Universidade de
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Poluição por microplásticos em ambientes dulçaquícolas: ingestão e efeitos em invertebrados bentónicos

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia e Ecologia das Alterações Globais (programa doutoral), realizada sob a orientação científica do Doutor João Luís Teixeira Pestana, (Investigador Auxiliar do CESAM e Departamento de Biologia da Universidade de Aveiro) e do Professor Doutor Carlos Alexandre Sarabando Gravato (Professor
Auxiliar da Faculdade de Ciências da Universidade de Lisboa)

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um âmago

no vulcão dos sentidos
a vida não é bem assim
não há princípio ou fim
só começa quando pegas
só acaba quando largas

não deixas nada ao mundo
nem coisa alguma levas
transformam-te energias
recebes e aprendes mais
do que pensas e ensinas

Joaquim Castro Caldas

o júri

presidente

Doutor **Rui Luís Andrade Aguiar**
Professor catedrático, Universidade de Aveiro

vogais

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palavras-chave

Macroinvertebrados aquáticos, microplásticos, stress oxidativo, ecotoxicologia sedimentar, ecossistemas dulçaquícolas.

resumo

Os microplásticos podem ser definidos como “qualquer partícula sólida de origem sintética ou matriz polimérica, de forma regular ou irregular e com tamanho entre 1 µm to 5 mm, tanto de origem de produção primária como secundária e que são insolúveis em água”.
A crescente procura por este tipo de produtos e consequentemente a sua maior produção, tanto à microescala (p. ex.: produtos de higiene pessoal), como à macroescala com descarte incorreto e consequente degradação no meio ambiente, tornaram os microplásticos num poluente emergente do ambiente aquático à escala global.
Assim sendo, os níveis de microplásticos que têm vindo a ser encontrados nos sedimentos de rios e lagos geram preocupação acrescida relativamente à sua potencial ingestão e efeitos gerados por esta em invertebrados bentónicos, particularmente aqueles que se alimentam de matéria orgânica particulada. Existe ainda pouca informação relativamente aos possíveis efeitos provocados pelos microplásticos nos diversos níveis de organização biológica (desde o nível suborganismal até à comunidade), bem como, em relação aos efeitos dos microplásticos em invertebrados sob cenários relevantes de exposição, em especial considerando as alterações climáticas.
Assim, o principal objetivo desta tese foi o de avaliar os efeitos de microplásticos com forma irregular de polietileno (um dos polímeros mais produzidos no mundo e também dos mais encontrados nos sedimentos de rios e lagos), usando uma abordagem holística que combina respostas ao nível sub-organismal, organismal, populacional e da comunidade, pretendendo deste modo facultar informação ecotoxicológica relevante que possa ser usada na avaliação de risco dos microplásticos em ambientes dulçaquícolas.
Para tal, a ingestão de microplásticos foi avaliada em termos quantitavos e qualitativos em duas espécies bentónicas -modelo, o díptero *Chironomus riparius* e o oligoqueta *Lumbriculus variegatus*, que sendo espécies que se alimentam de matéria particulada, estarão mais suscetíveis à ingestão de microplásticos. Paralelamente à avaliação da ingestão de microplásticos, foram também avaliados os efeitos (tanto a nível sub-celular como ao nível do organismo) numa perspetiva de relacionar ingestão e efeitos. Os principais efeitos observados prenderam-se assim com a ativação da resposta imune (medida através da atividade da fenoloxidase), stress e dano oxidativo, bem como efeitos ao nível do ciclo de vida em *C. riparius* (diminuição do crescimento larvar e atraso no desenvolvimento). Estes efeitos foram assim relacionados com a maior ingestão de microplásticos, quando comparada com a ingestão observada em *L. variegatus*, que somente apresentou sinais ligeiros de stress oxidativo e ausência de efeitos na reprodução.
A espécie mais sensível, *Chironomus riparius*, foi então exposta a microplásticos em combinação com diversos stressores naturais. Os stressores naturais escolhidos foram a temperatura, salinidade e limitação da disponibilidade de alimento, sendo estes relevantes em cenários de alterações climáticas, e capazes de alterar a ingestão e consequentemente os efeitos provocados pelos microplásticos.

resumo (cont.)

Os resultados demonstram que as interações decorrentes da exposição conjunta de microplásticos com os stressores naturais são complexas, sendo que os efeitos observados foram maioritariamente aditivos ou antagonísticos. Contudo, sob determinadas condições como baixa temperatura e significativa limitação da disponibilidade de alimento, os efeitos sub-letais dos microplásticos nas populações naturais de *C. riparius* poderão ser mais severos que o previsto pelos ensaios ecotoxicológicos padronizados.

Estes efeitos combinados revelaram ainda, que sob determinadas condições ambientais, os efeitos tóxicos dos microplásticos poderão não ser simplesmente um reflexo das concentrações de microplásticos encontradas no interior dos organismos.

A avaliação dos efeitos ecológicos da exposição a microplásticos é possivelmente, a principal lacuna que subsiste na investigação. Assim, esta tese procurou colmatar essa lacuna, com recurso a um ensaio usando rios artificiais (mesocosmos), em que foram estudados os efeitos na estrutura da comunidade de invertebrados bentónicos, bem como nas funções providenciadas pelo ecossistema, vitais para uma melhor avaliação de risco. Esta abordagem demonstrou que a exposição a microplásticos de polietileno de diversos tamanhos afetou a estrutura da comunidade, sobretudo através da redução da abundância de invertebrados coletores de depósito e raspadores. A ingestão de microplásticos pelos diferentes grupos funcionais de invertebrados correlacionou-se positivamente com os efeitos, uma vez que coletores de depósito e raspadores apresentaram o maior número de microplásticos no seu interior. Foi ainda observada uma ligeira redução na produção primária que poderá estar relacionada com um efeito direto dos microplásticos no crescimento do perifíton. De igual modo, observou-se apenas uma ligeira redução na decomposição da folhada, sendo este efeito indicativo de que a exposição a microplásticos de polietileno poderá não afetar significativamente o comportamento alimentar dos fragmentadores, ou em alternativa o tempo de exposição poderá ter sido demasiado curto para que estes efeitos indiretos se possam ter manifestado.

Em conclusão, a presente tese demonstra que os microplásticos podem ser ingeridos pelos macroinvertebrados de água doce e representar um risco para as populações naturais. Apesar de se ter verificado uma considerável variação interespecífica na sensibilidade aos microplásticos de polietileno, a sua presença nos sedimentos pode alterar a estrutura das comunidades de macroinvertebrados bentónicos e alterar o funcionamento dos ecossistemas a longo prazo. A presente tese comprova também que a resposta imune e o stress oxidativo são eventos-chave de início a nível molecular na toxicidade dos microplásticos, e demonstra que a avaliação das concentrações internas dos microplásticos é crucial para uma correta avaliação dos efeitos nos organismos. Salienta-se ainda a necessidade da incorporação de mais espécies de invertebrados, assim como, de cenários relevantes de exposição para uma avaliação de risco mais precisa dos microplásticos nos ecossistemas dulçaquícolas.

keywords

Aquatic macroinvertebrates, microplastics, oxidative stress, sediment ecotoxicology, freshwater ecosystems

abstract

Microplastics can be defined as “any synthetic solid particle or polymeric matrix, with regular or irregular shape and with size ranging from 1 µm to 5 mm, of either primary or secondary manufacturing origin, which are insoluble in water”. The growing demand and consequent production of plastic products both at microscale (e.g., personal care products) and at macroscale with inappropriate disposal subsequent degradation in the environment has turned microplastics into global emergent pollutants of the aquatic environment. In fact, the levels of microplastics that have been found in river and lake sediments, raise concern about ingestion and potential negatives effects of microplastics in aquatic benthic invertebrates, particularly those who feed on particulate matter. Possible effects at different levels of biological organisation (sub-organismal up to community levels) are still overlooked, as well as the study of the toxic effects of microplastics to invertebrate biota under relevant exposure scenarios, especially considering climatic change. Therefore, the objective of this thesis was to evaluate the effects of irregularly shaped polyethylene microplastics (one of the polymer type microplastics most produced and found in aquatic sediments) using a holistic approach that combines sub-organismal, organismal, population and community level responses, aiming to produce relevant ecotoxicological data for risk assessment of microplastics in freshwaters.

First, a quantitative and qualitative assessment of the ingestion of microplastics was evaluated in two model aquatic species, the dipteran *Chironomus riparius* and the endobenthic oligochaete *Lumbriculus variegatus*, as these species feed on particulate matter, being prone to ingest microplastics. Along with the assessment of the ingestion of microplastics, the effects (both at sub-cellular and organismal level) were assessed in an attempt to link ingestion and potential deleterious effects induced by the particles *per se*. Activation of immune response (phenoloxidase activity), oxidative stress and damage, as well as, effects on life-history traits (larval growth and development), were observed in *C. riparius* and associated to the augmented ingestion and retention of microplastics in their gut when compared to *L. variegatus*, which only presented slight evidence of oxidative stress and no effects on reproduction.

The combined exposure of microplastics with natural stressors was then performed in *Chironomus riparius (*the most sensitive species). Temperature, salinity and food shortage were used as natural stressors relevant under climate change scenarios and that could modulate the ingestion and induced effects of microplastics in relevant exposure scenarios.

The results show that interactions between microplastics exposure and these natural stressors are complex and mainly additive and antagonistic effects were observed. However, results also identified conditions (low temperatures and severe food shortage) where sub-lethal effects of MPs to natural populations of *C. riparius* can be stronger than predicted by standard toxicity assays. These combined exposures revealed that under different environmental conditions, toxicity of MPs is not simply a reflection of internal concentrations of particles.

abstract (cont.)

Conceivably the main research gap that subsists is the investigation on the ecological effects of microplastics. For that purpose, this thesis performed a mesocosms approach addressing the effects at the community structure and ecosystem functioning, vital to deliver higher-tier risk assessments. This higher-tier approach (i.e., using artificial streams inoculated with natural benthic invertebrate communities) showed that the exposure to a pool of different-sized polyethylene microplastics affected the macroinvertebrate community structure, mainly by reducing the abundance of deposit-feeders and grazers. The ingestion of microplastics by different invertebrate feeding groups correlated positively with the effects as deposit-feeders and grazers presented the higher number of microplastics inside the organisms' guts. A slight reduction in primary production was observed and may be a possible direct effect of microplastics on periphyton growth. Only slight effects were observed in terms of leaf litter decomposition signalling that the exposure to polyethylene microplastics might not have affected feeding behaviour of shredders or in turn that exposure period was too short to reveal trait-mediated indirect effects.

In conclusion, the present thesis demonstrates that microplastics can be ingested by some aquatic macroinvertebrates and that enhanced ingestion can potentially pose a risk to natural invertebrates' population. Despite considerable interspecific variation in sensitivity to polyethylene microplastics, their presence in sediments can alter community structure and represent a risk to ecosystem functioning at the long-term.

The present thesis confirms that immune responses and oxidative stress as key molecular initiating events in microplastic toxicity, shows that assessing the internal concentrations of plastic particles is critical for a correct evaluation of effects in biota and highlights the need to incorporate more invertebrate species and relevant exposure conditions for an accurate risk assessment of microplastics in freshwaters.

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Chapter 1

General Introduction

General Introduction

1. (Micro)plastics: origin, sources, and pollution

The word “Plastic” is derived from the Latin *plasticus* and the Greek *plastikos*, both meaning ‘able to be moulded, pertaining to moulding’. The first plastic was developed in 1855 to replace ivory, prior to modern plastic manufacturing. Presented at the 1862 Great Exhibition in London by Alexander Parkes, the plastic named *parkesine* won the third prize (Friedel, 1979).

Nowadays the term ‘plastic’ is used to define a sub-category of the larger class of materials called polymers. Polymers are very large molecules that have a characteristically long chain-like molecular architecture and therefore very high average molecular weights. The first synthetic plastic was invented in 1909 by Leo Hendrik Baekeland and was called Bakelite. Developed from phenol and formaldehyde, bakelite is considered the first true plastic (first fully synthetic plastic — meaning it contained no molecules found in nature) (Bijker, 2012).

The production of plastics and plastic-based products has been growing since the 1950s and so is their use and disposal (GESAMP, 2016). Ever since the beginning of the mass production of plastics, the production volume of synthetic polymers increased more than 239-fold to 359 Million tons (Mt) per year until 2018 (PlasticsEurope, 2019). Although the demand for plastics in Europe has been stabilizing and even slowly decreasing in recent years due to EU directives (e.g. European Directive 2019/904 on the reduction of the impact of certain plastic products on the environment), the demand continues to grow in markets like China and North America (PlasticsEurope, 2019). Furthermore, phenomena like the on-going Covid-19 pandemic is and will lead to a much higher production of plastics used for personal protection equipment (gloves, face masks, air-purifying respirators, goggles, face shields, respirators, and gowns) (Livingston et al., 2020; Patrício Silva et al., 2020, 2021).

Plastics are made of virgin plastic resin pellets or resins mixed (or blended) with numerous additives to enhance the material properties. The most common additives include fillers, plasticizers, colorants, stabilizers, and processing aids (Wensing et al., 2005). In 2018, plastic production has led to 6.9 Mt of plastic waste (~3.2 Mt for short-life products). The management of such plastic waste remains unsustainable, with a considerable part (42% in 2018) still ending-up inefficiently treated or irresponsibly and incorrectly discarded (i.e., either littered or inadequately disposed of in dumps or open land-fills) (de Souza Machado et al., 2018; Hahladakis and Iacovidou, 2018; Strungaru et al., 2019).

Once discarded, plastic items undergo deterioration and fragmentation processes, originating plastic debris of smaller size. Among them are microplastics (MPs), defined as *“any synthetic solid particle or polymeric matrix*, *with regular or irregular shape and with size ranging from 1 µm to 5 mm*, *of either primary or secondary manufacturing origin*, *which are insoluble in water*” (Frias and Nash, 2019). Despite the wide consensus in this definition, some institutions like the European Food and Safety Authority extend the definition of microplastic size down to 100 nm (EFSA, 2016). MPs resulting from the above described processes, i.e., fragmentation of large

plastic debris, are designated “secondary MPs” and are frequently reported as being mainly fibres or fragments (Browne et al., 2011; Cole et al., 2011). Plastic particles can also be intentionally produced at the microscale being denominated primary MPs (Fendall and Sewell, 2009; Cole et al., 2011; Horton et al., 2017b; Brennholt et al., 2018; Lambert and Wagner, 2018). Examples include microbeads for use as scrubbers in cosmetics or in abrasive products; or pellets used as feedstock for plastic production).

Taking into account the production and life-cycle of plastics it is expected that higher amounts of plastic debris and MPs end-up at shorelines near plastic processing plants where scrubbers or microbeads are present in industrial and domestic wastewater, entering the marine system via rivers and estuaries (Hidalgo-Ruz et al., 2012). Along with these primary MPs, fragments originated from larger plastic debris such as fishing nets, line fibres, films, industrial raw materials, consumer products and household items, and raw industrial pellets are ubiquitously found in marine systems (Hidalgo-Ruz et al., 2012; Free et al., 2014). In fact, the amount of plastic debris that goes into the seas and oceans is estimated to be 4–12 million tonnes/year, with a prediction for them to outweigh the amount of fish in 2050 (Picó and Barceló, 2019).

2. Environmental concentrations of microplastics in freshwater systems

Early in the XXI century, plastic debris was identified as a main environmental concern and reported as potentially implicated in the loss of biodiversity (Gall and Thompson, 2015; Sutherland et al., 2016). Characterised by a small size, MPs meet no boundaries, and have been found even in the most remote areas (Thompson et al., 2004; Lusher et al., 2013; Law and Thompson, 2014; Gall and Thompson, 2015). The majority of monitoring studies on MPs started on marine environments (Andrady, 2011; Hidalgo-Ruz et al., 2012; Wang et al., 2016; Auta et al., 2017), but recent attention has been driven to freshwaters as they constitute the main repositories and “highways” of MPs to marine environments (Lebreton et al., 2017). A survey quantified microplastic litter entering the Black Sea coming from the Danube River to be at a rate of 4 ton day^{-1} (Lechner et al., 2014). Human population and especially big urban and human consumption centres are known to be located closer to shore and to freshwater bodies. Thus, waste water treatment plants, household sewage discharge (Fendall and Sewell, 2009; Karlsson, 2015; Sun et al., 2019; van Emmerik and Schwarz, 2020), and plastic production plants (Lechner et al., 2014; Zbyszewski et al., 2014; Karlsson et al., 2018; van Emmerik and Schwarz, 2020) are among the main sources of MPs to freshwater ecosystems. These primary and secondary MPs are therefore not only transported to marine environment but also likely to have long residence times in freshwater systems (Browne et al., 2011; Eerkes-Medrano et al., 2015; van Emmerik and Schwarz, 2020). Especially since the 2010s, reports of MPs contamination within freshwater ecosystems include rivers and lakes across America (Castañeda et al., 2014; Anderson et al., 2017), Europe (Lechner et al., 2014; Klein et al., 2015; Hurley et al., 2018), Asia (Su et al., 2016; Wu, C., Zhang, K., & Xiong, 2018) and Africa (Biginagwa et al., 2016; Akindele et al., 2019) and include both plastics from primary and secondary origin (Lasee et al., 2017). Degradation of larger plastic debris occurs

in freshwater environment just like in the marine environment and is driven by physical, mechanical, and chemical degradation phenomena, oxidative weathering and biological/biochemical degradation (Fig. 1) (Kowalski et al., 2016; Kaiser et al., 2017).

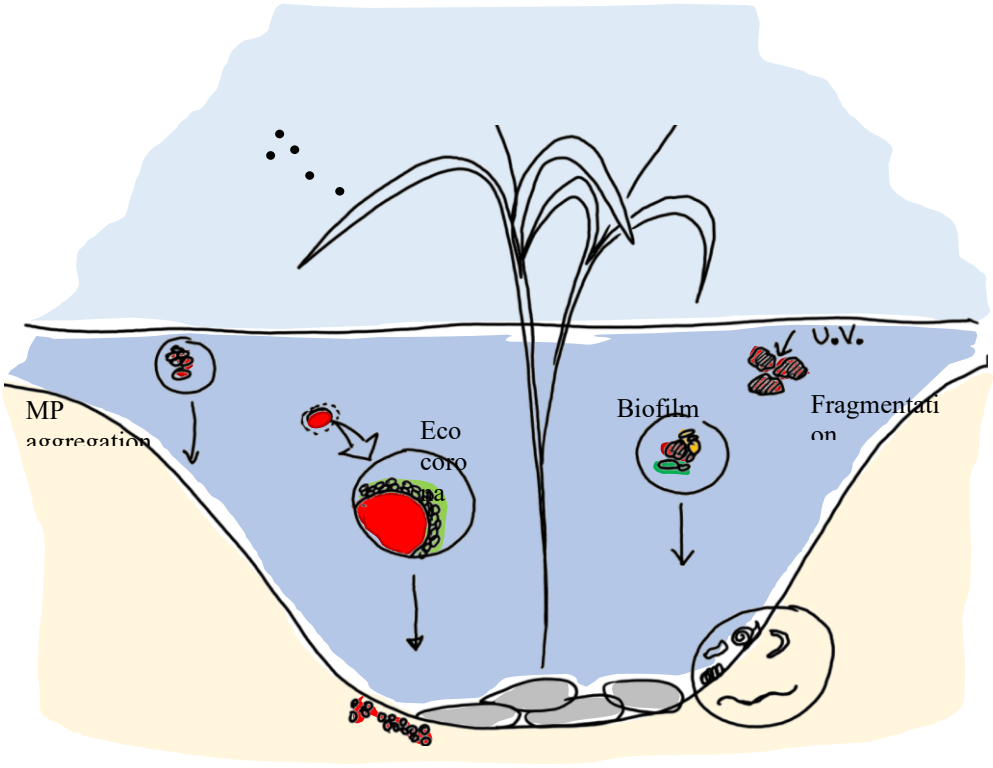

Fig. 1. Degradation processes of larger plastic litter that produce microplastics (physical, chemical and biological/biochemical degradation) and processes that allow for microplastics' deposition in freshwater sediments (aggregation, colonization and weathering).

Freshwater ecosystems are, thus, susceptible to microplastic pollution as they have highly populated areas and plastic production plants nearby (Mani et al., 2015; Murphy et al., 2016; Ziajahromi et al., 2016; Wang et al., 2017). Since most rivers and lakes have intense human activity near its shores it is not surprising that recent reports of microplastic occurrence in freshwater bodies show levels comparable to marine shoreline contamination hotspots (Hurley et al., 2018).

Dispersal and deposition of MPs within freshwater ecosystems depend on many factors. Some of the most important include river currents, flow, water depth, substrate type, bottom topography and seasonal variability (Simpson et al., 2005; Moore et al., 2011; Eriksen et al., 2013; Rocha-Santos and Duarte, 2015). Factors that may have a temporal aspect include tidal cycle (in estuaries); storms; floods, or anthropogenic activity (e.g., dam release) (Moatar et al., 2006; Kessarkar et al., 2010; Hurley et al., 2018). Despite all external factors, MPs characteristics like size, shape and density can play a decisive role in their dispersal, potential deposition and settling location within freshwater bodies. Common plastics' density ranges from 0.85 to 1.41 g cm^{-3}, with polypropylene and low/high density polyethylene (LDPE, HDPE) plastics having densities lower than 1g cm^{-3}, and polystyrene, nylon, polyvinyl chloride (PVC), and polyethylene terephthalate

(PET) having densities higher than 1 g cm^{-3}. It would be expected that plastics under 1g cm^{-3} would be transported in water column and eventually ending up in the ocean without significant deposition in freshwater sediments. However, polypropylene and polyethylene (density lower than 1g cm^{-3}) are among the most commonly found MPs in freshwater sediments (Yuan et al., 2019; B. Zhang et al., 2020; Scherer et al., 2020). Hydrodynamic conditions, biogeochemistry of rivers, colonization of MPs by microbial communities and microalgae for example can increase their density and be a decisive factor for their deposition (Kaiser et al., 2017; Hoellein et al., 2019). All these processes that increase the MPs deposition, make freshwater sediments not only a repository of high-density plastic debris (1.2–1.8 g cm^{-3}), but also of the ones with low-density (< 1.025 g cm^{-3}) (Hurley et al., 2018).

MPs concentrations on river water column and sediments are very heterogeneous. Concerning European rivers, MPs concentrations in water between as less as 0.03 (Mani et al., 2019) and up to 187,000 MPs m^{-3} have been found (Leslie et al., 2017). MPs in river water can also present a broad range of shapes including spheres, fibres, fragments and foils with varying relative abundances. Fragments and spheres are mostly predominant in European rivers (Lechner et al., 2014; Mani et al., 2015, 2019). In terms of polymer types, the buoyant MPs (polyethylene – PE; polypropylene -PP; and polystyrene -PS) are naturally predominant in the water column (Mani et al., 2015, 2019; Schmidt et al., 2018). Likewise, in European river sediments, the concentrations of MPs found are also very diverse ranging from as low as 18 particles kg^{-1} sediment (Rodrigues et al., 2018) to studies reporting MP concentrations around or higher than 1 g kg^{-1} or 4000 particles kg^{-1} (Klein et al., 2015; Scherer et al., 2020) reaching 72,400 particles kg^{-1} sediment (around 9 g kg^{-1} sediment) (Hurley et al., 2018) with fragments, fibres and spheres constituting the major fractions (Klein et al., 2015; Horton et al., 2017a; Leslie et al., 2017; Hurley et al., 2018; Rodrigues et al., 2018; Tibbetts et al., 2018; Blair et al., 2019). These high MP levels have also been reported in African and North American river and lake sediments (Castañeda et al., 2014; Merga et al., 2020). Polymer type composition found in sediments is commonly more diverse than in the water column, comprising buoyant-polymer types such PE, PP and PS, but denser polymers like polyvinyl chloride (PVC), polymethyl methacrylate (PMMA) and even dye particles (Klein et al., 2015; Horton et al., 2017a; Tibbetts et al., 2018) are also found. Buoyant MPs can sink into sediments as they can undergo biological and physical processes such as biofouling and homo- or hetero-aggregation (Lagarde et al., 2016; Lambert et al., 2017) that are known to modify MPs' density and floatability and to accelerate their deposition in sediments (Fig. 1) (Corcoran, 2015). Thus, buoyant MPs like PE-MPs are frequently a major fraction of the MPs found in freshwater sediments (Rodrigues et al., 2018; Li et al., 2019; Scherer et al., 2020).

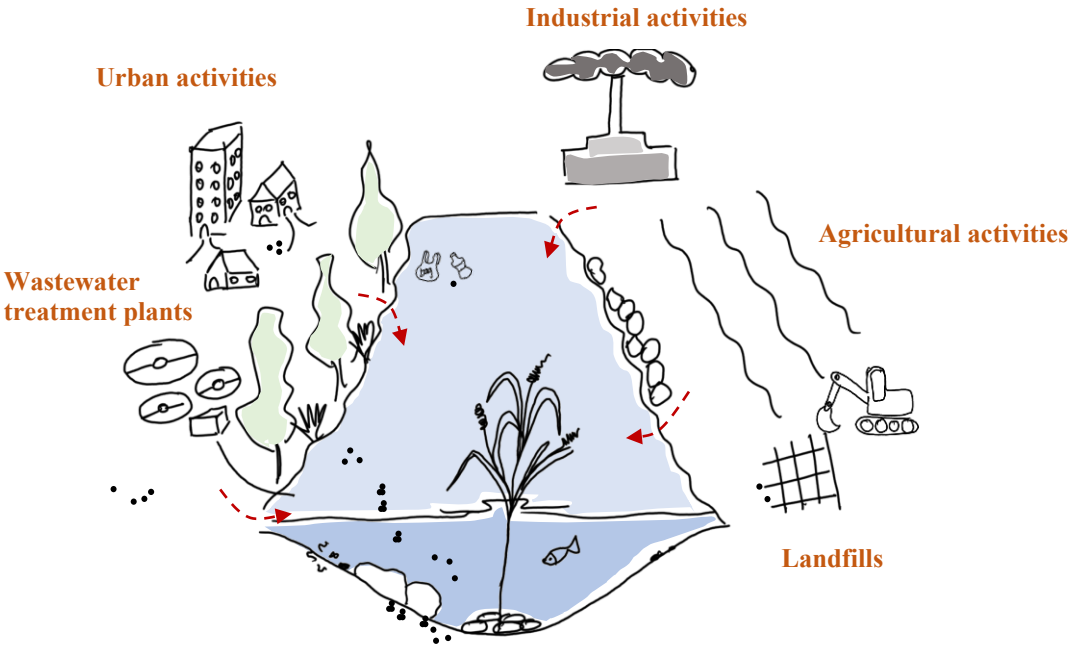

Fig. 2. Main sources and drivers of microplastics to the freshwater environment.

The existence of MPs contamination hotspots in European rivers have been associated with multiple pollution sources related to urbanization (Mani et al., 2015; Schmidt et al., 2018; Tibbetts et al., 2018), industry (Mani et al., 2015) and land run-offs (Horton et al., 2017b). Wastewater treatment plants (WWTPs) and landfill leachates have been pointed as another relevant source of MPs, mostly associated with inefficient MPs removal, particularly of small-sized MPs (i.e., < 10-12 μm in size; Fig. 2) (Mani et al., 2015; Leslie et al., 2017; Praagh et al., 2018; Schmidt et al., 2018; Silva et al., 2021). However, other studies found high concentrations of MPs in sediments in small rivers far from densely populated areas, industries, or wastewater treatment plants (Klein et al., 2015). Hydrodynamic processes seem to explain the possibility of MP high deposition areas distant to the direct sources (Alam et al., 2019; Li et al., 2020). In addition, meteorological events may strongly impact MP levels in European river systems, driving the dynamics of MPs accumulation in river sediments and release to the oceans (Hurley et al., 2018; Schmidt et al., 2018).

Analytical methods for MP sampling, extraction and quantification are lacking optimized procedures, and generally tend to underestimate small sized MPs (Fendall and Sewell, 2009; Prata et al., 2019a). For example, sampling MPs in water is performed by making use of a great variety of nets that have different mesh sizes and therefore allow the quantification of larger rather than smaller size MPs. The use of pumps theoretically allow to estimate all MPs size, but have limitations in terms of the volume of water that can be sampled (Prata et al., 2019a). A wide set of methodologies is also used to sample MPs in sediments. Instruments like corers with different diameters, box corers and grabs are some of the most commonly used for sampling (Vianello et al.,

2013; Castañeda et al., 2014; Naidoo et al., 2015). The area or volume of sediment sampled can also be very different (Imhof et al., 2013; Faure et al., 2015) and the same can be said concerning the water sampling (McCormick et al., 2014; Dris et al., 2015; Karlsson et al., 2017; L. Zhang et al., 2020). Sediment samples need a further digestion to eliminate the organic fraction necessary for MPs extraction. Digestion procedures include the use of nitric acid (Avio et al., 2015), hydrochloric acid (Cole et al., 2014), sodium hydroxide (Hurley et al., 2018), potassium hydroxide (Cole et al., 2014), hydrogen peroxide (Stock et al., 2019), proteinase-k and protease (Cole et al., 2014; Li et al., 2018; Stock et al., 2019), and also combination of the above mentioned digestion agents (Lusher et al., 2017). Same procedures are also applied to biological samples. Each method used to extract MPs has their pros and cons, which compromise MP extraction and counting. For instance, the use of acids are quick procedures but can deteriorate MPs or alter their surface, leading to an underestimation of the particles. Conversely, the use of enzymatic protocols does not affect MPs surface and integrity, allowing a good counting; but they are also quite expensive, time consuming, and require optimization for each type of sample (e.g., biological samples rich in chitin or cellulose or calcium carbonate). After the digestion process is complete, a density separation process can be required in sediment samples to discriminate the MPs from the rest of the particles. Again, multiple protocols are used but the most common use sodium chloride (Corcoran, 2015), zinc chloride (Stock et al., 2019), or sodium iodide (Stock et al., 2019) for the density separation. The use of a hypersaline sodium chloride solution seems the best compromise between MP extraction efficiency and costs, but high-density MPs are often overlooked. After this clean-up process, samples are usually filtrated, and MPs get retained in the filters. Again there is not a consensus in the type of filters to use: nitrocellulose, silicon, polycarbonate, anodisc, or glass fibre (Campanale et al., 2020). Usually, the choice of the filtration process is based on the requirements of chemical characterization and/or objective of the study (e.g., size of MPs to be counted).

The counting process of larger MPs can be performed under a stereomicroscope (Song et al., 2015) or making use of automatic image analysis approaches with and without previous staining procedures (e.g., Nile Red and Rose Bengal dyes) (Prata et al., 2019b; Primpke et al., 2019; Lorenzo-Navarro et al., 2020). The main advantage attributed to the automatic image analysis is to reduce the data calculation time and human bias during manual data analysis.

After the estimation of the number of MPs, occasionally there is a need to confirm and characterize the MPs in terms of their chemical composition. The identification of MPs makes use of a wide range of complementary analytical technologies, namely thermo-analytical techniques (pyrolysis–gas chromatography–mass spectrometry, Py–GC–MS; and thermal extraction desorption–gas chromatography–mass spectrometry, TED–GC–MS), and the more commonly used spectroscopic techniques (Raman and Fourier-transform infrared spectroscopy, FTIR) (Hermabessiere et al., 2018; Xu et al., 2019). The spectroscopic techniques are non-damaging for the particles and allow for further process with other techniques (e.g., Py–GC–MS) to obtain additional and complementary information on the composition of plastic polymers including the identification of additives or other compounds that are adsorbed to MPs (Fries et al., 2013;

Peñalver et al., 2020). Attenuated total reflectance (ATR) technique coupled with FTIR spectroscopy (ATR-FTIR) is usually used to characterize larger MPs, while smaller MPs require the use of μ-FTIR (or μ-Raman). In this case, usually μ-FTIR is coupled with focal plane array detector that enables a much faster generation of chemical imaging of MPs by simultaneously scanning thousand spectra within a single measurement (Chen et al., 2020). Traditional spectroscopic techniques have as main disadvantages, the absence of information about associated organic additives to MPs, and possible overlap of polymer bands given by organic and inorganic contaminations that can disturb identification of particles (Käppler et al., 2018). On the other hand, thermo-analytical methods (Py–GC–MS and TED–GC–MS), less conventional than spectroscopic alternatives, are destructive techniques that produce, by pyrolysis, decomposition products characteristic of each polymer, that get trapped on a solid-phase adsorbent (Hendrickson et al., 2018). The volatile marker compounds are then separated by gas chromatography and identified by mass spectrometry. As previously mentioned, these techniques allow not only to identify the polymer itself, but also other compounds present in samples, such as additives and contrarily to Raman or FTIR that only investigate the surface of a particle, the Py–GC–MS analyses the whole particle (Hermabessiere et al., 2018). Whole particle analysis is of great value and Py-GC-MS can also allow the MPs mass determination. Among the disadvantages of Py–GC–MS, is the destruction of the particles and the limit in the selection of the particle size class (~50/100 μm). In addition to these traditional techniques, other techniques have emerged and gained relevance in the characterization of MPs. A novel technique is the use of isotope ratio mass spectrometry (IRMS) to characterize polymers (Birch et al., 2021). Among the advantages listed, IRMS data seems to be more reliable in polymer identification than traditional FTIR and Raman microscopy alone, and for being sensible to distinguish weathered samples by using the simulated weathering conditions of ultraviolet (UV) light and heat, IRMS provides unique information about age and geographic origin of polymer hindered progress (Birch et al., 2021).

In resume, the continuous search for better and optimized methodologies for sampling, counting and characterizing the MPs will in the near future assure comparability between studies and overcome some limitations that still exist. For example, MP levels are sometimes substantively underestimated as a result of sampling procedures that frequently exclude MPs under 500 μm (Castañeda et al., 2014); 150 μm (Merga et al., 2020) or 63 μm (Klein et al., 2015; Hurley et al., 2018). It is essential to correctly address the problem of deficient sampling and identification of the smaller microplastic fraction since smaller MPs (<300 μm) are frequently reported to be the major fraction in European rivers such as the Rhine (Mani et al., 2019) and Elbe (Scherer et al., 2020) rivers. As an example, the average abundance of the different size MPs for the Elbe river is presented in Figure 3 (Scherer et al., 2020).

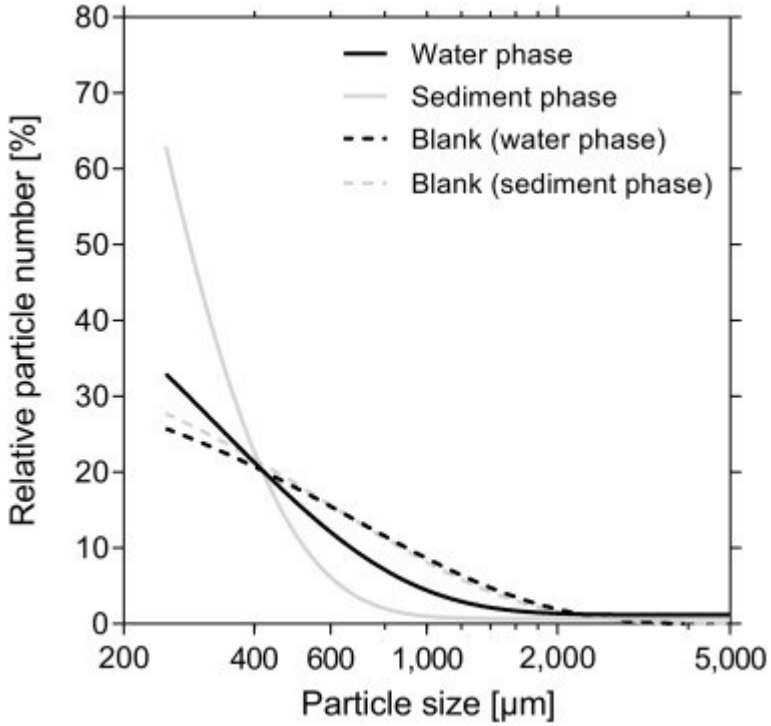

Fig. 3. Average size distributions of tentative MPs in the water and sediment samples from the Elbe river and the corresponding blanks. For better comparability, only particles with a size of 150-5000 µm were included. Source: Scherer et al., 2020

3. Ingestion of MPs by freshwater biota

The uptake of MPs occurs by invertebrate biota can generally occur through the adherence of MPs to soft tissue in organisms such as mussels, clams and copepods (Cole et al., 2013; Li et al., 2016; Kolandhasamy et al., 2018a; Su et al., 2018), and more frequently for accidental or intentional ingestion and likewise be transferred via food webs (Wang et al., 2019; Merga et al., 2020). As MPs are present in both the water column and the sediment, their uptake by ingestion is verified in pelagic and benthic vertebrates and invertebrates (Lusher et al., 2017; Scherer et al., 2017; Silva-Cavalcanti et al., 2017; Nel et al., 2018; Windsor et al., 2019; Zhang et al., 2019).

Most research addressing the presence of MPs inside freshwater biota have been focused on vertebrates, namely fish and with less studies assessing the presence of MPs in invertebrates (Table 1). All of these investigations showed evidence of MP ingestion, either through direct consumption or via trophic transfer. Considering invertebrates, considerable higher levels of MPs have been observed in annelids, dipteran larvae and bivalves species; thus, being proposed as bioindicators of MP pollution in freshwater environments (Hurley et al., 2017; Hu et al., 2018; Nel et al., 2018; Su et al., 2018) (Table 1). Aside from MPs size, shape and composition, organisms feeding behaviour, ecophysiology, and habitat (particularly benthic) are pointed as key factors that modulate species susceptibility to MPs ingestion and to their potential toxic effects (Hurley et al., 2017; Hu et al., 2018; Windsor et al., 2019). For instance, despite feeding by filtration and being able to partially regulate and select filtered particles based on their size, shape, nutritive value or chemical component, bivalves still accumulate MPs probably due to their hydrophobicity and

tendency to agglomerate in the gut (Kolandhasamy et al., 2018b). Annelids and dipteran larvae ingest organic matter along with sediments to fulfil their nutritional requirements. With such procedures, these sediment-dwelling and sediment-ingesters can also ingest a considerable number of MPs. The capacity of egesting these particles rely on their organisms' gut-physiology (as further explored in section 1.4).

Table 1. Field studies reporting MP particles in freshwater biota along with element(s) sampled in each organism, reporting unit, and size ranges.

Organism	Type of biological sample	Reporting unit	Size range	Reference
Vertebrates (*Gobio gobio*)	Gastrointestinal tract			(Sanchez et al., 2014)
Vertebrates (multiple species)	Gastrointestinal tract		All (<5mm)	(Phillips and Bonner, 2015)
Vertebrates (multiple species)	Gastrointestinal tract	MPs/organism		(Faure et al., 2015)
Vertebrates (multiple species)	Gastrointestinal tract	MPs/organism	50 µm to 5 mm	(Holland et al., 2016)
Vertebrates (*Lepomis macrochirus*; *Lepomis megalotis*; *Centrarchidae*	Gastrointestinal tract	Mean MPs/organism		(Peters and Bratton, 2016)
Vertebrates (*Lates niloticus*; *Oreochromis niloticus*)	Gastrointestinal tract			(Biginagwa et al., 2016)
Vertebrates (*Hoplosternum littorale*)	Gastrointestinal tract	MPs/organism	1-12mm	(Silva-Cavalcanti et al., 2017)
Vertebrates (multiple species)	Gastrointestinal tract	Mean MPs/organism		(Campbell et al., 2017)
Vertebrates (multiple species)	Gastrointestinal tract	MPs/organism	60 µm to 4.7 mm	(Pazos et al., 2017)
Vertebrates (multiple species)	Gastrointestinal tract	Mean MPs/organism		(Vendel et al., 2017)
Vertebrates (*Platichthys flesus; Osmerus eperlanus*)	Gastrointestinal tract	Mean MPs/organism		(McGoran et al., 2017)
Vertebrates (multiple species)	Gastrointestinal tract	MPs/organism	40 µm to 5 mm	(Jabeen et al., 2017)
Vertebrates (*Rutilus rutilus*)	Gastrointestinal tract	MPs/organism	All (<5mm)	(Horton et al., 2018)
Vertebrates (*Squalius cephalus*)	Gastrointestinal tract, liver, and muscle tissue	g/stomach content	390 µm to 7.38 mm	(Collard et al., 2018)
Vertebrates (*Neogobius melanostomus*)	Gastrointestinal tract	MPs/organism	<1.5 mm, 1.6–3.2 mm, >3.3 mm	(McNeish et al., 2018)
Vertebrates (*Gobio gobio; Rutilus rutilus*)	Gastrointestinal tract	MPs/organism		(Kuśmierek and Popiołek, 2020)
Vertebrates (*Clarias gariepinus*; *Cyprinus carpio*; *Carassius Carassius*; *Oreochromis niloticus*)	Gastrointestinal tract	MPs/organism		(Merga et al., 2020)
Vertebrates (multiple species)	Whole body	MPs/organism	<0.5 mm	(Hu et al., 2018)
Vertebrates (multiple species)	Whole body	MPs/organism	<1 mm	(Kolenda et al., 2020)
Invertebrates (*Tubifex tubifex*)	Whole body	MPs g^{-1} (wet weight)	55 µm to 4.1 mm	(Hurley et al., 2017)

Invertebrates (*Chironomus* spp.)	Whole body	MPs mg^{-1} (wet weight)		(Nel et al., 2018)
Invertebrates (*Corbicula fluminea*)	Soft tissue	MPs g^{-1}	0.021–4.02 mm	(Su et al., 2018)
Invertebrates (multiple species)	Gastrointestinal tract/Soft tissue	MPs/organism	41 µm to 9 mm.	(Bour et al., 2018)
Invertebrates (Baetidae; Heptageniidae; Hydropsychidae)	Whole body	MPs mg^{-1}	500 µm to 5 mm	(Windsor et al., 2019)
Invertebrates (*Lanistes varicus*; *Melanoides tuberculata*)	Whole body	Mean MPs g^{-1} (wet weight)		(Akindele et al., 2019)
Invertebrates (*Chironomus* spp.)	Whole body	MPs g^{-1} (wet weight)		(Dahms et al., 2020)

4. Ecotoxicological effects of MPs on benthic invertebrates

Along with the recognition of benthic invertebrates as putative indicators of MPs contamination, a need to understand ecotoxicological effects due to exposure and ingestion of plastic particles has been growing. The establishment of relevant exposure scenarios is hampered by the MPs heterogeneous presence in freshwater sediments and specific characteristics such as shape, size, and polymer composition (O'Connor et al., 2020). Additionally, the key-factors triggering effects and the mechanisms involved in MPs toxicity are not yet fully understood. Nevertheless, exposure to MPs and related effects have been studied for a broad range of species (Cole et al., 2015; Ziajahromi et al., 2017; Murphy and Quinn, 2018; Redondo-Hasselerharm et al., 2018b, 2018a; Weber et al., 2018; Stanković et al., 2020).

Despite this growing number of publications, most research has tested concentrations relatively higher than the levels reported in the field (Redondo-Hasselerharm et al., 2018b; Weber et al., 2018; Bhattacharya and Khare, 2020). Identified negative effects of MPs on invertebrates cover various levels of biological organization ranging from suborganismal level (e.g., inflammation, reduced antioxidant capacity, immune response, neurotoxicity, oxidative damage, alterations in energy reserves and energy metabolism and changes in gut microbiome) (Oliveira et al., 2013; Wright et al., 2013b; Avio et al., 2015; Jeong et al., 2017; Ribeiro et al., 2017; Espinosa et al., 2018; Gardon et al., 2018; Lei et al., 2018; Lu et al., 2018); individual level (e.g., survival, growth, feeding, emergence) (Redondo-hasselerharm et al., 2018; Cole et al., 2019; Stanković et al., 2020); population level (e.g., abundance, biomass and reproduction) (Bosker et al., 2019), and to community level effects (mainly community structure) (Green, 2016; Redondo-Hasselerharm et al., 2020). Conversely, other studies reported no significant effects on apical endpoints like growth, development, reproduction and survival (Imhof and Laforsch, 2016; Khosrovyan and Kahru, 2020) and at suborganismal level (e.g., no changes in gut microbiome, oxidative stress related endpoints or energy reserves) (Horton et al., 2020; Weber et al., 2021).

Most of the studies link individual and suborganismal level effects to physical damage (i.e., blockage of gut passage or mechanical damage of gut epithelium), leading to a feeling of satiation, and reduced feeding (Straub et al., 2017; Ziajahromi et al., 2017, 2018; Magara et al., 2019) while

others link the effects to chemical composition of the polymers and their leachates (Au et al., 2015; Blarer and Burkhardt-Holm, 2016).

From almost all ecotoxicological investigations, it is clear that the most important limitation identified has hampering the adequate risk assessment of MPs is the lack of consistency and standardization of microplastic dosing and test methods necessary to characterize dose–response for specific endpoints (de Ruijter et al., 2020). Recent reviews have been focused on the systematic documentation of the main effects induced by plastic particles (Kögel et al., 2020) and on the establishment of MPs Adverse Outcome Pathways (AOPs) knowledgebase (Hu and Palić, 2020). The establishment of these AOPs requires the joint-knowledge of the molecular initiating events (e.g., formation of reactive oxygen species) that lead to adverse outcomes (e.g., reduced growth) through a series of key-events such as oxidative stress cascades and inflammatory responses. Therefore, assessing the effects at molecular, cellular, organ/tissue, and individual/population level is key to understand the pathway that leads to the adverse outcomes (individual/population).

Molecular biomarkers have been extensively used in ecotoxicological assays and they provide information below-individual level, measured inside an organism or in its products (urine, faeces, hairs, feathers, etc.), indicating a departure from the normal status, that cannot be detected from the intact organism (Van Gestel and Van Brummelen, 1996). So, the use of biomarkers provides information on the molecular initiating events, the first link in the chain that leads to possible individual and population level events. Biomarkers are thus frequently used as early warning indicators of effects at higher levels of biological organization. Concerning ecotoxicological evaluation of MPs, a set of possible biomarkers should be used, not only to address the knowledge gaps relatively to the biochemical effects but also considering molecular initiating events that have been already proposed for MPs such as the formation of reactive oxygen species/oxidative stress and also weaker evidence of other possible key molecular events such as inflammation and energy metabolism (Jeong and Choi, 2019; Hu and Palić, 2020).

