and additive effects in fish embryos (Dai, J. Zhao, C. Sun, Diying Li, et al. 2022). Surprisingly, despite their frequent occurrence in aquatic environments, the combined toxicity of these PFAS has not been investigated in *Daphnia*. Our research represents a pioneering effort in quantifying the combined effects of PET with PFAS on the sentinel species *Daphnia* through the application of a null model of additivity, adapted from ecological literature. This model enables to quantitatively assess synergistic, antagonistic and additive effects of multiple stressors (Côté, Darling, and Brown 2016). Our findings indicate that chemical mixtures involving two and three compounds exhibited 60% additive and 41% synergistic effects, with fecundity demonstrating entirely additive effects. These results corroborate meta-analyses suggesting that up to 50% of combined stressors' effects in freshwater and marine environments are synergistic (Holmstrup et al. 2010). The significant level of additivity observed in our study underscores the critical need to understand the impacts of chemical mixtures on wildlife and human health. This highlights the necessity for incorporating toxicity assessments of unintentional mixtures into regulatory frameworks. Recent advances in untargeted

approaches and machine learning algorithms make it feasible to uncover complex interactions between non-targeted fingerprinting of real-world chemical mixtures and their biological effects. Consequently, a revision of outdated toxicity assessment methodologies is both possible and imperative (M. Abdullahi 2023).

Our study is timely in addressing the combined effect of PFAS and MP, which commonly co-occur and whose presence in aquatic environments has significantly increased over the past 15 years (De Sá et al. 2018). Our results showing synergistic and additive effects of PFAS and MP could be explained by adsorption, which would align with literature evidence indicating that MP provides a substrate for the adsorption of other environmental contaminants (e.g. heavy metals, (Ashton, Holmes, and Turner 2010) and PFCs; (Dai, J. Zhao, C. Sun, Diying Li, et al. 2022; F. Ribeiro et al. 2023). However, because we did not measure sorption directly, we are not able to confirm this mechanism in our study. The compounded effects of microplastics and PFAS have been shown to be more severe for irregularly shaped and aged microplastics, which may explain the more severe effect of mixtures on ecological endpoints observed in our study (Bhagwat et al. 2021). The higher impact of mixtures can be explained by enhanced affinity due to the differing charges of the chemicals. For example, PFAS adsorb very effectively onto positively charged MP, and cationic PFAS and negatively charged microplastics exhibit high affinity (Dai, J. Zhao, C. Sun, Diying Li, et al. 2022). This may be compounded by biofilm formation on the microplastic surface, which can generate a chemosensory response, rendering the MP bioavailable and palatable for ingestion by wildlife, thereby leading to bioaccumulation across the food web (Vroom et al. 2017).

4.4.3 Genotype-Specific Toxic Response is Determined by Previous Exposure to Chemical Stress

In contrast to conventional ecotoxicological studies, which frequently utilise a single commercially available *Daphnia* strain, two genotypes with distinct histories of exposure to chemical pollutants were employed. This approach allowed us to discern genotype-specific effects of PET on age at maturity and intervals between asexual reproductions. Also, genotype-specific effects was observed in the PFOS and the combined PFOS and PFOA exposures. However, in the three-way exposure, only size at maturity exhibited a significant genotype-by-treatment interaction.

In all instances where genotype-specific effects were identified, the genotype with historical exposure to chemical stress experienced the greatest fitness cost, except for mortality rates, which were higher for the naive genotype for both PFOS and PET exposures. These findings corroborate previous studies indicating that *Daphnia* genotypes naive to chemical stress exhibit greater tolerance to novel chemical stress, whereas genotypes with a history of chemical exposure demonstrate reduced tolerance to new chemicals (M. Abdullahi, Stead, et al. 2023; M. Abdullahi, J. Zhou, et al. 2022; Noyes et al. 2009). This reduced tolerance is likely due to cumulative fitness costs accrued from prior exposures, leading to compromised tolerance when encountering new chemical challenges. Our study and a few others using multiple genotypes emphasise the critical need to consider genetic diversity and historical exposure in ecotoxicological assessments. The observed genotype-specific responses highlight the complex interplay between genetic background and pollutant exposure, underscoring the importance of incorporating multiple genotypes in environmental risk assessments to accurately predict the ecological impact of chemical pollutants.

4.5 Conclusions

The current study elucidates the effects of environmentally relevant concentrations of PFAS and MP and quantifies the mechanisms of their mixtures using an additivity model derived from ecological literature. The assessment focused on fitness-related endpoints, revealing significant impacts on fecundity and age at maturity. Specifically, the study observed a markedly reduced number of offspring coupled with developmental failure, leading to egg abortion within the brood pouch. Mortality rates increased notably in treatments involving PFOS and PET. These results, coupled with molecular signatures of gene and metabolite dysregulation, can reveal the underpinning mechanisms of toxicity of chemical mixtures involving chemicals of very high concern. Due to the exceptional surface-active and resilience properties of PFAS, manufacturers have rapidly adopted new alternatives or next-generation PFAS to replace restricted compounds such as PFOS and PFOA. These alternatives often resemble some precursors byproducts of longer-chain PFAS. Although the toxic effects of these new PFAS remain largely unknown, emerging studies suggest they likely possess similar toxic profiles (Coperchini et al. 2020; Ragnarsdóttir, Abdallah, and Harrad 2024). Consequently, it is imperative to continue investigating the toxicological impacts of these substances on wildlife to inform regulatory and conservation efforts.

Our study provides critical insights into the chronic toxicity of PFAS and MP on the ecological endpoints of the sentinel species *Daphnia*. In addition, we quantify the combined effect of these chemicals on ecological endpoints, revealing synergistic and additive effects. This research significantly advances the understanding of the toxicity of these chemicals of high concern for environmental and human health.

Chapter 5

Isolation, Characterisation and Identification of Ampicillin-Resistant Bacteria from Domestic Water Systems

5.1 Introduction

This chapter investigates water contamination from a biological perspective, focusing on antibiotic-resistant bacteria (ARB) as ubiquitous and emerging biotic contaminants in the environment. Although the occurrence of ARB in domestic water supplies is documented, far less is known about the variation in species and levels of bacterial contamination from different households and water sources in community settings. Therefore, the starting point for the current project was to compare the range of bacteria isolated from households. This chapter investigates the presence

and prevalence of ARB in household plumbing systems to answer the third research question in this thesis: Does the presence and prevalence of ARB vary across residences? What are the common bacterial species found in household plumbing systems, and which sites are the most contaminated within each household? Three sites in each house were selected and analysed: kitchen taps, bathroom taps, and showerheads across 30 households in the city of Birmingham, the West Midlands region of the UK. These findings offer valuable insights into microbial loads and antimicrobial resistance profiles in the domestic environment that will enhance our understanding of their environmental and public health implications. Additionally, these results reveal key information regarding factors such as the safety of drinking water systems and improving wastewater treatment and contribute to the global fight against antibiotic resistance.

5.2 Materials and Methods

5.2.1 Sample Collection

Water samples were collected directly from thirty (N=30) residences in the city of Birmingham, West Midlands, UK, from three locations within each house: the kitchen tap, bathroom tap, and showerhead. The households were randomly selected on a single occasion from January to March 2022. The sampling technique was grab sampling. The cold tap was turned on for about 1 minute to drain the stagnant water and any influence from the plumbing system. 50 mL of each sample was collected in sterile Falcon bottles in three replicates. Then, all samples were transferred to the laboratory and stored at 4 °C for further analysis.

5.2.2 Chemical and Growth Media Preparation

Antimicrobial Stock Solution

The antimicrobial agent ampicillin (AMP) was purchased from Sigma-Aldrich (Dorset, UK). A stock solution was prepared in sterile deionized water. The solution was filter sterilised using a 0.22 µm pore syringe filter (Millipore, UK) and stored at -20°C until use. A final concentration of 100 µg/mL ampicillin was used in the experiments.

Agar Base: Middlebrook 7H11 Media Supplemented with Ampicillin

10.25 g of 7H11 agar base (Sigma-Aldrich) and 450 mL of distilled water were added together before autoclaving (121 °C for 15 min). The autoclaved agar was then placed in a water bath at 50-55 °C for 20 minutes to cool before adding 50 mL of filter-sterilised oleic acid-albumin-dextrose-catalase (OADC) supplement (10%), 5 mL of 50% glycerol (w/v) (Sigma, Dorset, UK) and ampicillin. Then, 7H11 agar was immediately poured into Petri dishes to a volume of approximately 20 mL per dish and was allowed to solidify at room temperature in a laminar flow hood. To prepare OADC, 5 mg oleic acid, 5 g bovine serum albumin fraction V, 2 g dextrose and 3 mg catalase were mixed in 100 mL of dH2O. Then, it was mixed until all components were dissolved and was filter sterilised with a 0.22 sterile filter. The OADC Enrichment was stored at 4°C.

Broth Base: Middlebrook 7H9 Broth

Middlebrook 7H9 broth was prepared following the manufacturer's instructions (Sigma-Aldrich) as follows: 2.35 g of 7H9 broth base and 450 mL of distilled water were

mixed and autoclaved at 121 °C for 15 minutes for sterilisation. After cooling, 50 mL of filter-sterilised ADC supplement (albumin, dextrose, and catalase; 10% v/v), 5 mL of 50% glycerol and 1.25 ml of 20% Tween 80 (mixing 20 mL Tween 80 with 80 mL distilled water) were added and gently mixed. For ADC preparation, 5 g of bovine serum albumin (BSA), 2 g of dextrose, 0.85 g of sodium chloride, and 3 mg of catalase were dissolved in 100 mL of distilled water and then was filter sterilised and stored at 4 °C.

5.2.3 Development of Optimal Technique for Isolation of ARB from Water Samples

Two techniques of membrane filtration and centrifugation were performed to concentrate bacterial load in drinking water samples before inoculating them onto selective agar Middlebrook 7H11 plates. Three water samples from a residence were tested using the following methods to confirm and identify the optimal technique for ARB recovery:

1) water samples were centrifuged at 15000 rpm for 30 minutes to have maximum bacterial loads at the bottom of a tube. After suspending the supernatant in 200 µl sterile water, 100 µl was directly spread on the surface of the agar plates.

2) Water samples were directly filtered, without dilution, using a 0.22 µm Steritop vacuum bottle-top filter using VWR vacuum gas pump. Then, filter paper was directly placed on the agar using a sterile tweezer and incubated at 37 °C for up to 5 days. The samples were in triplicates.

5.2.4 Isolation and Characterisation of Bacterial Strains

Bacteria were isolated in the laboratory with 100 µg/mL ampicillin concentration in agar plates. Cultured plates with no observed growth within 48 h were incubated for up to 5 days. The plates were regularly examined during the incubation period. The average number of colonies was determined by counting colonies on plates from three replicates of each sample, and CFU/mL in each sample was calculated using the following formula:

$$CFU(\text{ml}) = \frac{\text{Average number of colonies on the plates}}{\text{Volume plated}} \tag{5.1}$$

Within 5 days of incubation, morphologically distinct bacterial colonies were selected, and repetitive streaking was performed on agar plates to obtain pure colonies. The purity of bacterial cultures was verified by visual observation of microbial growth followed by microscopic observation of Gram-stained cultures. Sterile distilled water was filtered, and the filter was placed on the surface of the growth medium (7H11) without any ampicillin as a negative control. Additionally, the same procedure was carried out with actual water samples as baseline controls that provide a clear picture of the bacteria naturally present in the water and establish a reference for assessing the impact of ampicillin on bacterial growth. One to three morphologically distinct colonies from each sampling site were selected for characterisation and identification.

5.2.5 Bacterial Isolates Preservation and Growth Conditions

Overnight fresh bacterial cultures in Middlebrook 7H9 Broth supplemented with ADC and 1% glycerol were prepared. Then, 1 mL of bacterial culture was mixed with an equal volume of the 50% glycerol solution. Gently mixed the samples using several pipetting. 1 mL of the glycerol-bacterial mixture was transferred into sterile

labelled cryogenic vials (Thermoscientific, UK). Finally, the vials were immediately placed into a -80°C freezer for long-term storage. For preparing fresh passage of the bacterial cultures, an inoculum of glycerol stock was streaked onto 7H11 agar plates supplemented with 10% of OADC using a sterile loop and were incubated at 37 °C for 72 hours or small amount of the frozen cultures were inoculated in 10 mL of 7H9 broth and placed in an orbital shaking incubator at 37 °C for 72 hours to use for further microbiology and molecular biology studies.

5.2.6 Characterisation of Bacterial Isolates

Phenotypic Characterisation

Shape, consistency, elevation, colour, size, aspect were used to characterise selected colonies phenotypically. Acid-fast staining was used to assess the presence of acid-fast bacteria using a light microscope (LABORLUX11) X4.

Ziehl-Neelsen (Acid-Fast) Staining

The staining procedure was performed according to the online website (https://microbeonline.com/ziehl-neelsen-technique-principle-procedure-reporting/). Briefly, a thin layer of bacterial smear was prepared on a glass slide and air-dried for about 30 minutes and was heat fixed. Then, the smear was covered with carbol fuchsin stain and heated until begins to rise. Allow the heated stain to remain on the slide and after 5 minutes, it was washed off with water. After that, the smear was covered with 3% (v/v) acid alcohol or 20% sulfuric acid for 2-5 minutes until the smear was sufficiently decolorised, which was followed by rinsing off the slide. Then, the smear was covered with a malachite green stain for 1-2 minutes. The slide was washed off

and air-dried, and finally, the smear was microscopically observed using the 100x oil immersion objective

5.2.7 Growth Curve and Colony Forming Units (CFU/mL)

Bacterial isolates were grown overnight in Middlebrook 7H9 broth supplemented with 10% ADC and 1% glycerol in a shaking incubator at 37 °C. Optical density (OD) was measured at 600 nm every 12 hours for 120 h to assess the growth kinetics of the isolates. Miles & Misra techniques (or surface viable count) was used as standard surface inoculation method in microbiology to determine number of variable bacteria or colony forming unit (CFU) on agar plates (Miles, Misra, and Irwin 1938). 7H Middle brook agar plates were prepared, and each plate was divided into seven (7) sections with a label of the dilutions. At 24 h intervals, fresh bacterial samples were taken from the incubated cultures and serially diluted up to 10^{-7} using phosphate-buffered saline (PBS) as the diluent. For each dilution, three separate 20 µl spots were placed on corresponding sections of an agar plate, spreading over an area of 1.5–2.0 cm in diameter (three replicates were performed for each dilution). The surface of the plates was adequately dried to ensure that a 20 µl drop was absorbed within 15–20 minutes. The plates were incubated at 37 °C for 24 h, and colonies were counted in the section with the fully formed, countable, discrete colonies at each specific time point, up to 120 h for each isolate. Only sections containing between 3 and 30 colonies were considered for counting. The average number of colonies was used to calculate the CFU/mL for each plate. The following equation was used to calculate the CFU/mL in the original bacterial culture per mL.

$$CFU\ (\text{ml}) = \frac{\text{Average Number of Colonies} \times \text{Dilution Factor}}{\text{Volume Plated}} \tag{5.2}$$

5.2.8 Identification of Isolates Using 16S rRNA Gene Sequencing

Genomic DNA Extraction

Genomic DNA extraction was carried out using the NucleoSpin® Food Kit (Macherey-Nagel, Düren, GER), where DNA is reversibly bound to a silica membrane. Briefly, Overnight bacterial cultures were centrifuged, and the pellets were resuspended in 550 µL CF lysis buffer (MACHEREY-NAGEL GmbH & Co. KG, Düren) and 10 µL proteinase K, were mixed well for a few second and incubate at 65 °C for 30 min. Then 10 µL RNase A was added, mixed well, and incubated at room temperature (18–25 °C) for 30 min and then proceeded with the centrifugation step. 300 µL of clear supernatant was transferred into a microcentrifuge tube. An equal volume (300 µL) of Buffer C4 (guanidine hydrochloride 50%) and an equal volume of ethanol were added. The mixture was vortexed for 30 seconds. A NucleoSpin® Food Column was placed in a collection tube. Then, 700 µL of the mixture was pipetted onto the column and centrifuged for 1 minute at 10000 rpm. The flow-through was discarded. This procedure was repeated to load the remaining sample. The extraction was followed by three washing steps as follows: 400 µL of Buffer CQW (Guanidinhydrochlorid 24–36% + Ethanol 24–36%) was added to the column and centrifuged for 1 minute at 10000 rpm. The flow-through was discarded. Then, 700 µL of buffer C5 (ethanol 96%) was pipetted onto the column and centrifuged for 1 minute at 10000 rpm and the flow-through was discarded. Then, 200 µL of Buffer C5 was added to the column and centrifuged for 2 minutes at 10000 rpm to ensure Buffer C5 was completely removed. Finally, the column was placed in a new 1.5 mL microcentrifuge tube, 100 µL Elution Buffer (5 mM Tris/HCl, pH 8.5, preheated to 70 °C) was added onto the membrane, incubated for 5 min at room temperature and centrifuged for 1 min at 10000 rpm to elute the DNA that was used as a template for PCR. Quality and quantity of the eluted DNA were checked by agarose

gel electrophoresis by visual inspection and comparison of the DNA bands with a pGEM plasmid DNA standard (200 ng/µl) and comparison with a molecular-weight size marker (GeneRuler 1kb Plus DNA-Ladder, Thermo Fisher Scientific, Waltham, MA USA).

