Fate and effects of microplastics in combination with pharmaceuticals and endocrine disruptors in freshwaters: Insights from a microcosm experiment

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Abstract

Microplastic contamination of freshwater ecosystems has become an increasing environmental concern. To advance the hazard assessment of microplastics, we conducted a microcosm experiment in which we exposed a simplified aquatic ecosystem consisting of moss and caddisflies to microplastics (polyethylene, polystyrene and polypropylene) and pharmaceuticals and personal care products (1H-benzotriazole, bisphenol A, caffeine, gemfibrozil, ketoprofen, methylparaben, estriol, diphenhydramine, tris (1-chloro-2-propyl) phosphate) over the course of 60 days. We monitored the flux of microplastics within the microcosm, as well as the metabolic and total protein variation of organisms. This study offers evidence highlighting the capacity of moss to act as a sink for free-floating microplastics in freshwater environments. Moss is also shown to serve as a source and pathway for microplastic particles to enter aquatic food webs via caddisflies feeding off of the moss. Although most ingested microparticles were eliminated between caddisflies life stages; however, a small fraction of microplastics was transferred from aquatic to terrestrial ecosystem by emergence. While moss exhibited a mild response to microplastic stress, caddisflies ingesting microplastics showed stress comparable to that caused by exposure to pharmaceuticals. The molecular responses that the stressors triggered were tentatively identified and related to phenotypic responses such as the delayed development manifested through the delayed emergence of caddisflies exposed to stress. Overall, our study provides valuable insights into the adverse effects of microplastics on aquatic species, compares the impacts of microplastics on freshwater biota to those of pharmaceuticals and endocrine disrupting compounds, and demonstrates the role aquatic organisms have in redistributing microplastics between aquatic and terrestrial ecosystems.

Keywords: multiple stressors, emerging contaminants, metabolomics, aquatic insects, trophic transfer

1. Introduction

The mass production of plastics for the past 70 years, coupled with inadequate waste management and slow degradation times, has led to increased plastic pollution in natural environments (Geyer et al., 2017; Wang et al. 2019). Of particular concern are microplastics (MPs) – plastic particles up to 5 mm in diameter (Arthur et al., 2009). Although recent studies have revealed the ubiquity of MP debris in marine and freshwater ecosystem alike (Egessa et al., 2020; McCormick et al., 2014, 2016), research on MPs has long been limited to marine environments, with freshwater habitats receiving less attention (Wagner et al., 2014). However, it is critical we investigate these ecosystems, as average concentrations of MPs confirmed in freshwater environments have been shown to reach 4.7×10^6 particles/L in surface waters (Di and Wang, 2018) and 11070 ± 600 particles/kg in sediments (Mani et al., 2019), with concentrations only expected to increase over time (Barnes et al., 2009).

MPs enter aquatic environment as two main sources: I) primary MPs, manufactured as pellets for commercial use and II) secondary MPs, formed by the chemical and/or mechanical degradation of larger plastic fragments (GESAMP, 2016). Untreated wastewaters, waste water treatment plant effluents, and atmospheric deposition are just some of the many routes of entry of MPs into freshwater environments (Koutnik et al., 2021). Once MPs enter aquatic environments, those most abundantly present – polyethylene (PE), polypropylene (PP), and polystyrene (PS) (Andrady, 2011; Enders et al., 2015; Rezania et al., 2018) – remain buoyant, suspended in the water column, and transported by water currents. The concentration of these particles decreases further from the source (Koutnik et al., 2021), which is usually attributed to the development of a biofilm layer on the surface of the MPs, i.e., biofouling (Semcesen and Wells, 2021). Biofouling can decrease the buoyancy of low-density MP particles, causing them to sink more rapidly,

eventually accumulating in the sediments (Kaiser et al., 2017; Semcesen and Wells, 2021; Van Cauwenberghe et al., 2015). In addition to biofouling, suspended MPs may be consumed and/or adsorbed by freshwater organisms, contributing to the redistribution of MPs within the water column. Due to this, it is important to evaluate role of biota in the flux of MPs within freshwater environments, and to understand the risk that MPs pose to organisms that live and feed in these habitats (Bellasi et al., 2020).

Some biota known to ingest MPs include planktonic organisms, planktivorous fish, and benthic invertebrates (Bessa et al., 2018; Sun et al., 2017; Von Moos et al., 2012). The ingestion of MPs can have severe consequences on biota – impacting their feeding rate, oxygen consumption, growth, and development (Hamed et al., 2019; Lei et al., 2018; Yin et al., 2021b). Most research examining the impacts of MPs on biota focus on fish species, with observed effects ranging from oxidative stress and disruption of the composition of metabolites (Lu et al., 2016), to strong inflammatory responses and tissue damage (Lei et al., 2018; Lu et al., 2016). Furthermore, the knowledge concerning the impacts of MPs on aquatic organisms is mainly limited to filter-feeding invertebrates, such as the cladocerans (Schwarzer et al., 2022) and mussels (Vinay Kumar et al., 2021). Research focusing on aquatic insects, the dominant component of freshwater benthic communities is still scarce; however, the little research available shows that MPs can cause physical damage, oxidative stress, growth inhibition, and reproductive impairment (Bellasi et al., 2020; Malafaia et al., 2020; Ziajahromi et al., 2019, 2017). Pharmaceuticals and endocrine disrupting compounds (PhACs-EDCs) have similarly been shown to exert negative impacts on freshwater organisms, such as decrease in reproduction and growth, oxidative stress, changes in behavior, disturbed circadian rhythm and decreased locomotion and decrease in survivor rate (Brooks et al., 2003; De Castro-Català et al., 2017;

Hazelton et al., 2014; Melvin, 2017; Nunes et al., 2014; Sehonova et al., 2018; Weinberger and Klaper, 2014). In contrast, macrophytes have not been shown to exhibit a significant response following the adsorption of MPs to their surface (Kukkola et al., 2021; Mateos-Cárdenas et al., 2020). There are still, however, some gaps in our knowledge regarding the flux of MPs in freshwater systems and the impact of MPs on freshwater biota. Moreover, we lack understanding on the differences in organismal responses between MPs and PhACs-EDCs, as well as in combination (i.e., a multi-stressor scenario).

We therefore set the following objectives for this study:

- Gain insights regarding the flux of MPs in freshwater environments, i.e., the spatio-temporal distribution of particles
- Assess the potential of macrophytes to adsorb MPs commonly found in freshwater environments and transfer them further up the food web (i.e., to primary consumers)
- Evaluate the role of emerging aquatic insects as primary consumers and potential vectors of MP transfer across ecosystem boundaries
- Characterize the effects of MPs commonly found in freshwater systems on both macrophytes and emerging aquatic insects on a molecular level
- Determine differences in stress response profiles between individual stressors (MPs and PhACs-EDCs), as well as in a multi-stressor scenario (i.e., MPs combined with PhACs-EDCs).

To achieve these aims, we conducted a 60-day microcosm experiment simulating the exposure of a simplified aquatic ecosystem consisting of caddisflies and moss (nonvascular macrophytes) to MPs and PhACs-EDCs – both separately and in combination to assess multi-stressor organism responses. We monitored the flux of microplastics within the microcosm, as well as the

metabolic and total protein variation of organisms. The MPs (high-density polyethylene – HDPE, low-density polyethylene – LDPE, polystyrene – PS, polypropylene – PP) and PhACs-EDCs (1H-benzotriazole, bisphenol A, caffeine, gemfibrozil, ketoprofen, methylparaben, estriol, diphenhydramine, tris (1-chloro-2-propyl) phosphate) used in the experiment were selected to reflect those most commonly found in natural environments.

