Development of phenotypic endpoints as metrics for the assessment of emerging pollutants in daphnids

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Thesis Submitted for the award of MSc

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August 2024

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Dissemination of works produced from this thesis

A list of publishable outcomes and presentation from this thesis is presented.

Published manuscripts from this MSc thesis

- Development and application of a sensitive feeding assay for daphnids based on the ingestion of fluorescent microparticles
  M Giannouli, K Panagiotidis, KD Rochfort, K Grintzalis
  Environmental Science: Advances, 2023. 2(10): p. 1351-1359
  DOI: 10.1039/D3VA00116D

Published manuscripts from collaborations

- Toxicity of “green solvents” - The impact of butyl methylimidazolium ionic liquids on daphnids
  A Michalaki, D Kakavas, **M Giannouli**, K Grintzalis
  Journal of Ionic Liquids, 2023. 3(2): p. 100059,
  https://doi.org/10.1016/j.jil.2023.100059

Oral presentations from this MSc thesis

- **03/04/2023- 05/04/2023**
  Development of a feeding assay as a phenotypic endpoint for ecotoxicology in daphnids
  Poster and Oral Presentation, Environ Conference 2023, ATU Donegal Letterkenny, Ireland
- **20/04/2023**
  The development of a feeding assay to assess the impact of pollution on daphnids.
  Oral presentation on the Research Day organized by the Biological Research Society (BRS) at Dublin City University
- **18/04/2024**
  The effects of exposure to cigarette and e-cigarette extracts on the physiology of daphnids
  Oral presentation on the Research Day organized by the Biological Research Society (BRS) at Dublin City University

Poster presentations from this MSc thesis

- **06/03/2023- 08/03/2023**

  A miniaturized feeding assay for daphnids based on the ingestion of microplastics

  Poster Presentation, UNESCO - EU H2020 Limnoplast Conference, Paris, France

- **03/04/2023- 05/04/2023**

  Development of a feeding assay as a phenotypic endpoint for ecotoxicology in daphnids

  Poster and Oral Presentation, Environ Conference 2023, ATU Donegal Letterkenny, Ireland

Conference participation from this MSc thesis

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Abbreviations

ALP	Alkaline phosphatase
ACP	Acid phosphatase
β-Gal	Beta-galactosidase
LDH	Lactate dehydrogenase
GST	Glutathione-S-transferase
GSH	Glutathione
CBB	Coomassie brilliant blue
ONPG	Ortho-Nitrophenyl-β-galactoside
DMSO	Dimethyl sulfoxide
CDNB	2,4-Dinitrochlorobenzene
PNPP	*p*-nitrophenyl phosphate
NADH	Nicotinamide adenine dinucleotide
CGM	*Chlamydomonas rheinhartii* growth media
OECD	Organisation for economic cooperation and development
EC_1	The concentration of a substance required to have a 1% mortality rate.
EC_5	The concentration of a substance required to have a 5% mortality rate.
EC_{10}	The concentration of a substance required to have a 10% mortality rate.
EC_{50}	The concentration of a substance required to have a 50% mortality rate.
WWTP	Wastewater treatment plant
NSAID	Non-steroidal anti-inflammatory drug
AIAPs	Anti-inflammatory and analgesic pharmaceuticals
CEC	Contaminants of emerging concern
CB	Cigarette bud/filter
BTEX	Benzene, toluene, ethylbenzene, and xylene
PAH	Polycyclic aromatic hydrocarbon
HNBT	Heat-not-burn tobacco
WHO	World Health Organization

Abstract

The increased population over the past years has resulted in elevated consumption of pharmaceuticals, items of lifestyle and release of industrial effluents containing pollutants (e.g. heavy metals). The improper disposal of these substances has raised concerns in the scientific community regarding their harmful effects on aquatic ecosystems. Conventional approaches for water quality assessment commonly rely on the detection of pollutants in the aquatic environment. However, these approaches are limited by their sensitivity thresholds. On the other hand, effect-based methods present sensitive tools that could support ecotoxicology and risk assessment, utilizing model organisms to examine the effects of pollutants commonly found in the environment. This thesis focuses on the development of a sensitive feeding assay as a phenotypical endpoint for toxicity assessment of emerging pollutants. In addition, it explores the application of phenotypical and physiological endpoints for the investigation of the impact of cigarette filter-derived pollutants on *Daphnia magna*.

Chapter 1

Freshwater ecotoxicology and risk assessment

1.1. Introduction to freshwater ecology and ecotoxicology

It is widely known that European surface waters contain various chemicals, numbering in the tens to hundreds of thousands. This chemical diversity includes pesticides, pharmaceuticals, personal care products, items of lifestyle and a multitude of transformation products resulting from chemical reactions and processes in the aquatic environment [1].

Conventional approaches for water quality assessment are focused on the detection of specific chemicals and pollutants present in the aquatic environment, employing methods based on analytical chemistry [2]. However, these conventional approaches have underlying limitations concerning the range of pollutants they can identify and their sensitivity thresholds. The practice of monitoring a limited selection of individual pollutants is becoming progressively less effective in providing insights into the potential harm posed by chemical mixtures. This approach is characterized by an increasing likelihood of missing significant risks, and the risk of overlooking substantial threats is rising. Consequently, there is a growing recognition of the potential advantages of employing effect-based methods to enhance our understanding of pollutant impacts in the aquatic environment. Effect-based methods offer a promising alternative as they provide mechanistic insight into the action of pollutants on organisms. They contribute to the development of a comprehensive understanding of contaminants and serve as more sensitive tools, facilitating precise predictions in pollution assessment. This complementary approach has the potential to improve the effectiveness of current water quality assessment methods and enhance our ability to monitor and manage environmental pollution [1].

1.2. Pathways of pollution in the aquatic ecosystems

Human activities such as farming, urban waste management, industrial effluents, and increased urbanization present major threats to the freshwater ecosystem in recent years. Wastewater, metals, industrial effluents, organic pollutants, and emerging contaminants such as pharmaceuticals contribute significantly to freshwater pollution [3]. Additionally, items of lifestyle, such as cigarettes are often discarded improperly after their consumption and find their way into freshwater environments through storm drains, rivers, and streams [4]. Composed of non-biodegradable cellulose acetate, these buds can persist for years, leaching toxic chemicals such as nicotine, lead, and arsenic into the aquatic environment [5, 6]. These pollutants harm aquatic life, disrupting ecosystems and contaminating drinking water [7].

1.2.1. Occurrence of pharmaceuticals in the aquatic environment

The significant increase in population over the past years has resulted in elevated demand for pharmaceuticals for human use, veterinary medications, and other personal care products. Pharmaceuticals have been established over the past years as contaminants of emerging concern (CECs), crucial for human health and illness relief. They are categorized into 24 therapeutic classes, including approximately 10,000 different drugs that contain around 3,000 to 4,000 distinct active ingredients [8].

Over the past few decades, there has been a notable rise in the consumption of pharmaceuticals due to various factors, including growth in the population worldwide, growing investment in health care, research and development advancement, and the aging population in industrialized countries [9]. The frequent consumption of these substances, in addition to over-prescription, self-medication, and misdiagnosis, has resulted in their environmental presence and concerns for harmful effects on aquatic environments and organisms [8, 10, 11].

According to a study conducted by Beek et. al., a significant number of research studies focus on the pharmaceutical substances found in surface waters (approximately 50%), including data from rivers and streams, followed by oceans and lakes. In the aquatic environment, in concentrations higher than the detection limits, more than 100 pharmaceutical compounds have been found in a variety of European countries and the United States [12]. The majority of pharmaceuticals are released

into the environment through their improper disposal and emissions from manufacturing sites, eventually contaminating freshwater systems, and disrupting entire ecosystems [10, 13]. In addition, individual residential activities, wastewater treatment plants (WWTPs), landfill sites, and agricultural farms remain sources of pharmaceutical pollution (Figure 1) [14].

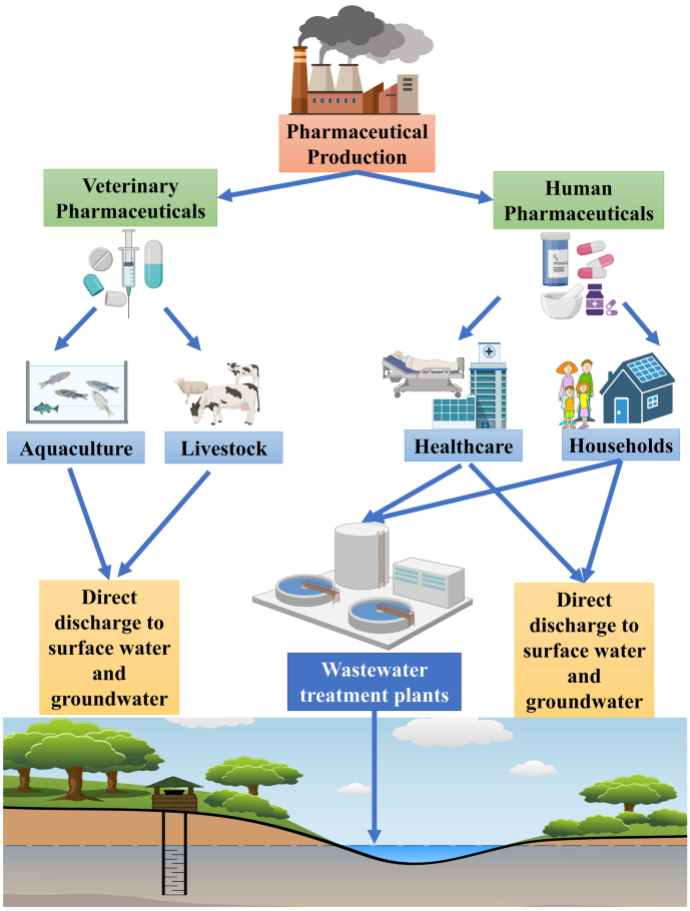

Figure 1. Pathways of pharmaceutical occurrence in the aquatic environment [10].

The factors that contribute significantly to the concentrations of pharmaceuticals in municipal wastewater include the total number of resident individuals, patterns of consumption, and improper disposal of leftover or expired drugs. In addition, the usage of pharmaceuticals may be affected by a variety of linked social and cultural factors, including improper usage, lack of financial resources, the non-enforcement of regulatory law, and inadequate levels of education and expertise [15]. Several studies have shown that hospital wastewater has greater per capita loads of medicines employed in laboratory and diagnostic procedures, including various antibiotics, anti-inflammatories, analgesics, and anticancer medications [16].

A high amount of pharmaceutical chemical ingredients is designed to be sufficiently water-soluble and is often presumed to have similar reactivity. They typically pass through the digestive systems of humans and animals before entering the environment. Specifically, pharmaceutical substances that are absorbed during the therapeutic phase are released into sewage systems and eventually find their way into the aquatic ecosystem [17]. These effluents may contain pharmaceutical products that are utilized to treat diseases in both humans and animals, including hormones, anti-inflammatory and antiallergic medications, antibiotics, β-blockers, and painkillers [8, 10].

The input concentrations of pharmaceuticals (detected from ng/L to μg/L) combined with potential toxicological effects on non-target organisms, bioaccumulation, and persistence have raised major concerns in the scientific community regarding the potential harmful effects in ecosystems [8, 18]. Several studies have examined the adverse effects of pharmaceutical pollution on aquatic organisms, including toxicity [19], alterations in heart rate, development, and physiology [20-23]. Notably, the studies that focus on the toxicity of these compounds for chronic exposure to low concentrations are limited, and a significant proportion of human pharmaceuticals lack comprehensive environmental toxicity data [10, 11].

Considering the significant impact of exposure to pharmaceuticals on organisms in the aquatic environment, pharmaceutical contamination is regarded as a serious hazard. Furthermore, the effective removal of pharmaceuticals from conventional treatment facilities is inadequate; as a result, various compounds have been found to be present in treated water worldwide [14]. However, the elimination of organic substances, including pharmaceuticals, was not the initial purpose of WWTPs. Consequently, complex mixtures of chemicals might be present in the discharges [10, 11].

1.2.2. Occurrence of metals in the aquatic environment

Heavy metal contamination presents a significant threat to terrestrial and aquatic ecosystems following its release from both anthropogenic and natural sources. More specifically, organic and inorganic pollutants are easily dissolved in aquatic ecosystems. Several studies highlight the significance of heavy metal pollution due to the environmental persistence, bioaccumulation, and biomagnification in the food

chains of the pollutant; thus, they pose a critical threat to aquatic organisms and human health [26, 27, 29]. Recent studies have shown that metals are acutely toxic [30, 31] and significantly impact the growth and ingestion rate of aquatic organisms [32, 33].

Heavy metals are commonly categorized as essential and non-essential based on their significance in biological systems. It has been recognized that essential metals contribute significantly to physiological and biochemical functions at lower concentrations. Exposure to non-essential metals and higher concentrations of essential metals could cause detrimental effects on the exposed organisms and ecosystems [26].

Several factors have contributed to heavy metal pollution and environmental contamination, such as rapid industrialization and urbanization, in addition to the increased mobilization and transport of these metals. The sources of the environmental presence of these pollutants include anthropogenic sources (industrial emissions, mining, smelting, agricultural practices, and fossil fuels) and natural sources (volcanic eruptions and metal-containing rocks) (Figure 2) [26-28]. In addition, heavy metals are released into the environment when wastewater, such as residential sewage and industrial effluents, is discarded [26].

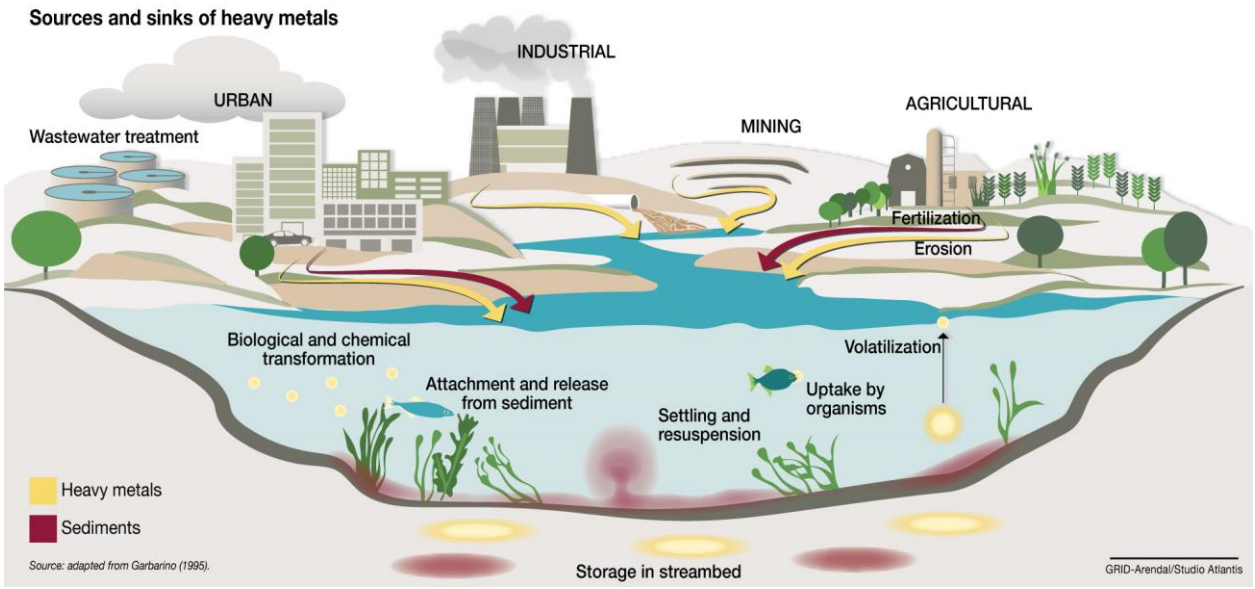

Figure 2. Sources of heavy metal pollution in the aquatic environment [34] , *https://www.grida.no/resources/13718.*

1.2.3. Cigarette filter pollution

The increasing cigarette consumption over the past few years has raised several public health and environmental concerns. The detrimental effects of cigarette smoking on human health have been extensively examined, and cigarette consumption has been linked to a significant impact on quality of life and various diseases. In addition to their negative impact on human health, cigarettes pose a significant threat to the environment [36, 37]. Following smoking bans in several countries, the industry has introduced alternative lifestyle products, electronic cigarettes (e-cigarettes), and heat-not-burn tobacco products (HNBT) (elements of traditional cigarettes and e-cigarettes by heating tobacco), which have gained popularity in recent years [37, 38].

It is estimated that trillions of cigarettes are consumed annually, resulting in the widespread disposal of cigarette buds (CB) into the environment [4, 39-41]. Cigarette filters constitute the most prevalent item in environmental litter, accounting for approximately 30% of the total [36, 41]. The improper disposal of cigarette filters leads to widespread environmental pollution, contaminating soil and waterways with toxic chemicals. Consequently, littered CB can be found in streets, urban areas, and the coast and due to their small size, they often become lodged in cracks in sidewalks, bushes, and beaches, making their removal a significant challenge (Figure 3) [4, 37, 42, 43]. In addition, rainfall, urban runoff, and flood events could transport CBs to drains, rivers, and aquatic ecosystems [44, 45].

Cigarette filters are intended to capture harmful chemicals derived during smoking, resulting in CBs containing mixtures of toxic substances. These mixtures can contain over 7,000 different compounds, with a minimum of 150 identified as toxic [38, 42]. According to studies, the complex mixtures present in CBs include highly soluble nicotine, aromatic amines, organic pollutants, nanoparticles, heavy metals (aluminium, zinc, nickel), and microfibers [4, 39, 40, 46-48]. Leachate from these toxic substances has been found to be harmful to a variety of aquatic organisms, with lethal and sublethal effects [48-51]. In addition, standard CBs are composed of cellulose acetate, a polymer characterized by slow biodegradation and influenced by environmental conditions [5]. This slow degradation contributes to the persistence of CBs in the environment and raises concerns about their potential chronic impacts on ecosystems [36, 52]. Companies have developed filters entirely made of cellulose as an alternative.

However, the study by Green et. al. demonstrates the harmful effects of biodegradable CBs on invertebrates, such as mortality and reduced activity [53].

Figure 3. Littered cigarette filters observed in Dublin streets, including Dublin City University.

(Photos: M. Giannouli, taken in Dublin, Ireland, 2024).

1.3. Sentinel species in toxicity assessment- the case of *Daphnia magna*

Despite the knowledge and technology that revolve around the harmful impacts on human health and ecosystems, chemical pollution monitoring remains inadequate. Based on the "3Rs Principle (Replacement, Reduction, and Refinement)", the use of vertebrate animals in research promotes the development and adoption of alternative methods in toxicity assessment [54]. Sentinel species are commonly applied to toxicity assessment and offer mechanistic insight into the impacts on ecosystems. *Daphnia magna* presents one of the most ecologically studied model organisms utilized to investigate the potentially toxic impact of chemical pollution based on their responses after exposure [55, 56].

