Universidade de Aveiro
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THE ROLE OF CONTAMINATED PREY ON PLANARIANS' BEHAVIOUR AND PHYSIOLOGY IN MICROPLASTICS RESEARCH

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Ecotoxicologia e Análise de Risco, realizada sob a orientação científica da Doutora Ana Luísa Patrício Silva, Investigadora Júnior do Departamento de Biologia e Centro de Estudos do Ambiente e do Mar da Universidade de Aveiro, e do Professor Doutor Carlos Alexandre Sarabando Gravato, Professor Auxiliar na Faculdade de Ciências da Universidade de Lisboa.

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Dedico este trabalho aos meus pais por me darem a oportunidade de seguir os meus sonhos sem nunca me pressionarem.

o júri

presidente	Professor Doutor Carlos Miguel Miguez Barroso Professor Auxiliar com Agregação, Departamento de Biologia, Universidade de Aveiro
Vogal - Arguente	Professora Doutora Vanessa Bezerra de Menezes Oliveira Professora Auxiliar, Universidade Federal de Tocantins
Vogal – Orientadora	Doutora Ana Luísa Patrício Silva Investigadora Júnior, CESAM & Departamento de Biologia, Universidade de Aveiro

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palavras-chave

Água doce, invertebrados, presa viva, poliuretano, ecotoxicidade, biomarcadores, parâmetros da história de vida, efeitos sub-letais

resumo

Os microplásticos (1 µm a 1 mm) tornaram-se um tópico preocupante na comunidade científica quando associados a uma má gestão de resíduos plásticos. Embora a presença e abundância destas partículas em ambientes de água doce tenha sido previamente reportada, ainda há um número insuficiente de estudos que fornecem informações sobre a potencial transferência trófica e as suas consequências nos organismos bentónicos de água doce. Este trabalho visou avaliar as respostas comportamentais e fisiológicas da planaria *Girardia tigrina* após o consumo de presas contaminadas - *Chironomus riparius* 4th instar larvae previamente expostas a microplásticos de Poliuretano (PU-MPs).

Para este efeito, foram efetuadas duas investigações. Na primeira investigação, foram avaliados os efeitos dos PU-MPs (concentrações até 750 mg/Kg) sobre os parâmetros da história de vida de *C. riparius* (tamanho larvar, emergência); e, quando expostos a uma concentração sem efeitos observados (NOEC), comportamento larvar (locomoção) e respostas bioquímicas (danos oxidativos, produção de energia aeróbica, e reservas energéticas). A exposição a PU-MPs não afetou o tamanho das larvas nem a emergência dos adultos. Não obstante, as larvas contaminadas revelaram um metabolismo aeróbio mais elevado sublinhado por uma maior atividade de locomoção (N.º de contrações corporais) mas sem comprometer as reservas energéticas.

Na segunda experiência, foram avaliados os efeitos do consumo de presas vivas contaminadas (expostas a PU-MP NOEC) num ensaio de alimentação de 3h sobre o comportamento das planárias (nº de presas consumidas, locomoção), regeneração, e fisiologia (reservas energéticas, metabolismo aeróbio, danos oxidativos). Após o ensaio de alimentação, as planárias consumiram mais 20% de presas contaminadas, sem alterações na sua locomoção. O consumo de presas contaminadas atrasou a regeneração das aurículas num dia, sem resultar em danos oxidativos, alteração do metabolismo aeróbico ou das reservas energéticas.

Estas investigações trazem novas informações sobre o papel das presas contaminadas no comportamento e fisiologia das planárias na investigação de microplásticos.

keywords

Freshwater, invertebrates, live prey, polyurethane, ecotoxicity, biomarkers, life-history endpoints, sub-lethal effects

abstract

Microplastics (1 µm to 1 mm) have become a concerning topic in the scientific community when associated with poor plastic waste management. Although the presence and abundance of these particles in freshwater environments have been previously reported, there is still an insufficient number of studies providing information about the potential trophic transfer and its consequences on freshwater benthic organisms. This work aimed to evaluate the behavioural and physiological responses of planarian *Girardia tigrina* after consuming contaminated prey - *Chironomus riparius* 4th instar larvae previously exposed to microplastics of Polyurethane (PU-MPs).

For this purpose, two investigations were carried out. In the first investigation, it was assessed the effects of PU-MPs (concentrations up to 750 mg/Kg) on *C. riparius* life history traits (larval size, emergence); and, when exposed to a no-observed-effect-concentration (NOEC), larvae behaviour (locomotion) and biochemical responses (oxidative damage, aerobic energy production, and energy reserves). Exposure to PU-MPs did not affect larval size nor imagoes emergence. Notwithstanding, contaminated larvae revealed higher aerobic metabolism underlined by a higher locomotion activity (curling/uncurling) but without compromising energy reserves.

In the second experiment, it was assessed the effects of the consumption of contaminated alive prey (exposed to PU-MP NOEC) in a 3h feeding assay on planarians' behaviour (nr of consumed prey, locomotion), regeneration, and physiology (energy reserves, aerobic metabolism, oxidative damage). After the feeding test, planarians consumed 20% more contaminated prey with no changes in their locomotion. The consumption of contaminated prey delayed auricles' regeneration by one day, without resulting in oxidative damage, altered aerobic metabolism or energy reserves.

These investigations bring new intel on the role of contaminated prey on planarians' behaviour and physiology in microplastic research.

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Outline of the thesis

This thesis is organized into four chapters, as follows:

Chapter I - introduces the problematic that underpin this research and major knowledge gaps. Here it is also highlighted the major objectives of this thesis, test hypotheses, and main tasks. It also provides an explanation and general description of the test species chosen for this research.

Chapters II and III – are related to two experiments performed to address the research questions. Here, a brief context, research objectives, materials and methods, results and discussion are provided in each chapter.

Chapter IV – provides an integrative discussion of the results obtained in chapters II and III, according to the main research gaps highlighted in the introduction and the raised hypotheses. It also discusses future research lines on the topic.

Chapter I

General introduction, main objectives of this thesis, and test species

I.1. Plastics – a double edged-sword to modern societies

I.1.1. Origin, characteristics, and major applications

Plastics are a material of choice in the global economy, essentially due to their durability, flexibility and low cost (Andrady, 2011; Lebreton et al., 2017; Rhodes, 2018), offering sustainable solutions to our fast-changing needs (PlasticsEurope, 2020). Since World War II, its global production has increased dramatically, reaching almost 370 million tons in 2019 (PlasticsEurope, 2020) (Figure 1).

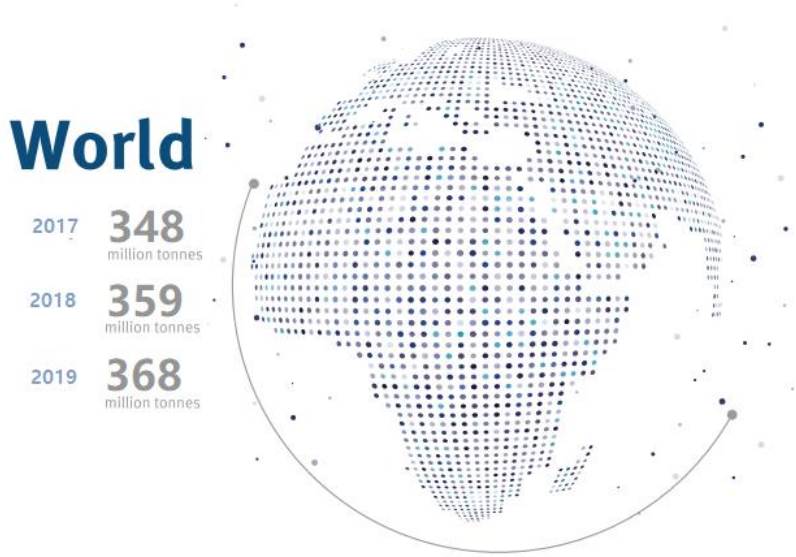

Fig. 1. Global plastic production in the years 2017, 2018 and 2019. Adapted from PlasticsEurope, 2020; PlasticsEurope & EPRO, 2019.

Plastics are defined as organic polymers of synthetic or semi-synthetic nature, typically formed from repeated smaller structural units called monomers (Bråte et al., 2017; GESAMP, 2016; Thompson et al., 2009). These polymers are derived from fossil (e.g. crude oil or natural gas) or natural materials (e.g. cellulose), being the second group theoretically more prone to degrade (Au, 2017; Rhodes, 2018; Rodrigues, 2018; Thompson et al., 2009).

This material can be classified by their i) polymeric constitution, ii) methods of synthesis (e.g. polycondensation), and iii) physical proprieties such as hardness, density or resistance to heat (Rhodes, 2018). During polymerization, several additives can be added to the plastic to optimize its performance and mechanical properties (Jacinto, 2018; Rodrigues, 2018). These additives can be divided into five categories: 1) functional, 2) colourants, 3) fillers, 4) reinforcement, and 5) NIAS (Non-Intentionally Added Substances) (Figure 2). Examples of some additives include several other classes of pollutants, such as flame retardants, stabilizers, plasticizers, and curing agents (UNEP, 2021a).

According to their capacity to be shaped when heated or cooled, plastics can be further divided into two categories: thermoplastics, which have a simpler molecular structure and can be moulded depending on the temperature whenever necessary; and, thermoset plastics, presenting a three-dimensional network arrangement of monomers that undergo irreversible chemical changes (decomposition) when heated (Jacinto, 2018; PlasticsEurope, 2020). Some examples of thermoplastics are polyethylene (PE), polypropylene (PP), polyethylene terephthalate (PET) and polystyrene (PS), which dominate the global demand. Thermosets include polyurethane (PU), epoxy resins, unsaturated polyesters and silicone (PlasticsEurope, 2020).

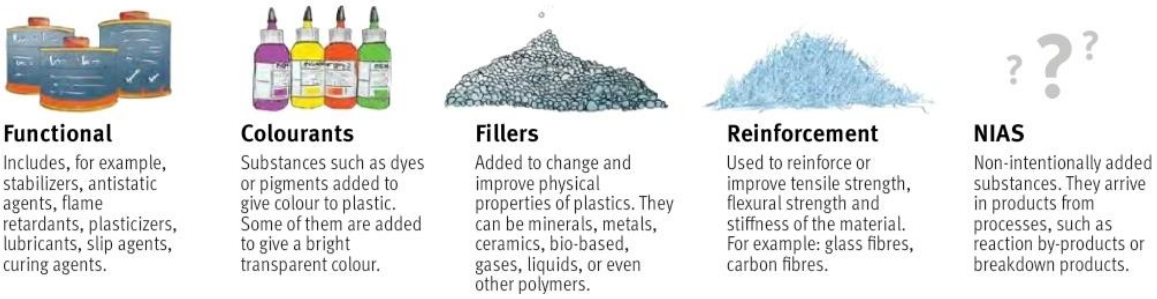

Fig. 2. Types of plastic additives. Taken from UNEP (2021a).

Due to its properties and its current consumption rates, this material is estimated to increase to an immense 33 billion tons of plastic by 2050 (Horton et al., 2017). The largest industrial sector is plastic packaging, designed mostly for single use, especially in business-to-consumer applications (UNEP, 2018). In the European Union, plastics are mainly use (Fig. 3) for i) packaging, to preserve and conserve the food (constituting 39.6 % of plastic demand); ii) building and construction for insulation, plumbing, window frames and decoration because it is easy to clean, has anti-corrosive properties, is fire resistant, among other reasons (constituting in 20.4 % of plastic demand); iii) automotive as a substitute for other materials reducing emissions and fuel consumption (constituting 9.6 % of plastic demand); iv) electrical and electronic applications because it doesn't conduct electricity (constituting 6.2 % of plastic demand); v) household, leisure and sports namely for clothes, shoes, sports equipment and toys (constituting 4.1 % of plastic demand); vi) agriculture in pesticides and fertilizers (constituting 3.4 % of plastic demand); vii) others (constituting 16.7 % of plastic demand) that Include appliances, mechanical engineering, furniture, medical, etc. (Horton et al., 2017; PlasticsEurope, 2020; Trindade, 2019).

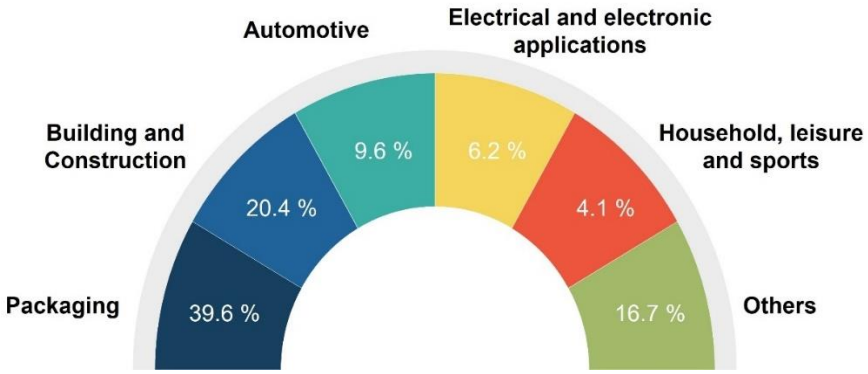

Fig. 3. Plastic demand (%) by sectors. Adapted from PlasticsEurope & EPRO (2019).

I.1.2. Plastics waste production and management

Currently, after being discarded, plastic waste can be recycled, incinerated with or without energy recovery, landfilled, dumped in uncontrolled sites, or littered in the environment (UNEP, 2018). According to Geyer (2017), cumulative waste generation of primary and secondary (recycled) plastic waste produced between 1950 and 2015 amounted to 6300 Mt. Of this amount, approximately 12% (800 Mt) of plastics have been incinerated, and 9% (600 Mt) have been recycled. Only 10% of recycled plastics have been recycled more than once. Thus, around 4900 Mt—60% of all plastics ever produced—were incorrectly discarded and accumulated in landfills or in the natural environment.

Since 2006, the recycling of post-consumer plastic waste has doubled (PlasticsEurope, 2020). This increase could possibly be related to the implementation of several strategies like consumer education and awareness or cleaning campaigns (Trindade, 2019). Notwithstanding, in 2019, the percentage of waste directed to landfills and incineration was still 49% and 19%, respectively. Both of these latter options posed great environmental disadvantages (OECD, 2022). Landfills, when poorly managed, can have items displaced or run off into the environment due to adverse weather conditions like wind and rain (Bråte et al., 2017). Incineration poses environmental and health risks as dioxins, and other toxic pollutants are emitted where combustions plants are poorly regulated (World Economic Forum, 2020).

The most sustainable option is recycling. Plastics that cannot be recycled sustainably can still provide valuable raw material to energy-efficient facilities for the production of electricity, heat or secondary fuel recovery (ANP & WWF, 2019). The current use of plastic must pass through

a better and more circular economy that facilitates the reuse, repair, re-manufacture and recycle plastic (GESAMP, 2016).

I.1.3. Economic, social, and environmental consequences of plastic waste

The high demand and production of plastic, associated with their incorrect disposal, has led to a significant accumulation of this material in natural environments, which is aggravated by its high persistence and low degradability. Plastic pollution was even exacerbated after the outbreak of coronavirus disease (COVID-19), with the intense use of single-use plastics and mandatory use of face masks by common citizens (Benson et al., 2021; Hassan et al., 2022). According to WHO (World Health Organization), 89 million medical masks and 1.6 million protective goggles were needed monthly in order to cope with the pandemic during the first trimester, but these numbers were suppressed significantly in the subsequent year (Anderson et al., 2021; Shen et al., 2021).