2. Materials and methods

2.1 Reagents and sampling

HPLC-grade solvents (methanol, water, and acetonitrile) were purchased from Fisher (Germany). The reagents used in the experiments (1H-benzotriazole, bisphenol A, caffeine, gemfibrozil, ketoprofen, methylparaben, estriol, diphenhydramine, tris (1-chloro-2-propyl) phosphate) were obtained from Sigma Aldrich (Germany). MPs were prepared using PP, HDPE, LDPE, and PS plastic resin pellets of 3-5 mm diameter. The pellets were shredded in liquid nitrogen using a home-made mill and consequently sieved into a fraction of less 500 μm. MPs were further characterized using a stereomicroscope as microbeads of irregular shapes, with 90% of the particles within a size range of 100-500 μm. MPs were counted in order estimate the number of MPs per gram.

2.2 Microcosm experiment

Our laboratory experiment was conducted in Spring 2019 at the Faculty of Science, University of Zagreb (Zagreb, Croatia). Water, sediment, and larger stones, together with moss (*Cinclidotus aquaticus* (Hedw.) Bruch and Schimper, 1842) and *Rhynchostegium riparioides* (Hedw.) Cardot, 1913) and caddisflies larvae (*Mycropterna nicterobia* McLachlan, 1875) were collected at the spring of the Krčić River – a river in southeastern Croatia minimally impacted by anthropogenic activity. 15 microcosms (30 × 20 × 15 cm aquaria) were randomly placed in three incubators (POL-EKO APARATURA, Poland), Fig. 1a. Each microcosm contained ~150 g sediment, with 1-3 larger stones, 30 caddisflies larvae, 3 tufts of moss and 3 L of spring water. The incubators were initially set at 9.5 °C, with the temperature increasing by 0.5 °C every 15 days to simulate

the natural water temperature regime of the Krčić River. Oxygenation and water mixing were achieved using aquaria air pumps and air stones. The aquaria were covered with glass slides to minimize evaporation, while the water level was kept constant by adding dechlorinated tap water. The microcosms were acclimatized for 7 days, after which they were exposed to the treatments. Due to the lack of standardized methods available for quantitatively determining an optimal acclimation period for lower trophic levels (i.e., macrophytes and insects), a 7-day acclimation period was selected based on prior studies that worked with invertebrates in micro- and mesocosms (Auffan et al., 2014; Previšić et al., 2021). Three aquaria were set as controls, while the rest (four aquaria per treatment) were exposed to the following treatments: MPs (a mixture of HDPE, LDPE, PP, and PS), a mixture of pharmaceuticals and endocrine disrupting compounds (PhACs-EDCs) (1H-benzotriazole, bisphenol A, caffeine, gemfibrozil, ketoprofen, methylparaben, estriol, diphenhydramine, tris (1-chloro-2-propyl) phosphate) and, lastly, MPs in combination with PhACs-EDCs (Fig. 1a). The concentration of the PhACs-EDCs in the experimental treatments was 500 ng L^{-1} for each compound. Taking into consideration knowledge gained from our previous experiments, the compounds were added daily to weekly (depending on the compound, please see Supporting Information (SI)) in order to compensate for abiotic attenuation (sorption and/or (photo)degradation) and maintain nominal concentrations of the compounds in the aqueous phase (Cetinić et al., 2022; Previšić et al., 2021). The concentration of MPs used in the experimental treatments was estimated to be 2000 MPs per L (6000 MPs in total in each aquarium; 1500 particles of each MP type) and was added once at the beginning of the experiment.

Moss and caddisflies were sampled at four time points after acclimatization – at day 0, day 15, day 30, and day 60 (Fig. 1b). Adult caddisflies were sampled as they emerged (from day 44 to

60). On each sampling date, replicate samples were taken from each microcosm (2 g of moss, and 3−14 caddisfly larvae); however, these were pooled per treatment per species to minimize the variability between the microcosms (Van Geest et al., 2010), and three analytical replicates for each sampling date were taken. For the purpose of testing the amount of MPs that pass through the digestive system of the caddisflies on a daily basis, half of the individuals were kept in clean aquaria for 24 h to allow for gut clearance prior to collection, while the rest were sampled directly. Sediments were sampled (i.e., removed) once, at the end of the experiment, to prevent disruption of the microcosms. All samples were then freeze-dried and stored at −80 °C until further processing.

Testing for the recovery rate of MPs during sample degradation and filtering showed that recovery of the particles varied greatly depending on the type of tissue examined. The average recovery rate of MPs in the sediments was 81%, in caddisfly tissues 82%, in caddisfly cases 95%, and in the moss 93%. Due to the high recovery rate, no corrections were applied to the data.

2.3 Extraction protocol

Samples were ground using a mortar (moss) or ball mill (caddisflies) in liquid nitrogen. 30 mg of ground tissue was dissolved in 1.5 mL of ice-cold acetonitrile and vortexed at medium speed (IKA® Vortex Genius 3, Germany) for 20 min. After vortexing, samples were left at -20 °C for 10 min to facilitate protein precipitation. Samples were centrifuged at 20 000 g for 10 min and the first supernatant was collected. The remaining pellet was resuspended in 1.5 ml of ice-cold acetonitrile and additional lysis was done via ultrasonic probe (Sonoplus HD4050, Bandelin

electronic GmbH, Germany) for 1 min at 50% intensity. Samples were vortexed for 5 min at medium speed, left in the fridge at -20°C for 10 min and centrifuged for 10 min at 20 000 g, after which the second supernatant was collected. The supernatants and pellet were evaporated to dryness. The supernatant extracts were used for metabolome analysis, while the remaining pellet was used for protein and MP extraction.

2.4 Metabolome analysis

The collected supernatants were additionally purified using Oasis HLB Prime cartridges (Waters Corporation, USA) as described in (Previšić et al., 2021). Extracts were evaporated to dryness under a gentle nitrogen stream and reconstituted with 0.5 ml of methanol/water (50:50, v/v) prior to mass spectrometric analysis. Non-target analysis of the metabolome samples was performed using a high resolution mass spectrometry system; LTQ-Orbitrap VelosTM (Thermo Fisher Scientific, USA) coupled with an ultra-performance liquid chromatography (UPLC) system (Ultimate 3000 RSLCnano system, Dionex, Amsterdam, Netherlands). Instrument parameters and UPLC gradients are provided in Supporting Information (SI). Data extraction, chromatographic deconvolution and final alignment were done using the MZmine program (Katajamaa et al., 2006). The exported .csv files were further filtered and sorted using modified parts of Bqunat script written in Mathematica (Wolfram Research Inc.,Campaign, IL, USA) (Rožman et al., 2018). Based on the exact mass match metabolite identification was performed by searching Metlin, Kegg, LipidMaps, PubChem, and HMDB databases. It is worth noting that the metabolites reported here are only the metabolites that were putatively annotated. Details about the procedures and parameters regarding data extraction and features identification are provided in SI.

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212 *2.5 Protein analysis*

213 Following metabolite extraction, the remaining pellet was re-suspended in 200 µL of protein
214 extraction buffer (0.1% RapiGest SF (Waters, UK) in 50 mM Tris-HCl, pH = 8). After 10 min of
215 incubation at room temperature, the samples were vortexed for 5 min and centrifuged at 15000g
216 for 5 min. Supernatants were placed in clean tubes and diluted in Milli-Q water. Protein
217 concentration was determined using the Bradford method (Bradford, 1976).

218

219 *2.6 Microplastic isolation and identification*

220 The pellet remaining after protein extraction for moss and caddisfly tissues was dried and
221 digested in a mixture of suprapur HNO_3 and 30% H_2O_2 (3:1). Samples were heated for 3.5 h at
222 85°C prior to filtration. The organic matter in the sediment and caddisfly cases was initially
223 dissolved in 30% H_2O_2, followed by density separation to isolate the MPs using an aqueous
224 solution of NaBr (ρ=1.266 g/mL). All processed samples (moss, caddisfly tissues and cases, and
225 sediments) were then filtered using Whatman Cyclopore polycarbonate membrane filters (φ =
226 4.7 cm, pore size 5.0 µm). MPs were counted using a stereo microscope (BTC STM-8, Hungary)
227 and identified by µRAMAN spectroscopy (HORIBA Jobin Yvon, France).