Reactive oxygen species (ROS) are a consequence of aerobic processes and the accumulation of ROS occurs when the equilibrium between their production and elimination is disrupted. The excessive formation and accumulation of ROS leads to oxidative stress and oxidative damage, i.e. to damage in the cellular constituents (Lushchak, 2011). To resist and remove excessive ROS, organisms have antioxidant defense systems, which are usually composed by antioxidant enzymes (e.g., superoxide dismutase, catalase, glutathione reductase, and peroxidase) and non-enzymatic (e.g., total glutathione, vitamin E and C) antioxidants (Valavanidis et al., 2006; Wang et al., 2015). As an example, catalase (CAT) is an antioxidant enzyme responsible to convert hydrogen peroxide (H_2O_2), toxic to cells, into water and oxygen, thus protecting the cells against hazardous reactive molecules (Jemec et al., 2010). Non-enzymatic antioxidant defenses, fat-soluble vitamins and water-soluble small molecules, such as ascorbic acid and reduced glutathione (GSH) that besides acting as ROS scavengers (Dickinson and Forman, 2002), also play an important role in reducing oxidative stress by directly interacting

directly with ROS and also operate as co-factor to several enzymes such as glutathione peroxidase (Lushchak, 2011; Park et al., 2017). Changes in CAT, and GSH levels were confirmed to be able to reflect the antioxidant response of organisms (Sun et al., 2012). Under standard physiological condition, ROS production and the antioxidant defense system are balanced, thus avoiding oxidative stress. When the rate of production of reactive oxygen species (ROS) exceeds the capacity of the antioxidant defense and repair mechanisms this leads to an imbalance, oxidative stress, which can lead to oxidative damage to biomolecules (Metcalfe and Alonso-Alvarez, 2010). Oxidative damage due to increase in ROS production after exposure to MPs can be noted in DNA damage (Avio et al., 2015; Jiang et al., 2020), and lipid peroxidation, one of the most frequent cellular injury processes, in which ROS react with membrane-associated lipids (Barboza et al., 2018). Glutathione-S-transferase (GST) is a phase II of biotransformation enzyme catalysing the conjugation of GSH with several compounds to facilitate its excretion by cells. Its involvement in MPs toxicity is unclear but some evidence of altered activity and modulation of the expression of GST-related genes was already found (Avio et al., 2015; Ribeiro et al., 2017).

One of the key-events that have been highlighted in the oxidative stress adverse outcome pathway is inflammation (Hu and Palić, 2020). Molecular inflammation is a potent mechanism elicited in response to pathogenic and xenobiotics. In recent years, engineered nanomaterials have entered the environment and can often be found in living organisms and could be considered as a special case of “particulate xenobiotics” that can interact with the immune system (Greven et al., 2016; Ng et al., 2018). The activation of the immune responses due to exposure to MPs has also been suggested by the upregulation of genes related to the immune system as observed in invertebrates such as mussels and oysters (Détrée and Gallardo-Escárate, 2017, 2018; Gardon et al., 2020). The activation of phenoloxidase (PO) is one of the mechanisms involved in invertebrates innate immune system, ultimately leading to encapsulation followed by melanisation of pathogens, parasitoids, damaged tissues (González-Santoyo and Córdoba-Aguilar, 2012), and potentially MPs. The activation of immune response is also frequently associated to the production of ROS and thus potentially a key event in MPs adverse outcome pathway.

On the other hand, energy-related biomarkers (energy reserves and energy consumption) proved to be altered in invertebrates after MP ingestion (Wright et al., 2013a; Gardon et al., 2018). For instance, energy reserves (i.e., carbohydrates, lipids and proteins contents) are dependent of the energy input (i.e., feeding) and metabolic expenditure of organisms while energy consumption, that can be measured at the cellular level through the electron transport system (ETS) activity, gives an insight into the aerobic energy production and of the energy expenditure in response to stress conditions (Sokolova et al., 2012). As such, alteration in energy homeostasis induced by exposure to MPs can also be evaluated and be used to predict consequences in terms of growth, development and reproduction of organisms and thus establish possible adverse outcome pathways (Sokolova et al., 2012; Jeong and Choi, 2019; Hu and Palić, 2020).

4.1. Relevant exposure scenarios in the study of ecotoxicological effects of MPs

Research on MPs and on their effects in freshwater biota is been growing exponentially in recent years (Fig. 4). Until now, researchers have been delivering valuable information not only on the presence of MPs in the environment, but also on the effects that MPs trigger on biota. Researchers recently recognised that research have been focused on "pristine" MPs and on unrealistic concentrations and should be oriented in the search for more relevant exposure scenarios.

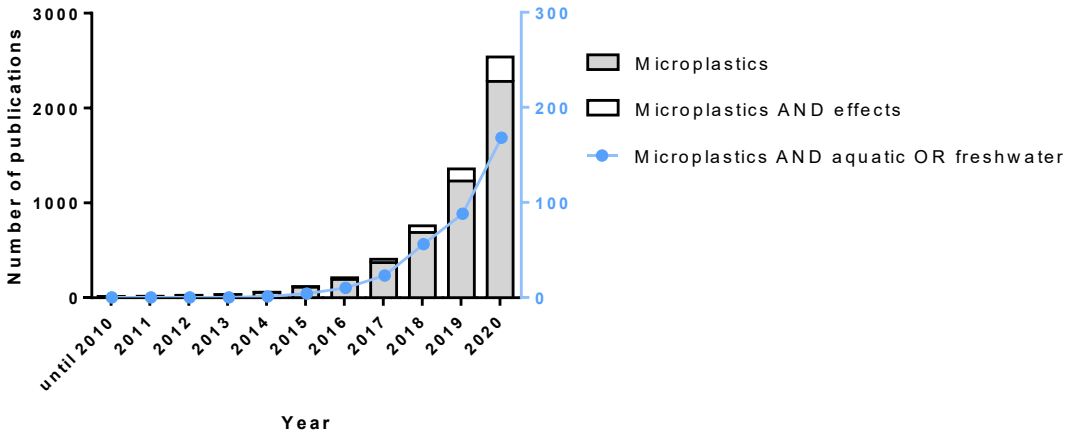

Fig. 4. Number of publications that have "Microplastics"; "Microplastics AND effects"; and "microplastics AND aquatic OR freshwater" words in the article title. The search was performed on Scopus database on December 14th, 2020 (cumulative frequency graph).

The use of realistic exposure scenarios remains overlooked and can provide complementary information that can be used to improve risk assessment. In this context, higher-tier tests (i.e., mesocosms or field-based methods), the employment of weathered MPs and studies addressing the combination of MPs with anthropogenic and natural stressors are research gaps that urge to be addressed.

One of the most referred approximation to more realistic circumstances is the use of weathered MPs in an attempt to provide an approximation to the aging process that MPs undergo in freshwater environment (Horton et al., 2017c). The use of weathered MPs can provide an accurate estimation of toxicological impacts, which are frequently underestimated when using "pristine" MPs (Horton et al., 2017c). This identified need has parallel in the use of environmentally "aged" nanomaterial forms (Judy et al., 2015; Lahive et al., 2017). Phenomena such as hetero- and homo-aggregation, usually associated to nanomaterials and known by the development of a surface 'corona' of associated macromolecules and chemicals may all also occur in MPs (Syberg et al., 2015). The use of weathered MPs is then needed to accurately identify the effects of MPs as other anthropogenic materials in real environments (Schultz et al., 2015). Their recent use in

laboratory conditions has already provided evidences of increased effects in biota as a result of leachates that are released during the aging process or due to increased ingestion promoted by colonization of their surface by microorganisms (Bejgarn et al., 2015; Gandara e Silva et al., 2016; Bråte et al., 2018; Hariharan et al., 2021).

Nevertheless, the use of more environmentally realistic conditions through the use of multispecies tests, pond mesocosms, artificial streams or field monitoring studies can provide more insight on the impacts (Boxall et al., 2002; Tilghman Hall et al., 2017). The use of mesocosms systems has proved to be a satisfactory approximation on the effects to natural ecosystems complementing standard laboratory tests (Abelho et al., 2016; Campos et al., 2020; Cañedo-Argüelles et al., 2017; Rodrigues et al., 2018). The use of mesocosm approaches allows for the assessment of responses of several species belonging to different trophic guilds and inhabiting an extensive range of habitat conditions and biological interactions that can modulate toxicity (Relyea and Hoverman, 2006; Pestana et al., 2009; Woodward, 2009). Community ecotoxicology testing is then commonly used to assess direct and indirect effects of contaminants by addressing higher level (community and ecosystem) responses replicating field conditions and, in this way, providing relevant data on the ecological impacts of contaminants (Lizotte et al., 2013)

That said, macroinvertebrate community structure is the parameter often used for determining the ecological status of freshwater ecosystems (Wallace and Webster, 1996; Relyea and Hoverman, 2006; Pestana et al., 2009; Stewart et al., 2013; Vidal et al., 2014; Dalu et al., 2017). In addition to the macroinvertebrate structure, a joint evaluation with ecosystem functional parameters constitutes a broader approach to evaluate direct and indirect effects of a certain stressor on key ecological processes of the ecosystems (Young et al., 2008; Woodward, 2009; Dalu et al., 2017). In summary, community ecotoxicology tests pretend to simulate field conditions to assess direct and indirect effects of a given stressor (e.g. MPs) delivering additional relevant information for environmental risk assessment since they usually provide community and ecosystem level responses (Lizotte et al., 2013). Invertebrates can ingest MPs and be affected very differently (Scherer et al., 2017; Redondo-Hasselerharm et al., 2018b, 2020a), which can also affect very dissimilarly distinct ecosystem functions (López-Rojo et al., 2020). Therefore, functional relevant parameters should be addressed along with macroinvertebrate community structure to evaluate the effect of MPs to the ecosystem functioning like previously performed for other stressors (Lizotte et al., 2013; Abelho et al., 2016; Cañedo-Argüelles et al., 2017; A. C. M. Rodrigues et al., 2018; Campos et al., 2020). Primary production is a key-process of ecosystem functioning, promoting the nutrients cycling and prompting carbon storage (Hooper et al., 2012). In small and medium size streams, the main element of primary production is periphyton (Tlili et al., 2017), a matrix composed by a cocktail of microorganisms including diatoms, algae, bacteria, fungi and organic and inorganic detritus (Guasch et al., 1998; Sabater et al., 2007; Battin et al., 2016). Used as an important biological indicator to classify water bodies (Guasch et al., 1998; Sabater et al., 2007; Battin et al., 2016), primary production (periphyton growth) is frequently evaluated

ecosystem functional endpoint as a potentially indicator of stress in streams (Guasch et al., 1998, 2016; Elias et al., 2017; Rodrigues et al., 2018).

Along with primary production, leaf litter decomposition is another key-process in freshwater ecosystems, especially in low order streams where detritivore macroinvertebrates are dominant in number and biomass and in which the main source of organic matter is the leaf litter from riparian vegetation (Abelho, 2001; Atkinson et al., 2017). The decomposition of the organic matter is of great importance transferring energy to higher trophic levels, and subsequently promoting the nutrient cycle. Leaf litter decomposition is mostly ensured by microorganisms and macroinvertebrates, especially shredders and collectors at a minor extent (Gessner et al., 1999; Seena et al., 2017). Leaf litter decomposition is then naturally influenced by the abundance of shredders, but also by the quality of litter and other factors like temperature (Graça, 2001; Dangles and Malmqvist, 2004; LeRoy and Marks, 2006; Friberg et al., 2009). Like macroinvertebrates community structure and primary production, leaf litter decomposition is also commonly used as an indicator of the ecosystems' ecological status (Young et al., 2008; Woodward et al., 2012), keeping in mind that anthropogenic pressure frequently affects organisms that control leaf decomposition thus indirectly affecting it (Rasmussen et al., 2012).

Limited studies addressed the effects of MPs at the macroinvertebrate community structure (Redondo-Hasselerharm et al., 2020b), and a significant knowledge gap remains in terms of joint evaluation of macroinvertebrate community structure and ecosystem functional parameters. The use of mesocosms approaches for the evaluation of effects of MPs on macroinvertebrate communities and on ecosystem functioning is of crucial importance since altered nitrogen cycling due to MPs exposure has already been shown (Huang et al., 2021). Community ecotoxicology testing is an important piece of the puzzle allowing simultaneous evaluation of structural and functional responses of benthic invertebrate communities and thus contributing to a better environmental risk assessment of MPs.

Recognisably, organisms are not exposed to MPs only in the environment, and the evaluation of combination of MPs with other stressors (multi-stressor exposure) also represents a step forward towards environmentally realistic approaches (Syberg et al., 2015; Thompson et al., 2018; Orr et al., 2020). In this context, it is crucial to study the effects of MPs combined with other stressors on freshwater invertebrates' apical and suborganismal endpoints. Recent research has been focused on "vector effect" delivering information on the combined exposure of MPs with anthropogenic contaminants such as: metals (Sıkdokur et al., 2020; Weber et al., 2021); polybrominated diphenyl ethers (Horton et al., 2020); polycyclic aromatic hydrocarbons (Magara et al., 2018; González-Soto et al., 2019); and pharmaceuticals like methamphetamine (Qu et al., 2020) and florfenicol (Guilhermino et al., 2018). However, the combination of MPs with environmental stressors (including biotic and abiotic factors) remains understudied, despite the recognition of its importance to provide realistic information on the effects of microplastic pollution under specific varying environmental conditions (Wagner and Lambert, 2018). Natural stressors like temperature and salinity are especially relevant under climate change scenarios as the

Intergovernmental Panel on Climate Change predicts that not only the average temperature will rise but also that extreme phenomena and processes like salinization of freshwater ecosystems will occur more often (IPCC, 2018; Cañedo-Argüelles et al., 2019; Carvalho et al., 2020). Some insights were recently given from research that evaluated the effects of different types of MPs in combination with thermal stress in different *daphnids* (Jaikumar et al., 2018) and in freshwater mussels (Weber et al., 2020). Acute sensitivity of daphnids *Daphnia magna* and *Daphnia pulex* to MPs increased sharply with temperature, whereas that of *Ceriodaphnia dubia* remained relatively stable across temperatures (Jaikumar et al., 2018), and for freshwater mussel *Dreissena polymorpha* no interactive effects were found (Weber et al., 2020).

5. Aim of the study and conceptual framework

The present thesis is focused on the ecotoxicological evaluation of MPs in freshwater ecosystems. In this respect, polyethylene particles were chosen due to their environmental relevance, since in most of the recent surveys , polyethylene and derivatives are the major fraction of MPs recovered from freshwaters (Imhof et al., 2013; Castañeda et al., 2014; Zbyszewski et al., 2014; Wang et al., 2017; M. O. Rodrigues et al., 2018), and from freshwater sediments (Li et al., 2019; Scherer et al., 2020). Therefore, the present study aimed to raise knowledge on some of the research gaps previously noticed: i) At which extent are MPs ingested by freshwater benthic invertebrates? ii) If so, at what extent are MPs ingested and/or retained inside invertebrates' gut? iii) Will this MP ingestion trigger suborganismal and life-history effects on these organisms? iv) Can natural stressors (e.g., temperature, salinity or food limitation) alter the effects of MPs on freshwater invertebrates? V) Can environmentally relevant concentrations of MPs affect the structure and function of freshwater benthic invertebrate communities?

To address these questions, individual exposures to different-sized PE-MPs were carried out using laboratory toxicity tests with the midge *Chironomus riparius* and the oligochaete *Lumbriculus variegatus*. The ingestion of different-sized PE-MPs was evaluated on both species, not only concerning the number of ingested particles but also in terms of size of ingested particles. This evaluation was performed after chronic (10 days) and acute, short-term(48h) exposures (chapter 2 and chapter 4, respectively) in *C. riparius* and after short (48h) and long-term (28 days) exposure for *L. variegatus* (chapter 3). Sub-lethal responses at the organism level like larval growth and emergence in *C. riparius* (chapter 4) and reproduction and biomass in *L. variegatus* (chapter 3) were assessed. At sub-organismal level, MPs effects on biomarkers related with oxidative stress (lipid peroxidation, catalase and glutathione-*S*-transferase activities, Total glutathione, neurophysiology (measuring Acetylcholinesterase activity), energy reserves (lipids, carbohydrates and proteins contents) and aerobic energy production (electron transport system activity) were evaluated in both *L. variegatus* and *C. riparius* (chapter 3 and 4, respectively). In addition to these "traditional" sub-cellular endpoints, effects on the innate immune response (phenoloxidase system) of *C. riparius* after ingestion of MPs was also evaluated (chapter 5). Combined effects of MPs and natural stressors such as temperature, salinity and food shortage were investigated to address

relevant exposure scenarios (chapter 6). In chapter 7 a mesocosms study was conducted, in which effects of two frequently reported concentrations plus a high concentration of a pool of PE-MPs were evaluated in terms of functional (leaf decomposition, primary production) and structural (benthic invertebrate community) parameters. Finally, on chapter 8, a general discussion of the major findings of the thesis and of prospective research concerning ecotoxicity of MPs within freshwaters is also presented.

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Chapter 2

Ingestion of small-sized and irregularly shaped polyethylene microplastics affect C*hironomus riparius* life-history traits

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Ingestion of small-sized and irregularly shaped polyethylene microplastics affect C*hironomus riparius* life-history traits

Abstract

Microplastics (MPs) are emerging contaminants of freshwater ecosystems. Once in aquatic systems, most of these plastic particles undergo processes of fragmentation, biofouling, and sedimentation, resulting in increased concentrations of smaller sized and irregularly-shaped particles in the sediment. High levels of MPs in freshwater sediments can denote a potential threat to benthic and sediment-dwelling organisms such as dipteran larvae. This study evaluates the ecotoxicological effect of three pools of irregularly-shaped polyethylene (PE) microplastics (pools containing 90% of the particles within 32-63 µm (size-class A), 63-250 µm (size-class B) and 125-500 µm (size-class C)), with concentrations ranging from 1.25 to 20 g Kg^{-1} sediment, on the dipteran *Chironomus riparius* life-history traits. After ten days of exposure, larvae ingested PE particles typically in the 32-63 µm range, even when 90% of the particles possessed higher size (i.e., in size-classes B and C) and the larvae mandible allowed the ingestion of such bigger-sized particles. Thus, the number of ingested particles was higher in size-class A, followed by B and C, and led to a significant reduction with similar magnitude on larval growth (Lowest Observed Effect Concentrations (LOEC) = 2.5 g Kg^{-1} sediment DW) and a significant delay on imagoes emergence (e.g., LOEC = 1.5 g Kg^{-1} sediment DW for females). The results from this study show that the ingestion and persistence of small-sized polyethylene microplastics caused significant impairments on life-history traits of *C. riparius*. Considering their role on freshwater food-webs and the potential persistence of small-sized PE particles in their larval gut, these results also point for the potential adverse effects of small-sized microplastics at the community and ecosystem level.

Keywords: Benthic macroinvertebrates; Emergence; Freshwaters; Insects growth; Pollution

1. Introduction

Microplastics (MPs, <5 mm in size) are an emergent environmental issue, and its research is rapidly advancing (Jiang, 2017; Alimi et al., 2018; Chae and An, 2018). Recent attention was driven towards primary MPs, i.e., plastic particles manufactured at the microscale (e.g., microbeads, capsules, fibers, pellets) that are being released continuously into the freshwater environments via sewage, runoff from inland activities, illegal industrial discharge and wind (Boucher and Friot, 2017). Once in the water systems, MPs are likely to undergo through processes of disintegration, fragmentation, and biofouling, resulting into a wide range of smaller particles that either remain in the water column or mainly settle in the freshwater sediments (Castañeda et al., 2014; Lagarde et al., 2016). Documented as important pathways and repository of plastic debris (GESAMP, 2015, GESAMP, 2016), freshwater compartments can contain MPs in concentrations in the order of grams of MP particles per Kg/cubic meter, or thousands of MP particles per kg/cubic meter of sediment (Klein et al., 2015; Hurley et al., 2018; Rodrigues et al., 2018; Wang et al., 2019) which can be directly comparable to concentrations of MP's found in shoreline ecosystems (McCormick et al., 2014; Dris et al., 2015; Anderson et al., 2017).

Despite freshwater sediments being recognized as central repositories of MPs (Hurley et al., 2018), most of the ecotoxicological studies performed so far have been focused on filtering invertebrates, such as daphniids and mussels (Ogonowski et al., 2016; Guilhermino et al., 2018), and fish (Silva-Cavalcanti et al., 2017), which are potentially exposed to buoyant MPs (Eerkes-Medrano et al., 2015; Ziajahromi et al., 2017). The few available studies on freshwater benthic invertebrates (mostly using oligochaete, amphipods and dipteran larvae) reported the ingestion of small MPs (size <300 µm). Along with the ingestion, these studies also point some evidences of higher retention time of microplastics in their gut when compared to other freshwater organisms and even to marine sediment-dwelling invertebrates (Courtene-Jones et al., 2017; Scherer et al., 2017; Al-jaibachi and Callaghan, 2018; Lei et al., 2018; Ziajahromi et al., 2018). In laboratory assays, the presence of MP particles has been shown to induce harmful effects on feeding and development in dipteran larvae (Scherer et al., 2017; Al-jaibachi et al., 2018; Ziajahromi et al., 2018). However, these investigations focused only on MPs with “bead” shape of specific sizes. In natural environments, MP beads are likely to undergo processes of degradation, eventually fragment and disintegrate forming particles consisting of a broad particle size distribution with a diverse range of particle shapes (Lambert et al., 2017). Furthermore, non-spherical MP shapes may cause higher toxicity than microbeads, as reported for marine amphipods (Au et al., 2015).

The non-biting midge larvae *Chironomus riparius* is one of the most abundant macroinvertebrate species in freshwater benthic ecosystems (Armitage et al., 1995). Inhabiting the uppermost layers of sediment, they act as deposit-feeders feeding on sedimented and deposited organic matter. Their feeding behavior is mostly non-selective determined by bioavailability, and (more rarely) selective, based typically on the nutritive value and food type (Rasmussen, 1984; Armitage et al., 1995). In sediments contaminated with MPs, we hypothesize that larvae will likely ingest such synthetic polymers as they ingest natural sediments and particulate organic matter

(detritus) of similar size, resulting in growth and emergence impairments. Nevertheless, such ingestion will likely be dependent on the larvae pre-mandible width and the size-range of the MPs present in the sediments. Typically, first instar larvae of *Chironomus riparius* ingest sediment particles up to 20 µm as part of their regular feeding activity. Conversely, final instars (3rd–4th instar) can ingest particles typically in the 60–200 µm fraction (Armitage et al., 1995; Ristola et al., 1999; Henriques-Oliveira et al., 2003). Therefore, the objectives of this study are: 1) to determine which size-range are preferably ingested by *C. riparius* larvae in the presence of a pool of particles of different size; 2) to evaluate if the ingestion is dose and pool dependent, and 3) to assess the ecotoxicological effects of MPs on *C. riparius* life history traits (growth and emergence). Thus, *C. riparius* larvae were exposed to increased concentrations of three size-classes (pools) of irregularly shaped polyethylene particles (PE, one of the most produced primary MP, Conkle et al., 2017) and effects of their ingestion on growth and emergence followed throughout their life cycle (~28 d) according to OECD guidelines.

2. Materials and methods

2.1. Test species and culture conditions

Chironomus riparius used in the bioassays were obtained from a culture established at the Department of Biology, University of Aveiro. Organisms are maintained in controlled conditions (16:8 h light-dark photoperiod, temperature of 20 ± 1 °C), with the larvae growing in 30× 20 cm polypropylene containers, and adults constrained within a120 × 60 × 40 cm acrylic cage. The polypropylene containers contain a layer of approximately 3 cm of previously burnt (500 °C for 4 h) inorganic fine sediment (<1 mm). The containers also contain American Society for Testing Materials (ASTM) hard water in 1:4 ratio (ASTM, 1980). Feeding for larvae is constituted by a suspension of macerated TetraMin (Tetrawerke, Melle, Germany) that is provided every two days (ad libitum). The medium (ASTM hard water) is changed every two weeks and the sediment every month. The life cycle of *C. riparius* in laboratory-controlled conditions is completed within 3–4weeks. Before experiments, egg ropes were collected and transferred to 50 mL glass vessels filled with ASTM hard water. First instar larvae (<48 h post-hatching) were used for the tests.

2.2. Polyethylene particles used for testing

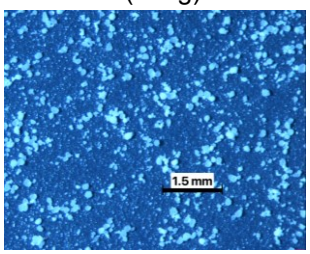
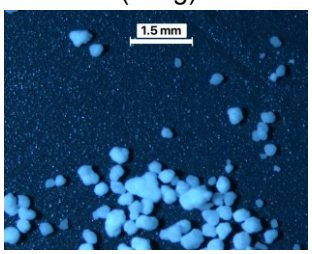
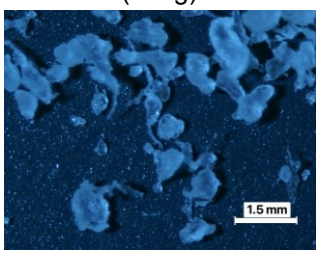
Commercially available polyethylene (PE) particles of three different size classes were purchased: 40–48µm (average size, ultra-high molecular weight powder, CAS No. 9002-88-4, Sigma-Aldrich UK); 125µm (average size, ultra-high molecular weight powder, CAS Number9002-88-4, Sigma-Aldrich UK) and 350µm (average size, medium-density polyethylene powder, CAS 708-316-83, Goodfellow). The size range of the polyethylene particles was chosen based on the size of typical food items ingested by the larvae of *C. riparius* (Epler, 1995; Ristola et al., 1999; Henriques-Oliveira et al., 2003). Particle size distribution of the purchased particles was determined by vibratory sieve shaking (mesh pore-sizes: 500, 250, 125, 63 and 32µm). Replicates

of 100 g of PE particles of each size class were sieved in a vibratory sieve shaker for particle size analysis within each size-class. The purchased PE particles presented a pool of particles with different sizes (Table 1). To avoid misleading, the results are presented according to three size-class: A, B, and C; characterized by≥90% of the particles with a size range of 32–63µm, 63–250µm and 125–500µm, respectively (highlighted in bold, Table 1). All equipment and test vials were acid washed and thoroughly rinsed with Milli-Q water before use in the tests. Plastic contamination via air exposure was minimized by covering samples and filters. Glassware was used instead of plasticware whenever possible. For MPs extraction and quantification, additional controls were used to account for possible airborne MPs contamination. The average blanks/controls were then subtracted from the particle quantification to correct for background contamination.

2.3. Polyethylene concentrations and sediment spiking

The tested concentrations were: 1.25, 2.5, 5, 10, 20 g PE Kg^{-1} sediment, for all the different PE particles. The concentrations range was set based on recent estimations of primary MPs (size<300µm) on freshwaters hotspots (Conkle et al., 2017). For each treatment, PE particles were directly mixed into the sediment (<1 mm, previously burnt at 500 °C). Each glass test vial contained 50 g of control (sediment with no MPs) or 50 g of sediment mixed with MPs and filled with ASTM hard water (~150 mL). The addition of ASTM hard water was performed by gently pouring the ASTM hard water to minimize resuspension of PE particles. The test vials were then covered with lids and allowed to equilibrate for 24 h.

Table 1: Optical appearance (magnification: 30×) and particle size distribution analysis of the purchased polyethylene (PE) particles (replicates of 100 g) after vibratory sieve shaking. The particle size distribution was measured through weighting (g) the resulting fractions present in each sieve mesh. Results are presented as mean ± standard error (*n*=2).

	Average size claimed by the supplier		
	40-48 μm	125 μm	350 μm
	(100g)	(100g)	(100g)
Particle size distribution			
∅ pore (μm)			
≤500	0	0±0	0.06±0.04
>500 - ≤250	0	1.15±0.25	**67.50±7.20**
>250 - ≤125	0	**50.15±2.35**	**26.30±5.70**
>125 - ≤63	0	**39.75±1.75**	5.35±1.05
>63 - ≤32	**94.36±1.36**	8.00±0.70	6.15±5.80
<32	5.54±0.96	0.90±0.10	0.00±0.00
Size-class code	A	B	C

2.4. *Chironomus riparius* partial life cycle test

First instar (less than 48 h post-hatching) *C. riparius* larvae were used in chronic 28-days partial life cycle assays (OECD, 2004). Each treatment condition and a control (uncontaminated sediment) consisted of thirteen replicates with five larvae each. During the experiment, organisms were fed every two days (0.5 mg of macerated TetraMin per organism per day), and the test conditions were the same as described for culturing. After ten days, larvae from five replicates of each treatment were sacrificed in ethanol 70%, counted, and measured (total length) using a stereo dissecting microscope fitted with a calibrated eyepiece micrometer. Larvae from three replicates of each treatment were carefully rinsed (three times) in Milli-Q water, checked under a stereoscopic microscope (for MP adhered to the integument), and frozen at -20°C for MPs quantification in the organisms. The remaining five replicates (of the initial thirteen) were used to follow the emergence of emerged adult insects (imagoes) until the end of the test. Imagoes were daily collected from emergence traps and placed in 5 mL tubes with ethanol 70% for correct

identification of the sex (male/female). Water quality parameters (pH, oxygen dissolved and conductivity) were evaluated every three days.

2.5. Extraction and quantification of PE particles in sediment, water and in chironomids

The quantification of PE particles in sediment and water column was performed for the two lowest concentrations (1.25 and 2.5 g Kg^{-1} sediment) before exposure to ensure real concentrations were not misrepresented comparing with nominal concentrations. Before the quantification of resuspended PE particles in the water column, the buoyant PE particles present in the water column were collected and filtered using pre-weighted (iW) black polycarbonate filters (PCTE, 0.2 μm pore size, 42 mm ∅, ref. 7063-4702, GE Healthcare Whatman™) to retain microplastics.

The extraction of PE particles present in the sediment followed the principle of density separation reported by Thompson et al., 2004, based on supersaturated NaCl solution (NaCl/ MilliQ, density ρ=1.2 g mL^{-1}) and a customized glassware designed by Karlsson and colleagues (Karlsson et al., 2017) onto pre-weighted PCTE filters.

After vacuum-filtration, all filters (from water and sediment samples) were treated with H_2O_2 (30%) to eliminate possible organic matter, dried at 25 ºC for 2-3 days, and weighed again (fW). The mass of PE particles in the sediment and resuspended in water was estimated by measuring the difference between the final (fW) and the initial weight (iW) of PCTE filters (using weight of blank filters for account possible microplastics' airborne contamination).

After exposure, the extraction and quantification of MPs from biological samples followed previous recommendations (Lusher et al., 2017). Briefly, biological samples were freeze-dried for 24h and weighed (dry weight, DW). Samples were transferred to glass flasks and carefully grounded with a small glass rod (which acted as a small mortar and pestle) to facilitate further digestion with 3 mL of HNO_3 for three hours at 60 °C. After cooling down to room temperature (RT), 2.6 mL of H_2O_2 (35%) was added to complete the digestion. After 24h, and if no visible oxygen bubbles were being released, samples were then gently diluted 1:10 with Milli-Q (at RT) and vacuum-filtered onto black PCTE filters. Retained biological material (in the filtration apparatus) was copiously flushed with Milli-Q. Membranes were transferred to glass petri-dish and allowed to dry in the oven at 25 °C for 2-3 days. Afterward, the number of particles ingested by the larvae were counted under a stereomicroscope. To assess the average size of the particles ingested by *C. riparius* larvae, the diameter (major axis when particles were fitted to an ellipse) of all particles found in each filter of the lowest concentration tested for all size-class was measured under a stereomicroscope (stereoscopic zoom microscope—SMZ 1500, Nikon Corporation) associated to NIS-Elements D 3.2 microscope imaging software.

2.6. Statistical analysis

Effects of PE-particles exposure on *C. riparius* growth were analyzed using parametric analysis of variance (ANOVA) with multiple comparisons examined by Dunnett's post hoc test. Normality of data and homogeneity of variances were assessed by performing Shapiro-Wilk and Levene's test, respectively. In case of non-normal distribution and the heterogeneous variances of the data set, non-parametric analysis was performed, i.e., Mann-Whitney tests and Kruskal-Wallis tests with Dunn's multiple comparison tests to assess significant differences.

Based on the time and the number of emerged imagoes, the mean emergence time (EmT_{50}) of *C. riparius* was calculated for each condition. The natural logarithm of time (in days) was taken, and the number of emerged imagoes was cumulated and normalized to percentages for each replicate. The mean emergence time (EmT_{50}) was determinate through dose-response analysis (survival curve), model Eq. Y=Bottom + (Top-Bottom)/(1– 10^((LogEC_{50}-x)*HillSlope)), and was statistically compared according to (Sprague and Fogels, 1976), based on Wilcoxon two-group test. The sex ratio of imagoes was calculated in each treatment as the number of males divided by the number of female organisms. A chi-square test was applied to evaluate the effect of dose and size of PE particles in sex ratio.

For all statistical tests, the significance level was set at $p<0.05$. Data were analyzed using GraphPad 7 (GraphPad Software Inc., La Jolla California USA), except for EmT_{50} values that were calculated with the statistical software R Ver 3.5.1. from The R Foundation for Statistical Computing (Vienna, Austria), using the "drc" package.

3. Results

3.1. Polyethylene characteristics and concentrations in sediment, water, and whole organisms

At the beginning of the exposure, more than 98% of polyethylene (PE) particles remained in the sediment while less than 2% resuspended in the water column (table 2).

Table 2: Nominal and measured concentration (g kg^{-1} sediment DW) of polyethylene (PE) particles in the sediment, and measured concentration in water column (mg PE per 150 mL ASTM) as result of resuspension process of PE particles, in the beginning of the test (see topic 2.5 in material and methods section). Measurements were performed in the two lowest concentrations of each PE size-class (*n*=3).

	Substrate		
	Sediment		**Water**
PE size-class	Nominal conc. (g kg^{-1} DW)	Measured conc. (g kg^{-1} DW)	Measured conc. (mg/150mL ASTM
A	1.25	1.23±0.01	0.9±0.3
(32-63 µm)	2.5	2.30±0.01	2.3±0.3
B	1.25	1.22±0.04	1.0±0.2
(63-250 µm)	2.5	2.47±0.02	1.06±0.07
C	1.25	1.18±0.07	0.15±0.07
(125-500 µm)	2.5	2.39±0.03	0.96±0.03

After ten days of exposure, larvae presented PE particles in their gut in all tested PE size-class (Figure 1A, B and C). Ingestion was size dependent, with larvae exposed to size-class A revealing higher internal PE concentrations than larvae exposed to size-class B and C ($F_{2,20}$ = 15.13, p<0.001). Besides, for larvae exposed to PE size-class A, significant dose-related ingestion of PE particles was observed (p<0.05), with larvae exposed to higher PE concentrations presenting more PE particles in their gut. The average size of the ingested particles was lower than the average size of the particles contained in the sediment (40.93 ± 0.34 µm, 57.08 ± 0.80 µm and 60.82 ± 1.59 µm for PE size-class A, B, and C, respectively, Figure 1D). The average size of the ingested particles corresponded to the fraction 32-63 µm, which in the size-class A included more than 90% or the particles, and in size-class B and C integrated less than 10% of the particles present.

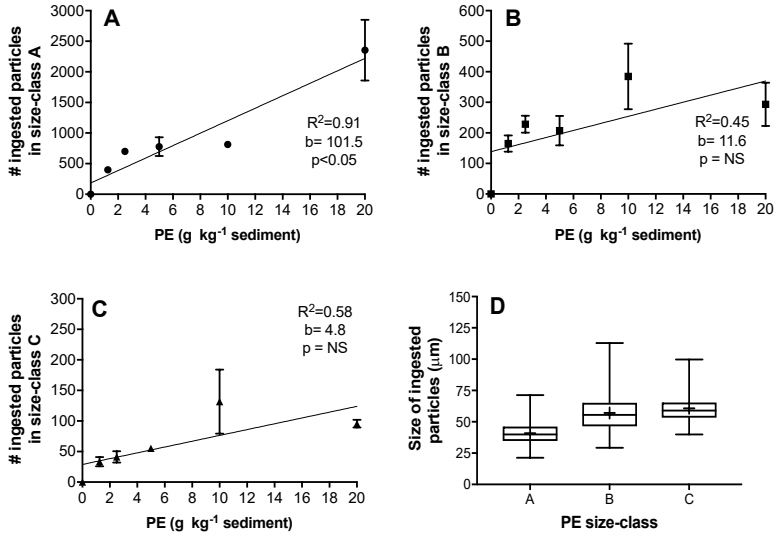


Figure 1: Number (1A, 1B and 1C) and size (1D) of Polyethylene (PE) particles ingested by *Chironomus riparius* larvae (DW), after ten days of exposure to three size-class (A: 32- 63 µm, B: 63-250 µm, and C: 125-500 µm). The number of ingested particles was counted in all tested PE concentrations. Such number is presented as mean ± standard error and described with linear regression (*n*=3). For some points, the error bars might be shorter than the height of the symbol. The size of the ingested particles was characterized in the larvae exposed to 1.25 g of PE kg^{-1} dry sediment and are presented as mean (+ symbol) with interquartile range, whiskers, and max/min outliers (*n*=3).

3.2. Life-history effects

During exposure the water quality parameters remained in the recommended range defined by OECD (OECD, 2004): temperature of 20.6 ± 0.6 ºC, pH at 8.34 ± 0.07, air saturation values between 70% and 90%. The survival and emergence of adults in control conditions were above 80%.

The effects of the ingestion of PE particles on larval growth can be observed in Figure 2. The ingestion of PE particles of all size-classes significantly reduced larval growth after 10 days of exposure in comparison with the control treatment (size class A: $F_{(5,23)}$ =61.417, p<0.001; size class B: $F_{(5,22)}$ =10.943, p<0.001; and size class C: $F_{(5,24)}$ =11.378, p<0.001). Stronger effects were observed for the smaller-sized PE class (size-class A) which caused a 10% reduction in larval length from the second tested concentration (2.5 g PE kg^{-1} DW sediment), and a 40% reduction at the highest tested concentration (20 g PE kg^{-1} DW sediment). For size-class B and C, significant effects were observed at the two highest concentrations tested with 9% and 11% reductions in larval growth at 10 g PE kg^{-1} DW sediment for size-class B and C, respectively; and 17% and 20% for 20 g PE kg^{-1} DW sediment for size-class B and C, respectively.

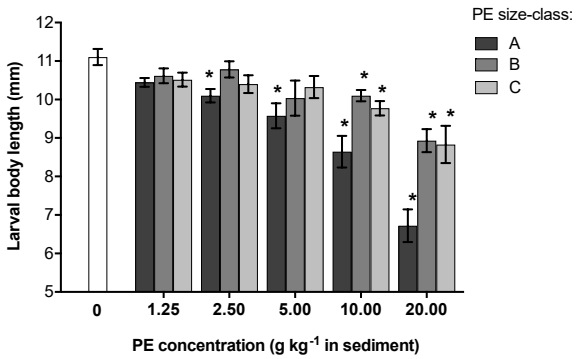


Figure 2: Effects of different size-class: A (32-63 μm); B (63-250 μm); C (125-500 μm), and dose of polyethylene (PE) particles on *Chironomus riparius* larval length (mm) after 10-days exposure. Values are presented as mean ± standard error (*n*=5). Asterisk (*) indicates significant differences ($p<0.05$)

The mean time to emergence (EmT_{50}) of imagoes in control conditions was around 16 days (males: 15.3±0.1; females: 16.6±0.1) (Figure 3; Table S1). In the presence of PE particles, a significant delay was observed with stronger effects for the size-class A (32-63 μm). The significant delay on females' emergence induced by these smaller sized particles was observed right from the first concentration tested (1.25 g Kg^{-1} sediment DW) while in males it was only observed from the third tested concentration (5 g Kg^{-1} sediment DW). The typical *C. riparius* emergence pattern, with females emerging consistently later than males, was observed in control (≈1 day) and PE treatments (≈2-4 days). The difference in time to emergence between males and females increased (2.1-2.5 times higher) for the two lower PE concentrations (1.25 and 2.5 g PE kg^{-1} sediment) of size class A and B.

There was no effect of PE particles size and concentration on the male/female ratio (chi-square tests, Supplementary data).

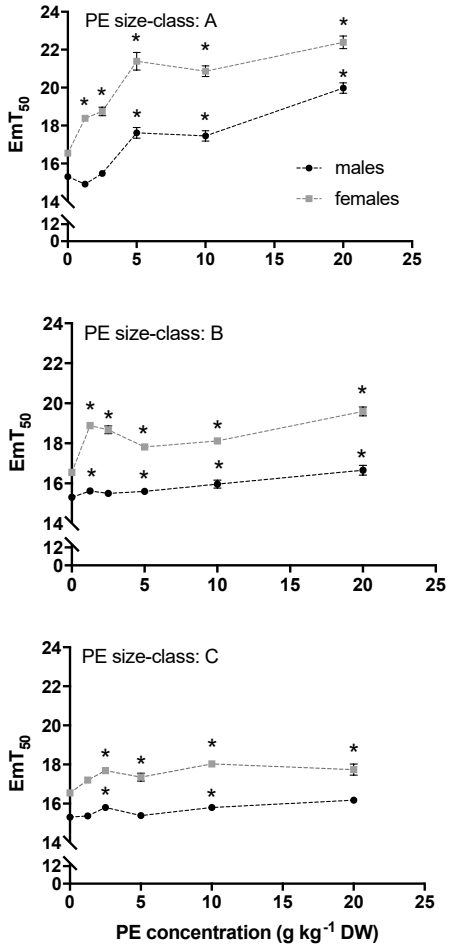


Figure 3: Effects of polyethylene (PE) particles of different size-class: A (32-63 μm); B (63-250 μm); C (125-500 μm), on *Chironomus riparius* emergence of males and females. Results are expressed as mean emergence time (EmT_{50}) ± standard error, in days (*n*=5). The asterisk (*) denotes significant differences compared to the control treatment (Sprague and Fogels, 1976). For some points, the error bars might be shorter than the height of the symbol. The original concentration-response curves are shown in Figure S1 in the Supplementary data.

4. Discussion

Microplastics (MPs) are emerging contaminants of freshwater sediments and may impose a risk to benthic invertebrate species. As initially hypothesized, *Chironomus riparius* ingested polyethylene particles present in sediments with size- and dose-related adverse effects on development (growth and emergence). Moreover, we observed an accumulation of small-sized particles (40-60 μm) in the gut of larvae. These results confirm the ingestion of polyethylene (and possibly of any king of MP) and stress the need to monitor their presence in freshwater sediments and potential ecological effects.

The predominant non-selective feeding behavior of chironomids seems to prevail in the presence of MPs (in this case PE particles) in sediments. Larvae seem to have no preference between MPs and natural sediments or particulate organic matter (detritus) of similar size (Scherer et al., 2017; Nel et al., 2018; Ziajahromi et al., 2018). Typically, *C. riparius* larvae in their final instar (4th instar) ingest sediment particles in the 60-200 μm fraction as part of their regular diet (Armitage et al., 1995; Ristola et al., 1999; Henriques-Oliveira et al., 2003). However, the size range of the PE particles found in their gut was smaller (average size-range of 40 μm to 60 μm), with very few plastic items reaching 125 μm when exposed to size-class B and C where > 90% of particles were indeed bigger than 63 μm (Figure 1D). The existence of smaller-sized particles in the 4th instar larval gut, compared to the average size of the plastic particles present in the sediment, may be a result of their ingestion and accumulation at early instars. The persistence of PE particles in the larval gut may be due to the lipophilic nature of this polymer which makes them to hetero-aggregate/compact in lipid-rich organs and gut (Paul-Pont et al., 2018). A clear relationship between the amount of ingested MP particles, MP size preference, and the organisms development stage has also been reported for other chironomids and sediment-dwelling annelids (*Tubifex tubifex, Lumbriculus variegatus)* and nematodes (*Caenorhabditis elegans)* (Hurley et al., 2017; Scherer et al., 2017; Lei et al., 2018; Nel et al., 2018; Redondo-Hasselerharm et al., 2018; Ziajahromi et al., 2018).

Despite their small size, the relatively high content of PE particles (< 63 μm) found along the gut of the *C. riparius* 4th instar larvae underline the low capacity of larvae to eliminate/egest PE microplastics as easily as sediment particles as pointed on previous studies (Scherer et al., 2017). The persistence of PE particles in *C. riparius* larval gut raises, therefore, its potential ecological impact when considering not only life-history traits, such as development and emergence but also suggesting insect larvae as vectors of microplastics to different trophic levels. The present study points that the ingestion and persistence of smaller-sized PE particles in the gut could likely cause a significant reduction in the ingestion of organic items and interfering in food processing (Au et al., 2015) and possibly to affect larval development and imagoes emergence. The larval growth and imagoes emergence revealed to be negatively affected by PE dose and size. However, and considering the size of the ingested particles and the percentage/availability of such particles in the sediment, the observed effects were mainly caused by PE size. Larvae exposed to size-class A encounter higher number of small particles (size < 60 μm, in all tested concentrations) compared with the ones exposed to the size-class B and C, which may explain the higher ingestion and persistence of such small particles in larval gut, and the consequent stronger effects on larval growth and emergence of adults. Naturally, such deleterious effects in larvae exposed to size-class B and C were only observed at higher concentrations, where the encounter with particles < 60 μm size was more likely to occur.

Additionally, the growth impairments at larval stages were translated in terms of emergence delay again with stronger effects being observed for smaller-sized PE class (size-class A), with a

delay in females' emergence observed at lower PE concentrations, resulting in a 2-3-fold higher difference in the mean emergence time between females and males. This delay may suggest that growth impairment can be more severe in females (the ones having a higher delay in EmT_{50}) than in males, at low (and environmental relevant) doses of small-sized MPs. A similar dose- and size-dependent effects of MPs on growth and emergence has also been reported for *Chironomus tepperi* (Ziajahromi et al., 2018), with higher effects for smaller sized MP particles at environmentally relevant concentrations. These results thus suggest negative consequences for the reproduction and population dynamics of midges inhibiting freshwater sediments with microplastics whose long-term effects deserve further study considering multigenerational setups.

The effects of microplastics particles seem to depend not only on features such as polymer size and dose but also on an organism's behavior and ecophysiology. As examples, the ingestion of MP particles (powder or microbeads, PE or PS) with a size range of 12-90 μm proved to affect feeding, growth, and emergence of imagoes, with potential for transference to higher trophic levels (this study, Scherer et al., 2017; Ziajahromi et al., 2017, 2018). On the other hand, the ingestion of MP particles with a size range of 2-12 μm by chironomid larvae appeared not to affect larval growth, imagoes emergence and oviposition, but have been shown to accumulate and be transferred ontogenically into the adult terrestrial life stage (Al-jaibachi et al., 2018; Cuthbert et al., 2019). In addition to the size and dose, microplastics shape might also likely play a role in the effects of microplastics in these organisms. Our study revealed significant effects of irregularly-shaped PE microplastics on chironomids life-history traits at the lowest tested concentration (1.25 g kg^{-1} PE sediment DW). However, to our best knowledge, there is no information on the effects of PE beads within the same (or similar) concentration and size range. Nevertheless, previous studies on marine amphipods pointed for the higher toxicity of irregular shaped MPs when compared to microbeads (Au et al., 2015), which underlines the importance of future studies on his topic.

Although arguable, the concentrations range used in this experiment was set based on recent estimations of primary MPs (size < 300 μm) on freshwaters hotspots (Conkle et al., 2017). Furthermore, the quantity of such small-sized microplastic particles (< 300 μm) described in field assessment studies seems to be continuously underestimated due to the applied methodologies (e.g. > 300 μm neuston net) (Klein et al., 2015; Conkle et al., 2017; Hurley et al., 2018), and the concentration of small-sized particles are expected to increase in the near future (Rochman et al., 2013). Likewise, contamination by microplastics is not monodisperse (Scherer et al., 2017; Brennholt et al., 2018) and it may reach extremely high concentrations in low river flows (inside of river bends/ curve-deposition) (Hurley et al., 2018), which is the optimal habitat for chironomid larvae and other sediment-dwelling macroinvertebrates such tricoptera, lumbriculidae and lumbricidae.