Besides plastic face masks, other plastic items were also used, such as gloves, protective aprons, face shields, safety glasses, safety shoes and medical gowns, which mostly contain polypropylene (PP) (Benson et al., 2021; UNEP, 2021a). Adding to the problem, hygiene concerns and the possibility of cross-contamination lead shops and supermarkets to a widespread practice of using single-use materials to wrap vegetables and fruit and to an increase in online shopping, which is impossible without packaging (Anderson et al., 2021; Hassan et al., 2022). Thus, a substantial contribution of single-use plastics (from PPE, packaging) ended up being mismanaged during the pandemic, with a decrease in recycling and an increment in landfill or incineration (Patrício Silva et al., 2020).

Once in open environments, plastic waste experiences processes of degradation, namely photo (e.g. solar UV radiation), thermal, mechanical (e.g. wave action or sand friction), chemical or biological degradation (microbial action), resulting in debris of various sizes (from macro to nano ranges, section 1.4.), densities, forms and colour (Andrady, 2011; Dusaucy et al., 2021; Jacinto, 2018). The accumulation of plastic waste in the most diverse environments has disastrous consequences at economic, social, and environmental levels. In 2020, the economic costs of marine plastic pollution were estimated to be at least 6-19 billion dollars (~ 6.13 to 19.41 billion euros) globally in tourism, fisheries, aquaculture and clean-up activities (UNEP, 2021b). At the same time, accumulation of plastic in the environment can lead to the creation of pools of

stagnant water/liquids that are a selective medium for the reproduction of disease vector insects (zoonosis), thus constituting a threat to animal and human health (Krystosik et al., 2020).

In natural ecosystems, plastic waste (including PPE) also poses threats to wildlife through voluntary or involuntary ingestion and entanglement, among other reasons, causing physical damage to various organs and tissues (Bråte et al., 2017). It is difficult to determine and quantify the causal links between mortality and ingestion of large plastic fragments; however, there are numerous studies that prove their existence in the guts and tissues of a wide range of species at all stages of their life cycle (UNEP, 2021b).

Plastic waste can also function as a host for a range of species, which potentially can lead to an increase in species' geographical ranges and consequentially spread invasive species and diseases (UNEP, 2021a). Besides, the accumulation of debris in marine sediments can also interfere with gas exchange between surface waters and sediment pores, leading to hypoxia or anoxia of benthic organisms (Derraik, 2002). In addition, plastic pieces can smother or cover habitats such as mangroves, mudflats and coral reefs, preventing oxygen and nutrient flow and blocking light (UNEP, 2021a). Thus, aquatic ecosystems (which provide numerous services (e.g. direct supply of food and resources, nutrient recycling, carbon storage, recreational opportunities, etc.)) are being seriously threatened by this pollution and potentially compromise and affect global well-being (ANP & WWF, 2019).

I.1.4. Plastic debris in the environment: source, fate, and implications

Currently, the designation used to describe plastic debris varies between macro, micro and nanoplastics, depending essentially on their size and the studies that carry them out. According to the latest definition from Hartmann et al. (2019), macroplastics are debris of dimensions larger than >1 cm in size, mesoplastics are between 1 mm and 1 cm in size, microplastics are between 1 µm to 1 mm, and nanoplastics are smaller than 1µm in size (Figure 4).

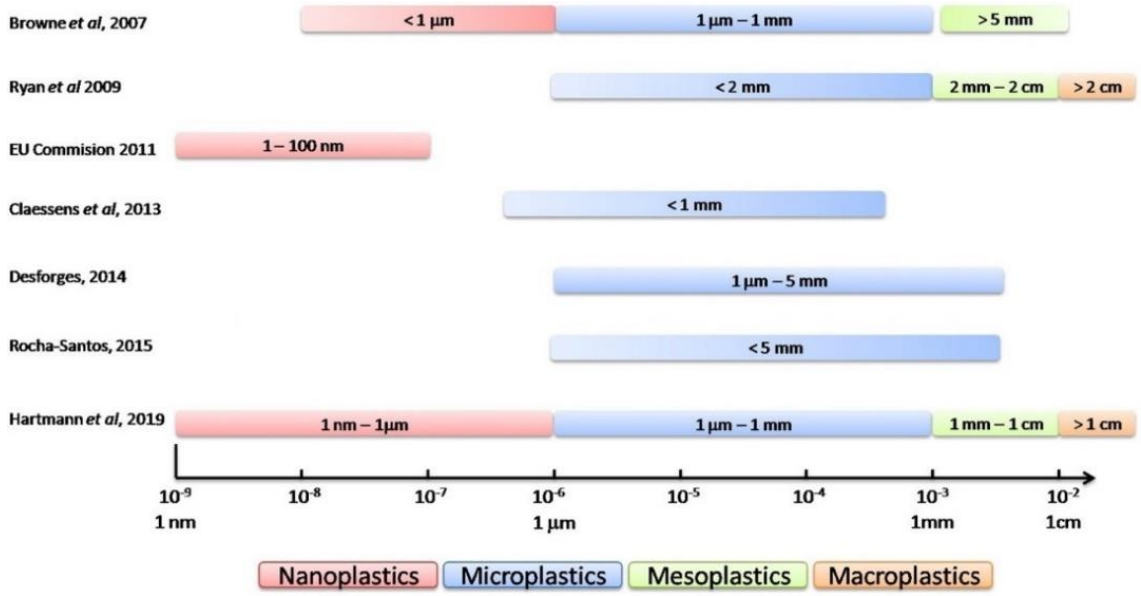

Fig. 4. Denomination of plastic debris according to its size, based on various studies. Adapted from da Costa et al. (2016).

Among plastic debris, microplastics (MPs) have received special attention from the media and the scientific community due to their ubiquity and unknown impacts in the most diverse natural environments. These plastic particles have dimensions between 1 µm and 1 mm (according to the consensus presented by Frias & Nash (2019); and may be of primary or secondary origin. Primary MPs are released into the environment in their small size, either because they were produced on a microscale (such as microspheres included in cleaning products and cosmetics) or resultant of abrasion phenomena (such as microfibers released from clothes during washing or dust resulting from car tires or from processing plastic materials and moulding). Secondary MPs result from the fragmentation and disaggregation of meso- and macroplastics by physical-chemical and biochemical processes, and this fragmentation occurs with greater speed in beach debris than in floating debris (Andrady, 2011; Eerkes-Medrano et al., 2015; Trindade, 2019). According to their shape and colour, MPs can be considered i) filaments/fibres, which are thin or fibrous, straight plastic; ii) films that are thin plane fragile plastic; iii) fragments that can be rounded, angular, hard and/or irregular plastic particle; iv) pellets that can be cylinders, disks, spherules opaque and/or transparent, hard plastic particle); or v) foam as lightweight, sponge-like plastic (Rodrigues, 2018).

Due to their small size, these do end up in aquatic ecosystems through a wide variety of land and marine sources (Fig. 5). Land sources include various sectors such as the plastics manufacturing and processing industry, agriculture (e.g. plates for greenhouses, irrigation pipes),

construction (e.g. abrasive jets for cleaning surfaces, insulating foam), transport (e.g. tires) or tourism (e.g. packaging) and marine sources rely on sectors such as fishing/aquaculture (e.g. nets, buoys, traps) and the naval and offshore industry (e.g. cargo waste, sewage) (GESAMP, 2016). In addition to these sources, MPs can also reach these environments through domestic sewage since the Wastewater Treatment Plants – WWTPs do not effectively remove MPs from the final effluents that are discharged to the receiving body (Raju et al., 2018).

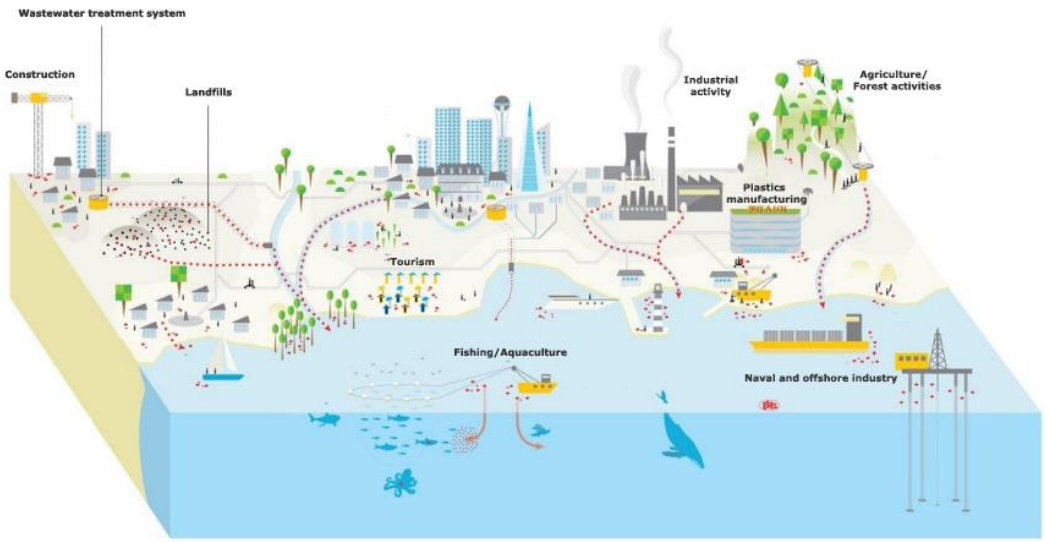

Fig. 5. Sources of microplastic pollution. Adapted from https://www.grida.no/resources/6922.

The physical and chemical properties of these debris are the main factors that determine MPs' fate in aquatic environments. For example, debris that are neutrally or positively buoyant (i.e., not very dense) tend to stay in the water column or on its surface, respectively; while those with negative buoyancy (denser) end up settling in the sediment (Horton et al., 2017). However, depending on the persistence (normally dependent on the polymeric chain) of these debris in the aquatic environment, fragments with neutral or positive buoyancy can suffer aggregation with other particles that are in suspension or colonization by algae or microorganisms (biofouling), which will increase their density and contribute to their deposition (Au, 2017). Also, particles on the surface will eventually sink due to thermohaline circulation (Jacinto, 2018). These are the main reasons why sediments can have considerably higher concentrations than the overlaying water (Sagawa et al., 2018; Scherer, Weber, et al., 2020). Furthermore, MPs can continue to undergo degradation that is classified according to the agent that causes them, namely mechanical degradation due to, for example, the action of waves or sand friction, biodegradation

by the action of living beings, photo-degradation by exposure to UV radiation, photo-oxidative degradation by exposure to moderate temperatures, and hydrolysis by the action of water. This deterioration causes a significant decrease in the molecular weight of the polymer and its destination in these environments (Andrady, 2011).

All these events (i.e., fragmentation/degradation, aggregation, biofouling, sedimentation, biological activity) occur at different speeds and times, creating phenomena of sinking and resuspension of MPs along the water column and sediments, allowing them to interact with pelagic organisms (inhabit the water column) but especially with benthic organisms (inhabitants of the bottom of the aquatic environment).

I.1.5. Politics and actions regarding the plastic debris

Considering their small size, MPs do not find barriers or borders, having been found even in remote environments (several lakes located in the Tibetan plateau, China; Xiong et al., 2018; Zhang et al., 2016). Several laws have been targeting single-use plastics and microbeads. For example, in Portugal, the Decree-Law No. 152-D/2017 of 11 December 2017 and Law No. 76/2019 of 2 September 2019 approved some measures to be implemented regarding the obligation of non-use of single-use plastics in activities concerning food, beverage and retail market, required suppliers to proceed to the respective adaptation within a year (i.e., until 3 September 2020). This deadline was, however, extended (until 1 July 2021) due to the COVID-19 pandemic by two Decree–Laws (No. 10-A/2020 and 22-A/2021) of 13 March 2021.

In addition, although not aiming specifically at MPs, water directives have been acting, such as the Water Framework Directive of the European Union (Directive 2000/60/EC of the European Parliament and of the Council, of 23 October 2000) and the Marine Strategy Framework Directive (Directive 2008/56/EC of the European Parliament and of the Council of 17 June 2008). Yet, in August 2022, the European Commission drafted a report aiming at amending Annex XVII to Regulation No 1907/2006 of the European Parliament and the Council concerning the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) regarding such synthetic polymer microparticles (Comitology Register, 2022); urging research on their potential behaviour, fate and effect on natural environments and biota for a proper risk assessment.

Concomitantly, the EU has increasingly tried to act preventively. To help European companies and consumers use plastics in a more sustainable way, the first European Strategy for

Plastics in a Circular Economy was adopted in 2018, which aims to improve the design of plastic products, introduce greener (biobased) alternatives, and increase recycling rates as well as their quality (European Commission, 2020). In addition to these legislations, more and more organizations are emerging with the aim of protecting systems from this material, and because of that, in 2011, The Declaration for the global Plastics Associations for Solutions on Marine Litter was created, which brings together 75 organizations and associations in 40 countries committed to acting and making measurable progress. In line with the EU Plastics Strategy, the European Commission has also requested the European Chemical Agency (ECHA) to prepare a dossier for restricting the inclusion of MPs (e.g., microbeads) in certain products (European Commission, 2020).

I.2. Freshwaters: a vital environment facing plastic pollution

Freshwater ecosystems provide important direct and indirect contributions to human well-being (aka ecosystem services), such as the provision of purified water, food source, nutrient mobilization, flood alleviation, and the opportunity for recreational activities (Maltby et al., 2022). However, freshwater biodiversity, particularly of macroinvertebrates that underpins the delivery of many of those ecosystem services, is under an additional threat caused by plastic pollution. In fact, freshwaters are among the major collectors and pathways of plastic debris to the sea. Here, plastic debris can reach the order of gram per litre in surface waters (e.g., 2.6 g/L in Australia, < 2mm, Kowalczyk et al. 2017) or thousands of particles per kilogram in sediments (e.g., 32947 items/kg dry sediment in a river network in eastern China, Wang et al., 2018) (more examples in C. Li et al. (2020)). In Portugal, the few studies on surface water reported levels of 17.06 MPs/100 m^3 in the Douro estuary for particles of higher size (Rodrigues et al. (2019) and 1.000 MP/m^3 in Vouga River and 18.000 MP/m^3 in Aveiro Lagoon, when including particles down to 1 um in size (Prata et al. (2020). Considering sediments, MPs levels were 18-629 particles per kg, in Antuã River, for particles of larger size and 133.3 particles per m^2 along the Portuguese coast (Martins & Sobral, 2011; Rodrigues et al., 2018).

The presence of MPs has also been reported in freshwater organisms. For example, Windsor et al. (2019) detected MPs in concentrations up to 0.14 MP/mg tissue in riverine organisms (Baetidae, Heptageniidae and Hydropsychidae) collected upstream and downstream of five UK Wastewater Treatment Works. MPs have also been detected in bivalves such as mussels (0.54-3.0 MPs/g) and clams (10.4-18.4 MPs/g), fish (1.67 ± 0.27 MPs/fish), and in sediment-dwelling organisms such as dipteran larva (0-5.04 particles/mg) and annelids (56-2543

particles/kg) (Bessa et al., 2018; Cozzolino et al., 2021; Hurley et al., 2017; Marques et al., 2021; Nel et al., 2018).