228

229 *2.7. Statistical analysis*

230 Using R software (R Core Team, 2021) for statistical analysis, we applied linear mixed-effects
231 (LME) models in the package 'lme4' (Bates et al., 2015) to test if concentrations of MPs

measured in moss and caddisfly tissues in experimental treatments differed over time. Due to the longitudinal (i.e., repeated measurements) nature of the data, we included time (for moss) and insect stage (for caddisfly tissues) as fixed effects, while subject was set as a random effect. The analysis was followed by Tukey's pairwise comparisons in the 'lsmeans' package (Lenth, 2016) whenever differences were found to be significant ($P < 0.05$).

The effect of treatments on the metabolome of caddisflies and moss over time was tested using principal response curves (PRC) constructed in R software using the 'vegan' package (Oksanen et al., 2022). PRC analysis was performed on $\log_{+1}$ transformed metabolite data. The significance of the results was tested using the Monte Carlo test in the 'permute' package, with 999 permutations, more specifically the significance of the 1st canonical axis of the PRC and the significance of sampling date/insect stage was tested. Metabolites were ranked according to the weight of each metabolite in the response observed by the PRC. Due to the large number of metabolites, metabolite weights were not included in the Figures. The metabolites exhibiting the greatest response to treatments were extracted and their putative identification was additionally verified by reviewing available literature and the STITCH database (Szklarczyk et al., 2016). Those metabolites whose presence was previously recorded in plants or insects were accepted by the identification check, with deviations from the reference values of m/z masses less than 20 ppm.

Differences in protein content in caddisflies and moss across treatments, time and sex were tested using repeated measures ANOVA (rANOVA) in SPSS Statistics ver. 27.0 (Corp, 2020). Prior to analyses, normality of distribution and homogeneity of variances of the data was confirmed using Shapiro-Wilk and Levene's test, respectively. If data violated the sphericity

assumption Huynh–Feldt correction was applied. rANOVA post hoc analysis was done by applying Bonferroni method.

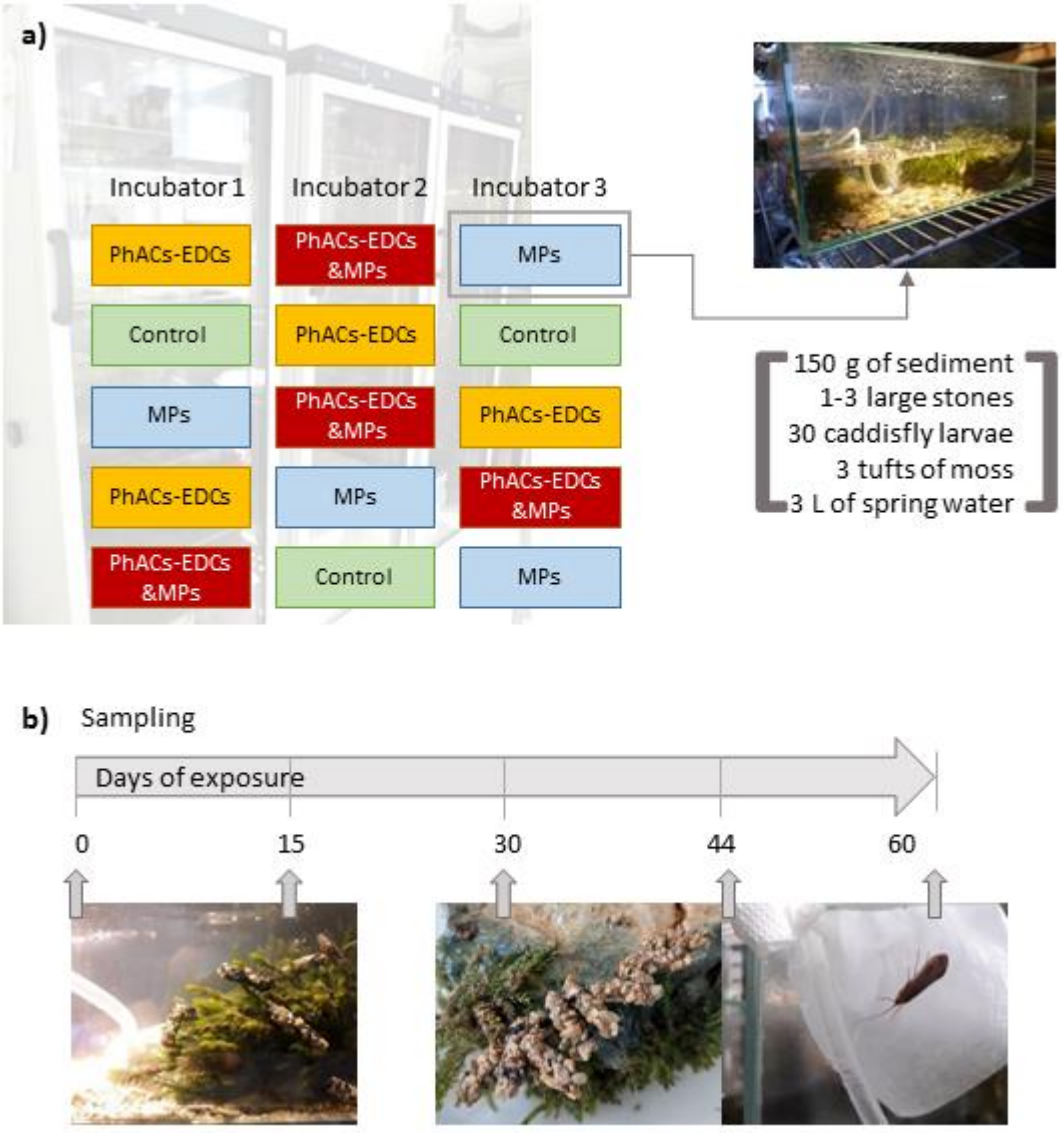


Fig. 1. A) Experimental design used in the study with moss and caddisfly *Micropterna nycterobia*, having four treatments: Control, PhACs-EDCs – pharmaceuticals and endocrine disruptors, MPs – microplastics, PhACs-EDCs & MPs - pharmaceuticals and endocrine disruptors & microplastics; b) Sampling scheme showing caddisfly stages (larvae, pupae, and

adults) sampled during 60 days of exposure. Sampling of moss followed the same sampling scheme.