In this context, the results described here are in line with several others describing deleterious effects of microplastics on aquatic invertebrates and potential for ecosystem-level consequences (Scherer et al., 2017; Ziajahromi et al., 2017, 2018). More research is needed in

terms of the effects of these microplastics and their different polymers on crucial physiological processes of freshwater invertebrates, mediating homeostasis such as immune responses and oxidative stress. Moreover, the described effects of MPs on freshwater sediment-dwelling invertebrates might be even serious since weathering and colonization of MPs by microorganisms in freshwaters seems to promote their ingestion by aquatic invertebrates (e.g., Vroom et al., 2017) and increases the molecular interaction with organic contaminants (e.g., Hüffer et al., 2018). Therefore, future studies should consider environmental aged particles combined with POPs, but also to assess effects at lower levels of biological organization (cell and subcellular level).

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Supplementary data

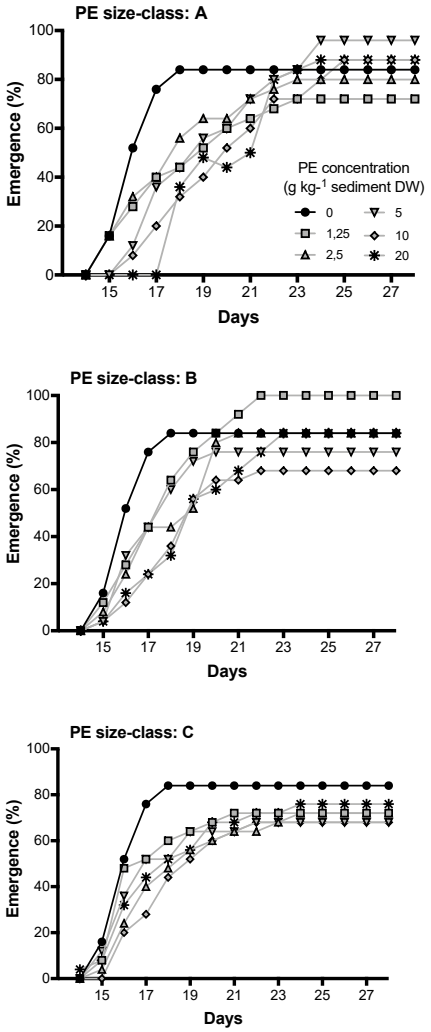

***Figure S1*:** Effects of different size-class (A: 32-63 µm, B: 63-250 µm, C: 125-500 µm) and dose of polyethylene (PE) particles on the emergence of *Chironomus riparius* imagoes. Cumulative percentage emergence was derived by dividing the number of adult midges by the number of introduced larvae after 28 days of exposure. Results are presented as average only.

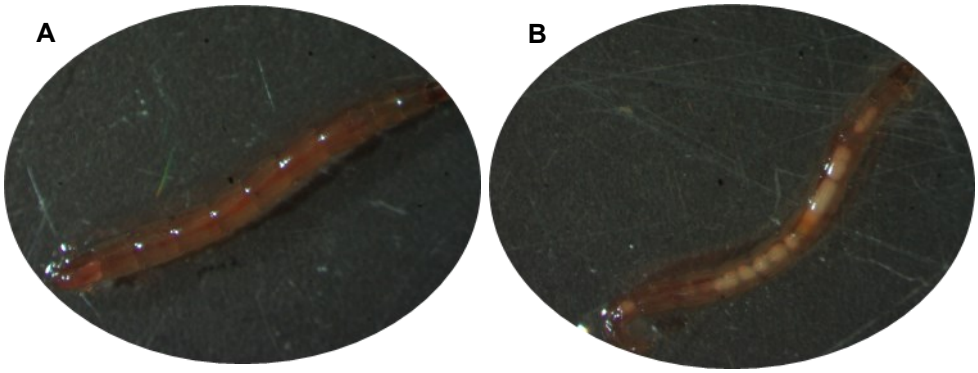

***Figure S2*:** Optical microscope images of *Chironomus riparius* larvae after ten days of exposure to (A) control conditions and to (B) 1.25 g particles per kg of sediment of polyethylene (PE) size-class A: 32-63 µm.

***Table S1*:** Output of the chi-square test with trend applied to sex ratio of emerged imagoes as the number of male-to-female organisms, to estimate the effect of size and dose of polyethylene (PE) particles.

	χ^2, df (1)	***P***
Effect of dose		
32-63 μm	0.119	0.730
63-250 μm	0.784	0.376
125-350 μm	0.556	0.456
Effect of size		
1.25 g PE kg^{-1}	1.782	0.182
2.5 g PE kg^{-1}	3.112	0.078
5 g PE kg^{-1}	0.021	0.885
10 g PE kg^{-1}	1.369	0.242
20 g PE kg^{-1}	0.100	0.752

Chapter 3

Lumbriculus variegatus (oligochaeta) exposed to polyethylene microplastics: biochemical, physiological and reproductive responses

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Lumbriculus variegatus (oligochaeta) exposed to polyethylene microplastics: biochemical, physiological and reproductive responses

Abstract

Freshwater riverbanks are a repository of microplastics (MPs) resulting from inland anthropogenic activities. Benthic invertebrates, particularly endobenthic sediment–ingesting species such as the annelid *Lumbriculus variegatus* (blackworm), are commonly found in contaminated sediments where they likely find and ingest MPs. In the present study, *L. variegatus* was exposed to concentrations between 0.51 and 20 g kg^{-1} dry sediment of four size-classes of irregularly-shaped polyethylene MPs (PE-MPs; size-class A: 32-63, B: 63-125, C: 125-250 and D: 250-500 µm) for 48h to assess their sub-cellular responses to particles ingested, and for 28 days to determine chronic effects on worm's reproduction and biomass.

After the short-term exposure (48h), blackworms presented PE-MPs in their gut, related to MPs concentration in the sediment. In general, PE-MP ingestion by blackworms induced depletion of their energy reserves (e.g., sugars in all size classes and lipids in the size-classes of PE-MPs > 125 µm), concomitant with the activation of antioxidant and detoxification mechanisms (increased level of total glutathione in all size-classes, and increased glutathione-*S*-transferase activity in PE-MPs > 250 µm), preventing lipid peroxidation. In addition, it was observed a reduction of aerobic energy production (decreased electron transport system activity) and a slight increase in neurotransmission (cholinesterase activity). After a long-term exposure (28d), the presence and ingestion of PE-MPs did not affect reproduction and biomass of *L. variegatus*.

The activation and efficiency of the antioxidant and detoxification mechanisms allied with the anatomy and physiology of *L. variegatus*, its feeding strategy and potentially dynamic ingestion/egestion capacity seem to be key features preventing MP deleterious effects under short- and chronic-exposures. Considering the MP levels reported in freshwater sediments, and despite evidence of MPs ingestion and some sub-organismal effects, our results suggest no adverse impacts of PE-MPs contamination on *L. variegatus* populations fitness.

This study applies an integrative approach in which data concerning the ingestion of different sized MPs and subsequent sub-cellular and apical responses are delivered, raising knowledge on endobenthic invertebrates' strategies to potentially overcome MP toxicity in field contaminated sites.

Keywords: Plastic pollution; Aquatic macroinvertebrates; Sublethal effects; oxidative stress; Microplastics ingestion

1. Introduction

Microplastics (MPs) are currently a recognised environmental problem for freshwaters that are simultaneously the main receptors of primary MPs from inland activities while also channelling MPs to coastal waters (GESAMP, 2015, 2016). River sediments are main repositories, in which MPs concentrations can reach 1-10 g kg^{-1} dry sediment (Castañeda et al., 2014; Klein et al., 2015; Hurley et al., 2018), with a major proportion of polyethylene polymer (PE-MPs) in European rivers (Scherer et al., 2020). MPs are thus a significant threat to biota, particularly benthic invertebrates since they have an increased risk of ingesting MPs due to their feeding behaviour and close contact to sediments (Scherer et al., 2020). The presence of MPs has been reported in many benthic and epibenthic invertebrates collected from natural habitats (Bour et al., 2018; Windsor et al., 2019). Several laboratory studies showed that MPs could be ingested by several freshwater invertebrates belonging to different functional feeding groups. Examples include filter feeders, such as *Daphnia magna* (Lambert et al., 2017; Al-jaibachi and Callaghan, 2018), shredders like *Gammarus pulex* (Lambert et al., 2017; Redondo-Hasselerharm et al., 2018b) and *Hyalella azteca* (Au et al., 2015), collector-gatherers such as *Culex pipiens* (Al-jaibachi et al., 2018), *Chironomus riparius* (Scherer et al., 2017; Silva et al., 2019), *Chironomus tepperi* (Ziajahromi et al., 2018), or *Lumbriculus variegatus* (Scherer et al., 2017), and also grazers like the snail *Physella acuta* (Scherer et al., 2017).

However, these studies address the effects of MPs on apical endpoints such as survival and reproduction (Au et al., 2015; Redondo-Hasselerharm et al., 2018a, 2018b; Ziajahromi et al., 2018; Silva et al., 2019). Although a minority, investigations on the sub-cellular effects due to the ingestion of MPs exist but are mostly focused on marine organisms (de Sá et al., 2018). Among the few studies available for freshwater invertebrates, reported effects include oxidative stress and damage (Jeong et al., 2016; Imhof et al., 2017; Silva et al., 2021) and alterations in energy metabolism (Lagarde et al., 2016; Trestrail et al., 2020a,b; Silva et al., 2021).

Assuming that *L. variegatus* are able to ingest polyethylene microplastics (PE-MPs) as observed for other MP polymers (e.g., polystyrene by Scherer et al., 2017), we hypothesised that several metabolic pathways might be altered to cope with ingestion of synthetic particles, gut obstruction and inflammation, and low nutrient uptake (Sidney et al., 2016), with consequences at the apical level as observed for sediment-dwelling dipteran larvae (Silva et al., 2019). Gut obstruction and inflammation can potentially induce an immune response that consequently may lead to increased levels of reactive oxygen species (ROS), increased antioxidant responses, and ultimately oxidative stress. Activation of immune and detoxification response may also imply energy costs and reserves depletion. To test these hypotheses, *L. variegatus* were exposed to four different-sized PE-MPs (A:32-63; B:63-125; C:125-250; D: 250-500 µm) in a wide range of concentrations (0.51-20 g kg^{-1}), for 48 hours and 28 days. The choice of different size-class followed previous studies showing that MPs size along with organisms feeding strategy and apparatus are essential factors for MPs ingestion (Sidney et al., 2016; Lehtiniemi et al., 2018; Windsor et al., 2019; Silva et al., 2021). The concentration range was chosen to include MPs levels

previously reported in sediments collected in the field (Castañeda et al., 2014; Klein et al., 2015; Hurley et al., 2018; Scherer et al., 2020); and high concentrations, similar to previously tested although with different polymer type (Redondo-Hasselerharm et al., 2018b), to investigate sub-cellular and reproductive endpoints.

Biochemical endpoints (48 h), as well as, effects on reproduction and growth (28 d) were determined on blackworm. Moreover, the number of particles ingested was determined on both acute and chronic bioassays. The set of biochemical endpoints analysed include antioxidant defences (total glutathione –TG- and catalase - CAT), conjugation enzyme (glutathione-*S*-transferase -), oxidative damage (lipid peroxidation - LPO), energy reserves (proteins, sugars, and lipids contents) and aerobic energy production (electron transport system - ETS).

2. Materials and methods

2.1. Microplastics and contamination contingency measures

Four different-sized polyethylene particles were used for testing. A vibratory sieve shaking separation was used to produce the four different size-class PE-MPS used in the tests: A: 32-63 µm (from PE average size 40-48 µm, CAS No. 9002-88-4, Sigma-Aldrich UK); B: 63-125 µm and C: 125-250 µm (PE 125 µm average size, CAS Number 9002-88-4, Sigma-Aldrich UK) and D: 250-500 µm (PE average size 350 µm, CAS 708-316-83, Goodfellow).

The test vials and equipment used were acid-washed (HNO_3 15%) and following washed with Milli-Q water prior using. The plastic contamination was minimised by using glass whenever possible, and airborne contamination was prevented by covering samples and filters with aluminium foil.

2.2. Test species

Lumbriculus variegatus were obtained from Mau river in central Portugal (40° 45'11.9 "N 8° 23'22.8 "W) and identification was performed by Dr Rüdiger Schemlz, University of A Coruña - Spain. Specimens were acclimated in laboratory conditions for several months before the experiments. The organisms were maintained under semi-static conditions in 4 L aquaria, at 20 ± 2 °C room temperature, and under a 16-/8-h light/dark photoperiod. Aquaria were filled with a 3 cm layer of commercially available river sediment (sieved <1 mm) and previously burnt at 500 °C to remove the organic matter. American Society for Testing and Materials (ASTM) hard water (ASTM, 1980) was used as a reconstituted medium with constant aeration. Blackworms were fed *ad libitum* twice a week by using a suspension of macerated TetraMin® (TetraWerke, Melle, Germany).

2.3. Acute exposure (48h) - physiological and biochemical responses

Lumbriculus variegatus of similar size and physiological state (i.e., adult worms showing no signs of recent fragmentation) were randomly picked and exposed to three PE-MPs nominal

concentrations (0.51, 3.2 and 20 g PE kg^{-1} dry sediment) of four different size-classes (A: 32-63 µm; B: 63-125 µm; C: 125-250 µm and D: 250-500 µm) plus the control condition (absence of PE-MPs). Polyethylene MPs were firstly thoroughly mixed in the bulk of sediment (< 1mm, previously burnt at 500 ºC for four hours) according to the target concentration. Afterwards, 50 g of sediment of each treatment was added to 300 mL glass vials and filled with 250 mL of overlying ASTM hard water medium (gently poured to minimise particles' resuspension). These procedures guarantee a good dispersion of the PE-MPs in the bulk sediment, its distribution in each vial (minimising differences between replicates potentially induced if individually spiked), and the permanence of the plastic particles in the sediment (and not their buoyancy) as reported in Silva et al., (2019).

Test vials were allowed to equilibrate for 24 h. After this period, seven blackworms were added to each vial. The test was run for 48 h without a food supply. Each treatment consisted of seven replicates (i.e., vials). After 48h, blackworms were picked out from the test vials, quickly rinsed with Milli-Q water, and gently dried with filter paper. Four blackworms per replicate were placed in 2 mL microtubes, weighted (fresh weight, FW), snap-frozen in liquid nitrogen and stored at -80 °C for biochemical analysis. The remaining three blackworms were weighted (FW) and used to estimate PE-MPs ingestion.

2.4. Biochemical analysis

The biochemical analysis follows the optimised protocol described in Rodrigues et al. (2015). Briefly, samples stored at -80 °C were homogenised in Milli-Q water using a tissuelyser II Qiagen. From each sample, two aliquots were taken for the energy reserves analysis (lipids; and sugars and protein contents) and another aliquot was used for the measurement of aerobic energy production (measured by estimating the electron transport system (ETS) activity). An extra aliquot was used for the determination of lipid peroxidation (LPO), in which 4 µL of 4 % BHT (2,6-Di-tert-butyl-4-methylphenol) in methanol was added prior to the processing of the samples. The remaining homogenate solution was diluted in an equal proportion of 0.2 M K-phosphate buffer, pH 7.4. The resulting solution was then centrifuged for 20 min at 9,000 g (4 °C) to separate the post-mitochondrial supernatant (PMS). The PMS was divided into microtubes and samples were stored at −80 °C until further enzymatic activity determination of catalase (CAT), glutathione-*S*-transferase (GST), total glutathione level (TG), and protein quantification.

The energy available (sugars, lipids, and proteins) and aerobic energy production (measured as ETS activity) were assessed following the method of De Coen and Janssen (De Coen and Janssen, 1997) with slight modifications (Rodrigues et al., 2015). Total lipid content was extracted by centrifugation of the 300 µL sample aliquots with a mixture of 500 µL of chloroform (119.38 M; ACS spectrophotometric grade, ≥99.8%) and 500 µL of methanol (32.04 M; ACS reagent, ≥99.8%). The organic phase of each sample was transferred to clean glass tubes prior to being acidified by using 500 µL of sulfuric acid (H_2SO_4). The absorbance was then measured at 375 nm.

In the second aliquot of the homogenate (300 µL), proteins were precipitated by trichloroacetic acid (15%), following by incubation at -20 °C. Samples were then centrifuged (1000 g for 10 min at 4 °C), being the supernatant collected to be used for sugar content determination. Samples for sugar determination as well as standard concentrations of glucose (300 µL) were incubated with phenol (5%) and sulphuric acid (H_2SO_4). The absorbance was read in the resulting solution at 492 nm to quantify sugars content. The pellets from this second aliquot were then resuspended in sodium hydroxide and incubated (30 min at 60 °C). After the incubation period, the solution was neutralised by using hydrochloric acid (HCl). Total protein content was then quantified following Bradford's method at 592 nm and using bovine serum albumin as the standard for quantification (Bradford, 1976).

The homogenate for ETS activity measurements (300 µL) was treated by adding 150 µL of homogenization buffer (0.3 M Tris base; 0.45 % (w/v) polyvinylpyrrolidone; 459 µM $MgSO_4$; 0.6% (v/v) Triton X-100 at a pH of 8.5), and centrifuged (10 min, 1000 g, 4 °C). On the 96 well-microplate, 50 µL of supernatant was incubated with 150 µL of buffered solution (0.13 M Tris base containing 0.27 % [v/v] Triton X-100; 1.7 mM reduced nicotinamide adenine dinucleotide; 274 µM reduced nicotinamide adenine dinucleotide phosphate; and INT (p-iodonitrotetrazolium; 8 mM solution). The kinetics of the reaction was followed by measuring the absorbance at 490 nm over a 3-min period.
Lipid peroxidation was determined by measuring the thiobarbituric acid-reactive substances (TBARS) at 535 nm, according to Bird and Draper (Bird and Draper, 1984). The GST activity determination was performed following the conjugation colourimetric reaction measured at 340 nm between GSH and 1-chloro-2,4-dinitrobenzene (Habig et al., 1974). The CAT activity was determined by measuring the decomposition of the substrate H_2O_2 at 240 nm (Claiborne, 1985). The ChE activity was measured by following Ellman's method (Ellman et al., 1961). The method uses acetylthiocholine as substrate, and the increase in absorbance was measured at 412 nm, according to Guilhermino et al., 1996. In the end, protein concentration was estimated by following the Bradford method, adapted from BioRad's Bradford microassay set up in a 96 well microplate, and bovine γ-globulin was used as standard (Bradford, 1976).

Total glutathione level (TG) was calculated after the addition of 250 µL of the reaction solution and 50 µL of PMS fraction according to the method expressed by (Baker et al., 1990). The reaction solution was prepared using 18 mL of Na-K phosphate buffer (0.2 M; pH 8.0), 3 mL of ß-nicotinamide adenine dinucleotide 2-phosphate reduced tetrasodium salt, 6 mL of 10 mM 5,5'-dithiobis(2-nitrobenzoic acid) and 1.5 mL of glutathione reductase (25 µL from stock with 1 U/mL). The absorbance was monitored at 412 nm for 3 min following the recycling reaction of reduced glutathione in excess of glutathione reductase. Total glutathione levels were then expressed as µmol per mg of protein after calculation using a standard curve with L-GSH as a standard.

2.5. Chronic exposure (28 days) - physiological and reproductive responses

The chronic test (28-days) was performed according to the OECD guideline (OECD, 2007) with minor adjustments. Briefly, inorganic fine sediment (<1 mm) previously burnt (500 °C for 4h) was used in the test vials, and organisms were fed with a suspension of macerated fish food (TetraMin) (3 times/week; 200 µg dry weight/worm according to Roman et al., 2007). Adult blackworms with the same physiological state were exposed to five PE-MP concentrations: 0.51, 3.2 and 20 g kg^{-1} (used in acute test), 1.28 and 8 g kg^{-1} (intermediate concentrations) of the above mentioned four different PE-MPs size-classes (A: 32-63 µm; B: 63-125 µm; C: 125-250 µm and D: 250-500 µm), plus a control condition.

Five replicates per condition were set up comprising ten blackworms each. After 28 days of exposure, blackworms were picked out of the sediment, rinsed in Milli-Q water, counted and placed in microtubes at -80 °C for further lyophilisation. At this point, reproduction (total number of surviving blackworms) and biomass in each replicate (dry weight after lyophilisation) were assessed. The freeze-dried blackworms were also used for the quantification of PE-MPs present inside the organisms.

2.6. Extraction and quantification of PE-MPs

The number of PE-MPs particles inside the blackworms was evaluated after 48 h and 28 d of exposure. The estimation after 48 h was performed using three blackworms per replicate (the other four blackworms per replicate were used in biochemical determinations) and the estimation after 28 d was performed using all surviving blackworms (used to evaluate reproduction and biomass).

The extraction and quantification of PE-MPs from biological samples were optimised for benthic invertebrates in previous work (Silva et al., 2019). Blackworms previously freeze-dried and weighed (dry weight, DW) were moved to glass flasks and incubated in nitric acid (HNO_3) 65% at 60 °C for three hours. After this period, hydrogen peroxide (H_2O_2) 35 % was added to each sample (1:1 v/v) and incubated for more 24 hours at room temperature. Samples were then diluted 1:10 with a and vacuum-filtered onto gridded cellulose ester filters (Whatman 10406972, Mixed Cellulose Ester Filter, 3.1 mm white/black grid, 0.45 µm pore size). Retained particles (in the filtration apparatus) were copiously flushed with Milli-Q water. Membranes were transferred to glass petri-dish and allowed to dry in the oven at 25 °C for 2–3 days. Afterwards, the number of particles ingested by the blackworms was estimated under a stereomicroscope (stereoscopic zoom microscope—SMZ 1500, Nikon Corporation).

2.7. Statistical Analysis

Non-parametric Spearman's rank correlation was used to analyse the relationship between the sediment concentration of MPs and the number of particles inside the worm gut for each size-class.

Reproduction and biomass endpoints, and also biochemical responses were previously analysed by Kolmogorov-Smirnov to check for normality and Levene's test to check for homogeneity of variances. Appropriate transformation of data (log transformation) was used whenever necessary. One-way analysis of variance (ANOVA) was used for each PE-MP size-class to analyse differences between treatments for each size-class. Dunnett's test was used to determine significant differences from control. Statistical differences were considered at $p<0.05$. SPSS 20.0 software was used for statistical analyses.

3. Results

3.1. Short-term exposure (48 h): physiological and biochemical responses

Lumbriculus variegatus was able to ingest PE-MPs from all tested class sizes, after 48 h exposure. The number of PE-MPs in their gut varied between a few particles (≤ 20 PE-MPs/worm) in treatments containing MPs larger than 63 µm (size-class B, C, and D), up to 71 PE-MPs per worm in the treatment containing MPs smaller than 63 µm (size-class A: 32-63 µm; Fig.1). The number of PE-MPs present inside blackworms gut was directly related to the concentration for three of the four PE-MPs size-classes tested (size-class A: 32-63 µm: $r=0.949$, $p=0.000$; size-class C: 125-250 µm: $r=0.741$, $p=0.011$; and size-class D: 250-500 µm: $r=0.858$, $p=0.002$; Fig. 1). The exception was the number of PE-MPs present inside blackworms' gut in worms exposed to size-class B (63-125 µm; $r=0.113$, $p=0.482$; Fig.1). Data on this size-class show increased variance, especially in worms exposed to 3.20 g kg^{-1} and possibly the absence of correlation is mostly related to the heterogeneity of data.

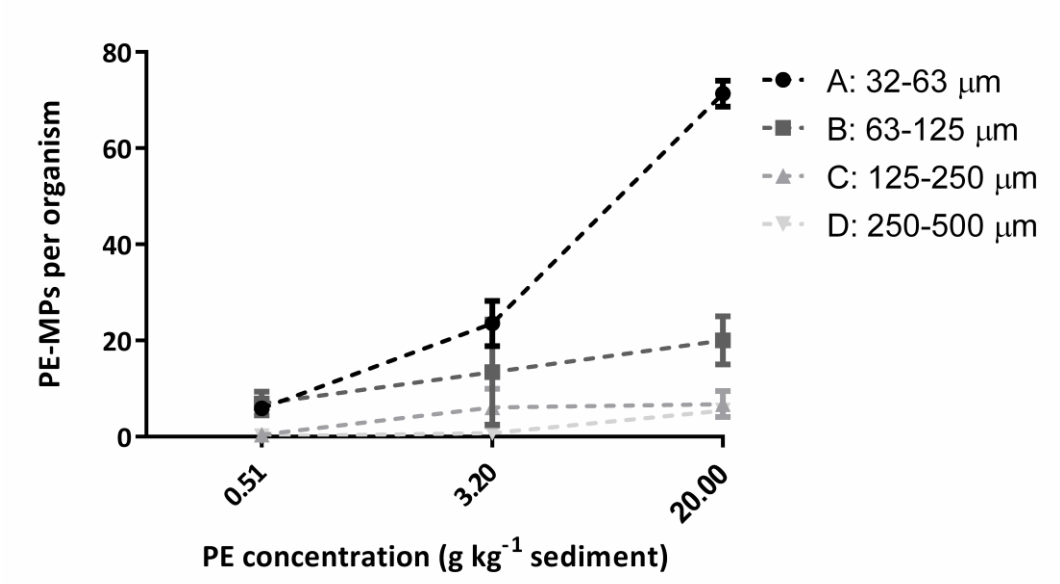

Fig. 1. Number of polyethylene microplastics (PE-MPs) present in the gut of the blackworm *Lumbriculus variegatus* (PE-MPs per organism) after 48 h exposed to different-sized PE-MPs (A: 32-63 μm; B: 63-125 μm; C: 125-250 μm; and D: 250-500 μm). Values are present as mean ± SE, *n*=3. The number of PE-MPs in the control condition was estimated and omitted as a result of a null value.

Lipid content was significantly decreased in blackworms exposed to larger-size particles (i.e., ≥63 μm) (size-class B: $F_{3,24}$=7.396, *p*=0.001; size-class C: $F_{3,24}$=4.950, *p*=0.008; size-class D: $F_{3,24}$=4.411, *p*=0.013). Levels of lipids decreased up to 60% in blackworms exposed to PE-MPs larger than 63 μm (Fig. 2). Moreover, levels of sugars in blackworms exposed to PE-MPs were generally lower compared to blackworms in control conditions (size-class A: $F_{3,24}$=5.083, *p*=0.008; size-class B: $F_{3,24}$=4.845, *p*=0.009) (Fig. 2). Conversely, exposure to PE-MPs did not alter protein content of blackworms (size-class A: $F_{3,24}$=2.594, *p*=0.076; size-class B: $F_{3,24}$=1.344, *p*=0.284; size-class C: $F_{3,24}$=1.155, *p*=0.348 and size-class D: $F_{3,24}$=1.394, *p*=0.269).

Aerobic energy production (ETS) was reduced in blackworms exposed to PE-MPs (size-class A: $F_{3,24}$=4.004, *p*=0.019; size-class B: $F_{3,24}$=6.010, *p*=0.003; size-class C: $F_{3,24}$=4.623, *p*=0.011 ; size-class D: $F_{3,24}$=5.147, *p*=0.007) when compared to blackworms in control conditions. This reduction in ETS activity was even more evident in blackworms exposed to particles larger than 125 μm (size-classes C and D), which showed a decreased aerobic energy production regardless of the concentration tested (Fig. 2 and Table 1). ETS activity decreased ~25% for 3.20 g kg^{-1} and 20 g kg^{-1} of PE-MPs under 125 μm (size classes A and B). Blackworms exposed to MPs larger than 125 μm (size classes C and D), regardless of the concentration tested, also revealed a decreased ETS activity of ~25 % (Fig. 2).

Cellular energy allocation (CEA) was not affected in blackworms exposed to MPs, due to the proportional decrease in the Energy Available (Ea) and the aerobic energy production (ETS) (Table 1).

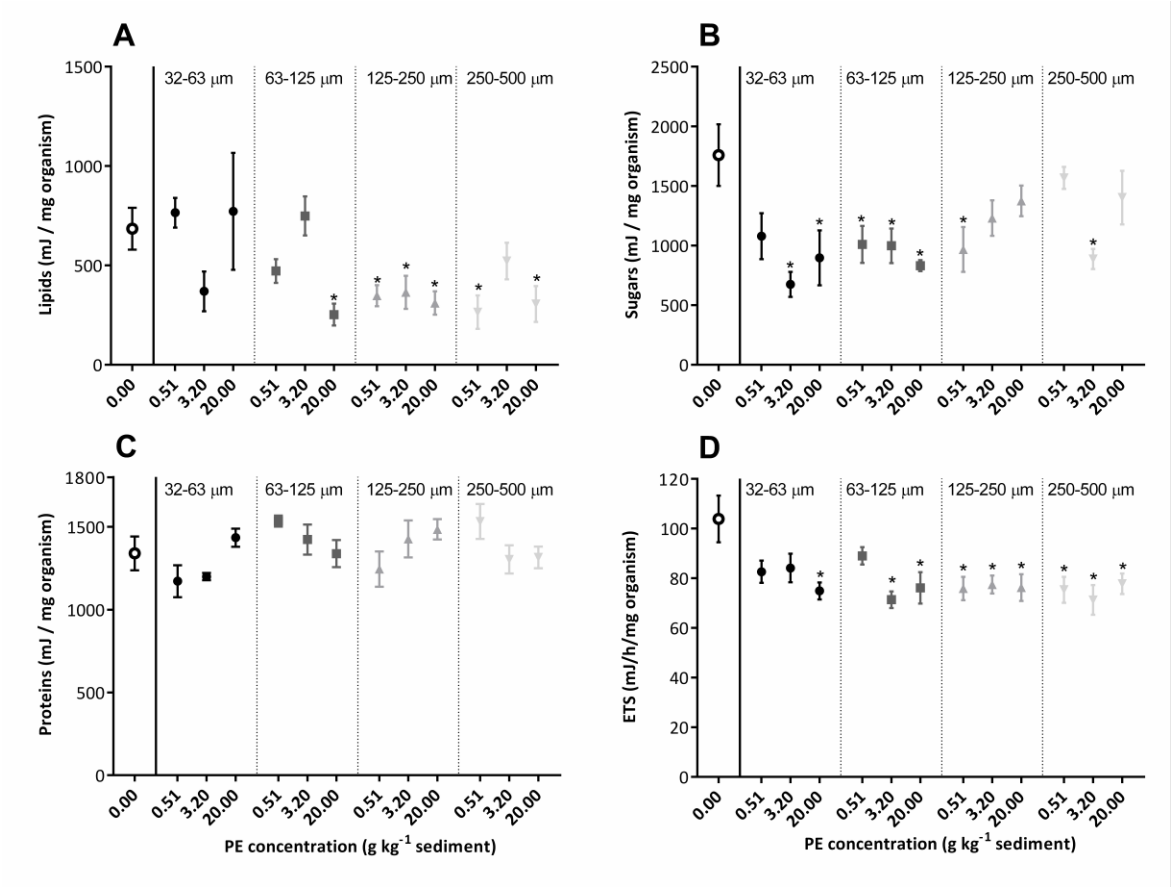

Fig. 2. Levels of energy reserves: A: lipids; B: sugars and C: proteins (mJ/mg organism); and D: aerobic energy production (Electron Transport System - ETS activity, mJ/h/mg organism) after short-term (48 h) exposure of *Lumbriculus variegatus* to polyethylene microplastics (PE-MPs). All values are presented as mean ± SE, *n*=7. Different-sized PE-MPs were tested: A-32-63 µm; B-63-125 µm; C-125-250 µm; and D-250-500 µm. *denotes a significant ($p < 0.05$) difference when compared with the control (0) treatment following ANOVA and post-hoc Dunnett's tests.

Table 1. Energy budget of *Lumbriculus variegatus* after short-term (48 h) exposure to polyethylene microplastics (PE-MPs). The total energy available, Ea, as a sum E_{lipids}, E_{sugars} and $E_{proteins}$ (expressed as mJ/mg organism); the aerobic energy production, ETS (expressed in mJ/h/mg organism); and the cellular energy allocation ratio (CEA = Ea/ETS). All values are presented as mean ± SE. Different-sized PE-MPs were tested: A-32-63 µm; B-63-125 µm; C-125-250 µm; and D-250-500 µm.

PE size-class	PE [g kg^{-1}]	Ea (lipids+sugars+proteins)	ETS	CEA (Ea/ETS)
	0	3763 ±379	104±9	36±5
32-63µm	0.51	3016±222	83±4	37±2
	1.28	2244±185 [a]	84±6	27±3
	20	3008±244	75±3 [a]	40±3
63-125µm	0.51	3013±180	89±3	34±3
	1.28	3170±187	71±3 [a]	45±2
	20	2423±118	76±6 [a]	33±3
125-250µm	0.51	2561±212	76±5 [a]	34±4
	1.28	3022±277	77±4 [a]	39±2
	20	3170±104	76±5 [a]	43±3
250-500µm	0.51	3365±62	75±5 [a]	46±3
	1.28	2714±198	71±6 [a]	39±3
	20	3024±236	78±4 [a]	39±3

[a] Denotes a significant difference compared to the control treatment (0 g kg^{-1})— (Dunnett's test, $p < 0.05$).

Table 1: Energy budget of *Lumbriculus variegatus* after short-term (48 h) exposure to polyethylene microplastics (PE-MPs). The total energy available, Ea, as a sum E_{lipids}, E_{sugars} and $E_{proteins}$ (expressed as mJ/mg organism); the aerobic energy production, ETS (expressed in mJ/h/mg organism); and the cellular energy allocation ratio (CEA = Ea/ETS). All values are presented as mean ± SE. Different-sized PE-MPs were tested: A-32-63 µm; B-63-125 µm; C-125-250 µm; and D-250-500 µm.

Total glutathione levels were significantly increased in blackworms exposed to 20 g kg^{-1} PE-MPs size-class A ($F_{3,24}$=7.483, p=0.001), and 0.51 and 3.20 g kg^{-1} PE-MPs size-class B ($F_{3,24}$=10.588, p=0.000) (Fig. 3) and blackworms exposed to all concentrations of MPs-PE larger than 125 µm (size-class C: $F_{3,24}$=11.613, p=0.000; size-class D: $F_{3,24}$=12.163, p=0.000; Fig. 2). Increased levels was observed for GST especially in blackworms exposed to PE-MPs size-class D (size-class D: $F_{3,24}$=3.542, p= 0.030, with significance for 0.51 and 3.20 g kg^{-1})(Fig. 3). Catalase levels were not affected by PE-MPs exposure (size-class A: $F_{3,23}$=2.471, p=0.087; size-class B: $F_{3,24}$=1.632, p=0.208; size-class C: $F_{3,24}$=1.760, p=0.182; size-class D: $F_{3,24}$=0.084, p=0.968; Fig. 3). Lipid peroxidation was not significantly affected in blackworms exposed to PE-MPs when compared to control conditions (size-class A: $F_{3,24}$=2.754, p=0.066; size-class B: $F_{3,24}$=1.644, p=0.201; size-class C: $F_{3,24}$=1.706, p=0.192; size-class D: $F_{3,24}$=0.557, p=0.648; Fig. 3).

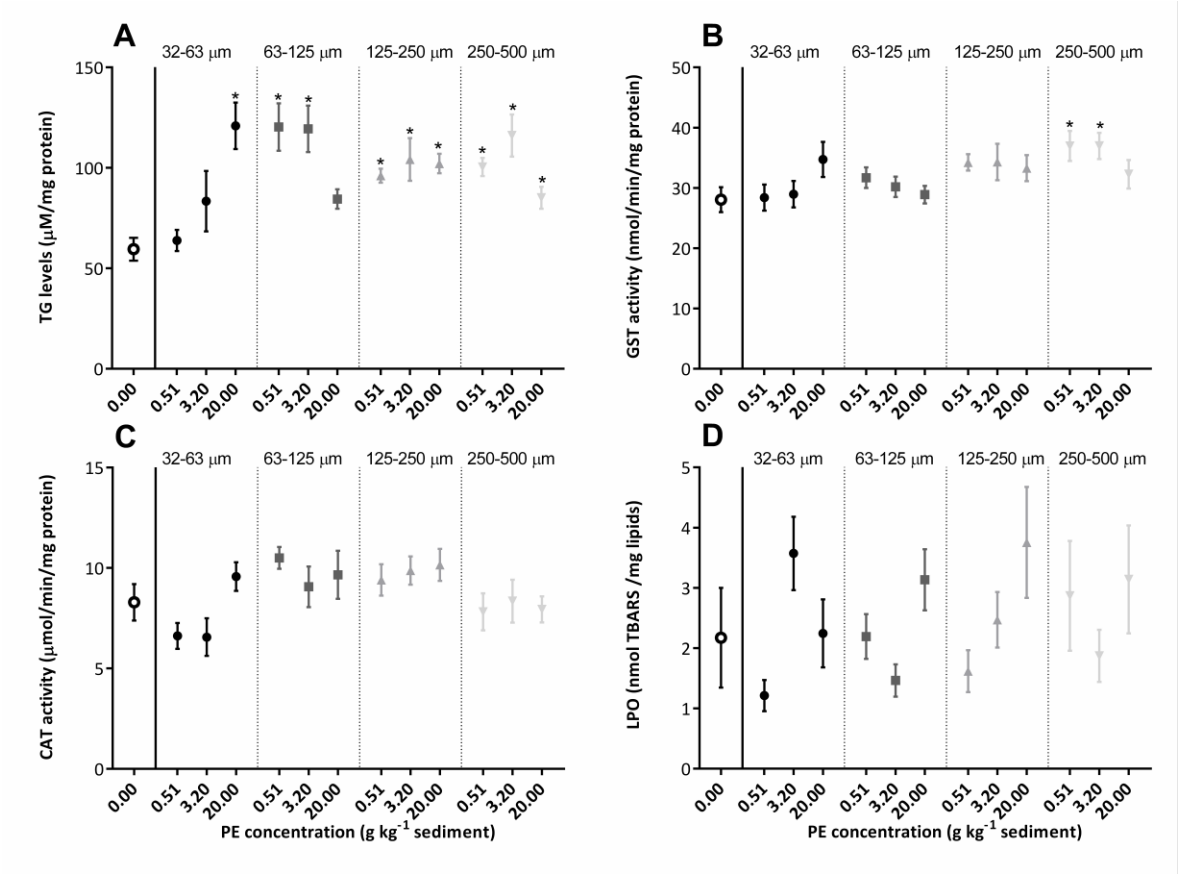

Fig. 3. Level of A: total glutathione (TG, µM/mg protein), activities of: B: glutathione-*S*-transferase (GST, nmol/min/mg protein); C: catalase (CAT, µmol/min/mg protein); and D: lipid peroxidation (LPO, nmol TBARS/mg lipids), after short-term (48 h) exposure of *Lumbriculus variegatus* to polyethylene microplastics (PE-MPs). All values are presented as mean ± SE, *n*=7. Different-sized PE-MPs were tested: A-32-63 µm; B-63-125 µm; C-125-250 µm; and D-250-500 µm. *denotes a significant ($p < 0.05$) difference when compared with the control (0) treatment following ANOVA and post-hoc Dunnett's tests.

Cholinesterase (ChE) remained similar between treatments (size-class A: $F_{3,24}$=1.902, p=0.156; size-class B: $F_{3,24}$=7.297, p=0.001; size-class C: $F_{3,24}$=2.497, p=0.084; size-class D: $F_{3,24}$=2.021, p=0.138; Fig. 4), except for blackworms exposed to 0.51 g kg^{-1} PE-MPs of size-class B (Fig. 4) where ChE levels were 36% higher than in control conditions.

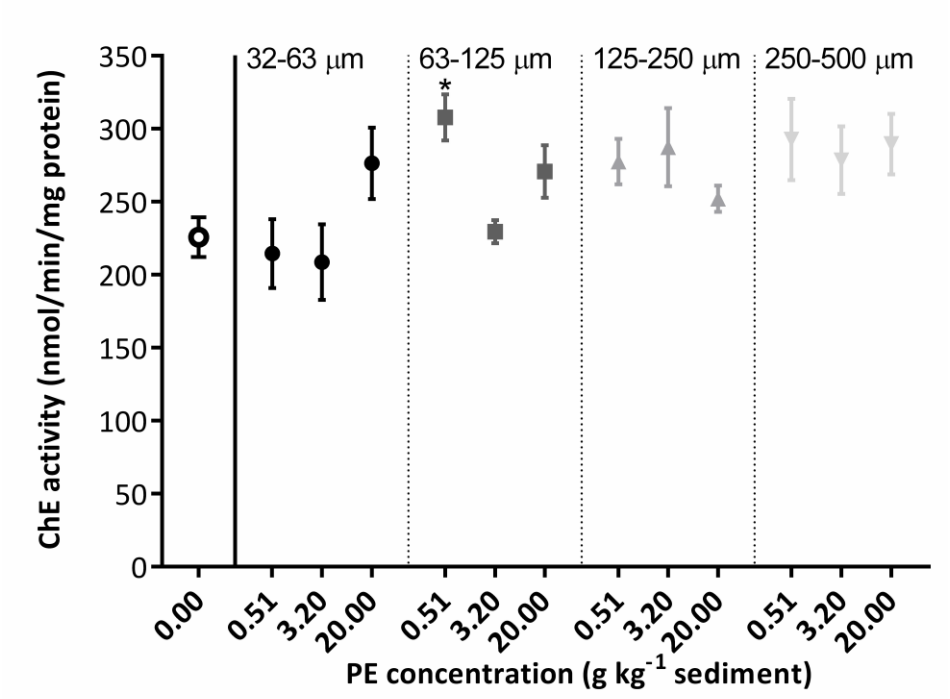

Fig. 4. Cholinesterase activity (ChE, nmol/min/mg protein) after short-term (48 h) exposure of *Lumbriculus variegatus* to polyethylene microplastics (PE-MPs). All values are presented as mean ± SE, *n*=7. Different-sized PE-MPs were tested: A-32-63 μm; B-63-125 μm; C-125-250 μm; and D-250-500 μm. *denotes a significant ($p < 0.05$) difference when compared with the control (0) treatment following ANOVA and post-hoc Dunnett's tests.

3.2. Long-term exposure (28 days): reproductive responses

The number of MPs in *L. variegatus* gut after 28 d was generally lower in organisms exposed to MPs > 63 μm size classes B, C and D (up to 4 PE-MPs per organism; Fig. 5) compared to organisms exposed to MP < 63 μm (up to 41 PE-MPs per worm in the highest concentration; Fig. 5). The number of PE-MPs ingested by the blackworms after 28 d was generally lower than those found after the 48h exposure. Also, like in the short-exposure bioassay, the number of ingested PE-MPs was directly related to their concentration (size-class A 32-63 μm: r=0.890; p=0.000; size-class B 63-125 μm: r=0.645; p=0.000; size-class C 125-250 μm: r=0.491; p=0.006), with the exception of blackworms exposed to the larger particles (size-class D 250-500 μm: r=0.044; p=0.418; Fig. 5).

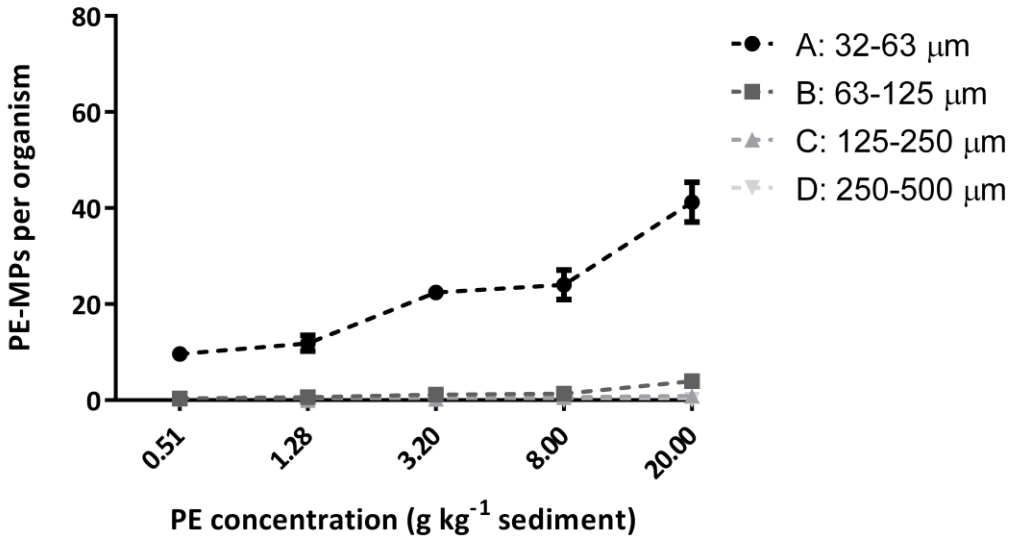

Fig. 5: Number of polyethylene microplastics (PE-MPs) present inside the blackworm *Lumbriculus variegatus* (MPs per organism) after 28 days exposed to different-sized PE-MPs. Values are present as mean ± SE, *n*=5. The number of PE-MPs in the control condition was estimated and was omitted as a result of a null value.

After 28 d of exposure, a factor of 1.8 was found in the number of living blackworms per replicate in control conditions (18 blackworms by the end of the test). Both this parameter and physicochemical parameters of the test complied the OECD criteria of the bioassay (OECD, 2007).

Reproduction of blackworms was not affected by PE-MPs size or concentration (size-class A: $F_{5,24}$=1.600, *p*=0.198; size-class B: $F_{5,24}$=1.765, *p*=0.158; size-class C: $F_{5,24}$=1.597, *p*=0.199; and size-class D: $F_{5,24}$=2.054, *p*=0.107; Fig. 5). However, a slight decrease in the reproduction rates was found in concentrations equal to, or higher than 1.28 g kg^{-1} for size-class A (32-63 μm) PE-MPs (Fig. 6A). Likewise, the biomass of living blackworms was not affected by the exposure to PE-MPs (size-class A: $F_{5,24}$=0.829, *p*=0.541; size-class B: $F_{5,22}$=2.002, *p*=0.118; and size-class D: $F_{5,24}$=0.984, *p*=0.448). For size-class C and although significance difference in biomass was detected among concentrations ($F_{5,24}$=3.211, *p*=0.023) post-hoc tests did not indicate any difference of any of the concentrations tested against the control treatment**.** The biomass measured as the dry weight per organisms varied between 0.6 and 0.8 mg/worm for all the conditions tested (Fig.6B).