The bioavailability of MPs uptake by invertebrates and vertebrates depends on particles abundance and size and the physiological and behavioural traits of the organism(e.g., feeding guilds) (Bellasi et al., 2020; Horton et al., 2017). The abundance of MPs increases the chance an organism will encounter MP particles, and smaller sizes make them more accessible to lower trophic organisms (Wright et al., 2013). Trophic roles also influence the MP burden. For instance, detritivores and predators may indirectly/involuntarily ingest/uptake MPs when consuming prey or scavenging detrital matter, respectively.

I.2.1. Microplastic toxicity in freshwater organisms

Ecotoxicology is a multidisciplinary science that studies the harmful effects of contaminants on ecosystems and their constituents, thus making it an ideal tool for studying the effects of MPs (Newman, 2014; C. H. Walker et al., 2012). Ecotoxicity of MPs have been addressed during the last three decades, but mostly on marine biota. Only in the last decade the number of studies addressing the MPs' ecotoxicological effects on freshwater biota have increased considerably, and their scientific conclusions regarding effects are similar to what has been described for marine biota.

The algae *Lemna minor* showed alterations in root growth and reduction of root cells viability after exposure to polyethylene (PE) microbeads (PE-MPs; 7 days; 4- 12 μm; 0, 10, 50, and 100 mg/L; Kalčíková et al. (2017)). Other ecotoxicological effects of exposure to polystyrene (PS-MPs) were observed. Li et al. (2020) observed a decrease in density in microalgae *Chlamydomonas reinhardtii*, along with a reduction in both chlorophyll *a* fluorescence yields and photosynthetic activities (PS-MPs; 10 days; 300- 600 nm; 5, 25, 50 and 100 mg/L). At a later stage, PS-MPs also inhibited microalgae settlement due to hetero aggregation with the MPs that inhibited extracellular polymeric substances secretion.

Considering macroinvertebrates, major effects involved behavioural distress, feeding and growth impairment, alterations in antioxidant and detoxification capacities, neurotoxicity, energy allocation, among others. For example, in studies with *Daphnia magna*, polyethylene terephthalate microfibers (PET-MPs) have been shown to be responsible for increasing mortality (PET-MPs; 48h; 62 μm - 1400 μm 12.5- 100 mg/L; Jemec et al. (2016)) while polyethylene particles showed an increase in organisms immobilization (PE-MPs; 96 h; 1- 4 μm and 90- 106 μm; 12.5

mg/L - 400 mg/L; Rehse et al. (2016)). Other studies evidenced changes to the morphology, reproduction and feeding rates of the cnidarian *Hydra attenuata* (PE-MPs; 30 and 60 min; <400 μm; 0.01, 0.02, 0.04, 0.08 g/mL; Murphy & Quinn (2018)) and increased enzymatic activity in the *Corbicula javanicus* when exposed to polyester (PET-MPs; 24h; > 0.45 μm; 8.1×10^4 fibres/L), although the opposite happened when exposed to high-density polyethylene (HDPE) fragments (HDPE-MPs; 24h; 5 mm to 1 μm; 0.01, 0.1 and 1.0 mg/L; Esterhuizen et al. (2022)). Decreased filtration rate was also observed in *Dreissena bugensis* at higher concentrations of high-density polyethylene powder (HDPE-MPs; 24h; 10 - 45 μm; 0.0 to 0.8 g/L; Pedersen et al. (2020)). Blarer & Burkhardt-Holm (2016) exposed *Gammarus fossarum* to polyamide (PA) fibres that significantly affected assimilation efficiency by inhibiting food assimilation (PA; 28 days; 500 × 20 μm; 100 - 13,380 fibres/cm^2).

Exposure to MPs also affected organisms with holometabolism (aka, complete metamorphosis, which includes four life stages: egg, larva, pupa, and imago or adult as observed in chironomids). For example, the chironomid *Chironomus riparius* revealed high sensitivity to the presence of MPs. When exposed to polyethylene MPs (PE-MPs; 48 h; 32−500 μm; 1.25, 5 and 20 g/kg) *C. riparius*'s larvae presented high amounts of particles in their guts that induced immune responses, oxidative damage and reduced aerobic energy production (Silva et al., 2021a; 2021b). Using the same concentrations of PE-MPs, but extending the period of exposure (10 days), Silva et al. (2019) reported a significant reduction in larval growth and a significant delay in imagoes emergence, which could impair its life-history traits. In accordance, Scherer et al. (2020) also detected a reduction in *C. riparius* emergence as well as a reduction in chironomids weight after exposure to polyvinyl chloride (PVC-MPs; 28 d; < 50 μm). Ziajahromi et al. (2018) exposed *C. tepperi* to polyethylene MPs (PE-MPs; 5 and 10 days; 1- 126 μm; 500 MPs/kg), and observed adverse effects on survival, growth (i.e. body length and head capsule) and emergence.

While using another polymer, polyethylene terephthalate (PET-MPs; 28 days; 14 μm; 500, 5000 and 50000 particles/kg), Setyorini et al. (2021) did not detect a significant effect on the time until emergence, weight or head capsule lengths in exposed organisms compared to control organisms. Carrasco-Navarro et al. (2021) studied gene expression in the insect and were able to detect alterations that correlate to cellular stress after exposure to polystyrene (PS-MPs) and tire rubber MPs (PS-MPs and microrubber; 36 h; 1 and 10 mg/L). Table 1 summarizes the key findings of studies aiming at addressing the ecotoxicity of MPs on freshwater biota.

Table 1. Summary of the impact of microplastics on freshwater organisms.

SPECIES	POLYMER	EFECTS	REFERENCES
Lemna minor	Polyethylene microbeads	Affected root growth Reduced the viability of root cells	(Kalčíková et al., 2017)
Chlamydomonas reinhardtii	Polystyrene	Decreased density Reduced chlorophyll *a* fluorescence yields and photosynthetic activities Inhibited settlement	(Li et al., 2020)
Daphnia magna	Polyethylene terephthalate textile microfibers	Increased mortality after 48 h	(Jemec et al., 2016)
	Polyethylene particles	Increased immobilisation	(Rehse et al., 2016)
Hydra attenuata	Polyethylene flakes	Changes to the morphology, reproduction and feeding rates	(Murphy & Quinn, 2018)
Corbicula javanicus	Polyester fibres	Increased enzyme activities	(Esterhuizen et al., 2022)
	High-density polyethylene microplastic fragments	Decreased enzyme activities	
Dreissena bugensis	High-density polyethylene powder	Decreased filtration rate	(Pedersen et al., 2020)
Gammarus fossarum	Polyamide fibres	Reduced assimilation efficiency	(Blarer & Burkhardt-Holm, 2016)
Chironomus riparius	Polyethylene	Induced oxidative damage and reduced aerobic energy production	(Silva et al. 2019)
		Reduced larval growth and delayed imagoes emergence	(Silva et al. 2021)
	Polyethylene terephthalate	Emergence, weight or head capsule lengths were not affected	(Setyorini et al. 2021)
	Polystyrene and tire rubber	Caused cellular stres Some alterations in the normal gene expression	(Carrasco-Navarro et al. 2021)
	Polyvinyl chloride	Reduced the emergence and weight	(Scherer et al., 2020)
Chironomus tepperi	Polyethylene	Affected survival, growth and emergence	(Ziajahromi et al. 2018)

A group of organisms that has been poorly investigated are the macroinvertebrate predators, such as planarians. These organisms occur in large numbers in lakes, streams, springs, and groundwaters, being a significant component of freshwater communities, particularly when top-down controlling prey populations (Barzaghi et al. (2021). Previous studies on the planarians, specifically *Dugesia japonica,* reported the uptake of PE-MPs (30 min; <10 µm in diameter) and PET-MPs (30 min; 14 µm diameter) in concentrations from 12 to 60 µg/mg in contaminated food (liver), without altering their feeding activity, food intake, and regenerative ability (Gambino et al., 2020). Notwithstanding, it induced a significant reduction of the gut epithelium thickness and lipid content of enterocytes, along with the induction of apoptotic cell death and reduced growth rate. In another study, such species revealed a decrease in the body and blastema areas upon exposure to polystyrene microspheres (PS-MPs; 21d; 0.1-1-10 µm in size in concentrations of 10, 50, or 100 µg/mg liver food), indicating a delay in their growth and regeneration (Gao et al., 2022). Concomitantly, stem cell proliferation and differentiation processes were inhibited, and the proportion of mitotic stem cells decreased (Gao et al., 2022). More recently, PS-MPs of ~1 µm (PS-MPs; 7d; 10 µg/L) in size seemed to induce oxidative stress on *D. japonica*, by significantly changing the levels of antioxidant enzymes, including superoxide dismutase, catalase, and glutathione S-transferase (Han et al., 2022).

Although these previous works provided important intel on the potential physiological and cellular effects on MPs in planarians, they are missing behavioural setups of environmental relevance. In natural environments, planarians display a preference for live prey that produce

disturbance in the water, such as chironomids with their curling swimming behaviour (as reviewed by Deochand et al. (2018)). When exposed to contaminants, these organisms often alter their energy allocation, suppress locomotor activity, and even compromise their regenerative ability (Knakievicz, 2014; López et al., 2021; Yuan et al., 2014). After consuming food, locomotion and responsivity to stimuli are often reduced; and if stressed, planarians can expel previously consumed food to enable an efficient evasion (Deochand et al., 2018).

Knowledge of the potential effects of MPs via contaminated prey remained (so far) a considerable knowledge gap. In addition, the potential trophic transfer of these particles along food chains, although expected, remains little explored. Concomitantly, the few studies carried out in this area show the trophic transfer of plastic debris between plankton of two different trophic levels (Setälä et al., 2014), between mussels and crabs (Farrell & Nelson, 2013; Watts et al., 2014), between *artemia* sp. and zebrafish (Batel et al., 2016) or between fish and captive grey seals (Nelms et al., 2018). Knowledge on the uptake and consequent transfer of MPs to organisms that rely on extracorporeal (followed by intracellular) digestion, as planarians, remains poorly addressed.

I.2.2. Biochemical biomarkers as early warnings of microplastics ecotoxicity

When considering concentrations of environmental relevance, most of the effects induced by the presence/potential uptake of MPs on biota have been demonstrated from the biochemical to physiological and behavioural levels. Most information comes from a range of biomarkers at lower levels of biological organisation, such as oxidative stress (e.g., lipid peroxidation, DNA damage, activation of antioxidant and detoxification enzymes) and immunological responses (e.g., signs of inflammation, and enzyme/protein response) (e.g., as reviewed by de Sá et al., 2018).

Biomarker research, which was initially applied in a medical context, evolved rapidly; and, since 1980s has been actively implemented in biology fields (Damásio, 2010; Ramakrishnan et al., 2017). In stress biology/ecotoxicology, such analysis became essential to better understand the influence of stressors or contaminant and their role in organism's biological processes. A biological marker (biomarker) is any, objectively measurable, biological alteration that can be indicative of biological responses (biochemical, cellular, physiological or behavioural changes) in organisms exposed to a stressor (Amiard-Triquet et al., 2012). This tool has generated a large

number of promising results, and it's considered an "early warning" regarding pollution-induced stress (Van Gestel & Van Brummelen, 1996).

There are numerous advantages to the use of biomarkers. These analyses offer great opportunities for early detection since they are short-term indicators of long-term adverse effects that may permit intervention before the effects become inevitable (Damásio, 2010; Hernández-Moreno et al., 2011). Additionally, biomarkers use standardized practices and come with the possibility of establishing a dose-response relationship between the toxic agent and the biomarker, which can be applied to several species (Travasso, 2011). Also, they can help understand the toxic action and molecular targets of pollutants within the organism (Damásio, 2010; Monteiro, 2019).

Several stressors, including MPs, induce an imbalance between the production and accumulation of reactive oxygen species (ROS) in cells and tissues and can lead to significant oxidative damage, possibly leading to enzyme inactivation, protein damage, DNA damage, lipid peroxidation and ultimately, cell death (Amiard-Triquet et al., 2012). These reduction products of molecular oxygen (O_2) include the superoxide anion radical (O_2^{-} •), hydrogen peroxide (H_2O_2) and the hydroxyl radical (•OH) (Damásio, 2010). ROS are normally produced by mitochondria and can play several physiological roles like oxidizing proteins, DNA and lipids of biological membranes. If the antioxidation mechanisms fail to eliminate the excess ROS production, oxidative stress can result in increased lipid peroxidation (LPO) and/or DNA damage (Amiard-Triquet et al., 2012).

Lipid peroxidation (indicative of oxidative damage) describes the process of mediating damage to cellular structures such as lipids containing carbon-carbon double bonds, namely polyunsaturated fatty acids (PUFAs) of phospholipids, which are extremely sensitive to oxidation (Ayala et al., 2014; Valko et al., 2006). These effects can lead to long-term consequences, including delayed development, growth impairment, reduced reproductive output and behavioural changes (e.g., Monteiro, 2019). An increase in lipid peroxidation levels was previously observed in *Chironomus riparius* under exposure to PE-MPs (Silva et al., 2020). Such oxidative damage was potentially triggered by the high retention time of PE-MPs uptake or obstruction of their gut (with consequent inflammation that leads to ROS increment in cells)(Silva et al., 2021b).

Concomitantly, and since biotransformation and some antioxidant defences require energy, another common approach is the estimation of the energy-related parameters of the organisms by measuring the electron transport system activity (ETS) (Smolders et al., 2004). Energy demand can also be increased due to stress resulting in a depletion of carbohydrates and

lipid reserves (Sokolova, 2013). The measurement of energy impairment is important since energy balance parameters can be extrapolated to predict effects on the growth and development of organisms (Sokolova, 2013). Alterations on energy acquisition (in terms of reserves) and expenditure were recently reported in *C. riparius* and *Lumbriculus variegatus* after a short-term exposure to LDPE-MPs, potentially related to the false sensation caused by MPs that accumulated in organisms GUT, activation of immune responses and/or activation of antioxidant/detoxification mechanisms (Silva et al., 2021a; Silva et al., 2021c).

I.2.3. Current knowledge gaps on microplastics research in freshwaters

Although the number of studies on the effects of MPs on freshwater organisms has grown substantially in the last 5 years (from ~301 to 2734 at the end of 2021; search for documents on SCOPUS.com; keyword "microplastics"), the truth is that there are still several knowledge gaps that must be addressed with high priority to obtain crucial information for the ecological risk assessment of MPs in freshwater environments. For example:

I. Increase the number of studies of MP effects considering environmentally relevant scenarios, including the natural nutritional needs and organisms' behaviour. For example, the only available (few) ecotoxicity tests do not consider the role of natural prey.

II. The studies carried out so far focus predominantly on PE, PP and polyester. Among the various types of MPs, polyurethane (PU) (included in paints for boats, cars, and houses) is one of the least studied. Esterhuizen & Kim (2022) tested the effects of six MPs, including PU-MPs, on lotus plants (*Nelumbo nucifera*), and plants revealed the greatest growth inhibition and lowest germination percentage in sediments contaminated with PU-MPs comparably to PVC, PP, PS, HDPE and PET . Zimmermann et al. (2019) also reported that extracts from PVC and PU were the most toxic, compared to PET and HDPE-MPs, when baseline toxicity, induction of oxidative stress, and endocrine activity were tested in bacterium *Aliivibrio fischeri*.