3. Results and discussion

3.1. Distribution of particles suggests moss acts as a sink for microplastics

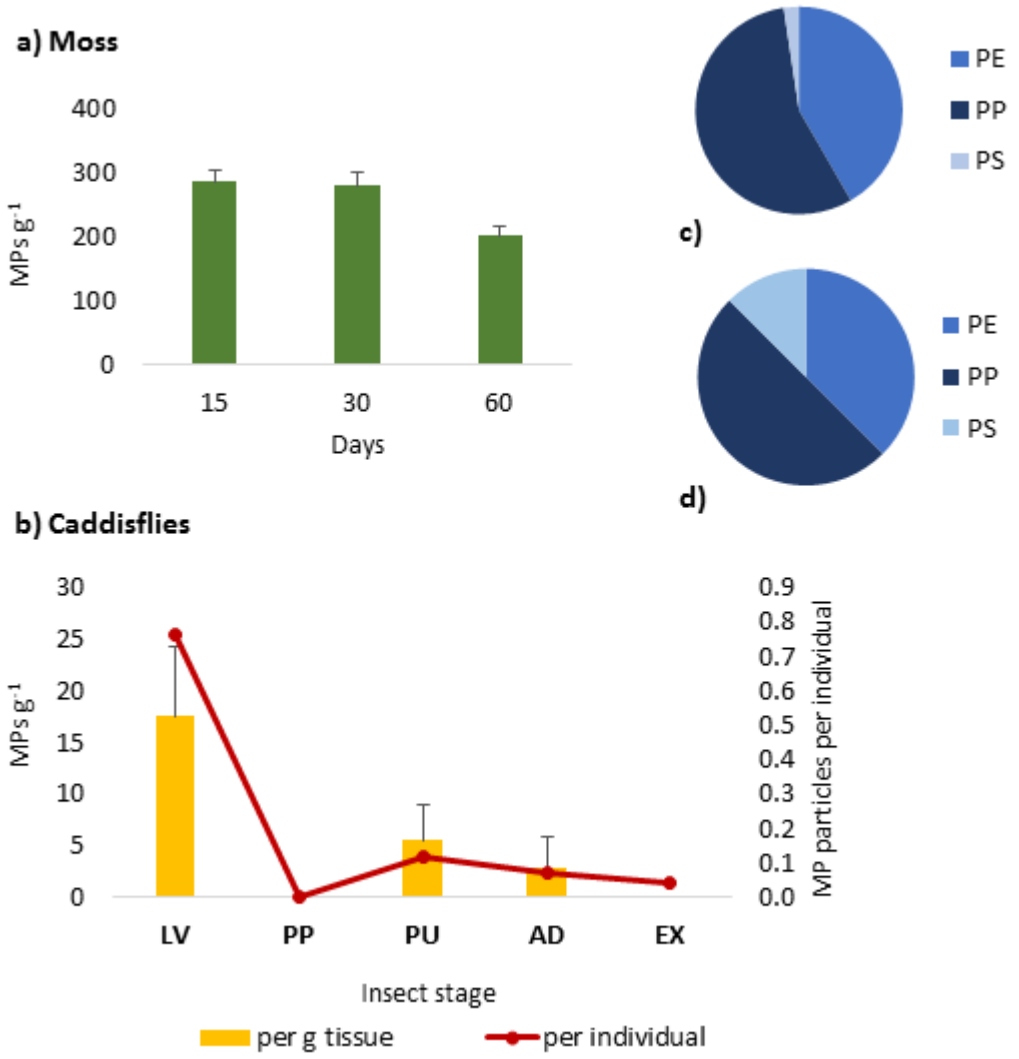
The presence and distribution of MPs was assessed from samples of sediments, moss and caddisflies collected from all mesocosm treatments. The largest portion of MPs added to each aquarium was concentrated in the moss (254.5 ± 14.08 particles per gram dry weight (DW)) (Fig. 2a). Concentrations of MPs in moss varied over time ($F_{(3,20)}$ = 61.71, P < 0.001; Table S1), with concentrations increasing rapidly to maximum measured levels on day 15 (284.65 ± 17.42 particles per g DW), remaining high at day 30 (278.31 ± 19.79 particles per g DW), and significantly dropping towards the end of the experiment (200.56 ± 16.08 particles per g DW) (Fig. 2a; SI Table S1). Adsorption onto the surface of the moss appeared to reach its saturation point by the first sampling date (Fig. 2a). This was likely due to the morphological characteristics of the moss, with its feathery leaf-like structures (Bhattacharya et al., 2010) and the positioning of the moss throughout the entire water column in the aquaria. In contrast, MPs accumulated in much lower concentrations in sediments, with an average of 1.19 ± 0.27 particles per g DW at the end of experiment (accounting for approximately 4% of total MPs added to the aquaria; SI Fig. S2).

The slow particle settling observed here could be attributed to the dynamics of fouling, as the development of a microscopic biofilm alone may not have been enough to increase the density of MPs to the point of sinking in the timeframe of our experiment (Fazey and Ryan, 2016; Kaiser et al., 2017; Miao et al., 2021). Although the sinking velocity of MPs has been measured to range between 0.0056 and 0.03 ms^{-1} in freshwater systems (Kowalski et al., 2016; Leiser et al., 2020), research has shown that this process can take over 7 weeks for particles with a lower density than water (i.e., PE), even when biofouling occurs (Amaral-Zettler et al., 2021;

Leiser et al., 2020). It therefore appears that most of the MPs remained buoyant within the water column, where they were adsorbed to the surface of the free-floating moss.

It is well-known that plants can adsorb air- and waterborne plastic debris in freshwater and terrestrial ecosystems (Dovidat et al., 2020; Liu et al., 2021; Mateos-Cárdenas et al., 2019; Yin et al., 2021a). However, only few studies have examined the adsorption and accumulation of nano- and MPs in mosses (Capozzi et al., 2018; Roblin and Aherne, 2020). As an estimated >50% of MPs were found adsorbed to the surface of the moss in the enclosures by the end of the experiment, our study offers evidence highlighting the capacity of moss to act as a sink for free-floating MPs in freshwater environments (SI Fig. S2). This could have significant ramifications for natural freshwater ecosystems containing moss. Although sediments are currently considered sinks for both high- and low-density MPs (Kabir et al., 2022; Kaiser et al., 2017; Thompson et al., 2004; Van Cauwenberghe et al., 2015; Vianello et al., 2013), we show that buoyant MPs (e.g., PE and PP) from contaminated water bodies may be removed from the water column via adsorption onto the surface of moss sooner than it would take for biofouling and subsequent sinking to occur.

Concentrations of MPs in the moss significantly declined by the end of the experiment ($F_{(3,20)}$ = 61.71, P < 0.001; Fig. 2a; SI Table S1); however, concentrations were still high compared to the distribution of particles elsewhere in the aquaria. This decline may have been due to a combination of (i) grazing by caddisfly larvae (*M. nycterobia* mainly feeds as a shredder; (Graf et al., 2022), resulting in a decrease of the leaf-like structures of the moss, in turn reducing the amount of surface area for adsorption (Gutow et al., 2016; Kalčíková et al., 2020), and (ii), biofouling, which may have increased towards the end of the experiment.



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310 Fig. 2. Concentration of microplastic particles a) adsorbed on moss and b) in caddisfly tissues
311 (LV-larvae, PP – prepupae, PU – pupae, AD – adult, EX – exuviae). Average ratio of polymer
312 types in c) moss and d) caddisfly samples across all sampling days.

313

314 *3.2. Microplastic particles rapidly eliminated across caddisflies life stages*

No MPs were detected in the control treatments of any caddisfly life stage (Fig. 2b). Concentrations of MPs varied significantly between life stages ($F_{(3,18)}$ = 5.66, P = 0.007), with average concentrations at least 7x higher in individual larvae compared to prepupae, pupae and adults (Fig. 2b; SI Table S2). Similar results were obtained when comparing concentrations per dry weight ($F_{(3,18)}$ = 4.34, P = 0.018), with MP levels detected in larvae higher than those measured in prepupae and adults (Fig. 2b; SI Table S3). Thus, our results suggest feeding as a major route of exposure of the shredding caddisfly species to MPs (Redondo-Hasselerharm et al., 2018; Scherer et al., 2017; Windsor et al., 2019), and indicate a trend of declining concentrations between feeding and non-feeding stages of caddisflies. Furthermore, the caddisflies larvae we left aside for 24 h to determine the impact of gut clearance on the accumulation of MPs measured no particles in their tissues, suggesting that MPs were ingested and, to a large extent, eliminated as waste. Although studies have confirmed that the amount of ingested MPs in aquatic invertebrates can be significantly reduced by gut clearance (Mateos-Cárdenas et al., 2020; Windsor et al., 2019), our results also highlight the possibility that life cycle-specific development may play a critical role in the reduction of accumulated MPs in caddisflies. Similar results were observed by (Al-Jaibachi et al., 2018), who noted a significant reduction in concentrations of MPs with each new life stage of *Culex* mosquitoes. The reduction of MPs between larvae and other caddisfly life stages observed in our study is therefore likely not directly linked to metamorphosis, but rather to behavioural changes related to successive life stages, as the larva is typically the only feeding stage in caddisflies (Thorp and Rogers, 2011). It is important to note, however, that although the number of particles declined between feeding and non-feeding stages of caddisflies, there were still particles present in both pupal and adult tissues, accounting for approximately 0.13% of all MPs added to the enclosures (SI Fig. S2). As