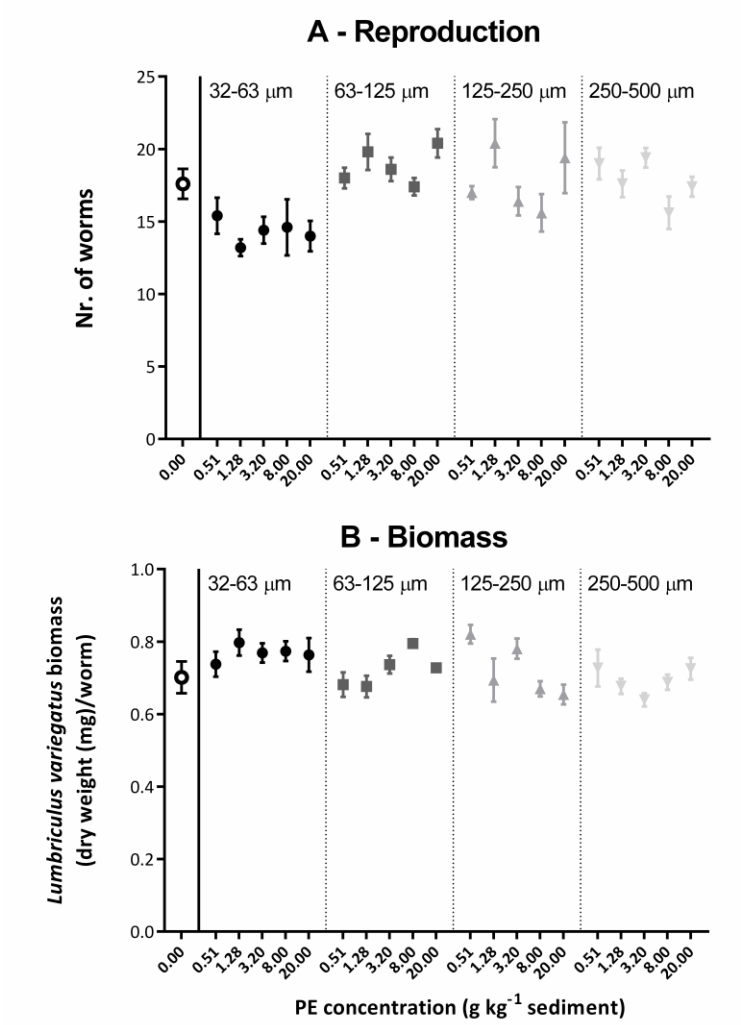

Fig. 6: *Lumbriculus variegatus* reproduction (A; the number of worms) and biomass (B; dry weight (mg) per organism) after 28 days exposed to different-sized polyethylene microplastics (PE-MPs): (A: 32-63 μm; B: 63-125 μm; C: 125-250 μm; and D: 250-500 μm). Values are present as mean ± SE, *n*=5.

4. Discussion

Different sizes of PE-MPs triggered distinctive biochemical alterations on *L. variegatus* after 48 h of exposure, mostly related to antioxidant and detoxification mechanisms and changes in energy metabolism (decreased energy reserves and a reduction of aerobic energy production). No significant effects were observed in terms of reproductive endpoints after 28 days of exposure.

After a short-term exposure of 48 h, blackworms presented PE-MPs in their guts. The ingested PE-MPs were within the size range of particles commonly ingested by blackworms, particularly in the 32-63 μm size-range (Leppänen and Kukkonen, 1998a, 1998c; Chapman, 2001; Sardo et al., 2007). Ingestion of MPs was previously observed for this species and also for other benthic/epibenthic freshwater invertebrates, such as *Chironomus riparius* (Scherer et al., 2017), *Chironomus tepperi* (Ziajahromi et al., 2018), *Hyallela azteca* (Khan et al., 2019), *Tubifex tubifex*

(Hurley et al., 2017) and *Gammarus pulex* (Scherer et al., 2017). The number of PE-MPs (up to 70 MPs per worm) found in the *L. variegatus* gastrointestinal tract (a proxy of ingestion) was dependent on the sediment concentrations used. Still, it was much lower than what was previously observed for other detritivore invertebrates that occupy the same habitat, such as, *C. riparius* larvae (2400 PE-MPs per larvae) after 48 h exposure to the same polymer of similar sizes (Silva et al., 2021). Such differences can be related to organisms' feeding strategy, including selectivity of food items and physiological limitations (mouth apparatus) (Redondo-Hasselerharm et al., 2018b; Silva et al., 2019). Blackworms are recognised sediment-ingesters, processing and recycling higher quantities of deposited material (compared to *C. riparius*) which may allow them to avoid agglomeration by decreasing residence time of inert particles in the gut (Leppänen and Kukkonen, 1998a, 1998b). This evidence seems to be also supported by the slightly lower number of PE-MP in *L. variegatus*' gut after 28 d exposure in the presence of food when compared to a short period (48 h) in the absence of food.

Nevertheless, despite the low number of PE-MPs ingested by blackworm, a general decrease in energy reserves (lipid and sugars content) was triggered by the presence of the inert plastic particles inside the gut. This depletion of energy reserves was also observed for other species as a response to MPs exposure (Wright et al., 2013; Deng et al., 2017; Veneman et al., 2017; Cole et al., 2019; Silva et al., 2021), and has been linked to energetic costs of immune system activation, altered activity, digestion and egestion of inert particles, and activation of detoxification and antioxidant mechanisms (Von Moos et al., 2012; Wright et al., 2013a, 2013b; Karami et al., 2016; Chen et al., 2020; Trestrail et al., 2020a,b). In our study, the depletion of energy reserves in *L. variegatus* could be related to the activation of detoxification mechanisms and specifically due to the oxidative challenges posed by microplastics ingestion which in our case was probably exacerbated under "starvation" (since no food was added in the 48h assay). General activation of antioxidant and detoxification mechanisms was observed, i.e., high levels of total glutathione in blackworms exposed to MPs (independently of the size) accompanied by the increased activity of glutathione-*S*-transferase (blackworms exposed to size-class D MPs >250 µm). Glutathione-*S*-Transferase upregulation after exposure to MPs was also reported in other invertebrates, such as *Caenorhabditis elegans* (Lei et al., 2018a, 2018b) and *Mytilus* spp. (Paul-Pont et al., 2016). Moreover, activation of antioxidants, like glutathione, was earlier depicted in other organisms (small vertebrates) after the ingestion of MPs (Deng et al., 2017). The activation of antioxidant and detoxification mechanisms observed in the present study, and especially the increase in glutathione levels due to *de novo* synthesis and recycling (Dickinson and Forman, 2002) allowed blackworms to cope with increased levels of ROS and prevent oxidative damage since lipid peroxidation was not increased. The production of ROS after exposure to MPs was reported before in invertebrates (for review see Trestrail et al., 2020a,b) like copepods (Jeong et al., 2017); bivalves (Von Moos et al., 2012; Paul-Pont et al., 2016; Ribeiro et al., 2017), crustacea (Yu et al., 2018) and nematodes (Lei et al., 2018b). Glutathione is known to play a more important role in antioxidant defence of L. variegatus than in other aquatic invertebrates, in response to other

chemical stressors (Kristoff et al., 2008) and its putative role as an antioxidant response in invertebrates following microplastic ingestion has been suggested (Trestrail et al., 2020a). The increased glutathione levels observed for blackworms, together with the increase of GST activity, in response to large microplastics, seem to be sufficient to prevent oxidative damage (lipid peroxidation). Along with the short residence time of particles in their guts, this might explain the low sensitivity of *L. variegatus* to PE MPs exposure when compared with other benthic species. For example, exposure to the same PE-MPs here tested lead to oxidative damage (increased lipid peroxidation) in *C. riparius* larvae showing a high number (~2400) of MPs ingested and retained in their gut (Silva et al., 2021). Accumulation of PE-MPs in the gut can induce an inflammatory response caused by the physical damage of the particles to the gut lumen. The tissue damage *per se* is enough to activate components of the immune system involved in wound repair (Rowley and Powell 2007; Grizanova 2014). Degrading proteins, and other molecules such as cytotoxic quinones, nitrogen intermediates and reactive oxygen species can harm the organism if produced in excess (González-Santoyo and Córdoba-Aguilar 2012). Thus, the induction of an immune response may itself partially explain the oxidative damage and antioxidant alterations previously observed for several organisms exposed to MP (Trestrail et al., 2020a; Silva et al., 2021).

Alongside with the depletion of energy reserves, likely a reflection of the induced antioxidant responses, exposure to PE-MPs also reduced the aerobic energy production (i.e., decreased electron transport system activity - ETS activity) in *L. variegatus*. This reduction in aerobic energy production seems to be balanced with the depletion of energy reserves resulting in similar values of energy budget (i.e. the quotient between energy available and energy consumption- CEA). This inhibition of ETS activity can be the result of two non-exclusive mechanisms i) a decrease in activity, metabolic rate and oxygen consumption, which has been observed before in response to microplastics ingestion (Kratina et al., 2019), maybe also as a consequence of energy reserves depletion due to the absence of food, since CEA was kept constant as previously shown; ii) increased anaerobic metabolism as a partial backup for energy production, despite the low anaerobic rate of energy metabolism known for *L. variegatus* (Penttinen and Kukkonen, 2000), but which has been reported before as a response to MPs in different organisms (Oliveira et al., 2013; Barboza et al., 2018; Rodríguez-Seijo et al., 2018). To further explore these two non-competing hypotheses, the evaluation of anaerobic pathway (e.g., by measuring lactate dehydrogenase activity – LDH) and the assessment of oxygen consumption and of the activity levels in exposed *L. variegatus* would be helpful to explore all possible effects of microplastics ingestion concerning energy metabolism.

In addition, there was a tendency for an increment in ChE activity that was significant particularly for *L. variegatus* exposed to larger PE-MP (>63 μm), which was also observed for *Chironomus riparius* exposed to particles of similar size (Silva et al., 2021) and in larval stages of *Amphibalanus amphitrite* barnacle exposed to polystyrene microplastics (PS-MPs) (Gambardella et al., 2017). A potential explanation might be related to an extra effort to egest MPs (related to peristaltic movements), and/or gut inflammation during egestion and consequent stress, which

agrees with the activation of antioxidant mechanisms in our study, as a response to the production of ROS. Inflamed cells and tissues have been related to a greater amount of acetylcholine compared to healthy ones (De Oliveira et al., 2012).

Reproductive endpoints (density and biomass) were not significantly affected on blackworms exposed for 28 days to PE-MPs. The same lack of effects was previously reported in blackworms after exposure to higher concentrations of similar-sized PS-MPs (e.g., 40% of MPs in sediment dry weight) (Redondo-Hasselerharm et al., 2018b). The absence of effects on reproductive output is probably linked to blackworms' physiology and feeding strategy that prevented long retention of MPs in their guts resulting in no observable effects. The continuous ingestion of sediment and food items (provided in the long-term exposure) accelerates the digestion process and facilitates the egestion of PE-MPs. Feeding is frequently linked to the stimulation of MPs' egestion and therefore to a shorter residence time of MPs in invertebrates' gut (Rist et al., 2017; Scherer et al., 2017) which may also explain the lower numbers of MPs registered after 28 days compared to the short-term exposure (48h). Besides, the food availability with high nutritional value during the chronic bioassay is likely to alter feeding activity and ingestion of plastic particles and also contribute to a replenishment of the energy reserves that are crucial to deal with stress. Likewise, blackworms' reproductive strategy can also limit the ingestion of MPs. During architomic reproduction, blackworms take several days to regenerate new segments and resume feeding after fragmentation (Leppänen and Kukkonen, 1998a, 1998b).

Final remarks

Despite no effects on reproduction after chronic exposures, our study provides evidence for the ingestion of PE-MPs by the freshwater invertebrate *Lumbriculus variegatus* and associated biochemical and physiological responses. Blackworms seem to ingest low numbers of PE-MPs both at short and long-term exposures compared to other sediment-dwelling invertebrates. The presence of PE-MPs in blackworms' guts triggered the activation of antioxidant and detoxification mechanisms that efficiently prevented oxidative damage. The activation of antioxidant mechanisms altered energy metabolism, accounting for the depletion of energy reserves and reduced aerobic energy production. Furthermore, blackworms' feeding strategy, including their high sediment processing rates, may shorten the residence time of MPs in their guts. It would be of interest to evaluate the ingestion/egestion of different sized-MPs to elucidate on their retention time in blackworms gut as well as the biochemical and reproductive effects induced by these particles under different food regimes.

Our results seem to indicate that this species is not a good bioindicator organism for environmental biomonitoring of MPs, as *L. variegatus* do not accumulate such particles in the gut and presented no oxidative damage even when exposed to significantly higher concentrations than reported in the field (worst-case scenario). Consequently, development and reproduction were not affected after chronic exposures indicating probable low interference of MPs ingestion in *L.*

variegatus population fitness. Nevertheless, the potential and continuous activation of the immune system of blackworms induced by ingestion of plastic particles, associated increased ROS and consequent energetic costs might lead to a higher susceptibility against other stressors such as pathogens and chemical pollutants.

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Chapter 4

Oxidative damage and decreased aerobic energy production due to ingestion of polyethylene microplastics by *Chironomus riparius* (Diptera) larvae

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Oxidative damage and decreased aerobic energy production due to ingestion of polyethylene microplastics by *Chironomus riparius* (Diptera) larvae

Abstract

Riverine sediments are major sinks of microplastics from inland anthropogenic activities, imposing a threat to freshwater benthic invertebrates. This study investigated the ingestion of three size-classes (SC) of irregularly shaped polyethylene microplastics (PE-MPs; SC I: 32− 63 μm; II: 63− 250 μm; III: 125− 500 μm) after 48 h by dipteran larvae (detritivore/collector) *Chironomus riparius*, and the consequent effects on neurotransmission, energy allocation and oxidative stress. The tested PE-MPs concentrations (1.25; 5; 20 g kg^{-1}) were within the range of concentrations reported in riverbanks from highly urbanised areas (1 - 9 g kg^{-1}), except for 20 g kg^{-1} representing the worst-case scenario. After exposure to SC I, larvae presented high amounts (up to ~2400 particles/organism) of PE-MPs in their guts, with an average size-range of 30− 60 μm. In the SC II and III, larvae presented PE-MPs of higher diameter (up to 125 μm) and a visible gut obstruction. The high number of particles in the larval gut (SC I) and/or difficulties for their egestion (SC I, II and III) induced oxidative damage and reduced aerobic energy production. In addition, larvae exposed to SC II and III revealed depletion in their total lipid reserves as a consequence of lacking nutrients, and the ones exposed to SC III presented a decrease in their detoxification capacity. These results highlight that freshwater detritivores with low selective feeding behaviour (e.g., chironomids) are more prone to ingest microplastics, with potentially adverse effects on cellular metabolism, redox status and antioxidant-detoxification defences. These harmful effects at lower levels of the biological organisation may ultimately affect organisms' physiology and fitness

Keywords: Benthic invertebrates; Energy reserves; Biomarkers; Aquatic insects; Acute effects

1. Introduction

Microplastics (MPs, synthetic or semi-synthetic polymer with size between 1 μm to 5 mm; Frias and Nash, 2019) reach freshwater ecosystems in its microscale (primary MPs) or as a result of degradation of larger pieces of plastic (secondary MPs). Once in freshwaters, MPs undergo biological and physical processes such as biofouling and homo- or hetero-aggregation (Lagarde et al., 2016; Lambert et al., 2017) that are known to modify MPs' density and floatability and to accelerate its deposition in sediments (Corcoran, 2015). Continuous release and long-term deposition make MPs present and persistent in freshwater sediments, especially near highly industrialised or densely populated areas (Klein et al., 2015; Castañeda et al., 2014) where they can reach levels up to 9 g kg^{-1}, as in Mersey/Irwell river, United Kingdom (Hurley et al., 2018).

The contamination of freshwater sediments by MPs is of special concern, as many key-benthic invertebrates (on nutrient cycling) feed on the particulate organic matter within the same size-range of most primary MPs (Henriques-Oliveira et al., 2003; Syrovátka et al., 2009; Conkle et al., 2017). The ingestion of MPs has been related to possible harmful developmental and reproductive effects on several freshwater benthic invertebrate species, such as *Chironomus tepperi* (Ziajahromi et al., 2018), *Chironomus riparius* (Scherer et al., 2019; Silva et al., 2019; Stanković et al., 2020; Khosrovyan et al., 2020), and *Hyalella azteca* (Au et al., 2015). Thus, the contamination of freshwater sediments by MPs is of special concern, since nutrient cycling would be impaired if key-benthic invertebrates feed on MPs instead of particulate organic. Also, the presence of MPs in freshwater sediments seems to alter benthic communities (Redondo-Hasselerharm et al., 2020). In contrast, other recent studies found limited to no effects on freshwater invertebrates' life-history traits (Weber et al., 2018; Khosrovyan and Kahru, 2020), but the studies were performed using different polymers (polyamide and polyethylene terephthalate) and did not address ingestion/egestion capacities. The effects of MPs at the cellular and sub-cellular levels on freshwater invertebrates are less studied and can represent early warning signals of sub-lethal effects providing valuable information concerning the mechanisms of action of MPs (Jeong and Choi, 2019). Despite this lack of information for freshwater biota, there are already some insights on biochemical responses triggered after exposure to MPs (González-Pleiter et al., 2019); but were mainly performed in filter-feeding organisms and most studies only report the ingestion of MPs, but do not quantify the particles present inside the gut of the organisms. Relevant physiological and biochemical responses induced by microplastics include (1) energy impairment (Wright et al., 2013a; Gardon et al., 2018); (2) neurotoxicity (Oliveira et al., 2013; Luís et al., 2015); (3) activation of immune system responses (Avio et al., 2015; Veneman et al., 2017); (4) oxidative stress (Jeong et al., 2016; Lu et al., 2016; Chen et al., 2017; Deng et al., 2017; Choi et al., 2018; Espinosa et al., 2018); and (5) changes in the microbiome (Jin et al., 2018; Lu et al., 2018).

Recognising that long-term ingestion of polyethylene microplastics (PE-MPs) cause significant alterations in life-history traits in *C. riparius* (Silva et al., 2019) and that polyethylene is one of the most common polymers found in freshwater sediments (Li et al., 2019), this study aimed to determine the number and size of ingested particles after short-term exposure to contaminated

sediments and to assess the alterations induced on key-physiological functions of *C. riparius* larvae that are triggered by the particles retained in the gut. For that, fourth instar *C. riparius* larvae were exposed for 48 h to three different concentrations (1.25; 5 and 20 g kg^{-1}) of PE-MPs of three different size-classes (I:32–63; II:63–250 and III:125−500 µm). The range of concentrations used for testing was set based on levels reported for freshwater sediments in highly urbanised areas (Hurley et al., 2018). Physiological responses were evaluated using biomarkers to assess the health condition of larvae including oxidative damage (lipid peroxidation, LPO), phase II biotransformation enzyme (glutathione-*S*-transferase, GST), enzymatic antioxidant defences (catalase, CAT), an enzymatic activity important for cholinergic neurotransmission (acetylcholinesterase, AChE), energy reserves (lipids, proteins and sugars), and electron transport system (ETS) activity (aerobic energy production).

2. Materials and methods

2.1. Organism culture conditions

Chironomus riparius larvae that were used in the experiment were reared at the University of Aveiro (Department of Biology). Briefly, *C. riparius* are maintained at 20 ± 1 °C under a 16:8 h light-dark photoperiod. Larvae are grown in glass aquaria containing a layer (~3 cm) of previously burnt (500 °C for 4 h) inorganic sediment (<1 mm) and American Society for Testing Materials (ASTM) hard water in a 1:4 ratio (ASTM, 1980), with continuum aeration. Glass aquaria used for rearing *C. riparius* larvae are confined within acrylic cage retaining the adults. *C. riparius* culture is fed (*ad libitum*) three times a week using a suspension of macerated TetraMin® (Tetrawerke, Melle, Germany). The ASTM hard water is biweekly renewed, and sediment is monthly replaced. *C. riparius* life cycle is finalised within 3–4 weeks in laboratory-controlled conditions.

Fourth instar larvae (12 days old post-hatching) from egg ropes isolated from the culture were used in the bioassays.

2.2. Polyethylene microplastics used in the experiment

Polyethylene (PE) microplastics of different size were purchased to be used in the experiments. The technical information on the PE-MPs used in the tests (size-classes I, II and III) and respective particle size distribution after vibratory sieve shaking is presented in Table 1. The particle size presented corresponds to ≥ 90 % of the particles present in the respective size-class.

Table 1. Technical information on the polyethylene microplastics used for testing.

Size-class	*Average size*	*≥ 90 % of particles size*	*CAS*	*Company*
I	40−48 µm	32−63 µm	9002−88-4	Sigma-Aldrich UK
II	125 µm	63−250 µm	9002−88-4	Sigma-Aldrich UK
III	350 µm	125−500 µm	708−316-83	Goodfellow USA

Polyethylene microplastics size-range was the same as previous tests (Silva et al., 2019), which is within the size of regular food items ingested by the *C. riparius* larvae (Epler, 1995; Henriques-Oliveira et al., 2003; Ristola et al., 1999).

Airborne plastic contamination was prevented by using glassware as a replacement for plasticware whenever possible. Thereby, technical controls were also adjoined to discount any possible airborne MP contamination during extraction and quantification procedures.

2.3. Short-time exposure (48 h) to PE-MPs contaminated sediment

For biochemical biomarkers and cellular energy allocation (energy reserves and aerobic energy production) measurements, fourth instar (12-day old) *C. riparius* larvae were exposed to a gradient of MP concentrations (1.25; 5 and 20 g kg^{-1} dry sediment) of PE-MPs of three different size-classes (I: 32−63 μm, II: 63−250 μm and III: 125−500 μm). It is important to note that these concentrations were chosen to include levels reported in the field (Hurley et al., 2018) and concentrations that are known to induce deleterious effects on *C. riparius* growth and emergence (Silva et al., 2019). Each condition plus a control treatment (uncontaminated inorganic sediment) demanded seven replicates, containing fifteen larvae each. Larvae were exposed in glass vials containing PE-MPs mixed (except for control treatment) in 50 g of fine sediment (<1 mm) and 150 mL of ASTM hard water. No food was provided during the experiment. After 48 h, larvae were washed using ultra-pure water to remove potential microplastic particles adhered to the larvae and quickly dried on filter paper. After collection, the larvae were immediately weighed, frozen in liquid nitrogen, and stored at −80 °C until use. Three additional replicates were used to estimate the MP ingestion by *C. riparius* larvae after the 48 h exposure period.

2.4. Biochemical biomarkers assessment in *C. riparius* larvae

All biochemical determination followed optimised protocols for a microplate reader (Campos et al., 2016; Rodrigues et al., 2015; 2015). Briefly, biological samples were homogenised (Qiagen TissueLyser II) for 30 s at 4 °C in 1600 μL of Milli-Q water in 2 mL microtubes. Two aliquots of 300 μL were collected from each sample for the analysis of energy reserves: one aliquot was used for the analysis of lipids content, and the other aliquot was used for the determination of sugars and proteins content. From the remaining homogenate, 300 μL aliquot was collected to estimate aerobic energy production - measured by evaluating the electron transport system (ETS) activity; and 200 μL aliquot was used for the determination of lipid peroxidation (LPO). The remaining volume of the homogenate (∼500 μL) was diluted in 500 μL of 0.2 M K-phosphate buffer, pH 7.4. The resulting solution was centrifuged at 9000*g* (4 °C) for 20 min, and the post-mitochondrial supernatant (PMS) split into microtubes for subsequent enzymatic determination. The resulting PMS samples were stored at −80 °C until enzymatic activity determination of catalase (CAT), glutathione-*S*-transferase (GST), acetylcholinesterase (AChE) activities, and also the quantification of protein levels in the samples.

The energy reserves (sugar, lipid, and protein contents), representative of the energy available as well as the aerobic energy production (ETS activity) were assessed following De Coen and Janssen (De Coen and Janssen, 1997). As for the total lipid content, aliquots were centrifuged after addition of chloroform (500 µL) and methanol (500 µL). After centrifugation, the organic phase of each sample and tripalmitin standard solution were moved to glass tubes and acidified using sulphuric acid (H_2SO_4; 500 µL). The absorbance was determined at 375 nm on the microplate reader.

Protein content was estimated after precipitation by 15 % trichloroacetic acid, followed by incubation at −20 °C. Samples were then centrifuged (1000 *g* for 10 min at 4 °C), and the supernatant was separated to be used in sugar measurement. For sugar content estimation, samples and glucose standard concentrations were incubated after adding 5% phenol and H_2SO_4. The absorbance was then read at 492 nm to quantify sugar content. In the same sample, the pellets were resuspended after sodium hydroxide (NaOH), incubated (60 °C for 30 min), and pH was neutralised using HCl. Total protein content was quantified by Bradford's method in which the absorbance was read at 592 nm and using bovine serum albumin for the standard concentration curve (Bradford, 1976).

The aliquot of the homogenate used for ETS activity measurements (300 µL) was treated by adding Tris base homogenisation buffer (0.3 M), polyvinylpyrrolidone (0.45 % (w/v), $MgSO_4$ (459 µM), Triton X-100 ph 8.5 (0.6 % (v/v), and centrifuged (1000*g* for 10 min at 4 °C). Before measurements, 50 µL of the resulting supernatant was incubated with a buffered solution (0.13 M Tris base with 0.27 % [v/v] Triton X-100, 1.7 mM reduced nicotinamide adenine dinucleotide, 274 µM reduced nicotinamide adenine dinucleotide phosphate, and INT (p-iodonitrotetrazolium; 8 mM solution). The oxygen consumption was estimated by following the kinetics of the absorbance at 490 nm throughout a 3-min period.

Lipid peroxidation was verified by assessing thiobarbituric acid-reactive substances (TBARS) at 535 nm (Bird and Draper, 1984), GST activity determination was performed by measuring the colorimetric reaction (340 nm) of GSH conjugation with 1-chloro-2,4-dinitrobenzene (Habig et al., 1974), and CAT activity was determined by measuring the colorimetric reaction of the H_2O_2 decomposition at 240 nm (Claiborne, 1985). Acetylcholinesterase activity was determined by following the absorbance at 412 nm using acetylthiocholine as substrate according to Ellman's method (Ellman et al., 1961). The protein levels in each sample were determined following the Bradford method, adapted from BioRad's Bradford microassay set up in a 96 well microplate and using bovine γ-globulin as a standard (Bradford, 1976).

2.5. Extraction and quantification of PE microplastics in *C. riparius* larvae

After exposure, five larvae of each replicate were acid digested and further used for the extraction and quantification of MPs inside the gut. Quickly, samples were lyophilised for 24 h and weighed (DW). After dehydration, larvae were placed into glass flasks and cautiously macerated

using a small glass rod (acting as a mortar). This procedure allowed biological samples to be easily digested latter. The digestion procedure was comprised after the addition of 3 mL of nitric acid (HNO_3; 65 %) and further incubation at 60 °C for three hours. After this incubation, samples were cooled down to room temperature (RT), and a volume of 2.6 mL of hydrogen peroxide (H_2O_2; 35 %) was added to complete the digestion (Lusher et al., 2017). After 24 h, and once no visible oxygen bubbles were being released (signal of successful digestion), the samples were diluted using Milli-Q water in a 1:10 ratio. The resulting solution was then vacuum filtered onto gridded cellulose ester filters (Whatman 10406972, Mixed Cellulose Ester Filter, 3.1 mm white/black grid, 0.45 µm pore size). Retained particles (in the filtration device) were rinsed using Milli-Q water to minimise estimation errors. Filter membranes containing the microplastics were transferred to glass Petri-dishes and left to dry at 25 °C for 2–3 days. Afterwards, the number of particles present inside the larvae was counted under a stereomicroscope. To calculate the average size of these particles, the major diameter (Fig. S1) of all microplastics located in five sorted squares of each filter was measured. A stereomicroscope (stereoscopic zoom microscope—SMZ 1500, Nikon Corporation) associated with NIS-Elements D 3.2 imaging software were used for the necessary measurements (check supplemental data).

2.6. Statistical analysis

Non-parametric Spearman correlation was used to analyse the relationship between MPs concentration and the number of microplastics inside larval gut for each pool.

Normality and variance homogeneity assumptions were confirmed by analysing the residuals. One-way analysis of variance (ANOVA) was used for each PE-MP size-class to analyse differences between treatments for each size-class. Dunnett's test was used to determine significant differences from control. Statistical differences were considered at $p < 0.05$. All statistical analysis was performed using R version 3.6.1 (Core Team R, 2019).

3. Results

3.1. Ingestion of PE-MPs by *Chironomus riparius* larvae after 48 h exposure time

The number of PE-MPs ingested by larvae was higher in size-class (SC) I that contained smaller-size PE-MPs < 63 µm. Thus, larvae exposed to SC I ingested an average of 525, 2047 and 2389 PE-MPs at concentrations of 1.25, 5 and 20 g kg^{-1} sediment, respectively; whereas larvae ingested 656–785 PE-MPs in SC II or approximately 75 PE-MPs in SC III, independently of the concentration tested. Moreover, the number of ingested particles was correlated to increasing PE-MPs concentration in sediments for SC I ($r = 1.000$, $p = 0.0417$) and II ($r = 1.000$, $p = 0.0417$), but not for SC III ($r = 0.400$, $p = 0.3750$) (Fig. 1A).

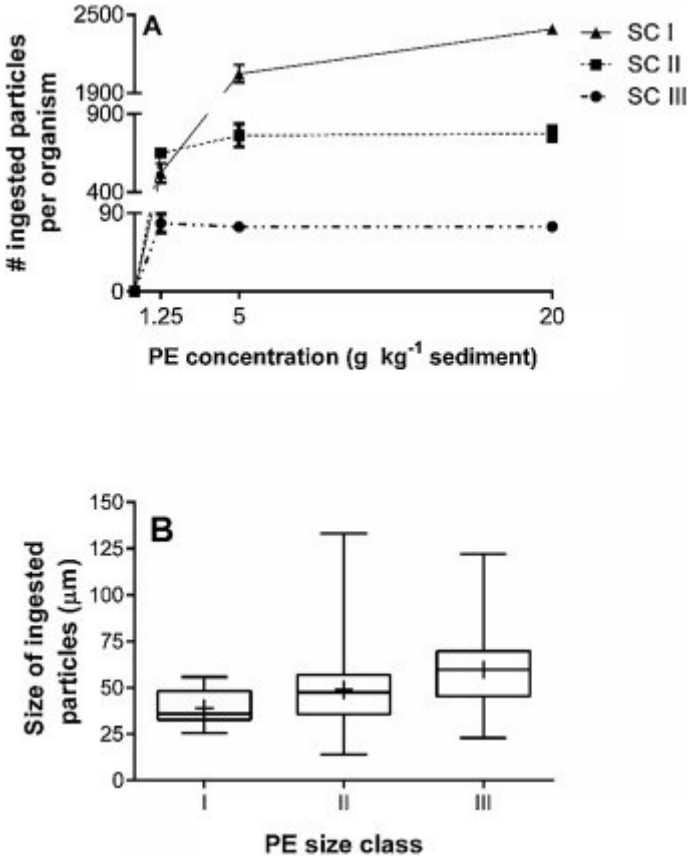

Fig. 1. Number (A) and size (B) of polyethylene microplastics (PE-MPs) of three size-classes (I, II and III) ingested by *Chironomus riparius* larvae, after 48 h of exposure. The number of ingested PE-MPs (A) is displayed as the mean ± standard error of the mean (n = 3). For some data, the error bars can be imperceptible. The size of the ingested PE-MPs (B) is presented as mean (± symbol) with interquartile range, whiskers, and maximum/minimum values.

The average size of PE-MPs ingested was higher on larvae exposed to any concentration of the SC II (48.78 ± 1.39 µm) and III (59.69 ± 2.16 µm) than in the ones exposed to SC I (38.76 ± 0.61 µm; Fig. 1B and Supplemental data Fig. S1). However, most of the ingested microplastics on larvae exposed to all SC of PE-MPs was within the 32−63 µm size-range, which in the case of SC I accounted for more than 90 % of the total particles, but less than 10 % for SC II and III (Table 1).

3.2. Energy reserves and aerobic energy production

Exposed larvae showed a decrease in aerobic energy production, regardless of PE-MPs SC (Fig. 2). The ETS activity significantly decreased in larvae exposed to 5 and 20 g kg^{-1} of SC I ($F_{3,24}$ = 7.810, p < 0.001;); all concentrations of SC II ($F_{3,24}$ = 18.05, p < 0.001) and 1.25 and 5 g kg^{-1} of SC III ($F_{3,23}$ = 5.447, p = 0.006;). Lipid content significantly decreased in larvae exposed to 1.25, 5 and 20 g kg^{-1} of SC II ($F_{3,24}$ = 6.045, p = 0.003) and III ($F_{3,24}$ = 4.677, p = 0.011) (Fig. 2). Sugar ($F_{3,23}$ = 0.8375, p = 0.487; $F_{3,24}$ = 3.989, p = 0.019; $F_{3,23}$ = 1.065, p = 0.383 for SC I, II and III respectively) and protein contents ($F_{3,24}$ = 0.878, p = 0.466; $F_{3,24}$ = 1.755, p = 0.183; $F_{3,23}$ = 0.818, p

= 0.497; for SC I, II and III respectively) did not vary between larvae exposed to control conditions and larvae exposed to any PE-MP treatments size-class (Fig. 2).

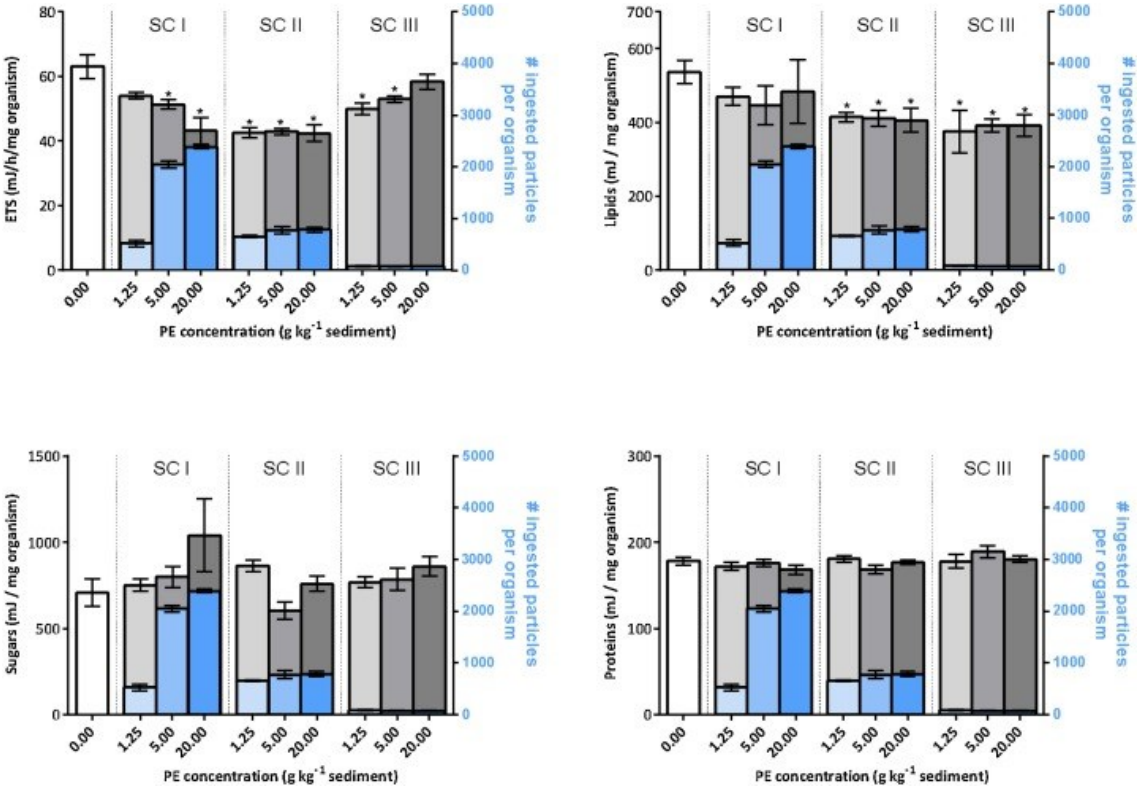

Fig. 2. Effect of short-term exposure (48 h) to polyethylene microplastics (PE-MPs) of three size-classes (I: 32–63 µm, II: 63–250 µm, and III: 125–500 µm, from left to right) on aerobic energy production (electron transport system, ETS, mJ/h/mg organism); lipids, sugars, and protein content (mJ/mg organism) of *Chironomus riparius* 4th instar larvae. All values are presented as mean ± standard error of the mean (n = 7). *denotes a significant (p < 0.05) difference when compared with the control (0) treatment following ANOVA and post-hoc Dunnett's tests. A gradient of blue bars represents the number of ingested MPs by larvae, as shown in Fig. 1A.

3.3. Oxidative stress and detoxification

Lipid peroxidation (LPO) significantly increased (approximately 70 %) on larvae of *C. riparius* exposed to all the concentrations of the SC I ($F_{3,24}$ = 26.57, p < 0.001), SC II ($F_{3,24}$ = 18.15, p < 0.001) and SC III ($F_{3,22}$ = 14.72, p < 0.001) PE-MPs (Fig. 3).

Catalase activity was significantly inhibited on larvae exposed to all concentrations of SC III ($F_{3,23}$ = 6.917, p = 0.002) and 5 g kg^{-1} of SC I ($F_{3,24}$ = 4.464, p = 0.013; Fig. 3). GST activity was significantly inhibited on larvae exposed to all concentrations of SC III ($F_{3,23}$ = 4.762, p = 0.010; Fig. 3). Acetylcholinesterase activity was significantly increased on larvae exposed to 20 g kg^{-1} of SC III ($F_{3,23}$ = 6.863, p = 0.002; Fig. 3).

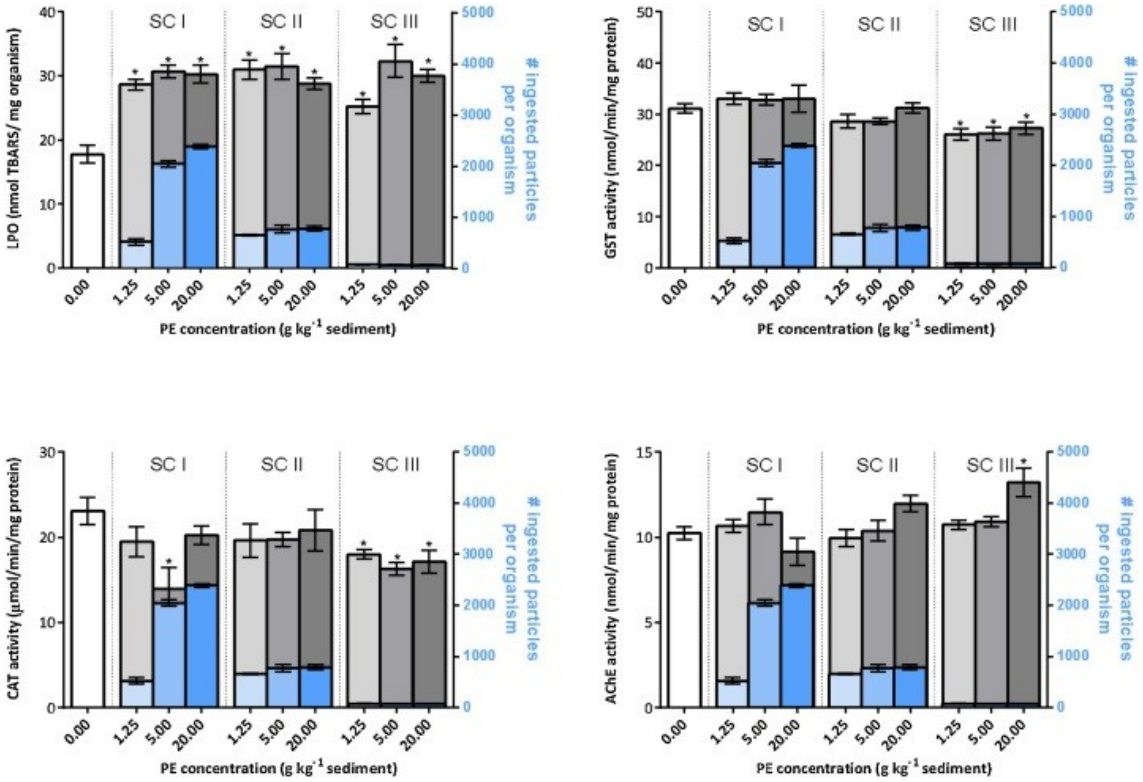

Fig. 3. Effect of short-term exposure (48 h) to polyethylene microplastics (PE-MPs) of three size-classes (I: 32–63 µm, II: 63–250 µm, and III: 125–500 µm, from left to right) on lipid peroxidation (LPO, nmol TBARS/mg protein), catalase activity (CAT, µmol/min/mg protein), glutathione-*S*-transferase levels (GST, nmol/min/mg protein) and acetylcholinesterase (AChE, nmol/min/mg protein) of *Chironomus riparius* 4th instar larvae. All values are presented as mean ± standard error of the mean (n = 7). *denotes a significant ($p < 0.05$) difference when compared with the control (0) treatment following ANOVA and post-hoc Dunnett's tests. The gradient of blue bars represents the number of ingested MPs by larvae, as shown in Fig. 1A.

4. Discussion

This study provides the first insight on the deleterious effects on cellular energy allocation, energy production, oxidative stress and neurotoxicity induced by the different size and number of microplastics ingested by larvae of the benthic freshwater insect *Chironomus riparius*.

As sediment-dwelling organisms, chironomid' larvae feed on the particulate organic matter without much selectivity (Armitage et al., 1995), which make them one of the most susceptible organisms to the potential ingestion of MPs that sink and accumulate in sediments. The ingestion of considerable amounts of microplastics was already reported in chironomid larva both from field samples (Nel et al., 2018) and from laboratory organisms after short-term (3 h) (Scherer et al., 2017) and long-term (≥ 5 days) (Ziajahromi et al., 2018; Silva et al., 2019) exposures. Given its non-selective feeding behaviour, the ingestion of MPs observed in larvae of *C. riparius* seems to be within the same range of the size of particles naturally ingested (up to 60 µm), and its accumulation in the gut is mostly dependent on organisms feeding apparatus and their egesting capabilities.

The number of PE-MPs inside *C. riparius* larval gut observed after 48 h agrees with our previous investigation (Silva et al., 2019). However, in such previous investigation, *C. riparius* larvae were exposed to PE-MP treatments (same concentrations and size-class) from their 1st instar and up to 10 days (so larvae were at their 4th instar after exposure period). The comparison of ingestion data and time of exposure from both studies suggests that: i) larvae ingest higher quantities of PE-MP particles in their 4th instar; ii) larvae may regularly be ingesting and egesting PE-MPs after an equilibrium that may be reached after 48 h in the 4th instar, resulting in a similar number of MPs inside the organisms on both studies and/or; iii) MPs have long residence times in the gut with slow or absent ingestion after some time that is dependent on the number of particles ingested reaching the maximal volumetric capacity of the digestive tract (SC I for concentration > 5 g kg^{-1}) and; iv) a complete obstruction seems to be occurring after ingestion of (although fewer) particles of a higher diameter of size-classes II and III, since the number of particles ingested was not sufficient to reach the maximal volumetric capacity of the digestive tract of larvae. The long residence time of particles can be caused by either aggregation of particles inside the larval gut (most likely for the smaller-size particles – SC I) or due to blocking of the gut passage (probable when ingesting particles closer to their physiological size-limit of ingestion and/or reduced egestion – SC II and III). MPs' aggregation inside invertebrates' gut have already been observed and associated with higher gut retention time (Ogonowski et al., 2016). Also, recent research has shown a particularly extended residence time of similar size polystyrene microplastics (10−90 μm) within chironomids' gut needing more than 24 h to egest at least 50 % of the ingested MPs (Scherer et al., 2017).

The fact that particles of SC II and III are not only larger (on average), but also more irregularly shaped, supports the idea of gut passage obstruction when compared to small and more spherical particles of SC I. Another indication of gut passage obstruction is the fact that the maximum number of particles ingested was reached for the lowest concentration tested of SC II (~785 particles) and III (~75 particles). Interestingly, AChE activity was significantly increased on larvae exposed to 20 g kg^{-1} of PE-MPs of SC III, which is in good agreement with obstruction and inflammatory processes, since acetylcholine levels are known to be increased in inflamed cells and tissues (De Oliveira et al., 2012; Gambardela et al., 2017). Moreover, an increase of peristaltic movements as an attempt to egest larger particles also explains the increased AChE activity observed in our current study. For SC I, the presence of MPs inside larval gut indicates a higher capacity for ingestion of smaller particles reaching a maximum number of ingested MPs of ~ 2400 for the highest concentration tested. Nevertheless, data also suggests that this number might be close to the maximal volumetric capacity of *C riparius* larval gut leading to increased retention time and potential aggregation, but not its severe obstruction.

Besides evidence of PE-MPs ingestion, retention and obstruction, our results show a significant increase of lipid peroxidation levels (LPO) on larvae of *C. riparius* exposed to all concentrations of all SC of PE-MPs, indicating that oxidative damage was triggered either by the high retention time of PE-MPs ingested (SC I) or obstruction (with potential inflammation) of their

gut by big and irregular particles ingested when exposed to PE-MPs of SC II and III. Studies addressing biological effects of PE-MPs have pointed out that physical stress (rather than the chemical toxic effect of PE monomers) is the main toxicity-related factor (Von Moos et al., 2012; Wright et al., 2013b; Karami et al., 2016; Rehse et al., 2016) since an additional effort is needed to digest inert material and maintain physiological homeostasis (Von Moos et al., 2012; Karami et al., 2016). In fact, the persistence of MPs in the gut, like other particles (e.g., nanoparticles), was already described to induce a false sense of satiation and likely-causing inflammation, with consequent alterations on organisms feeding behaviour and physiological status (Von Moos et al., 2012; Wright et al., 2013a; Gall and Thompson, 2015). Inflammation-induced by PE-MPs retention in *C. riparius* larval gut is the most probable cause for redox unbalance explaining the formation of reactive oxygen species (ROS) leading to oxidative damage, but further research concerning the immune response of larvae to MPs would be of great interest. Production of excessive ROS after exposure to MPs was previously reported in a variety of organisms, such as algae (Bhattacharya et al., 2010; González-Pleiter et al., 2019); copepods (Jeong et al., 2017); mussels (Von Moos et al., 2012; Paul-Pont et al., 2016), bivalves (Ribeiro et al., 2017), fish (Barboza et al., 2018), crustacea (Yu et al., 2018; González-Pleiter et al., 2019) and nematoda (Lei et al., 2018). The cascade prompted by the production of ROS, triggering oxidative stress and its related pathway is the main suggested MPs' toxicity mechanisms (Bhattacharya et al., 2010; Avio et al., 2015; Jeong et al., 2016, 2017; Paul-Pont et al., 2016; Alomar et al., 2017; Imhof et al., 2017; Veneman et al., 2017; Yu et al., 2018; Choi et al., 2018; Espinosa et al., 2018). Overall, ROS formation has also even been pointed out to be the key molecular initiating event of MPs toxicity (Jeong and Choi, 2019). Regardless of MPs size, effects triggered by the ingestion of particles by larvae *C. riparius* were severe. As explained, our results suggest that not only the number (SC I) but also the size of ingested particles (SC II and III) can mediate the oxidative stress effects observed in *C. riparius* larvae.