Polymerase Chain Reaction (PCR) Amplification and Products Purification

PCR amplification was performed using GoTaq HotStart Green MasterMix from Promega (Madison WI, USA). For PCR reactions, peqStar 96 HPL (VWR International GmbH, Darmstadt, Germany) thermal cycler was used (Appendix D, Table 10). Amplification of the 16S rRNA gene was performed using the universal primer set 27f: (5'-GAGTTTGATCMTGGCTCAG-3') and 1492r (5'- TACGGY-TACCTTGTTACGA - 3'), PCR master mix (2x) 10 µL, Forward Primer (27f) 2 µL, Reverse Primer (1492r) 2 µL, template DNA 1µL, and water (PCR grade) 20 µL (Appendix D, Table 11). Successful PCR amplification was verified, and PCR product quantity was checked by 1% (w/v) agarose gel electrophoresis by visual observation and comparison of the DNA bands with a molecular-weight size marker (GeneRuler 1kb Plus DNA-Ladder, Thermo Fisher Scientific, Waltham, MA USA). Additionally, PCR products were purified by performing a precipitation step and applying polyethyleneglycol (PEG). The quality of the purified template DNA was assessed by 1% (w/v) agarose gel.

Agarose Gel DNA Electrophoresis

The quality and quantity of PCR products were checked by Agarose Gel DNA Electrophoresis. 1x ethidium bromide (EtBr) as a loading dye was added to DNA samples and loaded onto 1% (w/v) TAE (Tris-Acetate-EDTA) agarose gel. 1 kb

ladder (New England Biolabs) was also loaded into a gel well. The gel was run in 1 x TAE buffer at 120 V for 40 minutes. The gel was then visualised under UV light and imaged.

16S rRNA Gene Sequencing

The PCR product was sent to Eurofins Scientific for sequencing, and the analysis are as follows: Approximately 10 ng of the PCR products were used as templates for sequencing reactions. PeqStar 96 HPL (PEQLAB Biotechnologie GMBH, Erlangen, Germany) thermal cycler was used for sequencing reactions. Sequencing reaction cleanup was performed either manually or on a Hamilton Starlet robotic workstation (Hamilton Robotics GmbH, Martinsried, Germany) by utilising the Xterminator Purification Kit, which separates excess dye terminators and other unwanted primers, nucleotides and polymerase by binding these selectively and subsequent precipitation (Eurofins Scientific SOP_SEQ_XTerminatorAufreinigung). Finally, all reactions were run on ABI3730xl capillary sequencers equipped with 50 cm capillaries and POP7 polymer (Thermo Fisher Scientific, Waltham, MA USA) (SOP_SEQ_ABI3730XL).

Phylogenetic Analysis

DNA sequence alignment editor software BioEdit version 5.0.9 was used to assemble and analyse raw sequencing data. The GenBank database using a BLAST search (http://www.ncbi.nlm.nih.gov/blast/) was used to compare the sequences. The 16S rRNA gene similarities were retrieved from the database, determining the most closely related strains. A phylogenetic tree was constructed by using the software package MEGA version 11 (Tamura et al. 2013). The similarity search of newly

generated 16S rRNA gene sequences was performed against the type strains of bacterial species with validly published names available in the NCBI database and ezbioClould (Yoon et al. 2017). The phylogenetic tree was reconstructed with the neighbour-joining and Kimura 2-parameter method (Saitou and Nei 1987), and bootstrap values were calculated from 1000 replicate runs. FigTree (version 1.4.4) was used as an editing tool to customize labels and apply colour coding to the isolates, enhancing clarity and improving the interpretation of the phylogenetic tree.

Sequence Data and Culture Deposition

The 16S rRNA gene sequences reported in this study were deposited in NCBI GenBank with accession numbers PQ678842-PQ678883, which correspond to the isolates defined in the phylogenetic tree.

5.3 Result

5.3.1 Optimisation of Techniques for Isolating ABR from Water Samples

Two commonly used methods, filtration and centrifugation, were investigated to identify the optimal technique for isolating antibiotic-resistant bacteria (ARB) from water samples. Following centrifugation and incubation time, An average of 0, 0.3, and 7.3 bacterial colonies was observed on Middlebrook 7H11 agar supplemented with 100 µg/ml of ampicillin, using three replicates for each sample. In contrast, when the same water samples were filtered, more visible bacterial colonies were observed on the filters, with averages of 3, 4.6, and 23.3 colonies. Filtration was ob-

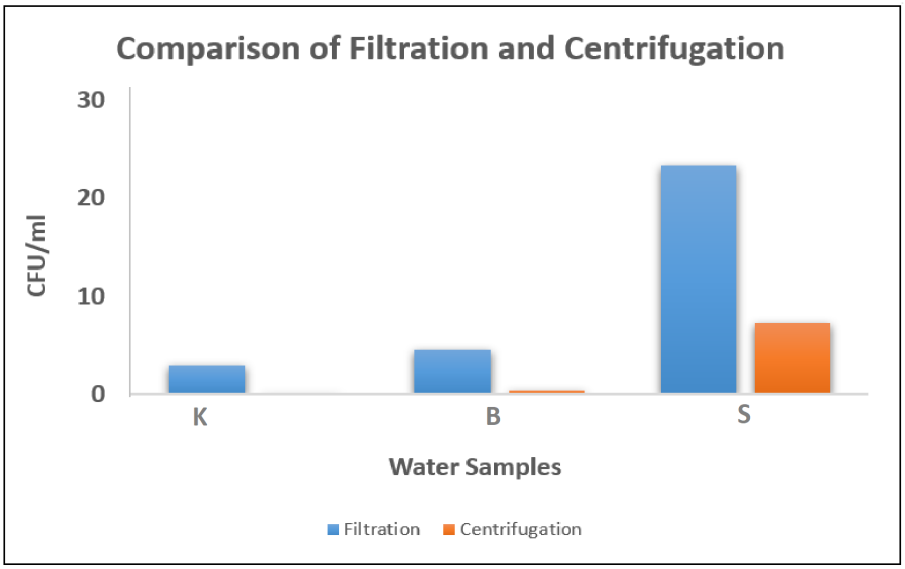


Figure 5.1: compares two techniques of filtration and centrifugation for concentrating water test samples and their corresponding bacterial recovery rates. The data highlights the effectiveness of filtration with higher CFU/ mL of each water sample. "K", "B" and "S" represent Kitchen, bathroom and showerhead.

served to be significantly more effective in terms of bacterial recovery rate and concentration efficiency, highlighting its enhanced ability to capture and isolate ARB. Based on these findings, the filtration technique was adopted to process the remaining water samples in the study Fig 5.1.

5.3.2 Isolation of Ampicillin-Resistant Bacteria from Domestic Showerheads

Thirty (30) water samples were collected from showerheads across various households. filtration technique was used to capture and recover bacteria from the samples. After incubating the agar plates supplemented with ampicillin with the filter on the surface for up to five days at 37°C, the number of colonies on each plate was counted. Table 7 in Appendix D lists the results for each of the 90 samples tested (30 samples in triplicates). Based on CFU/mL results, the highest level of contam-

ination was found in sample 26, with a value of 5.2 CFU/mL (Table 7, Appendix D). This sample was collected from a flat occupied by a couple, where the shower is not frequently used because the flat is not their permanent residence. However, the lowest level of contamination was observed in sample 10, with a value of 0.18 CFU/mL (Table 7, Appendix D). This sample was collected from a household with four members, where the shower is used more frequently. No bacterial colonies were observed in 50% of these samples after placing the filters on agar plates, even after five days of incubation. However, bacterial growth was observed in the remaining 15 samples, with at least a single phenotypic morphology of bacterial colonies on the filters. In some cases, colonies with different phenotypic morphologies were observed on the same filter or across the three replicates (Fig 5.2A) 5.2. Filtered sterile distilled water was used as a negative control (Fig 5.2B) 5.2). In Fig 5.2C 5.2, the filters were inoculated with the actual water samples; the filter placed on antibiotic-free agar plates (baseline controls) that exhibited significant bacterial overgrowth, often with densely crowded and mixed colonies that were too many to count. This observation indicates that a wide range of bacteria in the water samples could grow uninhibited. In contrast, agar plates supplemented with ampicillin, inoculated with the same water samples, showed significantly reduced bacterial growth in most of the cases. This reduction suggests that ampicillin effectively inhibited the proliferation of ampicillin-sensitive bacteria, allowing only resistant strains to grow, which resulted in fewer visible colonies. Distinct bacterial colonies with diverse morphotypes were then selected and purified from the same sample (Appendix D, Fig 4).

Overall, the initial experiments provided strong evidence that bacteria resistant to Beta-lactam antibiotics are frequently present in domestic showerhead systems. 25 ampicillin-resistant bacteria were isolated from 30 showerhead samples. However, the extent of contamination varied significantly among households.

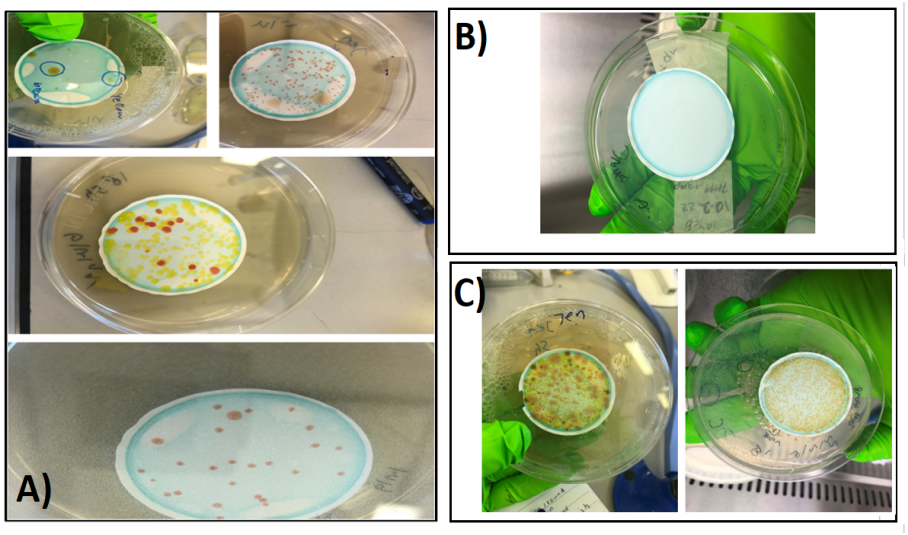


Figure 5.2: Shows examples of bacterial growth on filter papers after filtering water samples collected from different showerheads (A), filtered sterile distilled water as a negative control (B) and the baseline control conditions (filtered water samples on antibiotic-free media) (C).

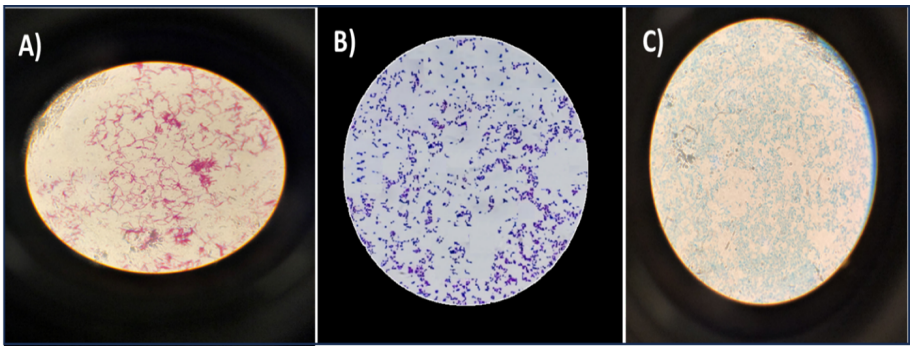


Figure 5.3: Depicts the microscopic observations of Gram staining and Acid-fast staining: (A) shows Gram-negative bacteria, (B) shows Gram-positive bacteria, and (C) shows non-acid-fast bacteria, indicated by a negative result in Acid-fast staining.

5.3.3 Preliminary Characterisation of the Isolates from Showerheads

The characterisation of the bacterial isolates recovered from showerheads focused on analysing their key morphological characteristics, staining properties, growth curve and CFU analysis. The results, including phenotypic morphology such as colony shape, texture, colour, size, and cell aspect, as well as Gram staining acid-fast staining outcomes, are summarised in Table 5.1 Among the 25 bacterial isolates,

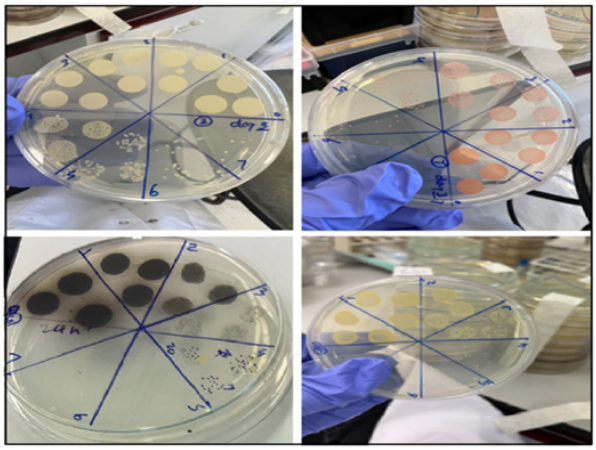


Figure 5.4: Shows examples of CFU/ml counts of bacterial isolates on Middlebrook 7H11 agar plates using the Miles & Misra technique.

24 isolates were Gram-negative, with one exception (isolate 23S), which was Gram-positive. All the isolates from showerheads tested negative for acid-fast staining, indicating that they do not belong to acid-fast mycobacterial species Fig 5.3.

The growth kinetics of the isolates were assessed over 120 h and showed a positive correlation with their CFU/mL results using Miles & Misra technique (Fig. 5.4 & 5.5). To better correlate CFU and optical density (OD), OD values at 12h time intervals were not included in the graph, as CFU data were collected every 24 hours. Among the isolates, 8S and 9S exhibited the highest growth rates, as indicated by their steep and elevated growth curves, reaching significantly greater OD values compared to the others. In contrast, isolates 12S2 and 27S displayed the lowest growth, with their OD values consistently remaining below the other isolates throughout the observation period. The remaining bacterial isolates demonstrated intermediate growth patterns, with average OD values falling between these extremes. These isolates exhibited moderate growth curves, suggesting balanced growth dynamics compared to the highly robust or minimal growth observed in the other groups.

Samples	Isolation Location	Code	Gram Staining	Acid-Fast Staining	Cell Shape (Aspect)	Colony Morphology
1	1S1	1S1	-	-	Rod	Round, smooth, flat, white, small
	1S2	1S2	-	-	Rod	Round, smooth, convex, cream, big
	1S3	1S3	-	-	Rod	Round, smooth, flat, cream, moderate
2	S	2S	-	-	Rod	Round, smooth, convex, light red, small
4	S	4S	-	-	Rod	Irregular, smooth, flat, white, big
5	S1	5S1	-	-	Rod	Round, smooth, flat, transparent, moderate
	S2	5S2	-	-	Rod	Round, smooth, convex, cream, big
8	S	8S	-	-	Rod	Round, smooth, convex, opaque white, small
9	S	9S	-	-	Rod	Round, smooth, flat, light Gray/cream, small
10	S1	10S1	-	-	Rod	Round, dry, convex, off-white, small
	S2	10S2	-	-	Rod	Round, smooth, convex, yellow, small
	S3	10S3	-	-	Rod	Round, dry, flat, cream moderate
12	S1	12S1	-	-	Rod	Round, smooth, convex, opaque white, small
	S2	12S2	-	-	Rod	Round, dry, convex, pink, punctiform
15	S	15S	-	-	Rod	Round, smooth, convex, white, small
19	S	19S	-	-	Rod	Irregular, smooth, convex, translucent moderate
22	S	22S	-	-	Rod	Round, dry, convex, white, moderate
23	S	23S	+	-	Rod	Round, dry, flat, dark pink, small
26	S1	26S1	-	-	Rod	Round, powdery, flat light yellow, small
	S2	26S2	-	-	Rod	Round, smooth, convex, dark pink, big
	S3	26S3	-	-	Rod	Undulate, smooth, flat, white, big
27	S	27S	-	-	Rod	Round, powdery, convex, light pink, small
28	S1	28S1	-	-	Rod	Round, smooth, flat, orange, pinpoint
	S2	28S2	-	-	Rod	Round, smooth, flat, lemon yellow, small
	S3	28S3	-	-	Rod	Round, smooth, convex, light brown, big

Table 5.1: Presents the characterisation result, including Gram staining, Acid-fast staining results, and phenotypic characteristics of the isolates from showerheads. “S” represents shower heads.