adults of *M. nycterobia* usually emerge on substrates (mostly plants) protruding above the surface of the water, where they shed their exuvia, the recorded particles present in adults were likely accumulated in the tissues. These findings demonstrate that aquatic insects with both aquatic and terrestrial life stages could incorporate and retain MPs throughout their entire life cycle (Al-Jaibachi et al., 2018), which could have important implications on the movement of MPs from aquatic to terrestrial ecosystems, as noted for some emerging contaminants such as metal ions and PhACs-EDCs (Cetinić et al., 2021; Previšić et al., 2021). Furthermore, flying adult caddisflies serve as prey for a number of terrestrial predators (e.g., adult dragonflies and damselflies, spiders, birds, and bats) (Thorp and Rogers, 2011), opening the possibility for the transfer of MPs further up the food web.

MPs were also found in the cases of caddisflies larvae and pupae (SI Fig. S1; Fig. S2). A greater number of particles was detected in pupal cases (0.58 ± 0.23 particles /case) than in larval cases (0.25 ± 1.3 particles/case), which is expected due to the longer timeframe for the larvae to build the pupal case. Moreover, pupal cases in *M. nycterobia* are not built *de novo*, but rather include larval cases of last larval instars. Although the concentrations of MPs in cases were found to be similar between these two life stages ($F_{(1,11)}$ = 1.69, P = 0.22; SI Table S4), it is possible that a significant difference between the groups would have been seen with a larger sample size. Recent studies have found that caddisflies can incorporate MPs into their cases (Ehlers et al., 2020; Gallitelli et al., 2021; Tibbetts et al., 2018). Although the concentrations of particles detected in the cases collected in our study were much lower compared to those reported in (Ehlers et al., 2020; Gallitelli et al., 2021), it is important to note that concentrations of MPs measured in the substrate of these studies were 2 – 40x higher than those used in our study. Regardless, we must not disregard this mechanism, as it may serve for retaining MPs in

freshwater environments. Moreover, as MPs have been shown to adsorb and accumulate other contaminants, such as organic contaminants and trace metals (Lionetto and Esposito Corcione, 2021; Munier and Bendell, 2018; Teuten et al., 2007), it is possible that MPs may, in turn, act as vectors for the transfer of these contaminants to aquatic organisms (Bradney et al., 2019; Walters et al., 2008; Wang et al., 2021).

3.3. Polymer types detected in biota indicate ingestion of particles from moss

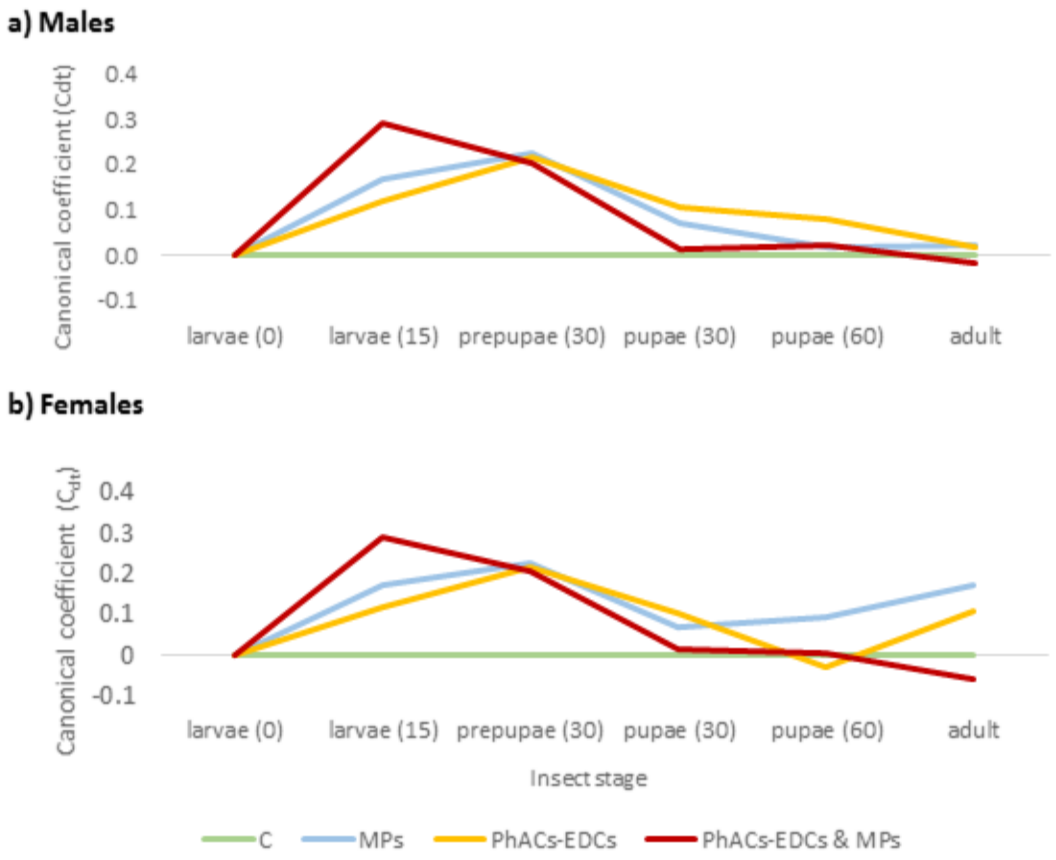
Samples of biota were further analyzed for the identification of polymer types. The predominant polymer types found in moss and macroinvertebrates were PP and PE, while PS was only rarely present in collected samples of biota (Fig. 2c&d; SI Table S5). Interestingly, however, the ratios of PP:PE:PS were similar across both moss and caddisflies (Fig. 2c&d), suggesting that caddisflies were inadvertently ingesting the adsorbed MPs when feeding off the moss, and offering further evidence that feeding was the primary exposure route of caddisflies to MPs (Foley et al., 2018; Nelms et al., 2018; Redondo-Hasselerharm et al., 2018; Scherer et al., 2017; Windsor et al., 2019). Although no studies have directly linked the transfer of MPs from moss to benthic macroinvertebrates, a recent study has found that the freshwater amphipod *Gammarus duebeni* ingested adsorbed PE particles by feeding on duckweed (*Lemna minor*) (Mateos-Cárdenas et al., 2022). Similarly, (Windsor et al., 2019) found evidence for the ingestion of MPs by caddisflies and mayflies in a natural riverine environment, while others have also reported on the uptake of MPs by freshwater invertebrates through feeding (Foley et al., 2018; Nelms et al., 2018; Redondo-Hasselerharm et al., 2018; Scherer et al., 2017). Our results show that moss may serve as a source and pathway for MPs to enter aquatic food webs due to

primary producer – herbivore interactions and emphasize the need of furthering our understanding of the role moss plays in the flux of MPs.