III. The main parameters evaluated in ecotoxicity studies include ingestion, survival, reproduction, and cellular stress. The potential trophic transfer and its effects on predator behaviour remaining unclear.

IV. The few studies developed so far that show evidence of trophic transfer focus on predators that ingest the prey fully or partially and present intracorporeal and extracellular digestion; there is no substantial information on predatory organisms that present extracorporeal and intracellular digestion, such as planarians.

I.3. Research objectives of this thesis

Considering the knowledge gaps mentioned in the previous section, this study aimed at addressing the behavioural, physiological, and biochemical responses of the planarian (as a predator) to MPs via the consumption of contaminated alive preys. The use of contaminated alive preys becomes more environmentally relevant, since it can trigger other processes in the predator's organism, such as chemical cues detection between predator and preys (Pijanowska & Kowalczewski, 1997) that can influence the amount of food that is effectively consumed by planarians and the amount of MPs that it will incorporate. It is hypothesized that, when consuming preys contaminated with small-sized MPs, planarians might alter their behaviour, aerobic metabolism, energy reserves, and regenerative ability.

For this study, the planarian *Girardia tigrina* was selected as test species, and the chironomid *Chironomus riparius* was selected as prey (explanations and brief description provided in the next section). The MP selected for this study was polyurethane (PU), due to the scarcity of studies involving this polymer despite its intense use in various types of coatings and paints as well as in foams for padding and insulation, that can easily find its way to aquatic environments like freshwater ecosystems.

To successfully achieve this goal, two bioassays were carried out:

1) A chronic exposure (28 days) to evaluate the effects of PU-MPs on *Chironomus riparius* larvae growth and imagoes emergence. From the chronic exposure, one concentration of PU-MP was selected to expose prey for the next experiment (Chapter III). At this concentration, larval behavioural (locomotion), potential PU-MP uptake and biochemical (oxidative damage, aerobic metabolism, and cellular energy reserves) responses were assessed (**Chapter II**).

2) A feeding assay (3 h) with *G. tigrina*, using contaminated/uncontaminated alive prey from the previous experiment (Chapter II), was performed to evaluate the effects of contaminated prey on planarians behavioural (number of consumed preys, locomotion), physiological (regeneration), and biochemical (oxidative damage, aerobic metabolism, and cellular energy reserves) responses, along with the potential PU-MP incorporation through prey consumption (**Chapter III**).

An integrative discussion of all the results and future research lines are proposed at the end of this thesis (**Chapter IV**).

I.4. Test species

I.4.1. Dipteran *Chironomus riparius*

Chironomus riparius belongs to the dipteran family Chironomidae (Table 2), which is the most distributed and frequently most abundant group of insects in freshwater environments, with representatives in terrestrial and also marine environments (Armitage et al., 1995; Pinder, 1986). They play an important role in detritus processing, trophic cycles (being preferred prey of planarians, fish, among others) and interactions in still waters (ponds, lakes) and in flowing waters (rivers, streams, torrents) (Armitage et al., 1995).

Table 2. Taxonomic characterization of the species used in this study as prey: *C. riparius.*

Taxonomic Characterization	
Kingdom	Animalia
Phylum	Arthropoda
Class	Insecta
Order	Diptera
Family	Chironomidae
Genus	Chironomus
Species	*C. riparius*

They are insects with holometabolic metamorphosis, developing from an egg, passing through four larval stages, a pupal stage and finally emerging as an adult individual (I. R. Walker, 2001). The eggs are placed in a protective gelatinous matrix that can contain between ten and several thousand eggs, depending on the species, thus forming the so-called egg masses (Vallenduuk & Moller Pillot, 2007). In general, each female produces only one egg mass (Oliver, 1971; Vallenduuk & Moller Pillot, 2007). Their shape varies greatly, and the eggs may be positioned peripherally- or randomly throughout the matrix (Oliver, 1971). The duration of this phase is usually brief but is temperature dependent and related to the overall length of the life cycle (Oliver, 1971; I. R. Walker, 2001).

Chironomus riparius, as all species of Chironomidae, has four larval stages. In the first stage (2 to 8 days), the larvae leave the egg mass and lead a typically planktonic existence, swimming using energy obtained from the consumption of algae and debris suspended in the water column as well as food derived from the remains of the egg (Oliver, 1971). At this stage, the larvae are more mobile than those found in higher stages and probably positively phototactic during the period of dispersal and habitat search (Vallenduuk & Moller Pillot, 2007). These eventually settle in the sediment and break free from a thin, chitinous cuticle that envelops the thorax and abdomen, moving on to the second larval stage, in which they already exhibit almost all the morphological and behavioural characteristics specific to the species (I. R. Walker, 2001). The second, third and fourth stages are not as studied, but growth and ecdysis (exoskeletal shedding) are known to occur and are typically benthic (I. R. Walker, 2001).

In the sediment, the larvae build a structure on or within the substrate using particles from the substrate and threads secreted by the salivary glands (Oliver, 1971). Larvae have hemoglobin characterized by having a “high affinity” in terms of oxygen concentration in the environment; that is, they tolerate poorly oxygenated conditions. This occurs through the saturation of the hemoglobin using the undulation of their bodies within the substrate structures that allow water to float on the surface of the substrate (Armitage et al., 1995). *Chironomus riparius*, specifically, acts as a deposit feeder, feeding on organic matter that exists on the bottom or that is sedimented using a non-selective feeding behaviour (Silva et al., 2019). This is the most common feeding method occurring at least at some point in larval development (Armitage et al., 1995). However, other Chironomidae can be 1) suspension feeders, 2) crushers, 3) scrapers, or 4) miners (Armitage et al., 1995).

During the second, third or fourth larval stages, *C. riparius* (as many other species of Chironomidae) have the ability to perform diapause that allows the larvae to survive in summer or winter so that their active period occurs in a favourable season (Vallenduuk & Moller Pillot, 2007).

Once the larvae have a clearly thickened thorax and abdomen, the individual is called a prepupa (Vallenduuk & Moller Pillot, 2007). Compared to the larval stage, the pupal stage is very short, lasting from a few hours to a few days (Armitage et al., 1995; Oliver, 1971). When it is mature, it receives a stimulus that causes it to rise to the surface of the water, where it hatches. This ascent is aided by the air accumulated inside the pupa and is marked by a series of difficulties due to possible predation by fish and birds (Oliver, 1971; Vallenduuk & Moller Pillot, 2007).

The emergence of the adult individual in aquatic environments occurs between 10 to 30 seconds, having immediately the ability to fly that allows them to find the place for mating, egg maturation and oviposition (Oliver, 1971). These organisms show sexual dimorphism (males are physiologically distinct from females) and semelparity, that is, females only reproduce once. Males emerge first (protandrous species) and form swarms with prominent antennae and are attracted to females that enter this swarm (Armitage et al., 1995; Vallenduuk & Moller Pillot, 2007). It is normal for the swarm not to move too far from the emergence site for the females to find them (Vallenduuk & Moller Pillot, 2007). The duration of this phase is ephemeral, lasting in maximum a few weeks (Oliver, 1971).

Species of the Chironomidae family, particularly *C. riparius*, are frequently used in ecotoxicological studies due to their diversity, high density, short life cycles and their tolerance to adverse conditions (Armitage et al., 1995; Pinder, 1986). These abundant midges also have a world-wide distribution, short-life cycle, easy culture under laboratory conditions. Under laboratory conditions, *Chironomus riparius* has a larval stage of 10 to 14 days, followed by 1-2 days for the pupal stage and 2-3 days for the adult stage. This species also has the particularity that its egg masses are always floating until the larvae hatch (Figure 6). In acute tests, the fourth stage ones are used more frequently because of their greater resistance (Armitage et al., 1995).

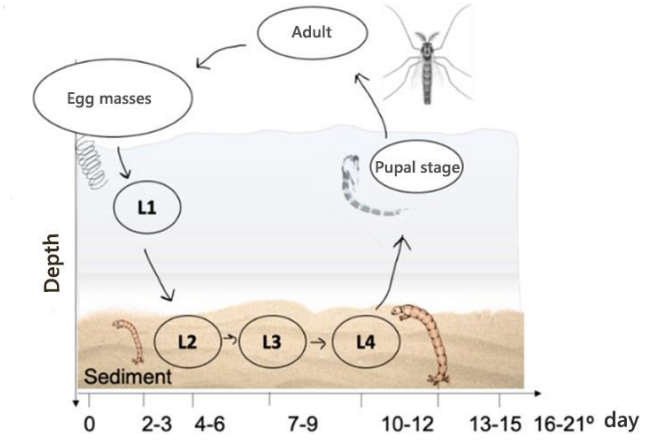

Fig. 6. Simplified life cycle of *Chironomus riparius.*

I.4.2. Planarian *Girardia tigrina*

Girardia tigrina is a benthic organism belonging to the phylum Platyhelminthes (Table 3) with wide geographic distribution, and can be found in rivers, streams, ponds or lakes (Rink & Vila-Farré, 2018; Vila-Farré et al., 2011; Wu & Li, 2018). They preferentially associate with firm substrates, such as rocks or aquatic plants, perhaps for two main reasons: i) their mode of locomotion, which involves the use of cilia on the ventral surface and mucus secretions and ii) derived from the fact that these types of substrate offer shelter from incident solar radiation, since they are negatively phototactic animals, that is, they hide in the dark during the day, to protect against possible predators and strong currents (Kenk, 1976; Rink & Vila-Farré, 2018). They preferably feed on alive prey, with preference for preys that produce disturbance in the water such as chironomids with their curling swimming behaviour (as reviewed by Deochand et al. (2018)).

Table 3. Taxonomic characterization of the species used in this study as a predator: *G. tigrina*.

Taxonomic Characterization	
Kingdom	Animalia
Phylum	Platyhelminthes
Class	Rhabditophora
Order	Tricladida
Family	Dugesiidae
Genus	Girardia
Species	*G. tigrina*

As in all planarians, the main sensory structures of *G. tigrina* are located on its head (Figure 7), which consist of a dorsally located ocellus (photoreceptors) and the auricles as extensions on the margins of the head (Rink, 2018). They have a flat body and a very simple body structure, but presenting some unique characteristics, such as the fact that they are acoelomates, which means they do not have a coelom (mesoderm epithelial layers surrounding inner cavities) and are instead filled with connective tissue or parenchyma (Hartenstein & Martinez, 2019; Wu & Li, 2018). Parenchyma is particularly important in planarians because, in addition to serving as a

matrix to maintain organization, it serves to protect neoblasts – populations of totipotent somatic stem cells crucial for planarian regeneration (Cebrià et al., 1997; Wu & Li, 2018). These organisms activate a complex molecular response after cutting, which allows the start of the regenerative process, through the formation of a blastema, and consequent cell formation, differentiation and morphogenesis (Gambino et al., 2020).

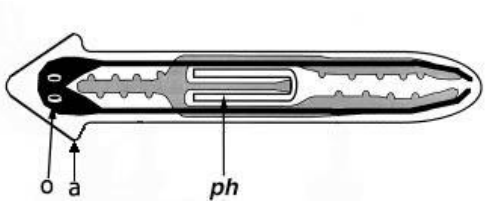

Fig. 7. Illustrative diagram of the basic structure of the *G. tigrina* planarian. (o - ocellus; a - auricles; ph - pharynx). Adapted from Bueno et al. (1997).

As soon as planarians find prey, the pharynx is inserted inside the prey's body and they initiate extracellular digestion by releasing digestive enzymes that act on the food material, breaking it down into small particles (Issigonis & Newmark, 2019; Markosova, 1984; Simão, 2022). This process occurs within the gastrovascular cavity, but once the food particles are transferred to the gastrodermal cells through endocytosis, it becomes intracellular digestion, with tissue and fluids being sucked in by the pharynx (Hartenstein & Martinez, 2019; Simão, 2022). Digestion is complete in digestive vacuoles where particles are broken down into food molecules (Hartenstein & Martinez, 2019). Any material that is not digested is expelled through the pharynx, which is located in the middle zone of the ventral surface (Simão, 2022).

Planarians can reproduce sexually or asexually, depending on the species (Issigonis & Newmark, 2019). In the case of *G. tigrina*, preferential reproduction is sexual, which normally involves copulation between two individuals in which the sperm of one is deposited in the copulatory bursa of the other (Kenk, 1976). Also, being hermaphrodites, any one of the *G. tigrina* individuals has a complete set of male and female reproductive organs and is, therefore, capable of performing any of the roles. However, under stress, this species can resort to asexual reproduction by fission, which consists of the voluntary cutting of the posterior portion of the body normally after the pharynx and is followed by the regeneration of the missing tissues. Each formed part represents a genetic clone of the original individual (Rink & Vila-Farré, 2018). Under laboratory conditions (photoperiod of 16 hours light: 8 hours dark and temperature 20°) *G. tigrina*

favours sexual reproduction and knows that, after hatching, this species takes 3-4 months to reach sexual maturity, 1-2 days for laying of cocoons after copulation (which can contain more than 3 eggs), which later take approximately 1 month to hatch (Figure 8).

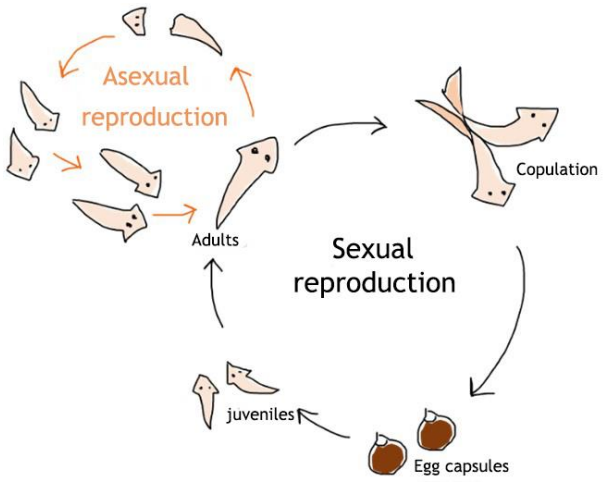

Fig. 8. Representative diagram of the reproduction of *Girardia tigrina.*

In addition to its special biological characteristics, *G. tigrina* has several other advantages when it comes to its use in toxicological studies, namely their representation in food webs; since planarians are predators and feed on other aquatic invertebrates, allowing therefore, a study of organisms located at multiple levels of the food chain (Wu & Li, 2018). Its easy acquisition also contributed to the choice of these organisms since they can be easily marketed or captured in the ecosystem using fresh liver bait traps. In addition, they are cultures that are easy to maintain and entail low costs for laboratories (Rodrigues et al., 2016; Wu & Li, 2018).