In addition to oxidative damage, evidence of deregulation in antioxidant defences was also found. First, a reduction of catalase (CAT) activity was observed in larvae exposed to PE-MPs (particularly evident for larvae that ingested larger particles). Catalase is involved in the removal of hydrogen peroxide (H_2O_2) (main precursor of hydroxyl radical), (Regoli and Giuliani, 2014) and similar inhibition of this enzyme was already observed for other invertebrates exposed to MPs (Paul-Pont et al., 2016). Authors suggested that CAT may follow a biphasic response in the neutralisation of the H_2O_2 production after MPs ingestion (Paul-Pont et al., 2016). Likewise, it is possible that CAT activation occurs in the first hours/first day of exposure followed by a decrease in activity (Regoli et al., 2011). Nonetheless, other authors suggest that catalase is not actively involved in response to MPs in invertebrates (Avio et al., 2015; Ribeiro et al., 2017).

Second, glutathione-*S*-transferase (GST) activity was also decreased on larvae exposed to larger particles (SC III) which might indicate its inactivation. This pattern of inhibition of CAT and GST was previously reported for other invertebrate species exposed to microplastics (Avio et al.,

2015; Ribeiro et al., 2017), suggesting that increased levels of ROS induced oxidative damage to proteins leading to enzyme inactivation.

Ingestion of non-nutritive particles and possible inflammation processes (with implications on oxidative stress and damage) observed here, can also imply energetic costs (Wright et al., 2013a). In the present study, the ingestion of PE-MPs induced energetic constraints to *C. riparius* larvae, namely by reducing lipid content (SC II and III) and the electron transport system (ETS) activity (all SC). The decrease of lipid reserves observed in the present study can be due not only to the inefficient and unprofitable digestion of non-nutritive particles (MPs) but also to energetic costs arising from physical stress and inflammation caused by large-size MPs. Like in the present study, other organisms such as marine worms presented depleted lipid reserves after MPs ingestion (Wright et al., 2013a). Besides, it has been shown in zebrafish larvae that MPs can activate a pathway for nuclear receptors involved in immunological recognition processes and lipid metabolism (Veneman et al., 2017). The decreased levels of lipids (and glycogen) during the larval stage may have consequences on Chironomidae life-history traits (Hamburger et al., 1996; Silva et al., 2019). Moreover, the decreased ETS activity demonstrates impairment of aerobic energy production and is often associated with a decline in aerobic metabolism, which can lead to partial anaerobiosis and consequent effects at higher levels of biological organisation (Sokolova et al., 2012). A decrease on aerobic metabolism was also observed on fish (e.g., European seabass) after the ingestion of MPs, alongside an increased anaerobic metabolism (Barboza et al., 2018). Some organisms may use anaerobic pathways of energy production to get additional energy to face chemical stress or stress caused by the ingestion of MPs (Fırat et al., 2011; Oliveira et al., 2013). Moreover, we cannot exclude the possibility that partial damage to the inner mitochondrial membrane due to overproduction of ROS (Roche and Bogé, 1993; Stolze and Nohl, 1994; Choi et al., 2001) may have also impaired the electron transport system and be linked to the reduction of ETS activity. Either way, overall energy produced by larvae exposed to PE-MPs was not sufficient to cope with stress, since oxidative stress was unequivocally observed for all the SC tested.

The lack of mechanistic studies onto MPs toxicity has been reported as one of the priority knowledge gaps that urge to be addressed (Jeong and Choi, 2019). The present study provides an insight on the sub-cellular effects triggered by a high number of MPs ingested and retained within the gut of larvae of *C. riparius* after short-term exposure. In contrast to our findings, other studies report limited or no effects of microplastics on apical and sub-cellular endpoints (Imhof and Laforsch, 2016; Beiras et al., 2018; Weber et al., 2018; Castro et al., 2020; Khosrovyan and Kahru, 2020), but factors like organisms behaviour, polymer-type, size of microplastics and mostly concentrations and exposure period have to be carefully taken into account for comparison of effects of microplastics. Moreover, the size of the mouth and digestive apparatus of each species used should also be carefully considered, since our results showed that this is an important factor for ingestion of particles (number and size of particles), retention time, obstruction and egestion. The concentrations of MPs used in this study are environmentally relevant (ranging from river hotspots to worst-case scenario) and induced biochemical effects that are associated with

deleterious effects in the lifecycle (growth and development impairments) induced by PE-MPs in *C. riparius* (Silva et al., 2019). Thus, oxidative stress biomarkers and metabolic responses can be used as early warning indicators of acute stress-induced by microplastic' pollution, to compare the sensitivity of different species and assess harmful effects of different polymers that a are now emergent contaminants of aquatic ecosystems.

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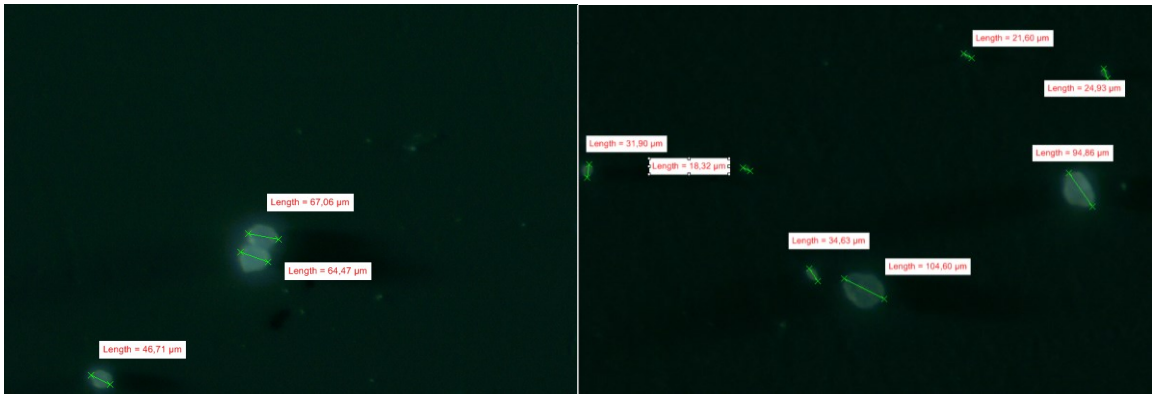
Supplementary material

Size-class I

Size-class II



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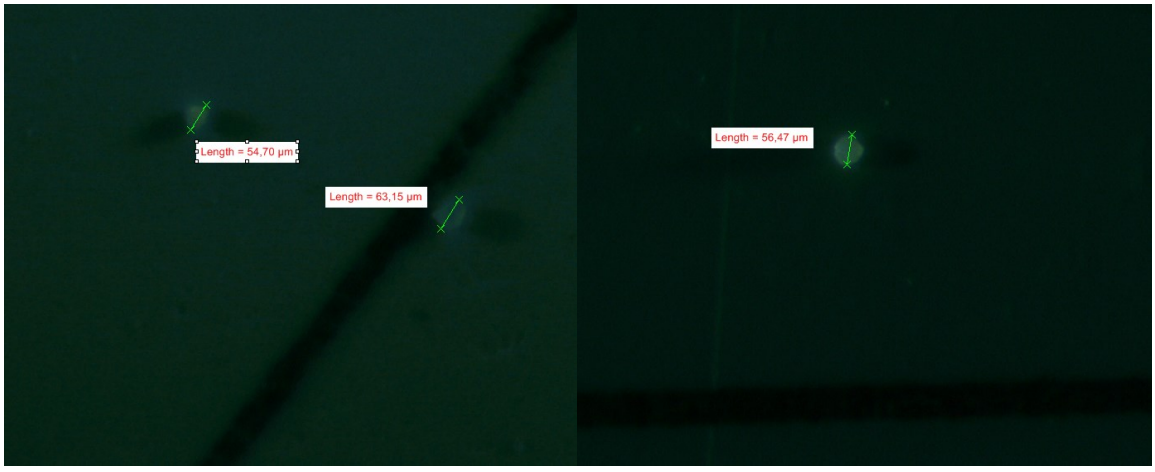

Fig.S1. Images (two images per PE size-class) used for the determination of ingested PE-MPs using stereomicroscope associated to NIS-Elements D 3.2 microscope imaging software.

Chapter 5

Immune response triggered by the ingestion of polyethylene microplastics in the dipteran larvae, *Chironomus riparius*

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Immune response triggered by the ingestion of polyethylene microplastics in the dipteran larvae, *Chironomus riparius*

Abstract

The activation of insects' immune system due to the ingestion of microplastics (MPs) has only been evidenced by the upregulation of specific genes. The activation of phenoloxidase (PO) system is one of the primary responses involved in the innate immunity of insects when facing parasites and pathogens, and ingestion of MPs can trigger similar process. This study aimed at addressing the activities of PO and total PO (PO+ prophenoloxidase - proPO), in *Chironomus riparius* larvae (a model species in ecotoxicology) exposed to sediments spiked with polyethylene microplastics (PE-MPs; size-range 32-63 µm; concentrations: 1.25; 5; to 20 g kg-1) for 48 h.

The retention in the gut of PE-MPs ingested by larvae triggered a significant increase of basal PO activity at 5 and 20 g PE-MPs kg^{-1}, by 26 and 29%, respectively, whereas total PO increased significantly in the latter (+48%), suggesting de novo synthesis of proPO by organisms. Considering the particle size, the activation of the immune response is probably linked to damage in the epithelial cells of the gut lumen. This research work provides the first evidence on the activation of the innate immune system of an insect after ingestion of MPs and underlines the PO activity as a good indicator of the immune response induced by ingestion of MPs.

Keywords: Innate immune system, phenoloxidase, biomarkers, invertebrates, plastic pollution

1. Introduction

The ingestion of microplastics (MPs) by freshwater benthic invertebrates, particularly detritivore-collector species, such as chironomid larvae and lumbriculids, has been previously observed and characterised (Scherer et al., 2017, 2019; Ziajahromi et al., 2018; Silva et al., 2019, 2021a). Although MPs ingestion rarely results in significant mortality in such invertebrates, it can reduce somatic growth (Silva et al., 2019) and developmental rates (Ziajahromi et al., 2018; Silva et al., 2019; Silva et al., 2021b). The observed impairments on organisms' life-history traits suggest that the ingestion of MPs can reduce feeding and/or alter resource trade-offs between maintenance and growth processes. In fact, ingestion of MPs seems to cause gut clogging or abdominal distention, particularly in chironomids which ingest high quantities of MPs (Nel et al, 2018; Silva et al., 2019), which could have affected feeding and digestion processes; thus, resulting in a deficit on energy acquisition/assimilation (Avio et al., 2015; Ziajahromi et al., 2018). Such energy constrains can partially explain why chironomids failed to prevent oxidative stress and damage (Silva et al., 2021a). In addition, the potential inflammation processes and activation of immune responses in organisms that ingest and accumulated MPs may also increase the energy requirements and add additional pressure on energy reserves that are being affected by suppressed feeding activity (Silva et al., 2021a, 2021b).

The activation of the immune responses due to exposure to MPs have only been suggested by the upregulation of genes related to the immune system as observed in mussels and oysters (Détrée & Gallardo-Escárate, 2017, 2018; Gardon et al., 2020), and with increased phagocytic activity of particles smaller than 10 μm as observed in polychaeta (Browne et al., 2013; Wright et al., 2013) and bivalves (Avio et al., 2015; Pittura et al., 2018). However, the pathway through which MPs elicit these sub-cellular responses is poorly documented, particularly in sediment-dwelling invertebrates that ingest a significant amount of MPs (Wright et al., 2013; Ziajahromi et al., 2018; Silva et al., 2019, 2021a; Khosrovyan & Kahru, 2020; Stanković et al., 2020). This study hypothesised that the retention or blockage of MPs in organisms' gut, particularly of particles larger than 10 μm and that cannot be phagocyted, may cause a mechanical/proteolytic damage in the epithelial cells of the gut lumen. Since the gut epithelium is an immunologically active tissue that produces PO (Wilson et al., 2001) is likely that the damage signals (by-products generated during mechanical/proteolytic damage) will trigger the activation of PO system (Krautz et al., 2014).

The activation of phenoloxidase (PO) is one of the mechanisms involved in invertebrates innate immune system, ultimately leading to encapsulation followed by melanisation of pathogens, parasitoids, damaged tissues (Davis and Engström, 2012; González-Santoyo & Córdoba-Aguilar, 2012), and potentially MPs. Phenoloxidase system is controlled by an inactive zymogen prophenoloxidase (proPO), mostly synthesised in haemocytes (Cerenius & Söderhäll, 2004) and activated to PO by numerous microbial surface components, such as peptidoglycans, β-1,3-glucan, lipopolysaccharide, and zymosan (Smith et al., 1984; Eleftherianos & Revenis, 2011) via controlled proteolysis controlled by the action of a serine protease cascade (Eleftherianos & Revenis, 2011). The PO activation ultimately leads to a melanisation process (González-Santoyo & Córdoba-

Aguilar, 2012). Phenoloxidase activity has been used as a biomarker of the immune response of several invertebrate species to organic contaminants, generally measured in haemolymph samples of organisms (Lee et al., 1998; Lilley et al., 2012; Yousefi-Lardeh & Zibaee, 2020). However, more recently, whole-body samples of chironomids were also used to assess the immune response of larvae to bioinsecticides (Bordalo et al., 2020) and ultraviolet filters (Muñiz-González & Martínez-Guitarte, 2020). Moreover, PO has also been used as a proxy for disease resistance of several invertebrate species (Cotter & Wilson, 2002; Aladaileh et al., 2007; van Ooik et al., 2007, 2008; Lilley et al., 2012).

Therefore, this study aimed to assess if microplastics (foreign and inert particles) ingested by insects trigger an immune response through the in vivo activation of phenoloxidase system. For this purpose, *Chironomus riparius* (Diptera: Chironomidae) was used as test species because its larvae are known to ingest and retain, in their gut, a considerable number of MPs (Silva et al., 2019, 2021a). *C. riparius* larvae were exposed for 48 h to irregularly-shaped polyethylene microplastics (PE-MPs) (32 to 63 µm) in sediments, considering concentrations previously determined in field-sediments (9 g kg-1; Hurley et al., 2018) up to a worst-case scenario (20 g kg-1). Moreover, in order to confirm that the ingestion of MPs triggered a true PO system by larvae, PO inhibition was estimated using an inhibitor of true PO, phenylthiourea (PTU). The activity of PO measured in whole-body samples was also optimised and characterised using in vitro activation by chymotrypsin as well as the response and sensitivity to pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) and zymosan (Zs).

2. Materials and Methods

2.1. *Chironomus riparius* culture conditions

Chironomus riparius were cultivated and maintained under controlled conditions at the Department of Biology, University of Aveiro. Organisms were reared in glass aquaria containing fine inorganic sediment (<1 mm), previously burnt at 500 °C (5h), and American Society for Testing and Materials (ASTM) hard water medium (ASTM, 1980). The culture room was kept at 20 ± 1 °C and under a photoperiod of 16 h light under constant aeration. Food was provided every two days in the form of macerated commercial food fish TetraMin® (Tetrawerke, Melle, Germany).

2.2. Detection and characterisation of phenoloxidase (PO)

2.2.1 Homogenisation and isolation of the samples

Fourth instar *C. riparius* larvae (twelve-days old) were selected and used for the characterisation of the PO system. For all determinations, each sample consisted of fifteen larvae that were cultivated in a separate container, being collected in the twelfth day, rinsed, quickly dried on filter paper, being subsequently frozen in liquid nitrogen and stored at -80 °C until further analysis. Briefly, samples were homogenised by sonication (Branson SONIFIER 250) on ice and

using 0.2 M phosphate buffer pH=7.4 (3200 µL). Homogenates were then centrifuged for 20 minutes at 9000 g (4 °C). After centrifugation, the supernatant was collected and reserved on ice.

2.2.2 Basal PO and total PO activity: activation of proPO using chymotrypsin

Five samples comprising 30 µL each of the above-mentioned supernatant were incubated for a 10 minutes period in the absence (50 µL of Milli-Q water); and presence of chymotrypsin (CAS Number: 9004-07-3 from Sigma-Aldrich) (50 µL of 0.5 mg chymotrypsin mL^{-1}), and 100 of 0.2 M phosphate buffer pH=7.4 (100 µL) at 25 °C. After incubation, 100 µL of sodium cacodylate (CAC, 10 mM) buffer (pH 7.4) and 20 µL of L-DOPA (CAS Number 59-92-7 from Sigma-Aldrich) (200 mM) were added (Pang et al., 2004), to start the reaction. Reaction blanks were added and consisted of phosphate buffer in substitution of the sample. Half of the replicates (of the same sample) were incubated in the presence of chymotrypsin, whereas the other half were incubated in its absence. After the addition of L-DOPA, the absorbance was instantly read at 490 nm (time 0) and monitored for up to 6 hours. Each sample was tested four times (technical replicates) for higher accuracy. All microplate readings were performed on Thermo Scientific Multiskan Spectrum microplate reader. The enzymatic activity was expressed as units, being one unit defined as 0.001 OD (Optical Density) change per mg protein (Pang et al., 2010).

2.2.3 Inhibition of Basal PO and total PO by phenylthiourea (PTU)

Larval basal PO was also analysed in the presence of a widely applied PO inhibitor – the phenylthiourea (PTU), which inhibition achieve high percentage when facing a true PO (Asokan et al., 1997; Pang et al., 2005). Five samples (30 µL each) were added in quadruplicate (four technical replicates for each sample) and incubated in the absence (50 µL of Milli-Q water) and presence of chymotrypsin (50 µL of 0.5 mg chymotrypsin mL^{-1}). Each condition was then tested in the absence and presence of PTU. To test PO inhibition by PTU, 100 µL of PTU solution (0.1 mg mL^{-1} PTU in 2M phosphate buffer pH 7.4) (Hellio et al., 2007), and incubated for 10 min at 25 ºC. In the samples tested for absence of PTU, the same volume of phosphate buffer (2M, pH 7.4) was added before incubation After incubation, 100 µL CAC buffer (10mM, pH 7.4) were added to the samples, followed by 20 µL of L-DOPA (200 mM) (Pang et al., 2004). Reaction blanks consisted in the replacement of the sample by 30 µL of phosphate buffer. The incubation and remaining procedure were performed as explained above for each condition (e.g., presence and absence of PTU). After the addition of L-DOPA, the absorbance was instantly measured (time 0) at 490 nm and monitored for up to 6 hours. PO activity was expressed as units min^{-1} mg $protein^{-1}$.

2.2.4 Effects of the addition of zymosan (Zs) and lipopolysaccharides (LPS) on PO activity

Four samples consisting of 30 µL of homogenate were added to a microplate containing 50 µL of Milli-Q water in the absence (100 µL of 2M phosphate buffer pH=7.4) and presence of zymosan (CAS Number 58856-93-2 from Sigma-Aldrich) (100 µL of 0.1 mg mL^{-1} zymosan in 2M phosphate buffer pH=7.4). The microplate was then incubated at 25 °C for 10 min. After incubation, 100 µL CAC buffer (10 mM, pH 7.4) and 20 µL of L-DOPA (200 mM) (Pang et al., 2004) were added. Reaction blanks were performed by replacing the sample volume by phosphate buffer (30 µL) followed by incubation as explained above. Four samples (30 µL of the homogenate) were added to a microplate performing four technical replicates per sample, followed by the addition of Milli-Q water (50 µL) in the absence (100 µL of 2M phosphate buffer pH=7.4) and presence of LPS solution (100 µL of LPS solution- 1 mg mL^{-1}, in 2M phosphate buffer pH=7.4) and incubated for 10 min at 25 °C. After incubation, CAC buffer (100 µL of 10 mM, pH 7.4) and L-DOPA (20 µL of 200 mM L-DOPA) were added (Pang et al., 2004). Reaction blanks were performed by replacing the volume of sample by equal volume of phosphate buffer followed by incubation as explained above. After the addition of L-DOPA, the absorbance was instantly measured (time 0) at 490 nm and monitored for up to 48 hours. PO activity was expressed as units min^{-1} mg $protein^{-1}$.

2.3. Basal PO and total PO after ingestion of microplastics

2.3.1 Short-time exposure to polyethylene microplastics (PE-MPs) contaminated sediment

The exposure of fourth instar *Chironomus riparius* larvae to PE-MPs followed the procedures of previous studies (Silva et al., 2019, 2021a). Briefly, *C. riparius* larvae (12-day old) were exposed for 48 h to three PE-MP concentrations (1.25 - 5 - 20 g kg^{-1} dry sediment) of PE-MPs pool of small-sized particles (32-63 µm; CAS No. 9002-88-4, Sigma-Aldrich UK). The PE-MP concentrations and size range were based on previous studies in which cellular and organismal level effects were described thus allowing for an integrative discussion of the obtained results (Silva et al., 2019, 2021a). Each PE-MP condition plus a control treatment (uncontaminated inorganic sediment) was prepared to contain seven replicates. Each replicate consisted of fifteen larvae exposed to PE-MPs. For exposure, PE-MPs were directly mixed (except for control treatment) in the sediment (50 g of fine sediment <1mm) and 150 mL of ASTM hard water. During the experiment, no food was offered to the larvae. After the exposure period (48 h), larvae were gently collected from the sediment, being briefly rinsed (Milli-Q water) to minimize the possibility of having microplastics adhered to the larval cuticle. After rinsing, larvae were instantly dried (using filter paper), they were weighed (fresh weight. – FW), and subsequently frozen in liquid nitrogen, being stored at –80 °C until further analysis.

2.3.2 Determination of basal PO and total PO in PE-MPs exposed larvae

Samples were homogenised by sonication (on ice) using 0.2 M phosphate buffer pH=7.4 (3200 µL) and homogenates were then centrifuged for 20 minutes at 9000 g (4 °C). After centrifugation, the supernatant was collected and reserved on ice. Each sample (30 µL) was incubated 10 minutes in the absence of chymotrypsin (using 50 µL of Milli-Q water) and in the presence of chymotrypsin (using 50 µL of 0.5 mg mL^{-1}chymotrypsin) for the determination of basal PO and total PO (PO+ProPO) activity, respectively. After incubation, 100 µL of sodium cacodylate (CAC, 10 mM) buffer (pH 7.4) and 20 µL of L-DOPA (200 mM) were added, to start the reaction. Reaction blanks were added and consisted of phosphate buffer in substitution of the sample. Half of the replicates (of the same sample) were incubated in the presence of chymotrypsin, whereas the other half were incubated in its absence. After the addition of L-DOPA, the absorbance was instantly read at 490 nm (time 0) and monitored for up to 6 hours. Each sample was tested four times (technical replicates) for higher accuracy. The enzymatic activity was expressed as units, being one unit defined as 0.001 OD (Optical Density) change per mg protein (Pang et al., 2010).

2.4. Protein determination

The determination of the protein levels on each sample followed the method described by Bradford (Bradford, 1976), adapted for microplate reader.

2.5. Data analysis

Data analyses were performed by using one-way analysis of variance (ANOVA), followed by Dunnett's *post hoc* test or by *t-tests* to identify significant differences between each condition tested and the respective control or between two conditions of the assay, respectively. The type of analysis is also signalised in the figure caption. The homoscedasticity and normality of data were assessed using Bartlett's and Brown–Forsythe tests and using residual probability plots. Significance level was set at $p \leq 0.05$ for all statistical tests. GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, California, USA) was used for statistical analyses.

3. Results

3.1. Basal PO and proPO activation by chymotrypsin

Values of PO activity (2.97 ± 0.92 Units/min/mg protein) on larvae of *Chironomus riparius* were significantly lower ($t_{(8)}$=5.568, p=0.0005) than the activity of total PO (9.34 ± 2.39 Units/min/mg protein) determined in the presence of 0.5 mg mL^{-1} chymotrypsin (Figure 1). This significant increase in the activity of total PO (PO+proPO) by chymotrypsin compared to basal PO (+218 ± 9%) suggests activation of ProPO by chymotrypsin through proteolysis of proPO (Figure 1).

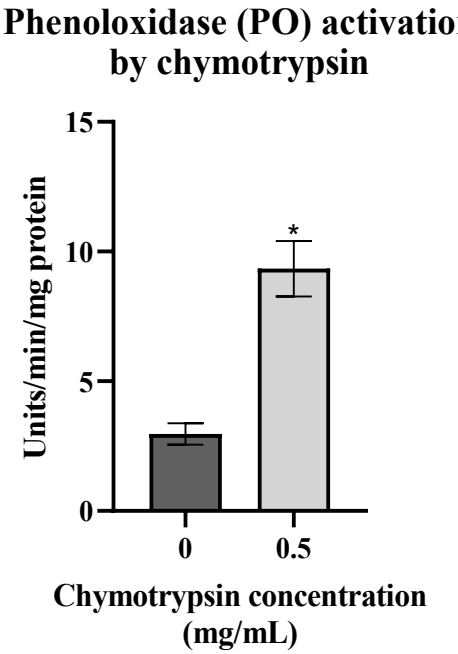

Fig. 1. Basal Phenoloxidase (PO) activity in the absence of chymotrypsin and total PO (PO+ProPO) after activation by 0.5 mg mL^{-1} chymotrypsin in fourth instar larvae of *Chironomus riparius*. Values are presented as mean of five samples± SEM. * symbolizes a significant difference compared to control (0) treatment at $p < 0.05$ (t-test).

3.2. Inhibition assay of basal PO activity by phenylthiourea (PTU)

The PTU significantly inhibited basal PO activity ($t_{(8)}$=3.571, p<0.0073) and total PO ($t_{(8)}$=8.358, p<0.0001) compared to control (Figure 2A and B). The presence of 0.1 mg mL^{-1} PTU inhibited ~50 ± 10% the activity of basal PO and more than 95 ± 1%, showing that all total PO measured in larvae of *Chironomus riparius* is due to a true PO activity (Figure 2B).

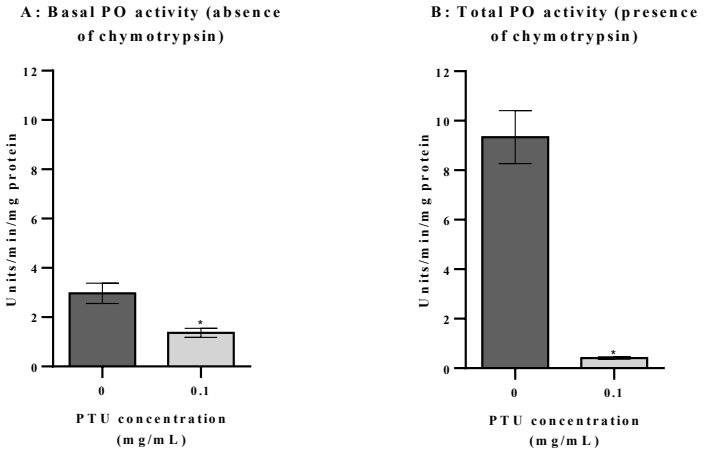

Fig. 2. Effect of PTU (0.1mg mL^{-1}) on basal phenoloxidase (PO) (absence of chymotrypsin) and total PO (PO+ProPO; 0.5 mg mL^{-1} chymotrypsin) activities of *Chironomus riparius*. Values are presented as the mean of five samples ± SEM. * symbolizes a significant difference compared to control (0) treatment at $p < 0.05$ (t-test).

3.3. Effects of zymosan (Zs) and lipopolysaccharides (LPS) on basal PO and proPO activation

Significant increases of basal PO were observed on larvae of *C. riparius* after *in vitro* treatment with 1 mg mL^{-1} LPS ($t_{(6)}$=5.849, p=0.0011; Figure 3A) and 0.1 mg mL^{-1} Zs (t (6)=3.156, p=0.0197; Figure 3B) compared to respective control. Basal phenoloxidase activity increased 100 ± 13 % in the presence of LPS and 52 ± 23 % in the presence of zymosan, suggesting activation of PO system in the presence of bacterium and fungi components (Figure 3A, B).

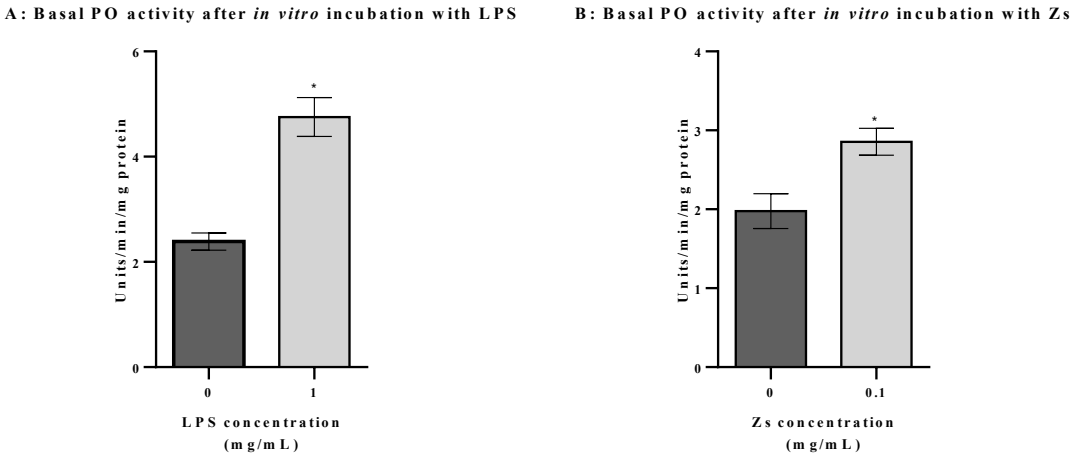

Fig. 3. Basal phenoloxidase (PO) activity on homogenates of *Chironomus riparius* larvae after *in vitro* exposure to 1mg mL^{-1} lipopolysaccharides (LPS) (A) and 0.1 mg mL^{-1} zymosan (Zs) (B). Values are presented as the mean of four samples ± SEM. * symbolizes a significant difference compared to control (0) treatment at $p < 0.05$ (t-test).

3.4. Basal PO and ProPO activities in *Chironomus riparius* larvae exposed to PE-MPs

Basal phenoloxidase activity was significantly increased in larvae exposed to 5 and 20 g kg^{-1} PE-MPs compared to control ($F_{(3,23)}$= 5,251, p=0.0066, Figure 4), reaching increases of 26 ± 8 % and 29 ± 7 %, respectively. The activity of total PO was significantly increased (48 ± 9 %) in larvae exposed to the highest concentration of PE-MPs (20 g kg^{-1}) tested compared to total PO activity of control ($F_{(3,23)}$= 9,071, p= 0.0004, Figure 4). Moreover, and considering all treatment conditions, the activity of total PO was always significantly higher than basal PO activity ($t_{(6)}$=6.130, p=0.0009). The activity of total PO observed in larvae of control and exposed to 1.25 and 5 g kg^{-1} was 6,41±0,33; 6,95±1,22 and 6,87±0,77 Units/min/mg protein, respectively, representing activation of +177; and +108 compared to basal PO activity, respectively. The activity of total PO observed in larvae exposed to 20 g kg^{-1} was 9,50± 1,92 Units/min/mg protein representing activation of +182 % compared to basal PO activity. The increased total PO activity (Figure 4) shows the activation of almost all existent proPO (+182%) and suggests its *de novo* synthesis (+48%).

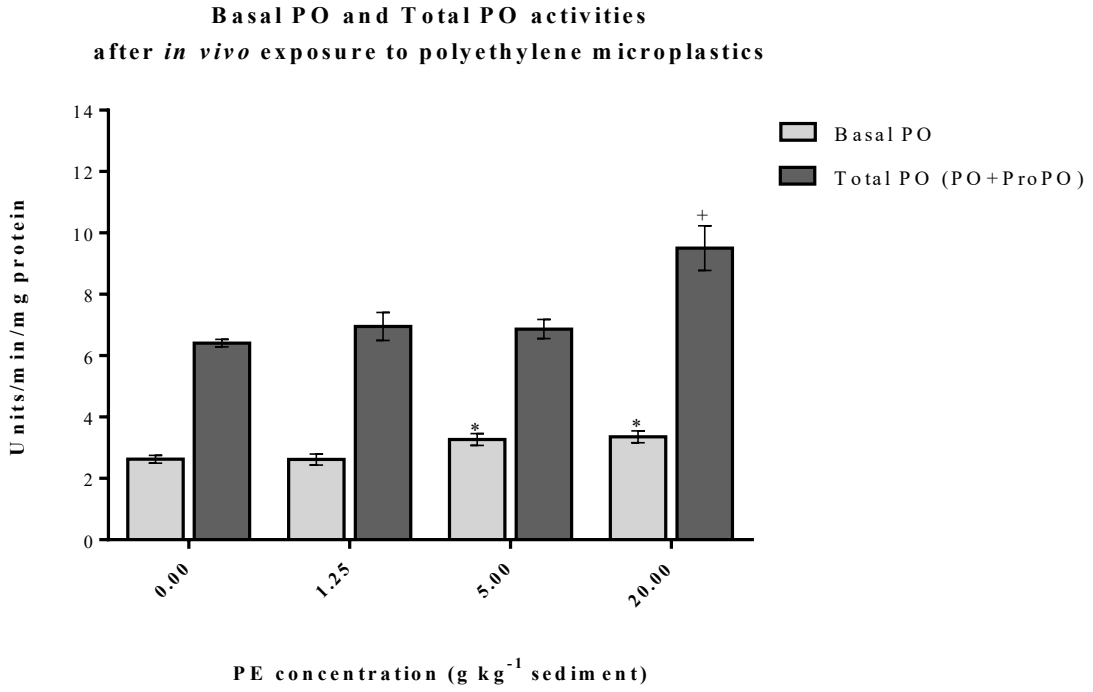

Fig. 4. Activities of basal phenoloxidase (PO) and total phenoloxidase (PO+proPO) in larvae of *Chironomus riparius* after *in vivo* exposure to PE-MPs (0, 1.25, 5 and 20 g kg^{-1} dry sediment). Values are the mean of seven replicates performed in quadruplicated ± SEM and expressed as Units/min/mg protein. * represents significant statistical differences (p<0.05) from control (PO) and (+) represents statistical differences from control (total PO).

4. Discussion

4.1. Phenoloxidase system in *Chironomus riparius*

The present study showed that phenoloxidase activity in *C. riparius* larvae (whole-body homogenates) was activated by chymotrypsin. In fact, *in vitro* activation of proPO by chymotrypsin was previously observed using whole-body samples of larvae of *Chironomus riparius* (Bordalo et al., 2020). However, the characterisation of this activity was not carried out by studying the effect of specific inhibitors of true type o-diphenoloxidase. Our results show that the typical o-diphenoloxidase inhibitor – PTU – significantly inhibited the activity of not only total PO (> -95 %) but also PO activity (~ -50%), which confirms the occurrence of PO system in *C. riparius* (Hellio et al., 2007) and that the measured PO activities of whole-body samples of larvae result mainly from a true type of o-diphenoloxidase. Phenoloxidase system is a key element of the immune response of many insects (González-Santoyo & Córdoba-Aguilar, 2012), including *C. riparius* (Lilley et al., 2012). As in all insect species (Ashida & Brey, 1995), *C. riparius* larvae hold a latent pro-enzyme (proPO). ProPO is activated by a cascade of enzymatic reactions that include pattern recognition proteins, proteinases, and regulatory factors (Cerenius & Söderhäll, 2004). Moreover, this PO system was significantly increased in *C. riparius* homogenates by the presence of LPS (+100.35 %) and Zs (+52.04 %) *in vitro*, confirming that phenoloxidase present in the homogenates is activated in the presence of bacterial and fungal PAMPs. This fact is in good agreement with a previous work that reported increased PO activity on whole-body samples of larvae of *C. riparius* exposed to bioinsecticides based on bacterial and fungal components (Bordalo et al., 2020). Other studies using hemolymph of insects exposed to bacteria, fungi, and viruses also showed increased of PO activity (Götz et al., 1987; Stączek et al., 2020). The use of whole-body measurement of phenoloxidase allows for simultaneous determination of several other parameters since it only needs a small volume aliquot of the homogenate and also reduces the number of organisms needed for the measurements of phenoloxidase activity.

4.2. Effects of PE-MPs on *C. riparius* PO system and potential physiological costs

The novelty of this study was the unequivocal activation of the immune system of larvae of *C. riparius* expressed in the PO system activation, probably triggered after mechanical damage caused by PE-MPs in their gut. Fourth instar *C. riparius* larvae are known to present a significant ammount of PE-MPs in their gut after 48 h exposure, which can be dependent on PE-MP concentration in the sediment if the particles size is compatible with larval mouth apparatus for their ingestion (up to ~2400 particles/organism, with the size range of 32-64 µm) (Silva et al., 2021a). The presence (and previously suggested potential accumulation) of such PE-MPs in the larval gut triggered the activation of the immune system of *C. riparius* observed as increased activity of PO in exposed organisms (PO system activation) to PE-MPs concentrations equal or higher than 5 g kg^{-1}. The activation of this immune response occurred in larvae that ingested large amounts of PE-MPs

(>2000 PE-MPs/larvae) (Silva et al., 2021a). Furthermore, an increased activity of total PO was observed on larvae exposed to 20 g kg^{-1}, suggesting the possibility of *de novo* synthesis of proPO. Additional ProPO synthesis indicates a possible attempt to refuel the zymogen levels, capable of being readily activated in the active form (PO) to be involved in the melanisation process. Likewise, increased levels of proPO after infection have already been reported in insects (Gillespie et al., 2000). The here observed activation of PO together with possible *de novo* synthesis of ProPO may indicate a stronger stimulation of immune response on larvae exposed to the highest concentration of PE-MPs (20 g kg^{-1}). This possibility is in good agreement with the maximum ingestion of PE-MPs (up to ~2400 PE-MPs/larvae) previously observed, which was related to potential gut passage block or retention (Silva et al., 2021a).

The activity of phenoloxidase and activation of the inactive form ProPO can be associated with encapsulation of foreign particles and/or immune response after mechanical damage to the epithelial cells of the gut lumen of larvae. The encapsulation of foreign bodies, like MPs into nodules, i.e., granulocytomas, has been observed after short exposure to MPs (size: < 10 µm) in the mussel *Mytilus edulis* (Von Moos et al., 2012). However, the MPs ingested by *C. riparius* were generally too large to cross the gut lumen and be encapsulated in the haemolymph (size range 32-63 µm). Thus, the observed immune response translated in basal and total PO activation may be linked to damage to the gut epithelium induced by PE-MPs retained after ingestion, which is a recognised immunologically active tissue (Wilson et al., 2001; Krautz et al., 2014). Mechanical damage to gut epithelium can also activate other components of the immune system, namely, haemocytes, which are involved in wound repair (Rowley & Powell, 2007; Grizanova et al., 2014). Several other studies have described immune responses mainly through increased phagocytic activity after exposure to MPs (Von Moos et al., 2012; Wright et al., 2013; Avio et al., 2015; Rodriguez-seijo et al., 2017). Moreover, a potential damage in epithelial gut' cells can also represent an entrance door for pathogens present in the gut, allowing them to enter the main body cavity. In response to this entrance, the immune system can also be activated, and the pathogens generally phagocytosed by immune cells (Rowley & Powell, 2007).

The damage of tissues inside the intestinal tract of invertebrates induced by microplastics has been associated with increased oxidative stress (Magara et al., 2019). The activation of PO system for the melanisation process involves the production and release of quinones, phenols, and reactive oxygen species (ROS) by haemocytes and can even harm the host if produced in excess (Siva-Jothy et al., 2005; Saad and Siva-Jothy, 2006; González-Santoyo & Córdoba-Aguilar, 2012; Stahlschmidt et al., 2015). The increased production of ROS as a product of PO activation may, at least partially explain, the alteration in antioxidant capacity and subsequent oxidative damage observed in *C. riparius* larvae exposed to similar sized PE-MPs (Silva et al., 2021a). This possibility has already been suggested after the reported oxidative damage that was linked to possible gut passage blocking and probable prolonged residence time of inert particles inside *C. riparius* larval gut (Scherer et al., 2017; Silva et al., 2021a).

It is thus clear that induction of the immune challenge triggered by MPs can further increase the energy constrains suffered by organisms since energy is diverted from growth/reproduction to maintenance, i.e., to sustain multiple energetically expensive processes related to melanogenesis and other cellular responses (Olsen et al., 2015; Wolowczuk et al., 2008) and thus may imply physiological costs (Siva-Jothy et al., 2005).

Physiological costs of immune responses and of increased phenoloxidase activity were shown before as the reduced growth of the damselfly *Coenagrion puella* exposed to non-pathogenic bacteria (Janssens & Stoks, 2014). and as the reduced longevity of the beetle, *Tenebrio molitor* after insertion of a inert sterile nylon monofilament (Armitage et al., 2003).

The activation of the innate immune response induced by MPs ingestion, here confirmed by the PO activation; the possible *de novo* synthesis of prophenoloxidase; the production of ROS and associated mechanisms of infection tolerance (e.g., glutathione synthesis to cope with increased ROS) are all energy-demanding processes that can carry physiological costs and affect life-history traits. In fact, reductions in growth, altered development rates or in reproduction have been observed before in *Chironomus tepperi* (Ziajahromi et al., 2018), *Chironomus riparius* (Scherer et al., 2019; Silva et al., 2019; Khosrovyan et al., 2020; Stanković et al., 2020), and *Hyalella azteca* (Au et al., 2015) after exposure to MPs. These effects are, at least in part, a likely consequence of the costs associated with the activation of immune response after mechanical damage induced by MPs.

5. Conclusion

This investigation provides the first evidence of the activation of insects' innate immune system after ingestion of MPs (via PO system), in response to foreign agents such as MPs. The ingestion and retention of MPs on *C. riparius* larval gut induced the immune response through the activation of phenoloxidase system. Nevertheless, the size of the PE-MPs ingested by *C. riparius* larvae was considerably large to cross biological barriers; thus, the activation of immune response – PO activation and the subsequent melanin production is possibly linked to mechanical/proteolytic damage of the gut epithelium rather than endocytosis. PO activation and melanisation process are linked to ROS production and consequent oxidative stress, which might explain the oxidative damage and deleterious effects in life-history traits observed before in *C. riparius* larvae exposed to these microplastics.

Additional studies, including co-exposure of MPs and pathogens, can be interesting to investigate immune responses under realistic exposure scenarios and its physiological consequences for invertebrates. Furthermore, additional studies should include the study of the isoforms of phenoloxidase present in *C. riparius* and analysis of gene expression using methodologies such as Reverse Transcription Polymerase Chain Reaction (RT-PCR) to confirm *de novo* synthesis of prophenoloxidase.

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Chapter 6

Effects of polyethylene microplastics on *Chironomus riparius* life-history traits under changing environmental conditions

Effects of polyethylene microplastics on *Chironomus riparius* life-history traits under changing environmental conditions

Abstract

Understanding the combined effect of microplastics (MPs) and natural stressors and thus under realistic exposure scenarios is of great importance in ecotoxicology and particularly for risk assessment. This study addressed the interactive effects of exposure to polyethylene microplastics (PE-MPs; 2.5 g kg^{-1}) in the midge *Chironomus riparius* life history-traits under different temperatures (15, 20 and 25 ºC), salinity (0, 1 and 3 g L^{-1} NaCl) and food shortage (0.5, 0,25 and 0.125 mg TetraMin $larva^{-1}day^{-1}$).

The complex interactions between microplastics and natural stressors difficult the interpretation of results and the establishment of mechanisms of toxicity. The present work reveals that under sub-optimal conditions (temperatures lower than 20 ºC and under severe food shortage) the effects of microplastics can be stronger than those observed at standard toxicity test conditions (20 ºC and food *ad libitum*).

As observed, MPs toxicity can me mediated by natural stressors, which underlines the importance of co-exposure studies. In this sense, this study is a steppingstone towards an accurate risk assessment and suggests that the deleterious effects of PE-MPs on *C. riparius* development will not be aggravated under climate change scenarios (increased temperature and salinisation of freshwaters).

Keywords: Natural stressors; combined exposures; temperature; salinity; food shortage

1. Introduction

Microplastics (plastic particles of 1 µm to 5 mm in size, Frias and Nash, 2019) are ubiquitous and persistent contaminants, particularly in freshwater environments that have been recently identified as major sinks of such particles (Castañeda et al., 2014; Klein et al., 2015; Wang et al., 2018; Scherer et al., 2020), with concerning levels reported in river sediments (e.g., up to around 9 g/kg in European river sediments; (Hurley et al., 2018). The literature addressing the ingestion and impact of microplastics on freshwater organisms has substantially increased in the last five years, reporting adverse physiological, biochemical, and reproductive outcomes on benthic and sediment-dwelling macroinvertebrates (Redondo-Hasselerharm et al., 2018, 2020; Silva et al., 2019, 2021b, 2021a; Stanković et al., 2020). For instance, dipteran larvae are able to ingest microplastic particles with size up to 125 µm, causing impairments on aerobic energy production and reduction of energy reserves, triggering oxidative stress, and compromising fitness by delaying development and causing body deformities (Scherer et al., 2017; Ziajahromi et al., 2018; Silva et al., 2019, 2021a; Stanković et al., 2020). Most research on microplastics ecotoxicity are still privileging optimized laboratory conditions, where a range of concentrations, different size, shape, or chemical composition of microplastics are tested. In natural environments, however, organisms rarely face optimal environmental conditions. Instead, they often deal with multiple stressors, including anthropogenic pollutants (e.g., microplastics and pesticides) and natural stressors (e.g., temperature variations, salinity and food shortages). In this context, multiple-stressor research represents a step forward towards environmentally realistic approaches (Thompson et al., 2018; Orr et al., 2020). The study of the combined effects of emergent contaminants and environmental stressors has been valued as crucial to provide realistic information on the effects of MP pollution under specific environmental conditions (Wagner and Lambert, 2018). These natural stressors (biotic/abiotic) can interact with microplastics, through either synergistic effects on organisms (i.e., with higher risks than the sum of the effects individually), additively (i.e., with effects corresponding to the sum of the effects individually), or having antagonistic effects (i.e., with weaker effects than the sum of the effects individually) (Weber et al., 2020).

The Intergovernmental Panel on Climate Change (IPCC) predicts that, along with the average temperature rising, salinization of freshwater ecosystems will occur more often (IPCC, 2014; Carvalho et al., 2020), imposing a serious risk of extinction to a large fraction of freshwater species. In addition to these climate change driven abiotic factors such as temperature and salinity, biotic stressors such as food shortage (common under intraspecific competition; Hooper et al., 2003) urge to be studied.

The present study focused on different natural stressors that are relevant for *C. riparius* natural populations: temperature (Müller et al., 2012), salinity (Venâncio et al., 2018) and food shortage (Postma et al., 1994). All these three stressors were tested with and without the presence of PE-MPs at a concentration of 2.5 g Kg^{-1}, the LOEC for larval growth determined on previous work (Silva et al., 2019), and that can be found in freshwater MPs contamination hotspots. Given that factors such as temperature and salinity are known to cause alterations in metabolism and

osmoregulation, and food shortage is known to cause delay in development it is hypothesized that natural stressors will interact with MPs, thus altering the effects triggered by PE-MPs alone in *C. riparius* key life-history traits.