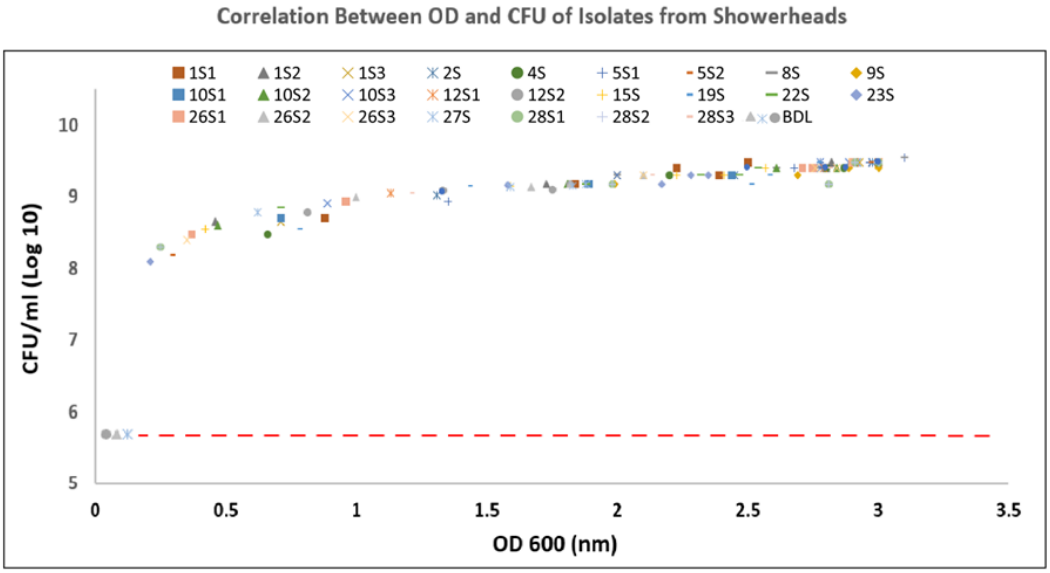


Figure 5.5: Compares and correlates the OD_{600} and CFU/mL values across 25 bacterial isolates from showerheads every 24 h. If no colony was observed on the plated dilutions, the data is presented as below the detection limit (BDL), as represented by the Miles & Misra technique. A logarithmic scale for CFU/mL was used to represent a wide range of values. The samples are shown with different coloured symbols. The red dashed line indicates the limit of detection (LOD) that is equal to 5.69897 5×10^5 CFU/ml.

5.3.4 Identification of Bacteria Isolated from Domestic Showerheads Using 16S rRNA Gene Sequencing

The quality of the extracted genomic DNA after PCR amplification and its purity after PEG purification were assessed by gel electrophoresis (Fig 5 & 6 in Appendix D). Phylogenetic analysis based on 16S rRNA gene sequences grouped three bacterial isolates (10S1, 1S2, and 26S3) within a clade closely related to the *Delftia* genus in the phylogenetic tree Fig 5.6. The isolates 10S2 and 26S1 were placed in the *Xenophilus aerolatus* cluster. Isolates 15S was closely associated with *Acidovorax-Betaproteobacteria* cluster. Six isolates (1S1, 2S, 5S1, 12S1, 19S and 28S3) were closely linked to the *Cupriavidus-Burkholderia* cluster. Although *Burkholderia* and *Cupriavidus* are separate genera, they are closely related. The isolates 4S, 8S, 9S were closely related to the *Pseudomonas* clade, which includes *Pseudomonas aeruginosa, Pseudomonas parafulva, Pseudomonas hunanensis, Pseudomonas putida*, and

other species of *Pseudomonas*. Isolate 22S were closely associated with the *Sphingomonas* cluster, which includes different species of *Sphingomonas*. 16S rRNA gene sequencing placed isolate 28S1 in a group with members of *Paracoccus*. The *Paracoccus* group was further divided into three subgroups; the first subgroup included *P. denitrificans* and *P. pantotrophus*; the second subgroup consisted of *P. thiocyanatus* and *P. yeei* and third subgroup comprised isolate 28S1 along with the type strain of *P. marinus*. Isolate 28S2 was closely connected to a group of *Caulobacter* species. Three ARB (1S3, 5S2, 10S3) clustered with *Bosea eneae* and other *Bosea* species. Isolates 26S2, 27S, and 12S2 were closely associated with the *Methylobacterium-Methylorubrum* cluster. Isolates 23S was closely similar to *Microbacterium oxydans*, belonging to the *Microbacterium* group. All the strains were closely related to their phylogenetic neighbours, showing 98.88% to 100% sequence similarity in their 16S rRNA gene sequences. The results of the identification revealed the most common bacterial genera as *Cupriavidus* (20%), *Pseudomonas* (12%), *Delftia* (12%), and *Bosea* (12%) in the showerhead systems. Fig 5.7 illustrates the prevalence and distribution of bacterial isolates across the showerheads.

5.3.5 Isolation of Ampicillin-Resistant Bacteria from Bathroom Taps

Three water samples were collected from the bathroom taps of Thirty (30) different houses. Bacteria in 50 ml of these samples were collected by filtration. Bacterial colonies were observed only in 8 of these samples after placing the filters on Middlebrook 7H11 agar plates supplemented with ampicillin, up to 5 days incubation at 37 °C. However, no bacterial growth was observed in the remaining 22 samples. The number of colonies on each plate was then counted, and CFU/mL was calculated (Appendix D, Table 8). Based on the observations, in most cases, bacteria with a single phenotype were grown. However, in a few samples, up to three colonies were

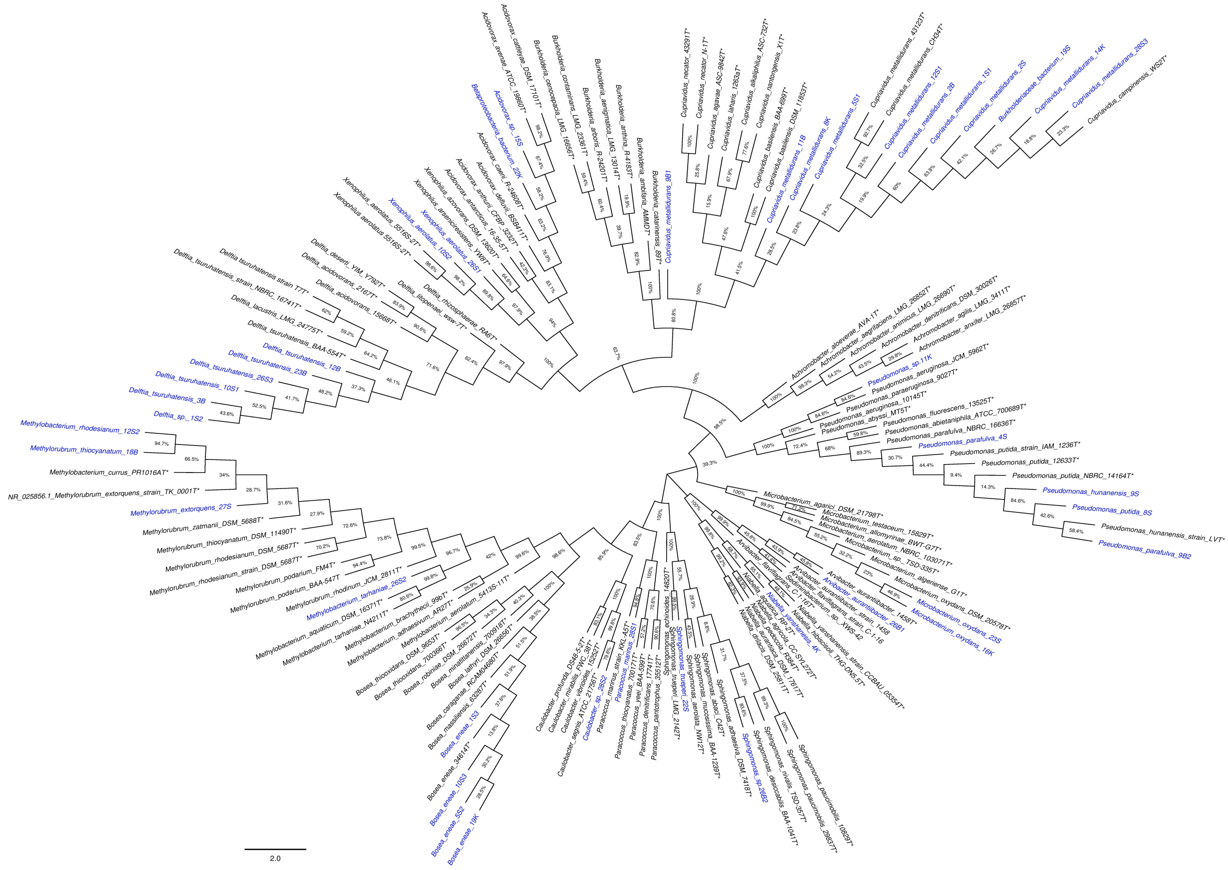


Figure 5.6: Illustrates the phylogenetic tree constructed based on 16S rRNA gene sequences of antibiotic-resistant bacteria and their closely related taxa (type strains) from GenBank. The tree was computed using MEGA 11 with the Kimura 2-parameter model (Saitou and Nei, 1987). Numbers at the nodes represent bootstrap values. The scale bar indicates 2.0 nucleotide substitutions per position.

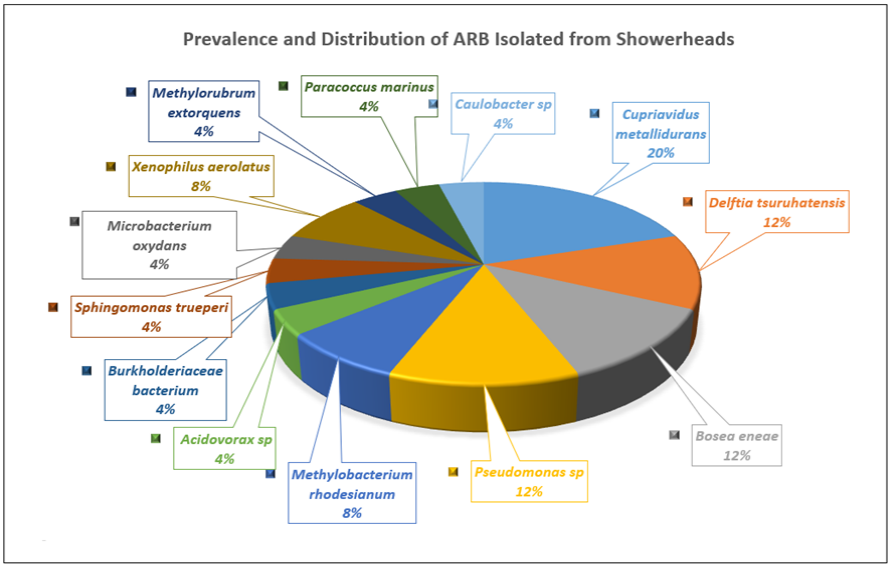


Figure 5.7: Demonstrates the prevalence and diversity of ARB from household showerheads and highlights the frequency of occurrence for each genus.

observed across the three replicates (Fig 5.8) Colonies were purified on agar using streak plating. Sample 26B exhibited the highest level of contamination, with a value of 6.19 CFU/mL. Notably, sample 26 also demonstrated the highest contamination levels in its corresponding showerhead. However, the lowest level of ARB contamination was observed in sample 18B, with a value of 1.14 CFU/mL. This sample was collected from a household occupied by two PhD students. Interestingly, no bacterial colonies were obtained from the corresponding shower sample.

The results of this experiment confirmed the presence of β-lactam antibiotic-resistant bacteria in this part of the domestic distribution system, in addition to showerheads. However, the level of contamination in bathrooms was relatively low compared to showerheads, as only 10 ampicillin-resistant bacteria were isolated from 30 water samples collected from bathrooms. Although variations in contamination levels were observed across bathroom samples, these variations were not significant, with the exception of sample B26.

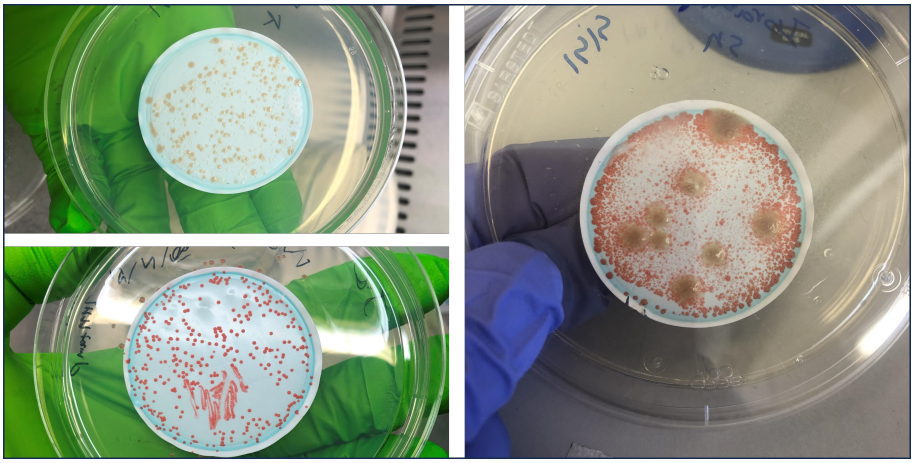


Figure 5.8: Depicts examples of bacterial colonies on filter papers after the filtration of water samples collected from various bathrooms and following an incubation period.

5.3.6 Preliminary Characterisation of Isolates from Bathroom Taps

The characterisation results for bacteria isolated from bathroom taps are presented in Table 5.2. The table summarised results for phenotypic characteristics, Gram and Acid-fast staining. All bacterial isolates were Gram-negative. All the isolates from bathroom taps tested negative for Acid-fast staining.

The growth curve of the isolates was assessed over 120 h of incubation at 37 °C and was positively correlated with their corresponding CFU/ml values Fig 5.9. Among the isolates, 11B and 9B2 exhibited the highest growth rates. However, isolate 18B showed the lowest growth, with its OD values falling below other isolates throughout the 120 h incubation period. The remaining bacterial isolates exhibited almost similar growth kinetics, with average OD values falling below isolate 11B. The growth curves provide useful insight into identifying slow- and fast-growing isolates among the tested isolates.

Samples	Isolation Location	Code	Gram Staining	Acid fast Staining	Cell shape (Aspect)	Colony Morphology
2	B	2B	-	-	Rod	Round, smooth, flat pigmented brown, big
3	B	3B	-	-	Rod	Round, smooth, convex, cream, punctiform
9	B1	9B1	-	-	Rod	Round, dry, convex, pink, small
	B2	9B2	-	-	Rod	Round, dry, flat, yellow, small
11	B	11B	-	-	Rod	Irregular, smooth, flat, light Gray, big
12	B	12B	-	-	Rod	Round, dry, convex, off white, small
18	B	18B	-	-	Rod	Round, smooth, convex, dark pink, small
23	B	23B	-	-	Rod	Undulate, dry, convex, light pink, small
26	B1	26B1	-	-	Rod	Round, flat, convex, yellow, small
	B2	26B2	-	-	Rod	Round, smooth, convex, orange, small

Table 5.2: Summarises the characterisation result including Gram staining, Acid-fast staining results, phenotypic characteristics of the isolates from bathroom taps and CFU/volume plated. "B" represents the bathroom.