Chapter II

Ecotoxicity assessment of polyurethane microplastics (PU-MPs) on *Chironomus riparius*

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II.1. Context of the research and main objectives

Chironomid larvae are among the favourite prey of planarians such as *Girardia tigrina*. The ecotoxicological effects of MPs on chironomids are mostly described for *Chironomus riparius*. According to the available literature, *C. riparius* larvae can easily uptake MPs instead of natural sediments and particulate organic matter with an equivalent size (Silva et al., 2019). When exposed to irregularly shaped polyethylene MPs (PE-MPs, up to 20 g/Kg sediment), the uptake of particles with the size range of 32-64 μm significantly decrease larval growth, and delayed female emergence (Silva et al., 2019). When considering irregularly-shaped co-polyamide (PA-MP), at considerably lower concentrations than previous studies (100 mg PA-MP/Kg sediment; 0 to 180 μm), *C. riparius* seemed not to reveal any effects on their life traits (Khosrovyan & Kahru, 2020). In the presence of a mixture of PET, PS, PVC, and PA (10-400 μm, 8- 80 g/ m^2 sediment), *C. riparius* revealed a tendency towards widening of the wings, changing the shape of the mandibles and elongation of the development time (Stanković et al., 2020). Knowledge of polyurethane small-sized MPs (< 10 μm in size) on chironomids remains, so far, uncovered.

This study aimed at addressing the effect of small-sized polyurethane MPs (PU-MPs) on *C. riparius* life-history traits (survival, larval growth, imagoes emergence) for 28 days. Afterwards, and considering a no-observed-effect-concentration (NOEC) obtained from the life-history assessment assay, the behavioural (locomotion) and biochemical responses (oxidative damage, aerobic energy production, and energy reserves) of 4th instar larvae (that will be used as prey to planarians – chapter IV) were characterised after 10 days of exposure. This knowledge was crucial to better understand the potential effects on the predator (planarian) that would feed on them (explored in Chapter III).

II.2. Materials and Methods

II.2.1. Culture conditions of *Chironomus riparius*

Chironomus riparius were cultivated and maintained under controlled conditions at the Technological Laboratories building - Chemistry Department, University of Aveiro. Cultures were maintained in reconstituted hard water (ASTM, 1996). A stock of 20 L of this medium (also used in the tests) consisted of 17 L of ultrapure water (which comes from the action of a distiller and a purifier with filters on the mains water), 200 ml of solution A (0.16 g/L KCl), 200 ml of solution B (4.91 g/L $MgSO_4{\cdot}7H_2O$), 200 ml of solution C (3.84 g/L $NaHCO_3$), 1.5 L of solution D (2.4 g/1.5L

$CaSO_4$) and 1 ml of vitamins (1.5 mg of thiamine HCl + 0.02 mg of cyanocobalamin + 0.15 mg of biotin in 1 ml of ultrapure water). The solution is finally adjusted to a pH between 7.3 and 7.5 with 5N HCl.

Organisms were reared in glass aquaria (30 x 20 cm) at 20 ± 1 °C under a 16:8 h light-dark photoperiod. Larvae were grown in a layer (~3 cm) of previously burnt inorganic sediment (500 ◦C for 4 h) and American Society for Testing Materials (ASTM) hard water in a 1:4 ratio (ASTM, 1996), with continuum aeration. Adults were confined within an acrylic cage covered with mesh netting. Feeding consisted of macerated commercial food fish TetraMin® (Tetrawerke, Melle, Germany) (nutritious food for aquarium animals) macerate that was provided three days a week (ad libitum).

II.2.2. Polyurethane microplastics used in the experiment

The polymer chosen for this study was Polyurethane (PU), an odourless white powder composed of aliphatic PU with a density of 1.05 (g/cc) and an average particle size between 7.0 and 9.0 μm, kindly provided by a local enterprise that prefers to remain anonymous. Such polymer is used in various types of coatings and paints, as they can modify their tactile properties while improving their ability to resist fire, scratches, and abrasion, and in foams for padding and insulation. These particles also can return to their original shape after being deformed when disturbed by a physical force, thus making them a polymer of choice in the paint and coatings industry.

II.2.3. A 28-day exposure and evaluated endpoints

The 28 d assay followed the international guidelines for sediment–water chironomid life cycle toxicity tests (OECD, 2010) with slight adaptations. Briefly, four *C. riparius* egg masses were isolated in glass flasks containing ASTM, until hatching. Then, first instar (<48 h post-hatching) larvae were used in chronic 28-day life cycle assays. The tested concentrations were: 93.5, 187.5, 375 and 750 mg of PU-MPs/Kg sediment. This concentration range was chosen to include the same order of magnitude of concentrations used on previous tests for comparison purposes (e.g., Silva et al., 2019; Khosrovyan & Kahru 2020), and concentrations expected in the field for small-sized MPs (Conkle et al., 2018). Each PU-MP condition and a control (uncontaminated sediment) consisted of ten replicates with 50 g of contaminated sediment (except for control treatment) that were gently filled with 150 mL of ASTM hard water. Such vials were allowed to equilibrate for 3 days prior to adding 5 larvae to each vial.

Five replicas of control and from each PU-MP concentration were used to measure the **total body length** of the surviving larvae after sacrificing them with 70% ethanol. Larvae body length was measured from the base of the head capsule to the anus using a microscope and a micrometre calibration ruler (Figure 9).

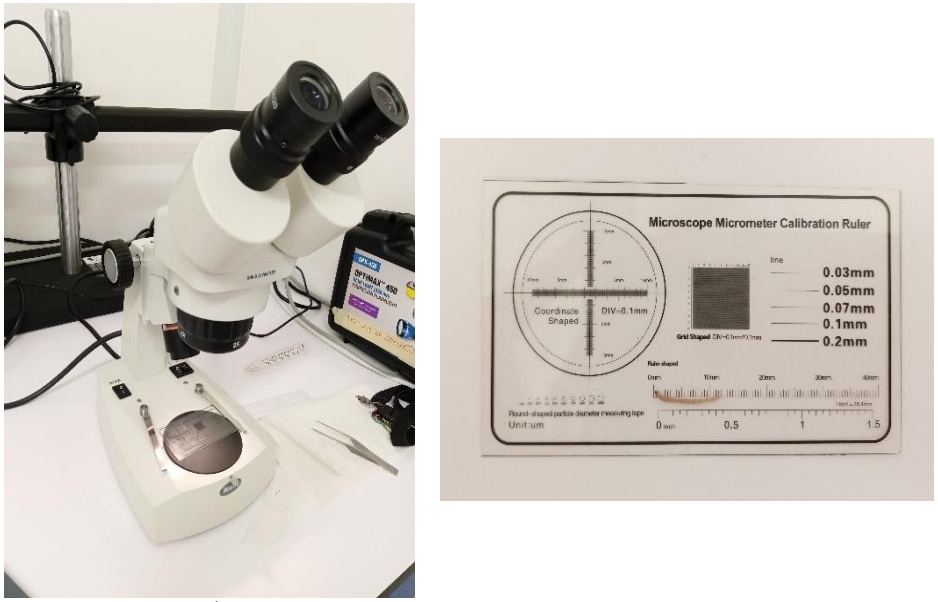


Fig. 9. *Chironomus riparius* 4th instar larvae being measured under a microscope and a micrometre calibration ruler.

The other five replicates of five organisms were used for analysing the **emergence of imagoes** (adult insects) until the end of the test. Such imagoes were trapped in plastic container inserted on top of the glass test vial (Figure 10). Daily emerged imagoes were collected and counted.

Fig. 10. Experimental setup for the 28-days life cycle bioassay.

Throughout the experiment, larvae were fed three days a week (0.25 mg of macerated TetraMin per organism per day), and the test conditions were the same as described for culturing. The life cycle experiment lasted a total of 28 days until the last adult emerged. From this experiment, a no-observed-effect-concentration (NOEC) was chosen for the short-term exposure assay (10 days, following section), to reduce animal experimentations to the minimum (European Parliament and Council of the European Union, 2010) and assess behavioural, physiological, and biochemical endpoints.

II.2.4. A 10-day exposure and evaluated endpoints

Similar to the previous assay, four *C. riparius* egg masses were isolated in glass flasks containing ASTM, until hatching. The larvae (< 48 h) were equally transferred to three glass aquaria (30 x 20 cm) containing 800 g of sediment with 2L hard water (control condition) or three glass aquaria containing 800 g spiked sediment with 2L hard water (375 mg PU-MP/kg – a NOEC; equilibrated for 3 days before larvae addition), for a period of 10 days at 20 ± 1 °C under a 16:8 h light-dark photoperiod.

Again, the addition of ASTM was performed by gently pouring the ASTM hard water to minimize the resuspension of PU-MPs mixed in the sediment. The test aquaria were then covered with lids and allowed to equilibrate for three days (a time consisting of the hatching of isolated egg masses). After the hatching of the masses, larvae were equally divided per aquarium, thus initiating the exposure of the larvae. Each aquarium was provided with aeration. Larvae were fed 3 days a week with macerated TetraMin® diluted in ultra-pure water, as described in the previous section.

After exposure, fifteen random larvae were collected from control and PU-MP treatment and sacrificed in ethanol 70%. Firstly, such larvae were measured (**total body length**) using a USB microscope and a micrometre calibration ruler (also addressed in the first test, as in Figure 9). This procedure was to confirm results obtained in the 28-day bioassay. After this procedure, the same larvae were used to confirm **larvae PU-MP uptake**. For this purpose, larvae were gently dried on filter paper and transferred to glass tubes, and 2 ml of nitric acid (HNO_3; 65%) was added for 3 hours at 60˚C. To complete the digestion process, the samples were cooled to room temperature, and a volume of 2 ml of hydrogen peroxide (H_2O_2; 35%) was added for 24 hours. Once the samples were cleared of visible oxygen bubbles, replicas were diluted using Milli-Q water in a 1:10 ratio (Silva et al., 2019; 2021b). Solutions resulting from digestion were filtered through a glass vacuum system using a kitasato flask, a glass büchner funnel and a black polycarbonate filter

(PCTE, 0.2 μm pore size, 47 mm Ø, ref. 7063 -4702, GE Healthcare Whatman™), stained with Nile Red (Sigma Aldrich, U.S.A.; 100 μg/ml in absolute ethanol) for 2 minutes, and washed abundantly with ultrapure water. Afterwards, filters containing PU-MPs were observed and photographed under a microscope (Olympus BX41; objective 10X) copulated with a Canon EOS 1200D camera. This entire procedure took place in a fume hood covering samples and filters to avoid potential airborne contamination by MPs. Additionally, all the equipment and test vials were acid-washed and thoroughly rinsed with Milli-Q water.

From each aquarium (the control or PU-MP treatment), four replicas of five larvae were also transferred to 250 mL glass vials containing 40 mL of ASTM hard water to evaluate **larvae behaviour (locomotion)**. Larvae were filmed for a period of 2 mins (exact time), and the number of curling and un-curling (typical behaviour of chironomid larvae while foraging) was recorded. Data were presented as number of curling/min per treatment.

Finally, another five replicates of fifteen larvae were collected from each aquarium (the control or PU-MP treatment), gently rinsed, dried on filter paper, put into 2 mL microtubes, snap-frozen in liquid nitrogen, and stored at -80 ˚C to further **assess oxidative damage (via LPO), energy reserves (lipids, proteins, carbohydrates) and energy consumption (via ETS)**.

Samples were defrosted on ice and homogenized using the sonicator Fisherbrand™ Model 120 Sonic Dismembrator in 1600 μl of ultra-pure water. From such homogenate, three aliquots of 300 μL were collected from each sample: one for analysis of lipid content, one for sugars and protein content and the other to evaluate ETS activity. For LPO determination, 200 μL aliquot was collected from the remaining homogenate, to which it was added 4 μL of 4% BHT (butylated hydroxytoluene). Measurement of lipid peroxidation was conducted according to Campos et al. (2017) using thiobarbituric acid-reactive substances (TBARS) and measuring LPO levels at 535 nm (Bird & Draper, 1984). Results are expressed in nmol TBARS/g organism.

Available energy (energy reserves) and aerobic energy production (ETS) were determined following the method by De Coen & Janssen (1997), following the adaptations by Campos et al. (2017). For total lipid content, one of the groups of 300 μL aliquots were centrifuged (1000 g or 3500 rpm for 5 min) after the addition of 500 μL of chloroform (≥ 99.8 %) and 500 μL of methanol (≥ 99.8 %) and the organic phase were then transferred to a glass tube, acidified using 500 μL sulphuric acid (H_2SO_4) and incubated at 200 ˚C for 15 min. After cooling, 1500 μL of ultra-pure water was added to each tube. The absorbance of samples and tripalmitin, for the calibration curve, was measured at 375 nm.

Carbohydrate and total protein content were determined after adding 15 % tri-chloroacetic acid (TCA) to one of the groups of 300 μL aliquots, followed by incubation at -20˚C for 10 min. The supernatant, resulting from centrifugation at 1.000 g for 10 min at 4 ˚C, was then collected to be used in carbohydrate measurement. The absorbance was read at 492 nm after samples and glucose standard concentrations were incubated for 30 min at room temperature by adding 200 μL of 5% phenol and 800 μL of H_2SO_4. The remaining pellet was then resuspended by adding 500 μL of sodium hydroxide (NaOH) and heated to 60°C for 30 minutes. For sample neutralization, 280 μL of Hydrochloric acid (HCL) was used to finalize the samples. Total protein content was obtained following Bradford's method adapted from BioRad's Bradford micro-assay set up in a 96-well flat bottom plate (Bradford, 1976), which uses bovine serum albumin for the calibration curve and a reading absorbance of 592 nm. Results of lipids, sugars and proteins are expressed in mJ/mg organism.

The last 300 μL aliquots were used to evaluate ETS activity. Following the method by De Coen & Janssen (1997), 150 μL of homogenization buffer (Tris base (0.3M); Polyvinylpyrrolidone (0.45 % (w/v)); 459 μM MgSO4; Triton X-100 (0.6 % (v/v)) at a pH of 8.5) was added to the samples that were later centrifuged at 1000 g for 10 min at 4˚C. In the multi-well plate, 50 μL of the resulting supernatant was incubated with 150 μL of a buffered solution (Tris base (0.13 M) with Triton X-100 (0.27 % [v/v]), 1.7 mM reduced nicotinamide adenine dinucleotide (NADH), 274 μM reduced nicotinamide adenine dinucleotide phosphate (NADPH)) and 100 μL of INT solution (p-iodonitrotetrazolium; 8 mM). Absorbance was estimated kinetically at 490 nm over a 3 min period. Results are expressed in mJ/h/mg organism.

All biomarker determinations were performed with the Microplate reader MultiSkan Spectrum (Thermo Fisher Scientific, USA).

II.2.5. Statistical analysis

For analysing the results from the 28 days exposure bioassay, the emergence ratio (ER), the mortality rate (M) and the percentage of emerged individuals per day (ED) were calculated according to Savić-Zdravković et al., 2018. To analyse the differences between the control group and the various concentrations of PU-MPs, a one-way analysis of variance (ANOVA) was used, followed by a Dunnett's post-hoc test. Results are presented as mean ± standard error of the mean (SEM).

For the second bioassay, related to the short-term (of 10 days) exposure, and considering that there were only two groups - control vs. PU-MPs, the difference in the multiple parameters (larval body length and movement and biochemical biomarkers), in *C. riparius* was evaluated by applying the unpaired two-tailed Student's t-test. Results are presented as mean ± SEM.

For all statistical tests, the significance level was set at $p < 0.05$. All data were analysed using the statistic software GraphPad Prism version 9.3.1 for Windows (GraphPad Software Inc., La Jolla, California, USA).