1.3.1. Daphnids in molecular ecology and ecotoxicology

Daphnia magna, commonly known as the water flea, plays a crucial role in both ecology and ecotoxicology due to its position in aquatic food webs and its sensitivity to environmental changes. In ecotoxicology, *Daphnia magna* is an important bioindicator species used to assess the impact of pollutants on aquatic ecosystems. Its sensitivity to changes in water quality and its rapid reproductive cycle make it an

ideal model organism for studying the effects of contaminants and evaluating the health of aquatic environments.

Daphnids are planktonic crustaceans forming part of the Phyllopoda group, sometimes referred to as Branchiopoda. They are characterized by their flattened, leaf-like legs, which are significant in generating a water current for their filtering apparatus. *Daphnia magna* occupies a taxonomic category known as Cladocera within the higher taxonomic classification of Branchiopods. Cladocerans have bodies enclosed by an uncalcified shell, known as the carapace, which consists of a double wall that allows haemolymph flow and is an integral part of the body cavity. Cladocerans possess up to 10 pairs of appendages, arranged from front to back as follows: antennules, antennae (the second antennae, used for swimming), maxillae, and mandibles (Figure 4). This arrangement is succeeded by five (as in *Daphnia magna*) or six limbs on the trunk, forming an apparatus for both feeding and respiration. At the end of the abdomen, a pair of claws is present. The body length of Cladocera individuals can range from less than 0.5 mm to over 6 mm. Distinguishing males from females involves noting the former's smaller size, larger antennules, modified post-abdomen, and first legs, which feature a hook used for clasping purposes. Adult daphnia individuals exhibit a size range from less than 1 mm to 5 mm, with smaller species generally inhabiting ponds or lakes where predation by fish is prevalent.

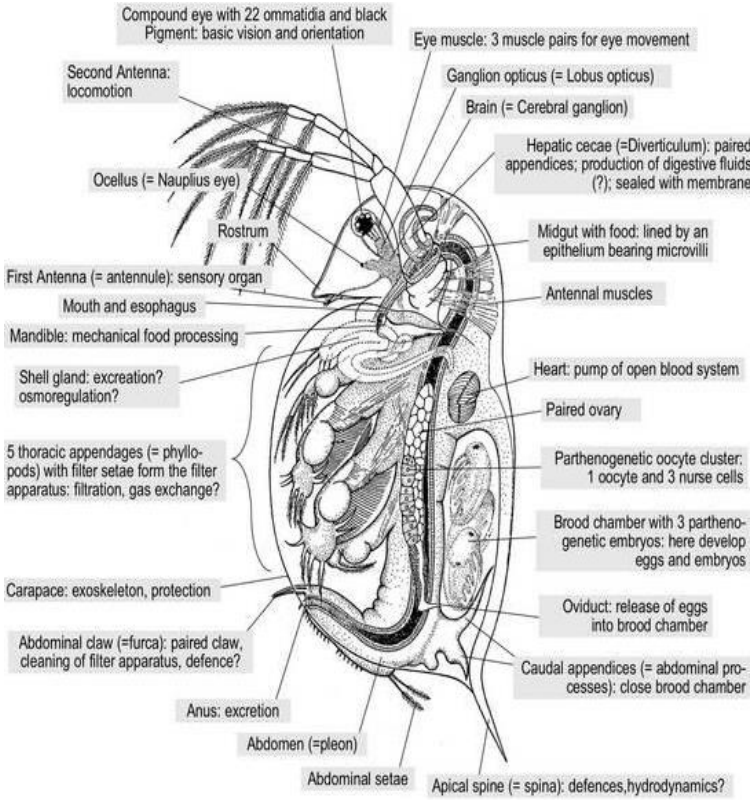

Figure 4. Anatomy of *Daphnia magna* [57].

Daphnia magna exhibits a feeding strategy focused on small, suspended particles within the aquatic medium. They function as suspension feeders, also termed filter feeders. The feeding rate in *Daphnia magna* is commonly utilized as a phenotypical endpoint for the assessment of emerging pollutants by measuring the reduction in feeding activity, which serves as an indicator of the toxic effects of these pollutants in the physiology of the exposed organisms.

Their feeding process involves the utilization of a specialized filtering apparatus comprised of phyllopods, which are leaf-like legs flattened in structure. These phyllopods serve to generate a water current that serves by gathering suspended particles that are transferred into a specialized food groove. While daphnids are characterized by a highly efficient feeding apparatus capable of collecting even bacteria, their primary dietary source typically comprises planktonic algae. Among these algae, green algae, such as Scenedesmus and Chlamydomonas, are considered excellent food sources and are frequently utilized in laboratory experiments due to their suitability for monoclonal chemostat cultivation. Daphnia usually consume particles ranging in size from approximately 1 µm to 50 µm, although

in the gut contents of larger individuals, particles of up to 70 μm in diameter can be encountered.

The genus *Daphnia* comprises over 100 recognized species of freshwater planktonic organisms distributed worldwide. These organisms inhabit various types of still freshwater environments, excluding extreme habitats like hot springs, and play a crucial role in the food web, mainly as the primary source of food for planktivorous fish. It is known that daphnia species found in lakes that are the habitat of fish present morphological differences that serve in the survival of the species. For instance, *Daphnia* species found in lakes that do not contain fish as predators, such as *Daphnia magna* and *Daphnia pulex* which are larger and less transparent than species found in lakes with intensive predation. The morphological differences, based on the survival of the species, contribute significantly to the evolution of the body size of *Daphnia magna*.

Daphnids are characterized by their asexual parthenogenetic mode of reproduction with the alternative of sexual reproduction under threatening environmental conditions (Figure 5). In conditions where there is a suitable food supply, female daphnids typically produce a clutch of parthenogenetic eggs following each adult molt. These eggs are placed in a specialized brood chamber, enclosed in the daphnids' carapace. The embryos within these eggs hatch after one day, but they remain in the brood chamber for about three more days to undergo further development before release. Daphnids recently hatched simulate adult animals, except for their undeveloped brood chamber. The first egg clutch is typically in their brood chamber when daphnids are between five to ten days old, whereas under unfavourable environmental conditions, this may occur later in their development.

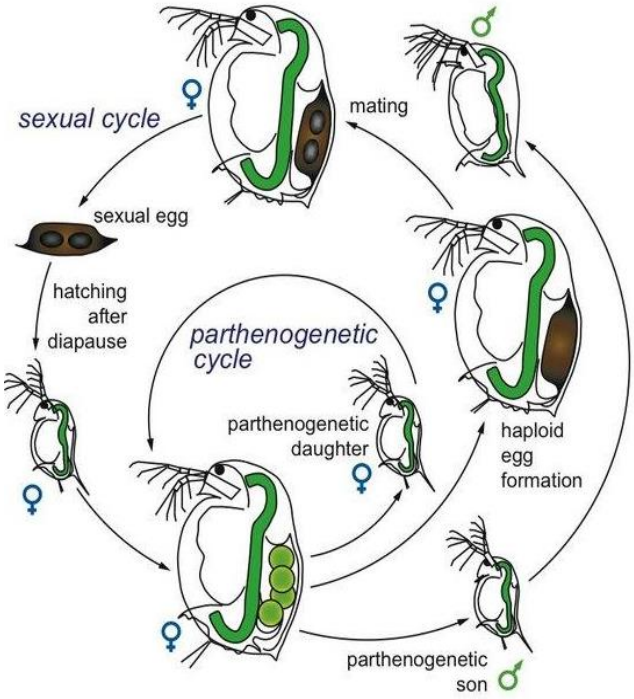

Figure 5. The life cycle of daphnids (sexual and parthenogenetic cycle) [57].

In addition to the parthenogenetic cycle of reproduction that allows the production of clones identical to their mothers, daphnids are defined by many characteristics that make them model species used in ecology and ecotoxicology [56]. Their short life cycle makes it easy to culture in the lab and for experimental manipulations. Sentinel species, including *Daphnia magna,* are commonly used in aquatic toxicity testing due to their sensitivity to chemical and physical pollution in aquatic environments; hence, the sublethal consequences of chemical pollution can be revealed [55].

Daphnids occupy a central role in the food web of aquatic environments, and they are responsive to pollutants entering the freshwater environment through their non-selective filter-feeding method [58]. Additionally, due to the anatomy and physiology of *Daphnia magna*, these organisms have become essential in the development of non-invasive methods for toxicity assessment. Their high sensitivity to environmental changes, coupled with their transparent exoskeleton, enables researchers to easily observe physiological responses. Among these methods, measuring the feeding rate of daphnids has emerged as a particularly effective non-invasive approach, providing an early indication of freshwater environment pollution. These characteristics make *Daphnia magna* a model organism for evaluating the effects of pollutants, offering valuable insights into the critical impacts of contaminants on aquatic ecosystems.

1.4. Research thesis outline

This research thesis focused on employing daphnids as bioindicators in ecology and ecotoxicology. The first chapter is an introduction that covers the necessary theoretical background to freshwater ecotoxicology, risk assessment, and the presence of common pollutants in the aquatic environment that will be discussed for their impact in the following chapters.

The main results of this study were divided in the development and application of a novel sensitive feeding assay and the toxicity assessment of emerging pollutants derived from cigarettes. This feeding assay (chapter 2) presents an innovative approach to assessing the ingestion rate of the tested organism in response to exposure to toxicants from different categories, commonly encountered in the aquatic environment.

The final third chapter of this study addresses the issue of cigarette pollution, which has escalated in recent years. The toxicity of discarded cigarette filters in the aquatic environment was evaluated through both acute and chronic scenarios, considering various developmental stages of daphnids.

Research questions

- How can a sensitive feeding method be developed and optimized for *Daphnia magna* to improve their use in ecotoxicological studies?
- What are the comparative toxic effects of cigarette and e-cigarette leachates on *Daphnia magna* using multiple biological endpoints?

Hypothesis

The developed feeding method provides a more accurate and reliable assessment of the toxicity of emerging pollutants in aquatic organisms compared to traditional assessment methods. Specifically, this optimized method will reveal significant differences in the toxicity profiles of cigarette and e-cigarette leachates when multiple biological endpoints are evaluated.

Chapter 2

Development and application of a simple feeding assay for daphnids as endpoint of physiology

Abstract

The assessment of toxicological hazards and safety relevant to chemical substances traditionally relies on animal testing, which consists of a comprehensive evaluation of mortality, phenotypic characteristics, and molecular endpoints. However, the ethical considerations of animal welfare and evolving societal concerns necessitate the exploration of novel approaches and methodologies within the field of risk assessment. Furthermore, such alternative tests have the potential to provide cost-effective outcomes, thereby enhancing the efficiency of risk assessment. Focusing on freshwater ecosystems, *Daphnia magna* has emerged as a model organism in toxicological studies. Following exposure to pollutants entering the freshwater environment, the feeding rate stands as an established phenotypic endpoint in ecotoxicology. Impairments in feeding rate provide early indications of physiological alterations in daphnids, even in non-lethal exposures. Conventional methods typically require extended incubation periods and significant volumes of culturing media during feeding. This results in increased waste generation and a higher demand for animals. In this study, we developed a robust and highly sensitive approach based on the tracking of the ingestion of fluorescent microparticles. The developed method minimizes the demand for animals, incubation periods, and media volume. Parameters such as the total volume of media, microparticle concentration, and number of animals were optimized for this feeding test to assess the impact of the selected pollutants on the feeding rate of daphnids. Our findings reveal that the number of animals used per replicate had a significant impact on the feeding rate, notably increasing the ingestion of microplastics, more so than the assay volume and microplastic concentration. In an attempt to evaluate the impact of chemical exposure on the feeding rate, a comprehensive selection of pollutants was employed, including metals (lithium

chloride, zinc sulfate heptahydrate, zirconium chloride, aluminium sulfate hexadecahydrate, cobalt nitrate hexahydrate), pharmaceuticals (diltiazem hydrochloride, propranolol hydrochloride, diclofenac sodium, metformin), and a stimulant (nicotine). The results indicate a concentration-dependent reduction in feeding rates, particularly following exposure to metals. This new method represents an efficient approach to toxicological assessment, offering valuable insights that can inform future studies, especially those involving environmentally relevant concentrations of chemicals.

2.1. Water quality assessment approaches

The assessment of water quality is commonly based on the detection of pollutants in the aquatic ecosystem. Recently, effect-based methods have additionally been employed due to their higher sensitivity limits and their improved insight to support the existing traditional approaches for toxicity assessment [1].

Conventional approaches to water toxicity assessment often use mortality as an endpoint to evaluate the effects of a wide range of chemicals entering the aquatic environment [30, 59-63]. Several studies focus on physiological and phenotypical alterations of the tested organism after exposure to pollutants, such as growth, reproduction, respiration, swimming activity, and feeding rate, which are more sensitive indicators than survival in aquatic organisms [64-68]. Feeding activity, specifically, is highly plastic and associated with physical and environmental changes such as the availability of resources and the presence of chemicals and pollutants [67, 69]. Algal cell counts, chlorophyll fluorescence, and radioactively labelled algae, dyes, or beads are commonly used to assess the feeding rate of daphnids [70, 71]. Each of the previously mentioned methods presents various limitations, such as utilising adult animals and high volumes, requiring continuous stirring to avoid algal sedimentation, minimal light conditions to prevent algal growth, and being time-consuming with long feeding incubation periods [69, 72, 73]. Therefore, it is crucial to establish advanced methods in terms of convenience and efficiency to evaluate the impact of commonly detected pollutants on the feeding rate of aquatic organisms.

The model organism used in this study, *Daphnia magna*, is a filter-feeder and, therefore, can ingest particles with sizes between 1-50 µm. In addition, it has been shown that acute exposure to low concentrations of microplastics does not significantly affect the survival and growth of daphnids [74, 75]. In this study, we assessed the impact of toxicants on the feeding rate of daphnids. The impact of common pollutants from two main categories (pharmaceuticals and metals) and a stimulant was examined. Several authors have stated that the toxicants used in this study occur very often in the aquatic environment and affect physiological and phenotypical endpoints of freshwater organisms [30, 76-78].

2.2. Materials and Methods

2.2.1. Materials

All chemicals used in this study were of the highest analytical quality. Diclofenac sodium (CAS RN: 15307-79-6), zinc sulfate heptahydrate (CAS RN: 7446-20-0), cobalt (III) nitrate (CAS RN: 10026-22-9), 1,1-dimethylbiguanidine hydrochloride (metformin, CAS RN: 1115-70-4), DL-propranolol hydrochloride (CAS RN: 318-98-9) and L-nicotine (CAS RN: 54-11-5) were purchased from Acros Organics. Aluminium sulfate hexadecahydrate (CAS RN: 1628-11-8) and lithium chloride anhydrous (CAS RN: 7447-41-8) were purchased from Thermo-Fisher. Zirconium (IV) chloride (CAS RN: 10025-11-6) was purchased from Alfa Aesar. Diltiazem hydrochloride (CAS RN: 33286-22-5) and fluorescent microparticles (carboxylate-modified fluorescent latex beads, CAT number: L3030, size: 2 µm in diameter) were purchased from Sigma.

2.2.2. Cultures of algae and daphnids

The cultures of daphnids were prepared according to OECD recommendations. Eighty animals were cultured in 4-liter beakers with OECD medium (final concentrations of 0.2 g $CaCl_2$ x $2H_2O$/L, 0.123 g $MgSO_4$ x $7H_2O$/L, 0.065 g $NaHCO_3$/L, 0.0058 g KCl/L, 2 g Na_2SeO_3/L, pH 7.7) under a 16h:8h light: dark at 21°C as described previously [76]. The primary source of food for daphnids is algae of the species *Chlamydomonas rheinhartii*. A semi-continuous stock culture of the algae was kept in Chlamydomonas growth medium (20 g NH_4Cl/L, 8 g $MgSO_4$ x $7H_2O$/L, 4 g $CaCl_2$ x $2H_2O$/L, 8.64 g K_2HPO_4/L, 8.4 g KH_2PO_4/L, 50/31 g EDTA/KOH/L, 4.98 g acidified iron/L, 11.42 g boric acid/L, 14.12 g $ZnSO_4$ x $7H_2O$/L, 2.33 g $MnCl_2$ x $4H_2O$/L, 2.54 g $CuSO_4$ x $5H_2O$/L, 0.82 g $Co(NO_3)_2$ x $6H_2O$/L, 1.92 g Na_2MoO_4 x $4H_2O$/L, pH 6.7) for feeding.

Centrifugation at 3,000 rpm for 10 minutes at room temperature was used to collect the algae. After centrifugation, the pellet was retained and then re-suspended in ddH_2O. In a specified volume, the absorbance was measured at 440 nm and the average value was calculated. This value was multiplied by the dilution factor, divided by 7, and a ratio greater than 1 was obtained. The concentrated algae volume was then multiplied by this ratio to determine the final volume of the algae. Finally, the required volume of ddH_2O was added to achieve the final algae volume.

Cultures were fed daily with the algal solution and dried baker's yeast (*Saccharomyces cerevisiae*) (2 mL from 100 mg/L ddH_2O). When starting a culture of daphnids and at renewal of media, a standard organic seaweed (*Acoplymun nodosum*) extract was added. This seaweed extract was prepared in water and diluted at an absorbance of 8A at 400 nm. Neonates from the first or second brood were discarded and not used for experiments. Males or ephippia were also discarded if found in parent cultures. For experiments, neonates (<24 hours) from the third brood of their mothers were used to start new cultures.

2.2.3. Toxicity exposures and feeding assay development

For toxicity curves, neonates (<24h) were collected from the third brood of their mother and cultured until four days old with algal supplementation (6 million cells/mL). After four days, fifteen animals were collected and used per replicate with a minimum of four replicates. For acute exposure to the chemicals, daphnids were exposed to 100 mL OECD media for 24h in the absence of food. Toxicity curves were plotted using the four-parameter logistic model and EC_{50} values were calculated.

For feeding experiments, neonates were collected from the third brood of their mothers and cultured until four days old. The feeding assay was developed with four-day-old daphnids following acute exposure of 24h to chemicals (Figure 6). For each concentration (5, 10, and 20 mg/L), a minimum of four replicates was prepared. Therefore, to simulate the exposure period, unexposed animals were incubated in OECD media for 24h to stimulate the absence of food during exposure. These control animals were used to develop and optimize the test parameters. The feeding rate of daphnids was measured from the ingestion of carboxy microplastics. Animals were incubated at 2, 4, 6, 8, or 10 mL of microplastic in 24, 12, 12, 6, or 6 well plates, respectively, to define the optimum vessel, volume, and concentration of microplastic and the number of daphnids, as it will be discussed in the results section. During the incubation feeding period of 1h, the concentration of the remaining microplastic was measured with fluorescence at an excitation of 560 nm and emission at 590 nm. To assess the ingestion rate of daphnids, the difference in fluorescence was converted to ingested microplastic using the corresponding standard curve. The final rate of feeding was expressed per animal and minute.