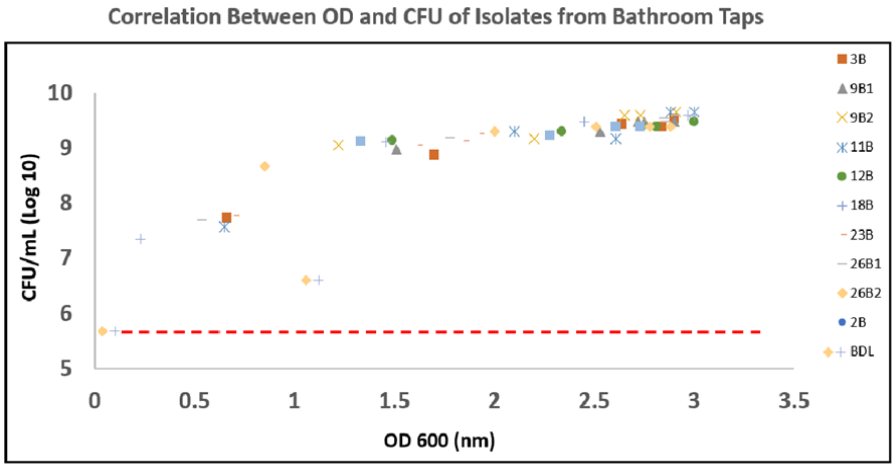


Figure 5.9: Compares and correlates the OD_{600} and CFU/mL values across 10 bacterial isolates from bathroom taps every 24 h. In cases where no colonies were observed on the plated dilutions, the data is represented as below the detection limit (BDL), following the Miles & Misra technique. A logarithmic scale is used for CFU/mL to accommodate the wide range of observed values. Different coloured symbols are used to represent the samples, while the red dashed line indicates the limit of detection (LOD), corresponding to 5.69897 (510) CFU/mL.

5.3.7 16S rRNA Gene Sequencing for Identification of Bacteria Isolated from Households Bathroom Taps

Phylogenetic analysis revealed that 3 isolates of 3B, 12B, 23B were closely related to *Delftia tsuruhatensis* and were grouped in *Delftia* cluster. Isolate 9B2 were closely related to the *Pseudomonas* clade, which includes different species of *Pseudomonas*. Isolates 2B, 9B1, 11B were closely linked to the *Cupriavidus-Burkholderia* cluster. The isolate 26B1 was placed in the *Arvibacter-Sediminibacterium* cluster and closely associated with *Arvibacter aurantiibacter, Arvibacter flaviflagrans*, and *Sediminibacterium* species. Isolates 26B2 and was closely associated with *Sphingomonas* cluster, which includes different species of *Sphingomonas*. 16S rRNA gene sequencing placed isolate 18B in *Methylobacterium-Methylorubrum* cluster. All the strains were closely related to their phylogenetic neighbours, showing high sequence similarity in their 16S rRNA gene sequences Fig (5.6). Fig 5.10 illustrates the prevalence and distribution of bacterial genera across bathroom taps.

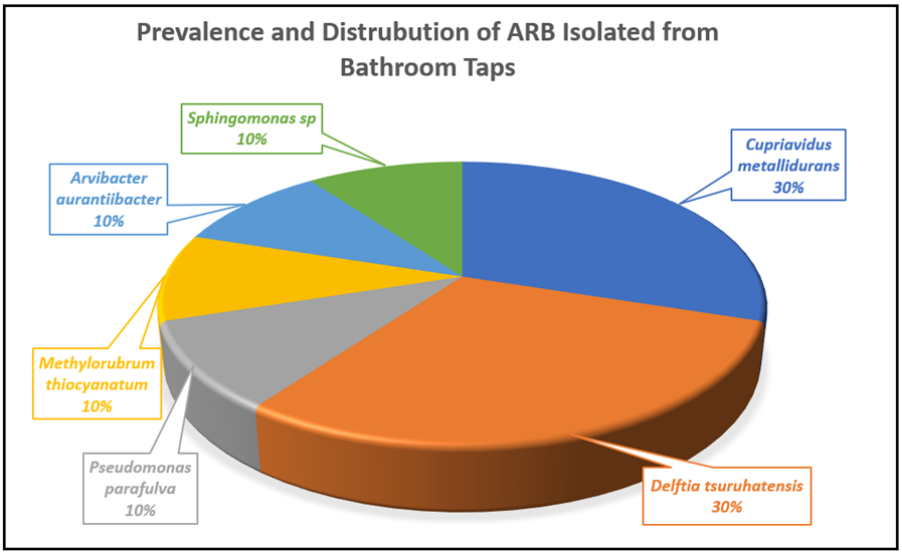


Figure 5.10: Visualises the prevalence and diversity of ARB isolated from household bathrooms and shows how frequently each genus appears in the samples.

5.3.8 Isolation of Ampicillin-Resistant Bacteria from Kitchen Taps

Three water samples were collected from kitchen taps in thirty (30) different households. A volume of 50 mL from each sample was filtered to concentrate the bacterial content. Out of 30 samples (30 samples in triplicate), only seven (7) water samples showed bacterial growth with visible colonies after the filters were placed on Middlebrook 7H11 agar plates supplemented with ampicillin and incubated at 37 °C for up to five days. However, no bacterial colonies were obtained from the remaining 23 samples. The CFU/mL was calculated based on the mean number of colonies counted from three replicates of each sample (Appendix D, Table 9). The density of observed bacterial colonies was relatively low on filters as compared to showerheads and bathroom taps (Fig 5.11). The highest level of contamination in the kitchen taps belonged to sample 8K, with a value of 3.7 CFU/mL (Appendix D, Table 9). Notably, sample 8K also exhibited a high bacterial density in its corresponding showerhead sample, with a CFU/mL of 3.18, while no bacterial colonies were obtained from the bathroom of this house. In contrast to the highest level of contamination

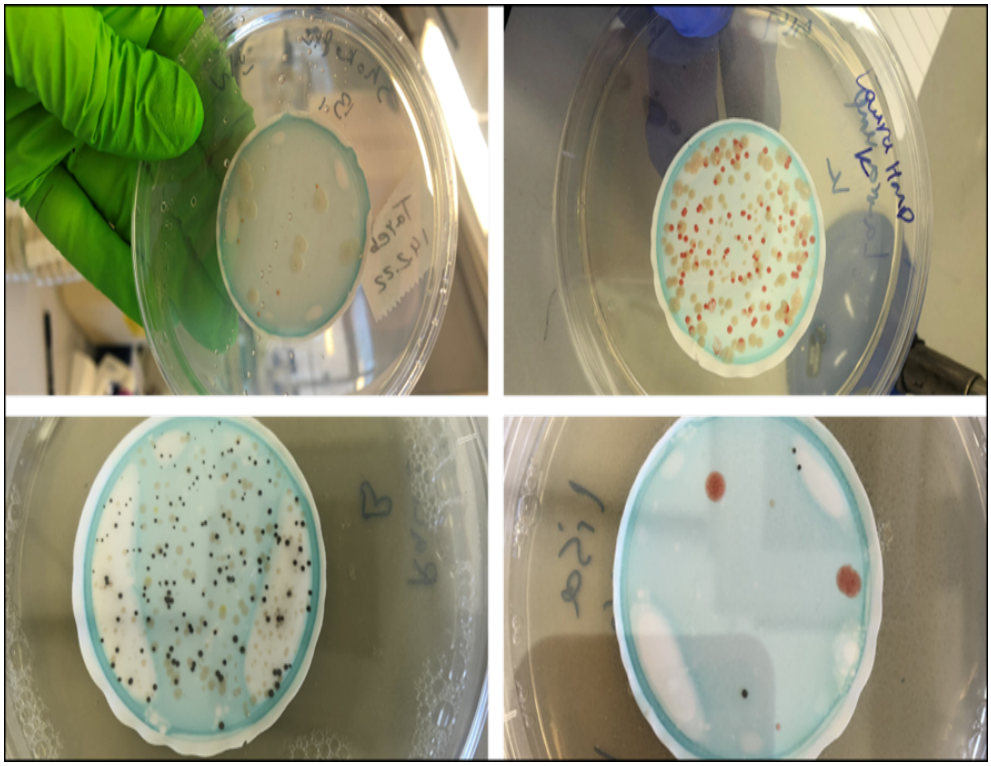


Figure 5.11: Shows examples of bacterial colonies on filter papers after the filtration of water samples collected from various kitchens and following an incubation period.

in kitchen taps, the lowest level of ARB contamination was observed in sample 16K, with a value of 0.1 CFU/mL. This sample was collected from a household occupied by an elderly woman. Interestingly, no bacterial colonies were obtained from the shower and bathroom of this house.

Overall, the results from water samples collected from kitchen taps confirmed the presence of β-lactam antibiotic-resistant bacteria, similar to bathrooms and showerheads. However, the level of contamination in this part of the household water distribution systems was relatively low and only comparable to bathroom samples, with only seven ampicillin-resistant bacteria isolated from 30 water samples. Although the number of positive samples for ARB was low in kitchen taps, significant variation was observed in the number of colonies in samples from different households.

Samples	Isolation Location	Code	Gram Staining	Acid-Fast Staining	Cell Shape (Aspect)	Colony Morphology
4	K	4K	-	-	Rod	Round, dry, flat, light red, moderate
8	K	8K	-	-	Rod	Undulate, smooth, flat, white, big
11	K	11K	-	-	Rod	Round, dry, convex, black, small
14	K	14K	-	-	Rod	Round, dry, convex, gray, small
16	K	16K	+	-	Rod	Round, powdery, convex, pink, small
19	K	19K	-	-	Rod	Round, smooth, convex, white, small
22	K	22K	-	-	Rod	Round, smooth, convex, cream, small

Table 5.3: Presents characterisation of isolates, including Gram staining results, Acid-fast staining results, and phenotypic characterisation of the isolates from kitchen taps across 30 houses. "K" represents kitchen tap.

5.3.9 Preliminary Characterisation of Isolates from Kitchen Taps

The bacterial colonies isolated from kitchen tap water samples were preliminarily characterised based on colony phenotypic morphology, Gram staining and Acid-fast staining results. The results are presented in Table 5.3. All isolates were Gram-negative, except for isolate 4K, which was Gram-positive. Acid-fast staining results were negative for all isolates. The growth kinetics of the isolates were assessed over a 120 h incubation period at 37 °C. Growth curves and CFU/mL values for the isolates were measured and compared at regular intervals (5.12). Among the isolates, 11K exhibited the highest growth rate, while isolate 4K displayed the lowest growth throughout the incubation time.

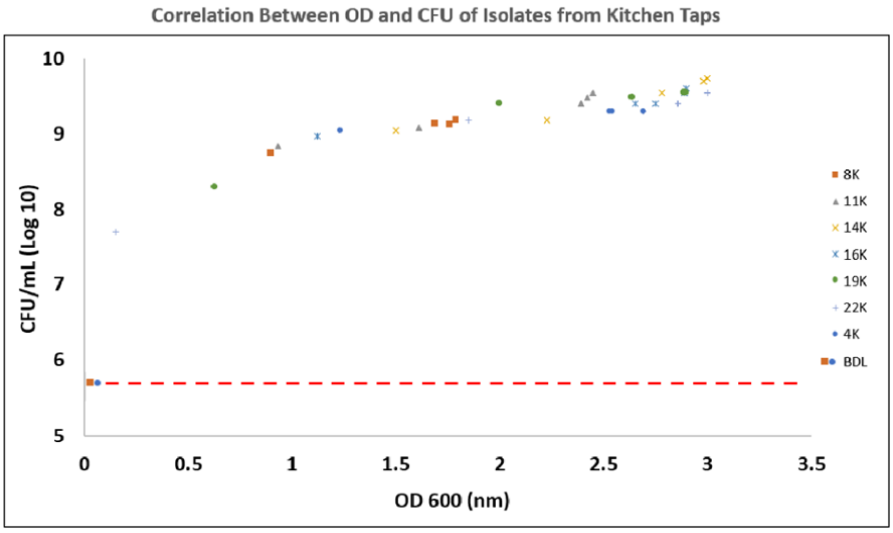


Figure 5.12: Compares and correlates the OD_{600} and CFU/mL values across seven (7) bacterial isolates from kitchen taps every 24h. In cases where no colonies were observed on the plated dilutions, the data is represented as below the detection limit (BDL), following the Miles & Misra technique. A logarithmic scale is used for CFU/mL to accommodate the wide range of observed values. Different coloured symbols are used to represent the samples, while the red dashed line indicates the limit of detection (LOD).

5.3.10 Identification of Bacteria isolated from Kitchen Taps and Phylogenetic Analysis

Based on phylogenetic analysis and 16S rRNA gene sequences, isolate 22K was closely associated with *Acidovorax-Betaproteobacteria* cluster. Two isolates of 8K and 14K were closely linked to the *Cupriavidus-Burkholderia* cluster. The isolate 11K were closely related to the *Pseudomonas* clade, which includes different species of *Pseudomonas*. Isolate 16K was closely similar to *Microbacterium oxydans*, belonging to the *Microbacterium* group. Isolate 4K was identified as *Niabella yanshanensis,* belonging to *Niabella* cluster. Isolate 19K was clustered with *Bosea eneae* and other *Bosea* species. All the strains were closely related to their phylogenetic neighbours, showing 98.88% to 100% sequence similarity in their 16S rRNA gene sequences with high bootstrap values (5.6). Fig 5.13 visually represents the occurrence and concentration of bacterial genera in kitchen taps collected across the residences.

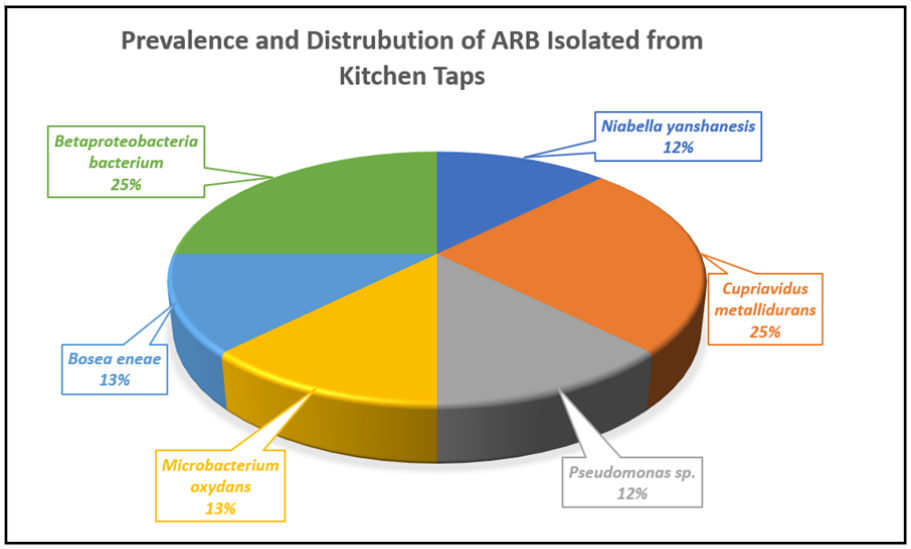


Figure 5.13: Illustrates the prevalence and diversity of ARB isolated from household kitchen taps and shows the frequency of occurrences of each genus.