2. Materials and Methods

2.1 Organism culture conditions

A laboratory culture of the macroinvertebrate *Chironomus riparius* is maintained in the Department of Biology, University of Aveiro. *Chironomus riparius* is reared under regulated conditions (16:8 h light-dark photoperiod and temperature of 20 ± 1 °C). In culture, larvae are allowed to grow in glass aquaria holding previously burnt mineral sediment (size<1 mm; 500 °C for 4h) and American Society for Testing Materials (ASTM) hard water in a 1:4 ratio (ASTM, 1980) in continuous aeration. Eight glass aquaria are located inside an acrylic cage constraining the adults and allowing the egg laying completing the cycle. The feeding of the larvae (macerated TetraMin®-Tetrawerke, Melle, Germany) is prepared and offered three times per week (*ad libitum*).

2.2 Polyethylene microplastics selection and preparation

Polyethylene microplastics (PE-MPs) tested were originated from commercially available polyethylene microplastics: average size 40-48 µm, CAS No. 9002-88-4, Sigma-Aldrich UK). A vibratory sieve shaking was used for size separation and the fraction of PE-MPs between 32-63 µm was used in the experiments. The PE-MPs concentration of 2.5 g Kg^{-1} dry sediment was chosen, based on previously determined LOEC for larval growth (Silva et al., 2019), being also a concentration regularly found on freshwater sediment microplastic hotspots (Klein et al., 2015; Hurley et al., 2018; Wang et al., 2018; Scherer et al., 2020).

2.3 Experimental design

All three experiments (temperature; salinity; and food shortage) were performed using first instar (<24 h post hatching) *C. riparius* larvae (OECD, 2004) and followed similar methodology as used in previous research (Silva et al., 2019). Briefly, each treatment condition and a control (uncontaminated sediment) consisted of ten replicates/vials containing five larvae in each vial. For each treatment, PE-MPs (2.5g Kg^{-1}) were directly mixed into the sediment (< 1mm, previously burnt at 500 °C) on a glass vessel and distributed to each 250 mL glass test vial. Each test vial contained 50 g of control (sediment with no MPs) or sediment containing MPs and filled with ASTM hard water (~150 mL). The addition of ASTM hard water was performed by gently pouring the ASTM hard water to minimize MPs resuspension following previous procedures (Silva et al., 2019, 2021a, 2021b). The test vials were then covered with lids and allowed to equilibrate for 24h. During the experiments (temperature, salinity and food shortage), organisms were fed every two days (0.5 mg of macerated TetraMin® per organism per day), except for food shortage test (specific food levels

explained below) and the tests were conducted at 20 °C (except for temperature tests). Water quality parameters (temperature, pH, dissolved oxygen, and conductivity) were evaluated every three days.

After ten days, larvae from five replicates of each treatment were sacrificed, counted, and placed in 70% ethanol. The total length of each larva was measured using a stereo dissecting microscope fitted with a calibrated eyepiece micrometer. These larvae were also used for the estimation of MPs inside the gut and followed procedure previously established for the estimation of MPs inside *C. riparius* gut (Silva et al., 2019). Briefly, organisms were placed in glass flasks and covered with aluminium foil to be left drying at room temperature overnight. Samples were then incubated after the addition of nitric acid (HNO_3) 65% at 60 °C for 3 h. After this time, samples were left at room temperature for cooling down followed by the addition of hydrogen peroxide (H_2O_2) at 35% to each sample (1:1 v/v) and incubated for more 24 h at room temperature. After this period, samples were diluted 1:10 using Milli-Q water and vacuum-filtered. Gridded cellulose ester filters (Whatman 10406972, Mixed Cellulose Ester Filter, 3.1 mm white/black grid, 0.45 µm pore size) were used to retain the microplastics. After, each filter was transferred to a glass petri-dish and left in the oven to dry at 25 °C for 2–3 days. After that, the number of PE-MPs ingested by each invertebrate was counted under a stereomicroscope (stereoscopic zoom microscope—SMZ 1500, Nikon Corporation).

The remaining five replicates were used to follow the emergence of emerged adult insects (imagoes) until the end of the test. The end of the test was determined after three days without emergence of organisms. Imagoes were daily collected from emergence traps, gender identified (male/female) and placed in microtubes containing ethanol 70% for storage. The determination of the weight of adult imagoes (dry weight) was performed after evaporation of the ethanol solution (dried 60 °C for three days).

In the three experiments, one of stress factors (temperature, salinity or food shortage) was altered while the other factors were kept constant (and based on regular test conditions: 20 °C, salinity 0 g L^{-1} NaCl and 0.5 mg TetraMin®$larva^{-1}day^{-1}$) with and without the addition of PE-MPs.

- **Temperature**

Larvae were exposed to three different temperatures: 15, 20 and 25 °C. The temperature was daily monitored, and every three days so were additional parameters such as conductivity, pH, and dissolved oxygen. Temperatures were chosen based on a frequently found temperature in Iberian rivers (15 °C), the standard laboratory used temperature (20 °C; control), and a higher temperature (25 °C) to simulate warming up to 5 °C and water temperature increase due to climate change and significant increase in the number of days above 20 °C - mean temperature (IPCC, 2014, 2018; Carvalho et al., 2020).

- **Salinity**

To test the effect of salinity, larvae were exposed to three different concentrations of NaCl: 0, 1 and 3 g L^{-1}. NaCl was added to the ASTM used to fill the test vials. Recognizing that salinity in freshwaters should be represented by other ions rather than Na^+ and Cl^-, NaCl (99.5% purity, Merck, Germany) was used as a proxy as these are the major ions in seawater (Cl^- = 18.9 mg/L; Na^+=10.6 mg/L in a total = 34.5 mg/L) (Venâncio et al., 2018). The three NaCl concentrations were chosen based to mimic conductivities measured in rivers suffering moderate and severe secondary salinization, including in Iberic rivers (Cañedo-Argüelles et al., 2013, 2019).

- **Food shortage**

Test larvae were fed using three food regimes: 0.5 mg TetraMin®$larva^{-1}day^{-1}$ (commonly used for testing) and two lower food regimes to simulate food shortage: 0.25 and 0.125 mg TetraMin®$larva^{-1}day^{-1}$.

2.4. Statistical Analysis

The data on the number of PE-MPs ingested by the larvae was checked for normality by performing Kolmogorov-Smirnov test and for homogeneity of variances by using Bartlett's test. One-way analysis of variance (ANOVA) was used to evaluate potential differences between treatments. Tukey's multiple comparisons test was used to determine significant differences between treatments. Statistical differences were considered at $p< 0.05$. GraphPad Prism 8.0 software was used for statistical analysis of MP ingestion.

Linear Mixed Models (LMM) were applied to test the effects of PE-MPs when combined with temperature, salinity, or food shortage on the studied endpoints: larval length, female/male day of emergence, female/male adult weight. In the first experiment, PE-MPs, temperature levels and their interaction were included in the model as fixed effects factors, while replicate was included as a random effect, to take into account the lack of independence between animals tested in the same vial. The same procedure was carried out for the effect of PE-MPs combined with different salinity levels and for the PE-MPs with food shortage. All the statistical analysis was performed in R v4.0.0 (Core Team, 2020) using lme4 package v1.1-23 (Bates et al., 2015).

In addition to statistical modelling, to further investigate the interaction type (additive, synergism or antagonism) between PE-MPs and the selected environmental stressors, a model of independent action (IA) was used, as described by Coors and DeMeester, 2008. Following this method, deviations from additivity (i.e., synergistic/ antagonistic effects) of the combined stressors are evaluated by comparing the observed responses to predicted responses given by IA reference model.

According to IA mathematical model, the combined effect in a two-factor combination is predicted from the respective observed single-stressor effects, following the equation:

$$E_{mix} = 1 - \prod^{i} (1 - E_i)$$

E_{mix} represents the effects of the combined stressors and E_i the observed effect of each single stressor *i.* Previously, observed absolute effects were converted into a proportional effect using the equation $E_i = \frac{(e_i - e_{control})}{(e_{max} - e_{control})}$, where e_i is the single-stressor effect in absolute units, $e_{control}$ is the effect observed in the control, also in absolute units, and e_{max} is the maximum possible effect of that single stressor, defined based on the range of the experimental data. The calculated E_{mix} is then rescaled again into absolute units to allow the comparison with the experimental observed joint effects.

Joint effects are considered significant (i.e. non-additive) if the IA predicted effects are not within the 95% confidence interval of the observed effects. According to Coors and DeMeester, 2008, a significantly stronger observed effects of the combined stressors than predicted in the IA model indicates a synergistic interaction, while a significantly weaker observed effects of the combined stressors than predicted indicates a antagonistic interaction.

The interaction type was determined for the two levels tested of each environmental stressor, since the type of the interaction might be different in each treatment.

3. Results

For clarity only data larval growth and female day of emergence and adult weight are presented and discussed in detail, because females were generally most affected. Data on males' time to emergence and males' adult weight can be found in supplementary material (Table S1-S7).

3.1. Combined effects of PE-MPs and Temperature

The larvae used for estimation of larval length presented PE-MPs on the larval gut in all temperatures tested (Fig. 1A). However, the number of PE-MPs found in larval gut after 10 days varied with the temperature and larvae exposed to 25 ºC presented significantly more PE-MPs particles than larvae growing at lower temperatures (15 ºC) ($F_{2,12}$=10.21; *p*= 0.0026; Fig. 1A). The number of PE-MPs inside the gut increased with increasing temperature as well as the larval length which also augmented with increasing temperature (Fig. 1A and 1B).

Without the presence of PE-MPs in the sediment, larvae exposed to higher temperatures showed an increase in the larval length after 10 days and a subsequent reduction in the time to emergence and females' weight (both males and females; Table S5 and Table 1 and Fig. 1, respectively). The larval length ranged from 5.20±0.14 (at 15 C) to 11.13±0.27 mm (at 25 C) (Fig. 1B, -MPs). When combined with PE-MPs, larval length (after 10 days of exposure) ranged from 4.76±0.17 to 10.83±0.20 mm from 15 C to 25 C (Fig. 1B, +MPs). According to the result of LMM analysis, no interaction was found between temperature and MPs (F=2.4196, p>0.05; Table 1). However, the results from the Independent Action (IA) model showed an antagonist interaction between the presence of MPs and high temperature (25 C), reflecting a weaker effect of MPs at 25 C (Table S1).

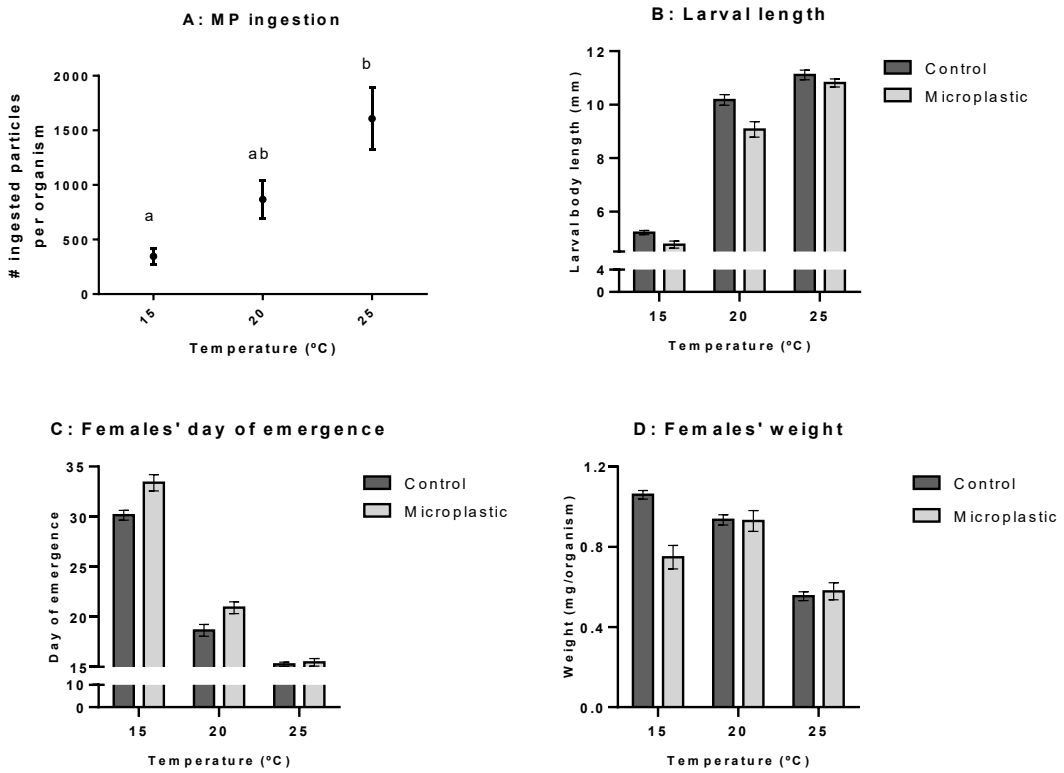

Fig.1. Number of polyethylene microplastics (PE-MPs) present inside the gut (A) after 10 days of exposure to PE-MPs in combination with temperature (MP ingestion) and effects of PE-MPs and temperature (15; 20 and 25 ºC) in: (B) larval length, (C) females' time to emergence and (D) females' weight. Means are plotted ± standard error of the mean and were calculated using a maximum number of 25 values per condition (each condition consisted of 5 replicates - vials with 5 larvae for a maximum of 25 organisms).

In the absence of MPs, both day of emergence and adults weight decreased from 15 ºC to 25 ºC for both females and males (Fig. 2C and Table 1; and Table S5, respectively). The day of emergence for female midges ranged from 30.14±0.49, at 15º C to 15.25±0.22 days at 25 C (Fig. 1C). In the presence of PE-MPs, this parameter ranged from 33.38±0.81, at 15 ºC to 15.44±0.38 days at 25 ºC (Fig. 1C). LMM indicates an interaction between temperature and the presence of MPs on "females' day of emergence" (F=3.6623, *p*=0.0312; Table 1). The IA model indicates an antagonistic interaction between MPs and high temperature (MPs x 25 ºC) for females' time to emergence (Table S1). Females' weight ranged from 1.059±0.022 at 15 ºC to 0.554±0.022 mg at 25ºC (Fig. 1D,). In the presence of PE-MPs, females' weight decreased from 0.748±0.059 to 0.578±0.042 mg from the lowest temperature (15 ºC) to the highest temperature (25 ºC; Fig. 1D). The presence of MPs had a marginally significant effect on weight of female adults (Table 1) and the variation of temperature strongly affected this parameter. LMM analysis indicates a significant interaction between MPs and temperature on females' weight (F=7.4060, *p*=0.0099; Table 1) and indicates the interaction to be synergistic interaction between MPs and the lowest temperature

(MPs x 15 ºC). Likewise, the IA model indicates a synergistic interaction between MPs and low temperature on females' weight (MPs x 15ºC; Table S1).

Table 1 – Results of the linear mixed model analysing the effect of exposure to PE-MPs and different temperatures on several life-cycle endpoints: Larval length; Females' time to emergence and Females' weight. (*) indicates the significative effect of MP; temperature or significant interaction between MP and temperature on an endpoint (p< 0.05).

Endpoint	Fixed factor	F value	Pr(>F)	Sig.
Larval length	Microplastic (MP)	14.9719	0.0007089	*
	Temperature	517.0172	< 2.2e-16	*
	MP x Temperature	2.4196	0.1098800	
Females' time to emergence	Microplastic (MP)	16.5174	0.0001342	*
	Temperature	465.7866	< 2.2e-16	*
	MP x Temperature	3.6623	0.0311966	*
Females' weight	Microplastic (MP)	3.8934	0.075136	.
	Temperature	33.0139	2.97e-05	*
	MP x Temperature	7.4060	0.009867	*

3.2. Combined effects of PE-MPs and Salinity

After 10 days of exposure *C. riparius* larvae exposed to 1 g L^{-1} NaCl presented a significantly higher number of particles in their gut ($F_{2,12}$=15.00; p= 0.0005; Fig. 2A). Effects on larval length were an increase at 1 g L^{-1} NaCl and a slight decrease in larvae exposed to 3 g L^{-1} NaCl when compared to larvae exposed to 0 g L^{-1} NaCl.

The exposure to increased salinity (g L^{-1} NaCl) *per se* caused a decrease in larval length (Table 2) ranging from 10.64±0.12 mm (0 g L^{-1} NaCl) to 8.64±0.18 mm (3 g L^{-1} NaCl) in the absence of MPs (Fig. 2B). When combined with PE-MPs, larval length ranged from 9.71±0.21 (0 g L^{-1} NaCl) to 9.29±0.24 mm from at 3 g L^{-1} NaCl (Fig. 2B). Results show only a slight tendency for reduction in larval length caused by the presence of MPs (Table 2), which was at salinity 0 g L^{-1} NaCl (Table S1). Additionally, LMM points out to a significant interaction between MPs and salinity on larval length (F=9.0153, p= 0.0002075; Table 2, MPs x High salinity; Table S2). The IA model supports this interaction and signalizes that the antagonistic interaction between MPs and salinity happens at both low and high salinities (Table S1).

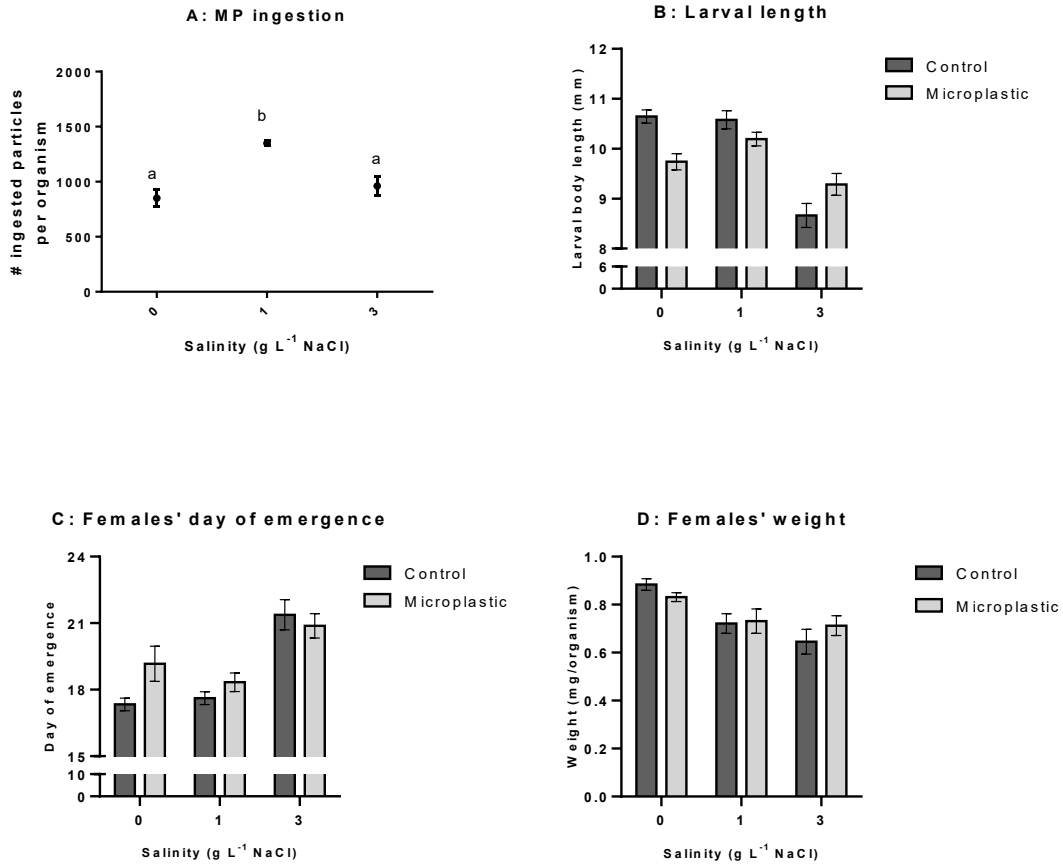

Fig.2. Number of polyethylene microplastics (PE-MPs) present inside the gut (A) after 10 days of exposure to PE-MPs in combination with salinity (MP ingestion) and effects of PE-MPs and salinity (0; 1 and 3 g L^{-1} NaCl) in: (B) larval length, (C) females' time to emergence and (D) females' weight. Means are plotted ± standard error of the mean and were calculated using a maximum number of 25 values per condition (each condition consisted of 5 replicates - vials with 5 larvae for a maximum of 25 organisms).

Increased salinity caused an increase in females' day of emergence (Fig. 2C and Table 2) and a reduction in adults' weight (both females and males; Fig. 2D, and Table 2; and Table S5, respectively) from the control (0 g L^{-1} NaCl) to the highest salinity tested (3 g L^{-1} NaCl). Females' day of emergence ranged from 17.33±0.28 to 21.38±0.68 days, in the control (0 g L^{-1} NaCl) and highest salinity conditions, respectively (Fig. 2C). In the presence of PE-MPs, the day of emergence for female imagoes ranged from 19.17±0.79 to 20.88±0.55 days, for 0 and 3 g L^{-1} NaCl, respectively (Fig. 2C, +MPs). LMM indicates no interaction between the stress factor (salinity) and the presence of MPs in the sediment (F=2.5626, p=0.0980; Table 2). Nevertheless, the IA model points out to an antagonistic interaction between MPs and salinity (both low and high) for females' time to emergence (Table S1). The weight of the female imagoes ranged from 0.884±0.024 to 0.645±0.052 mg in 0 to 3 g L^{-1} NaCl, respectively (Fig. 2D). In the presence of PE-MPs, the females weight ranged from 0.831±0.059 to 0.712±0.041 mg in the lowest salinity condition (0 g L^{-1} NaCl) and the highest salinity tested (3 g L^{-1} NaCl; Fig. 2D). The presence of MPs did not affect the

females' time to emergence nor the females' weight (Table 2), contrary to salinity which affected both females' time to emergence (Table 2) and females' weight (Table 2). No interaction was found between the presence of MPs and the stress factor (salinity) concerning females' weight (F=0.9656, *p*=0.39755; Table 2). However, the IA model detected an antagonistic effect between MPs and the highest salinity tested (MPs x Salinity 3) in females' weight (Table S1).

Table 2 – Results of the linear mixed model (LMM) analysing the effect of exposure to PE-MPs and salinity on several life-cycle endpoints: Larval length; Females' time to emergence and Females' weight. (*) indicates the significative effect of MP; salinity or significant interaction between MP and salinity on an endpoint (*p*< 0.05).

Endpoint	Fixed factor	F value	Pr(>F)	Sig.
Larval length	Microplastic (MP)	2.2636	0.1347000	
	Salinity	35.3762	3.672e-13	*
	MP x Salinity	9.0153	0.0002075	*
Females' time to emergence	Microplastic (MP)	2.9087	0.10098	
	Salinity	23.6719	2.075e-06	*
	MP x Salinity	2.5626	0.09797	
Females' weight	Microplastic (MP)	0.0003	0.98652	
	Salinity	6.1875	0.00796	*
	MP x Salinity	0.9656	0.39755	

3.3. Combined effects of PE-MPs and Food shortage

The combined exposure of *C. riparius* larvae to PE-MPs and different food levels to simulate food shortage led larvae to present different PE-MPs ingestion. Larvae with more food available (0.5 mg TetraMin®/larva/day) presented more PE-MPs in the gut. Larvae that had only 0.125 mg TetraMin®/larva/day of food available presented significantly less PE-MPs inside the gut ($F_{2,12}$=8.580; *p*= 0.0049; Fig. 3A). Concomitantly, larvae that had the more food available (0.5 mg TetraMin®/larva/day) presented significantly higher larval length than larvae that under food shortage.

The reduction of the food level to simulate food shortage caused a decrease in larval length from 10.88±0.13 to 7.79±0.21 mm in larvae fed with 0.5 and 0.125 mg TetraMin®/larva/day, respectively (Fig. 3B). When combined with PE-MPs, larval length decreased from 9.95±0.13 to 6.80±0.07 mm from 0.5 to 0.125 mg TetraMin®/larva/day (Fig. 3B, +MPs). No interaction was found between the presence of MPs and food shortage (F=0.0224, *p*=0.9778), despite the significant effects of both the presence of MPs in the sediment and food shortage (Table 3). The IA model indicates no interaction (additive effect) between MPs and moderate food shortage (0.25 mg TetraMin®/larva/day) but signalizes a synergistic interaction between MPs and severe food shortage (0.125 mg TetraMin®/larva/day) on larval growth (Table S1).

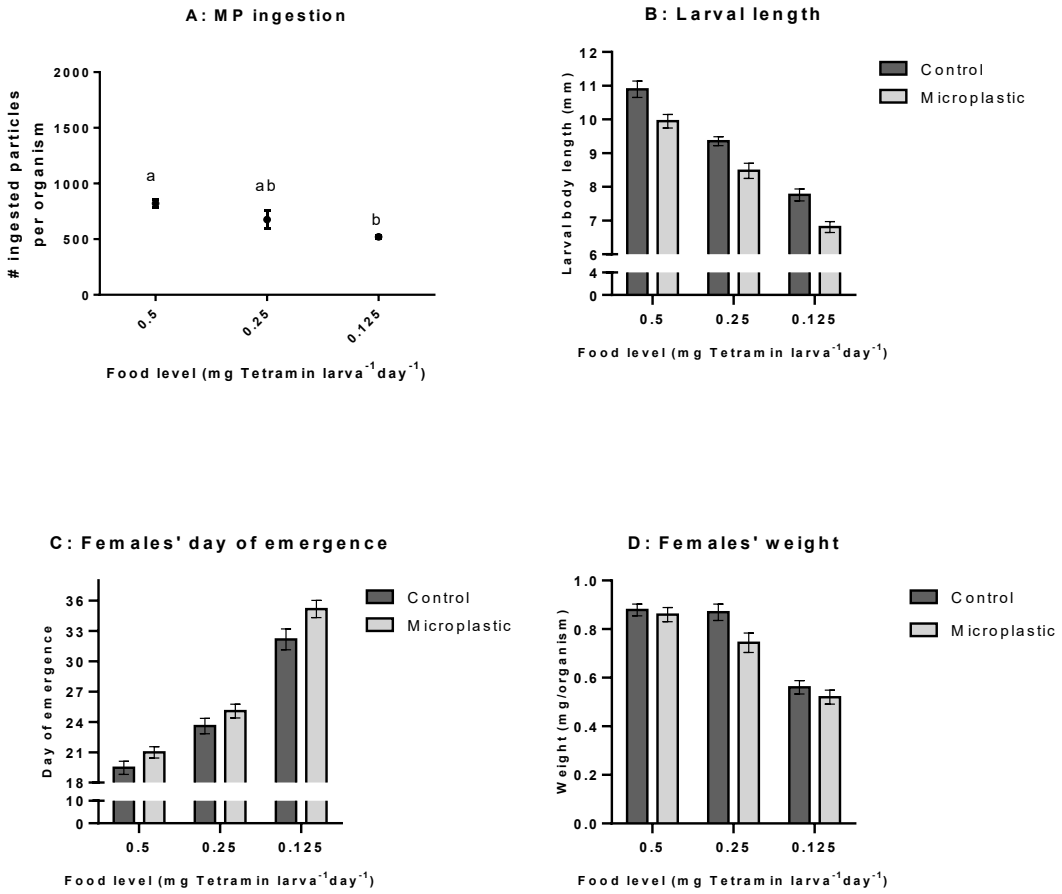

Fig.3. Number of polyethylene microplastics (PE-MPs) present inside the gut (A) after 10 days of exposure to PE-MPs in combination with food shortage (MP ingestion) and effects of PE-MPs and food shortage (food level: 0.5, 0.25 and 0.125 mg TetraMin® larva^{-1}day^{-1}) in: (B) larval length, (C) females' time to emergence and (D) females' weight. Means are plotted ± standard error of the mean and were calculated using a maximum number of 25 values per condition (each condition consisted of 5 replicates - vials with 5 larvae for a maximum of 25 organisms)

Females' time to emergence was significantly altered by the food level (Table 3) and ranged from 19.46±0.65 to 32.17±1.03 days, from the highest to the lowest food levels, respectively (Fig. 3C). In the presence of PE-MPs, the time to emergence for female imagoes was also significantly altered (Table 3) and ranged from 21.00±0.56 to 35.18±0.85 days, from 0.5 to 0.125 mg TetraMin®/larva/day, respectively (Fig. 3C, +MPs). Likewise, food shortage caused a delay in the emergence in males in the absence and presence of MPs (Table S6). LMM indicates no interaction between the stress factor (food shortage) and the presence of MPs in the sediment for both males' ($F= 1.6724$, $p= 0.1977$; Table S6) and females' time to emergence ($F=0.6397$, $p=0.5303$; Table 3). This absence of interaction was confirmed by the IA model which indicates no interaction (only additive effect) between MPs and food shortage for both males' and females' time to emergence (Table S1).

The weight of the female imagoes decreased from 0.879±0.024 to 0.560±0.027 mg under food shortage (from 0.5 to 0.125 mg TetraMin®/larva/day, respectively) in the absence of MPs (Fig. 3D, -MPs). In the presence of PE-MPs, females' weight decreased from 0.860±0.029 to 0.520±0.028 mg from the highest food level (0.5 mg TetraMin®/larva/day) to the lowest food level tested (0.125 mg TetraMin®/larva/day; Fig. 3D, +MPs). The presence of MPs did not affect the males' weight (Table S6) nor the females' weight (Table 3). The stress factor (food shortage) affected both males' (Table S6) and females' weight (Table 3). No interaction was found in the LMM between the presence of MPs and the stress factor (food shortage) concerning both males' (F=0.6641, *p*=0.5191; Table S6) and females' weight (F=0.9697, *p*=0.40236; Table 3). The IA model indicates no interaction between MPs and food shortage, except for MPs and severe food shortage (0.125 mg TetraMin®/larva/day) on males' weight in which an antagonistic interaction was identified (Table S1).

Table 3 – Results of the linear mixed model (LMM) analysing the effect of exposure to PE-MPs and food shortage on several life-cycle endpoints: Larval length, Time to emergence (males and females) and adults' weight (males and females). (*) indicates the significative effect of MP; food level or significant interaction between MP and food level on an endpoint ($p < 0.05$).

Endpoint	Fixed effect	F value	Pr(>F)	Sig.
Larval length	Microplastic (MP)	34.9237	2.52e-08	*
	Food shortage	131.3795	< 2.2e-16	*
	MP x Food shortage	0.0224	0.9778	
Females' time to emergence	Microplastic (MP)	9.9188	0.002349	*
	Food shortage	167.3193	< 2.2e-16	*
	MP x Food shortage	0.6397	0.530326	
Females' weight	Microplastic (MP)	3.4893	0.08195	.
	Food shortage	48.9767	3.405e-07	*
	MP x Food shortage	0.9697	0.40236	

4. Discussion

This study aimed to investigate how thermal stress, increased salinity and food shortage could alter the toxicity of polyethylene microplastics to *Chironomus riparius* developmental endpoints. Despite the expected negative effects of the chosen environmental stress factors observed in *C. riparius* life-history traits, results suggest complex effects of stressor combination but that, in general, effects triggered by PE-MPs are not amplified under stressful environmental conditions such as elevated temperatures, salinity exposure or conditions of food shortage.

4.1 Combined effects of PE-MPs and Temperature

As expected, under higher temperatures and in the absence of microplastics, *C. riparius* larvae grew faster and consequently emerged earlier than larvae exposed to lower temperatures. Fast developing *C. riparius* midges presented reduced adult weight (both males and females) which has been previously related to shortened feeding time/period (Vogt et al., 2007b). At higher temperatures the number of eggs per egg rope decreases (usually associated to low weight of female adults). With the accelerated *C. riparius* development at higher temperatures and mainly the decreased mean emergence time, the population growth rate also increases, compensating for the negative effect on the clutch size (Vogt et al., 2007b).

When presented an additional exposure to MPs, *C. riparius* responded in a complex way. Negative effects of MPs such as reduced larval growth, delayed development and reduced adult weight were mainly observed at lower temperatures (15 ˚C) but not at elevated temperatures (25 ˚C). It is important to note however that both lower and higher temperatures, i.e. 15 ˚C and 25 ˚C represent stressful conditions since chironomids used in the experiments are reared under constant temperatures of 20±1 ˚C.

Despite no interactive effects observed for larval growth, LMM and IA models detected significant interaction of temperature and MP exposure for development rate (time to emergence) and for female adult weight suggesting synergistic effects only at low temperatures (15 ˚C) and antagonistic effects at 25˚C. The observed lower reduction in *C. riparius* females' weight caused by MPs exposure at higher temperatures can therefore be a result of physiological limitation. Generally, female imagoes do not present weight lower than 0.35mg, even when exposed to chemicals that induce deleterious reproductive effects (Vogt et al., 2007b, 2007a) or exposed to toxicants that cause severe effects on emergence (Rodrigues et al., 2015; Campos et al., 2016). This minimum female weight hypothesis is supported by the fact that the emergence of female *C. riparius* imagoes depends on reaching a critical size (Stanko-Mishic et al., 1999).

Besides this antagonistic effect for female adult weight our results further indicate synergistic effects for time of emergence when *C. riparius* were simultaneoulsy exposed to MPs and low temperatures. This result seem contradictory with the higher number of particles found in larval guts observed for higher temperatures. However, as *C. riparius* growth and development is reduced at 15 ˚C, such larvae are likely exposed to MPs for a longer period before reaching pupal

stage. Moreover, lower metabolism can also reduce egestion of these inert particles and thus may contribute to increase the residence time of MPs in *C. riparius* larvae guts which has been suggested and associated to its triggered effects on previous investigations (e.g., Scherer et al., 2017). In the same way, the possible higher metabolic rates at higher temperature (25 ˚C) may have increased feeding and perhaps egestion rates of MPs since higher ingestion rates of nutritive particles (food) have been associated to a faster egestion of non-nutritive particles (e.g. MPs) (Wright et al., 2013; Scherer et al., 2017) which can, to some extent, reduce the physiological/biochemical deleterious effects on *C. riparius* larvae.

This might explain why effects were stronger at 15 ˚C where lower number of MP particles were observed in larval guts after 10 days of exposure and not under exposure to higher temperatures where these numbers were much higher. The higher number of PE-MPs found inside the larvae growing at 20 and 25 ˚C is also related with physiology. Growing at higher temperatures larvae reach later instars faster which means a wider mouth apparatus and ingestion of high amounts of MPs. Other sediment-ingesters benthic invertebrates (e.g., lumbriculidae) have a more dynamic ingestion/egestion, which avoid the deleterious effects of MPs (Silva et al., 2021b).

However, and since effects of PE-MPs on *C. riparius* have been previously related with activation of immune responses (Silva et al., submitted), altered energy metabolism and oxidative stress and damage (Silva et al., 2021a) we cannot exclude the possibility that responses to warmer temperatures could allow them to mobilize antioxidant stress machinery more readily to cope with the stress caused by MP ingestion.

Recent research reported increased sensitivity in freshwater invertebrates such as *Daphnids* when exposed to microplastics combined with thermal stress (Jaikumar et al., 2018). This exacerbated sensitivity to higher temperatures is often related to an increase in metabolic turnover (Baas and Kooijman, 2015). Enhanced metabolic rates could also cause faster use of lipid-reserves, resulting in elevated feeding and ventilation rates (Heugens et al., 2003). Therefore, Jaikumar et al., 2018 relates the elevated feeding with increased ingestion of microplastics or accelerated clogging of respiratory apparatus in exposed invertebrates. Yet, for other invertebrate species like the freshwater shredder *Gammarus pulex* changes in metabolism caused by temperature variation are not always associated with changes in the feeding rate (Kratina et al., 2019). In the present study, and although we can hypothesize that ingestion was in fact altered by warmer temperatures (number of MPs found in *C. riparius* larval gut were higher in higher temperature treatments) that alone cannot explain the observed effects. Results show nevertheless that effects of MPs present in sediments can be stronger when tested at lower temperatures (in comparison with standard toxicity tests).

4.2 Combined effects of PE-MPs and Salinity

Under increased salinity and in the absence of microplastics, *C. riparius* larvae grew less, emerged later and imagoes presented reduced weight (both males and females). The reduction in

larval growth caused by salinity alone is in line with previously observed for similar conditions (Hassell et al., 2006; Venâncio et al., 2018). Increased energy requirements for osmoregulation as salinity increase are suggested as explanation for the reduction in larval growth and increased times to emergence in chironomids under osmotic stress (Hassell et al., 2006).

In addition, *Chironomus riparius* response to increased salinity is also related to a decrease in K^+ absorption, which seems to compromise the activities of Na^+/K^+-ATPase and V-type H+-ATPase (also known as VA activity) (Jonusaite et al., 2011, 2013). In addition to a severe decline in VA activity, *C. riparius* exposed to increased salinities (0, 1, and 3 g L^{-1} NaCl) also revealed increased levels of Na^+ and Cl^- in hemolymph, and a decrease in hemolymph pH. The decline in VA activity when *C. riparius* larvae are transferred to brackish water has also been associated to alterations in the gut physiology and Malpighian tubule tissue (Jonusaite et al., 2011).

Considering PE-MPs as only stressor, the effects of PE-MPs with size < 63 µm have been associated to the activation of antioxidant defenses, oxidative damage, energy impairments, with consequent decreased larval growth, and delayed in emergence (Silva et al., 2019, 2021a). Being homo-polymeric, free of plasticizers and pristine (presumably free of hazardous environmental chemicals and microbes), the observed effects were mostly related with the physical effects of the particles (impairing feeding, energy acquisition, inducing gut clogging, among others). In our case, at a concentration of 2.5 g/kg of sediment, PE-MPs slightly decreased larval growth and adult's emergence, corroborating our previous investigations (Silva et al., 2019). Considering the effects of each stressor (salinity and PE-MPs) alone, one would expect an additive or synergistic effect when both stressors were combined, as the mechanisms behind them are differently triggered (chemical *vs* physical). Instead, it was observed an antagonistic interaction on larval growth (estimated in larval growth endpoint) and in females' time to emergence and partially (only at high salinity) in females' weight. The negative effect of PE-MPs on growth and emergence was slightly attenuated, particularly at the highest salinity level.

Because MPs ingestion leads to activation of immune responses, to ROS production and to oxidative stress and damage (Jeong et al., 2016; Imhof et al., 2017; Silva et al., 2021a, 2021b) these antagonistic effects on life history might be partially explained by effects of salt exposure that have been reported in invertebrates namely the increase in oxidative defences (Patang and Soegianto, 2020) or decrease of immune responses (Mangahas et al., 2019) that can contribute to reduce ROS in *C. riparius* exposed to MPs. These hypotheses need confirmation with the simultaneous evaluation of biochemical endpoints

4.3 Combined effects of PE-MPs and Food shortage

Experiment on food shortage was performed following OECD recommended food level (0.5mg TetraMin®$larva^{-1}day^{-1}$) and following research that suggested this food level as being ad libitum and food levels of 0.2 mg TetraMin®$larva^{-1}day^{-1}$ or below to represent food shortage (Péry and Garric, 2006; Heye et al., 2019). Food shortage is intuitively a factor that is known to regulate

development and reproduction. Several studies confirm this assumption reporting reductions in offspring, egg production and growth as well as constrains in imagoes' weight and population growth (Ankley et al., 1993; Postma et al., 1994; Péry et al., 2002; Hooper et al., 2003). Likewise, food shortage can increase the organisms' sensitivity to xenobiotics and other stressors with repercussions on organisms' metabolism physiology and capability to maintain homeostasis (Heugens et al., 2001, 2006). In the present experiment, the IA model indicates that an additive effect explains most of the effects and only a synergistic interaction was found between MPs and severe food shortage in terms of larval growth.

Under food shortage (0.125 and 0.25 mg TetraMin®larva^{-1}day^{-1}) and in the absence of MPs, *C. riparius* larvae grew less and emerged later than larvae fed ad libitum (0.5 mg TetraMin®larva^{-1}day^{-1}). Under severe food shortage and besides reduced developmental rates, midges also presented reduced weight in adults (both males and females).

Moreover, effects of MPs were similar (reduction in larval growth, delay in emergence and slight reductions in female adult weight) but no significant interaction were found between these stress factors, indicating that the deleterious effects of MPs were not general not exacerbated under food shortage despite an indication of synergism observed for male imagoes weight. Under stressful conditions like food shortage, organisms use a major part of energy for maintenance, limiting energy available for development, growth, and reproduction (Koehn and Bayne, 1989; Sokolova et al., 2012). Thus, under severe food restrictions (0.125 mg TetraMin®larva^{-1}day^{-1}), and since food availability (and not food quality) can be detrimental for development and emergence of *C.riparius* (Goedkoop et al., 2007), larvae exposed to MPs had potentially even less energy available not only for development but also to sustain the biochemical responses induced by MP ingestion. More than additive, synergistic effects were to be expected but again, only observed for male imagoes weight.

Besides, and also contrary to what we expected, under some degree of starvation the ingestion of non-nutritive particles (MPs) was expected to be higher and to cause prolonged residence time of MPs in larval gut (Scherer et al., 2017) that could also contribute to stronger effects. Our results showed, however, that the number of MPs in larval gut was similar across treatments with a slight reduction in the severe food shortage treatment. Most likely, the strong impairments in development caused by severe food shortage could led to reduction in MPs intake since developing slower, larvae take more time to reach third instar in which they potentially ingest most of the MPs present in the sediment. That could, at least in part, compensate for the lack of additional energetic constrains arising from reduced feeding and increase expenditure for MPs induced antioxidant and immune responses. Moreover, and as explained for temperature experiments, physiological limitations concerning minimum size of female adults under severe food shortage could have prevented the estimation of the expected synergistic effects

Final Remarks

The need to address relevant exposure scenarios is of crucial value to evaluate the possible impacts of the exposure of microplastics to freshwater macroinvertebrate communities. In this respect, the effects of MPs evaluated under non-ideal conditions: temperatures lower than 20 ºC, some degree of food shortage or face increased salinity, provides valuable ecotoxicological data for an accurate risk assessment.

However, the interactions between microplastics and these natural stressors are recognizably complex, hampering the establishment of mechanisms of toxicity and therefore the results should be carefully considered. That said, the results show that under specific conditions such as low temperatures and severe food shortage, the sub-lethal effects of MPs to natural populations of *C. riparius* can be stronger than previously predicted by standard toxicity assays. Moreover, under different environmental conditions, MPs toxic effects are not simply a consequence of the number of MPs inside Chironomids's gut.

The use of multi-generational assays to evaluate reproduction and possible transgenerational effects as well as the use of sub-organismal (i.e., biochemical) endpoints should deliver valuable data for the interpretation of these interactive effects between microplastics and natural stressors.

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Supplementary Material

Table S1. Results of the independent action (IA) models identifying the interaction type between temperature; salinity; food shortage and microplastics (MPs): (A) larval growth, (B) adult weight - females (C) adult weight - males, (D) day of emergence – females and (E) day of emergence - males. If the predicted value is not within the range of the 95% confidence interval of the observed value, there is a non-additive effect (highlighted in bold). Medium salinity corresponds to 1 g L^{-1} NaCl and High salinity corresponds to 3 g L^{-1} NaCl.

	Predicted	*Observed*	*95% CI Observed*	*Interaction effect*
Temperature				
a) Larval growth				
MPs x 15°C	*4.642*	*4.761*	*[4.422 – 5.101]*	*Additive*
MPs x 25°C	*9.932*	*10.828*	*[10.434 – 11.222]*	***Antagonistic***
b) Weight (females)				
MPs x 15°C	*1.132*	*0.832*	*[0.632 – 1.033]*	***Synergistic***
MPs x 25°C	*0.601*	*0.634*	*[0.502 – 0.767]*	*Additive*
c) Weight (males)				
MPs x 15°C	*0.545*	*0.516*	*[0.461 – 0.571]*	*Additive*
MPs x 25°C	*0.360*	*0.392*	*[0.357 – 0.426]*	*Additive*
d) Day of Emergence (females)				
MPs x 15°C	*34.491*	*33.450*	*[23.132 – 34.768]*	*Additive*
MPs x 25°C	*17.533*	*15.063*	*[14.160 – 15.965]*	***Antagonistic***
e) Day of Emergence (males)				
MPs x 15°C	*32.493*	*29.517*	*[28.613 – 30.421]*	***Antagonistic***
MPs x 25°C	*14.928*	*14.333*	*[11.613 – 17.053]*	*Additive*
Salinity				
a) Larval growth				
MPs x Medium Salinity	*9.652*	*10.193*	*[9.887 – 10.499]*	***Antagonistic***
MPs x High Salinity	*7.880*	*9.290*	*[8.816 – 9.764]*	***Antagonistic***
b) Weight (females)				
MPs x Medium Salinity	*0.740*	*0.724*	*[0.583 – 0.865]*	*Additive*
MPs x High Salinity	*0.603*	*0.745*	*[0.632 – 0.857]*	***Antagonistic***

c) Weight (males)				
MPs x Medium Salinity	*0.405*	*0.376*	*[0.358 – 0.395]*	***Synergistic***
MPs x High Salinity	*0.403*	*0.370*	*[0.354 – 0.386]*	***Synergistic***
d) Day of Emergence (females)				
MPs x Medium Salinity	*19.640*	*18.375*	*[17.640 – 19.110]*	***Antagonistic***
MPs x High Salinity	*22.908*	*20.800*	*[20.135 – 21.465]*	***Antagonistic***
e) Day of Emergence (males)				
MPs x Medium Salinity	*16.658*	*16.450*	*[15.624 – 17.276]*	*Additive*
MPs x High Salinity	*17.735*	*16.967*	*[15.743 – 18.191]*	*Additive*
Food Shortage				
a) Larval growth				
MPs x Food shortage (0.25mg)	*8.560*	*8.475*	*[8.352 – 8.600]*	*Additive*
MPs x Food shortage (0.125mg)	*7.128*	*6.799*	*[6.670 – 6.927]*	***Synergistic***
b) Weight (females)				
MPs x Food shortage (0.25mg)	*0.866*	*0.811*	*[0.673 – 0.948]*	*Additive*
MPs x Food shortage (0.125mg)	*0.559*	*0.558*	*[0.457 – 0.659]*	*Additive*
c) Weight (males)				
MPs x Food shortage (0.25mg)	*0.443*	*0.459*	*[0.412 – 0.505]*	*Additive*
MPs x Food shortage (0.125mg)	*0.250*	*0.298*	*[0.280 – 0.316]*	***Antagonistic***
d) Day of Emergence (females)				
MPs x Food shortage (0.25mg)	*24.648*	*24.933*	*[23.872 – 25.995]*	*Additive*
MPs x Food shortage (0.125mg)	*34.963*	*35.010*	*[33.367 – 36.653]*	*Additive*
e) Day of Emergence (males)				
MPs x Food shortage (0.25mg)	*21.195*	*20.438*	*[19.299 – 21.576]*	*Additive*
MPs x Food shortage (0.125mg)	*27.933*	*30.083*	*[25.345 – 34.820]*	*Additive*

Note: synergistic (stronger effect than predicted by a null model) or antagonistic (weaker effect than predicted by a null model).

Table S2. Estimates of the linear mixed model analysing the effect of different temperatures (15, 20 and 25 ˚C) in the presence and absence of polyethylene microplastics (PE-MPs) on several life-history traits: larval length, day of emergence (males and females) and adults' weight (males and females).