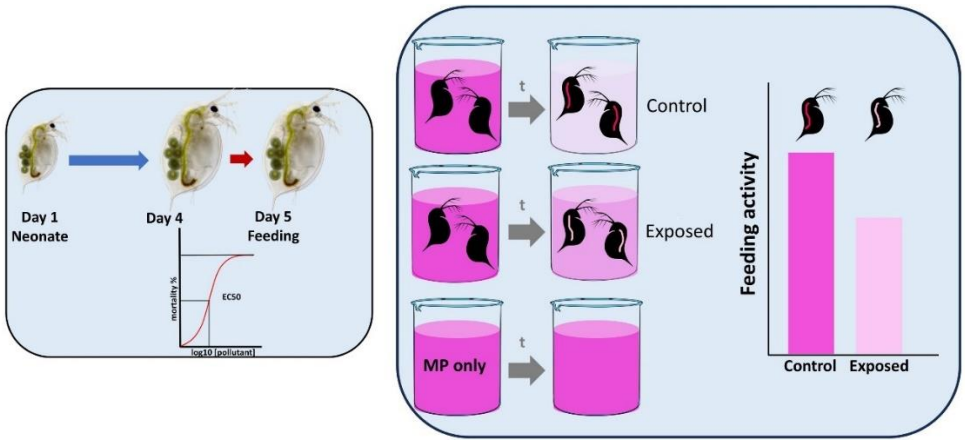

Figure 6. Experimental design of acute toxicity exposures and feeding assay development.

2.3. Statistical analysis

Data were presented as mean±standard deviation (SD) and were analyzed and plotted with the GraphPad Prism 6 software (Dotmatics). Statistically significant differences were identified with One-Way ANOVA corrected with post-hoc Dunnett's test.

2.4. Results

The toxic effects of a wide range of chemicals in the aquatic environment are commonly detected by traditional approaches which present limitations in the spectrum of chemicals they can detect. Effect-based methods can provide an improved insight to support the current approaches. This sensitive feeding assay exhibits a promising approach to detecting the toxic effects of the feeding rate of daphnids after exposure to pollutants entering the aquatic environment.

2.4.1. Optimization of the conditions of the feeding assay

Previous studies assessed the feeding rate to evaluate the impact of pollutants on the physiology of aquatic organisms, commonly utilized algal cell counts, chlorophyll fluorescence, radioactively labelled algae, etc. However, these methods pose several limitations, as mentioned previously. More specifically, the utilization of algae requires extensive consideration, given that algal growth and sedimentation might occur and require time-consuming incubation periods [69, 72, 73]. Therefore, the development of novel assays that minimize the mentioned limitations is essential. Several parameters were tested and optimized for this feeding assay to establish a new approach to evaluate the feeding rate of daphnids.

For feeding experiments, neonates were cultured until four days old, prior to acute exposure to chemicals for 24h. Sublethal concentrations were selected to perform the feeding assay to prevent high mortality rates. Toxicity curves were plotted after 24h of exposure to chemicals for four-day-old animals, and EC values were calculated (Table 1). For each concentration of the selected chemicals (5, 10, and 20 mg/L), a minimum of 6 replicates were prepared. However, to simulate the exposure of 24h, four-day-old daphnids were incubated in OECD media in the absence of food as “control” animals to develop and optimize the parameters of the feeding test. The feeding rate of daphnids was measured from the ingestion of carboxy microplastics based on their fluorescence at excitation of 560 nm and emission of 590 nm. Different numbers of animals, volumes, and concentrations of the microparticles were used in a series of experiments to define the optimum conditions of the developed feeding assay. Finally, for feeding experiments, a minimum of three replicates with the absence of animals were incubated, with a consistent concentration of microplastics to ensure that the final concentration of microplastics remained constant over time.

The first parameter assessed was the number of animals used in each replicate for the feeding assay. One to nine animals were incubated for one hour in a volume of 6 mL at a concentration of 13 mg/L. Keeping the volume and concentration of the microplastic consistent per replicate, the impact of the number of animals in the ingestion of microplastic and the feeding rate per individual animal was determined. The increase of animals per replicate increased the feeding rate of the group of animals per replicate but decreased the feeding rate expressed per individual animal (Figure 7). This result indicates that a higher number of animals used for the test rapidly consumed the microplastics in the media, following the absence of microplastics after one hour. In addition, with over a certain number of animals per replicate, the ingestion reached a plateau. In contrast, a low number of animals per replicate would be restricted from consuming a high amount of microplastics to observe significant differences between exposed and unexposed animals. Furthermore, a low number of animals used per replicate increases the variance, which is essential to avoid during the optimization of the feeding assay. For the reasons mentioned above, the optimum number of daphnids per replicate to perform this test is between four and six animals.

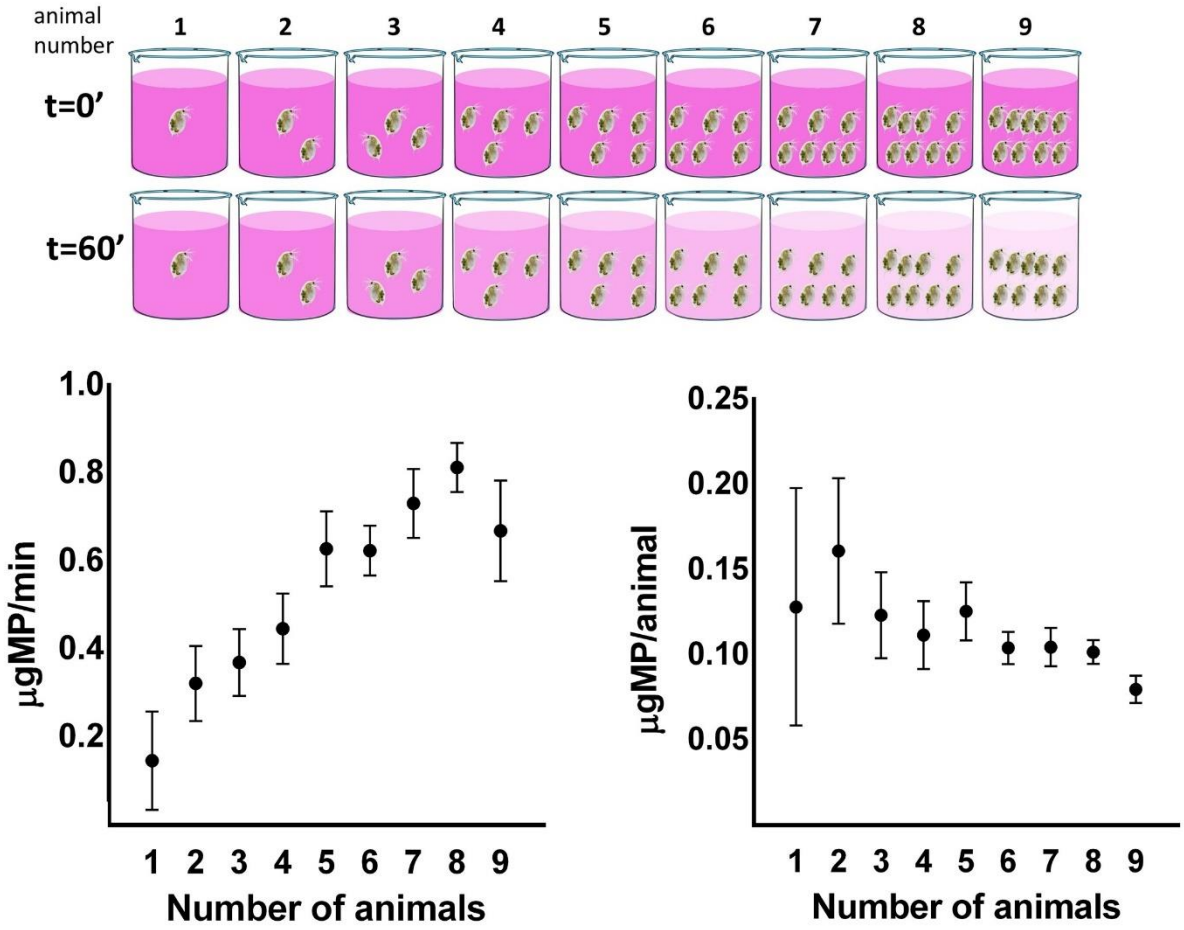

Figure 7. Experimental design and impact of animal number on the feeding rate. One to nine animals were incubated in 6 mL of microplastic at a final concentration of 13 mg/L. The concentration of microplastic in the supernatant was measured in 10-minute intervals for 60 minutes, and the feeding rate was calculated based on the linear part of the ingestion of microparticles.

The second parameter assessed was the impact of the concentration of microplastic. Keeping the volume of the microparticles and the number of animals consistent, the effect of microplastic concentration on the feeding rate of daphnids was examined. In a constant volume (6 mL) and number of animals (6 per replicate), the increase in the concentration of microplastic (from 1.3 to 52 mg/L) increased the feeding rate of daphnids (Figure 8). A maximum plateau was observed in the feeding rate for high concentrations of microplastics (>19.5 mg/L). This can be explained as daphnids operating non-selective filtration for any particle in their media. Therefore, in low concentrations, all amount of the microparticle was ingested quickly, resulting in the absence of its excess in the media; consequently, the feeding rate reached a plateau early in the test. In contrast, concentrations of microplastic above 19.5 mg/L had little

to no effect on the feeding rate of the animals, considering that the food intake remained constant after a critical concentration. Subsequently, 13 mg/L was the selected concentration to perform the following tests for the optimization of the assay, considering that a high feeding rate and minor variance are crucial.

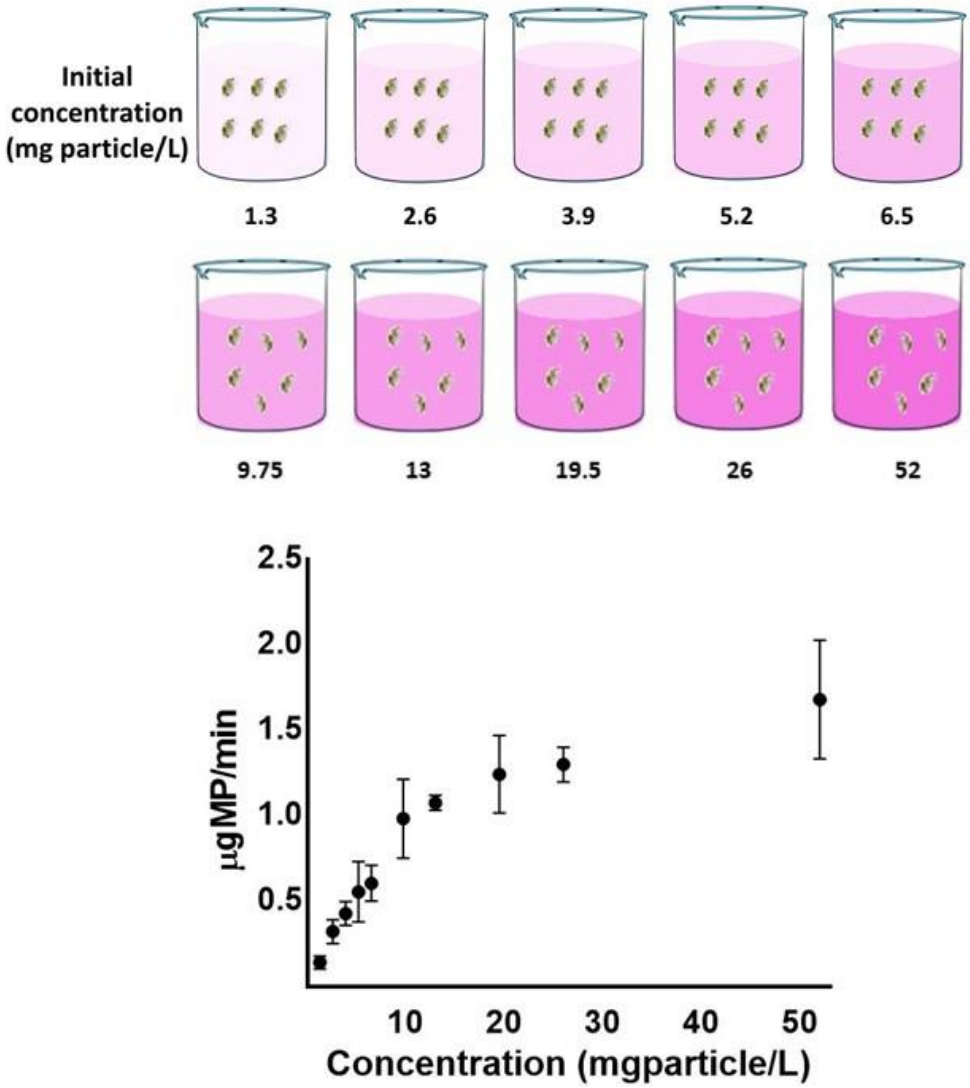

Figure 8. Experimental design and impact of concentration of microplastic on the feeding rate. Six animals were incubated in 6 mL of different concentrations of microplastic. The concentration of microplastic in the supernatant was measured in 10-minute intervals for 60 minutes, and the feeding rate was calculated based on the linear part.

The impact of volume on the feeding rate of daphnids was also assessed using various approaches. First, a constant number of animals and concentration of microplastics were used to determine the effect of the volume on the feeding rate of daphnids (Figure

9). Four animals per replicate were incubated in different volumes (2 to 10 mL) of microplastic at an initial concentration of 13 mg/L. Increasing the volume of microplastic results in a proportional increase in the absolute amount of microparticles, as the concentration used was constant. The increase in volume had little to no effect on the feeding rate of daphnids.

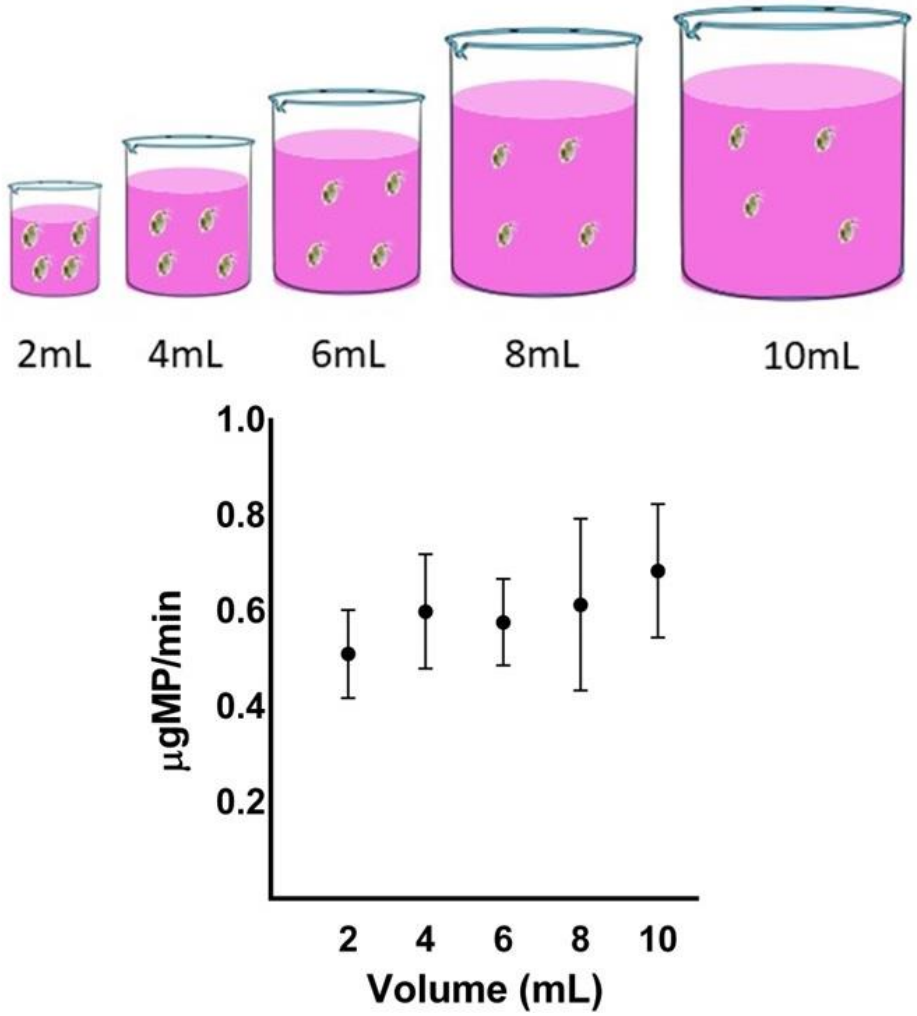

Figure 9. Experimental design and impact of volume on the feeding rate. Four animals were incubated in different volumes of well plates with the same initial concentration of microplastic (13 mg/L). The concentration of microplastic in the supernatant was measured in 5-minute intervals for 30 minutes, and the feeding rate was calculated based on the linear part.

The impact of volume at the feeding rate was also assessed using a different number of animals with a volume-to-animal ratio equal to one. Two to ten animals were incubated in 2 to 10 mL of microplastic, respectively, while the concentration of microplastic was constant (13 mg/L) (Figure 10). While increasing the volume of the

microparticles at a constant concentration, the absolute amount of microparticles increased respectively; hence, there was a higher availability of microparticles in higher volumes. As could be expected, the feeding rate increased correspondingly by increasing the number of animals used per replicate. This appears to be a result of the animal number rather than the volume increase, as mentioned earlier. Nevertheless, at higher volumes, lower consumption per animal was observed. However, using lower volumes increases the variance. Due to that fact, volumes between four and six were selected to perform the test to improve accuracy and minimize the variance of the feeding assay.