II.3. Results and discussion

II.3.1. Effects on *C. riparius* life history traits after 28-d exposure

The exposure to the various concentrations of PU-MP did not cause significant differences in larval body length in comparison with the control treatment, indicating that growth was similar in the presence or absence of PU-MPs when achieving the 4th instar (one-way ANOVA, $F_{(4, 20)} = 1.190$, $p= 0.3454$, Figure 11). The control group presented an average body length of 10.5 ± 0.4 mm (mean ± SEM). For the treatment groups, the average body length was 11.1 ± 0.2, 10.8 ± 0.3, 10 ± 1 and 9.7 ± 0.7 at concentrations of 93.5, 187.5, 375 and 750 mg PU/Kg sediment, respectively. These results were important to determine the exposure concentration that would not interfere with larval growth since they would serve as prey for the planarians in the next experiment (Chapter III).

Our results contrast (to some extent) with previous investigations, where exposure to sediments contaminated with small-sized PE-MPs (32–64 µm) affected *C. riparius* larval body length (a proxy of growth), although at concentrations considerably higher and bigger sizes than the ones tested in this investigation (Silva et al., 2019). The same species exposed to a mixture of polymers (PA, PE, PET, PS, PVC, and concentrations up to 80 g/cm2) revealed an increase in larval body mass and length (Stanković et al., 2020). In another chironomid, *C. tepperi*, exposed to PE-MP concentrations even smaller than those used in the present study (size ranges of 1–4, 10–27, 43–54, and 100–126 µm), also showed a reduction in larval body length (Ziajahromi et al., 2018). Such differences in MPs effects on chironomids larval growth are related not only to the number and size of incorporated MPs, but also to polymer type, its potential additives, and organisms' capacity to deal with physical and chemical hazardousness caused by MPs.

Imagoes emergence passed the validity criteria, with an emergence ratio in controls of at least 70% (OECD, 2010). There was no statistically significant difference in the emergence ratio (ER) or larvae mortality (M) between treatments (one-way ANOVA, $F_{(4, 20)} = 1.400$, p= 0.27; Table 4). Adults emerged per day were also analysed and did not vary significantly, with an average of approximately three individuals per day (one-way ANOVA, $F_{(4, 20)} = 1.190$, p =0.3454). In fact, the study carried out by Stanković et al. (2020) obtained similar results, with no differences regarding larvae emergence and mortality ratio in *C. riparius* when exposed to a mixture of MPs (PET, PS,PVC and PA, 10 - 400 μm, 8- 80 g/m^2 sediment). Notwithstanding, some deformities were observed in female imagoes wings.

The mean time until first emergence can be observed in Fig. 12. In control group, first emergence was noted after 11 ± 1 days of exposure, while in the presence of PU-contaminated sediment, first emergence occurred after 9 ± 0 when exposed to 93.5 mg/Kg, 9.4 ± 0.2 days at 187.5 mg/Kg, 9.4 ± 0.2 days at 375 mg/Kg and 9.6 ± 0.4 days at 750 mg/Kg. These results, although not significant (one-way ANOVA, $F_{(4, 20)} = 1.663$, p= 0.1980), showed an anticipation in the first emergency of exposed larvae. These results contradict other studies, such as Silva et al. (2019), who observed a delay in the emergence of females right from the first concentration tested (1.25 g/Kg sediment, which is considerably higher than the ones from this study), resulting in a 2–3-fold higher difference in the mean emergence time.

The results on these organismal parameters suggest that this particular polymer size and concentration did not seem to affect the reproduction (and eventual population dynamics) of *C. riparius* midges inhabiting contaminated freshwater sediments. However, it should be noted that the effects of environmental stressors such as MPs are, in fact, dependent on features like polymer size and dose. Using PE as a test polymer, Silva et al. (2019) revealed that imagoes emergence was negatively affected by smaller-sized PE (32–63 μm) for concentrations higher than 1 g/kg, which contained a larger number of small particles.

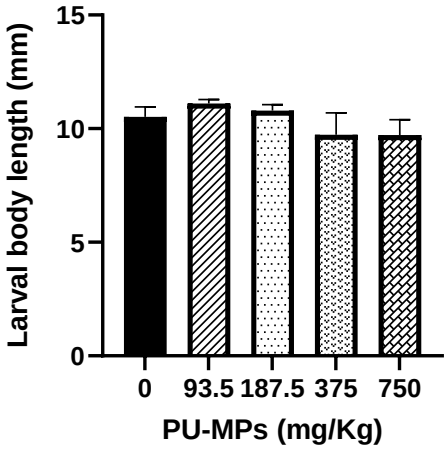


Fig. 11. Effect of different concentrations of polyurethane microplastics (PU-MPs) on *Chironomus riparius* larval body length (n = 5). All values are presented as mean ± standard error of the mean. No significant differences between treatments were denoted.

Table 4. Effect of different concentrations of polyurethane microplastics (PU-MPs) on *Chironomus riparius* life cycle endpoints: Emergence ratio (ER) - % of emerged individuals at the end of the experiment; Mortality rate (M) - % of dead individuals at the end of the experiment and average % of emerged individuals per day (ED). All values are presented as mean ± standard error of the mean. No significant differences between treatments were denoted.

Parameters	PU-MPs (mg/Kg)				
	0	93.5	187.5	375	750
Emergence ratio (ER) ± SEM	84 ± 7	92 ± 5	84 ± 7	100 ± 0	92.5 ± 3.66
Mortality (M) ± SEM	16 ± 7	8 ± 5	16 ± 7	0 ± 0	8 ± 4.90
Emerged individuals/day (ED) ± SEM	2.3 ± 1	3 ± 1	2.3 ± 0.9	2.8 ± 0.9	2.6 ± 0.6

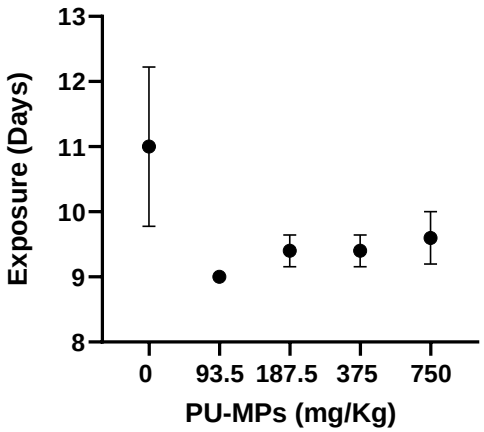
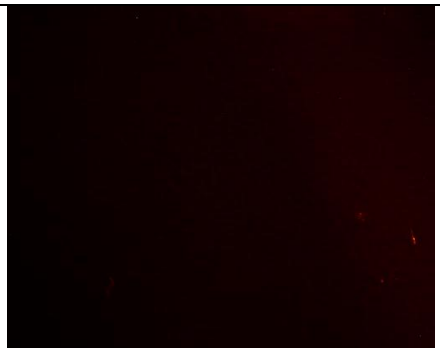


Fig. 12. Effect of different concentrations of polyurethane microplastics (PU-MPs) on *Chironomus riparius* time to first emergence (n = 5). All values are presented as mean ± standard error of the mean. No significant differences between treatments were denoted.

II.3.2. Effects on larvae behaviour and homeostasis after 10-d exposure

Chironomid larvae were able to incorporate PU-MP, as several thousands of particles were observed in their gut after 10 days of exposure to PU-MP NOEC (375 mg/kg) (Fig. 13). However, their quantification was compromised by a high number of particles and, due to microplastics' physicochemical characteristics (Drago et al., 2020), high aggregation. Chironomids, particularly *C. riparius*, incorporated and accumulated (to some extent) a considerably high number of PE-MPs of 32–63 µm (up to 2500 particles per organism) (Silva et al., 2019). Scherer et al. (2017) also reported the uptake of polystyrene (PS) beads of 1, 10 and 90 µm by *C. riparius*, with a consumption of 226 particles per hour. Another chironomid species, *Chironomus tepperi*, also incorporated PE-MPs with a strong tendency to agglomerate in the gut, visible using a stereo-microscope (Ziajahromi et al., 2018). The smaller the microplastics' size, the higher tendency to their accumulation (and translocation) within tissues (J. C. Prata et al., 2022; Su et al., 2019). Although no quantification is provided in this study, it is unquestionable the high amount present in their digestive tract that could (to some extent) induce sub-lethal effects, as discussed further.

PU-MP (mg/Kg)	Microscope photos
0	 

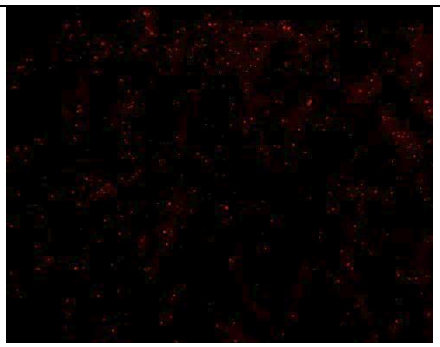
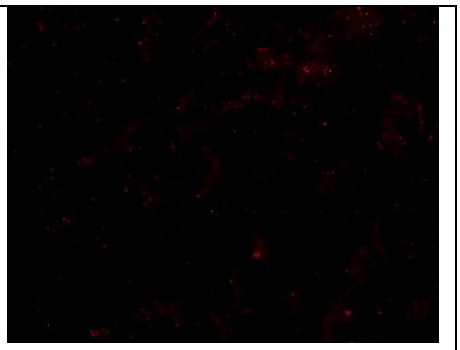
375		

Fig. 13. Polyurethane microplastics (PU-MPs) present in *C. riparius* four instar larvae digestive track after a 10-day exposure to 0 (control condition, two random filters) and 375 mg PU-MP/kg of sediment (two random filters) (proof of concept). Particles presenting red fluorescence are PU-MPs. Note: The presence of MPs in controls might be due to potential cross contamination or airborne contamination. Nonetheless, the number of particles were negligible compared to PU-MP treatments. Eyepiece magnification: ×10; Objective: ×40.

The effects of the uptake of PU-MPs on larval growth corroborate the results found during the life cycle test performed in the previous section. The exposure to 375 mg/kg of PU-MPs and its ingestion did not cause significant differences in larval body length in comparison with the control treatment, and the growth was similar in the presence or absence of PU-MPs ($t= 0.2248$, $df=34$, $p = 0.8235$; Figure 14). The control group presented an average body length of 10.2 ± 0.2 mm (mean ± SEM), and the contaminated group had an average body length was 10.3 ± 0.2 mm. This parameter remained around 10 mm for both treatments, which is not very different from Stanković et al. (2020), which presented the same larval length in the control group. These results were to be expected since it is probably a reason for the outcome observed in larval emergence in the previous section, which proved to also not be affected by PU-MPs.

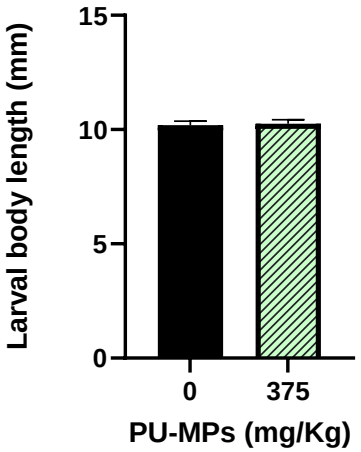


Fig. 14. Effects of polyurethane microplastics (PU-MPs) on *Chironomus riparius* larvae body length after 10 days of exposure (n= 15). All values are presented as mean ± standard error of the mean. No significant differences between treatments were denoted.

In Fig. 15 can be depicted the number of curlings and un-curlings (larval movement) executed per minute, comparing the results from the control and contaminated group. Conversely, contaminated larval presented significantly higher locomotion ($t = 2.989$, $df = 6$, $p = 0.0244$), with a mean difference of 60.53 curlings per minute between the contaminated and the control group (mean of the control group = 29.35; mean of contaminated group = 89.88). Such greater activity from contaminated larvae can be related to a potential increase in acetylcholinesterase activity (AchE, as a proxy of "organisms' activity/movements" in macroinvertebrates) as observed in *C. riparius* larvae exposed to PE-MPs < 64 µm in size (Silva et al., 2021b). Authors attributed the increment in AchE to the peristaltic movements promoted by larvae as an attempt to egest microplastics retained in their gastrointestinal tract. This increase in larvae locomotion can, therefore, be reflected in energy expenditure.

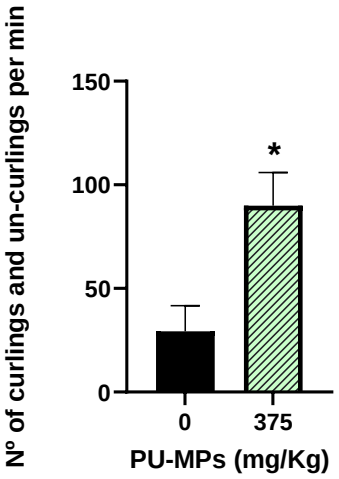


Fig. 15. Effects of polyurethane microplastics (PU-MPs) number of curlings and un-curlings per minute of *Chironomus riparius* larvae after 10 days of exposure (n= 4). All values are presented as mean ± standard error of the mean. * denotes significant different from the control ($p < 0.05$).

In fact, larvae exposed to PU-MPs revealed activation of aerobic energy production (in other words, one can infer in terms of activation on the metabolism, also reflecting an increase in energy expenditure) compared to controls (Figure 16A). The fact that contaminated larvae were more active while foraging (curling/un-curling more) partially explains such energy consumption. However, as despite the higher locomotion activity, contaminated larvae did not reveal significant energy reserves shift, i.e., in terms of proteins ($t= 0.8437$, $df=8$, $p= 0.4234$, Fig. 16B), lipids ($t= 1.549$, $df=8$, $p= 0.1600$, Fig. 16C) and carbohydrates ($t= 1.179$, $df=8$, $p= 0.2724$, Fig. 16D). A higher energy consumption (increased metabolism) can also be related with an activation of immune responses and consequent trigger of antioxidant defences to fight reactive oxygen species (ROS). Silva et al. (2021b) used a different MP polymer (PE; 32-63 µm) and provided evidence of activation of the *C. riparius* immune system due to damage of the epithelial cells of the gut lumen. Effective activation of response mechanisms against ROS can be related to the absence of lipid peroxidation, as observed in Figure 17. Levels of LPO were not significantly altered with exposure to PU-MPs ($t= 0.4500$, $df=8$, $p= 0.6647$; Fig. 17). Yet, to confirm such a hypothesis, an assessment of the antioxidant and detoxicant capacities of *C. riparius* larvae is required (e.g., assessment of catalase, glutathione peroxidase, glutathione-S-transferase activities, among others). The expression of these antioxidant enzymes was previously observed by Silva et al. (2021b) in *C. riparius* who registered an inhibition of CAT and GST activities after exposure to PE-MPs (particularly evident for larvae that ingested larger particles, 125–500 µm). A different response

was observed in the annelid *Lumbriculus variegatus* after exposure to PE-MPs, that showed an increase in total glutathione in all size classes (PE-MPs; size-class A: 32–63, B: 63–125, C: 125–250 and D: 250–500 μm) and GST levels only on particles > 125 μm. CAT levels were not affected. This is evidence that the responses of these enzymes are highly dependent on, not only the species, but also the organism's capacity and mechanisms efficiency to manage the uptake of MPs.