Experiment: Temperature							
		Estimate	Std. Error	df	t value	Pr(>|t|)	Signif.
Larval length	(Intercept)T. 20 ºC	10.1731	0.1961	25.0446	51.867	< 2e-16	*
	Microplastic (MP)	-1.1041	0.2722	23.4533	-4.056	0.000474	*
	Temp. 15 ºC	-4.9613	0.2774	25.0446	-17.886	8.91e-16	*
	Temp. 25ºC	0.9380	0.2747	24.1840	3.415	0.002257	*
	MP x Temp 15 ºC	0.6534	0.3849	23.4533	1.697	0.102840	
	MP x Temp 25 ºC	0.8072	0.3936	25.1384	2.051	0.050877	.
Day of emergence - Males	(Intercept)T. 20 ºC	16.5354	0.6145	20.0493	26.908	< 2e-16	*
	Microplastic (MP)	1.6880	0.8718	19.9532	1.936	0.06714	.
	Temp. 15 ºC	12.7799	0.9370	22.6911	13.640	2.03e-12	*
	Temp. 25ºC	-2.9064	0.8777	21.0873	-3.312	0.00331	*
	MP x Temp 15 ºC	-1.6964	1.3001	22.1384	-1.305	0.20536	
	MP x Temp 25 ºC	-1.1988	1.3238	20.6197	-0.906	0.37561	
Day of emergence - Females	(Intercept)T. 20 ºC	18.6364	0.5847	64.0000	31.871	< 2e-16	*
	Microplastic (MP)	2.2727	0.8270	64.0000	2.748	0.00777	*
	Temp. 15 ºC	11.5065	0.7814	64.0000	14.725	< 2e-16	*
	Temp. 25ºC	-3.3864	0.8095	64.0000	-4.183	8.93e-05	*
	MP x Temp 15 ºC	0.9690	1.1144	64.0000	0.870	0.38778	
	MP x Temp 25 ºC	-2.0783	1.1896	64.0000	-1.747	0.08543	.
Adults weight (Males)	(Intercept)T. 20 ºC	0.464798	0.019156	18.731637	24.264	1.3e-15	*
	Microplastic (MP)	0.002619	0.027179	18.739833	0.096	0.924264	
	Temp. 15 ºC	0.083982	0.030356	17.449249	2.767	0.012973	*
	Temp. 25ºC	-0.105501	0.027468	20.781205	-3.841	0.000964	*
	MP x Temp 15 ºC	-0.032098	0.041235	18.660034	-0.778	0.446075	
	MP x Temp 25 ºC	0.031176	0.041068	22.548908	0.759	0.455626	
Adults weight (Females)	(Intercept)T. 20 ºC	0.93027	0.05001	13.36367	18.601	6.1e-11	*
	Microplastic (MP)	0.01456	0.06975	12.38942	0.209	0.838004	
	Temp. 15 ºC	0.12723	0.06720	10.80787	1.893	0.085407	.
	Temp. 25ºC	-0.37386	0.06818	12.27880	-5.484	0.000129	*
	MP x Temp 15 ºC	-0.30287	0.09523	10.43754	-3.181	0.009315	*
	MP x Temp 25 ºC	0.02393	0.10008	11.57560	0.239	0.815211	

Table S3. Estimates of the linear mixed model analysing the effect of salinity on several life-history traits: larval length, day of emergence (males and females) and adults' weight (males and females) in the presence and absence of polyethylene microplastics (PE-MPs). The level of significance was

set at $p < 0.05$. Medium salinity corresponds to 1 g L^{-1} NaCl and High salinity corresponds to 3 g L^{-1} NaCl.

		Estimate	Std. Error	df	t value	Pr(>|t|)	
Larval length	(Intercept) Salinity 0	10.64583	0.18297	140.00000	58.183	< 2e-16	*
	Microplastic (MP)	-0.90625	0.25876	140.00000	-3.502	0.000619	*
	Medium Salinity	-0.06783	0.25616	140.00000	-0.265	0.791545	
	High Salinity	-1.98313	0.25876	140.00000	-7.664	2.72e-12	*
	MP x Medium Salinity	0.52125	0.36226	140.00000	1.439	0.152419	
	MP x High Salinity	1.52771	0.36594	140.00000	4.175	5.22e-05	*
Day of emergence - Males	(Intercept)	16.7578	0.4503	18.7028	37.211	<2e-16	*
	Microplastic (MP)	0.3387	0.6369	18.7028	0.532	0.601	
	Medium Salinity	-0.1480	0.6804	19.0965	-0.218	0.830	
	High Salinity	0.9702	0.6701	23.2691	1.448	0.161	
	MP x Medium Salinity	-0.5549	0.9293	18.9195	-0.597	0.557	
	MP x High Salinity	-1.0274	0.9306	21.6093	-1.104	0.282	
Day of emergence - Females	(Intercept) Salinity 0	17.3147	0.4213	14.7418	41.100	< 2e-16	*
	Microplastic (MP)	1.8316	0.7161	24.4051	2.558	0.0172	*
	Medium Salinity	0.2864	0.5871	13.1813	0.488	0.6337	
	High Salinity	4.0145	0.6606	18.2056	6.077	9.18e-06	*
	MP x Medium Salinity	-1.0964	1.0077	23.5339	-1.088	0.2876	
	MP x High Salinity	-2.2907	1.0122	24.7715	-2.263	0.0326	*
Adults weight (Males)	(Intercept)	0.47742	0.01550	22.38259	30.794	< 2e-16	*
	Microplastic (MP)	-0.00256	0.02116	20.37496	-0.121	0.90490	
	Medium Salinity	-0.07226	0.02317	21.79752	-3.118	0.00505	*
	High Salinity	-0.07187	0.02317	27.97127	-3.102	0.00436	*
	MP x Medium Salinity	-0.02516	0.03113	20.83501	-0.808	0.42807	
	MP x High Salinity	-0.03411	0.03150	24.67651	-1.083	0.28932	
Adults weight (Females)	(Intercept)	0.87838	0.04287	15.21518	20.488	1.71e-12	
	Microplastic (MP)	-0.05027	0.06892	20.51111	-0.729	0.4740	
	Medium Salinity	-0.12472	0.06032	14.70873	-2.068	0.0567	.
	High Salinity	-0.23195	0.06576	16.92065	-3.527	0.0026	*
	MP x Medium Salinity	0.02524	0.09728	20.25325	0.259	0.7979	
	MP x High Salinity	0.12761	0.09733	20.51575	1.311	0.2043	

Table S4. Estimates of the linear mixed model analysing the effect of food shortage (food level: 0.5, 0.25 and 0.125 mg TetraMin larva^{-1} day^{-1}) in the presence and absence of polyethylene microplastics (PE-MPs) on several life-history traits: larval length, day of emergence (males and females) and adults' weight (males and females).

		Estimate	Std. Error	df	t value	Pr(>|t|)	
Larval length	(Intercept) Food level 0.5	7.760417	0.192567	139.000000	55.380	< 2e-16	*
	Microplastic (MP)	-0.953125	0.272331	139.000000	-3.436	0.000778	*
	Food level 0.125	1.594583	0.269594	139.000000	-11.383	< 2e-16	*
	Food level 0.25	3.133396	0.275275	139.000000	-5.646	8.92e-08	*
	MP x Food level 0.125	0.073125	0.381263	139.000000	-0.019	0.985132	
	MP x Food level 0.25	0.007229	0.387222	139.000000	0.172	0.863778	
Day of emergence - Males	(Intercept) Food level 0.5	25.9000	0.6915	52.0000	26.404	< 2e-16	*
	Microplastic (MP)	3.7000	1.1976	52.0000	1.729	0.08982	
	Food level 0.125	-6.6500	0.9362	52.0000	9.862	1.67e-13	*
	Food level 0.25	-9.2333	0.9362	52.0000	2.894	0.00555	*
	MP x Food level 0.125	-2.7500	1.5202	52.0000	1.322	0.19180	.
	MP x Food level 0.25	-2.0333	1.5375	52.0000	-0.533	0.59613	
Day of emergence - Females	(Intercept) Food level 0.5	32.1667	0.8156	75.0000	24.836	< 2e-16	*
	Microplastic (MP)	3.0098	1.0653	75.0000	1.458	0.148931	
	Food level 0.125	-8.5667	1.2097	75.0000	11.233	< 2e-16	*
	Food level 0.25	-12.7051	1.1310	75.0000	3.482	0.000833	*
	MP x Food level 0.125	-1.5329	1.5960	75.0000	0.981	0.329555	
	MP x Foodlevel 0.25	-1.4713	1.4992	75.0000	-0.039	0.969212	
Adults weight (Males)	(Intercept) Food level 0.5	0.50417	0.02493	52.00000	20.224	< 2e-16	*
	Microplastic (MP)	-0.04728	0.03808	52.00000	-1.242	0.220	
	Food level 0.125	-0.01492	0.03526	52.00000	-0.423	0.674	
	Food level 0.25	-0.23087	0.03698	52.00000	-6.244	7.92e-08	*
	MP x Food level 0.125	0.02693	0.05308	52.00000	0.507	0.614	
	MP x Food level 0.25	0.06998	0.06072	52.00000	1.152	0.254	
Adults weight (Females)	(Intercept) Food level 0.5	0.876793	0.036233	12.639342	24.199	5.76e-12	*
	Microplastic (MP)	-0.015987	0.049760	13.982315	-0.321	0.753	
	Food level 0.125	-0.005783	0.054152	14.696665	-0.107	0.916	
	Food level 0.25	-0.314193	0.051379	15.085247	-6.115	1.93e-05	*
	MP x Food level 0.125	-0.097525	0.073413	14.872689	-1.328	0.204	
	MP x Food level 0.25	-0.019281	0.069763	14.009896	-0.276	0.786	

Table S5. Significance of the linear mixed model analysing the effect of exposure to PE-MPs and different temperatures on life-cycle endpoints: Day of Emergence (males) and adults' weight (males). (*) indicates the significative effect of MP; temperature or significant interaction between MP and temperature on an endpoint (p< 0.05).

Endpoint	Fixed effect	F value	Pr(>F)	Sig.
Day of emergence (males)	Microplastic (MP)	1.7533	0.1992	
	Temperature	280.0295	3.221e-16	*
	MP x Temperature	0.9186	0.4140	
Adults weight (males)	Microplastic (MP)	0.0182	0.8941	
	Temperature	26.3163	1.953e-06	*
	MP x Temperature	1.0484	0.3682	

Table S6. Significance of the linear mixed model analysing the effect of exposure to PE-MPs and salinity on life-cycle endpoints: Day of Emergence (males) and adults' weight (males). (*) indicates the significative effect of MP; salinity or significant interaction between MP and salinity on an endpoint (p< 0.05).

Endpoint	Fixed effect	F value	Pr(>F)	Sig.
Day of emergence (males)	Microplastic (MP)	0.2421	0.6278	
	Salinity	1.6947	0.2082	
	MP x Salinity	0.6134	0.5511	
Adults weight (males)	Microplastic (MP)	2.9616	0.09845	.
	Salinity	21.1474	5.833e-06	*
	MP x Salinity	0.6485	0.53201	

Table S7. Significance of the linear mixed model analysing the effect of exposure to PE-MPs and food shortage on life-cycle endpoints: Day of Emergence (males) and adults' weight (males). (*) indicates the significative effect of MP; food level or significant interaction between MP and food level on an endpoint (p< 0.05).

Endpoint	Fixed effect	F value	Pr(>F)	Sig.
Day of emergence (males)	Microplastic (MP)	12.3129	0.0009375	*
	Food level	93.4137	< 2.2e-16	*
	MP x Food level	1.6724	0.1977285	
Adults weight (males)	Microplastic (MP)	0.3993	0.5302	
	Food level	25.8946	1.571e-08	*
	MP x Food level	0.6641	0.5191	

Chapter 7

Microplastics in freshwater sediments: effects on benthic invertebrate communities and ecosystem functioning assessed in artificial streams

Microplastics in freshwater sediments: effects on benthic invertebrate communities and ecosystem functioning assessed in artificial streams

Abstract

The high levels of microplastics (MPs) found in freshwaters, particularly in riverine sediments, may impose a threat to the macroinvertebrate communities with possible consequences at ecosystem-level. The present study aimed to assess the effects of a pool of polyethylene microplastics (PE-MPs) on the composition and structure of macroinvertebrate communities and key-functions, such as primary production and leaf litter decomposition. Microplastics were mixed in the sediment at three different concentrations (0.1; 1; 10 g kg^{-1}) already found in freshwater sediments to enhance the relevance of the work. After eight days of exposure to PE-MPs, the observed changes in macroinvertebrate community structure were mostly due to the reduction in the abundance of deposit-feeders and grazers, which concomitantly presented higher MP ingestion. The alterations in the community structure did not translate into marked impairments in the functional endpoints analysed. Leaf litter decomposition and primary production showed a tendency for reduction, indicating potential sub-lethal effects on detritivores and also possible direct impact of PE-MPs on periphyton.

This study represents the first assessment of microplastic effects on freshwater macroinvertebrate community structure and associated ecosystem-level functional measures, raising knowledge on potential ecological consequences of MPs to natural benthic macroinvertebrate communities.

Keywords: Mesocosms; Leaf litter decomposition; Primary production; Polyethylene; Plastic pollution

1. Introduction

Freshwaters are recognized sinks of microplastics (MPs, < 5mm in size) (Hurley et al. 2018), in both highly urbanised (Castañeda et al., 2014; Klein et al., 2015; Zhang et al., 2015; Hurley et al., 2018; Tibbetts et al., 2018; Scherer et al., 2020) and remote (less-urbanised) areas (Horton et al., 2017a, 2017b; Li et al., 2019). Although most MPs possess low density (i.e., high buoyancy), its majority eventually end up sinking due to weathering, biofouling and hetero-aggregation processes (Corcoran, 2015; Chubarenko et al., 2016). For this reasons, riverine sediments frequently present higher MP levels than water column (e.g., up to 600,000-fold in European riverbanks) (Scherer et al., 2020). The levels of MPs found in river sediments worldwide are estimated in the order of thousands of particles per kg of sediment (or grams per kg of sediment) (Klein et al., 2015; Hurley et al., 2018; Wang et al., 2018). In addition, most of MPs found in riverine sediments are small-sized particles (<300 μm) with low density (e.g., polyethylene) (Wang et al., 2018; Scherer et al., 2020).

Organisms depending on freshwater sediments can therefore be highly susceptible to get in close contact with MPs. Among these organisms, benthic invertebrates are particularly vulnerable since many of them ingest fine particulate matter that besides being similar in size can also be surrounded by biofilm as MPs. Therefore, it is not surprising the ingestion of MPs that have been observed in the field (Bour et al., 2018; Windsor et al., 2019), and under MPs exposure in laboratory tests with deleterious effects at sub-organismal and organismal levels (Besseling et al., 2013; Imhof and Laforsch, 2016; Hurley et al., 2017; Scherer et al., 2017; Ziajahromi et al., 2017; Al-jaibachi and Callaghan, 2018; Redondo-Hasselerharm et al., 2018; Silva et al., 2019, 2021b). Traditional ecotoxicology assays (i.e., single-species) have proven that similar exposure conditions (i.e. same polymer type and MP concentration) can affect different invertebrate species at different magnitudes (Redondo-Hasselerharm et al., 2018), indicating feeding strategy and habitat as key-traits that modulate species susceptibility to MP ingestion and their potential toxic effects (Hurley et al., 2017; Scherer et al., 2017; Hu et al., 2018; Windsor et al., 2019). This diverse sensitivity to MPs exposure allied to differences in MPs ingestion can lead to changes in the macroinvertebrate community structure (Blijswijk et al., 2004; Redondo-Hasselerharm et al., 2020) and, consequently, to alterations in the ecosystem functioning (López-Rojo et al., 2020). Up to now, only one study addressed the potential effects of MP exposure at ecosystem-level (Redondo-Hasselerharm et al., 2020). However, the approach used only allowed to evaluate the effects on the invertebrate community structure, lacking important information on ecosystem functions. A joint evaluation of community structure and functional ecosystems level endpoints can provide a more accurate assessment of the implications of MP pollution on fundamental ecological processes and consequently on the ecosystem health (Young et al., 2008; Dalu et al., 2017). Functional parameters such as primary production and leaf decomposition are representative endpoints of primary energy sources in freshwater ecosystems and proven sensitive and trustworthy indicators of ecosystem health (Peters et al., 2013; Evans et al., 2014; Abelho et al., 2016; Campos et al., 2020). The interlink between producers and consumers is evident since effects on specific

macroinvertebrates groups such as detritivores and grazers can frequently lead to alterations in the leaf decomposition and primary production (Dossena et al., 2012; Peters et al., 2013; Gessner and Tlili, 2016; Hasenbein et al., 2017). Alternatively, direct effects on producers might impact specific feeding-type macroinvertebrate groups altering the macroinvertebrate community (Fleeger et al., 2003). In this way, effects on important ecosystem functions like primary production or leaf decomposition can also affect the energy transfer efficiency across trophic levels (Palmer et al., 2000). Anthropogenic perturbations that alter macroinvertebrate community structure and ecosystem functions can alter energy fluxes well beyond ecosystem boundaries and potentially alter the carbon balance between aquatic and terrestrial ecosystems (Greig et al., 2012).

The present work investigated the effects of polyethylene microplastics (PE-MPs) on freshwater ecosystems. A pool of different size polyethylene microplastics was used since this polymer is one the most found in freshwater sediments (Klein et al., 2015; Rodrigues et al., 2018; Scherer et al., 2020), trying to mimic the MPs' size distribution encountered in the field (Adam et al., 2019) and the concentrations tested include environmentally relevant (0.1 g Kg-1; McCormick et al., 2014; Klein et al., 2015); concentrations frequently found in North-America and European rivers (1 g Kg-1; Castañeda et al., 2014; Klein et al., 2015) and a concentration that is found in contamination hot spots areas – 10 g Kg-1 Hurley et al., 2018; Wang et al., 2018b). Moreover, the production of microplastics is expected to grow, so the concentrations found in the environment and thus in riverine sediments are expected to increase (Geyer et al., 2017). Endpoints like macroinvertebrate community structure; total macroinvertebrates abundance; abundance of different feeding groups (deposit-feeders, filter-feeders, grazers, shredders, and predators); and ecosystem functional parameters: leaf decomposition (measured through leaf mass loss), and primary production (measured through chlorophyll a content) were assessed. Additionally, ingestion of MPs was estimated in organisms of the main represented feeding groups: deposit-feeders, grazers, shredders, and predators to establish connections between MP ingestion and lethal and sub-lethal toxicity that can lead density and trait-mediated effects on ecosystem important functions.

2. Materials and methods

2.1 Tested polymer and concentrations

Irregularly shaped polyethylene microplastics (PE-MPs) were used in the present experiment following earlier studies that investigated effects of MPs in suborganismal endpoints and life-history traits of two invertebrate species (Silva et al., 2019, 2021a, 2021b). A pool of different sized PE-MPs was created by mixing different sized PE-MPs (PE average size 40-48 µm, CAS No. 9002-88-4, Sigma-Aldrich UK; PE 125 µm average size, CAS Number 9002-88-4, Sigma-Aldrich UK; and PE average size 350 µm, CAS 708-316-83, Goodfellow) and used to contaminate sediments. The MP particle size distribution was determined by vibratory sieve shaking (mesh pore-sizes: 500, 250, 125, 63 and 32 µm) and can be verified on Table S1. MP size distribution used in this study

can be considered environmentally relevant, being most MP particles below 250 µm (Klein et al., 2015; Hurley et al., 2018; Wang et al., 2018; Scherer et al., 2020).

The concentrations tested include environmentally relevant (0.1 g Kg^{-1}; McCormick et al., 2014; Klein et al., 2015); concentrations frequently found in North American and European rivers (1 g Kg^{-1}; Castañeda et al., 2014; Klein et al., 2015) and a concentration that is found in contamination hot spots areas – 10 g Kg^{-1} Hurley et al., 2018; Wang et al., 2018b).

2.2 Experimental design (Macroinvertebrates collection, mesocosms treatments and setup)

The macroinvertebrate community used in the experiment was sampled in Troço river, a Vouga river tributary, near the river mouth at São Pedro do Sul, Portugal (40°45'19.0"N, 8°03'35.2"W). One week before starting the experiment, samples of the macroinvertebrate community in Troço river were obtained using a Surber sampler to estimate the exact composition and density to be used as a reference in the indoor artificial stream mesocosm.

The experiment took place in indoor artificial streams (mesocosms system), under a controlled temperature of 15 ± 1 °C and a photoperiod of 16h light: 8h dark reproducing field conditions (Rodrigues et al., 2018; Campos et al., 2020). A total of twelve artificial streams were used (2 m length, 0.200 m width, and 0.225 m depth), with three replicates (3 artificial streams) per treatment (control plus three PE-MPs concentrations - 0.1; 1; and 10 g Kg^{-1}).

The PE-MPs were mixed to the sediment consisting in 1% of autoclaved peat and 99% or mineral sediment, previously burnt at 500ºC for 3 h (three replicates – streams were prepared per condition). The mixture of MPs with the sediment was performed individually for each individual stream (replicate). After MPs contamination this artificial sediment was placed in the indoor artificial streams and distributed uniformly along the artificial stream.

The medium used in the streams was artificial pond water (APW) (Naylor et al., 1989), enriched with Dipotassium hidrogenophosphate (K_2HPO_4; 0.008g/L), Sodium nitrate ($NaNO_3$; 0.085g/L) and Sodium metasilicate nonahydrate ($NaSiO_3.9H_2O$; 0.028g/L). Besides approximately 300 L of enriched APW, each artificial stream contained 7 kg of sediment previously prepared, five leaf packs (10 mm mesh size) to assess leaf litter decomposition derived from macroinvertebrates feeding, three leaf packs (250 µm mesh size) for the determination of leaf litter decomposition derived from microbial decomposition and five unglazed ceramic tiles (20 cm^2) for periphyton primary production estimation. In each stream, a constant flow rate of approximately 4 L/min was operated to recirculate water. Each 10 mm mesh size leaf pack contained around 1.0 g of alder (*Alnus glutinosa)* leaves while 250 µm mesh size leaf packs contained an average of 0.25 g; all leaf packs were previously conditioned for two weeks in aerated river water collected in Troço river before being placed in the artificial streams (at day 0). The alder leaves were previously collected from the riparian vegetation at São Pedro de Alva, Portugal (40°16'38.8"N, 8°11'52.8"W) during autumn, air-dried and stored in the darkness until being used. Ceramic tiles for posterior periphyton

sampling were conditioned for the same 2-week period in enriched APW at an artificial stream, constantly aerated after inoculation with natural biofilm collected at Troço river. The inoculate was obtained after scraping stones from the Troço River using toothbrushes and washed with water from the site to obtain a concentrated biofilm solution. At the experiment kick-off, macroinvertebrates were collected by kick-sampling from riffle habitats in the Troço river and transported to the laboratory in river water 20 L containers. Once in the artificial stream mesocosm facility, the organisms were sorted by taxa and equally distributed to each artificial stream. In total, 228 benthic organisms representing a total of 21 taxa were placed in each artificial stream according to their natural densities, being the majority, in terms of abundance, grazers (38.60%), followed by deposit-feeders (32.46%); shredders (23.25%), filter-feeders (3.95%) and lastly predators (1.75%) (table S6). The classification in deposit-feeders, filter-feeders, grazers, shredders, and predators was based on macroinvertebrates feeding habits (Charvet et al., 2000; Usseglio-Polatera et al., 2000), which is a key factor for the ingestion of MPs (Scherer et al., 2017; Redondo-Hasselerharm et al., 2018). Flow rate and physicochemical water parameters (pH, temperature, conductivity, and dissolved oxygen) were measured every three days.

Before the start of the experiment, two different samples (per stream) were collected in two different spots of the stream for MPs extraction and quantification.

2.3 Macroinvertebrate community responses

2.3.1 Macroinvertebrate community structure

At the end of the experiment (8 days), all the macroinvertebrates were carefully collected, removed from the leaf packs, the ceramic tiles and the sediment was sieved to ensure that all the remaining living macroinvertebrates were collected. All the organisms present in the leaf packs, ceramic tiles, water column and sediment of each artificial stream constituted a macroinvertebrate sample which was preserved in 70% ethanol for counting and identification under a stereomicroscope (MS5, Leica Microsystems, Houston, USA). For the macroinvertebrate community structure, the organisms were identified, counted and data were used to estimate different endpoints: macroinvertebrates abundance; and the abundance of invertebrates with different feeding habits: deposit-feeders; grazers; shredders; and predators

2.3.2 Ecosystem functioning (leaf litter decomposition and primary production)

Leaf packs were carefully removed from the artificial streams and carefully cleaned with soft paintbrushes to remove attached organisms, microplastics and sediment particles. Leaves were then left to dry for three days at 50 °C and weighed. Additional leaf packs were used and removed after conditioning (at the beginning of the experiment) and left to dry as above-mentioned. These were used as correction factors to correct for weight loss due to leaching. Analysis of leaf

decomposition (both mediated by microorganisms and by macroinvertebrates) can be found in Table S4. For the determination of leaf mass loss in the experiment, the leaf mass loss that occurred during the 2 weeks conditioning process was deducted from the mass loss verified in the experiment.

For the determination of primary production, ceramic tiles were collected from each artificial stream and scrubbed using a soft brush, before being rinsed in water. The same procedure was applied to additional tiles collected at the beginning of the experiment (to account for the primary production at the experiment kick-off). The obtained samples were filtered with GF/C filters (1.2 µm) and stored in the darkness at -20 °C until further analyses. To estimate primary production, the chlorophyll *a* content retained in the filters was extracted using 90 % acetone and measured spectrophotometrically (Jeffrey and Humphrey, 1975).

2.4 Extraction and quantification of PE-MPs in the sediments and ingested by macroinvertebrates

Before adding the invertebrate communities to artificial streams, sediment samples were collected from each stream (in duplicate) in different spots in each artificial stream and used for estimation of initial MPs concentration. Likewise, at the end of the experiment, sediment samples were collected in each stream (in triplicate) and used for estimation of MP concentration. In these samples, sediments were searched after collection, and possible organisms present in sediment samples were collected in 70% ethanol for identification and incorporation in macroinvertebrate community structure assessment. Sediment samples dried completely for three days at 65 ºC.

Total organic matter (TOM) content was then analysed in these sediment samples using loss on ignition (3 hours, 600°C) to determine the plastic content through thermal degradation. Nominal MP concentrations were verified by subtracting the %TOM obtained in controls from those in the treatment artificial stream, nominal plastic concentrations could be verified in the mixtures as the thermal degradation of polyethylene occurs below 600°C (Ranzi et al., 1997) and following similar procedures performed by Redondo-Hasselerharm (Redondo-Hasselerharm et al., 2020).

For the estimation of MPs inside macroinvertebrates, organisms representative of different feeding guilds were chosen for the determination of ingestion of PE-MPs. *Chironomus* sp.*e* was chosen as representative of the deposit-feeders; *Ephemerella* sp., *Potamopyrgus antipodarum* and *Baeti*s sp. as grazers; *Sericostoma vittatum* and *Allogamus ligonifer* as shredders and lastly *Onychogomphus uncatus* as representative of predators for this determination of PE-MPs ingestion.

One organism of each one of these groups was picked in each stream for the determination of PE-MPs' ingestion. Filter-feeders were not analysed for the ingestion of MPs due to their reduced numbers at the end of the experiment and to their feeding habits having into account that the contamination was performed in the sediment and low number of MPs were resuspended to the water column.

The extraction and quantification of PE-MPs in these organisms followed previously optimised methodology for benthic invertebrates (Silva et al., 2019). Briefly, organisms previously preserved in 70% ethanol were moved to empty glass flasks and covered with aluminium foil to be left drying at room temperature for more than 12 hours (overnight). Samples were then incubated in nitric acid (HNO_3) 65% at 60 °C for 3 h. Hydrogen peroxide (H_2O_2) at 35% was added to each sample (1:1 v/v) and incubated for more 24 h at room temperature. Samples were then diluted 1:10 using Milli-Q water and vacuum-filtered. Gridded cellulose ester filters (Whatman 10406972, Mixed Cellulose Ester Filter, 3.1 mm white/black grid, 0.45 µm pore size) were used to retain the microplastics. Filtration apparatus was flushed with Milli-Q water after the filtration of each sample to minimise the possibility of MPs getting retained in the filtration apparatus. After, each membrane was transferred to a glass petri-dish and left in the oven to dry at 25 °C for 2–3 days. After that, the number of PE-MPs ingested by each invertebrate was counted under a stereomicroscope (stereoscopic zoom microscope—SMZ 1500, Nikon Corporation).

Samples of PE-MPs directly from the package (pristine) were analysed by Fourier Transform Infrared Spectroscopy-Attenuated Total Reflectance (FTIR-ATR) to verify the polymer nature and purity. Additional samples of pristine PE-MPs were subjected to the digestion procedure (HNO_3 + H_2O_2; see above) to be characterised by FTIR-ATR and to analyse possible polymer degradation by the digestion and extraction procedure. Likewise, PE-MPs found inside the macroinvertebrates were also characterised by FTIR-ATR. The resulting spectra can be found in the supplemental material (Fig. S1).

2.5 Statistical Analysis

Effects of PE-MPs exposure on primary production (chlorophyll a content) and leaf decomposition were analysed trough LMM (Linear Mixed Model) using MP concentrations as fixed effect factor and stream/replicate as a random factor, to take into account the lack of independence of leaf packs and tiles placed in the same stream. Macroinvertebrates community (i.e., the total macroinvertebrates abundance), family richness (i.e., number of different taxa) and number of macroinvertebrates belonging to the most represented functional feeding groups (deposit-feeders; grazers; shredders; and predators) were evaluated using GLM (Generalised Linear Models) with Poison Distribution, which is more appropriate for count data, considering MPs concentration as a factor. Differences on the global structure of the benthic macroinvertebrate communities between different MP treatments were assessed by using permutational multivariate analysis of variance, PERMANOVA (Anderson, 2001), performed based on a Bray-Curtis dissimilarity matrix, and its significance was tested using Monte Carlo permutation method with 999 permutations. A similarity percentage analysis (SIMPER) was used to determine the relative contribution of each individual taxa to the Bray-Curtis dissimilarity observed between treatments.

All data were analysed using R software 4.0.0 (R Core Team, 2020), including lme4 package (Bates et al., 2015) for LMM and vegan package (Oksanen et al., 2019) for PERMANOVA and SIMPER.

3. Results and discussion

The present study used a mesocosms approach (indoor artificial stream) to assess the effects of microplastics (polyethylene microplastics – PE-MPs) on freshwater benthic invertebrate community and lotic ecosystem functioning. Eight days of exposure to PE-MPs altered the macroinvertebrate community, by affecting macroinvertebrates' abundance (namely deposit-feeders and grazers). Despite these effects in the macroinvertebrates' community structure, only a tendency for reductions in the leaf decomposition and primary production was observed in this short exposure period. The effects on the macroinvertebrate community structure, mainly noticed by a decrease in the abundance of grazers and deposit-feeders, is an indication of possible adverse ecological effects of MPs in freshwaters. Moreover, the limited effects in terms of leaf litter decomposition were likely linked to the absence of lethal effects on shredders. The decrease in primary production by periphyton can be related to and possible direct negative effects of MPs on periphyton growth.

The estimated levels of MPs in the sediments at the beginning of the experiment is in good agreement with nominal concentrations for the three used concentrations (0.1; 1 and 10 g Kg^{-1}; Fig. 1A) demonstrating the homogeneity and accuracy of the preparation of the plastic-sediment mixtures added to the artificial streams. The MP levels present at the end of the experiment show that the major fraction of the PE-MPs remained in the sediment. Less than 15% of the MPs were resuspended to the water column (via invertebrates' bioturbation of the sediment), resuspension due to water flux, or ingested by organisms (Fig.1B).

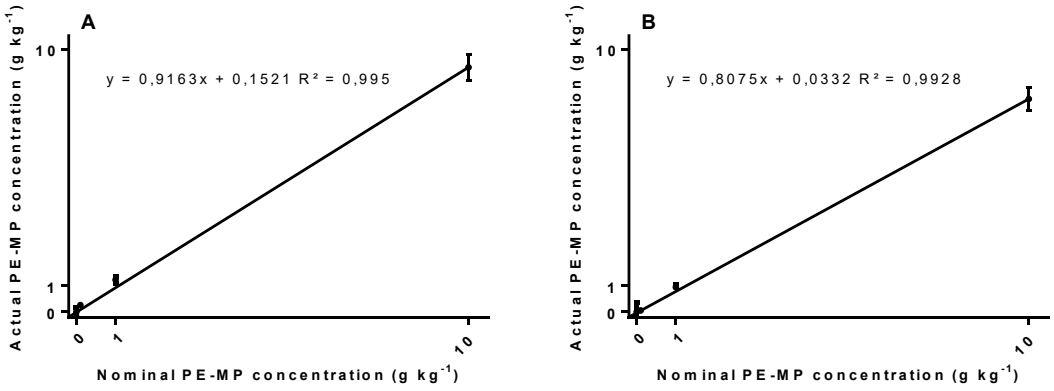

Fig. 1. Measured (actual) *vs* Nominal PE-MP concentrations. PE-MP concentrations measured in the plastic-sediment mixtures at time zero (A) and in streams after 8 days (B) after subtracting the %TOM in controls from the measured %TOM in the stream as a function of the nominal MP concentration (as g kg^{-1} sediment dry weight). For the starting concentration, average ± SD (n = 6) was based on two samples per stream taken from the initial concentrations prepared. Values after the 8 days exposure (B) the average ± SD (n = 9), which correspond to three samplings per stream.

The exposure to PE-MPs, affected the macroinvertebrates community structure (PERMANOVA; Pseudo-$F_{3,8}$ = 2.5394; p = 0.021; Table S3). The SIMPER analysis (Table S7) indicates that the abundances of deposit-feeders and grazers feeding groups were the most affected by MPs exposure, and taxa such as *Chironomus* sp. (deposit-feeder), *Baetis* sp. and *Ephemerella* sp. (grazers) contribute with around 60 – 70% of the community dissimilarity observed between treatments (Table S7). Our results partially agree with the alteration in macroinvertebrate community noticed after long-term (15 months) exposure to MPs, in which the abundance of the deposit-feeders Naididae (oligochaeta) was significantly decreased. Our results also show that the exposure to PE-MPs reduced the total abundance of macroinvertebrates exposed to 1 g PE-MPs kg^{-1} sediment (p=0.0202) and 10 g kg^{-1} (p=0.0005; Fig. 2 and Table S2). Also, a tendency for reduction in macroinvertebrate abundance was found at the lowest concentration: 0.1 g kg^{-1} (p=0.0565; Fig. 2 and Table S2). The abundance of deposit-feeders was reduced in all concentrations tested: 0.1 (p<0.0001), 1 (p=0.0076), and 10 g kg^{-1} (p<0.0001; Fig. 3A and Table S2); and the grazers abundance was reduced when exposed to 1 g kg^{-1} (p=0.0458; Fig. 3B and Table S2) and slightly reduced when exposed to 10 g kg^{-1} (p=0.0813; Fig. 3B and Table S2). However, in the lowest concentration tested (0.1 g kg^{-1}), an opposite tendency was observed as grazers abundance presented a tendency to increase (p=0.0819; Fig 3B and Table S2) when compared to control. The abundance of other feeding functional groups such as shredders (Fig. 3C and Table S2) and predators (Fig. 3D and Table S2) was not affected by the exposure to PE-MPs.

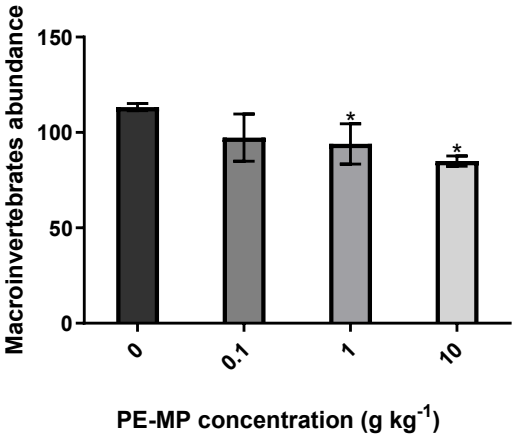

Fig. 2. Macroinvertebrates abundance after 8-day exposure to PE-MPs in an artificial stream system. All values are presented as mean ± SEM. * denotes significant differences comparing with control treatments (0); p < 0.05.

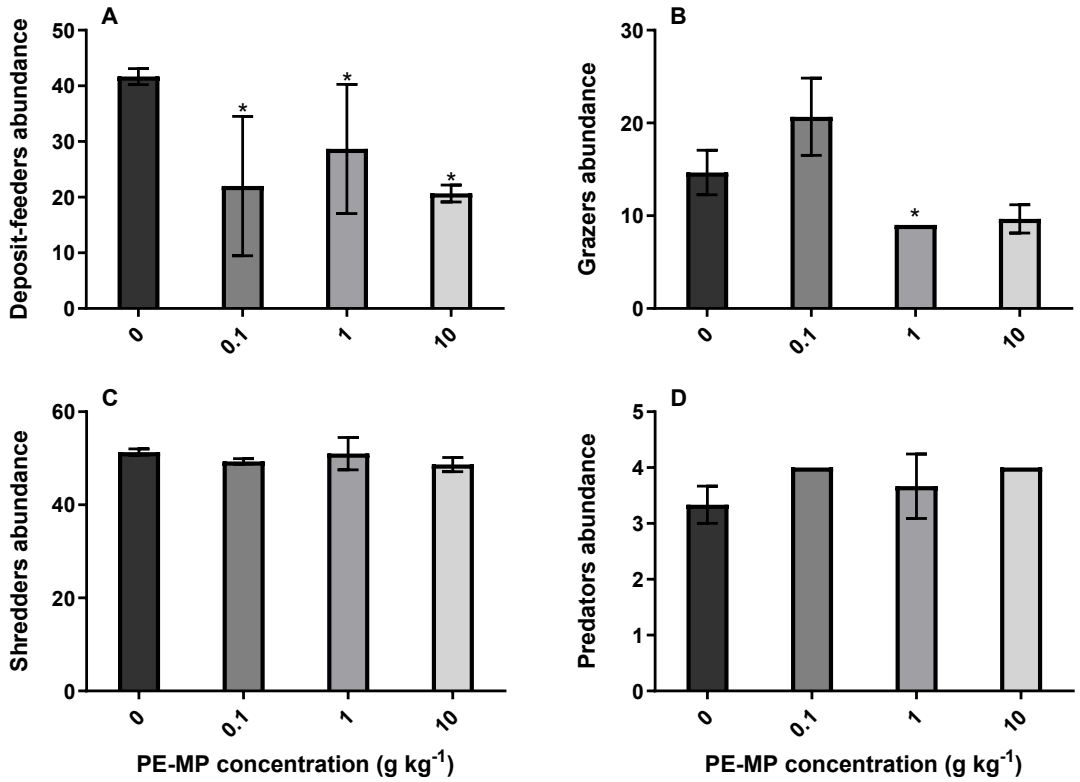

Fig. 3. Effects of PE-MP exposure on A) Deposit-feeders abundance (total number); B) grazers abundance (total number); C) shredders abundance (total number); and D) predators abundance (total number) upon an 8-day exposure in an artificial stream system. All values are presented as mean ± SEM. * denotes significant differences comparing with control treatments (0); $p < 0.05$. The absence of error bars in some conditions is due to null variance.

Alterations in the community structure can be translated into ecosystem-level effects since ecosystem functions like organic matter processing (e.g., leaf litter decomposition) and primary production are biologically driven processes controlled by several microorganisms and invertebrates (Wallace and Webster, 1996; McKie and Malmqvist, 2009; Gessner et al., 2010; Woodward et al., 2012; Abelho et al., 2016). Leaf litter processing, for example, is mainly controlled by shredders which feed on large organic plant substrates such as leaf litter producing smaller organic particles and contribute to nutrient recycling (Cummins et al., 1989). In our study, the leaf litter decomposition mediated by microorganisms (estimated in coarse mesh leaf pack bags) was not affected by the presence of MPs (Table S4). Moreover, only a very slight decrease in the leaf litter processing rates (leaf decomposition mediated by invertebrates and microorganisms) was observed in the MPs treatments (a 10% reduction was observed in the highest MP concentrations tested) (Fig. 4A; Table S4). This result (lack of significant differences) agrees with the results concerning no mortality of shredders observed due to MPs exposure in shredders (Fig. 3C). Although leaf litter decomposition could also be changed through trait-mediated effects such as changes in the feeding rates of detritivores due to MPs exposure and ingestion these results are

also in agreement with other studies where the exposure and even the ingestion of MPs have been reported not to affect shredders' feeding activity in species such as *Gammarus pulex* (Weber et al., 2018) and *Gammarus fossarum* (Blarer and Burkhardt-Holm, 2016). It is important to note that, in our case, the numbers of MPs particles found in shredders species used in artificial streams were very low in comparison with other groups (Fig. 5). *Allogamus* presented up to 43 PE-MPs per organism inside the gut, whilst *Sericostoma* presented up to 45 PE-MPs/organism (MP ingestion was only verified in the highest concentration tested (Fig. 5). These two caddisflies were the most common shredders in our experiment, and their behaviour might have limited the ingestion of MPs. First, these caddisflies larvae were mostly found attached to the leaf packs (feeding on the leaves) on our artificial streams, maybe as avoidance behaviour, that can contribute to reduce their direct contact with the contaminated sediment by lowering the direct contact to MPs as previously suggested for other compounds (Campos et al., 2020). Also, another crucial aspect for the lower MP ingestion, was that the larvae collected for our experiment were large (last larval stages) and these larvae do prefer larger particulate organic matter over fine particles that can also contribute to lower MP ingestion as observed in other shredder species (e.g., *Gammarus pulex*) (Weber et al., 2018). Nevertheless, MP ingestion by different shredder invertebrates has been linked to the presence of MPs adhered to the leaves (Straub et al., 2017; Redondo-Hasselerharm et al., 2018; Weber et al., 2018; López-Rojo et al., 2020). In agreement with our results, i.e., no alteration in shredder abundance in experimental streams, the ingestion of MPs by shredders, both in the presence (Redondo-Hasselerharm et al., 2018) and in the absence of sediment (López-Rojo et al., 2020) was only associated to sub-lethal effects whereas no lethal effects have been recorded (Redondo-Hasselerharm et al., 2018; López-Rojo et al., 2020).

Primary production, on the other hand, is mainly controlled by grazing activity as grazers use periphyton as a primary food source (Feminella and Hawkins, 1995). Changes on periphyton biomass and productivity are then frequently attributed to indirect effects of contaminants through changes on grazers abundance or behaviour (Abelho et al., 2016; Rogers et al., 2016). In the present study, the higher numbers of grazers in the lowest concentration tested (0.1g kg^{-1}) in comparison with the control treatment had a direct correspondence in the noticed reduction in the periphyton productivity, shown in the reduction of the chlorophyll a content (p=0.04220; Fig. 4B and Table S5). For the intermediate and highest concentrations tested (1 and 10 g kg^{-1}), the primary production also showed reductions in comparison with the control treatment (20% and 34% respectively) (Fig. 4B and Table S5). However, for these concentrations, the grazers abundance showed marked reductions (Fig. 3B and Table S2) in comparison with the control treatment thus suggesting that the exposure to MPs might have a possible direct negative effect on periphyton. Supporting this possibility, significantly lower periphyton growth due to MPs was found after exposure (and adherence to colonized tiles) of polystyrene MPs (10µm) for 14 days (comparable to the present study - 8 days period) (Boyero et al., 2020). Also, Vosshage et al. (2018) have shown that plastic substrates alter the quality of periphyton with a possible consequence for grazers (Vosshage et al., 2018) and other research demonstrated that exposure to a pool of MPs within the

same size range of the one used in the present study, caused a reduction in photosynthetic activity and chlorophyll *a* concentrations of the freshwater microalgae *Chlorella pyrenoidosa* and *Microcystis flos-aquae* (Wu et al., 2019). In our case, and despite using contaminated sediment only, we cannot exclude the possibility that some MPs end up adhered to leaves and tiles colonized by periphyton and could thus affect primary production. Additionally, the relatively short duration of the experiment could have been insufficient for the subsequently expected increment in primary production due to density mediated effects on grazers.

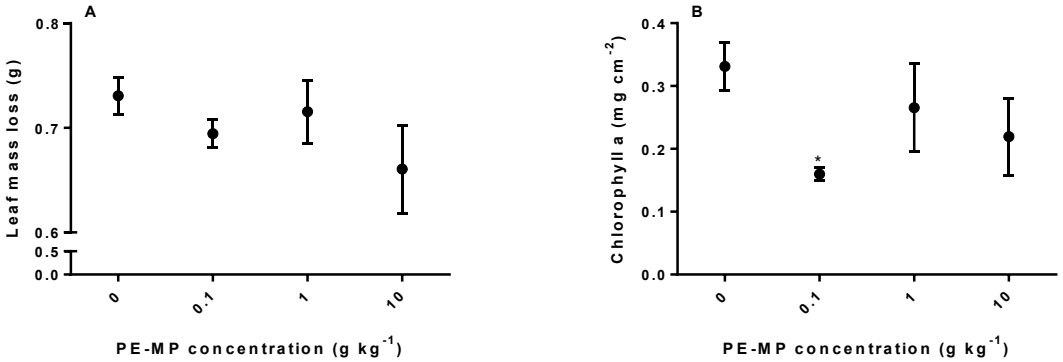

Fig. 4. Effects of PE-MP exposure on A) leaf mass loss (g); and B) chlorophyll a concentration (mg cm^{-2}) upon an 8-day exposure in indoor artificial stream system. All values are presented as mean ± SEM. * denotes significant differences comparing with control treatments (0); $p < 0.05$.

The observed reduction in the abundance of two specific feeding groups (deposit-feeders and grazers) is likely linked to higher rates MP ingestion and associated toxicity, which is mostly dependent on invertebrates feeding habits (Scherer et al., 2017). In fact, our results show that the number of ingested MPs varies considerably amongst feeding groups. The organisms presenting a higher number of MPs in the gut among all sampled organisms were the deposit-feeder *Chironomidae* (up to 143 MPs/organism at 10g kg^{-1}) and the grazers *Baetis* (up to 208 PE-MPs/organism), and *Ephemerella* (up to 523 PE-MPs/organism). Interestingly, the abundance of these two feeding groups: deposit-feeders (all concentrations) and grazers (concentrations higher than 1 g kg^{-1}) was reduced after exposure to PE-MPs (Fig. 3 – abundance; Fig. 5 - ingestion).

These results reflect the non-selective feeding strategy of most deposit-feeders, which involves the ingestion of fine particulate matter and has been pointed as a key-factor for ingestion of MPs and therefore for the generally higher toxicity observed in these organisms (Hurley et al., 2017; Scherer et al., 2017; Ziajahromi et al., 2018; Silva et al., 2019, 2021a). The non-selective ingestion of MPs by deposit-feeders like *Chironomus* spp. has led them to be proposed as indicators of local microplastic sediment pollutant levels (Nel et al., 2018). The ingestion of MPs and associated higher mortality observed for Chironomidae in our experimental treatments may have partially contributed for the slight reductions in leaf processing rates since chironomids can

also feed on coarse organic matter and leaves in particular (Callisto et al., 2007; Campos et al., 2014; Bordalo et al., 2018).