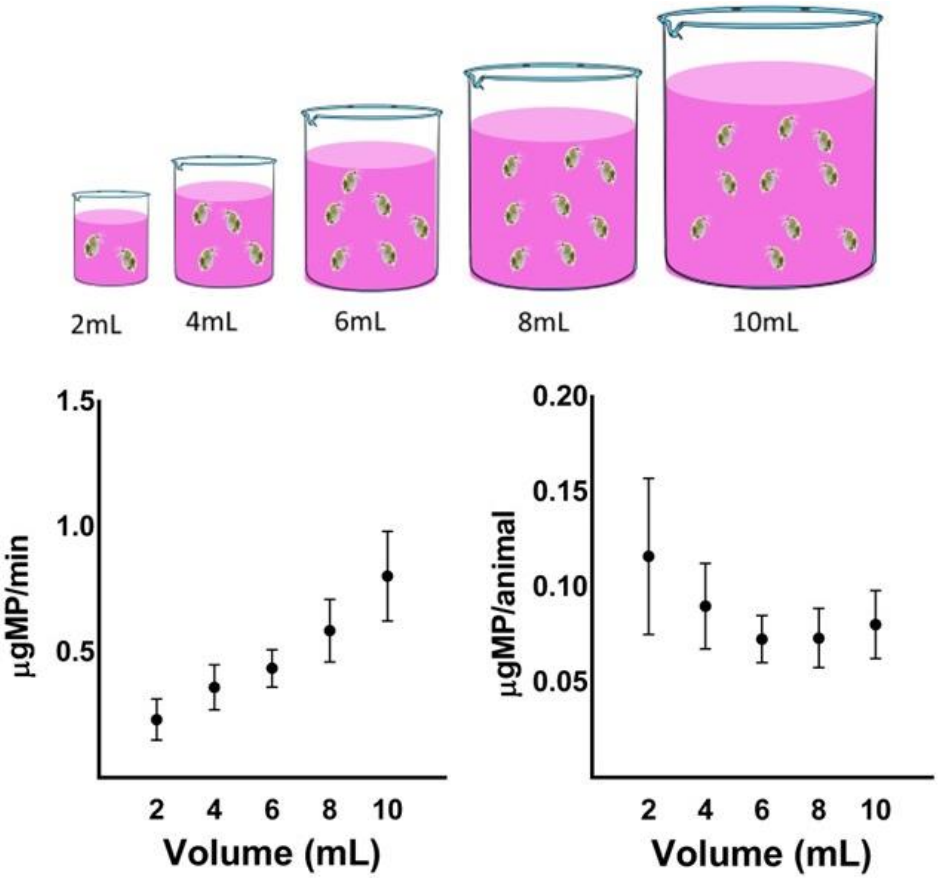

Figure 10. Experimental design and impact of volume on the feeding rate. A different number of animals were used (with ratio volume/ animals = 1) for different volumes (2, 4, 6, 8, and 10 mL) with the same concentration of microplastic (13 mg/L). The concentration of microplastic in the supernatant was measured in 10-minute intervals for 60 minutes, and the feeding rate was calculated based on the linear part.

Finally, another approach to the impact of the volume of microparticles on the feeding rate was the following: Varying volumes and concentrations were used to achieve the same absolute amount of microplastics in every condition. The exact final amount of microparticles was achieved by increasing the volume and decreasing the

concentration per replicate. First, the same number of animals were incubated in 2, 4, and 6 mL of microplastic concentrations of 39, 26, and 13 mg/L, respectively. Following, 2, 4, and 6 animals were incubated in 2, 4, and 6 mL of microplastic with concentrations of 39, 26, and 13 mg/L, respectively (Figure 11). There was little to no effect on the feeding rate using the same number of animals and the same absolute amount of microparticles. However, when the volume-to-animal ratio was equal to one, there was a significant difference in the feeding rate. As mentioned above, this result indicates that the number of animals is a crucial parameter of the feeding assay rather than the volume and concentration of microplastics. Therefore, including the results from the previous experimental designs, the selected optimal animal number and volume for the developed feeding assay are between four and six respectively; to such an extent that the number of animals is not restrictive and achieves a minor variance.

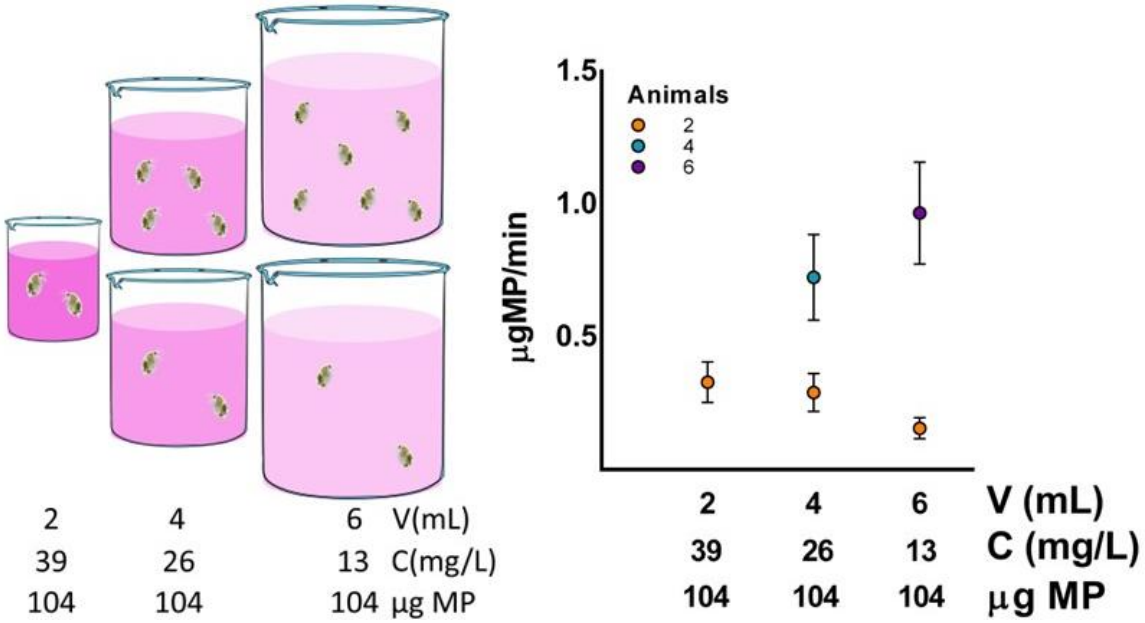

Figure 11. Experimental design and impact of volume on the feeding rate. The experiment includes two parts of different volume/ animal ratios. First, the same number of animals were used for different volumes and concentrations of microplastic. Following, a different number of animals were used (with ratio volume/ animals = 1) for different volumes (2, 4, and 6 mL) and concentrations (39, 26, 13 mg/L) of microplastic. The concentration of microplastic in the supernatant was measured in 10-minute intervals for 60 minutes, and the feeding rate was calculated based on the linear part.

2.4.2. Impact of selected pollutants on the feeding rate of daphnids

To assess the impact of exposure to chemicals on the feeding rate of daphnids, exposure concentrations of 5, 10, and 20 mg/L were selected to ensure a range of

doses that would reflect both sublethal and potentially more severe effects. These concentrations were chosen to capture a spectrum of chemical exposure, allowing for a comprehensive evaluation of any dose-dependent changes in feeding behavior. By using these concentrations, we can expect comparable results for the toxicity of the chemicals, providing a clear understanding of how different levels of exposure impact daphnid feeding rates and enabling us to determine the threshold at which chemicals begin to affect their behavior significantly (Table 1).

Neonates were cultured until four days old, prior to exposure to the chemicals. Four-day-old animals were exposed to 5, 10, and 20 mg/L for 24h, and a minimum of 3 replicates were used to perform the feeding assay. As mentioned previously, having optimized the conditions of the feeding assay, we performed the test in 50 mL falcon tubes, by increasing the parameters of the feeding assay proportionally. Fifteen animals per replicate were used for each condition, and the test was performed in 18 mL of microplastic at 13 mg/L. The feeding rate of daphnids was measured from the ingestion of carboxy microplastics based on their fluorescence at excitation of 560 nm and emission of 590 nm.

Table 1. EC values (mg/L) for acute toxicity in day four animals.
Values were calculated from corresponding toxicity curves (with N=4 replicates per concentration).

Category	Chemical	EC_{50}	(Min-max)	Hill slope	EC_1	EC_5	EC_{10}
Ca- channel blocker	Diltiazem hydrochloride	80.82	78.99-82.70	16.37	61.04	67.52	70.67
β-blocker	DL- Propranolol hydrochloride	83.62	78.04-89.61	3.864	25.46	39.03	47.35
NSAID	Diclofenac sodium	84.86	81.01-88.89	5.292	35.61	48.65	56.03
Type-2 diabetes drug	Metformin	145	142.6-147.5	9.534	89.55	106.47	115.15
Stimulant	L-nicotine	455	450.8-459.2	14.76	333.28	372.71	392.07
Metal	Lithium chloride	93.65	91.49-95.86	9.354	57.30	68.36	74.05
	Zinc sulfate heptahydrate	29.75	27.26-32.47	3.858	9.04	13.87	16.83
	Zirconium chloride	26.96	24.74-29.38	4.740	10.23	14.49	16.96
	Aluminium sulfate hexadecahydrate	59.39	56.85-62.04	5.282	24.88	34.01	39.18
	Cobalt nitrate hexahydrate	90.53	82.93-98.82	5.813	41.11	54.55	62.03

Acute exposure to the selected chemicals resulted in decreased feeding rates, based on the ingestion of microplastic, with the exception of diclofenac (Figure 12). Exposure to diltiazem decreased feeding rate; however, this was only significant for 20 mg/L by

70%. On the other hand, exposure to 5, 10, and 20 mg/L of propranolol reveals a dose-dependent decrease of ingested microplastic per animal by 46%, 57%, and 82%, respectively. The feeding rate of daphnids was significantly decreased by 15% and 34% after acute exposure to 5 and 20 mg/L of metformin, respectively. Exposure to 5, 10, and 20 mg/L of nicotine resulted in a decrease in the feeding rate of daphnids by 76% (5 mg/L) and 99% (10 and 20 mg/L). For daphniids exposed to metals, the impact on feeding rate was enhanced compared to pharmaceuticals. The only exception was lithium chloride, where a significant increase in the ingestion of microplastics was observed. Specifically, exposure to 5 and 10 mg/L of lithium increased the feeding rate by 50% and 52%, respectively. Exposure to zinc sulfate decreased ingestion by 35%, 75%, and 77%, at 5, 10, and 20 mg/L, respectively. Zirconium chloride had a more gradual dose-dependent decrease in feeding rate by 33%, 42%, and 69%. Finally, aluminium sulfate decreased the feeding rate by 67%, 63%, and 76%, and cobalt nitrate by 60%, 78%, and 98%, respectively, revealing a concentration-dependent reduction. Changes in the feeding rate after exposure to the selected chemicals were also confirmed with fluorescent microscopy (Figure 12).

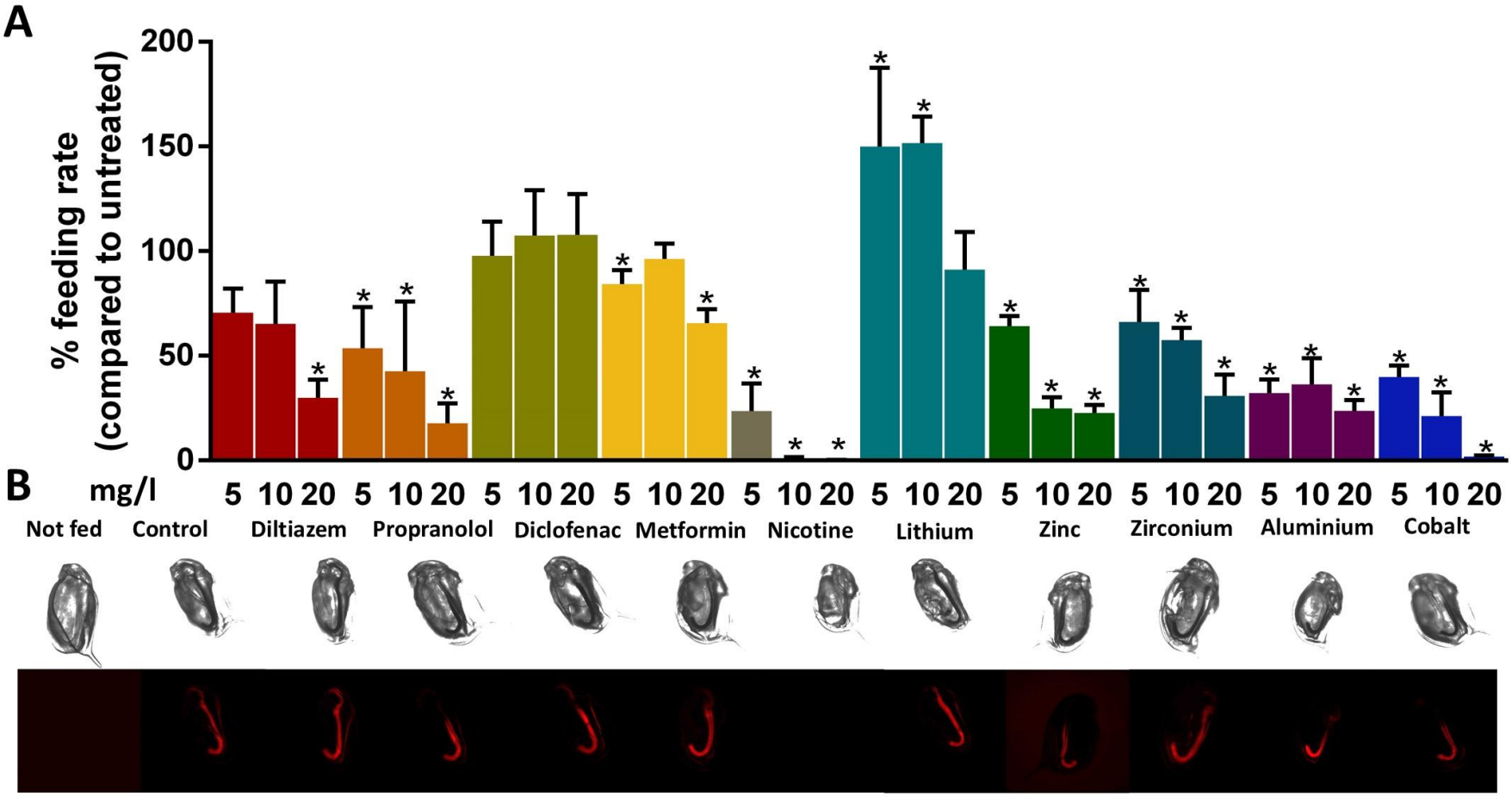

Figure 12. The impact of pollutants on feeding rate. (A) The feeding rate was assessed in daphnids exposed to 5, 10, and 20 mg/L of pollutants. Data expressed as % difference from control represent average±standard deviation (N=3). The asterisk (*) indicates a statistically significant difference in Student's t-test compared to the control. (B) The ingestion of microparticles was also visualized after exposure to 5 mg/L with bright field and fluorescence.

2.5. Discussion

2.5.1. Effects of exposure to individual pollutants

In the context of this research, we investigated the effects of a spectrum of frequently encountered pollutants at sublethal concentrations on feeding activity, to prevent high mortality rates. The sublethal concentrations (5, 10, and 20 mg/L), which were of comparable molecular concentrations, were determined after exposure to the selected pollutants, and EC values were calculated. Overall, exposure to these chemicals led to a decrease in feeding rates, with the notable exception of diclofenac.

2.5.1.1. Diltiazem hydrochloride

Diltiazem hydrochloride is a calcium channel blocker commonly used to treat patients with hypertension and rhythm disorders by altering the calcium balance. During depolarization, it acts by inhibiting calcium influx into both cardiac and smooth muscles. Calcium is essential during the contraction of smooth and cardiac muscles and acts by increasing blood pressure and the heart's workload. Therefore, decreased calcium decreases blood pressure by inhibiting muscle contractions [79]. Diltiazem hydrochloride presents one of the most frequent pharmaceuticals entering the aquatic environment. It is found in the freshwater environment through wastewater following the improper disposal of expired drugs. This can potentially affect non-target aquatic organisms by inhibiting calcium channels, posing a risk to the ecosystem. The reported concentrations vary and depend on several factors, such as the rate of removal, the daily intake, and the properties of the chemical [80]. A recent study has established that the maximum concentration found in freshwater is 130 ng/L and has also been found in several species. More specifically, acute exposure to diltiazem has been shown to delay the development of freshwater organisms, and when exposed to higher concentrations, immobility was observed [20].

Especially in *Daphnia magna*, several physiological processes, such as the adjustment of heart rate, and muscular activities that are responsible for the motion of the second antennae, thoracic limbs, and enteric peristaltic contraction, are calcium dependent. In our study, exposure to concentrations of diltiazem was only significant at the highest concentration (20 mg/L), revealing a concentration-dependent trend of decrease in the feeding rate. A study by Michalaki et. al. has shown that acute

exposure to diltiazem impacts the physiology of daphnids by altering the levels of enzyme activities [76]. Additionally, a study by Steinkey has shown that exposure to this pharmaceutical resulted in increased heart rate and oxygen consumption and altered (enhanced) the chance of survival through decreased reproduction and increased offspring size in response to environmental stress, but the feeding behavior of the animals remained consistent, probably due to the fact that these cells lack the potential Ca^{2+} channels, that are the main target of diltiazem [79].

2.5.1.2. Propranolol hydrochloride

Propranolol hydrochloride is a non-specific β-blocker that was primarily used to treat patients with cardiac disorders such as hypertension, angina, and irregular heart rate, by decreasing the heart rate, and cardiac output, and increasing the heart volume. In the past years, this pharmaceutical has also been commonly used for essential tremors and anxiety by controlling the symptoms of sympathetic overactivity [81, 82]. It is a β1- and β2-receptor antagonist, but it also acts as an antagonist to β3- or 5-HT (hydroxytryptamine) receptors, which are found in fish and wildlife, respectively.

It has been detected in significant quantities within the freshwater environment, highlighting the potential risks associated with its presence. Several studies have examined the occurrence of the drug in the aquatic environment, and the reported concentrations vary between 4 ng/L and 1.9 μg/L in wastewaters and 8-590 ng/L in surface waters [18, 83, 84]. The concern arises from the possibility of propranolol accumulating and concentrating in aquatic organisms, which could lead to ecological consequences [85, 86].

Studies involving *Daphnia magna*, a crucial species in aquatic ecosystems, have revealed that exposure to propranolol induces adverse effects similar to its known pharmaceutical actions. However, interestingly, the mechanism driving these effects remains a subject of debate. Some arguments hypothesize that propranolol may act as a narcotic, inducing non-specific effects on non-target organisms rather than being driven by a specific mode of action [87, 88]. Several studies that examined the effect on the physiology of daphnids after exposure to propranolol revealed that it can affect the swimming behaviour and fertility of the animals [77, 89]. Another study by Michalaki et. al. reported adverse effects on various biochemical markers [76]. Finally, in our study, acute exposure to 5, 10, and 20 mg/L of propranolol decreased the feeding rate

of daphnids revealing a concentration-dependent model. More specifically, the ingestion of microparticles was decreased by 46%, 57%, and 82%, after exposure to 5, 10, and 20 mg/L of propranolol, respectively (Figure 12).

