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PET microplastics do not negatively affect the survival, development, metabolism and feeding activity of the freshwater invertebrate *Gammarus pulex*☆

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ABSTRACT

Over the past decade, microscopic plastic debris, known as microplastics, emerged as a contaminant of concern in marine and freshwater ecosystems. Although regularly detected in aquatic environments, the toxicity of those synthetic particles is not well understood. To address this, we investigated whether the exposure to microplastics adversely affects the amphipod *Gammarus pulex*, a key freshwater invertebrate.

Juvenile (6–9 mm) and adult (12–17 mm) individuals were exposed to irregular, fluorescent polyethylene terephthalate fragments (PET, 10–150 μm; 0.8–4,000 particles mL^{-1}) for 24 h. Results show that body burden after 24 h depends on the dose and age of *G. pulex* with juveniles ingesting more microplastics than adults. After chronic exposure over 48 d, microplastics did not significantly affect survival, development (molting), metabolism (glycogen, lipid storage) and feeding activity of *G. pulex*.

This demonstrates that even high concentrations of PET particles did not negatively interfere with the analyzed endpoints. These results contradict previous research on marine crustaceans. Differences may result from variations in the exposure regimes (e.g., duration, particle concentrations), plastic characteristics (e.g., type, size, shape, additives) as well as the species-specific morphological, physiological and behavioral traits. As a detritivorous shredder *G. pulex* is adapted to feed on non-digestible materials and might, therefore, be less sensitive towards exposure to synthetic particles. Accordingly, we argue that the autecology needs to be taken into account and that research should focus on identifying traits that render species susceptible to microplastic exposure.

1. Introduction

Plastic debris, especially its microscopic form termed microplastics (MP, commonly defined as < 5 mm in diameter), accumulates in aquatic ecosystems and is of concern due to their persistence, mobility and increasing abundance in marine and freshwater waterbodies (Auta et al., 2017; Eerkes-Medrano et al., 2015; Thompson et al., 2004). Due to their capacity to interact with or to be ingested by aquatic organisms, MP might be able to negatively affect ecosystem (reviewed by Cole et al., 2011; Eerkes-Medrano et al., 2015). So far, laboratory and field studies reported that over 160 different marine species ingest MP, including invertebrates, reptiles, fish, birds and mammals (reviewed by Lusher, 2015). In contrast, MP ingestion was confirmed in 39 freshwater species only, including crustaceans, annelids, insects, mollusks and fish (Scherer et al., 2017).

Ingested MP can trigger molecular, cellular or physiological effects (Browne et al., 2015). First investigations in freshwater amphipods and fish point towards particle-induced alterations of behavior, physiology and reproduction (Au et al., 2015; Carlos de Sá et al., 2015; Mattsson et al., 2015). Still, knowledge on the biological impact of MP is limited and sometimes conflicting, preventing a science-based risk assessment of synthetic polymers in freshwater systems (Wagner et al., 2014). To address this gap of knowledge, we investigated the uptake (i.e., body burden) as well as the effects of MP in the freshwater amphipod *Gammarus pulex*. The species is

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vastly distributed throughout European rivers and lakes (Engelhardt et al., 2015; Hynes, 1955) and widely used in ecotoxicological studies (De Lange et al., 2006; McCahon and Pascoe, 1988).

Laboratory studies have mainly analyzed the uptake and toxicity of uniform, spherical MP made of polyethylene (PE) or polystyrene (PS, Lusher, 2015). While PE and PS are among the most abundant polymers in the environment (Wagner et al., 2014), the impacts of other plastic types remain mostly unstudied. The same is true for other particle shapes: Because fragments, foams and pellets are more abundant in freshwater ecosystems than plastic spheres (Klein et al., 2015; Mani et al., 2015; Moore et al., 2011), we used irregular polyethylene terephthalate (PET) particles in this study. PET is predominantly used as packaging material and makes up to 7.1% of the total European plastic consumption (Plastics Europe, 2014). Studies by Klein et al. (2015) and Gasperi et al. (2014) highlight that PET MP, though not as dominant as the polymer materials PE and polypropylene (PP), make up an important part of the overall MP load in large European river systems. Furthermore, PET MP have been detected in several lake systems worldwide (Corcoran et al., 2015; Imhof et al., 2016; Zbyszewski and Corcoran, 2011; Zhang et al., 2016). Due to its density >1 g cm^{-3}, PET sinks rapidly and is especially available for benthic species such as *G. pulex*.

We investigated the ingestion of MP by the shredder *G. pulex* over a period of 24 h. We hypothesized that (1) *G. pulex* ingests MP and that (2) the body burden after 24 h of exposure depends on the particle concentration (2a) as well as the age (2b). Chronic toxicity was evaluated over 48 d. Based on the results of previous studies (Cole et al., 2015; Hämer et al., 2014; Lee et al., 2013; Rosenkranz et al., 2009) we hypothesized that (3) MP uptake affects the feeding activity which subsequently alters the metabolism (energy reserves) and development (molting) of *G. pulex*, ultimately resulting in a lower survival.