Along with deposit-feeders, grazers presented high MP ingestion and despite these organisms usually do not actively feed on the sediments, they can still ingest MPs on the sediment surface and adhered to periphyton and leaf litter (both present in our artificial streams). The higher MP ingestion by grazers corroborates the reduction in the abundance in the intermediate and highest PE-MP concentration. Likewise, significant MP ingestion was observed in snail grazers like *Physella acuta* (Scherer et al., 2017), *Radix balthica* (Vosshage et al., 2018) and *Potamopyrgus antipodarum* (Imhof and Laforsch, 2016) in laboratory conditions. Curiously, we did not find evidence of high ingestion and accumulation of MPs in the snail *Potamopyrgus* in our experiment (up to 25 PE-MPs/organism) which might be related with its behaviour in our artificial streams since they were found often attached to the glass walls of the artificial streams and not in close contact with the sediment or periphyton tiles.

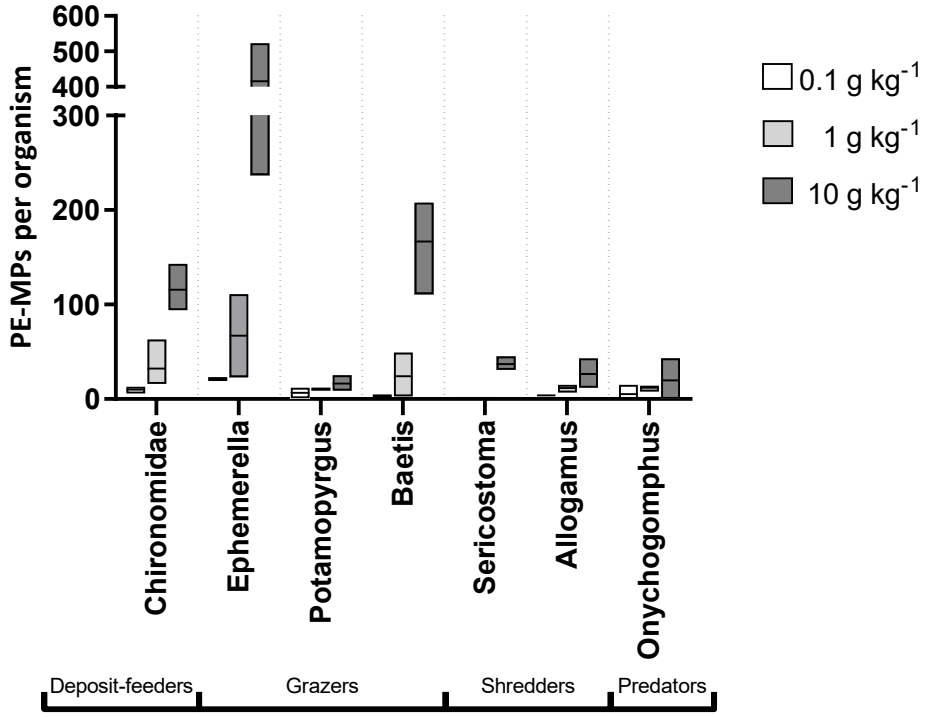

Fig. 5. Number of polyethylene microplastics (PE-MPs) present in the gut of the different organisms (PE-MPs per organism) from the macroinvertebrate community tested (PE-MPs per organism) after 8 days exposed to a pool of different-sized PE-MPs. One organism per stream (3 streams in each PE-MP concentration) representative of different feeding guilds (deposit-feeders, grazers, shredders and predators. Values (boxes) represent the minimum, median and maximum values of PE-MPs present inside the gut.

Concerning predator species, in our study we evaluated ingestion of MPs on Odonate nymphs (*Onychogomphus* genus) that can prey on a number of different invertebrates. Results showed that some organisms exhibited a low number of PE-MPs inside the gut while others did

not, suggesting that ingestion of MPs (trophic transfer) through predation can occur. This is because predator species like dragonflies behave as ambush predators and not feed on particulate matter but can ingest microplastics while consuming prey (i.e., trophic transfer) or scavenging detrital matter (e.g., faecal pellets, carcasses) (Lusher et al., 2016; Wagner and Lambert, 2018).

Is also plausible that the reduction in the abundance of deposit-feeders, and grazers may be linked to behavioural changes (i.e., reduced ability to escape from predators) triggered after ingestion of MPs, since organisms belonging to these feeding groups presented the higher ingestion of PE-MPs amongst all feeding groups.

4. Conclusions

Our results showed that the presence of microplastics in freshwater sediments can induce significant changes of freshwater benthic macroinvertebrate communities, with deposit-feeders and grazers being the invertebrate feeding groups especially affected. Results also showed a good agreement between numbers of MPs found in the gastrointestinal tracts of organisms and associated mortality, giving support for monitoring of effects based on the internal concentration of microplastics in different invertebrate species. Conversely to what was hypothesised, we did not find significant reductions in leaf decomposition or increased primary production in experimental treatments that were expected to reflect both density- and trait - (feeding behaviour) mediated effects of microplastics on shredders and grazers, respectively. This can be a sign of the short exposure period used in our experiment, but we cannot disregard potential adverse and direct effects of MPs on periphyton growth and quality. Given the ubiquity of plastic pollution in freshwater systems, it is critical to continue using relevant scenarios of exposure to better assess its ecological effects. Mesocosm systems allow for invertebrate communities to be exposed to microplastics as a whole thus integrating biotic stressors (competition, predation, among others), while at the same time allow for the assessment of ecological processes like organic matter decomposition and primary production that are critical for ecosystem functioning. Future research should employ longer exposure periods and different conditions (temperature, salinity, flow, etc.) that, despite challenging given the short-life cycles and possible emergence of many insects, can deliver valuable insights on long-term direct and indirect effects of microplastic pollution on natural communities but also on ecosystem-level functional endpoints including primary production.

In addition, and given the wide variability of effects and sensitivities already being described, the use of different polymer types, different sizes and shapes of plastic particles and also the use of weathered microplastic particles is also needed. Further research should always consider monitoring invertebrate's internal concentrations of these plastic particles so as to evaluate trophic transfer not only within the aquatic systems but also to the terrestrial system. This is even more probable, if considering small microplastics and nanoplastics that an adult, terrestrial insect stages, can accumulate and transfer. Therefore, it is relevant to evaluate impacts across ecosystem boundaries considering aquatic-terrestrial subsidies and the potential transfer of MPs from freshwaters to terrestrial environments.

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Supplementary Material

Table S1. Particle size distribution analysis of the pool of polyethylene (PE) particles (replicates of 100 g) determined by mass quantification after vibratory sieve shaking. The particle size distribution was measured through weighting (g) the microplastics retained in each sieve mesh. Results are presented as mean ± standard deviation (n = 2).

Particle size distribution Ø pore (µm)	Pool used in the experiment (per 100 g)
1000 - 2000 µm	0.41±0.30
500 – 1000 µm	0.78±0.11
250 – 500 µm	18.23±1.41
125 – 250 µm	37.51±9.18
63 – 125 µm	26.01±6.02
32 – 63 µm	16.34±4.65
≤ 32 µm	0.68±0.17

Table S2. Estimates of the general linear mixed model analysing the effect of exposure to PE-MPs on macroinvertebrates community (Total Macroinvertebrates abundance), EPT´s abundance (i.e. total number of Ephemeroptera, Plecoptera and Trichoptera specimens) and number of macroinvertebrates belonging to the most represented functional feeding groups (deposit-feeders; grazers; shredders; and predators) (*) indicates the significative effect of MP on an endpoint ($p < 0.05$). Significant differences compared to control are highlighted in bold.

Endpoint	[PE-MPs] (g kg^{-1})	Estimate	Estimate Std. Error	z value	Pr (>F)	Signif.
Total Macroinvertebrates abundance	0	4.73033	0.05423	87.223	< 2e-16	*
	0.1	-0.15219	0.07979	-1.907	0.056456	.
	1	-0.18704	0.08054	-2.322	**0.020222**	*
	10	-0.28768	0.08284	-3.473	**0.000515**	*
Deposit-feeders abundance	0	3.72970	0.08944	41.699	< 2e-16	*
	0.1	-0.63866	0.15216	-4.197	**2.70e-05**	*
	1	-0.37397	0.14010	-2.669	**0.0076**	*
	10	-0.70118	0.15534	-4.514	**6.36e-06**	*
Grazers abundance	0	2.6856	0.1508	17.814	<2e-16	*
	0.1	0.3429	0.1971	1.740	0.0819	.
	1	-0.4884	0.2445	-1.998	**0.0458**	*
	10	-0.4169	0.2392	-1.743	0.0813	.
Shredders abundance	0	3.938340	0.080582	48.87	<2e-16	*
	0.1	-0.039740	0.115110	-0.345	0.730	
	1	-0.006515	0.114147	-0.057	0.954	
	10	-0.053346	0.115511	-0.462	0.644	
Predators abundance	0	1.20397	0.31623	3.807	0.00014	*
	0.1	0.18232	0.42817	0.426	0.67025	
	1	0.09531	0.43693	0.218	0.82732	
	10	0.18232	0.42817	0.426	0.67025	

Table S3. Results of the analysis on global macroinvertebrates community structure (PERMANOVA)

	Df	SumsOfSqs	MeanSqs	F.Model	R^2	Pr(>F)	
Treatment	3	0.071892	0.0239640	2.5394	0.48778	**0.021**	*
Residuals	8	0.075494	0.0094368		0.51222		
Total	11	0.147386			1.00000		

Table S4. Estimates of the linear mixed model analysing the effect of exposure to PE-MPs on coarse mesh leaf pack bags (mesh size 1 cm) to assess decomposition of leaf material from invertebrate feeding and microbial activity and fine mesh leaf pack bags (size 0.250 mm) to assess microbial decomposition of leaf material (*) indicates the significative effect of MP on an endpoint ($p< 0.05$). Significant differences compared to control are highlighted in bold.

Endpoint	**[PE-MPs] (g kg-1)**	**Estimate**	**Std. Error**	**df**	**z value**	**P value**	**Signif.**
Coarse mesh leaf pack bags (leaf Decomposition derived from invertebrates feeding and microbial activity)	0	0.41965	0.03374	56.000	12.438	<2e-16	*
	0.1	0.01174	0.04772	56.000	0.246	0.807	
	1	-0.01024	0.04772	56.000	-0.215	0.831	
	10	-0.03767	0.04772	56.000	-0.789	0.433	
Fine mesh leaf pack bags (leaf Decomposition derived from microbial decomposition)	0	0.027297	0.007256	32.000	3.762	0.00068	***
	0.1	0.006255	0.010262	32.000	0.610	0.54648	
	1	-0.004988	0.010262	32.000	-0.486	0.63025	
	10	0.005690	0.010262	32.000	0.555	0.58309	

Table S5. Estimates of the linear mixed model analysing the effect of exposure to PE-MPs on chlorophyll a content (*) indicates the significative effect of MP on an endpoint ($p< 0.05$). Significant differences compared to control are highlighted in bold.

Endpoint	**[PE-MPs] (g kg-1)**	**Estimate**	**Std. Error**	**df**	**z value**	**P value**	**Signif.**
Chlorophyll a content	0	0.30338	0.04719	9.18851	6.430	0.00011	*
	0.1	-0.15140	0.06673	9.18851	-2.269	**0.04889**	*
	1	-0.04745	0.06673	9.18851	-0.711	0.49469	
	10	-0.11036	0.06673	9.18851	-1.654	0.13185	

Table S6. Macroinvertebrates community composition: number and relative percentage (%) of organisms at the beginning of experiment per artificial stream and final number of organisms (mean and standard deviation (SD)) per taxa per treatment.

Organism	Feeding group	Initial number	%	Final number of organisms per taxa per treatment							
				0		0.1		1		10	
				Mean	SD	Mean	SD	Mean	SD	Mean	SD
Chironomidae	Deposit-feeder	71	31.14	38.67	2.05	9.84	12.06	26.67	8.73	18.33	2.05
Oligochaeta	Deposit-feeder	3	1.32	3	0.00	2.33	0.47	2	0.82	2.33	0.94

Ephemerella	Grazer	17	7.46	3	0.82	2.67	2.05	1.67	1.25	1.67	0.94
Baetis	Grazer	59	25.88	4.33	3.09	9	2.45	2.67	1.	3	2.65
Habroleptoides	Grazer	3	1.32	1.33	1.25	2	0.00	0.33	0.47	0.67	0.94
Ecdyonurus	Grazer	3	1.32	0.67	0.94	1.67	0.47	0.33	0.47	1	0.82
Potamopyrgus	Grazer	6	2.63	5.33	0.94	5.33	0.47	4	0.82	3.33	0.94
Limnephilus	Shredder	3	1.32	3	0.00	3	0.00	3	0.00	2.67	0.47
Allogamus	Shredder	15	6.58	15	0.00	14.33	0.47	14.67	0.47	15	0.00
Lepidostoma	Shredder	2	0.88	2	0.00	2	0.00	1.33	0.47	1.67	0.47
Sericostoma	Shredder	24	10.53	23.67	0.47	23.33	0.94	23.67	0.47	23.33	0.47
Calamoceras	Shredder	2	0.88	1	0.00	1	0.00	0.67	0.47	1	0.00
Ephemera	Shredder	2	0.88	1.33	0.94	2	0.00	2	0.00	1	0.82
Isoperla	Shredder	3	1.32	1.67	1.25	0.67	0.47	1.33	0.94	2	0.82
Halesus	Shredder	2	0.88	2	0.00	2	0.00	2	0.00	2	0.00
Philopotamus	Filter-feeder	4	1.75	0.33	0.47	0	0.00	0	0.00	0.33	0.47
Hydropsyche	Filter-feeder	5	2.19	2	0.82	1.33	0.47	1.67	0.47	1.67	0.47
Onychogomphus	Predator	1	0.44	1	0.00	1	0.00	1	0.00	1	0.00
Cordulegaster	Predator	1	0.44	1	0.00	1	0.00	1	0.00	1	0.00
Atherix	Predator	1	0.44	1	0.00	1	0.00	1	0.00	1	0.00
Dugesia	Predator	1	0.44	0.33	0.47	1	0.00	0.67	0.47	1	0.00
Total		228		111.7	3.1	96.3	12.3	91.7	11.5	84.7	2.5

Table S7. Results of SIMPER analyses showing the similarities and dissimilarities among treatments in terms of macroinvertebrates relative abundance.

Contrast: Control (A)_MP 0.1 g kg^{-1} (B)						
	Average	Standard deviation	ratio	Average A	Average B	cumsum
Chironomus	0.092505	0.054972	1.6828	38.6667	19.6667	0.5205
Baetis	0.023560	0.019261	1.2232	4.3333	9.0000	0.6530
Ephemerella	0.008904	0.006047	1.4726	3.0000	2.6667	0.7031
Isoperla	0.006802	0.003893	1.7471	1.6667	0.6667	0.7414
Habroleptoides	0.006362	0.002469	2.5769	1.3333	2.0000	0.7772
Ecdyonurus	0.005824	0.004117	1.4147	0.6667	1.6667	0.8100
Ephemera	0.005178	0.004136	1.2520	1.3333	2.0000	0.8391
Hydrosphyche	0.004292	0.003869	1.1092	2.0000	1.3333	0.8632
Potamopyrgus	0.004234	0.002921	1.4497	5.3333	5.3333	0.8871
Sericostoma	0.003736	0.004002	0.9335	23.6667	23.3333	0.9081
Lepidostoma	0.003599	0.003003	1.1987	2.3333	2.0000	0.9283
Oligochaeta	0.003272	0.002456	1.3324	3.0000	2.3333	0.9467
Dugesia	0.003155	0.002374	1.3287	0.3333	1.0000	0.9645
Allogamus	0.003150	0.002375	1.3267	15.0000	14.3333	0.9822
Halesus	0.001604	0.002410	0.6655	3.3333	3.0000	0.9912

Philopotamus	0.001559	0.002342	0.6656	0.3333	0.0000	1.0000
Cordulegaster	0.000000	0.000000	NaN	1.0000	1.0000	1.0000
Onychogomphus	0.000000	0.000000	NaN	1.0000	1.0000	1.0000
Limnephilus	0.000000	0.000000	NaN	3.0000	3.0000	1.0000
Calamoceras	0.000000	0.000000	NaN	1.0000	1.0000	1.0000
Atherix	0.000000	0.000000	NaN	1.0000	1.0000	1.0000
Contrast: Control (A)_MP 1 g.kg^{-1} (B)						
	Average	Standard deviation	ratio	Average A	Average B	cumsum
Chironomus	0.062711	0.042431	1.4780	38.6667	26.6667	0.4375
Baetis	0.016537	0.006745	2.4517	4.3333	2.6667	0.5528
Ephemera	0.008084	0.008776	0.9211	1.3333	3.0000	0.6092
Ephemerella	0.007700	0.006802	1.1321	3.0000	1.6667	0.6629
Potamopyrgus	0.007502	0.004861	1.5433	5.3333	4.0000	0.7153
Isoperla	0.005929	0.005379	1.1023	1.6667	1.3333	0.7566
Habroleptoides	0.005872	0.005807	1.0112	1.3333	0.3333	0.7976
Oligochaeta	0.004952	0.004261	1.1621	3.0000	2.0000	0.8321
Lepidostoma	0.004775	0.003339	1.4300	2.3333	1.3333	0.8654
Ecdyonurus	0.003802	0.004022	0.9454	0.6667	0.3333	0.8920
Hydrosphyche	0.003789	0.003332	1.1373	2.0000	1.6667	0.9184
Dugesia	0.002669	0.002539	1.0515	0.3333	0.6667	0.9370
Halesus	0.002163	0.002568	0.8423	3.3333	3.3333	0.9521
Sericostoma	0.002125	0.002526	0.8412	23.6667	23.6667	0.9669
Calamoceras	0.001640	0.002460	0.6666	1.0000	0.6667	0.9784
Philopotamus	0.001582	0.002376	0.6659	0.3333	0.0000	0.9894
Allogamus	0.001520	0.002280	0.6666	15.0000	14.6667	1.0000
Cordulegaster	0.000000	0.000000	NaN	1.0000	1.0000	1.0000
Onychogomphus	0.000000	0.000000	NaN	1.0000	1.0000	1.0000
Limnephilus	0.000000	0.000000	NaN	3.0000	3.0000	1.0000
Atherix	0.000000	0.000000	NaN	1.0000	1.0000	1.0000
Contrast: Control (A)_MP 10 g kg^{-1} (B)						
	Average	Standard deviation	ratio	Average A	Average B	cumsum
Chironomus	0.102634	0.016361	6.2732	38.6667	18.3333	0.5469
Baetis	0.016800	0.011743	1.4307	4.3333	3.0000	0.6364
Potamopyrgus	0.010014	0.007022	1.4260	5.3333	3.3333	0.6898
Ephemerella	0.007841	0.005143	1.5244	3.0000	1.6667	0.7316
Halesus	0.006779	0.005153	1.3155	3.3333	2.0000	0.7677
Habroleptoides	0.006676	0.005567	1.1994	1.3333	0.6667	0.8033
Isoperla	0.006171	0.004912	1.2564	1.6667	2.0000	0.8361
Ephemera	0.005059	0.004402	1.1492	1.3333	1.0000	0.8631
Ecdyonurus	0.005046	0.004339	1.1629	0.6667	1.0000	0.8900
Hydrosphyche	0.003941	0.003423	1.1515	2.0000	1.6667	0.9110
Oligochaeta	0.003345	0.005018	0.6666	3.0000	2.3333	0.9288
Dugesia	0.003343	0.002508	1.3328	0.3333	1.0000	0.9466

Lepidostoma	0.003315	0.003491	0.9493	2.3333	1.6667	0.9643
Sericostoma	0.002798	0.002655	1.0538	23.6667	23.3333	0.9792
Philopotamus	0.002240	0.002658	0.8428	0.3333	0.3333	0.9911
Limnephilus	0.001664	0.002497	0.6666	3.0000	2.6667	1.0000
Cordulegaster	0.000000	0.000000	NaN	1.0000	1.0000	1.0000
Onychogomphus	0.000000	0.000000	NaN	1.0000	1.0000	1.0000
Allogamus	0.000000	0.000000	NaN	15.0000	15.0000	1.0000
Calamoceras	0.000000	0.000000	NaN	1.0000	1.0000	1.0000
Atherix	0.000000	0.000000	NaN	1.0000	1.0000	1.0000

Contrast: MP 0.1 g kg^{-1} (A)_MP 1 g kg^{-1} (B)

	Average	Standard deviation	ratio	Average A	Average B	cumsum
Chironomus	0.061996	0.049878	1.2429	19.6667	26.6667	0.3722
Baetis	0.033217	0.015787	2.1040	9.0000	2.6667	0.5716
Ephemerella	0.010931	0.008308	1.3157	2.6667	1.6667	0.6372
Habroleptoides	0.008859	0.002994	2.9589	2.0000	0.3333	0.6904
Ephemera	0.007722	0.007468	1.0341	2.0000	3.0000	0.7367
Ecdyonurus	0.007050	0.003810	1.8503	1.6667	0.3333	0.7791
Potamopyrgus	0.006989	0.005384	1.2981	5.3333	4.0000	0.8210
Isoperla	0.005752	0.002823	2.0376	0.6667	1.3333	0.8556
Lepidostoma	0.004720	0.004145	1.1387	2.0000	1.3333	0.8839
Sericostoma	0.004086	0.004534	0.9011	23.3333	23.6667	0.9084
Oligochaeta	0.004032	0.003386	1.1909	2.3333	2.0000	0.9326
Allogamus	0.002942	0.002805	1.0491	14.3333	14.6667	0.9503
Hydrosphyche	0.002927	0.002783	1.0516	1.3333	1.6667	0.9679
Halesus	0.001784	0.002682	0.6653	3.0000	3.3333	0.9786
Calamoceras	0.001784	0.002682	0.6653	1.0000	0.6667	0.9893
Dugesia	0.001784	0.002682	0.6653	1.0000	0.6667	1.0000
Cordulegaster	0.000000	0.000000	NaN	1.0000	1.0000	1.0000
Onychogomphus	0.000000	0.000000	NaN	1.0000	1.0000	1.0000
Limnephilus	0.000000	0.000000	NaN	3.0000	3.0000	1.0000
Philopotamus	0.000000	0.000000	NaN	0.0000	0.0000	1.0000
Atherix	0.000000	0.000000	NaN	1.0000	1.0000	1.0000

Contrast: MP 1 g kg^{-1} (A)_MP 10 g kg^{-1} (B)

	Average	Standard deviation	ratio	Average A	Average B	cumsum
Chironomus	0.045804	0.048219	0.9499	26.6667	18.3333	0.3535
Baetis	0.011791	0.007689	1.5335	2.6667	3.0000	0.4445
Ephemera	0.011368	0.010010	1.1357	3.0000	1.0000	0.5323
Halesus	0.007548	0.005773	1.3073	3.3333	2.0000	0.5905
Ephemerella	0.007486	0.004945	1.5140	1.6667	1.6667	0.6483
Isoperla	0.006309	0.005433	1.1611	1.3333	2.0000	0.6970
Potamopyrgus	0.006291	0.005234	1.2018	4.0000	3.3333	0.7455
Oligochaeta	0.005655	0.004825	1.1718	2.0000	2.3333	0.7892
Ecdyonurus	0.004945	0.004284	1.1543	0.3333	1.0000	0.8273
Habroleptoides	0.004285	0.004721	0.9077	0.3333	0.6667	0.8604

Sericostoma	0.003156	0.003001	1.0517	23.6667	23.3333	0.8848
Lepidostoma	0.003105	0.002954	1.0510	1.3333	1.6667	0.9087
Hydrosphyche	0.002525	0.002998	0.8422	1.6667	1.6667	0.9282
Calamoceras	0.001905	0.002858	0.6666	0.6667	1.0000	0.9429
Dugesia	0.001905	0.002858	0.6666	0.6667	1.0000	0.9576
Philopotamus	0.001898	0.002852	0.6655	0.0000	0.3333	0.9723
Limnephilus	0.001846	0.002773	0.6656	3.0000	2.6667	0.9865
Allogamus	0.001745	0.002618	0.6666	14.6667	15.0000	1.0000
Cordulegaster	0.000000	0.000000	NaN	1.0000	1.0000	1.0000
Onychogomphus	0.000000	0.000000	NaN	1.0000	1.0000	1.0000
Atherix	0.000000	0.000000	NaN	1.0000	1.0000	1.0000

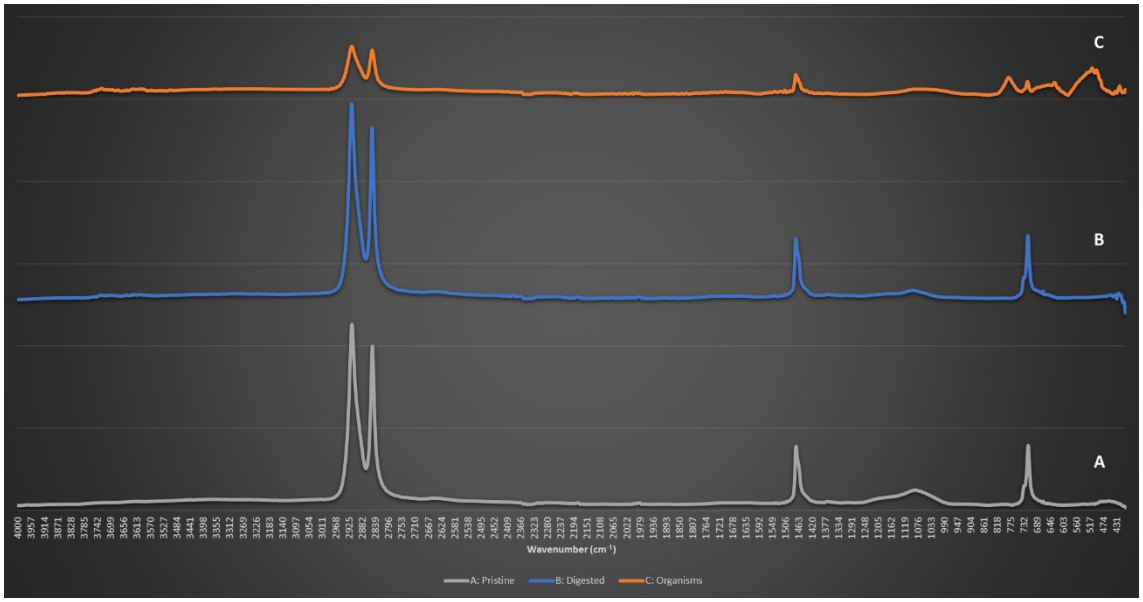

Fig. S1. Polyethylene microplastics (PE-MPs) characterization by infrared spectra by Fourier Transform Infrared Spectroscopy-Attenuated Total Reflectance (FTIR-ATR). Pristine PE-MPs (A) used in the experiment were taken from the packaging and directly analysed, as well as PE-MPs subjected to the same digestion (nitric acid + hydrogen peroxide) and extraction procedures (B) as PE-MPs found inside the organisms (C). The analysis was carried out at Perkin Elmer (USA) Spectrum BX FTIR instrument (32 scans, 4 cm^{-1} and 4000–431 cm^{-1}).

Chapter 8

General discussion and future research

General discussion and future research

The levels of microplastics (MPs) in the environment have become a major concern and the assessment of MPs especially in the freshwater environment remains overlooked (Bhattacharya and Khare, 2020). Although insufficient, the available reports on the levels of MPs on freshwaters sets them to be equal or higher than those found in the marine environment (Wang et al., 2018a; Scherer et al., 2020), which led to a general awareness on the susceptibility of freshwater biota to MP exposure. Corroborating this perspective, field and laboratory studies have unveiled the widespread ingestion of MPs by freshwater fauna at different trophic levels (Wang et al., 2019). Moreover, environmental MPs concentrations are likely to increase in the future, due to the persistence and continued input of plastics in the environment (Rochman et al., 2013; Adam et al., 2019), and in freshwaters with a special emphasis on smaller size fraction MPs (Imhof et al., 2013; Besseling et al., 2017). MP levels found in freshwater sediments are much higher than levels on the water column (Scherer et al., 2020), and conspicuous when considering their smaller fraction (<300 μm) (Wang et al., 2018b; Scherer et al., 2020). Not surprisingly, macroinvertebrates that live in close contact with these sediments and that feed on particulate matter within the same size range are prone to ingest an increased number of small-sized MPs, as confirmed by several studies both in laboratory and in the field (Scherer et al., 2017; Nel et al., 2018; Redondo-Hasselerharm et al., 2018).

Within this framework, one of the actual research focus is not only to report MPs ingestion by aquatic benthic macroinvertebrates but also to estimate it since quantitative measurements of particle ingestion are analytically challenging (Rist et al., 2017). Quantitative assessments of MPs ingestion are of special importance in biota prone to ingest significant number of microplastic particles (Scherer et al., 2017; Straub et al., 2017), and particularly as an attempt to link MPs ingestion to effects on the organism. These quantitative assessments should also help to verify if feeding type and organisms' behaviour are main drivers of MPs ingestion (Scherer et al., 2017). Also, the use of macroinvertebrates as test species will evaluate the potential effects of plastic particles on different biochemical and organismal level endpoints and the assessment of effects on populations and ecosystems.

Another important priority in assessing ecotoxicological effects of MPs is the use of realistic exposure scenarios (Horton et al., 2017; Paul-Pont et al., 2018; Schür et al., 2020). To increase the realism in testing and ecological relevance, the employment of MPs levels that have been found in the environment, and the use of different polymer types , the use of weathered and different-sized, different-shaped MPs is desired (Schultz et al., 2015; Gray and Weinstein, 2017a; Horton et al., 2017; Lehtiniemi et al., 2018a). Likewise, the exposure of MPs in combination with other stressors (anthropogenic/natural) is needed to represent an approximation to the mixture of stressors that may interact with MPs and alter biota's toxic effects (Lambert and Wagner, 2018). In this sense, and although many studies are being performed evaluating the interaction of MPs with anthropogenic contaminants (e.g., metals, polybrominated diphenyl ethers, polycyclic aromatic

hydrocarbons and pharmaceuticals) (Guilhermino et al., 2018; Magara et al., 2018; González-Soto et al., 2019; Horton et al., 2020; Qu et al., 2020; Sıkdokur et al., 2020; Weber et al., 2021), the evaluation of combined effects of MPs under different natural environmental conditions has been lagging behind (Jaikumar et al., 2018; Weber et al., 2020).

The different sensitivity of macroinvertebrates to MPs exposure can then alter the macroinvertebrate community structure by affecting some taxa more than others (Redondo-Hasselerharm et al., 2018). Moreover, these effects on the community structure can also lead to alterations in ecosystem functioning (Dossena et al., 2012) and MPs can affect directly certain ecosystem functions (e.g., reduced primary production by affecting periphyton growth) (Boyero et al., 2020). Up to now, almost no studies exist on the effects of MPs at the ecosystem level (Redondo-Hasselerharm et al., 2020) and the combined assessment of effects on macroinvertebrate community structure and on ecosystem functioning was not yet performed . This combined assessment will provide integrative data allowing for an accurate evaluation of the implications of MP contamination on fundamental ecological processes and hence on ecosystem health (Young et al., 2008; Dalu et al., 2017).

This thesis provides the first evidence on how and to which extent the presence of polyethylene PE-MPs affect aquatic benthic macroinvertebrates (known to accumulate MPs in the field and also model species in ecotoxicology) at sub-organismal and organismal levels. Also, relevant exposure scenarios were considered as were the effects at the community and ecosystem levels. Therefore, in addition to the traditional assessment of effects using single-species standard tests, both addressing biochemical biomarkers and life-history traits, a combined exposure of PE-MPs with natural stressors was used to verify their toxicity under different environmental conditions. Finally, a mesocosms approach was used to test the effects of PE-MPs exposure on a freshwater macroinvertebrate benthic community and key ecosystem endpoints like organic matter decomposition and primary production. A quantitative assessment of PE-MPs ingestion by invertebrates was performed as an attempt to link uptake and subsequent effects.

Some of the main results and highlights of this thesis are summarized below, followed by an integrative and critical discussion, with the identification of potential research lines that should be pursued in near future:

- *Benthic invertebrates indeed ingest MPs, but the toxic effects of these particles depend on the organism. Considerable interspecific variation exists even within the same functional feeding group*.

The ingestion of MPs by benthic invertebrates has been confirmed by several laboratory and field studies (Scherer et al., 2017; Nel et al., 2018; Redondo-Hasselerharm et al., 2018), and quantified in the present study for *Chironomus riparius* and *Lumbriculus variegatus* after short and

long-term exposures to PE-MPs (chapter 2 to 4). Despite confirming that these two invertebrate species easily ingest irregularly-shaped PE-MPs, *L. variegatus* presented significant lower ingestion than *C. riparius* (chapter 2-4).

Ingestion constitutes the main route for MPs uptake by most invertebrates. Conversely to chemical contaminants, MPs possess tridimensionality, and their toxicity (particularly for pristine particles) is intimately related with their levels and residence time inside the organism. As observed in chapter 2 to 5, *C. riparius* presented higher numbers of MP compared to *L. variegatus*; with concomitant reduced larval growth and delayed development, while *L. variegatus* presented no effects on development and reproduction (chapter 2 and 4). These data seem to corroborate data on the ingestion of fluorescent polystyrene spheres by the same two species, in which *C. riparius* ingested significant more MPs than *L. variegatus* (Scherer et al., 2017). All these research point feeding type and organisms' morphology as key-drivers of MP ingestion by invertebrates (Setälä et al., 2016; Scherer et al., 2017; Redondo-Hasselerharm et al., 2018). Therefore, although *L. variegatus* seemed less sensible to PE-MPs than *C. riparius*, this can change in the presence of other MP factors that drive their toxicity. Other key- issues can alter MP ingestion and toxicity in invertebrates such as MPs size (Gray and Weinstein, 2017b; Lehtiniemi et al., 2018b; Silva et al., 2019), polymer-type and shape (Gray and Weinstein, 2017b). The use of different-sized and shaped- polymers (representative of MPs found in the environment) and the use of weathered particles allowing for colonization and adsorption of other compounds are key areas for future research that can contribute for a more accurate risk assessment of plastic particles in the freshwater environment (Lehtiniemi et al., 2018b; Ho et al., 2020). Risk assessment can also be improved with more ecotoxicological data produced in single-species tests using also non-model freshwater invertebrates that might also present sensitivity to plastic particles (chapter 6). In this way species sensitivity distribution (SSD) approach can also be applied to MPs (Gottschalk and Nowack, 2013), should also be encouraged in the future (Adam et al., 2019).

Other effective approaches that increase realism for an accurate ecotoxicological evaluation of MPs to aquatic invertebrates and that are still overlooked is the use of behavioural endpoints such as swimming velocity or avoidance, as well assays that allow to evaluate multi-generational effects of plastics in invertebrates (Chen et al., 2017; Seuront, 2018; De Felice et al., 2019; Ju et al., 2019; Schür et al., 2020) and how these particles can disrupt predator–prey interactions (Seuront, 2018; Cuthbert et al., 2019) that are directly related to possible consequences on population dynamics and fitness.

- *Immune responses, oxidative stress biomarkers, energy reserves and consumption revealed to be early warning indicators of MP toxicity*

Microplastic toxicology research have been mainly focused on apical endpoints and few studies deal with toxicity mechanisms (Jeong and Choi, 2019). Among the studies considering sub-

cellular endpoints, the most reported toxicity mechanism involved in MPs toxicity is the production of reactive oxygen species (ROS), which was proposed as molecular initiating event in the establishment of the most probable Adverse Outcome Pathway in microplastic toxicity (Lei et al., 2018; Jeong and Choi, 2019).

The present study (chapter 3 and 4) confirms this and provides evidence of oxidative stress in both *C. riparius* (considerable effects) and *L. variegatus* (slight effects) induced by PE-MPs. The strong responses observed in *C. riparius*, including oxidative damage, along with the depletion of energy reserves and energetic costs to keep to redox balance and activate the immune response were markedly linked to the verified adverse outcome (reduced growth and delayed development), while in *L. variegatus* the minor evidence of oxidative stress did not have correspondence at the organismal level.

The physical effect of MPs inside the chironomids' gut likely caused damage in gut epithelium similarly to what was also found by other authors who associated oxidative stress to the physical presence of MPs damaging tissues inside the intestinal tract of the invertebrates (Magara et al., 2019; Wang et al., 2019). Damage to the gut epithelium can induce immune responses that also increase ROS concentrations (Dupré-Crochet et al., 2013). In chapter 5, the immune response assessed through phenoloxidase activity was indeed activated in *C. riparius* after MP ingestion. The observed oxidative damage in *C. riparius* (chapter 4) is then at least partially, linked to the activation of the immune response triggered after damage to the gut epithelium caused by accumulation of MPs.

Following this process, different-sized MPs can be ingested at very different magnitudes (Lehtiniemi et al., 2018b; Silva et al., 2021), and different-shaped MPs can be ingested and produce damage to gut epithelium, like it was observed already in zebrafish larvae (Qiao et al., 2019).

The measurement of biochemical biomarkers and analysis of gene expression involved in oxidative stress response should be a fruitful area of research in the near future, helping to unveil the mechanism of toxicity of MPs in different invertebrate species (Cheng et al., 2020). For this focus should be placed on sub-organismal endpoints (e.g., endpoints related to ROS formation and subsequent oxidative stress) including the assessment of the immune-related factors involved in the immune response to MPs ingestion, (Liu et al., 2019). This evaluation will evidently benefit with the consideration different-sized and shaped MPs, and of how can other MP characteristics like the polymer-type or different colonized MPs (produced by different weathering processes), can mediate these biochemical responses and alter fitness of benthic invertebrates that are naturally exposed to other stressors including parasites.

The ingestion and accumulation of MPs in the gut can also cause significant alterations in gut microbiome that have been mostly studied in terrestrial invertebrates and zebrafish (Jin et al., 2018; Zhu et al., 2018; Ju et al., 2019), but are still overlooked in aquatic invertebrates. The possible disruption of the symbiosis between host and the natural gut (Skillings and Hooks, 2018)

caused by exposure and ingestion of MPs remains relatively neglected (Fackelmann and Sommer, 2019).

- *The importance of characterising the ingestion of MPs by aquatic macroinvertebrates*

The number of MPs present inside the macroinvertebrates gut was used as proxy of ingestion after the short and long-term exposures in single-species tests for both *C. riparius* and *L. variegatus* (chapters 2-4). The direct correspondence and agreement of PE-MPs concentrations in sediments with ingestion data (especially for smaller-size MPs) and triggered effects (sub-organismal and organismal level) was one of the main findings of the present work. Not only the number of MPs but the consideration of their size since a lower ingestion of larger-size MPs can produce effects similar to a higher ingestion of small-size MPs (chapter 4). Likewise, macroinvertebrates that presented reduced abundance in the mesocosms experiment were the organisms presenting the higher number of MPs inside the gut (chapter 7).

The present work highlights the importance of a good characterisation of the ingestion of MPs by invertebrates. Most studies only address ingestion on a qualitative basis and most verify the ingestion of MPs using fluorescent MPs (Lusher et al., 2016).

A good characterisation of ingestion by invertebrates, however, includes quantitative and qualitative assessment considering intrinsic characteristics of the MPs (polymer-type, size and shape) and its ingestion by different species. In this context, a species can present higher ingestion of determined plastic particles being this preference related not only to the size or shape of the particle but also due to different palatability of different polymers.

In addition to the characteristics of the MPs, the organism' anatomy and physiology can also be crucial (e.g., size of the mouth apparatus) and development stage (e.g., invertebrate larvae at initial stages ingest smaller particles due to morphological restrictions (reduced size of mouth)(Scherer et al., 2017). The ingestion of MPs can also be altered due to weathering of particles (potential increase in palatability due to biological colonization) and external factors to the organisms and the particles. Properties of the sediment, environmental factors (like it was observed in chapter 6) and the presence of other contaminants associated to MPs can also modulate ingestion and/or toxicity (e.g., the presence of other contaminants may not alter ingestion, but still increase the toxic effects). Despite the direct effects (e.g., physical and/or nutritional) induced by MPs *per se* after ingestion, these may be exacerbated by the presence of plasticizers and other toxic pollutants adhered to the particles' surface (Harmon, 2018).

The characterization of MPs ingestion must also include the time factor. The evaluation of residence time of MPs inside the gastrointestinal tract of organisms is of utmost importance to understand the time needed to ingest (and egest) the particles and the potential existence of gut clogging. Bioaccumulation tests with exposure to MPs and subsequent transfer to clean media may unveil how can the presence of other factors such as the presence of food items, and of natural

and chemical stressors alter MPs ingestion and residence time and change internal concentrations of MPs through time (Scherer et al., 2017).

However, the lack of standardized protocols (Prata et al., 2019a), makes it difficult to ensure comparability between studies which disallows an accurate assessment of the impacts and risks that MPs pose to biota, (Lusher et al., 2017). The same lack of consistency is verified in sampling and quantifying MPs in water column and sediments (Prata et al., 2019a; Zhang et al., 2020). For the extraction and quantification of MPs, multiple methods are applied in all phases: digestion (Cole et al., 2014; Avio et al., 2015; Lusher et al., 2017; Hurley et al., 2018; Li et al., 2018; Stock et al., 2019); density separation (Corcoran, 2015; Stock et al., 2019); filtration (Campanale et al., 2020); counting (manual and making use of automatic image analysis) (Song et al., 2015; Prata et al., 2019b; Primpke et al., 2019; Lorenzo-Navarro et al., 2020); and characterization of the chemical composition of plastic particles (most used techniques are pyrolysis–gas chromatography–mass spectrometry, and thermal extraction desorption–gas chromatography–mass spectrometry, Raman and Fourier-transform infrared spectroscopy and novel techniques such as isotope ratio mass spectrometry) (Fries et al., 2013; Hermabessiere et al., 2018; Xu et al., 2019; Peñalver et al., 2020; Birch et al., 2021).

- *The importance of considering relevant exposure scenarios and how MP effects can be modulated by different concomitant stressors*

Research on effects of MPs (particularly within aquatic environments) is now decisively trying to incorporate relevant exposure scenarios to improve the accuracy in risk assessment. Relevant exposure conditions addressed have been mainly focused on the use of environmental relevant concentrations of plastic particles (Weber et al., 2018; Ziajahromi et al., 2018), and the role they might have as possible vectors for chemical contaminants (e.g., metals, polybrominated diphenyl ethers, polycyclic aromatic hydrocarbons, pesticides and pharmaceuticals) (Guilhermino et al., 2018; Horton et al., 2018, 2020; Magara et al., 2018; González-Soto et al., 2019; Qu et al., 2020; Sıkdokur et al., 2020; Weber et al., 2021). However, it is also of utmost importance to assess not just the effects of MPs under predicted conditions under the current predictions of global climatic change (Convey and Peck, 2019; Horton and Barnes, 2020). Limited research have been performed up to now on the effects of MPs in combination with natural stressors and almost all of it is focused on the combination of MPs with temperature (Jaikumar et al., 2018; Weber et al., 2020). Future studies must be carried out varying environmental conditions not only considering combined exposures of MPs and abiotic stressors (e.g., different temperature and temperature variability, salinity, pH and light conditions) but also including relevant scenarios in terms of biotic stressors including for example the challenges posed by predation, competition and parasitism (Agnew et al., 2004; Fonte et al., 2016; Wen et al., 2018). The predictive capacity of these kind of studies can be also be increased by investigating the individual responses under the different environmental conditions and additional stressors that may be encountered now or in the future. These studies

may allow for identification of responses and causal biological mechanisms, and enable an understanding of specific individual and community responses to MPs (Horton and Barnes, 2020).

In the present work, the effects triggered by MPs on the dipteran *C. riparius* were investigated under several abiotic conditions expected to be altered by climate change such as temperature and salinity and a biotic condition (food limitation). Results showed complex patterns of response (from additivity to synergism and antagonism – although mostly antagonism) to combinations of stressors and suggest that effects of MPs can be stronger in conditions of lower temperatures (in comparison with the temperature tested in standard laboratory assays, i.e., lower than 20ºC) or under food stress. Results also show that under different environmental conditions toxicity of MPs are not simply a reflection of internal concentrations of particles.

It is clear that more research is needed to understand if and how changes in metabolism and gut physiology can explain these interactions and how temperature and salinity stress (and of other stressors) modulate MPs ingestion and effects. This research will benefit from the use of more invertebrate species and also the assessment of effects in wide array of physiological and life-history parameters.

- *Exposure to MPs can lead to population and community level effects*

In the present work, delays in development of *C. riparius*, particularly evident by the increased time to emergence were observed after exposure to PE-MPs in concentrations similar to those found in MP contamination hotspots in freshwaters (chapter 2). Delayed emergence can naturally lead to population level effects but investigation using reproductive parameters and multigenerational experiments are needed.

This thesis and particularly the mesocosms experiment (chapter 7) demonstrated that sediment contamination by PE-MPs can also lead to detrimental effects on benthic invertebrate community and ecosystem level. The exposure and pronounced MP ingestion in specific taxa (mainly grazers and collectors) were directly linked to community-level effects). Both the effects observed in the macroinvertebrate community structure and the suggestion of possible direct and indirect, trait-and density-mediated effects on ecosystem functional parameters such as leaf litter decomposition and primary production can carry significant ecological consequences.

Given the ubiquity of plastic pollution and having in consideration their heterogeneous distribution in freshwater systems, it is of critical importance to increase the monitoring of MPs in the environment. Concomitantly, the correlation between MP levels in the environment and local macroinvertebrate community and functional parameters that can be measured *in situ* are an important approach to better understand possible ecological effects of MP exposure in freshwaters. Multispecies assays and community ecotoxicological experiments using mesocosms are a valuable tool to understand and predict the long- term ecological effects of plastic particles in freshwater ecosystems.

Moreover, the monitoring of the levels of smaller particles and nanoplastics in freshwater sediments is still inexistent, as the evaluation of nanoplastics can be particularly challenging, mostly due to difficulties in the extraction from sediments, without losing or destroying the nanoplastics (Mintenig et al., 2018). Although there is no data on the nanoplastics levels in the environment, some studies suggest that their levels in seawater can be even higher than the levels of MPs (Bergmann et al., 2017) and so a similar trend must be expected for freshwater environments, also based on the distribution of MPs per size found in freshwater sediments (Scherer et al., 2020). Therefore, a better understanding of the real distribution and characteristics of plastic particles in the environment will allow to design appropriate exposure regimes to be used in ecotoxicological tests (Kögel et al., 2020). Furthermore, the capability of nanoplastics to cross biological barriers and their potential to be biomagnified and transferred throughout the food chain and from the aquatic to the terrestrial ecosystem pose new challenges for environmental scientists but is necessarily a research gap that needs to be addressed in the near future.

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