2.5.1.3. Diclofenac sodium

Diclofenac sodium is a non-steroidal anti-inflammatory drug (NSAID) widely used in humans and animals to treat pain and inflammatory diseases. Diclofenac sodium, classified within the class of anti-inflammatory and analgesic pharmaceuticals (AIAPs), is characterized by its elevated acute toxicity and presents a potential hazard to non-target organisms even at concentrations below 1 μg/L [90]. Notably, diclofenac has been associated with adverse effects on fish and bird populations, resulting in renal failure, according to studies conducted by Oaks and Hoeger, underlying the substantial ecological impact of this pharmaceutical [23, 91]. Additionally, it has been found that it exhibits higher chronic toxicity relative to its acute toxicity and may induce toxic effects on non-target organisms, a finding that underscores the need for comprehensive assessments of its long-term ecological implications [19, 92]. Focusing on the adverse effects of exposure to diclofenac on *Daphnia magna*, studies have demonstrated that it induces biochemical and behavioural changes, such as reductions in growth, reproduction, heart rate, filtration, and ingestion rates, revealing a dose-dependent model [22, 93]. Furthermore, it was observed that exposure to diclofenac led to the inhibition of oxidative stress-related enzymes, which could potentially be associated with reduced ingestion rates [94]. It is important to note that our findings appear to be at odds with previous studies, likely due to the variations in exposure durations and the age of individual animals.

2.5.1.4. Metformin

Metformin (1,1-dimethylbiguanide hydrochloride) has been established as the primary choice oral medication to treat patients with type 2 diabetes by lowering blood glucose concentrations, but it is also used as an anti-cancer agent and a treatment for polycystic ovary syndrome [95, 96]. It is important to highlight that metformin is highly consumed, and alongside its minimal metabolism within the human body and the high concentrations required for therapeutic effects, can enter the environment through various routes. Moreover, it exhibits the potential for bioaccumulation in edible plant and fish species, raising the possibility of contaminating the food chain. The results of

previous investigations indicate that the levels of this pharmaceutical in aquatic environments are notably elevated in contrast with its removal rate. Consequently, the exposure concentrations are significantly higher and have the potential to affect aquatic organisms [95, 97, 98]. More specifically, it has been found that exposure to environmentally relevant concentrations of metformin has adverse effects on fish fecundity, and higher concentrations increase the heart rate, mortality, and reproduction of several aquatic species [21, 99]. Other studies that examined the adverse effects of this pharmaceutical on *Daphnia magna,* demonstrated that acute and chronic exposure decreased the activity of several enzymes, and increased the activity of detoxification enzymes, respectively [76, 100]. In this study, acute exposure to metformin significantly decreased the feeding rate of daphnids. The concentrations of pollutants examined in the newly developed feeding assay were considerably lower in comparison to the effective concentrations reported in previous studies [101]. This discovery serves as robust evidence for the remarkable sensitivity of the method.

2.5.1.5. Nicotine

Nicotine is considered a drug that is the primary component of tobacco, found in cigarettes, and widely used as a lifestyle product. Recently, the sales of tobacco products have demonstrated consistent growth [102]; thus, several studies have provided evidence of a substantial prevalence of discarded cigarette buds in the environment, solidifying their status as a globally widespread form of waste [4, 103-105]. While the effect of smoking cigarettes on human health has been extensively investigated [106], the impact of cigarette-derived pollution on the environment remains an active area of investigation [37, 107]. Cigarette buds, owing to the potential presence of toxic compounds such as nicotine, represent a significant source of pollution with substantial consequences for the environment. Nicotine, the main component of tobacco and a central nervous system stimulant, has detrimental effects on both human health and aquatic organisms. Several studies have suggested that even at sublethal concentrations, nicotine has the potential to disrupt the physiology of various aquatic species. Acute exposure to nicotine has been linked to alterations in the reproductive and nervous systems of *Daphnia magna*, resulting in the production of male offspring and inducing mobility [108, 109]. Moreover, other studies that examined the impact of nicotine on the physiology of daphnids, revealed that acute

exposure decreased the heart rate and the activity of several biomarkers [22, 76]. Notably, neonicotinoids, a class of insecticides structurally resembling nicotine, have also been associated with reduced swimming and thoracic limb activity in *Daphnia magna* [65]. In our study, nicotine emerged as the most significant inducer of reduction in feeding rates among the tested pollutants. This reduction is likely attributed to its influence on swimming and thoracic limb activity, both of which play a pivotal role in the ingestion process [110].

2.5.1.6. Metals

Metals can be introduced into aquatic ecosystems through various sources, including natural processes such as the release of metals from rock formations and volcanic eruptions, agricultural activities involving the application of metal-containing fertilizers, and industrial discharges from mining and smelting operations. Certain heavy metals, termed "essential," have vital physiological roles in organisms when present at low concentrations. However, they can become hazardous when the organisms are exposed to high concentrations. On the other hand, "non-essential" heavy metals are toxic and can disrupt various biological processes in both humans and aquatic organisms. Both essential and non-essential metals have the potential to accumulate in aquatic organisms as they are transferred through the food chain. This bioaccumulation can have adverse consequences for both human health and the overall health of aquatic ecosystems. Specifically, toxic heavy metals can have adverse effects on the survival, growth, and population dynamics of aquatic organisms [26, 29, 30].

Although various studies have already assessed the toxic effects of metals, the greatest number concentrate on survival as the primary indicator of their toxicity in aquatic organisms [30, 111, 112]. To our knowledge, there is limited research that employs alternative physiological parameters, like feeding behavior, to evaluate the effects of exposure to sublethal concentrations of pollutants. Nevertheless, evidence indicates that exposure to metals, even at lower concentrations, significantly influences phenotypic and physiological endpoints in *Daphnia magna*.

2.5.2. Sensitivity of the developed feeding assay

By referencing the geometric mean EC_{50} values from the ECOTOX database for daphnids, we highlighted the differences in sensitivity between our four-day-old daphnids and those reported in the literature. Our findings show that the concentrations tested in our study were notably low, often below the EC_1 for four-day-old daphnids, underscoring the high sensitivity of the feeding assay. Additionally, we compared the immobilization data to standard EC_{50} values, revealing whether our daphnids are less or more sensitive than typical results. This comparison allows us to quantify the increased sensitivity of the feeding rate assay, demonstrating it to be two to three times more sensitive than traditional methods, thus emphasizing the assay's efficacy.

In a study contacted by Kim et. al. EC_{50} values of 28 mg/L and 8.2 mg/L after 48h and 96h of acute exposure of neonates to diltiazem were reported, respectively [80]. In our study, the EC_{50} value for day-four animals exposed to diltiazem for 24h was determined to be 80.82 mg/L, demonstrating a higher sensitivity of neonates to diltiazem exposure compared to day-four animals. Moreover, the concentration used in the feeding assay (20 mg/L) was 1.4 times lower than the EC_{50} value reported in the previous study. The ECOTOX database indicated that EC_{50} values for propranolol after 24h of acute exposure were below 10 mg/L, significantly lower than the EC_{50} value obtained in our study (83.62 mg/L). During the feeding assay, acute exposure to 5 mg/L of propranolol resulted in a significantly lower feeding rate, highlighting the method's sensitivity, which was twice as high as the immobilization test on neonates. Cleuvers et. al. reported EC_{50} values of 68 mg/L and 64 mg/L for diclofenac sodium and metformin, respectively, after 48h of acute exposure [19, 113]. In our study, the EC_{50} values for diclofenac sodium and metformin after 24h of acute exposure in day-four animals were 84.86 mg/L and 145 mg/L, respectively. The feeding assay conducted on four-day-old animals exhibited nearly thirteen times higher sensitivity than the immobilization test on neonates. Furthermore, the EC_{50} value for nicotine after acute exposure was below 10 mg/L, contrasting with our study's findings for day-four animals (455 mg/L), and the feeding rate was twice as sensitive as the immobilization test. Okamoto et. al. documented EC_{50} values of 6.3 mg/L and 2.9< mg/L for lithium chloride and zirconium chloride, respectively, which were significantly lower than the values obtained for four-day-old animals in our study (93.65 mg/L and

26.96 mg/L, respectively) [114]. Finally, in a study contacted by Brun et. al. EC_{50} value of 44.27 mg/L was observed for neonates exposed to aluminium sulfate for 24h (EC_{50}=59.39 mg/L for four-day-old animals) [115]. The feeding assay for four-day-old animals exhibited nine times higher sensitivity than the immobilization test on neonates during acute exposure to aluminium sulfate. In conclusion, neonates displayed higher sensitivity to the selected chemicals during acute exposure compared to four-day-old animals, whilst the developed feeding assay proved to be a more sensitive endpoint for most of the chemicals investigated.

Chapter 3

The impact of extracts from cigarettes and e-cigarettes on the physiology of daphnids

Abstract

During the past decades, cigarette and e-cigarette sales have increased as they present popular lifestyle products worldwide. The occurrence of discarded cigarette filters presents a significant environmental challenge, consisting of various harmful substances such as nicotine and polycyclic aromatic hydrocarbons, contributing to environmental pollution. Since the harmful effects of cigarette and e-cigarette use on human health have been widely examined, it is crucial to investigate the effects of cigarette and e-cigarette pollution on the aquatic environment and organisms. Recently, a few studies have reported the potentially toxic effects of cigarette leachate on a variety of aquatic species, highlighting the impacts on physiology, behavior, and development even at sublethal concentrations. In this study, the impact of cigarette and e-cigarette filter extracts on the aquatic organism *Daphnia magna* was examined. To assess the effects on the mortality of the animals, toxicity curves were plotted, and effective concentrations were calculated. To assess the sublethal effects on the physiology of the animals, acute, chronic, and transgenerational exposures were conducted. The effects of the cigarette extracts on feeding, growth rate, survival, and key enzymes of metabolism were examined. E-cigarette extracts were shown to have a stronger effect on the feeding, growth, and survival of the animals at higher concentrations. On the contrary, the cigarette extract has significantly affected the activity of key enzymes after exposure for one generation and in the fourth generation (recovery). To conclude, this study aims to assess the possible ecological risks of cigarette pollution on aquatic organisms utilizing the model organism *Daphnia magna*.

3.1. Occurrence of cigarette and e-cigarette filters in the aquatic environment

Cigarettes and e-cigarettes are commonly acknowledged as items of lifestyle for a global base of customers [116, 117]. Sales of tobacco products have increased in recent years, especially considering the substantial rise that has occurred in e-cigarette consumption among young individuals [102, 118, 119]. E-cigarettes are characterized as battery-operated devices generating an aerosol from a water-based solution. In addition, heat-not-burn tobacco (HNBT) is commonly characterized as a hybrid between traditional and e-cigarettes [120-122]. Considering the relevance of HNBTs as substitutes for traditional cigarettes, the World Health Organization (WHO) has acknowledged the necessity of monitoring the adverse effects and risks of novel tobacco products [123].

Several studies indicate that discarded cigarette buds are a highly prevalent and commonly encountered type of waste in the environment [4, 45, 103-105]. The studies by Novotny et. al. and Araújo et. al. have demonstrated that trillions of cigarettes (approximately 6 trillion) are consumed globally, with the majority being discarded into the environment (approximately 4.5 trillion) [116, 124]. Despite efforts to mitigate pollutants in natural ecosystems, the issue persists [125]. Although cigarette filters are widespread as litter in the environment, their impacts on marine, freshwater, and terrestrial habitats remain insufficiently investigated [53]. Numerous research has concentrated on the effects of smoking cigarettes on human health; however, the consequences of cigarette-derived pollution on the environment remain under investigation [37, 106, 107, 126]. Due to the fact that cigarette filters may contain toxic compounds including nicotine [40, 104, 127, 128], polycyclic aromatic hydrocarbons [127, 129, 130], heavy metals [129, 131-137], nitrosamines, carbonyls, phenols [127, 128], carbonyls [138], insecticides [139-141], and BTEX [142], cigarettes represent a significant source of environmental pollution. More specifically, smoked cigarette filters contain a variety of toxic chemicals resulting from smoking, and an excessive amount of these chemicals has been identified as harmful to aquatic organisms [143]. According to recent studies, cigarette leachate has the potential to be hazardous for a variety of aquatic species, having both lethal and sublethal effects. The physiology, behavior, and development of the exposed organisms might alter as a result of cigarette filter exposure [144]. In addition, studies indicate the harmful compounds

contained in cigarette buds may bioaccumulate in organisms, which could have a detrimental impact on the entire food chain [124, 145, 146]. Insufficient documentation on environmental contamination arising from the use and disposal of cigarettes and e-cigarettes requires greater consideration, especially given the rising prevalence of these products. Addressing this issue is essential to comprehend the possible adverse effects on the environment and public health. Additional research and consistent monitoring are critical to evaluate the scope and implications of pollution thoroughly and to formulate effective strategies for the management and reduction of this environmental issue [37].

The traditional methods commonly applied to evaluate water quality rely primarily on identifying particular compounds and contaminants present in the aquatic environment. However, these methods present limitations, detecting only a narrow range of substances and relying on specific sensitivity thresholds. In contrast, effect-based methods present a promising alternative for supporting our comprehension of pollutant effects in the aquatic environment, acting as a complement to existing approaches. These methodologies offer detailed mechanistic insights into how pollutants interact with organisms to assist in the establishment of a more comprehensive understanding of contaminant behavior [1]. Furthermore, they serve as increasingly sensitive tools, enabling predictions and precise evaluations of pollutant levels. Therefore, integrating effect-based techniques with conventional methods can provide more thorough and enhanced insights that could support water quality assessment efforts.

Daphnids, along with other sentinel species, are frequently utilized in aquatic toxicity assessments due to their sensitivity to both chemical and physical changes in the freshwater environment [55]. Their widespread geographical distribution and key role in freshwater food chains underscore their significance in ecosystem dynamics. In addition, their adaptability across various environmental conditions enables them to serve as effective indicator species, uncovering subtle effects arising from chemical pollution within freshwater environments [58, 147, 148]. Moreover, their filter-feeding behavior makes them highly responsive to pollutants, as they non-selectively ingest substances present in the freshwater environment. Furthermore, daphnids have various characteristics that make them easy to culture in the lab. Notably, their short life cycle and capability for parthenogenetic reproduction contribute to ensuring

consistent and uniform responses among individual daphnids when exposed to pollutants entering the aquatic ecosystem. Consequently, these attributes provide a reliable and replicable system for research purposes [56].

The primary objective of this study was to evaluate the impact of cigarette and e-cigarette (HNBT) extracts on the physiology of *Daphnia magna* after exposure to non-lethal concentrations. In contrast to prior research focusing on mortality as an indicator of water toxicity [20, 30, 59, 60, 149], this study employed multiple endpoints, including transgenerational exposures, growth, feeding, and survival (Figure 14). To our knowledge, this is the first study that examines the effects of cigarette and e-cigarette filter extracts on the physiology of *Daphnia magna*, considering various phenotypical and physiological endpoints.

3.2. Materials and Methods

3.2.1. Materials

All chemicals used in this study were of the highest analytical quality. *p*-nitrophenyl phosphate (pNPP, CAS RN 4264-83-9), ammonium acetate (CAS RN: 631-61-8), boric acid (CAS RN: 10043-35-3), sodium hydroxide (CAS RN: 1310-73-2), hydrochloric acid (CAS RN: 7647-01-0), dimethyl sulfoxide (CAS RN: 67-68-5), and sodium pyruvate (CAS RN: 113-24-6) were purchased from Thermo Fisher. L-Leu-4-nitroanilide (CAS RN: 4178-93-2) and β-nicotinamide adenine dinucleotide reduced disodium (CAS RN: 606-68-8) were purchased from Alfa Aesar. o-nitrophenyl-β-galactoside (CAS RN: 369-07-3), 1-chloro-2,4-dinitro-benzene (CAS RN: 97-00-7), L-glutathione reduced (CAS RN: 70-18-8), Coomassie Brilliant Blue G250 (CAS RN: 6104-58-1), carboxylate-modified fluorescent latex beads, p-nitrophenyl butyrate (CAS RN: 2635-84-9), and sodium phosphate dibasic (CAS RN: 7558-79-4) were purchased from Sigma Aldrich.

3.2.2. Collection and preparation of cigarette and e-cigarette extracts

Cigarettes and e-cigarettes were naturally smoked by individuals and cigarette and e-cigarette filters were collected. We tested one brand of cigarettes and e-cigarettes, the filters were collected randomly, placed in clean plastic bags, and transported to the laboratory for further processing. The extracts were prepared from smoked cigarette and e-cigarette filters (smoked filter and tobacco) by adding one cigarette and e-

cigarette filter in a falcon with 10 mL (100 CBs/L) of daphnid culture media, respectively (final concentrations 0.29 g $CaCl_2$ x 2H2O/L, 0.123 g $MgSO_4$ x $7H_2O$/L, 0.065 g $NaHCO_3$/L, 0.0058 g KCl/L, 2 µg Na_2SeO_3/L, pH 7.7). Following, the falcons were left under continuous shaking (120 rpm) overnight (>24h). Extracts were cleared by filtering (0.8 µm), the clear extract was collected, and dilutions of the initial extract were used for acute, chronic, and transgenerational exposures (Figure 13).

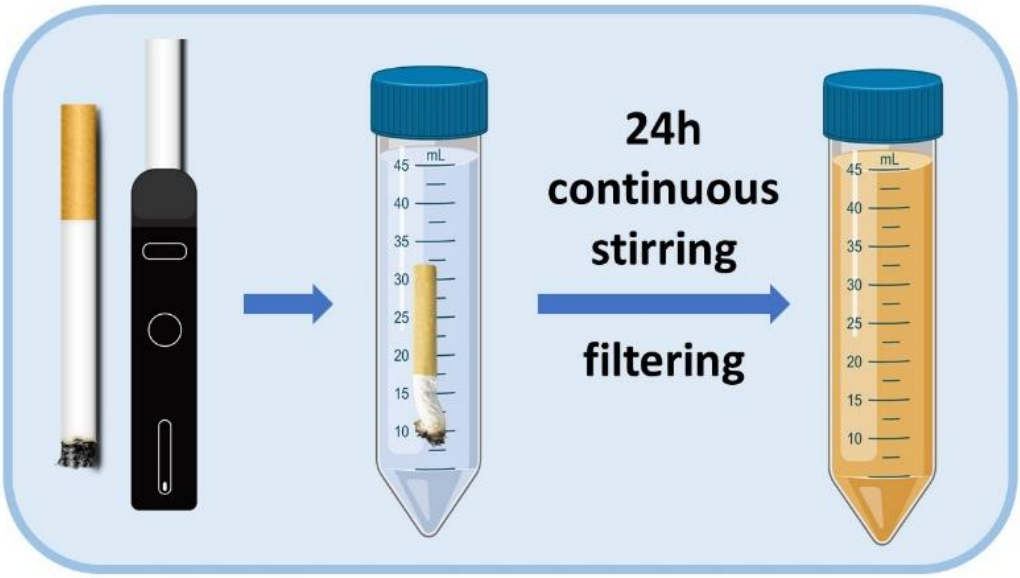

Figure 13. Preparation of cigarette and e-cigarette extracts. One cigarette bud was incubated in 10 mL media for >24h under continuous stirring. Following the extract was filtered and collected.