According to Sokolova et al. (2012), organisms facing stress allocate their energy for survival instead of storage, activity, or reproduction. However, it has been proved that the energy used is not proportionally deviated from those physiological processes. Indeed, it has been shown that larvae of chironomids exposed to paraquat prefer to allocate energy for reproduction, whereas planarians decrease the reproductive outputs to ensure their survival (Saraiva et al., 2020). In our particular case, it seems that chironomids use energy for their curling activity, probably trying to egest microplastics from inside their guts.

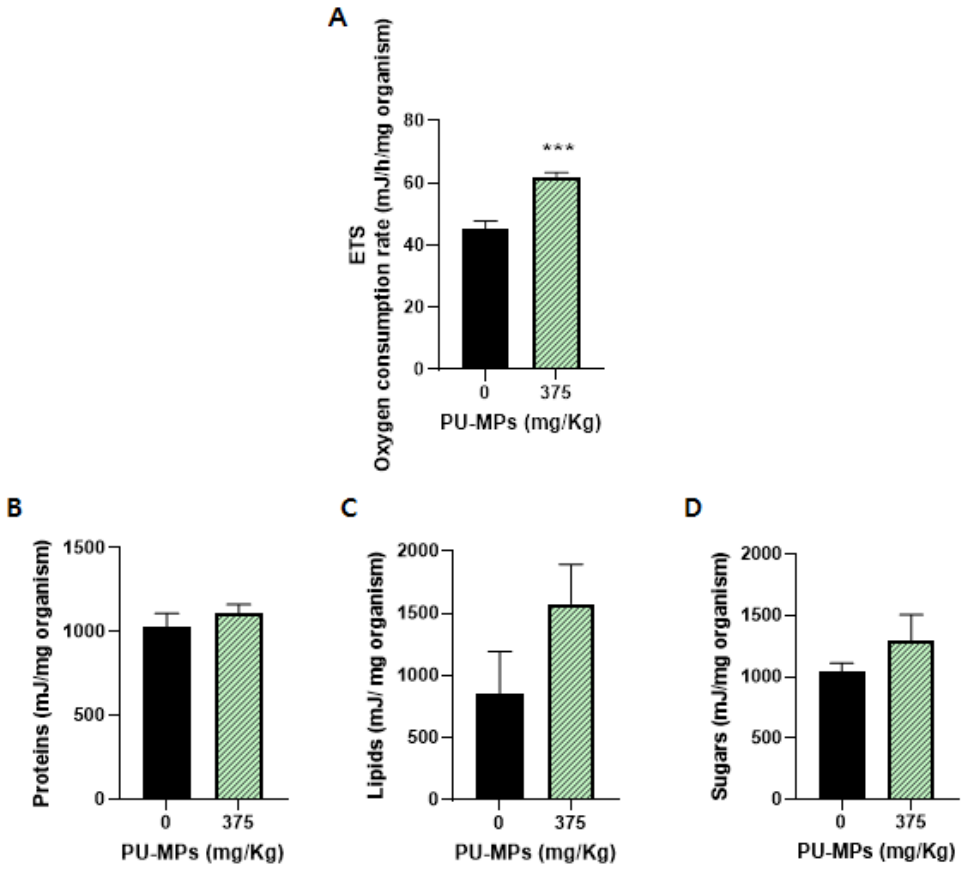


Fig. 16. Effect of polyurethane microplastics (PU-MPs) on (A) aerobic energy production (electron transport system, ETS, mJ/h/mg organism), (B) proteins, (C) lipids, and (D) sugars content (mJ/mg organism) of

Chironomus riparius larvae after 10 days of exposure (n=5). All values are presented as mean ± standard error of the mean. *** denotes significant different from the control ($p < 0.001$).

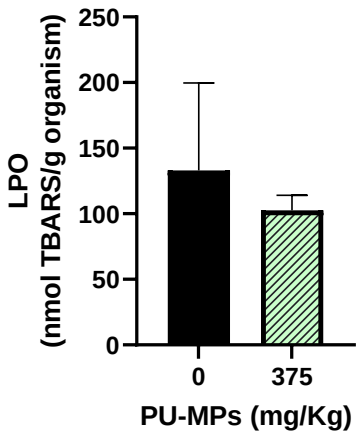


Fig. 17. Lipid peroxidation (LPO) levels in *Chironomus riparius* larvae after 10 days of exposure to polyurethane microplastics (PU-MPs) (n= 5). All values are presented as mean ± standard error of the mean. No significant differences between treatments were denoted.

Chapter III

Behaviour, physiological and biochemical responses on planarians after exposure to contaminated prey – evaluation via trophic transfer

Resulted in the article under review in the International Journal of Science of The Total Environment

STOTEN-D-22-33034

III.1. Context and main objectives

Most studies on the effects of MPs focus predominantly on marine environments compared to freshwater environments, creating knowledge gaps that must be addressed with high priority to obtain crucial information for the ecological risk assessment of MPs in freshwater environments. The studies conducted so far considering freshwater organisms focus predominantly on detritivores (e.g., chironomids, Carrasco-Navarro et al., 2021; Scherer et al., 2020; Silva et al., 2019, 2020, 2021b) or filter-feeders (e.g., clams, Esterhuizen et al., 2022), without great attention to predators (such as planarians) and the potential trophic transference of MPs along the food chain. The main endpoints assessed include uptake, survival, reproduction and cellular stress, while the potential trophic transfer and its effects on the behaviour of predators, in particular predators with extracorporeal and intracellular digestion like the planarians, remains unclear.

When fed with contaminated food (liver), the planarian *Dugesia japonica* seemed to uptake polyethylene microspheres (PE-MPs, <10 µm in diameter) and polyethylene terephthalate microfibers (PET-MPs, 14 µm large and 5/6 µm length) in concentrations from 12 to 60 µg/mg food, without altering their feeding activity, food intake, and regenerative ability (Gambino et al., 2020). Notwithstanding, it induced a significant reduction of the gut epithelium thickness and lipid content of enterocytes, along with the induction of apoptotic cell death and reduced growth rate. Such species also revealed a decrease in the body and blastema areas upon exposure to polystyrene microspheres (PS-MPs, 0.1-1-10 µm in size, at concentrations of 10, 50, or 100 µg/mg food), indicating a delay in their growth and regeneration (Gao et al., 2022). Concomitantly, the proliferation and differentiation processes of stem cells were inhibited, and the proportion of mitotic stem cells decreased (Gao et al., 2022). PS-MPs of ~1 µm in size also induced oxidative stress on *D. japonica*, by significantly changing the levels of antioxidant enzymes, including superoxide dismutase, catalase, and glutathione S-transferase (Han et al., 2022).

Although these previous works provided important intel on the potential physiological and cellular effects on MPs in planarians, they are missing behavioural setups of environmental relevance. In natural environments, planarians display a preference for live prey that produce disturbance in the water, such as chironomids with their curling swimming behaviour (as reviewed by Deochand et al., 2018). When exposed to contaminants, these organisms often alter their energy allocation, suppress locomotor activity, and even compromise their regenerative

ability (Knakievicz, 2014; López et al., 2021; Yuan et al., 2014). After ingesting food, locomotion and responsivity to stimuli are often reduced; and if stressed, planarians can expel previously ingested food to enable an efficient evasion (Deochand et al., 2018). Knowledge of the potential effects of MPs via contaminated prey remains uncovered.

To fill some of these knowledge gaps, the present study aimed to evaluate the predatory ability, locomotion, physiological and biochemical responses of the freshwater macroinvertebrate, planarian *Girardia tigrina*, with extracorporeal digestion, after consuming larvae of *Chironomus riparius* contaminated with polyurethane small sized MPs (375 mg of PU-MP/Kg sediment; as described in Chapter II).

III.2. Materials and Methods

III.2.1. Culture conditions - *Girardia tigrina*

A culture of sexually reproducing *Girardia tigrina* individuals were maintained under controlled conditions at Technological Laboratories building - Chemistry Department, University of Aveiro. Organisms were grown in glass containers with ASTM, and their photoperiod was eliminated, as they were protected from the light given their lucifugal (i.e., negative phototaxis) behaviour., except during feeding and media replacement. Food was supplied once per week with either chicken liver (preserved frozen at -20°C until use) or alive chironomid larvae for periods of 3h, followed by the renewal of ASTM medium. Media was also replaced two-three days after feeding. Before the start of bioassays, planarians were not fed for seven days, and active organisms with similar physiology and state of maturity (i.e., approximately 1 cm in height, juveniles, with no apparent lesions) were used for experiments.

III.2.2. Feeding assay and behavioural assessment

To assess the effects of contaminated (or not) prey with PU-MPs on *Girardia tigrina* feeding, locomotion and regeneration, individual planarians were placed in Ø 50 mm crystallisers containing 20 mL of ASTM hard water (N=17 crystallisers per group) along with ten *C. riparius larvae* obtained from control group or contaminated group and allowed to feed for three hours (Simão et al., 2021). After this period, the number of consumed prey was registered. Then, for each group (control and contaminated), i) 6 planarians were used to assess the regeneration of photoreceptors and auricles during the following 7 days; ii) 6 planarians were used to assess

locomotor activity through the gridline method; and iii) 5 planarians were used for further histological assessment (out of the time set for this thesis).

To assess the effects of contaminated (or not) prey with PU-MPs on *Girardia tigrina* energy reserves (total content of sugars, lipids and proteins), aerobic metabolism (estimated using electron transport system (ETS) activity), and oxidative damage (here, lipid peroxidation - LPO), planarians were placed individually in Ø 50 mm crystallisers containing 20 mL of ASTM hard water (N=15 per group) along with five *C. riparius* larvae obtained from control group or contaminated group and allowed to consume them (4 hours were enough). After this period, planarians of each group were pooled in three organisms (n=5 of 3 organisms), rinsed with ultrapure water, gently dried on filter paper, placed in microtubes, weighted, and deep-frozen in liquid nitrogen until further analysis.

III.2.3. Regeneration assessment

Regeneration was evaluated by choosing 6 planarians of each condition, with a similar number of larvae consumed, to be decapitated. For this, a sterilized scalpel was used to make a cut between the pharynx and the auricles, and its regeneration was monitored daily until complete regeneration (up to 8 days) with the help of a digital USB microscope (1600x, Figure 18). The absence or presence of photoreceptors was recorded in each replicate, and the results were presented as an average of days needed for photoreceptors formation.

Fig. 18. Method used to monitor planarian *Girardia tigrina* regeneration. Digital photographs were taken using an USB microscope (1600x).

III.2.4. Locomotor activity assessment

The effects in planarians' movement were determined individually by placing the organism in a clear glass container with ASTM hard water covering the bottom on top of a grid sheet with 1x1 cm gridline spacing (adapted from López et al., 2021). Locomotor velocity was measured as the number of crossed and re-crossed gridlines over a recording period of 2 min. Results were expressed as the mean number of gridlines crossed by planarians per minute in each condition.

III.2.5. Biochemical biomarkers assessment

Oxidative stress (LPO), energy reserves (sugar, lipid, and protein contents) and aerobic energy production (ETS activity) were assessed on planarians. Briefly, samples were kept on ice and subsequently homogenized with 1200 µl of ultra-pure water. This homogenate was divided into portions for LPO (200 µL), ETS (300 µL), and energy reserves (300 µL sugars, 300 µL lipid and 300 µL proteins). All biomarker determinations were performed with the Microplate reader MultiSkan Spectrum (Thermo Fisher Scientific, USA) and as described in the previous chapter.

III.2.6. Statistical analysis

Statistical differences between the two groups (control/PU-MPs) were tested using the independent samples two-tailed Student's t-test. Results are presented as mean ± SEM.

For all statistical tests, the significance level was set at $p < 0.05$. All data were analysed using the statistic software GraphPad Prism version 9.3.1 for Windows (GraphPad Software Inc., La Jolla, California, USA).

III.3. Results and discussion

III.3.1. Feeding behaviour

The feeding behaviour of *G. tigrina* was significantly affected by the type (contaminated or uncontaminated) of prey ($t=5.394$, $df=58$, $p< 0.0001$, Figure 19). Over a 3-hour feeding period, planarians consumed approximately 20% more contaminated than uncontaminated larvae, with an average of 13.43 ± 0.32 and 11.23 ± 0.26 consumed larvae, respectively. This could be due to the more active behaviour displayed by contaminated larvae (see chapter II, section II.3.2). Planarians are driven to prey that produce greater disturbance in the water (as reviewed by Deochand et al., 2018).

In Gambino et al. (2020), MPs in food did not affect the feeding of the planarian *Dugesia japonica*. However, it should be noted that in Gambino et al. (2020), the contaminated chicken liver was used as food while live prey was used in our study, which is more environmentally relevant because of the potential to trigger increased visual and chemical cues stimulating planarians to exhibit a more ferocious predator behaviour (Pijanowska & Kowalczewski, 1997), and influencing the final amount of food consumed and concomitantly the number of MPs it will potentially incorporate.

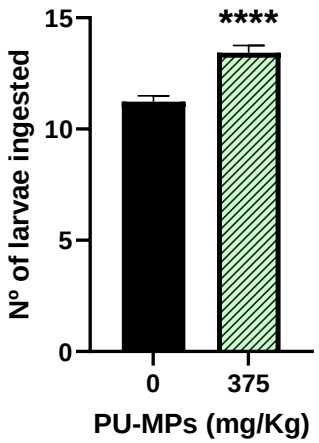


Fig. 19. Number of *Chironomus riparius* larvae consumed by *Girardia tigrina* after 3 hours of exposure (n=17). All values are presented as mean ± standard error of the mean. **** denotes significant different from the control ($p < 0.0001$).

III.3.2. Regeneration

Figure 20 shows the effect of PU-MPs on the well-known planarian capacity to regenerate any missing body parts such as photoreceptors and auricles. Planarians exposed to contaminated prey formed photoreceptors from the 6th day following and did not show effects in comparison with time for photoreceptors (ocellus) regeneration observed in the control treatments ($t= 2.000$, $df=10$, $p= 0.0734$, Fig. 20A). Auricles regeneration of *G. tigrina* control group started 5.83 ± 0.17 days after decapitation. However, in planarians fed with PU-MP contaminated prey, auricle regeneration was significantly delayed for at least 24h when compared to the control treatment ($t= 3.814$, $df=10$, $p= 0.0034$, Fig. 20B).

Regeneration-related responses have been used as sensitive endpoints to test the effects of natural and chemical environmental stressors (Córdova López et al., 2019). The effect of

delayed regeneration of auricles observed in this study is in line with other investigations. Briefly, Gambino et al. (2020), for small PE-MPs within the size range of PU-MPs used (< 10 µm in diameter), showed a significant reduction in body and blastema size of *Dugesia japonica*, reflecting a reduced growth. Accordingly, Gao et al. (2022), also indicated a reduction in *D. japonica* regeneration with increasing concentrations of PS-MPs (10, 50, and 100 µg/mg) indicating that planarian regeneration would be repressed with increased PS-MPs uptake by planarians.