3.4. Ingestion of microplastics causes stress comparable to PhACs-EDCs exposure in caddisflies

To broaden our understanding of the impact MPs have on moss and caddisflies following observations of their interactions with the particles (i.e., adsorption and ingestion, respectively), we examined changes in the metabolic profiles of moss and caddisflies and measured total protein content as early warning signs of stress. Our results show that MPs induced significant changes in the metabolic profiles of caddisflies throughout the experiment (Fig. 3). Compared to the control treatments, the largest deviations were observed in larval and prepupal stages, while these differences were less pronounced in pupal stages, Fig. 3. This is somewhat expected as larval stages feed extensively, causing MPs to pass through their digestive system, in turn leading to stress and alterations of their metabolism. This is in accordance with findings from (Silva et al., 2021), who found that the ingestion of polyethylene MPs by the dipteran larvae *Chironomus riparius* triggered a stress response likely linked to damage in the epithelial cells of the gut lumen. Although prepupal stages do not feed and gradually become inactive, differences in the metabolism with respect to the controls were still high, suggesting active stress response mechanisms. The smaller differences in the pupal stage may indicate a stabilization of the physiological condition of the insects, or perhaps that responses to environmental stress were obscured by the intense metabolic changes brought on by the onset of metamorphosis. However, one cannot rule out either interpretation, nor perhaps that a combined effect of both processes occurred.

It is interesting to note that exposure to PhACs-EDCs exhibited a similar variance in metabolite datasets as did exposure to MPs (Fig. 3), suggesting that stress induced by microplastics ingestion is comparable to that induced by a mixture of PhACs-EDCs. Although research has shown that PhACs-EDCs pollution induces stress in aquatic macroinvertebrates (Gómez-Canela et al., 2016; Previšić et al., 2020), our current findings expand this prior knowledge by exhibiting the dynamics of this stress-induced physiological adjustment of caddisflies. While the individual effects of MPs and PhACs-EDCs on metabolite variability were similar, there is indication of an additive effect between MPs and PhACs-EDCs, Fig. 3. However, the additive effect was only observable at the beginning of the experiment (larval stage, 15 days) and diminished at the prepupal stage, suggesting, on one hand, intense metabolic activity, as well as increased levels of stress when organisms are exposed to multiple stressors and, on the other hand, a fast rate of adaptation to a multiple-stressors environment (Overgaard and Sørensen, 2008; Pallarés et al., 2017). Studies on the effects of MPs in combination with other stressors are slowly emerging (e.g., at elevated temperatures the negative impact of MPs on the growth and survival of shellfish increases (Green et al., 2019)). Unfortunately, as virtually none of the studies examine the combined effects of MPs and PhACs-EDCs in freshwater insects, it is challenging to draw comparisons. We can, however, offer evidence in support of the potential of MPs to induce combined effects in combination with certain stressors.



424

425 Fig. 3. PRC of changes in the metabolic profiles of caddisflies life stages (a) males and (b)
426 females exposed to treatments in relation to control (males - $F_{(1,48)}$ = 76.15, females - P<0.05
427 $F_{(1,48)}$ = 80.89, P<0.05, SI Table S6). Numbers in brackets denote days of exposure.
428 Abbreviations of treatments are as follows: C – control, MPs - microplastics, PhACs-EDCs -
429 pharmaceuticals and endocrine disrupting compounds, and PhACs-EDCs & MPs -
430 pharmaceuticals and endocrine disrupting compounds combined with microplastics.

431

432 *3.5 Female caddisflies exhibit stronger individual responses to stress than males*

433 Towards the completion of metamorphosis, it was possible to determine the sex of individual
434 caddisflies, allowing the possibility of observing sex-specific responses and interactions among

stressors which may have been obscured by the limited "sex resolution". Female late pupae (60 days) and adults displayed a greater variance of metabolites compared to males in stressed and multiple stressed treatments (Fig. 3a&b). These findings are in agreement with previous studies that showed a stronger response of female insects compared to males when exposed to stressful conditions such as parasite infections, predators, food quality, chemical stress, and impacts of climate change (Lindsey and Altizer, 2009; Slos et al., 2009; Stillwell and Davidowitz, 2010). This can be explained by the different evolutionary roles of males and females, which have led to the development of different stress-defense mechanisms. The increased response of females is most likely related to their functions following metamorphosis, which are both much more complex and more energetically demanding than those of males. Since the fitness of females in most insects is closely related to life expectancy and the number of offspring, greater benefits are achieved by developing a defense mechanism against stress. On the other hand, the fitness of males is related to the number of matings (Boots and Begon, 1993; Schmid-Hempel and Ebert, 2003; Slos et al., 2009), forcing males to compromise between a developmental mechanism that will allow them to survive longer and an investment in fitness components that will allow them to mate (compete) (McKean and Nunney, 2005).

3.6 Delayed development of individuals exposed to microplastics might be associated with changes in the metabolism of juvenile hormones

Metabolites belonging to fatty acids, glycolipids, terpenoids and carboxylic acid showed the most significant changes in abundance with respect to stressor treatments (SI -Fig. S3), with the most abundant classes being fatty acids and conjugated fatty acids. In our study, we recorded an increase in the abundance of stearolic and tetradecanoic acid (myristic acid), especially in the

larval and prepupal stages of *M. nycterobia* across all three treatments (MPs, PhACs-EDCs and MPs & PhACs-EDCs) (SI Table S7). Similarly, elevated concentrations of tetradecanoic acid were observed in aphid insects exposed to thermal stress (Chen et al., 2005). Besides playing a crucial role in energy storage, membrane structure and regulatory physiology in invertebrates (Silva et al., 2017), lipids are highly involved in metabolic pathways, hence, they are usually used as biomarkers of stress (He and Ding, 2020; Tagliaferro et al., 2022). Furthermore, profile alterations in saturated fatty acids have been reported as an effect or an adaptive response of exposure to contaminants, starvation and increased temperature (e.g. Fokina et al., 2013; Guerzoni et al., 2001; Rocchetta et al., 2006).

Another interesting member of the lipid class of metabolites, jasmonic acid (JA) and its precursor 12-oxo-phytodienoic acid (OPDA), recorded increased concentrations in caddisflies exposed to PhACs-EDCs and PhACs-EDCs & MPs treatments (SI Table S7). JA and OPDA are well-known phytohormones of the jasmonate family, that play a central role in mediating plant defense responses against insect herbivores, slowing down their growth and development (Chen et al., 2019). As *M. nycterobia* larvae are herbivores, feeding mainly through shredding moss, the presence of defense chemicals is expected. However, as moss showed higher levels of stress in treatments with PhACs-EDCs and MPs & PhACs-EDCs (see Section 3.8), the increased levels present in *M. nycterobia* may be an indication of a stress response to abiotic stress in moss, rather than the feeding itself. It is interesting to note that only the plastidic part of the JA biosynthesis route is present in moss, resulting in the JA precursor OPDA, while the peroxisomal route genes are absent (Monte et al., 2018). This explains the lack of detectable JA in our moss samples (see Section 3.9). The source of JA in our insect samples is unknown, however it is worth noting that JA itself is non-toxic to insects, while OPDA is potentially toxic (Shabab et al.,

2014). Here we speculate that the metabolite tentatively identified as JA might be produced from moss-synthesized OPDA during detoxification in insects, similarly to the suggested inactivation of OPDA by isomerization (Shabab et al., 2014).

In addition to changes in lipid metabolism, juvenile hormones (members of the terpenoid class) increased in stressor treatments (Table S7). Juvenile hormones are very important metabolites, as they regulate many aspects of insect physiology, e.g., postembryonic development and reproduction of adult insects (Wheeler and Nijhout, 2003). In many insects, juvenile hormones have been shown to play a role in postponing metamorphosis (Bounhiol, 1938; Smykal et al., 2014; Wigglesworth, 1934), until individuals have reached an appropriate body size and stage (Smykal et al., 2014). Increases in the concentrations of juvenile hormones early in the last instar larvae phase block metamorphosis causing additional molting, while the untimely removal of these hormones causes the formation of precocious adult characteristics such as external genitalia, wings, or miniature pupae in holometabolic insects (Smykal et al., 2014). Accordingly, the increased concentrations of juvenile hormones (I and III) in *M. nycterobia* in our experiment may have led to the delayed development of individuals exposed to PhACs-EDCs and MPs. This is in accordance with observed delays in the emergence of individuals from treatments exposed to MPs, PhACs-EDCs and MPs & PhACs-EDCs compared to controls (SI Fig. S4).