5.3.11 Comparison of Prevalence, Distribution, and Concentration of ARB Across three Sampling Sites

A total of 42 opportunistic ampicillin-resistant bacterial isolates, representing different morphotypes, were isolated from 30 houses across three sites per residence. Isolated bacteria belonged to 15 distinct genera. It was found that a maximum number of bacteria were isolated from showerheads (59%), followed by the bathroom (24%) and kitchen (17%) (Fig 5.14). Showerheads and kitchens exhibited maximum and minimum levels of contamination across the houses. The overall mean bacterial counts (total CFU) for water samples collected from showers, kitchens and bathrooms were 56, 30.25, and 23, respectively. The calculated average CFU offers insight into the overall level of contamination in these environments. No bacterial growth was detected at any of the three sites (kitchen tap, bathroom tap, and showerhead) in 10 samples (samples 6, 7, 13, 17, 20, 21, 24, 25, 29, and 30). In contrast, in the remaining houses, at least one, two, or all three tested sites showed the presence of ampicillin-resistant bacteria. Gram-negative bacteria were the most commonly isolated from all three sites. 40 out of the 42 bacteria were Gram-negative with two

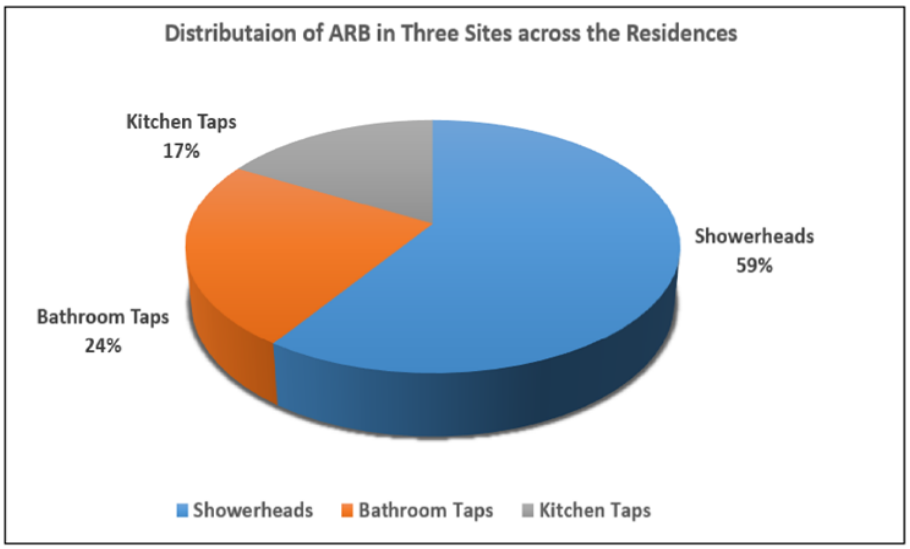


Figure 5.14: Provides a visual representation of the occurrence of bacterial isolates at each site that enables a comparative analysis of microbial contamination across different household water sources.

exceptions (isolate 16K and 23S), which were Gram-positive. All the isolates in this study tested negative for acid-fast staining, suggesting that they do not belong to acid-fast mycobacterial species. Among the genera, *Cupriavidus* and *Pseudomonas* were the dominant genera and were found at all three sites.

5.4 Discussion

Our finding after using filtration as an optimal method to isolate ABR was consistent with the study by Thomson et al. 2008, who compared filtration and centrifugation for processing potable water samples to isolate nontuberculous mycobacteria (NTMs). They stated that filtration is a more effective and accurate method for concentrating mycobacteria than centrifugation in water samples. In addition, El Boujnouni et al. 2022 compared membrane filtration, filtration on the gauze pad and centrifugation for bacterial recovery rate. Their result showed that the membrane filtration protocol yielded the best recovery rate and concentration of microorgan-

isms, followed by filtration on a gauze pad. This methodological shift enhanced the reliability of bacterial isolation and improved the overall quality and consistency of the data collected. This efficiency is likely due to the effective retention of bacteria on filter membranes. In contrast, centrifugation may result in bacterial loss or damage due to potential mechanical stress (centrifugal force), high-speed processing, inconsistent pellet formation, and variability, affecting bacterial viability and recovery (El Boujnouni et al. 2022). The result of the current chapter confirmed that the presence of antibiotic-resistant bacteria is common in household fixtures, raising concerns about their potential impact on public health. However, the level of contamination varied across the houses significantly. There are many factors that can contribute to the variation in ARB contamination levels observed across different households. For instance, these factors include the quality of the incoming water supply (e.g., municipal vs. private well), the geographical location of the houses, the material and condition of the plumbing system (e.g., plastic vs. metal, old vs. new), the number of occupants in each house, and the presence or absence of water treatment devices, such as filters or purifiers. Bacterial prevalence and diversity were higher in showerheads compared to bathroom and kitchen taps. This finding highlights the role of showerheads as potential reservoirs of microbial contamination in domestic water systems, and this phenomenon can be attributed to the formation of biofilms, which occur due to stagnant water and the infrequent use of pipes leading to the showerhead. When water remains stationary for extended periods, pipelines can provide an ideal environment for extensive biofilm growth on the pipe walls. Opportunistic antibiotic-resistant bacteria can persist in showerhead biofilms due to the protective environment provided by the biofilm matrix, which protects them from stressors like disinfectants and the unique dynamics of water flow. However, the kitchen and bathroom sinks are used more frequently, which prevents the water from remaining stagnant for long periods. The continuous water flow in these taps might disrupt the bacterial growth cycle, reducing the chances for bacteria to

form biofilms on the pipe surfaces, which justifies our result. Schages et al. 2020 introduced shower drains as an important source of ARGs and ABR in private households. Factors such as temperature and water residence time influence biofilm formation in pipelines. The duration of time that water remains in the distribution system (water age) directly impacts its chemical composition, potentially promoting the growth of pathogenic bacteria and ARB (Venâncio et al. 2023). The material of pipelines is a critical factor that needs to be taken into consideration. Introducing materials that minimise biofilm formation on pipe walls could effectively improve water safety for consumers. Plastic pipes are the most commonly used material in drinking water distribution systems. However, plasticizers, which are key components of these pipes, play a significant role in enhancing biofilm formation and the growth of ARB (Kalu et al. 2024). Understanding how biofilm formation facilitates the transmission of the resistome in water is crucial. This knowledge can offer valuable insights into evaluating biofilm-associated risks and optimising pipe material selection to enhance biofilm control in drinking water distribution systems (Goraj et al. 2021). Despite the high temperature and pressure within showerhead systems that inhibit bacterial growth, many bacteria can adapt by forming biofilms, entering dormancy, or resisting stress. This allows them to survive unfavourable conditions and reactivate when conditions improve, explaining the abundance and diversity of bacteria in showerheads. As a result, while bacterial proliferation may be inhibited, their persistence in biofilms poses a potential risk of intermittent release into water streams during use (Makris, Andra, and Botsaris 2014). The presence of these bacteria in drinking water highlights the importance of monitoring and maintaining water quality, especially for vulnerable populations. A primary intention for this study was to isolate and identify nontuberculous mycobacteria (NTMs) from household plumbing systems, as these specific opportunistic, antibiotic-resistant bacteria are known to pose significant health risks, particularly to immunocompromised individuals. NTMs are highly resistant to commonly used water disinfectants like

chlorine, and their ability to persist in urban water supplies may be a contributing factor to the rising incidence of NTM lung disease across the world, presenting a substantial public health challenge (Falkinham 2015). Although several studies have reported the isolation of NTM from water samples collected from various sources (Cerna-Cortes et al. 2019; Maranha et al. 2024; Dowdell et al. 2019; Vaerewijck et al. 2005; Moradi et al. 2019; Maleki et al. 2017) no NTMs were detected in the water samples collected from the households, which was against our initial expectation that NTM could be isolated from household water systems. The findings suggest that isolating NTM from household water is unlikely under the conditions studied. Despite using selective growth media designed explicitly for isolating NTM (Middlebrook 7H9 broth and 7H11 agar) and performing Acid-fast staining, which is a technique widely recognised for the identification of mycobacteria, we were unable to detect any NTMs. These negative results indicate that, at least in our study, NTMs are not commonly found in household water systems, or they may be present at levels too low to be detected using the methods employed. Instead, other opportunistic bacteria that exhibited ampicillin resistance were detected. These isolated bacteria pose significant health risks due to their ability to resist ampicillin. Nearly all the isolates have previously been reported as antibiotic-resistant, particularly to ampicillin. Notably, based on our knowledge, *Methylorubrum extorquens* and *Paracoccus marinus* were found to be ampicillin-resistant for the first time. Infections caused by antibiotic-resistant bacteria are becoming a serious healthcare concern worldwide, and their presence in drinking water further exacerbates the problem. For instance, *Pseudomonas aeruginosa* can cause Pseudomonas infections, *Burkholderia mallei* can lead to Glanders disease, and *Sphingomonas* species are associated with sepsis, meningitis, endocarditis, visceral abscesses, enteritis, osteoarticular, urinary, skin, or soft tissue infections (Kalu et al. 2024). Growing evidence suggests that current therapies are often insufficient to effectively combat infections caused by ABR. Therefore, there is an urgent need to develop safe and effective treatments to

address this global issue.

Cupriavidus metallidurans is one of the robust opportunistic pathogens (OP) that is commonly found in drinking water systems and potentially contributes to the dissemination and persistence of antibiotic-resistance in drinking water systems (Charnock and Nordlie 2016; Khan, Knapp, and Beattie 2016; M. Zhang et al. 2018). This bacterium exhibits strong resistance to various environmental stresses, making it a resilient inhabitant in these aquatic environments (Mijnendonckx et al. 2013). Its ability to thrive in drinking water highlights its adaptability and potential impact on water quality and safety (M. Zhang et al. 2018). This fact may support this study, as 25% of the total isolated bacteria belong to this genus, and they were found throughout all three locations within the studied residences. M. Zhang et al. 2018 isolated *C. metallidurans* from a drinking water filter and showed that the organism is extremely resistant to chlorine and chloramine. They also confirmed that multi-resistance traits and the resistance megaplasmids were effectively preserved in the bacteria, which makes them dormant reservoirs of resistance genes in the environments.

Delftia tsuruhatensis is another emerging opportunistic healthcare-associated pathogen, particularly in immunocompromised patients (Ranc et al. 2018). It causes various infections, including bacteremia, endocarditis, and urinary tract infections (Ranc et al. 2018). Initially, it was isolated from environmental samples and studied for its environmental roles (bioremediation of organic pollutants), unlike now, which is associated with human infections and can pose health risks under specific conditions, particularly in vulnerable populations (Juárez-Jiménez et al. 2010). In addition to *D. tsuruhatensis, D. acidovorans* have also been identified as clinically significant in both immunocompromised and immunocompetent patients (Bilgin et al. 2015). (Ranc et al. 2018) documented a case of pneumonia caused by *D. tsuruhatensis* in an infant following cardiac surgery. The antibiotic susceptibil-

ity of *Delftia* species varies widely. Most isolates are sensitive to broad-spectrum cephalosporins, carbapenems, and fluoroquinolones, but they tend to show resistance to aminoglycosides (Lang, Chinzowu, and Cann 2012). This variability in susceptibility, combined with their potential to acquire and transfer antibiotic resistance genes, is particularly concerning in water systems. *Pseudomonas aeruginosa* and *Burkholderia* are another group of opportunistic, drug-resistant pathogens responsible for complex infections in cystic fibrosis (CF) patients and can also cause infection in other immunocompromised individuals (Bartell 2015). Antibiotic-resistant *Pseudomonas* species were isolated from tap water sourced from a karstic springs system during turbidity events in Le Havre, France (A. F. Ribeiro et al. 2014). Su et al. 2018 confirmed that Bacteria and ARGs were still present in tap water after treatment; however, their percentage significantly declined. They observed increased presence and proliferation of *Pseudomonas* parallel to increased dissemination of ARGs in the filtration stage of drinking water treatment. They suggested that *Pseudomonas* could transfer resistant genes to other abundant bacteria. Based on the study by Kalu et al. 2024, almost 97% of OP like *P. aeruginosa* was significantly removed in disinfected water. Surprisingly, they reappeared within the distribution system and were detected in the tap water (J. Huang et al. 2021). Ramalingam 2013 isolated *Xenophilus sp* and *Methylobacterium sp* from domestic drinking water in Sheffield, UK. Bridgeman et al. 2015 analysed over 200 samples collected from source water to customer taps across three UK water companies in 2015 and reported the presence of *Xenophilus* and *Methylobacterium*. Z. Han et al. 2020 found that the most abundant bacteria in tap water were classified as *proteobacteria*, including α, β, and γ-*Proteobacteria*. They also confirmed that, compared to source water, the relative abundance of α- and β-Proteobacteria increased 66.98% and 146.88% in tap water, respectively. However, the relative abundance of other bacterial groups declined. Falcone-Dias, Vaz-Moreira, and Celia M Manaia 2012 isolated 238 ABR from bottled mineral waters, with the majority showing resistance to mul-

tiple antibiotics like *Betaproteobacteria* and *Acidovorax*. It was found that *Bosea* was among the genera exhibiting higher levels of multi-resistance. In Glasgow, Scotland, Khan, Knapp, and Beattie 2016 discovered antibiotic-resistant *Burkholderia* and *Sphingomonas*, with other bacteria carrying ARGs, in tap water. Perkins et al. 2009 demonstrated that membrane-integrated showerheads significantly reduce the microbial load in indoor air from shower water and aerosolised mist compared to conventional showerheads. Their study confirmed that these advanced showerheads can effectively protect immunocompromised patients from waterborne infections in a stem cell transplant unit. This protection is due to their ability to efficiently capture large numbers of potentially pathogenic bacteria from hospital water, making them a viable mitigation strategy in healthcare settings. Addressing ARB and ARGs in water systems requires a comprehensive approach that includes improved detection methods, advanced water treatment technologies by prevention and control strategies, regular monitoring, antibiotic stewardship, and public awareness campaigns. Effective strategies to protect public health and ensure safe water supplies can be developed by understanding the sources, mechanisms, and health implications of these bacteria.

In real-world environments, biotic and abiotic contaminants coexist. Certain chemical pollutants in water can, directly and indirectly, promote the spread of ARB and ARG through mechanisms such as selective pressure, co-selection, enhanced gene transfer, and biofilm promotion (O'Neill 2016). Numerous studies have shown that various contaminants besides antibiotics, such as metals (X. Ji et al. 2012), detergents (Najim 2017), biocides (Adkin et al. 2022), nanomaterials (Z. Qiu et al. 2015), pesticides (D. Qiu et al. 2022), PFAS compounds (PFOS) (L. Yin et al. 2023), and MP (Y. Liu et al. 2021) can promote the dissemination of ARGs. These compounds act as emerging carriers for ARGs, facilitating the transfer of ARB and ARGs across various environments due to their mobility and ubiquity, resulting in

significant ecological impacts (Dong et al. 2021). However, the mechanism through which opportunistic pathogenic bacteria obtain the resistance, as well as their prevalence and ultimate fate in the environment, are still not well understood and need more investigation. PFOA and PFOS, as ubiquitous contaminants in the environment, can increase the risk of bacterial infections in humans by promoting the growth of pathogen bacteria in water distribution systems (Bulka, Avula, and Fry 2021). H. Yin et al. 2023 reported that PAEs (plasticizers) and PFAS coexistence affects bacterial communities and pathogens in rural drinking water distribution systems. Their findings revealed that the combination of PAEs and PFAS increased the growth of potential human pathogens, including *Burkholderia mallei* and *Pseudomonas aeruginosa*. Qing Wang et al. 2021 found that the selective pressure from heavy metals contributes to the rise of ampicillin-resistant opportunistic pathogens in the Xiangjiang River, China. This influence of heavy metals may ultimately lead to the prevalence of multidrug-resistant pathogenic bacteria by facilitating the horizontal transfer of conjugative plasmids. They confirmed that the presence of β-lactam resistance genes in opportunistic pathogenic bacteria was 2-10 times higher in groups exposed to heavy metals compared to the control group. This finding may support our finding, as a high proportion of isolated bacteria have been reported for heavy metal degradation. The co-selection mechanism provides a plausible explanation for this correlation (N. Bai et al. 2020; Doolotkeldieva, Bobusheva, and Konurbaeva 2024; Awasthi et al. 2015).

5.5 Conclusion

Although the presence of ARB in water supplies is well recognised, there is limited information about the extent of their presence in different households and specific water sources, as well as how this contamination varies across community envi-

ronments. Addressing this knowledge gap is critical for public health, as it can provide insights into the factors influencing bacterial diversity and resistance in domestic water systems. This chapter aimed to address the third research question in my thesis: How do bacterial genera/species and contamination levels vary across households in a community setting, and what implications does this have for the spread of antibiotic resistance? Bacteria were isolated from 30 households in the West Midlands region of the UK. Sampling was conducted across three key sites: showerheads, bathroom taps, and kitchen taps. A total of 42 ampicillin-resistant bacterial isolates, identified as opportunistic pathogens, were recovered from these household water systems. These isolates may pose a potential risk to human health, particularly to immunocompromised individuals. The results indicated that showerheads exhibited the highest levels of bacterial contamination. However, kitchen taps showed the lowest levels of contamination. 16S rRNA gene sequencing was employed to identify these isolates. *Cupriavidus, Delftia, Pseudomonas*, and *Bosea* were the most frequently occurring bacterial genera. These genera were almost consistently detected across all three sampling sites within the households, suggesting their widespread distribution in domestic water systems. The observed variation in contamination levels between the different water sources highlights the need for further research. Factors such as household characteristics, including demographics, water usage patterns, pipeline materials, plumbing system conditions, and water source quality, may influence the presence and distribution of antibiotic-resistant bacteria in domestic water systems. In addition to household characteristics, other environmental contaminants, such as MP and PFAS, which are ubiquitously present in the environment, contribute to the proliferation and dissemination of ARB. MP provide surfaces for biofilm formation, which creates an environment for bacteria and enhances their ability to exchange antibiotic resistance genes within bacterial communities. Similarly, PFAS, due to their persistence and toxicity, can disrupt bacterial communities in water systems, leading to imbalances that favour resistant

strains. These chemical contaminants not only exacerbate the proliferation of ARB but also increase the risk of resistance transfer within microbial populations. Future studies should aim to explore the variables in detail to better understand the factors driving ARB prevalence and distribution in household water. Such investigations could provide valuable insights for developing strategies to mitigate ARB contamination and reduce its potential risks to public health and the environment.