2. Materials and methods

2.1. Microplastics preparation

The MP particles were prepared from green fluorescent soft drink bottles made of PET (fluorescence excitation at 465–495 nm). The polymer type of the bottles was confirmed by ATR-FTIR spectroscopy (PerkinElmer, Spectrum Two, Waltham, Massachusetts). In brief, 1 g bottle material was snap frozen in liquid nitrogen and ground in a swing mill to produce irregular MP particles with a size of ≤150 μm in the largest diameter (for details see 2.1 in the Supplementary data (SD)). The surface structure of the particles was analyzed qualitatively by scanning electron microscopy (SEM, Hitachi, S4500, Krefeld, Germany).

2.2. Preparation of stock suspensions

A 40,000 particles mL^{-1} (p mL^{-1}) MP stock suspension was prepared by suspending 1.59 g MP in 1.5 L ISO medium (ISO, 1996). 300 mg L^{-1} cetyl alcohol pellets were added as water-insoluble surfactant to facilitate homogenous particle distribution. Further suspensions with nominal concentrations of 4, 40, 400 and 4,000 p mL^{-1} were prepared by serial dilution. Particle concentrations in the 40,000 and 4,000 p mL^{-1} suspensions as well as total particle volume in the 40,000 p mL^{-1} suspension were measured with a Coulter Counter (Beckman Coulter, Multisizer 3, Krefeld, Germany; software version 3.53, see 2.2 in SD). For the 4, 40 and 400 p mL^{-1} suspensions, actual particle concentrations were determined by filtering 1 mL on Metricel Black PES membrane filters (Ø 25 mm, Pall Corporation, Dreieich, Germany; pore size: 0.8 μm) and counting particles under a fluorescent microscope. Particle concentrations refer to MP in a size range between 10 and 150 μm. Particles <10 μm in the stock suspensions were not further examined due to analytical limitations of the fluorescence microscope methodology (see 2.3.1 in SD).

2.3. Uptake of microplastics

The uptake study included the test variables *particle concentration* (0.4, 40 and 4,000 p mL^{-1}) and *age* with two classes: juveniles (6–9 mm length) and adults (12–17 mm length, length measured from rostrum to telson). We here defined the smaller size class as "juveniles" although some of the larger individuals in this class may already be sexually mature (Welton and Clarke, 1980). Testing each exposure concentration with both adults and juveniles resulted in a total of six treatment groups with ten replicates of one individual each.

G. pulex was collected on May 1st, 2015 in the Urselbach near Frankfurt/Main, Germany (N 50° 10.249′ E 8° 37.100′) and kept for 7 d in a 20 L aerated aquarium with ISO medium at a temperature of 16 ± 1 °C and a 16:8 h light-dark cycle. Collected oak leaves (N 50° 12.797′ E 8° 30.819′) were cleaned twice with distilled water and provided as food source *ad libitum*.

24 h before the experiment *G. pulex* individuals were transferred to a separate 20 L aquarium with ISO medium to allow gut clearance from remaining food particles. 200 mL screw top glasses were filled with 45 mL ISO medium and 5 mL MP suspension each resulting in nominal exposure concentrations of 0.4, 40 and 4,000 p mL^{-1}. *G. pulex* individuals were exposed for 24 h at a temperature of 16 ± 1 °C, a 16:8 h light-dark cycle and constant aeration via glass pipettes. Aeration intensity was adjusted to a level that allowed PET particles to settle at the bottom of the glass vessel becoming available for *G. pulex*. Afterwards, individuals were removed from the vessels, cleaned from attached MP in 100 mL ISO medium and directly frozen at −80 °C. We determined the abundance of MP in the digestive tract of *G. pulex* qualitatively by direct examination under the fluorescent microscope (Olympus, BX50, Hamburg, Germany, Narrow Band (NB) filter, 100× magnification) as well as quantitatively by lysing the individuals enzymatically (for methodology details see 2.3.1 in SD). The lysates were filtered on black PES membrane filters (see 2.2), fixed on microscope slides and analyzed under a fluorescent microscope with the image analyzer software ImageJ (National Institute of Health, version: 1.46r, Rockville Pike, Maryland, USA). We examined both the total particle number on the filter surface as well as the size of the particles. Due to high concentrations of heterogeneously distributed particles overlapping each other on the filter surface, we could not accurately determine particle abundance in six individuals with the analyzer software. These replicates were consequently excluded from analysis (one adult in the 0.4 p mL^{-1} treatment, three juveniles in the 440 p mL^{-1} treatment, two adults in the 4,000 p mL^{-1} treatment, compare Table S1).

In the same way, we lysed four *G. pulex* individuals which were not previously exposed to PET particles to determine background contamination caused by lysis and analysis. In average, four green fluorescent particles per individual were observed in unexposed animals. Subsequently, all particle abundance results in the study were corrected by this blank value. In addition, the size distribution of particles in the 40,000 p mL^{-1} stock suspension was examined using the same analytical method as for lysates (see 2.3.1 in SD).

2.4. Effects of chronic microplastic exposure

In the effect study we tested the same variables as in the uptake study (particle concentration, age), but we used five MP concentrations (0.4, 4, 40, 400 and 4,000 p mL^{-1}) as well as a negative and