3.7 Stressor treatments nonspecifically increase total protein concentration in caddisflies

The temporal dynamics of total protein levels in caddisflies matched those of metabolites, suggesting increased cellular activity during stress responses. Significant differences were observed between treatments but also between life stages ($F_{(3,16)} = 4.18$, $P < 0.05$ and $F_{(5,80)} = 61.25$, $P < 0.05$ respectively). It is important to note that the differences in total protein

concentration observed between larvae collected from days 0 and 15 were most likely due to stress caused by the experiment. Furthermore, an interaction effect was observed between treatments and life stages ($F_{(15,80)}$ = 2.19, P < 0.05), suggesting that the total protein concentration in different experimental treatments was different across developmental stages. In particular, larvae sampled on day 15 exhibited higher total protein concentrations in stressed treatments than in the controls (Fig. 4). These higher protein concentrations recorded in stress treatments align with previous observations (Browne et al., 2014; Shen et al., 2014; Zielińska et al., 2021) that show an increase in total protein concentration following exposure to stress, most likely due to the activation of intracellular defense mechanisms consisting of a strong protein base. Nonspecific defense mechanisms, as the dominant system response found in insects (Rowley and Powell, 2007), may explain why there were no statistical differences observed among stress treatments (SI Table S8). However, it is worth noting that only protein characterization (which was not the focus of this study) may distinguish the specificity of the insects' system response to stressors. Similar to metabolite dynamics, total protein concentrations of later developmental stages (prepupae and pupae) in stressed treatments were not different from the control and among stages, suggesting similar activity on both metabolite- and protein-level. The temporal pattern of proteins recorded in our study, i.e., the increase in total protein concentration (compared to controls) followed by depletion, has been shown to occur in fishes exposed to heavy metal salts (Gopal et al., 1997), albeit on a shorter time scale. Regarding the sex-specific response, we found that females had a higher total protein concentration than males (($F_{(1,16)}$ = 11.89, P < 0.05; Fig. 4), however with no interaction with treatment ($F_{(3,16)}$ = 0.91, P > 0.05). This is in alignment with the higher protein content found in

female insects due to a higher protein "investment" in reproductive organs and imaginal tissues (Tojo, 1971).

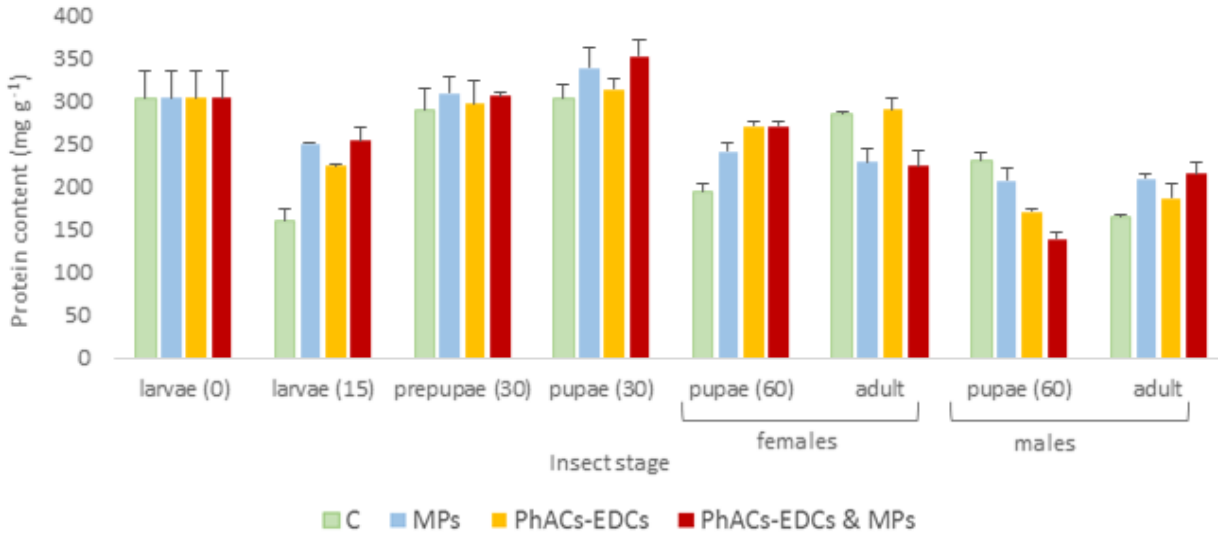
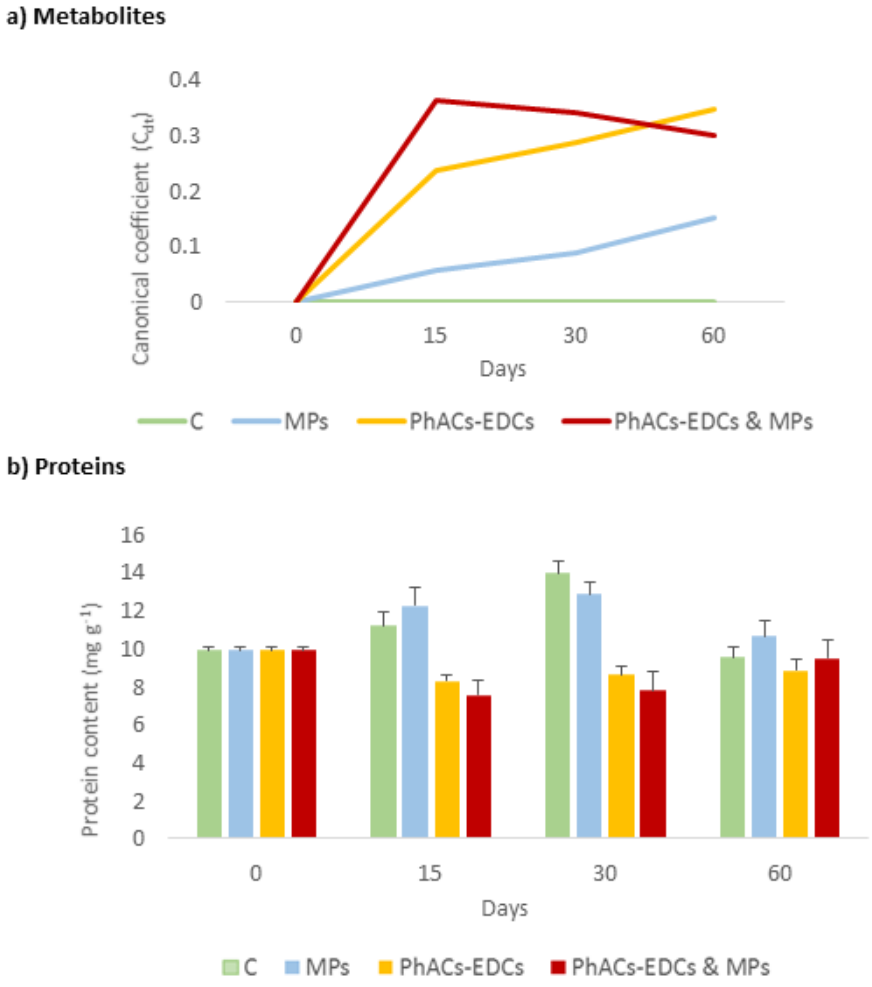


Fig. 4. Total protein content in different life stages of caddisflies in control samples (C) and samples treated with: MPs – microplastics, PhACs-EDCs – pharmaceuticals and endocrine disrupting compounds, and PhACs-EDCs & MPs – pharmaceuticals and endocrine disrupting compounds combined with microplastics. Numbers in brackets denote days of exposure. Accompanying statistics can be found in SI Table S8.