This research can contribute as baseline data to the development of guidelines for the responsible and optimal use of antibiotics. Future studies should focus on understanding the evolutionary mechanisms of resistance to ARB in water systems, the concentration of antibiotics in water and the potential risks posed by the consumption of contaminated water. Additionally, it is crucial to share the findings from studies like this with relevant stakeholders and impacted communities to ensure informed decision-making and awareness.

Chapter 6

Key Research Findings, Synthesis, and Future Directions

This chapter presents the results and key findings of this thesis, integrating them with relevant literature to provide a comprehensive analysis. It begins with a discussion of the proof-of-concept study conducted in Chapter 3, which served as a critical preliminary step before advancing to the chronic exposure experiments in Chapter 4. This study focused on the feeding behaviour of *Daphnia magna*, specifically investigating the ingestion, retention, and egestion of four common microplastics (MP). MP were characterised, a specific MP type was refined and selected, and the experimental design was optimised before proceeding with the chronic exposure experiments. This chapter addresses a part of research objective 1 (RO 1). Chapter 4 investigates the toxicological effects of MP, PFOA, and PFOS on two distinct genotypes of *Daphnia magna* with different histories of chemical exposure. It also explores two-way and three-way interactions among these contaminants, assessing their combined impact on *Daphnia's* overall health. The findings in this chapter address the first and second research objectives.

Additionally, the current chapter summarises the findings from Chapter 5 and correlates them with current literature. This chapter investigated the presence and prevalence of antibiotic-resistant bacteria (ARB) in household plumbing systems, aligning with the third research objective.

Finally, the chapter outlines future research directions, highlighting potential advancements and recommendations for further studies to build upon the findings of this thesis.

The following sections provide a detailed discussion of each chapter along with its corresponding research objective (RO). Chapter 4 consists of three sections, each addressing different aspects of the study. As this chapter contributes to answering two specific research objectives, its sections are structured to systematically present the findings and their relevance to these objectives

6.1 RO1: Factors Influencing the Ingestion and Egestion of Microplastics by *Daphnia*

In Chapter 3, a detailed proof-of-concept study offers valuable insights into the ability of *Daphnia magna*, to ingest, retain and egest four distinct types of microplastics (MP), both with and without algae as a food source. Four common types of plastics, representative of materials widely used in various sectors such as medical, engineering, construction, and electrical industries, were selected. Considering the widespread occurrence of residual microplastics from these sources in the environment, this research investigates the interaction between *Daphnia magna* and these microplastics, providing valuable insights into their potential ecological effects. Our own-made irregularly shaped MP, produced by cryogenically grinding and sieving

to achieve specific size ranges (ingestible by *Daphnia*), were used in the study. The experiment used dynamic light scattering (DLS) and microscopy as MP characterisation techniques. Our initial analysis revealed that all the tested MP were negatively charged and primarily consisted of fibres and fragments, the common shapes of MP found in the environment. MP particles that exhibited a broad spectrum of average sizes and various shapes were analysed, highlighting the considerable variability in their physical characteristics. *Daphnia* ingested and egested the MP that mimic the natural environment in terms of shape and concentration. However, the retention time in the gut varied depending on the type of MP. PE and PS were extracted from *Daphnia* gut within 72 h, exhibiting the longest retention time. However, PET showed the shortest retention time (24 h). In addition, PMMA was excreted within the first 48 h from *Daphnia's* guts. The observed differences in egestion timing reflect a combination of particle morphology and size-dependent gut-processing efficiency, which together influence how long MP remain within *Daphnia* and their potential biological impacts. Despite these differences in retention time, no distinct pattern was observed in the ingestion process. The animals' guts were filled with MP particles at comparable levels under conditions with different types of MP in the presence and absence of algae. It demonstrates *Daphnia's* filter-feeding behaviour, showing that even in the presence of algae in their environment, they non-selectively ingested particles. However, their egestion rate varied depending on the different types of MP. Exposure of *Daphnia manga* to these MP at a concentration of 100 mg/l did not cause significant mortality, indicating that the organisms exhibited a notable tolerance to the MP under the experimental conditions. This suggests that *Daphnia magna* can survive in environments with similar microplastic concentrations without immediate lethal effects. However, while no mortality was recorded, further investigation is needed to assess potential sub-lethal impacts, such as changes in behaviour, reproduction, or physiological stress, which may not be immediately apparent but could have long-term ecological and biological implications that are

assessed in Chapter 4. This finding underscores the importance of evaluating both acute and chronic effects of MP on aquatic organisms to better understand their overall ecological impact. Overall, this chapter highlights the importance of considering the physical characteristics of MP when assessing their effects on aquatic life. Our findings contribute to advancing the understanding of how MP characteristics such as size, shape, and surface properties may influence their biological effects on aquatic organisms. This highlights an important direction for future research, where the physical traits of MP should be more thoroughly considered alongside their chemical composition. The results of this study strengthen the growing argument that MP toxicity is not solely chemically driven but also mediated by physical interactions, which represents an emerging area of concern in ecotoxicology. Based on our findings, a specific type of MP, polyethylene terephthalate (PET), was selected for the subsequent study phase that focuses on a chronic exposure experiment to investigate the long-term impacts of MP and PFAS exposure on *Daphnia magna* more comprehensively.

6.2 RO 1: Toxicological Impact of MP, PFOA and PFOS on *Daphnia*

It is vital to monitor and understand the impacts of anthropogenic chemicals, as they can cause unintended, long-lasting effects on non-target organisms, even at concentrations below established regulatory thresholds. The aim of this study was to address key challenges in monitoring chemical pollution by utilising *Daphnia* as a bioindicator. *Daphnia* can detect and signal the presence of toxic chemicals in aquatic environments as a diagnostic agent, providing valuable insight into pollution levels and ecosystem health. Chapter 4 investigates the toxicity of MP, PFOA,

and PFOS as emerging and persistent chemicals identified in international regulatory frameworks and their combined effects on *Daphnia magna* under chronic exposure conditions. An important first step was taken to investigate the long-term effects of seven stressor chemicals, representing different combinations of MP, PFOA, and PFOS (single, two-way, and three-way combinations of MP, PFOA and PFOS) that include MP, PFOA, PFOS, MP+PFOA, MP+PFOS, PFOA+PFOS and MP+PFOA+PFOS. The toxicological impact of these chemicals on key life-history fitness traits of two genotypes of *Daphnia magna* with distinct histories of chemical exposure was studied. The study focused on the seven tested stressors that significantly affected the overall fitness of two genotypes of *Daphnia*, and these effects were driven by different combinations of the life history traits in the seven stressors. It was confirmed that when *Daphnia* are exposed to even low, environmentally relevant concentrations of these chemicals for a long time, the chemicals can affect *Daphnia's* overall health, like their growth, survival, and reproduction. Significant negative impacts on various occasions were observed that could be due to treatments (T), genotype (G) and/or (G x T) effects that were driven by a smaller size, delayed maturation, reduced fecundity and longer intervals between broods. The findings revealed that exposure to PFAS and MP causes developmental failure and significant delays in sexual maturation and stifle growth. These effects highlight how the compounded presence of PFAS and MP in aquatic environments can profoundly disrupt biological functions critical for survival and reproduction. The disruption caused by these contaminants can lead to serious consequences for the health of aquatic life and the stability of ecosystems. The PFOS treatment caused a decrease in number of offspring and developmental problems, leading to a high number of aborted broods. Mortality rates were not significant across both genotypes tested, indicating a limited lethal effect under the experimental conditions. However, an exception was observed in the genotype LRII36_1, where exposure to PFOS resulted in a notable increase in mortality. This suggests that this genotype may

exhibit increased sensitivity to PFOS exposure compared to another genotype, potentially due to genetic or physiological differences. Further investigation is needed to determine the underlying mechanisms driving this increased susceptibility. Since *Daphnia* are primary consumers and a critical food source for fish and other invertebrates, any adverse impacts on their populations could disrupt food webs, reduce fish populations, lower biodiversity, and alter ecosystem functions through cascading effects. These findings contribute to a deeper understanding of the ecological implications of MP and PFAS and inform strategies for environmental management and pollution mitigation. Using *Daphnia* as a sentinel species helps to identify bioactive components in chemical mixtures and accurately determine their toxicity targets. These bioactive components are linked to potential molecular initiating events (MIEs). In chemical toxicology, MIEs are the first interactions between a chemical and a biological target that can trigger a chain of biological responses that may ultimately lead to harmful effects in an organism. As an initial step in the adverse outcome pathway (AOP), MIEs link molecular-level changes to broader toxic outcomes, forming the foundation for understanding how chemicals cause adverse effects. MIEs can disrupt normal functions, have a cascade of downstream effects, and eventually result in visible toxic impacts like developmental failure or immune dysfunction. Recognising MIEs that are evolutionarily conserved allows scientists to predict chemical risks, design toxicity tests, and better understand pollution's ecological effects across other species (Krewski et al. 2020).

6.3 RO 1: Naive Genotype Showed Greater Fitness in Response to Novel Chemical Stressors

The approach of utilising two genotypes of naive and experienced *Daphnia*, provides insights into how a history of pollution affects their tolerance and how well they can tolerate or are susceptible to new mixtures of pollutants during chronic exposure. It was first expected that *Daphnia* genotype with a history of exposure to environmental contaminants would exhibit a higher overall fitness, likely underpinned by an enrichment of genes or pathways involved in detoxification. Such genetic enrichment may have evolved as a response to prolonged exposure, providing these genotypes with enhanced biochemical tools to manage and mitigate the harmful effects of pollutants. This adaptation would suggest that the "experienced" genotype is better equipped to cope with new chemical challenges, showing greater tolerance due to its refined detoxification mechanisms and possibly other protective pathways. In contrast, it was expected that "naive" genotype (those without prior exposure to contaminants) would demonstrate a lower tolerance to these novel chemical stressors. Without the evolutionary advantage conferred by prior adaptation, these naive genotypes may lack the specific detoxification capabilities that experienced genotypes possess, rendering them more susceptible to the adverse effects of new chemical environments. This anticipated difference in tolerance levels between the experienced and naive genotypes highlights the role of environmental exposure in driving evolutionary tolerance and adaptation in *Daphnia*. Our results revealed significant fitness differences between the two genotypes that might be driven by variations in pathways enriched for detoxification, catabolic, and metabolic functions between experienced and naïve genotypes. Contrary to our expectation, on occasions where genotype-specific effects were observed, the naive genotype (LRII36_1) displayed greater tolerance to novel chemical stressors, along with lower fitness costs,

as indicated by ecological endpoints such as mortality rates and changes in phenotypic trajectories, however, the genotype with a history of chemical stress exposure (LRV0_1) experienced the most significant fitness costs, except mortality rates, which were higher in the naive genotype during both PFOS and PET exposures which are linked to reduced genetic diversity in detoxification, catabolism, and endocrine genes in the naive genotype. These results align with previous research, suggesting that *Daphnia* genotypes that are naive to chemical stress exhibit greater tolerance to new chemical stressors, while the experienced genotype showed lower tolerance to novel chemicals (A. M. Abdullahi et al. 2023; M. Abdullahi, X. Li, et al. 2022 and Noyes et al. 2009). For example, A. M. Abdullahi et al. 2023 used the same genotypes of *Daphnia* and exposed them to five different chemicals, including pharmaceuticals. Their results showed that naïve genotypes exhibited greater tolerance to chemical exposure, which supports and validates the findings presented in this study.The reduced tolerance observed in the experienced genotype suggests that prior exposure to contamination has lasting biological impacts, potentially making some populations more susceptible to the cumulative or compounded effects of persistent pollutants over time. Also, a diminished capacity to adapt to changes in environmental conditions, such as detoxifying new chemical stressors, and past exposure to certain chemicals may have favoured the selection of genotypes adept at handling those specific chemicals while potentially compromising their ability to manage novel chemical stressors. Our study and a few others that investigated more than one genotype highlight the importance of considering genetic diversity and historical exposure in ecotoxicological evaluations. These findings reveal the drawbacks of relying solely on single laboratory strains for toxicity testing and emphasise the transformative impact of renewable energy (RE) in ecotoxicology by supporting sustainable practices, reducing pollution, or contributing to innovative environmental assessment and management methods. By integrating population-wide responses and historical exposure information into chemical risk assessments, RE enhances

the accuracy of toxicity evaluations and advances the development of the emerging field of paleoecotoxicology. The observed genotype-specific responses revealed the intricate relationship between genetic background and pollutant exposure, highlighting the necessity of including multiple genotypes in environmental risk assessments to predict the ecological impacts of chemical pollutants accurately.

6.4 RO 2: Combined Effects and Interactions of Microplastics, PFOA, and PFOS on *Daphnia*

Naturally, contaminants from various sources co-occur in the environment and interact with one another. Their interactions can be classified as additive, where their combined toxicity equals the sum of each chemical's effect; synergistic, where the combined impact exceeds what would be expected from each pollutant alone; or antagonistic, where the interaction reduces their overall effect. Surprisingly, despite the widespread coexistence of MP, PFOA, and PFOS in aquatic environments, their combined toxicity in aquatic biota remains unexplored. By taking advantage of *Daphnia's* strong sensitivity to environmental pollutants, it was aimed to identify the risks from toxic chemical mixtures and toxicity targets in water ecosystems. This strategy improves our ability to identify hazardous pollutants and supports the development of more effective prevention measures, ultimately protecting ecosystem health and reducing chemical risks. This holistic strategy makes chemical safety checks more accurate and effective, improving environmental protection and public health outcomes. Based on our knowledge, this is the first study that reports the combined effects of MP and PFAS on *Daphnia* as sentinel species.

The interactions between pollutants were quantitatively assessed using a null additive model to identify potential synergistic, antagonistic, or additive effects. The

impact and interactions of two-way and three-way chemical mixtures on the life-history traits of *Daphnia* were evaluated. Our results revealed that these chemical mixtures demonstrated additive and/or synergistic interactions that affected the organism's physiology, behaviour, and survival. Such findings emphasise the need for integrated environmental policies and ongoing research to address the unique risks posed by these persistent chemical mixtures, particularly for aquatic species crucial to ecosystem health. Assessing these interactions provides critical insights into how these widely prevalent environmental contaminants may jointly impact marine ecosystems, a topic not explored in previous studies. As a key species in the aquatic food web and an essential ecosystem bioindicator, the impact of environmental stressors on *Daphnia* can trigger cascading effects throughout freshwater ecosystems, potentially affecting a wide range of organisms. Beyond this indirect influence via the trophic chain, analysing the conservation of biological pathways across the Tree of Life could identify whether the same pathways are vulnerable in other species. This broader understanding would help determine if similar toxicological impacts observed in *Daphnia* could also occur in other organisms, further emphasising the ecological importance of protecting this sentinel species that helps to better understand the potential hazards of environmental mixtures to other animals. Although our model can predict potential chemical mixture hazards for various species, it is crucial to understand that common mechanisms of action do not always equate to expected adverse effects. The proposed model can predict the potential hazards of chemical mixtures to various species. In the context of evolutionary mechanisms that underpin responses to complex environments, there is still no agreement on how multiple stressors influence adaptive pathways in natural populations.

6.5 RO 3: Antibiotic-Resistant Bacteria as Emerging Biological Contaminants in Domestic Water

In chapter 5, the presence, prevalence and distribution of ABR in household plumbing systems was investigated. This part of this thesis provides valuable insights into how these resistant microorganisms persist and spread in everyday environments by investigating ARB within domestic water networks. The findings underscore the need to monitor ARB in water systems to mitigate their impact on ecosystem health and human well-being. Forty-two (42) strains of ampicillin-resistant bacteria (belonging to 15 distinct genera) were isolated, characterised, and identified from three locations of showerheads, bathroom sinks and kitchen taps within 30 houses. The findings indicate that showerheads serve as a favoured location for the proliferation of ABR and are considered the most contaminated site across the houses. This trend corresponds with biofilm formation in plumbing systems, particularly parts that experience stagnant water due to infrequent use. However, the prevalence of ARB was lowest in kitchen taps, followed by bathroom sinks. This observation suggests that a higher frequency of use in these areas reduces the likelihood of biofilm formation within the plumbing, thus mitigating the potential for bacterial growth and antibiotic resistance. Furthermore, a consistent pattern was observed, revealing the recurring presence of specific bacterial genera across all analysed samples. This finding indicates that these particular bacteria are widespread and likely integral components of the microbial community within drinking water systems. Their repeated detection suggests they play a common role in the dynamics of the ecological system or could indicate environmental or operational factors that consistently support their growth and persistence in such settings.