3.2.3. Culturing of algae and daphnids

Algae of the species *Chlamydomonas rheinhartii* is the main source of food for *Daphnia magna*. A semi-continuous stock culture of algae was kept in Chlamydomonas growth medium (20 g NH_4Cl/L, 8 g $MgSO_4$ x $7H_2O$/L, 4 g $CaCl_2$ x $2H_2O$/L, 8.64 g K_2HPO_4/L, 8.4 g KH_2PO_4/L, 50/31 g EDTA/KOH/L, 4.98 g acidified iron/L, 11.42 g boric acid/L, 14.12 g $ZnSO_4$ x $7H_2O$/L, 2.33 g $MnCl_2$ x $4H_2O$/L, 2.54 g $CuSO_4$ x $5H_2O$/L, 0.82 g $Co(NO_3)_2$ x $6H_2O$/L, 1.92 g Na_2MoO_4 x $4H_2O$/L, pH 6.7) for feeding.

Centrifugation at 3,000 rpm for 10 minutes at room temperature was used to collect the algae. After centrifugation, the pellet was retained and then re-suspended in ddH2O. In a specified volume, the absorbance was measured at 440 nm and the

average value was calculated. This value was multiplied by the dilution factor, divided by 7, and a ratio greater than 1 was obtained. The concentrated algae volume was then multiplied by this ratio to determine the final volume of the algae. Finally, the required volume of ddH2O was added to achieve the final algae volume.

The cultures of *Daphnia magna* were prepared according to OECD recommendations. Eighty animals were cultured in 4-liter beakers with OECD medium (final concentrations of 0.2 g $CaCl_2$ x $2H_2O$/L, 0.123 g $MgSO_4$ x $7H_2O$/L, 0.065 g $NaHCO_3$/L, 0.0058 g KCl/L, 2 g Na_2SeO_3/L, pH 7.7) under a 16h:8h light: dark at 21°C as described previously [76]. The algal solution (6 million cells/mL) and dried baker's yeast (*Saccharomyces cerevisiae*) (2 mL from 100 mg/L) were the primary food for daphnid cultures, while standard organic seaweed (*Acoplymun nodosum*) extract that had an absorbance of 8A after dilution to ddH_2O was added for medium renewal and the setup of new cultures. Neonates from the first and second brood of their mothers, males, and ephippia were removed and not used for the experiments, whereas neonates (<24h) from the third brood were used to set up new cultures.

3.2.4. Culturing of daphnids for toxicity and chronic exposures

For toxicity exposures, neonates (<24h) were collected from the third brood of their mothers. Fifteen neonates per replicate were exposed to 50 mL of aqueous media with a minimum of four replicates in different concentrations of cigarette and e-cigarette extracts (Figure 14). All plots were calculated using the Four parameter logistic (4PL) model, following the equation Span = Top − Bottom and Y = Bottom + (Top-Bottom)/(1 + 10^((LogIC50-X)*HillSlope)), using the GraphPad Prism 6 software (Dotmatics). The EC_1, EC_5, EC_{10}, and EC_{50} values were calculated, and a non-lethal concentration was selected to initiate the chronic and transgenerational exposures. Thirty-six neonates were cultured in 900 mL of culturing media and supplemented with 1.35 mL standard organic seaweed extract (*Acoplymun nodosum*), and 2.7 mL of algae was added daily. Media, cigarette and e-cigarette extracts, and seaweed extract were renewed twice a week (Tuesday and Friday), and samples were collected after fourteen days of exposure for biochemical assays. Neonates released from the third brood of the first generation were used to initiate the second generation of exposure.

3.2.5. Survival analysis

Exposures for survival analysis were set up as a scaled-down version of chronic exposures. Ten neonates (<24h) were collected from the third brood of their mothers and cultured in 250 mL aqueous media and 0.375 mL seaweed extract with daily supplementation of 0.75 mL algae. To assess the impact of cigarette and e-cigarette extract on the survival of daphnids, the animals were exposed to different concentrations of extract in triplicates, and the average was recorded. Median survival days were reported, and Kaplan-Meier curves were plotted (Figure 14).

3.2.6. Sample homogenization

Six fourteen-day animals per replicate were pooled together and homogenized in 1 mL of ddH_2O with a microtube tissue grinder pestle. The average number of animals used per replicate was sufficient to ensure enough tissue was available for all biochemical assays and to reduce individual variance. The homogenate was centrifuged (13,000 g for 5 minutes), and the clear supernatant was collected and assessed for protein and biochemical markers activity immediately.

3.2.7. Protein quantification

A sensitive method was used to quantify the total protein of the homogenates, which is based on the electrostatic reaction of proteins with Coomassie Brilliant Blue (CBB) G-250 reagent [150, 151]. A solution of CBB reagent (60 mg CBB dissolved in 100 mL 2 M HCl, left under continuous stirring for 40 min and cleared by filtration to remove undissolved dye particulates) was diluted with 2 M HCl (1:1) before use. The standard curve was prepared by dilutions (2-20 µg/mL) of bovine serum albumin (BSA) in ddH_2O. For the protein quantification assay, the unknown samples were appropriately diluted in ddH_2O, and 200 µL of the sample, BSA standards, or ddH_2O as blank was used were mixed with 50 µL of the CBB: 2 M HCl reagent. The reaction was incubated at room temperature for 10 minutes, and the absorbance was measured at 610 nm. The corresponding BSA standard curve was used to convert the final sample net absorbance to protein concentration equivalents.

3.2.8. Responses in the physiology of daphnids

For feeding experiments, neonates were collected from the third brood of their mother and cultured until 4 days old. Following, day 4 animals were exposed to sublethal

concentrations of cigarette and e-cigarette leachate to prevent high mortality rates, with a minimum of four replicates. According to our novel feeding assay [152], daphnids were exposed to different concentrations of cigarette and e-cigarette extract for 24h. Following the exposures, animals were collected in clear media, and replicates were prepared. For the feeding assay, fifteen animals were used for each replicate in a final volume of 18 mL microparticles at a final concentration of 13 mg/L. Daphnids were incubated in microplastic and media was collected after ten minutes. To assess the microplastic consumed by daphnids, incubations in the absence of animals were used to determine the feeding rate expressed over time and per animal (Figure 14).

To assess the growth rate of the animals, ten daphnids were exposed to 250 mL of media with various concentrations of cigarette and e-cigarette extract, with 0.375 mL seaweed extract and daily supplementation of 0.75 mL algae. After seven and fourteen days of exposure, animals were collected, and size was measured as the distance from the eye to the beginning of the spine. Images were collected with a stereoscope and analyzed with the ImageJ software, and size was expressed as absolute values and as growth rates (GR) using the Chopelet incremental growth rate (Figure 14). Different approaches were used, and the growth rate was expressed as follows, using the mathematical formulae below: from neonates to day 7, and neonates to day 14 (1st) and from day 7 to day 14 (2nd), to evaluate the significance of multiple timepoints or timelines in the growth rate of the animals.

1st approach: $GR_7 = \frac{ln_7 - ln_{\text{neonate}}}{7}$ and $GR_{14} = \frac{ln_{14} - ln_{\text{neonate}}}{14}$

2nd approach: $GR_7 = \frac{ln_7 - ln_{\text{neonate}}}{7}$ and $GR_{14} = \frac{ln_{14} - ln_7}{7}$

Animal physiology and response to external stimuli depend mainly on cellular defense mechanisms and enzyme function. While other enzymes (such as phosphatases, β-galactosidase, and lipase) are more critical to metabolism and animal physiology, antioxidant enzymes (glutathione-S-transferase) eliminate ROS, detoxify xenobiotics, and repair cellular damage. Kinetics measures the photometric (or other) activity of enzymes. Acid phosphatase (ACP), alkaline phosphatase (ALP), β-galactosidase, and lipase are examples of endpoint kinetics, where a one-time point is measured and used to calculate the rate. Glutathione-S-transferase (GST) and lactate dehydrogenase (LDH) are examples of continuous kinetics, where continuous

monitoring of a substrate's consumption or the product's generation is measured to calculate the relevant activity rate. The activities of ACP and ALP were measured from the production of *p*-nitrophenol from *p*-nitrophenyl phosphate (pNPP). The acid and the alkaline phosphatase reaction used citric (for acid) or boric acid (for alkaline), correspondingly. 200 μL of sample appropriately diluted in buffer and 50 μL of 8 mM pNPP were mixed and incubated at room temperature for 30 minutes. The reaction was alkalined with the addition of 4 M hydroxide, which allows *p*-nitrophenol to absorb higher in an alkaline environment. The absorbance was measured at 405 nm and converted to nmoles *p*-nitrophenol from a standard curve of pNPP. The activity was converted to units of *p*-nitrophenol per minute. The activity of β-galactosidase was measured from the conversion of o-nitrophenyl-β-galactoside (ONPG) galactose after alkalinization. 200 μL of sample appropriately diluted in phosphate buffer and 50 μL of ONPG (8mM) were mixed and incubated at room temperature for 30 minutes. The reaction was alkalined with the addition of 4 M hydroxide, allowing the o-nitrophenol produced to absorb higher in an alkaline environment. Absorbance was measured at 405 nm and was converted at nmoles of o-nitrophenol produced. The activity was converted to units of p-nitrophenol per minute. Lipase activity was measured from the conversion of p-nitrophenyl butyrate to p-nitrophenol. 200 μL of sample appropriately diluted in phosphate buffer and 50 μL of 2.8 mM p-nitrophenyl butyrate were mixed and incubated for 30 minutes at room temperature. This reaction does not require alkalinization but can be monitored at 405 nm in 5-minute intervals. The absorbance was measured and was converted at nmoles of o-nitrophenol produced and then units of p-nitrophenol per min. Aminopeptidase activity was measured from the hydrolysis of L-Leu-4-nitroanilide and the production of 4-nitroaniline. 200 μL of sample appropriately diluted in phosphate buffer and 50 μL of L-Leu-4-nitroanilide (8 mM, diluted at DMSO) were mixed. The production of 4-nitroaniline was monitored continuously for 10 minutes every 30 seconds at 418 nm and converted to nmoles of 4-nitroaniline from a standard linear curve. The activity of glutathione-S-transferase was measured from the reaction of reduced glutathione with 1-chloro-2,4-dinitro-benzene (CDNB), resulting in the production of S-2,4-dinitrophenyl-glutathione (S-DNP-GS) [153]. 200 μL of sample appropriately diluted in phosphate buffer and 50 μL of 2 mM CDNB:6 mM GSH (1:1) were mixed and measured continuously at 340 nm. The lactate dehydrogenase activity was measured continuously from the consumption

NAD(P)H, which can be monitored from the decrease of the absorbance measured at 340 nm. A 1:1 mixture of 40 mM pyruvate and 0.5 mM NADH was used as the substrate. 50 µL of this substrate was combined with 200 µL of a sample that had been appropriately diluted in phosphate buffer. Data were presented as mean±standard deviation (SD) with four replicates per condition and were analyzed with the GraphPad Prism 6 software (Dotmatics). Statistically significant differences were identified with One-way ANOVA.

3.3. Statistical analysis

Data were presented as mean±standard deviation (SD) and were analyzed and plotted with the GraphPad Prism 6 software (Dotmatics). Statistically significant differences were identified with One-Way ANOVA corrected with post-hoc Dunnett's test.

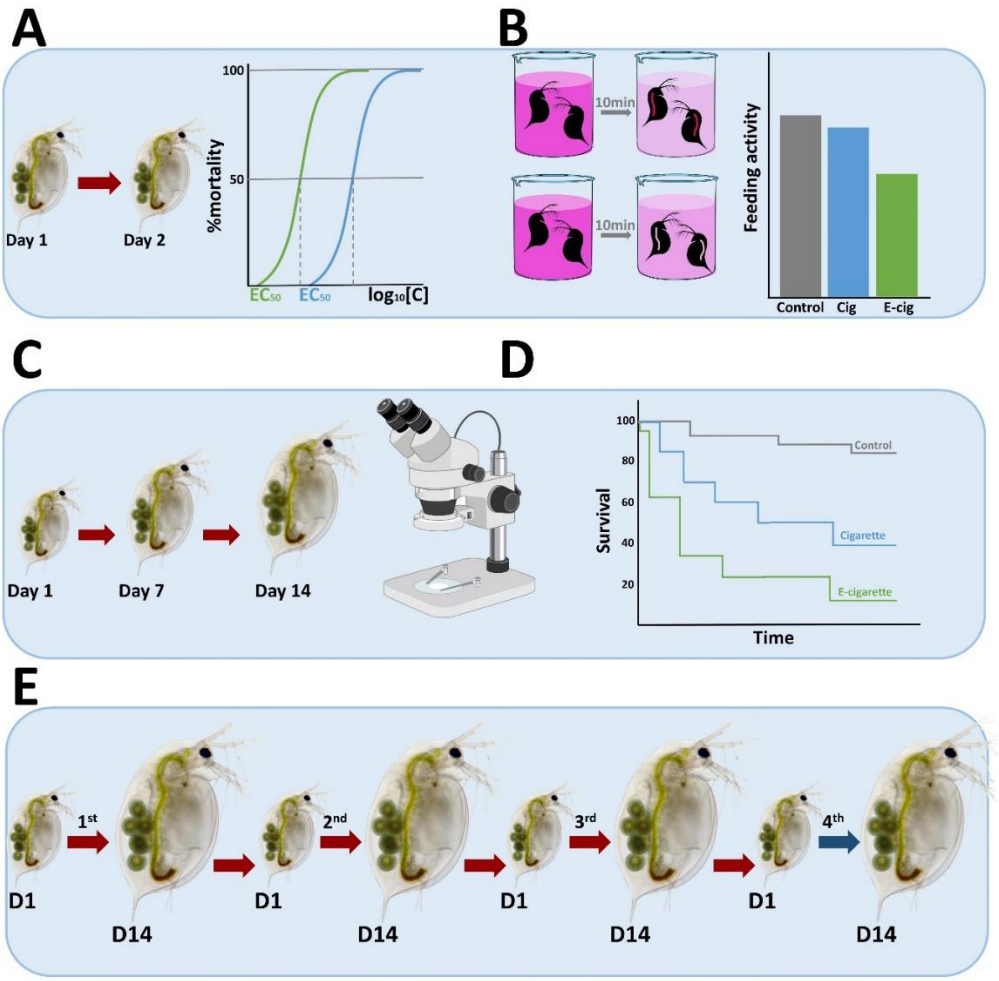

Figure 14. Experimental design of acute, chronic, and transgenerational exposures . Acute toxicity curves were plotted after acute exposure in neonates (A), feeding experiment was assessed on day 5 animals, after acute exposure in day 4 animals (B). Growth rates were measured after exposure for 7 and 14 days (C). Survival curves were plotted after chronic exposure to examine the effect of cigarette and e-cigarette extract on the life span of the animals (D). Chronic and transgenerational exposures (1^{st}, $3^{rd,}$ and 4^{th} generation) were conducted to assess the impact of cigarette and e-cigarette exposure on biochemical markers of physiology in daphnids (E). Red arrows indicate exposure to cigarette/ e-cigarette bud extract, and blue arrows indicate the absence of the extract for recovery generation.

3.4. Results

3.4.1. The impact of cigarette and e-cigarette extracts on mortality and survival of daphnids

The study examined the acute toxicity of cigarette and e-cigarette filter extracts from the assessment of toxicity curves in neonates after 24h of exposure (Figure 15). EC_{50} values were calculated for each extract. Notably, the EC_{50} value of e-cigarette leachate (12.44 ml extract/L) was significantly lower than conventional cigarettes (31.26 ml extract/L), indicating a more toxic effect to daphnids of e-cigarettes compared to cigarettes, after 24h of exposure.

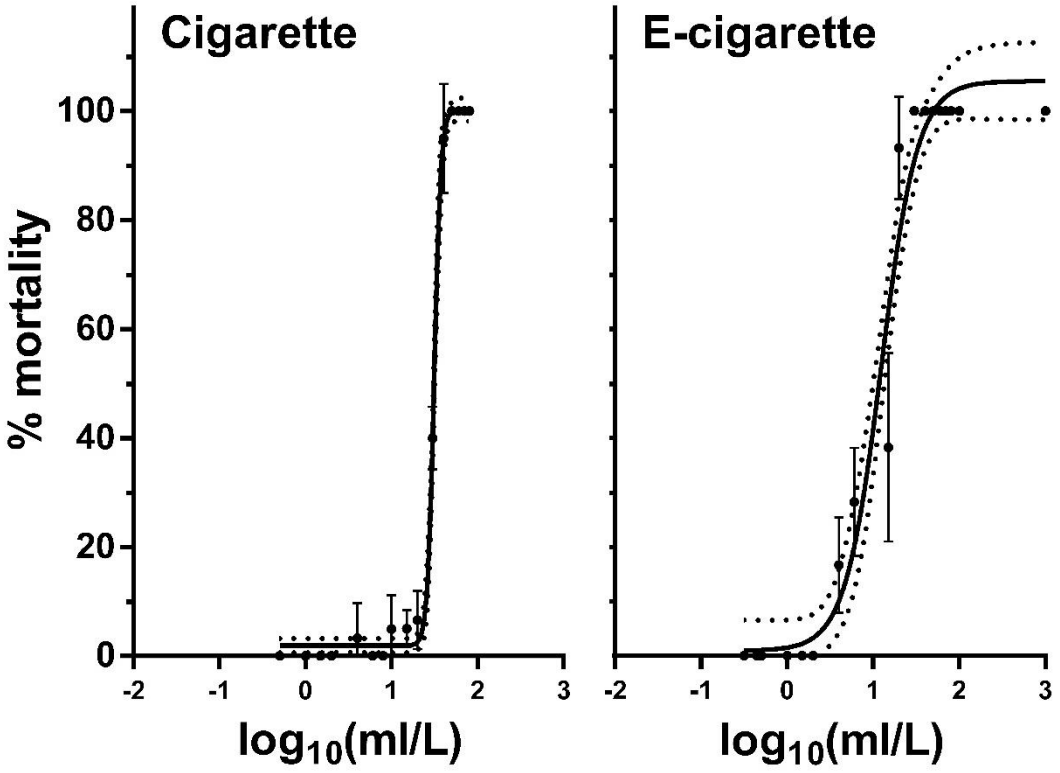

Figure 15. Acute toxicity curves for cigarette and e-cigarette extracts. Data represent average±standard deviation (N=4 replicates).