3.8 Moss exhibits a mild response to microplastic stress

In contrast to what was observed in caddisflies, here we observed a significant difference in metabolic profiles of moss exposed to MPs vs. PhACs-EDCs (Fig. 5a). The MP treatments triggered a mild metabolic response in moss, while the remaining treatments (PhACs-EDCs, and the combination of PhACs-EDCs and MPs) induced more intense changes (Fig. 5a). This shows that the impact caused by a stressor depends on the properties of both the stressor and receptor.

PhACs-EDCs are dissolved in water and can be easily absorbed by both caddisflies and moss, thus causing stress and affecting metabolic profiles (Matich et al., 2019; Previšić et al., 2020)(Matich et al., 2019; Previšić et al., 2020)(Matich et al., 2019; Previšić et al., 2020)(Matich et al., 2019; Previšić et al., 2020)(Matich et al., 2019; Previšić et al., 2020)(Matich et al., 2019; Previšić et al., 2020). On the other hand, MPs remains at the surface of moss (Yin et al., 2021a), while caddisflies ingest MPs, which can in turn have various adverse effects – slowing down digestion and causing physical damage – *vide supra* and references (Pittura et al., 2018; Straub et al., 2017). These effects highlight that two receptors can differ largely in their response to the same stressor. While there is a difference in magnitude of the response, there is also a difference in the dynamic of the response to stressor treatments. Moss exhibited an intense response at the beginning of the experiment, followed by stabilization, primarily observed in the PhACs-EDCs, and MPs & PhACs-EDCs treatments, and less so in the MPs treatments (Fig. 5a). On the other hand, a much greater variability was observed in caddisflies, suggesting that sensitivity in the case of some receptors is not fixed and instead differs throughout their life span and between different life stages.



556

557 Fig. 5. a) PRC of changes in the metabolic profiles of moss exposed to treatments over time of
558 the experiment (P<0.05 $F_{(1,40)}$ = 105.08, P<0.05, SI Table S9). b) Changes of total protein
559 content in moss exposed to treatments. Accompanying statistics can be found in SI Table S10.
560 Abbreviations of treatments are as follows: C – control, MPs – microplastics, PhACs-EDCs –
561 pharmaceuticals and endocrine disrupting compounds, and PhACs-EDCs & MPs -
562 pharmaceuticals and endocrine disrupting compounds combined with microplastics.

563

564

3.9 Stressor treatments in moss induce changes in lipid metabolism

Fatty acids and their metabolites were the most abundant class of metabolites that exhibited change in experimental treatments relative to the controls (SI Fig. S3; Fig. S5). The changes in fatty acids metabolism observed here may have been a response of moss to the stressor treatments, as was seen in caddisflies. Fatty acids and their metabolites are involved in cell regulation and the signaling of various stress responses in plants (Okazaki and Saito, 2014). More specifically, we observed a change in concentration of alpha-linolenic acid and OPDA, metabolite members of the octadecanoid pathway formed in plastids (SI Table S11). OPDA is an important signaling molecule in the coordination of the response of moss to herbivores (Shabab et al., 2014). However, altered concentrations of OPDA observed in treatments with PhACs-EDCs and MPs & PhACs-EDCs (compared to the controls) may suggest an OPDA-mediated response against abiotic stress (i.e., PhACs-EDCs), rather than just responses against herbivore pressure. OPDA was also observed in caddisflies, suggesting that this metabolite may be food-derived in caddisflies. Metabolites of the peroxisome part of the octadecanoid pathway (e.g., JA) were not detected, which is in line with the absence of the peroxisomal route genes in moss (Monte et al., 2018). Regarding the changes in fatty acids metabolism, it is important to note that gemfibrozil, one of the PhACs-EDCs used in the treatments, participates and affects lipid acid metabolism (STITCH) (Szklarczyk et al., 2016). Thus, we cannot completely rule out the effect of gemfibrozil on the regulation of fatty acids and their metabolites in the treated samples.

587

588 *3.10 Microplastic treatments did not change total protein concentration in moss*

589 There was a significant main effect of experimental treatments on total protein concentration in
590 moss ($F_{(3,8)}$ = 11.71, P < 0.05; Table S1). However, pairwise comparisons revealed that these
591 differences were only seen in moss exposed to PhACs-EDCs and a combination of MPs and
592 PhACs-EDCs (Fig. 5b), with no significant changes observed in treatments exposed to MPs.
593 These findings differ from the trends we recorded in caddisflies but are congruent with
594 observations on metabolites (Fig. 5a), further supporting our observations of the mild impact of
595 MPs on moss. Furthermore, a significant interaction effect between time and treatment was
596 observed ($F_{(9,24)}$ = 6.39, P < 0.05; SI Table S10), indicating that the moss exposed to PhACs-
597 EDCs and a combination of MPs and PhACs-EDCs measured significantly lower protein
598 concentrations at days 15 and 30 (Fig. 5b). These results suggest that exposure to PhACs-EDCs
599 in general may reduce the expression of proteins and/or enhance proteolytic degradation in moss.
600 Most of the research examining the impacts of stress on plants (e.g., heat stress, drought, heavy
601 metals, pharmaceuticals) reports a strong increase in defense and heat shock proteins and a
602 decrease in total protein content (Akhzari and Pessarakli, 2016; Esposito et al., 2012; Gorovits et
603 al., 2020; Gulen and Eris, 2004; He and Huang, 2007). More specifically, observed activity in
604 the octadecanoid pathway may be linked with lower protein concentration, since in moss OPDA
605 is found to suppress the expression of a broad range of proteins (Toshima et al., 2014). However,
606 confirmation of these links calls for a thorough proteomics examination, which is beyond the
607 scope of this manuscript.

608

Conclusions:

This study shows that aquatic organisms play an important role in the flux of MPs in freshwater environments. Free-floating moss may have the capacity to remove MPs from the water column, in turn becoming a potential vector for the trophic transfer of MPs to caddisflies and other herbivorous organisms. With caddisflies retaining MPs through metamorphosis, these particles could also cross ecosystem boundaries, transferring from aquatic to terrestrial systems. Our study also found that exposure to MPs elicited effects on the metabolome and total protein content of moss and caddisflies. While moss exhibited a mild response to MP stress, caddisflies showed levels of stress comparable to exposure to PhACs-EDCs, as well as the potential for an additive effect when exposed to both stressors. These responses in caddisflies differed between adult males and females, likely due to differences in evolutionary roles. This study also tentatively identified metabolic alterations mainly related to lipids, such as the delayed emergence of caddisflies. Our findings advance the understanding of the flux of MPs in freshwater environments and contribute to the understanding of molecular mechanisms that were triggered by stressors.

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6. Competing Interests:

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

7. CRediT authorship contribution statement

Ivana Grgić: Investigation, Methodology, Formal analysis, Database search, Data curation, Visualization, Writing - original draft, Writing - review & editing

Katarina Cetinić: Investigation, Formal analysis, Writing - original draft, Writing - review & editing.

Ana Previšić: Conceptualization, Investigation, Methodology, Visualization, Writing - review & editing.

Zrinka Karačić: Investigation, Methodology, Writing - review & editing

Marko Rožman: Conceptualization, Methodology, Formal analysis, Software, Visualization, Supervision, Writing - original draft, Writing - review & editing, Funding acquisition

7. Supporting information:

Supporting info.pdf

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