By integrating the diverse elements of this research presented in chapters 3 to 5, our understanding of the ecological consequences associated with MP, PFAS and

ABR contamination is significantly enhanced. Our findings emphasise the need for developing more laboratory- based toxicity testing protocols that better reflect the complexities of natural environments. These improved methods would produce more accurate, relevant, and applicable toxicity data, essential for assessing the broader ecological implications of such pollutants by mirroring real-world scenarios. These findings call for more comprehensive studies in toxicity assessments, emphasising the need to account for the combined and long-term effects of pollutants on aquatic systems. Moreover, The findings of this study offer valuable baseline data that can be used to assess the safety of chemicals before their introduction into the market and, ultimately, into natural environments. This data is a critical reference point for evaluating potential risks and impacts associated with new chemicals, ensuring they meet safety standards and minimise adverse effects on the environment and living organisms. Utilising this information enables regulatory bodies and researchers to make well-informed decisions about the approval and use of such chemicals. This approach promotes sustainable and responsible chemical management by ensuring thorough testing to identify toxicological risks and systematically evaluate potential environmental and health impacts. Additionally, it supports a safer and more sustainable marketplace for consumers, ecosystems, and wildlife and will establish clear protection goals, further ensuring long-term ecological balance and the preservation of natural systems.

6.6 Recommendation for Future Research

Interdisciplinary collaborations are the ideal way to comprehensively evaluate various stressors' impacts on aquatic environments. By combining expertise from various disciplines, researchers can assess the full scope of these impacts more effectively. This research opens several avenues for future exploration, providing a foundation

for further studies in various fields. At the molecular level, studies may investigate the genetic and physiological mechanisms underlying the organism's response to pollutants, including gene expression, stress response pathways, and adaptive tolerance mechanisms. On a broader scale, these findings can potentially impact environmental applications significantly. They could inform the development of innovative, nature-based solutions for managing and mitigating pollution in aquatic ecosystems. By bridging molecular biology and environmental science, the findings of this thesis could act as a stepping stone for multidisciplinary research, promoting collaboration between fields to address pressing ecological and public health challenges.

Our findings highlight the significant impact and toxicity of MP and PFAS, providing an analytical and conceptual foundation for future omics research where molecular-level insights can deepen our understanding of these pollutants. Traditional ecotoxicology often misses early warning signs of toxicity, focusing on ecological effects that only appear after visible impacts, such as reproductive issues or death. This approach overlooks sublethal effects and the underlying mechanisms leading to these outcomes, limiting efforts to prevent harm to wildlife. *Daphnia* can be used to address these gaps to detect molecular responses to chemical mixtures even at very low concentrations. With its high sensitivity to pollutants and genomic pathways conserved with humans, *Daphnia* enables early detection of toxicity, linking environmental risks to potential human health concerns due to its greater sensitivity to chemicals compared to humans and other animals and its clear, measurable responses to toxic exposures. By linking molecular responses to ecological outcomes, this approach aims to bridge the gap between laboratory-based molecular insights and real-world ecological implications, ultimately enhancing our understanding of how these pollutants affect aquatic ecosystems over time.

The gut microbiota is likely sensitive to these chemical pollutants and other environmental stressors, making it a promising area for further research. The functional

biological pathways impacted by these pollutants will be explored by employing advanced multi-omics techniques, such as gut microbiome profiling and comprehensive transcriptomic analysis (gene expression) in two genotypes. Analysing gene expression helps researchers understand the functional activity of genes in response to these triggers. Additionally, machine learning modelling approaches will be implemented to predict ecotoxicity and uncover the connections between the microbiome and host functional pathways, providing deeper insights into how pollutants affect microbial communities and the host organism's biology. This approach will offer a more holistic understanding of the molecular and physiological mechanisms underlying *Daphnia's* response to environmental stressors.

Also, it is crucial to develop precise methods to accurately quantify the amounts of MP and PFAS ingested by animals using a mass balance approach. This quantification is essential for understanding not only the ingestion rates of these pollutants but also how they vary across different *Daphnia* genotypes. Measuring ingestion levels will allow us to examine potential differences in uptake and retention among genotypes more precisely. Quantifying ingested MP and PFAS in *Daphnia* has significant implications. First, it would enhance our ability to evaluate the effectiveness of *Daphnia* as bioindicators for environmental pollution and pollutant filtration. Second, the data would be critical for assessing the feasibility of using *Daphnia* on a larger scale in wastewater treatment systems to remove MP, PFAS and other pollutants known for their persistence in the environment. This future research will not only expand our knowledge of ecotoxicology but also contribute to the development of more effective and safe environmental management strategies.

The potential application of *Daphnia* in the bioremediation of these contaminants will be another key focus to explore in future research. The *Daphnia*-based technology can effectively remove excess nutrients and persistent pollutants, such as pharmaceuticals, pesticides, and industrial chemicals that are resistant to tra-

ditional treatments. This innovative approach helps meet regulatory standards, including those for emerging contaminants, and supports near net-zero water reuse goals. Combining renewable energy (RE) with bioremediation provides a practical, sustainable, and scalable solution to global water quality issues, broadening the scope of RE beyond its traditional uses. A key objective will be to investigate whether *Daphnia magna*, as a filter feeder, can play a role in mitigating the spread of these contaminants in aquatic ecosystems. Specifically, we will assess *Daphnia's* ability to filter out ARB, MP, and PFAS from water systems and whether it can break down or degrade any components of these pollutants directly or through interactions with its microbiome. This research could be expanded to a larger scale, aiming to address significant environmental, ecological, and public health challenges. Moreover, our findings could serve as a foundation and provide valuable insight into identifying genotypes of *Daphnia* that exhibit greater tolerance to chemical pollutants. This discovery could play a crucial role in scaling up their application in wastewater treatment plants as a natural, bioremediation-based solution. Integrating these genotypes of *Daphnia* as an additional "polishing" step in conventional wastewater treatment processes could enhance treated effluent quality. This improved treatment method reduces the need for extra treatment steps, yielding water that meets higher standards for reuse in applications such as irrigation, industrial processes, and even specific household uses like toilet flushing. Incorporating *Daphnia* as a natural, biological step in the treatment process supports a shift toward sustainable and eco-friendly wastewater management. This nature-based solution enhances overall treatment efficiency and aligns with environmental goals by reducing the toxicity of synthetic chemicals and energy-intensive processes. Promoting safer, high-quality water recycling contributes to more sustainable water resource management and can help meet growing demands for reclaimed water in an environmentally responsible way. Through this innovative application, *Daphnia* is a powerful partner in safeguarding aquatic ecosystems and promoting sustainable en-

vironmental management practices. Conducting large-scale exposure studies will provide valuable data for monitoring pollution in freshwater systems. It will also investigate the long-term accumulation of MP and PFAS in aquatic environments, offering insights into their persistence, potential for bioaccumulation, and biomagnification, as well as their broader ecosystem impacts over time. This approach will enhance our understanding of the prolonged environmental consequences of these contaminants.

Furthermore, another avenue to investigate will be to integrate biotic and abiotic stressors and explore the synergistic interactions between PFAS, MP, and ABR under chronic exposure conditions using *Daphnia* as sentinel species to understand their complex dynamics and combined ecological impacts better, mirroring the natural environments where biotic and abiotic stressors coexist and interact. Also, to investigate the impact of ARB on gut microbiome in *Daphnia*.

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Appendices

Appendix A

Supplementary Information for Chapter 2

Table 1: Summary of common microplastic associated chemicals (MPACs).

Common additives groups	**Compounds**	**Uses/Application**	**References**
Flame retardants (FRs)	**Metal hydrate FRs;** e.g., Magnesium hydroxide ($Mg_2(OH)_4$) and aluminium trihydrooxide (Al (OH_3))	They Reduces flammability of electric cables and other electrical plastic appliances. They release water molecules during the combustion process of plastic products.	(Ambrogi et al. 2017, X. J. Luo et al. 2010b, Xiao, D. Liang, and H. Shen 2016)
	Phosphorus-based FRs; known as char formers	Used in computer connectors, cables, electrical wires, and roofing materials(plastics). They produce chars and phosphoric acid during the burning to protect substrates.	
Continued on next page			

Table 1 – continued from previous page

Common additives groups	Compounds	Uses/Application	References
	Halogenated FRs; (Group VII periodic table). These are F, Cl, Br, and I)	Used in mostly electronics like TV, phones, computers, and in ABS resin. They work in a gaseous state or vapour and interfere with the burning process. They replace all hydrogen atoms with the halogen ones during the combustion. This group is the most effective FRs but harm the environment especially the Brominated FR. In general, they all reduce the flammability of plastics.	
Antioxidants	di-tert-butyl phenol (DTBP)	This provides the plastics with the Long-term thermal stability by inhibiting the thermal oxidation reaction.	(El Mansouri, Yagoubi, and Ferrier 1998)

Continued on next page

Table 1 – continued from previous page

Common additives groups	Compounds	Uses/Application	References
	Tinuvin 326 Irganox1010, irgafos 168 and ultranox 626	UV absorbers and heat stabilizers Prevent thermos oxidative degradation of plastics.	
Primary antioxidants	Phenols, amines	In synergy, they both act as decomposers of hydroperoxide, thereby making the plastics more thermally stable. In general, they slow the rate of oxidative degradation of plastic polymers)	(Ambrogi et al. 2017)
Secondary antioxidants	Thioester, phosphites		
Plasticisers	**Phthalate:** Phthalic acid esters	Provide flexibility to plastics, processability, and extensibility, slows down the velocity of melting of plastic polymers, like hoses, medical PVC, pipes, buildings	

Continued on next page

Table 1 – continued from previous page

Common additives groups	Compounds	Uses/Application	References
	Non phthalate: bisphenols, benzoates, phosphates	PVC rigids as in cable wiring, packaging, magnetic tapes etc	
Colourants, pigments, and dyes	Carbon black, organic and inorganic pigments, titanium oxide,	Opaque colouring of polymers	
	Azodyes	Give bright colours to the plastic polymers	(Singh et al. 2012)
	Photochromic and thermochromic pigments	Give colour changes of plastics when expose to light or certain temperature	
UV filters/ stabilisers	salicylates, phenyl ketones, benzotriazoles, substituted acrylonitrile, triazines	absorb UV light or reduce the transmission of UV light. It is capable of energy conversion, converting high-energy ultraviolet light into the form of heat or non-destructive longer light waves to release the energy, thereby protecting polymers from	(Khare et al. 2023)

Continued on next page

Table 1 – continued from previous page

Common additives groups	Compounds	Uses/Application	References
Lubricants	fatty acid esters (e.g., glyceryl monostearate). Hoechst-Wax.	Reduces the external intern friction in the molten plastic polymers. They serve as mould release agents which help in plastic injection moulding. They serve as polymer processing AIDS by optimizing the processing properties of plastics. Serve as antistatic agents, this can be seen to prevent electric charges in the isolation cables	(Hahladakis et al. 2018)
Fillers	Calcium carbonate, Kaolin, Talcum, Barium sulfate, Wollastonite, Magnetite	Improve the material's properties, reduce costs, and make the plastic easier to process.	(Hahladakis et al. 2018)

Appendix B

Supplementary Information for Chapter 3

Table 2: Presents borehole water chemical analysis

Borehole Water Chemisty

Analysis covers the following determinands, listed in the table below.

Determinand	Detection limit
Unionised ammonia	-
Ammoniacal nitrogen, as N	15 µg/L
Ammoniacal nitrogen, as NH_3	15 µg/L
Ammoniacal nitrogen, as NH4	15 µg/L
Nitrate, as NO_3	0.05 mg/L
Nitrite, as NO_2	5 µg/L
Orthophosphate, as PO_4	62 µg/L
Total oxidised nitrogen (TON)	0.02 mg/L
Total organic carbon (TOC)	0.1 mg/L
Chemical oxygen demand (COD)	2 mg/L
Alkalinity, as $CaCO_3$	3 mg$CaCO_3$/L
Hardness – total, as $CaCO_3$	1 mg$CaCO_3$/L
pH	± 0.1 pH units
Electrical conductivity	10 µS/cm
Fluoride	50 µg/L
Mercury (total)	0.05 µg/L
Potassium (total)	0.025 mg/L
Chlorine (total)	0.05 mg/L
Cadmium (total)	0.02 µg/L
Copper (total)	0.5 µg/L
Iron (total)	0.004 mg/L
Lead (total)	0.2 µg/L
Manganese (total)	0.05 µg/L
Nickel (total)	0.5 µg/L
Zinc (total)	0.5 µg/L
Organochlorine pesticides	10 – 20 ng/L
Organophosphorus pesticides	10 – 20 ng/L
PCBs	0.02 µg/L

Appendix C

Supplementary Information for Chapter 4

Table 3: Results on QA/QC conducted on PET, PFOS and PFOA are shown. Method blanks were run for each batch of five samples, whereas recovery samples and field blanks were run for each batch of 20 samples. Method and field blank concentrations are shown in mg/L for PET and ng/L for PFOS and PFOA. Recovery is expressed in percentage.

Sample Type	PET (mg/L)	PFOS (ng/L)	PFOA (ng/L)
Method Blank 1	ND*	ND	ND
Method Blank 2	ND	0.5	ND
Method Blank 3	ND	ND	ND
Method Blank 4	0.4	ND	ND
Method Blank 5	ND	ND	ND
Method Blank 6	0.7	ND	ND
Method Blank 7	ND	0.7	0.1
Method Blank 8	ND	0.8	ND
Method Blank 9	0.8	0.5	ND
Method Blank 10	ND	ND	ND
Method Blank 11	ND	ND	0.2
Method Blank 12	0.6	ND	ND
Method Blank 13	ND	ND	ND
Method Blank 14	0.8	ND	ND
Field Blank 1	ND	0.8	ND
Field Blank2	ND	0.9	0.2
Field Blank 3	0.9	ND	0.3
Field Blank 4	ND	ND	0.2
		Recovery %	
Recovery sample 1	88	105	86
Recovery sample 2	92	97	82
Recovery sample 3	79	115	97
Recovery sample 4	107	104	90

Table 4: PET characterisation. Microscopic characterisation of MP using OPTIKA fluorescent microscope. Shape, average particle size, standard deviation (SD), maximum and minimum size, and the proportion of fibres and fragment particles are shown. These estimates are based on 300 randomly selected particles

Shape	**Av. particle size (μ m)**	**Min size (μ m)**	**Max size (μ m)**	**SD**	**Proportion (%)**
Fiber	20.03	7.23	54.72	5.72	33
Fragment	14.93	8.93	45.91	1.27	66

Table 5: Average concentrations of PFOS and PFOA in single chemical and mixtures solutions were quantified in spiked medium without *Daphnia* using LC-MS liquid Chromatography equipped with a Sciex API 2000 mass Spectrometer following (Abdullahi et al., 2023; Harrad et al., 2019). Two replicates per sample were used.