Table 2. EC values (ml/L) for acute toxicity in neonates. Values were calculated from corresponding toxicity curves (with N=4 replicates per concentration).

Extract	EC_1	EC_5	EC_{10}	EC_{50}
Cigarette	20.62	23.94	25.62	31.26
E-cigarette	1.34	2.99	4.29	12.44

To further investigate the previous findings in a chronic context, survival was assessed at a range of extract concentrations, and Kaplan-Meier curves were plotted with three independent replicates per concentration (Figure 16). The mean values from these assessments were compared with those of the unexposed control group over five weeks. The findings revealed that lower concentrations of both cigarette and e-cigarette extracts did not significantly affect the survival of the animals, showing comparable survival rates to those of the unexposed animals. However, at higher concentrations of e-cigarette extract (>6,000 µL extract/ 900 mL media), a noticeable toxic effect on the survival of daphnids was observed. Specifically, daphnids exposed to concentrations equal to or higher than 6,000 µL extract/ 900 mL media of e-cigarette leachate exhibited a median survival of only two days, while those exposed to concentrations equal to or higher than 9,000 µL extract/ 900 mL media showed a median survival of one day. Similar outcomes were noted following exposure to concentrations equal to or greater than 20,000 µL extract/ 900 mL media of cigarette leachate.

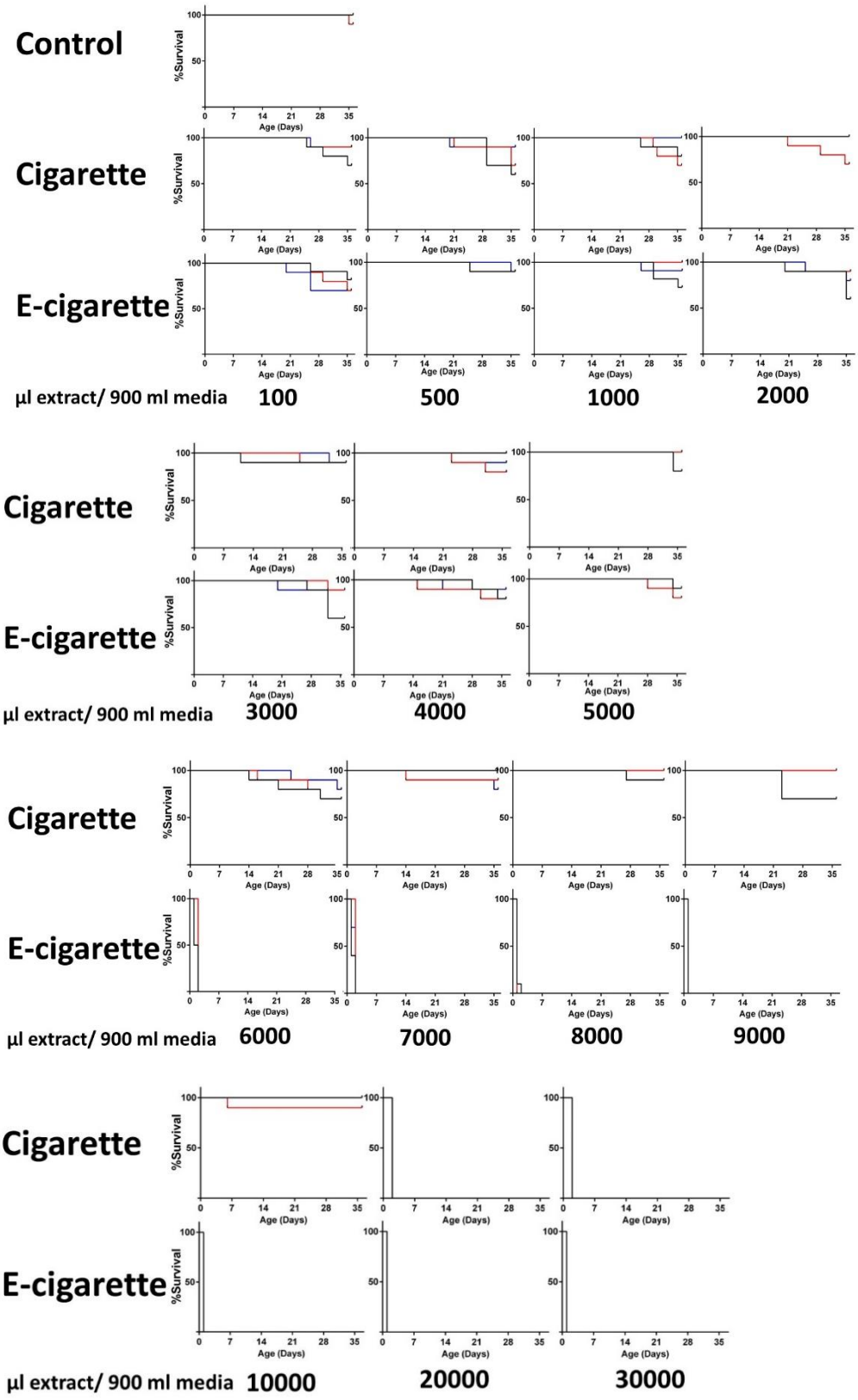

Figure 16. Kaplan Meier curves for survival after exposure to cigarette and e-cigarette filter extracts. Data represent three independent replicates.

3.4.2. The impact of cigarette and e-cigarette extracts on feeding and growth rates of daphnids

After establishing the effects of cigarette and e-cigarette extracts on the mortality and survival of daphnids, animals were exposed to non-lethal concentrations to evaluate the effect on growth and feeding rates. The objective was to assess growth and feeding rates as phenotypic endpoints using non-invasive methods. To examine the impact of cigarettes and e-cigarettes on the size and growth rate of the animals, daphnids were exposed for seven and fourteen days to various concentrations of extracts (100-3000 µL/ 900 mL media). Following, daphnids were collected, size was measured, and growth rates were calculated as described previously. The impact of cigarette and e-cigarette extracts on the feeding rate of daphnids was examined with an innovative protocol that quantified the ingestion of fluorescent microparticles [152].

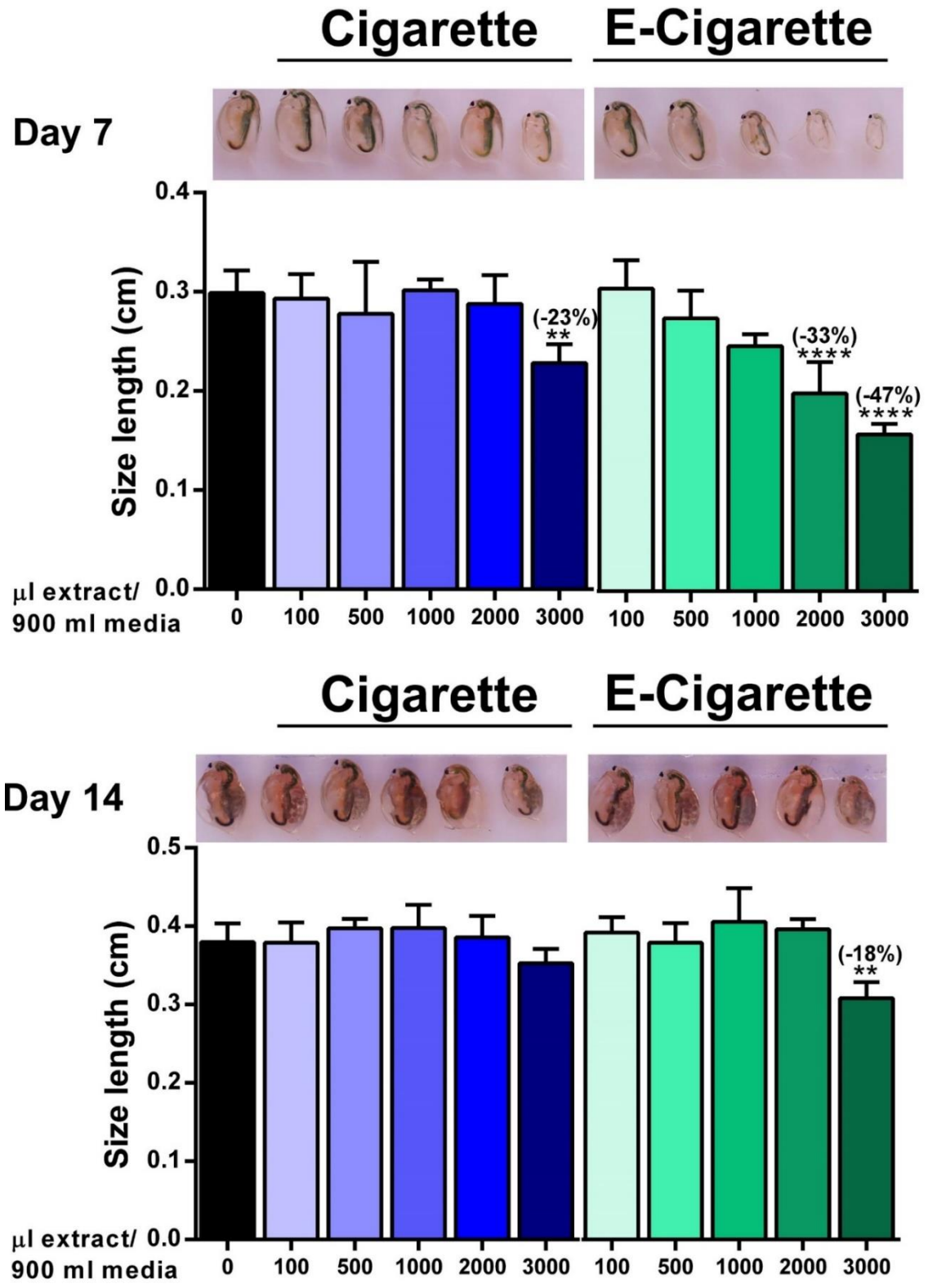

Figure 17. The impact of cigarette and e-cigarette extracts on growth. Size was measured in animals exposed for 7 and 14 days to the extracts. Data represent average±standard deviation (N=4). The asterisks indicate a statistically significant difference by One-way ANOVA compared to the unexposed control for **p<0.01, ****p<0.0001.

Table 3. The impact of cigarette and e-cigarette extracts on the **size** of daphnids. Size was calculated at 7 and 14 days daphnids. Data represent average±standard deviation (N=4). Bold font indicates a statistically significant difference by One-way ANOVA compared to the unexposed control.
The concentration of extracts was measured in µl/900 mL media.

Age (days)	Control	Extract	100	500	1000	2000	3000
D7	0.3±0.02	Cigarette	0.293±0.025	0.278±0.052	0.302±0.011	0.288±0.029	**0.228±0.019 (-23%)**
		E-cigarette	0.305±0.029	0.275±0.028	0.247±0.012	**0.2±0.032 (-33%)**	**0.158±0.011 (-47%)**
D14	0.38±0.023	Cigarette	0.379±0.026	0.397±0.012	0.398±0.03	0.386±0.028	0.353±0.018
		E-cigarette	0.392±0.02	0.379±0.025	0.406±0.043	0.396±0.013	**0.308±0.021 (-18%)**

Growth was measured as size at 7 and 14 days old and it was notable that high concentrations of the extract had a negative effect on the size of the animals. Specifically, a concentration-dependent decrease was recorded for e-cigarette extract and a reduction in the size which was only significant for the highest concentration of the cigarette extract in the first 7 days of exposure. After 14 days of exposure to cigarette and e-cigarette extract, the decrease in the size was only significant in the highest concentration of exposure to cigarette and e-cigarette extract, and a notably higher decrease after exposure to e-cigarette extract (Figure 17, Table 3).

Table 4. The impact of cigarette and e-cigarette extracts on the **growth rate** of daphnids
. Size and growth rates (GR) were calculated at 7 and 14 days daphnids. Data represent average±standard deviation (N=4). Bold font indicates a statistically significant difference by One-way ANOVA compared to the unexposed control.
Growth rate at 14 days is calculated with t1=day 1 and t2=day 14.
The concentration of extracts was measured in µl/900 mL media.

Growth rate	Control	Extract	100	500	1000	2000	3000
D7	0.2±0.013	Cigarette	0.19±0.014	0.18±0.034	0.2±0.006	0.19±0.017	**0.15±0.014 (-22%)**
		E-cigarette	0.2±0.016	0.18±0.016	0.17±0.009	**0.13±0.026 (-35%)**	**0.09±0.011 (-53%)**
D14	0.11±0.009	Cigarette	0.11±0.01	0.11±0.005	0.11±0.011	0.11±0.01	0.1±0.007
		E-cigarette	0.11±0.004	0.11±0.005	0.11±0.008	0.11±0.003	**0.09±0.005 (-14%)**

The differences in size were also reflected in the growth rates of animals (Table 4). Notably, high concentrations of the extract had a negative effect on the growth of the animals, providing that the growth rate of the animals was measured using day 1 as the initial time point. Specifically, similar to the size of daphnids, after exposure to the

highest concentration of cigarette extract in the first 7 days, the growth rate of the animals was significantly decreased by 22%. A significant concentration-dependent decrease was noted after exposure to e-cigarette extract for 7 days. More specifically, the growth rate of the animals after 7 days of exposure to e-cigarette extract at 1000, 2000, and 3000 µl /900 mL media was decreased by 16%, 35%, and 53%, respectively. After 14 days of exposure, the negative effect of cigarette and e-cigarette extract exposure was diminished, demonstrating no significant differences after exposure to cigarette extract, and a significant decrease of 14% after exposure to 3000 µl /900 mL media of e-cigarette extract.

Table 5. The impact of cigarette and e-cigarette extracts on the **growth rate** of daphnids. Size and growth rates (GR) were calculated at 7 and 14 days daphnids. Data represent average±standard deviation (N=4). Bold font indicates a statistically significant difference by One-way ANOVA compared to the unexposed control.
The concentration of extracts was measured in µl/900 mL media.

Growth rate	Control	Extract	100	500	1000	2000	3000
D7	0.2±0.013	Cigarette	0.19±0.014	0.18±0.034	0.2±0.006	0.19±0.017	**0.15±0.014 (-22%)**
		E-cigarette	0.2±0.016	0.18±0.016	0.17±0.009	**0.13±0.026 (-35%)**	**0.09±0.011 (-53%)**
D14	0.03±0.009	Cigarette	0.04±0.01	**0.05±0.005 (+54%)**	0.04±0.011	0.04±0.01	**0.06±0.007 (+81%)**
		E-cigarette	0.04±0.007	0.05±0.01	**0.07±0.015 (+114%)**	**0.1±0.005 (+202%)**	**0.1±0.01 (+190%)**

Following, the growth rate of the animals was assessed at a 7-day interval after 7 and 14 days of exposure to cigarette and e-cigarette filter extract. Interestingly, a contrasting effect on growth rates was observed. Exposure to cigarette extract notably enhanced the growth rate of daphnids at concentrations of 500 and 3000 µL / 900 mL by 54% and 81% respectively. However, exposure to e-cigarette extract demonstrated a concentration-dependent effect on growth rate enhancement across most concentrations. Specifically, exposure to concentrations 1000, 2000, and 3000 µl / 900 mL increased the growth rate of the animals by 114%, 202%, and 190% respectively (Table 5).

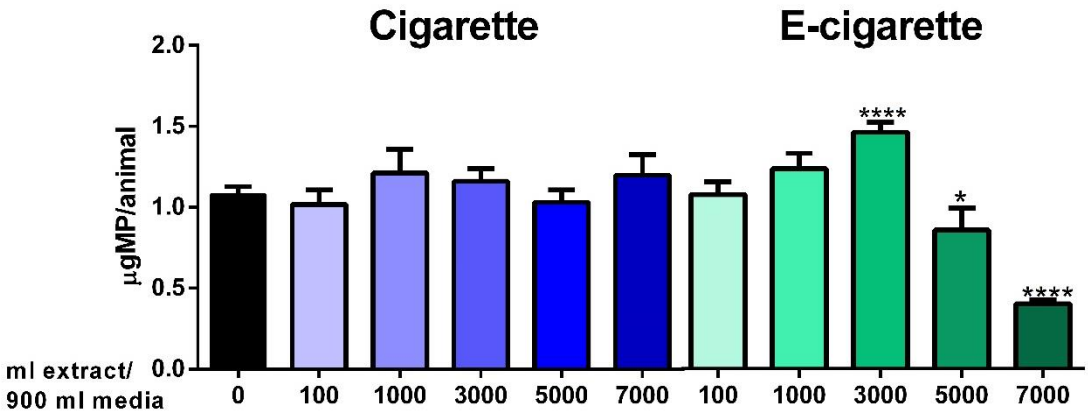

Figure 18. The impact of cigarette and e-cigarette extracts on feeding rate. The feeding rate was assessed in d4 animals (<24h) exposed to each extract. Data expressed as µg microplastic/animals represent average±standard deviation (N=4). The asterisks indicate a statistically significant difference by One-way ANOVA compared to the unexposed control for *p<0.05, ****p<0.0001.

The feeding rate was significantly affected only by exposure to e-cigarette filter extracts for day-four animals (Figure 18). A notable effect was demonstrated on the feeding rate of daphnids following exposure to e-cigarette leachate. Specifically, exposure to 100 and 1000 µl/ 900 mL media did not significantly alter the feeding rate of the animals. However, exposure to 3000 µl/900 mL media increased the feeding rate of daphnids. Conversely, exposure to 5000 and 7000 µl/900 mL media led to a concentration-dependent decrease in the feeding rate of daphnids, as shown above.

3.4.3. The impact of cigarette and e-cigarette extracts on the physiology of daphnids

Chronic and transgenerational exposures were conducted to assess the impact of cigarette and e-cigarette extracts on the physiology of daphnids utilizing biochemical markers. The non-lethal concentrations of exposure were based on previous experiments to prevent mortality. An additional chronic exposure was conducted to investigate the potential for recovery following the stress of the initial exposure. Enzyme activities were evaluated in the first generation of animals after 14 days of exposure. Subsequently, their offspring were exposed to a second and third generation, with enzyme analysis conducted on animals from the third generation (Table 6). Finally, a recovery generation was initiated from the third generation, and enzyme activities were assessed in the fourth generation.

Table 6. The impact of extracts on key enzyme activities of daphnids after exposure to cigarette and e-cigarette extracts. Data represent average±standard deviation (N=4). Enzyme activity was expressed as units/mg protein for phosphatases (ACP; acid, ALP; alkaline), lipase (LIP), β-galactosidase (βGAL), peptidase (PEP), lactate dehydrogenase (LDH), and glutathione-S-transferase (GST). Bold font indicates a statistically significant difference by One-way ANOVA compared with the unexposed control.
The concentration of extracts was measured in µl/900 mL media.