Nevertheless, the absence of effects on photoreceptors regeneration in this study can be justified by the presence and effective action of neoblasts, which have a very high mitotic rate and are present in large quantities in these organisms, making them highly resistant to adverse conditions. The lack of literature regarding the evaluation of planarians' regeneration after exposure to MPs, especially exposure via contaminated live prey can justify the differences in results. Despite these aspects, our current results showed a delay in auricles' regeneration, which contradicts the results of photoreceptor regeneration and previous assumptions. Nevertheless, auricles of planarians are important structures enabling the organisms to detect chemical cues present in water (Reho et al., 2022). Therefore, it seems that a physical impairment in planarians exposed to MPs (via contaminated food) will negatively affect the perception of their habitat, which might compromise their feeding, escaping and reproductive behaviour.

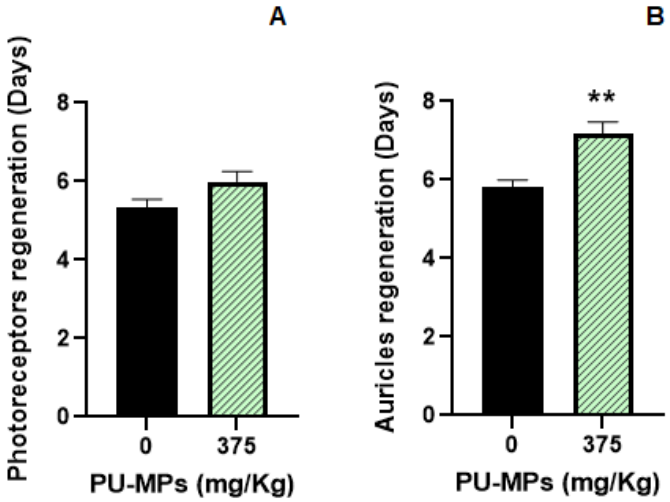


Fig. 20. Effect of contaminated prey on *Girardia tigrina* regeneration. (A) Days until photoreceptors regeneration; (B) Days until auricles regeneration (n= 6). All values are presented as mean ± standard error of the mean. **denotes significant different from the control (p <0.01).

III.3.3. Locomotor activity

The locomotor activity of *G. tigrina*, measured as gridlines crossed per min, covered in the 2 minutes of the test was similar in the two groups, which is evident in the graph in Figure 21, which shows that the group contaminated with PU-MPs moved a total average of 4.5 lines/min and the control group an average of 5.8. The statistical analysis performed with a Student t-Test confirmed that there were no significant differences (t= 1.964, df=10, p= 0.0780).

The locomotor activity of planarians was measured using a simple but quantitative method that has proven sensitive to contaminant exposure (Córdova López et al., 2019; Ofoegbu et al., 2019; Silva et al., 2022), where the quantitative metrics simply compares the distance travelled by planarians in a given time, either by counting the crossing of squares on a grid (as in this study) or by measuring the total distance travelled by video tracking (as reviewed by Reho et al., 2022). Planarians are known to reveal a clear change behaviour when exposed to a harmful stimulus (either mechanical, chemical, or electrical) (as reviewed by Reho et al., 2022). Such changes in behaviour can encompass increased mucus production, a decrease in gliding smoothly, and adoption of various body shapes. In our case, the lower number of crossed gridlines by planarians that fed on contaminated prey reflect a delay in the movements (kind of confounded); but once they started gliding, they followed the same pattern as planarians fed on uncontaminated larvae (mostly in one random direction), along with similar body shape.

The potential effect of microplastics on planarians locomotion (generalising to flatworms) remained (up to now) unreported. Notwithstanding, changes in locomotor activity on organisms exposed to (or fed on contaminated prey) have been observed in vertebrates, such as *Danio rerio* larvae (Chen et al., 2017) and adult fish (Chen et al., 2020), and invertebrates such as *Crassostrea gigas* (Bringer et al., 2020), *Daphnia magna* (De Felice et al., 2019), and *Artemia franciscana* (Gambardella et al., 2017). In a meta-analysis with aquatic biota (Sun et al., 2021) organisms showed a significant decrease in locomotor activity by 6% after exposure to MPs when considering environmentally relevant concentrations (≤ 1 mg/L) compared with the control, manifested as the obvious reductions of average speed, and moved distance (by 5% and 8%, respectively). A change on locomotor activity of predators can further produce a negative effect

on their hunting, behaviour, exploration, competence, and escape capability to other predators, reducing their fitness and potentially impairing the ecosystem function. Thus, despite the lack of significant differences, the reduction of planarians locomotion (which was ~25% after 3 hours of feeding) can compromise their development/sustenance if a longer-term exposure (i.e., continuous feeding on contaminated prey) is considered.

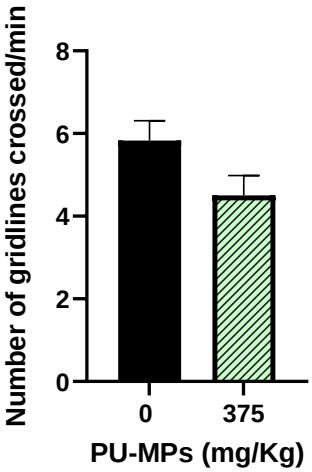


Fig. 21. *Girardia tigrina* locomotor activity, as the number of gridlines crossed per min, after exposure to contaminated prey (n= 6). Values are presented as mean ± standard error of the mean.

III.3.4. Biochemical biomarkers

Oxidative stress/damage and changes in metabolism and energy allocation are common biochemical responses observed in benthic invertebrates exposed to MPs (Li et al., 2022). Notwithstanding, in our case, no lipid peroxidation (indicative of oxidative damage) was observed between control and treatment groups (t= 0.4898, df=8, p= 0.6375, Figure 22). The absence of oxidative damage indicates that planarians were able to successfully counteract reactive oxygen species (ROS) through activating their antioxidant defence mechanisms (i.e., catalase and/or glutathione-S-transferase activity, which were not assessed in the present study, but triggered in previous studies on planarians). Han et al. (2022) examine the effect PS-MPs (1.0-µm; 10 µg/L) on the antioxidant defence system in *Dugesia japonica* and detected an increase in GST activity which is an important detoxification enzyme that is involved in the removal of harmful xenobiotics and endogenous compounds (Liu et al., 2020). After a 10 day exposure to PS-MPs

mixed in liver homogenate, the CAT activity in *D. japonica* increased, indicating a stress response to 10 µm PS-MPs (Gao et al., 2022).

In addition, figure 23 shows the energy reserves available, in combination with ETS, in planarians. Aerobic metabolism (estimated by ETS activity) increased by ~30% and carbohydrates (sugars) increased by ~50% in planarians fed on contaminated prey, which differences are marginally significant (ETS: t= 2.047, df=8, p= 0.0749, Fig. 23A; sugars: t= 2.185, df=8, p= 0.0604, Figure 23D). Proteins and lipids remained similar between planarians after consuming contaminated or uncontaminated prey (t= 1.225, df=8, p= 0.2554, Figure 23B; t= 0.2656, df=8, p= 0.7972, Figure 23C, respectively). These results were to be anticipated since planarians possess large reserves of proteins and fat as well as hydrocarbon reserves dispersed throughout the parenchyma and in the gastrodermis cells that allows them to sustain starving planarians for several months (Simão, 2022).

The existing majority of biochemical studies in planarians have evaluated oxidative stress responses and/or antioxidant enzymes activity but not energy-related biomarkers, especially on contaminants such as MPs, resulting in the current lack of literature.

Although future studies are needed, the combination of the results from the biochemical analysis indicates that planarians are not affected by this exposure to contaminated prey. This could be expected since planarian tissues are highly dynamic due to the presence of an abundant population of totipotent somatic stem cells that can make this system highly resistant to physical and chemical damage (Gambino et al., 2020). Moreover, the increased consumption of prey, although promoting the potential incorporation of more particles, also promote the input of more nutritional values that are able to cope with extra stress produced by MPs. This higher consumption is also obviously in good agreement with high activity of ETS and levels of proteins, sugars and lipids whose availability was sufficient to avoid oxidative damage to lipids since LPO was even slightly lower than control.

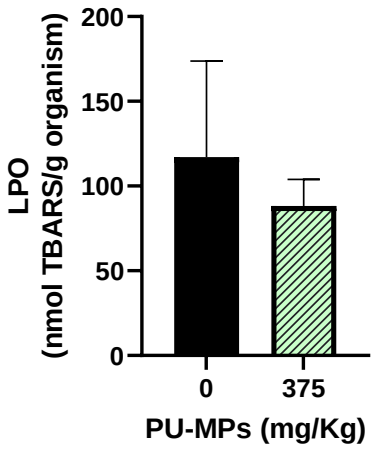


Fig. 22. Lipid peroxidation (LPO) levels in *Girardia tigrina* planarians after exposure to contaminated prey (n=5). All values are presented as mean ± standard error of the mean.

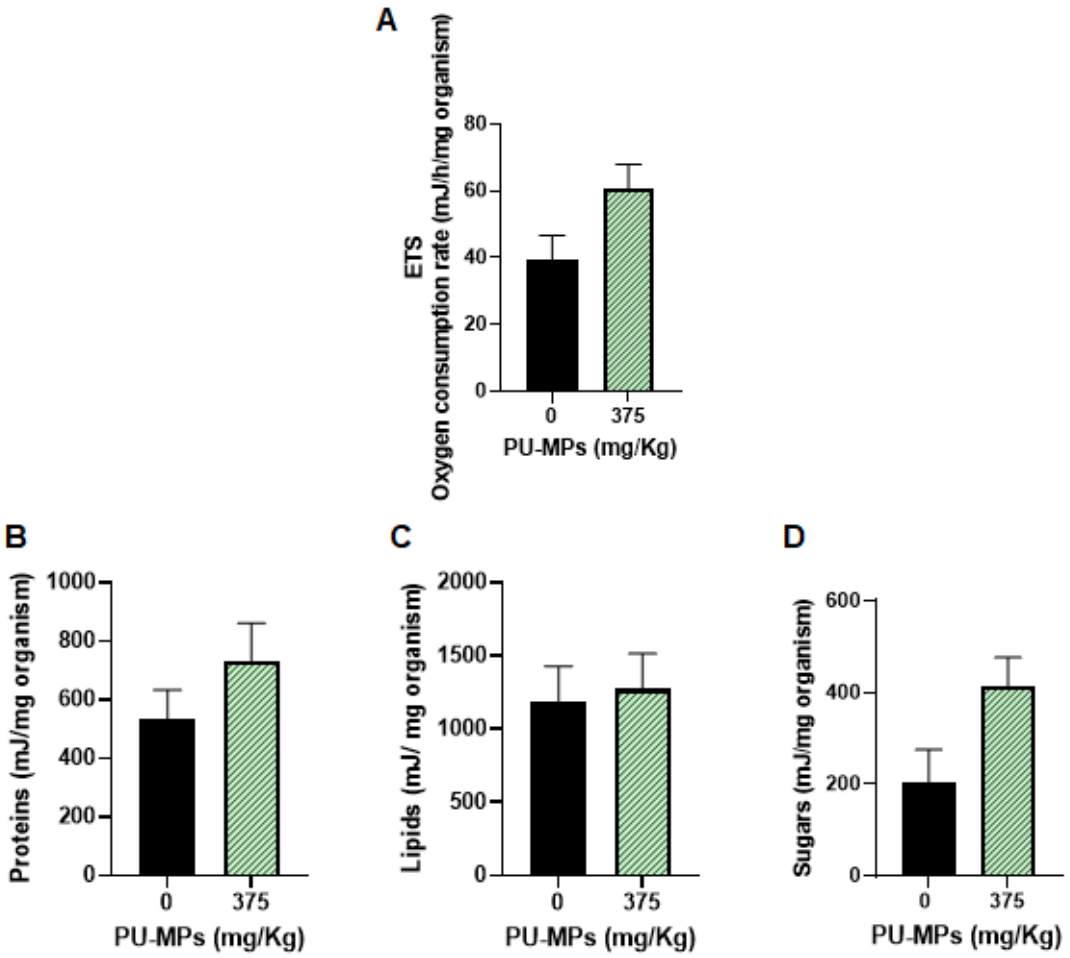


Fig. 23. Effect of polyurethane microplastics (PU-MPs) on (A) aerobic energy production (electron transport system, ETS, mJ/h/mg organism), (B) proteins, (C) lipids, and (D) sugars content (mJ/mg organism) of *Girardia tigrina* planarians after exposure to contaminated prey (n= 5). All values are presented as mean ± standard error of the mean.

Chapter IV

Results integration and conclusions

Final remarks and perspectives

IV.1. Results integration and conclusions

Integrating all the results obtained in this study, *C. riparius* larvae exposed to 375 mg PU-MPs/kg and size between 7.0 and 9.0 μm could ingest a considerably higher number of PU-MPs. They reflected slight changes in behavioural (locomotion) and biochemical (energy consumption) responses. Contaminated larvae revealed to be more active when foraging, which was reflected in the activation of their aerobic metabolism. Notwithstanding, in a short-term exposure of 10 days, PU-MPs did not originate quantifiable energy costs neither alterations of energy reserves on contaminated larvae. In view of this observation, that may justify the absence of effects on larval growth and emergence.

In a feeding assay, planarians presented a higher consumption (by ~20%) of contaminated prey than uncontaminated prey. This could be related to i) the potential lower nutritional value of the contaminated larvae prey that resulted in higher consumption of these (which was not observed in chapter II); or ii) the increased movements displayed by contaminated larvae (which was confirmed in chapter II). This change in larvae behaviour influenced planarian predatory behaviour also. As top predators with chemical and visual stimuli in their habitats, planarians may have been stimulated to predate more larvae due to the increase in curling and un-curling activity in contaminated chironomids. Behaviours like this are observed in organisms with predatory behaviour that tend to prefer slightly more active prey than larvae that shows lethargy (as reviewed by Deochand et al., 2018).

The consumption of more contaminated prey seems to be in good agreement with biomarkers assessed in planarians, since more nutritional value was reflected on higher aerobic energy production and levels of energy available to prevent oxidative damage that could have been triggered by the uptake PU-MPs (preliminarily confirmed via histological assessment – not presented on this thesis). This reinforces the relevance of the present study since it is the first to use live prey instead of contaminated chicken liver. Another innovative factor is the use of PU-MPs, which despite having the same range of sizes, is still a different polymer that can trigger different chemical responses.

Notwithstanding, but interestingly, the increased availability of reserves and capacity to aerobically produce energy, which seems to be allocated to compensate for potential oxidative damage, were insufficient to avoid a total lack of alterations on locomotor activity (a decrease of 25% on the distance/grids crossed) and regeneration of photoreceptors (delay of ~1 day) of exposed animals (despite the lack of statistical support). In addition, energetic requirements

allocated to detoxification and antioxidant processes significantly impaired the formation of auricles.

IV.2. Final remarks and future perspectives

In this study, it was possible to identify that at concentrations that were even much higher than those mentioned in the environment (i.e., 750 mg PU/Kg sediment), PU-MPs did not seem to derail survival and life history traits of *Chironomus riparius*. Notwithstanding, the few sub-organismal changes on chironomid larvae involved behavioural alterations in *Girardia tigrina* planarians. It is important to note that continued exposure could lead to other consequences. Gao et al. (2022) conducted a study which used contaminated food for 21 days and showed significant changes in the growth, regeneration and levels of antioxidant enzymes of planarians. Therefore, it is crucial to perform not only long-term studies involving a multigenerational assessment considering environmental relevant scenarios (including alive prey); but also, to study early-warning biochemical and physiological changes that can underpin microplastics toxicity.

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