Chemical(s)	**PFOS concentration (ng/l)**	**PFOA concentration ng/l**
PFOA		6.6
PFOS	69.3	
PET		
PFOA + PET		6.8
PFOS + PET	66.8	
PFOS + PFOA	70.3	6.3
PFOS + PFOA + PET	68.6	6.9

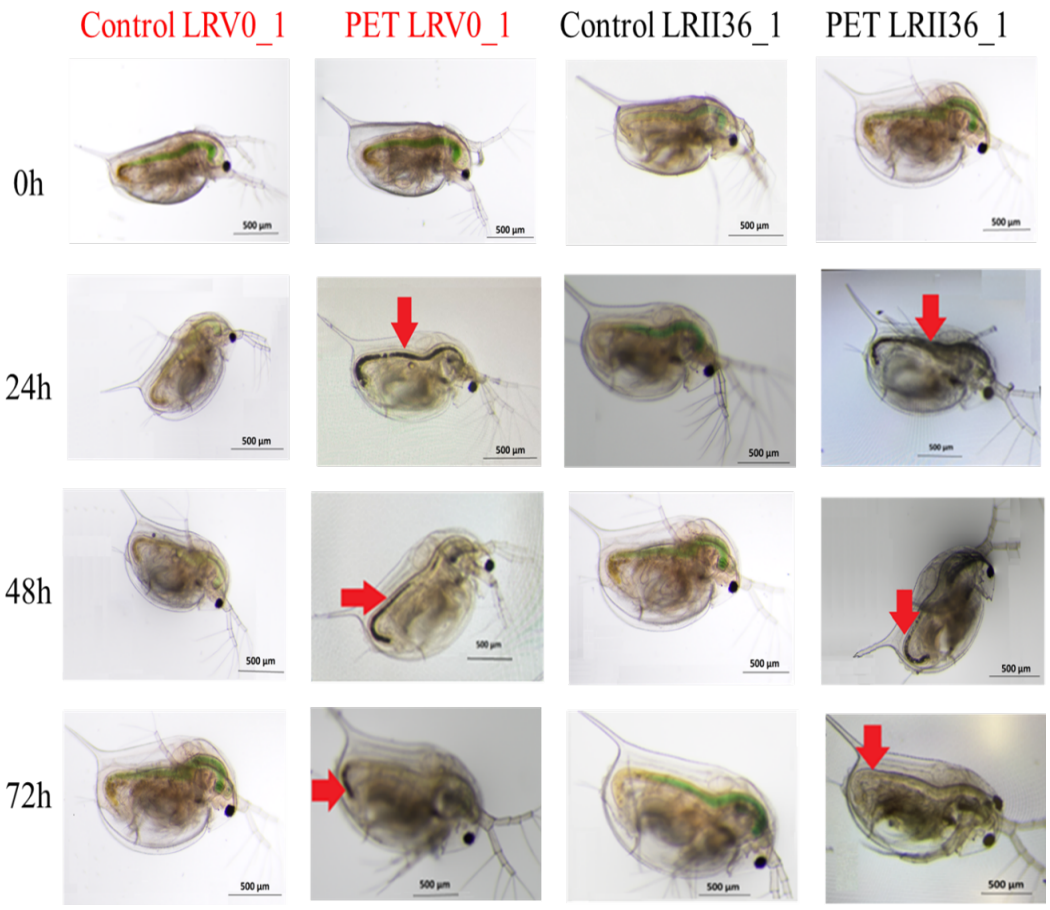

Figure 1: Ingestion and egestion of PET by *Daphnia* is shown for the two genotypes used in this study (LRV0_1 and LRII36_1) at time zero (0h) as compared to a control group fed on *Chlorella vulgaris*. Also, egestion of MP is shown at intervals of 24h up to 72h. Red arrows point to the MP in the gut, showing their progressive egestion over the 72h of the experiment.

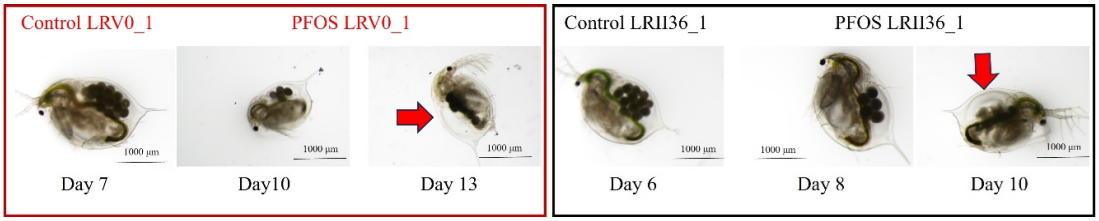

Figure 2: Developmental failure in PFOS exposures. Control and PFOS exposed female *Daphnia* are shown for the two genotypes used in this study. Exposed *Daphnia* are shown with the first brood in the brood pouch (day 10 for LRV0_1 and day 8 for LRII_36) and again 48 to 72h hour later when offspring were expected to be released. On day 13 and 10, LRV0_1 and LRII_36 show an empty brood pouch and no offspring. The red arrows point to the empty brood pouch where parthenogenetic eggs were observed between 48 and 72h earlier.

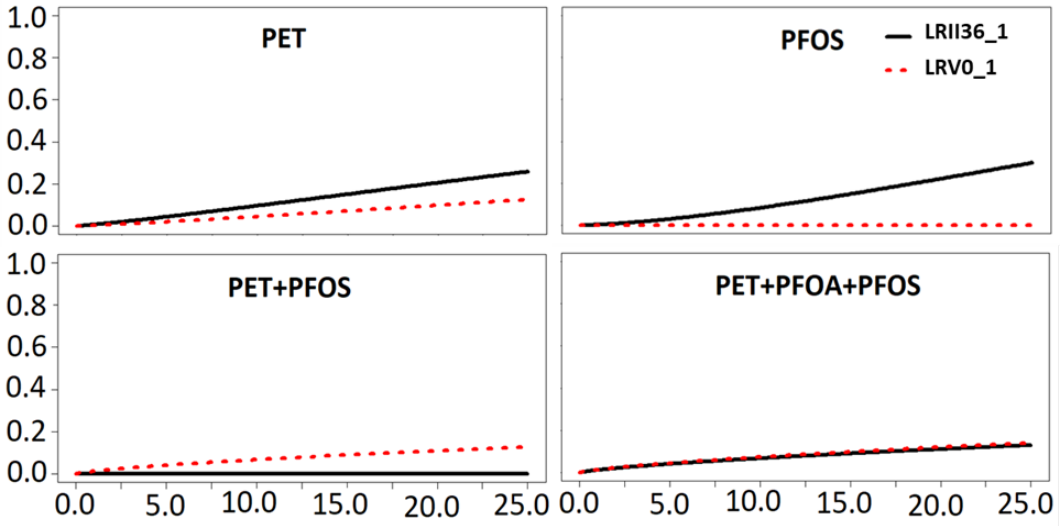

Figure 3: Survival of the *Daphnia* genotypes following chronic exposure to PET, PFOS, PFOA and their two-way and three-way mixtures are shown. Survival was calculated with survival model fit using the 'psm' function in the 'rms package in R. The genotypes are colour coded as in Figure 1. Treatments not shown had no mortality.

		age at maturity (days)			size at maturity (mm)			fecundity (1st&2nd brood)			interval between broods (days)		
Treatment	**Genotype**	***t*-test**	***p*-value**	**Effect**	***t*-test**	***p*-value**	**Effect**	***t*-test**	***p*-value**	**Effect**	***t*-test**	***p*-value**	**Effect**
PET-PFOA	LRV0_1	-0.31	0.77	add	-9.19	**0.00**	syn	0.27	0.80	add	1.43	0.22	add
	LRII36_1	-12.87	**0.00**	syn	-12.87	**0.00**	syn	0.19	0.86	add	0.77	0.49	add
PET-PFOS	LRV0_1	0.79	0.47	add	-9.37	**0.00**	syn	0.32	0.76	add	0.96	0.39	add
	LRII36_1	5.64	**0.00**	syn	-16.96	**0.00**	syn	0.20	0.85	add	0.85	0.44	add
PFOA-PFOS	LRV0_1	5.64	**0.00**	syn	-12.31	**0.00**	syn	0.30	0.78	add	2.24	0.07	add
	LRII36_1	2.78	**0.04**	syn	-13.76	**0.00**	syn	0.27	0.79	add	0.87	0.43	add
PET-PFOA-PFOS	LRV0_1	0.51	0.63	add	-19.02	**0.00**	syn	0.36	0.73	add	5.52	**0.01**	syn
	LRII36_1	5.99	0.07	add	-22.27	**0.00**	syn	0.43	0.69	add	2.32	0.06	add

Table 6: Synergistic, antagonistic and additive effects of chemical mixtures. T-test results of life history trait values (effect size) per genotype and treatment testing their departure from a null model of additivity in the analysis of chemical mixtures to identify synergistic, antagonistic and additive effects. Treatments are as in Table 1. add = additive; syn = synergistic.

Appendix D

Supplementary Information for Chapter 5

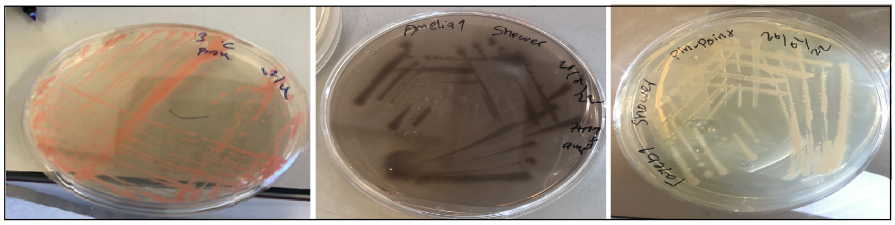

Figure 4: Shows pure bacterial colonies obtained after isolation on the filter and subsequent streaking on agar plates.

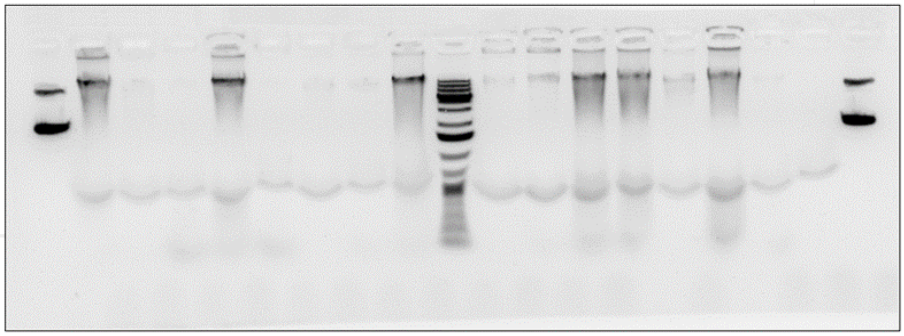

Figure 5: Visualises PCR products and compares them with a standard DNA ladder.

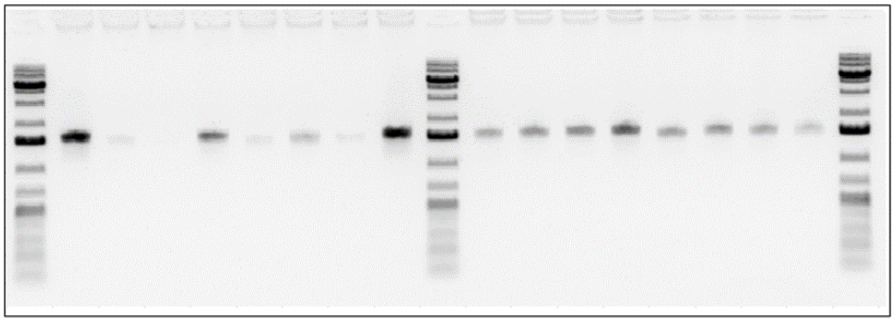

Figure 6: Visualises the PCR products after PEG purification in the presence of standard DNA ladder

Samples	Growth	R1/Plate A	R2/Plate B	R3/Plate C	Average Number of Colonies	CFU/mL
1S	+	120	100	84	128	2.56
2S	+	160	TNTC	TNTC	160	3.2
3S	NG	0	0	0	0	0
4S	+	100	98	102	100	2
5S	+	114	112	125	117	2.34
6S	NG	0	0	0	0	0
7S	NG	0	0	0	0	0
8S	+	159	167	152	159	3.18
9S	+	122	122	131	125	2.5
10S	+	9	9	9	9	0.18
11S	NG	0	0	0	0	0
12S	+	62	62	59	61	1.22
13S	NG	0	0	0	0	0
14S	NG	0	0	0	0	0
15S	+	66	71	58	65	1.3
16S	NG	0	0	0	0	0
17S	NG	0	0	0	0	0
18S	NG	0	0	0	0	0
19S	+	90	89	86	88	1.76
20S	NG	0	0	0	0	0
21S	NG	0	0	0	0	0
22S	+	230	199	201	210	4.2
23S	+	67	63	66	65	1.3
24S	NG	0	0	0	0	0
25S	NG	0	0	0	0	0
26S	+	260	TNTC	TNTC	260	5.2
27S	+	55	56	48	53	1.06
28S	+	71	88	79	79	1.58
29S	NG	0	0	0	0	0
30S	NG	0	0	0	0	0

Table 7: Illustrates growth/no growth on agar, the number of colonies in three replicates of each sample from showerheads, their average, and the CFU/mL. “S”, “+”, “NG”, and “TNTC” present the showerhead, with distinct visible bacterial colony (growth), not growth, and too numerous to count.

Samples	Growth	R1/Plate A	R2/Plate B	R3/Plate C	Average Number of Colonies	CFU/mL
1B	NG	0	0	0	0	0
2B	+	100	110	108	106	2.12
3B	+	91	93	92	92	1.84
4B	NG	0	0	0	0	0
5B	NG	0	0	0	0	0
6B	NG	0	0	0	0	0
7B	NG	0	0	0	0	0
8B	NG	0	0	0	0	0
9B	+	87	99	75	87	1.74
10B	NG	0	0	0	0	0
11B	+	99	147	162	136	2.72
12B	+	123	105	132	120	2.4
13B	NG	0	0	0	0	0
14B	NG	0	0	0	0	0
15B	NG	0	0	0	0	0
16B	NG	0	0	0	0	0
17B	NG	0	0	0	0	0
18B	+	48	59	64	57	1.14
19B	NG	0	0	0	0	0
20B	NG	0	0	0	0	0
21B	NG	0	0	0	0	0
22B	NG	0	0	0	0	0
23B	+	135	105	118	120	2.4
24B	NG	0	0	0	0	0
25B	NG	0	0	0	0	0
26B	+	300	319	TNTC	309.5	6.19
27B	NG	0	0	0	0	0
28B	NG	0	0	0	0	0
29B	NG	0	0	0	0	0
30B	NG	0	0	0	0	0

Table 8: Illustrates growth/no growth, the number of colonies in three replicates of each water sample collected from bathroom taps, their average, and the CFU/mL. “B”, “NG”, and “TNTC” present distinct visible bacterial colonies (growth), not growth and too numerous to count.

Samples	Growth	R1/Plate A	R2/Plate B	R3/Plate C	Average Number of Colonies	CFU/mL
1K	NG	0	0	0	0	0
2K	NG	0	0	0	0	0
3K	NG	0	0	0		0
4K	+	106	147	122	125	2.5
5K	NG	0	0	0	0	0
6K	NG	0	0	0	0	0
7K	NG	0	0	0	0	0
8K	+	198	161	196	185	3.7
9K	NG	0	0	0	0	0
10K	NG	0	0	0	0	0
11K	+	127	89	115	110	2.2
12K	NG	0	0	0	0	0
13K	NG	0	0	0	0	0
14K	+	10	12	14	12	0.24
15K	NG	0	0	0	0	0
16K	+	4	7	5	5	0.1
17K	NG	0	0	0	0	0
18K	NG	0	0	0	0	0
19K	+	71	64	71	78	1.42
20K	NG	0	0	0	0	0
21K	NG	0	0	0	0	0
22K	+	159	168	162	162	3.26
23K	NG	0	0	0	0	0
24K	NG	0	0	0	0	0
25K	NG	0	0	0	0	0
26K	NG	0	0	0	0	0
27K	NG	0	0	0	0	0
28K	NG	0	0	0	0	0
29K	NG	0	0	0	0	0
30K	NG	0	0	0	0	0

Table 9: Illustrates growth/no growth, the number of colonies in three replicates of each kitchen sample, their average, and the CFU/mL. “K”, “+”, “NG”, and “TNTC” present kitchen, distinct visible bacterial colony (growth), not growth and too numerous to count.

Step no	Steps	Temperature & duration
1	Denaturation	95 °C for 2 minutes
2	Denaturation	95 °C for 1 minutes (35 cycles)
3	Annealing	58 °C for 30 seconds (35 cycles)
4	Extension	72 °C for 3 minutes (35 cycles)
5	Repeat the cycles from step 2 X 35	
6	Final extension	72 °C for 10 minutes
7	Cooling	4°C

Table 10: Presents the PCR cycles, temperature and time for amplification of 16S rRNA genes.

Step no	Compounds	Volume
1	Template DNA	1:100 µl
2	Primer 27F (10pmol/µl)	2 µl
3	Primer 1492R (10pmol/µl)	2 µl
4	PCR master mix (2X)	10 µl
5	Final extension Water (PCR grade)	20 µl

Table 11: Presents the components in PCR reaction for amplification of 16S rRNA genes.