	Enzyme	Control	Cigarette		E-cigarette	
			100	1000	100	1000
1st generation	ACP	2.88±0.16	**3.63±0.26 (+25%)**	**4.11±0.33 (+42%)**	3.5±0.36	3.42±0.16
	ALP	4.03±0.17	**4.51±0.1 (+11%)**	4.43±0.07	4.33±0.14	**5.04±0.2 (+25%)**
	βGAL	2.27±0.14	**2.66±0.14 (+16%)**	**2.88±0.17 (+26%)**	2.29±0.2	**2.92±0.1 (+28%)**
	LIP	39.63±0.88	45.46±1.21	**52.4±2.9 (+32%)**	42.21±3.1	40.05±2.7
	PEP	3.14±0.11	**3.49±0.11 (+11%)**	3.32±0.22	3.02±0.17	**2.78±0.11 (-11%)**
	GST	0.13±0.02	**0.17±0.01 (+32%)**	**0.16±0.02 (+22%)**	**0.14±0.03 (+11%)**	**0.17±0.01 (+31%)**
	LDH	213.16±20.88	**165.79±9.9 (-22%)**	182.08±10.89	**120.4±6.71 (-43%)**	196.82±27.46
3rd generation	ACP	1.59±0.04	**1.82±0.09 (+14%)**	**2.04±0.14 (+28%)**	1.46±0.12	1.66±0.17
	ALP	3.08±0.31	3.35±0.29	**4.25±0.36 (+38%)**	3.11±0.19	**3.72±0.11 (+20%)**
	βGAL	2.18±0.21	2.76±0.39	**3.54±0.4 (+62%)**	2.55±0.2	**2.93±0.27 (+33%)**
	LIP	94.68±2.93	89.68±6.06	**121.87±10.93 (+28%)**	**106.07±7.25 (+12%)**	96.8±3.17
	PEP	3.39±0.29	**4.42±0.24 (+30%)**	**5.45±0.37 (+60%)**	**3.84±0.21 (+13%)**	**4.21±0.23 (+24%)**
	GST	0.32±0.04	**0.46±0.04 (+43%)**	0.34±0.02	**0.28±0.02 (-13%)**	**0.27±0.01 (-15%)**
	LDH	130.91±10.66	**183.15±12.54 (+39%)**	**203.39±20.93 (+55%)**	**150.04±21.09 (+14%)**	**160.53±23.14 (+22%)**
Recovery 4th generation	ACP	1±0.13	0.8±0.05	**0.65±0.09 (-34%)**	0.81±0.18	1.02±0.13
	ALP	3.46±0.08	**3.07±0.16 (-11%)**	**3.17±0.04 (-8%)**	3.41±0.05	3.52±0.22
	βGAL	2.89±0.23	**2.29±0.27 (-20%)**	**2.43±0.11 (-16%)**	2.72±0.14	2.94±0.3
	LIP	52.81±2.14	**43.38±4.8 (-17%)**	**45.08±3.68 (-14%)**	50.36±0.76	49.75±4.06
	PEP	2.4±0.13	**2±0.1 (-16%)**	**2.09±0.03 (-13%)**	**1.84±0.05 (-23%)**	2.3±0.13
	GST	0.2±0.01	**0.22±0.02 (+12%)**	0.19±0.01	**0.27±0.03 (+36%)**	**0.27±0.01 (+34%)**
	LDH	168.48±13.6	**113.11±5.02 (-32%)**	**77.94±9.92 (-53%)**	**125.95±8.1 (-25%)**	**95.04±8.25 (-43%)**

Exposure to cigarette and e-cigarette leachate over 14 days (1st generation), as well as across 3rd and 4th (recovery) generations, resulted in notable alterations in the physiology of daphnids, characterized by significant changes in enzyme activities (Table 6).

Following exposure to cigarette and e-cigarette extracts for 14 days, the activity of ACP exhibited significant changes. More specifically, ACP activity was increased by 25% and 42% after exposure to 100 and 1000 µl/ 900 mL of cigarette extract, respectively. However, the increase in ALP enzyme activity was significant only after exposure to low concentrations of cigarette extract and high concentrations of e-cigarette extract. Specifically, ALP activity increased by 11% after exposure to 100 µl/900 mL of cigarette extract and by 25% after exposure to 1000 µl/900 mL of e-cigarette extract. The enzyme β-galactosidase increased by 16%, 26%, and 28% after exposure to 100 and 1000 µl/900 mL of cigarette extract and 1000 µl/900 mL of e-cigarette extract, respectively. However, lipase activity was significantly increased only after exposure to 1000 µl/900 mL of cigarette extract, rising by 32%. Peptidase activity increased by 11% after exposure to 100 µl/900 mL of cigarette extract but decreased by 11% after exposure to 1000 µl/900 mL of e-cigarette extract. GST activity showed increases under all conditions; exposure to 100 and 1000 µl/900 mL of cigarette extract increased GST activity by 32% and 22%, respectively, while exposure to 100 and 1000 µl/900 mL of e-cigarette extract increased GST activity by 11% and 31%, respectively. Lastly, LDH activity was significantly decreased after exposure to 100 µl/900 mL of both cigarette and e-cigarette extract, declining by 22% and 43%, respectively (Table 6).

Transgenerational exposure spanning three generations to cigarette and e-cigarette extracts resulted in statistically significant differences in enzyme activities among daphnids. ACP activity was notably increased only following chronic exposure to cigarette extract, rising by 14% and 28% after exposure to 100 and 1000 µl/900 mL extract, respectively. ALP and β-gal activities were significantly increased after exposure to the highest concentrations of both cigarette and e-cigarette extracts. Specifically, exposure to 1000 µl/900 mL of cigarette and e-cigarette extracts elevated ALP activity by 38% and 20%, respectively, while β-gal activity increased by 62% and 33% after exposure to 1000 µl/900 mL of cigarette and e-cigarette extracts, respectively. Lipase activity increased after exposure to 100 µl of e-cigarette extract

(12%) and after exposure to 1000 µl of cigarette extract (28%). Peptidase activity was increased after exposure to all conditions (100 and 1000 µl extract of cigarette and e-cigarette), showing rises of 30%, 60%, 13%, and 24% respectively. GST activity significantly increased after exposure to 100 µl/ 900 mL of cigarette extract (43%) but decreased after exposure to 100 and 1000 µl/900 mL of e-cigarette extract (13% and 15% respectively). Finally, LDH activity exhibited significant increases after exposure to all conditions. Specifically, LDH activity increased by 39%, 55%, 14%, and 22% after exposure to 100 and 1000 µl/900 mL of cigarette and e-cigarette extracts, respectively (Table 6).

Neonates from the third generation of exposure were collected and cultured in the absence of cigarette and e-cigarette extracts (4^{th} generation) to investigate the potential for recovery following a single generation. In contrast to exposures during the 1st and 3^{rd} generations, the 4^{th} generation exhibited significant decreases in the activity of various enzymes, notably ACP, ALP, β-gal, lipase, and peptidase, particularly among animals previously exposed to cigarette extract in the preceding generations. Specifically, ACP activity decreased significantly by 34% after exposure to 1000 µl/900 mL of cigarette extract, while ALP activity decreased by 11% and 8% after exposure to 100 and 1000 µl/900 mL of cigarette extract, respectively. β-gal and lipase enzyme activities were notably decreased after exposure to both 100 and 1000 µl/900 mL of cigarette extract, with reductions of 20%, 16%, 17%, and 14%, respectively, as indicated in the accompanying table (Table 6). Furthermore, peptidase activity showed significant decreases of 23% after exposure to 100 µl of e-cigarette extract, and 16% and 13% after exposure to 100 and 1000 µl/900 mL of cigarette extract, respectively. GST activity exhibited increases of 36% and 34% after exposure to 100 and 1000 µl of e-cigarette extract, and by 12% after exposure to 100 µl of cigarette extract. Lastly, LDH activity was significantly decreased under all tested conditions, with reductions of 32% and 53% (100 and 1000 µl of cigarette extract) and 25% and 43% (100 and 1000 µl of e-cigarette extract), respectively.

3.5. Discussion

Cigarette and e-cigarette filters present a commonly encountered pollutant in the aquatic environment. Several studies have shown that discarded cigarette filters constitute approximately 30% of litter along shorelines, waterways, and terrestrial environments in the United States [41].

A significant proportion of smoked cigarettes are discarded onto the ground as cigarette buds (CBs), which may contain unburnt or partially burnt tobacco. These buds often end up in marine environments. Even without remaining tobacco, CBs contain toxic chemicals in their filters from the smoking process. Standard CBs are made of plasticized cellulose acetate, ash, unburned tobacco, and filter [154]. Therefore, CBs pose serious environmental threats due to their resistance to biodegradation [36, 44].

In this study, the acute and chronic effects of cigarette and e-cigarette leachate were assessed, using a combination of physiological and biochemical markers. To assess the impact of the selected pollutants on physiological markers, growth and feeding rate, survival, and mortality were used. For the assessment of biochemical markers, phosphatases, β-galactosidase, lipase, peptidase, glutathione-S-transferase, and lactate dehydrogenase activity were examined.

Although numerous studies from previous years have explored the impacts of nicotine (cigarette compound) on *Daphnia magna* [22, 108, 109, 155-157], there is limited research specifically investigating the effect of cigarette and e-cigarette leachate on aquatic organisms, with particular emphasis on daphnids. The toxicity of nicotine in aquatic organisms offers valuable insight, however, does not adequately represent the toxicity of cigarettes due to the complex composition of this pollutant, which consists of numerous hazardous compounds apart from nicotine [158].

Previous studies have demonstrated that residual tobacco within cigarette filters is the primary factor contributing to mortality, although compounds present in smoked cigarette filters also exhibit lethal effects on organisms [159]. Another study revealed that the brand of cigarette can impact the concentration of toxic chemicals in CB leachates and the toxicity response of different organisms. It was suggested that the toxicity towards certain species was associated with specific cigarette brands and their tobacco residue content [51]. However, contradictory findings have been reported,

indicating that differences in cigarette brands are not directly associated with the observed toxicity [160]. Moreover, there is no standardized quantity of cigarette filters utilized in the preparation of extracts for exposure. The number of cigarette filters employed in the existing literature commonly ranges from two to twenty [50, 51, 53, 136, 160-164]. In this study, we utilized one brand of cigarette and e-cigarette smoked filters (no residual tobacco), and one filter was added to 10 mL of media, to prepare the initial extract, which was subsequently diluted for exposure.

Studies focusing on the toxicity of cigarette leachate on aquatic organisms commonly assess the impact of the pollutant on the mortality of aquatic organisms. Different EC_{50} values have been reported for different types of cigarette filter leachate. For instance, Lee et. al. indicated that low concentrations of smoked and unsmoked cigarette filters affected the development of fish embryos, and high concentrations of the extracts were acutely toxic. In addition, embryonic growth was negatively impacted after exposure to low concentrations of the leachate [160]. Other studies indicated that different types of leachates were acutely toxic and exhibited varying levels of toxicity to aquatic organisms, with unsmoked filters being less toxic than smoked filters, and smoked cigarettes exhibiting the highest toxicity [50, 165]. More specifically, Slaughter et. al. was the first to study the toxic impact of CB leachate on marine and freshwater species, bioindicators for aquatic environments. CB leachates were prepared in various concentrations, and several factors were examined (smoked cigarette filters, unsmoked cigarette filters, and smoked cigarette buds with remaining tobacco) [50]. Several studies have demonstrated that CB leachates were detected to be acutely toxic for aquatic organisms [51, 53, 163, 166, 167]. Wright et. al. indicated that exposure to cigarette filter extracts resulted in growth rate inhibition and increased DNA damage in marine worms [164]. The outcomes from previous research are in line with our study, which indicates that cigarette and e-cigarette filter extract is acutely toxic for *Daphnia magna*. Furthermore, lower concentrations of the leachates negatively affect the physiology and behavior of daphnids, resulting in reductions in growth and feeding rates. To our knowledge, there is no available biochemical data for the impact of cigarette and e-cigarette extracts on daphnids. In our study, cigarette and e-cigarette extracts were found to be strong stressors, with cigarettes decreasing enzyme activities, indicating a more significant impact on daphnids.

A recent study that examined the impact of conventional, electronic, and menthol cigarettes on *Xenopus laevis* embryos, indicated that cigarette filters were acutely toxic, while menthol cigarette filter exposure resulted in higher teratogenicity [162]. In our study, e-cigarette filters indicated a higher toxicity, and decrease in growth and feeding rate of the animals. The variance in observations could potentially originate from differences in the source of e-cigarettes utilized in the prior study, given its nature as an electronic device with liquid content. In addition, some studies have investigated the impact of cigarette filters on plant physiology [168], with recent ones incorporating electronic cigarettes as a source of pollutants [161]. Findings suggested that exposure to cigarette buds had detrimental effects on plant physiology, and certain effects observed were contradictory when comparing cigarette and e-cigarette filters.

Previous studies that examined the impacts of cigarette filter exposure on aquatic organisms, including *Daphnia magna*, indicated that acute toxicity was observed, even at the lower concentrations of the leachate, and physiological and heart rate alterations were also observed [125, 159]. Overall, in our study, exposure to cigarette and e-cigarette leachate significantly altered the physiology and behavior of the tested organism. More specifically, a decrease in survival, growth, and feeding rate was observed after exposure to higher concentrations of cigarette and e-cigarette filter extract. The impact of e-cigarette extract on daphnids' physiology is notably higher in comparison with cigarette extracts, except for molecular biomarkers. This finding highlights the need for further investigation into the specific mechanisms involved.

The authors of studies that examine the impact of cigarette pollution on aquatic organisms emphasized that leachates from cigarette filters consist of complex mixtures containing diverse classes of chemicals. These chemical compositions may account for the observed toxic effects on the tested organisms [166]. However, it remains crucial to consider the multitude of factors that could affect the toxicological effects of cigarette filter pollution when comparing findings from different studies. Apart from species distinctions, these factors include the method of smoking (mechanized or human), the source of cigarette filters, variations in cigarette types and brands (which may change over time), the specific endpoints under examination, and the duration of exposure, among others [160].

3.6. Conclusions

Cigarette and e-cigarette extracts present a commonly encountered pollutant in natural environments and one of the most common types of waste in the last few decades. Past studies have shown that approximately 30% of global waste consists of discarded cigarette filters [116]. Cigarette filter leachates are complex mixtures that consist of harmful substances highly concentrated after the consumption of the cigarette [41, 50], including nicotine, which is water-soluble [40]. The harmful effects of cigarette exposure on human health, which include cancer risk and heart and lung diseases, among others, have been widely investigated [42]. However, there is a gap in knowledge regarding the effects of cigarette pollution on the physiology and behavior of aquatic organisms, and little data is available today. This study examined the impact of cigarette and e-cigarette leachate on the physiology of the aquatic organism *Daphnia magna*. Additional research is required to thoroughly identify the ecological hazards posed by cigarette pollution in aquatic environments. Considering the existing literature on the harmful effects of discarded cigarette filters on organisms and natural environments, it is essential to apply measures to reduce cigarette pollution. Several actions have been proposed to reduce cigarette filter pollution. Public education campaigns emphasizing the environmental hazards of improper cigarette disposal can raise awareness and promote responsible behavior among smokers. Legislative measures, such as banning single-use cigarette filters or imposing fines for littering, can provide a regulatory framework to discourage improper disposal. In addition, increasing the availability of disposal bins and ashtrays in public areas and enhancing urban waste management systems can facilitate proper disposal. Finally, investing in the development and promotion of biodegradable or less harmful alternatives to traditional cigarette filters can significantly reduce the long-term environmental impact [36, 43, 52].

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Supplementary figures and tables

Figure 19. Visualization of the ingestion of microparticles after exposure to 5,10, and 20 mg/L with bright field and fluorescence.

Appendices

Appendix A. *Daphnia magna* culturing protocols

OECD standard media. OECD media were prepared in a final volume of 52 L ddH_2O in a tank. The final concentrations of salts were 0.29 g $CaCl_2$ x $2H_2O$/L, 0.123 g $MgSO_4$ x $7H_2O$/L, 0.065 g $NaHCO_3$/L, 0.0058 g KCl/L, 2 µg Na_2SeO_3/L. The pH was adjusted to 7.77 and the media were aerated for 24h.

Seaweed extract supplement. The standard organic seaweed (*Acophylum nodosum*) extract is added to the modified media on culture setup only when the media is renewed. The concentrated extract was diluted in ddH_2O to an absorbance of 8A at 400 nm.

Proposed plan for culturing daphniids. The following culturing timeline is followed to generate clonal populations of mothers which will breed animals (neonates) for experiments.

Daphnid age (days)	Procedure	OECD	Yeast	Fresh algae	Seaweed extract
1	**Set up new culture**	4 L	2 mL	4 mL	12 mL
2			2 mL	4 mL	
3			2 mL	4 mL	
4			2 mL	6 mL	
5			2 mL	6 mL	
6	**Change media**	4 L	2 mL	6 mL	12 mL
7	**Remove neonates and discard them**		2 mL	6 mL	
8			2 mL	8 mL	
9			2 mL	8 mL	
10			2 mL	8 mL	
11			2 mL	8 mL	
12			2 mL	8 mL	
13	**Change media**	4 L	2 mL	8 mL	16 mL
14	**Remove neonates for culturing experiments**		2 mL	8 mL	
15			2 mL	8 mL	
16			2 mL	8 mL	
17			2 mL	8 mL	
18			2 mL	8 mL	
19			2 mL	8 mL	
20			2 mL	8 mL	

Appendix B. Chlamydomonas reinhardtii culturing

A semi-continuous stock culture of the algae in *Chlamydomonas* growth medium (CGM) was maintained for feeding. Algae were collected by centrifugation at 3,000 rpm for 10 minutes at room temperature and re-suspended in ddH_2O at a suspension of 7A at 440 nm.

Chlamydomonas growth medium (CGM)

Using a volumetric cylinder, in 5.25 L ddH_2O the following volumes from stock solutions were added under continuous stirring.

Stock	Concentration (g/L)	mL to add
NH_4Cl	20	140
$MgSO_4$ x $7H_2O$	8	70
$CaCl_2$ x $2H_2O$	4	70
K_2HPO_4	8.64	70
KH_2PO_4	8.4	35
EDTA/KOH	50/31	2.85
Acidified iron	4.98	5.7
Boric acid	11.42	5.7
$ZnSO_4$ x $7H_2O$	14.12	3.56
$MnCl_2$ x $4H_2O$	2.33	3.56
$CuSO_4$ x $5H_2O$	2.54	3.56
$Co(NO_3)_2$ x $6H_2O$	0.82	3.56
Na_2MoO_4 x $4H_2O$	1.92	3.56
Acetic acid	100%	5.7

Following, 3 g MOPS buffer and 3.4 g NaOH were added, and the pH was adjusted to 6.7. Media were stored in the cold room to avoid microbial growth.