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Modulation of PAH toxicity on the freshwater organism *G. roeseli* by microparticles☆

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ABSTRACT

Polycyclic aromatic hydrocarbons are widespread and environmentally persistent chemicals that readily bind to particles in air, soil and sediment. Plastic particles, which are also an ubiquitous global contamination problem, may thus modulate their environmental fate and ecotoxicity. First, the acute aqueous toxicity of phenanthrene in adult *Gammarus roeseli* was determined with a LC_{50} of 471 μg/L after 24 h and 441 μg/L after 48 h. Second, considering lethal and sublethal endpoints, effects of phenanthrene concentration on *G. roeseli* were assessed in relation to the presence of anthropogenic and natural particles. The exposure of gammarids in presence of either particle type with phenanthrene resulted after 24 and 48 h in reduced effect size. Particle exposure alone did not result in any effects. The observed reduction of phenanthrene toxicity by polyamide contradicts the discussion of microplastics acting as a vector or synergistically. Especially, no difference in modulation by plastic particles and naturally occurring sediment particles was measured. These findings can most likely be explained by the similar adsorption of phenanthrene to both particle types resulting in reduced bioavailability.

1. Introduction

Polycyclic aromatic hydrocarbons (PAH) are widespread and environmentally persistent chemicals of concern. Their sources and distribution are well understood and documented (Abdel-Shafy and Mansour, 2016; Baek et al., 1991; Lima et al., 2005; Rabodonirina et al., 2015). Small quantities of the PAHs can be of biological origin, but most enter the environment from anthropogenic sources (Menzie et al., 1992). The highest quantity of PAHs is emitted into the atmospheric environment, where further distribution to soil and water occurs. Because of their lipophilic characteristics, PAHs readily bind to particles in air, soils and sediments, and tend to be rarely found dissolved in water (Abdel-Shafy and Mansour, 2016; Baek et al., 1991; Srogi, 2007). PAHs are known to cause potential adverse health effects for humans and animals (Abdel-Shafy and Mansour, 2016) and a wide range of toxicity-tests for various PAHs have been conducted (Barata Martí et al., 2005; Besse et al., 2013; McConkey et al., 1997). With respect to the lipophilic characteristics and known distribution of PAHs (Srogi, 2007), many of these studies focused on PAHs adsorbed to natural particles like sediment (Landrum et al., 1994; Lankadurai et al., 2011; Lotufo and Fleeger, 1997; Verrhiest et al., 2001). Thus, the effects of PAHs sorbed to sediment particles are well-known for many species. Due to the increasing occurrence in the environment, research has recently focused on the effects of chemicals associated with anthropogenic plastic particles (Kleinteich et al., 2018; Karami et al., 2016; Browne et al., 2013). Likewise to PAHs, plastic particles are ubiquitous in lake and stream ecosystems worldwide (Rezania et al., 2018; Sharma and Chatterjee, 2017; Strungaru et al., 2019). As reviewed by Dris et al., (2018), a median of 0.0026 particles/L surface water with a size of >50 μm was found in 63 rivers. In beach sediments, even 333 particles/kg dry weight in a size range between 0.5 and 5 mm were detectable. Water samples from

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anthropogenically influenced sites proved to be more burdened with microplastic in concentrations around 2.5 and 2.9 particles/L in China and 7.9 ± 7.3 microparticles/L in Germany compared to natural sites (Dris et al., 2018; Schmidt et al., 2018).

PAHs adsorb to and desorb from plastic (Alimi et al., 2017; Bakir et al., 2014a; Browne et al., 2013; Rios et al., 2010). Comparing the chemical properties, sorption capacity of microplastic seems to be slightly higher than for sediment, while adsorption kinetics of PAHs on microplastic or sediment can vary depending on many properties such as the chemicals hydrophobicity or sediment organic carbon content. Like for sediment, partitioning coefficient of the specific chemicals is one of the main drivers for sorption on microplastic and vice versa. However, the release of the chemicals from plastic particles seems to be faster (Wang and Wang, 2018). As a result, a discussion about possible synergistic effects of PAH and plastic particles evolved, specifically concerning the role of plastic particles to act as a vector (Alimi et al., 2017). Nowadays, nearly 80% of examined PE-microparticles from the north pacific central gyre were associated with PAHs (Rios et al., 2010). In samples from the Bejing river in the Feilaixia reservoir, all the microplastic particles found were associated with PAHs ranging from 282.4 ng/g for PP and 364.2 ng/g for PE (Tan et al., 2019). As many organisms ingest plastic particles (Cole et al., 2013; Cole and Galloway, 2015; Foley et al., 2018; van Cauwenberghe et al., 2015), a high potential for adverse effects exists evoked by transported chemicals due to the large surface of microparticles (Bakir et al., 2014a; Batel et al., 2016; Browne et al., 2013). However, modelling studies assume a negligible impact from plastic particles as vector, because of their insignificant small amount in the environment (Bakir et al., 2016; Koelmans et al., 2013). Yet, a tipping point may be reached at which the role of plastic in sorption, cleaning and transport of chemicals can become important (Koelmans et al., 2016) depending on the plastics' characteristics (Lee et al., 2018; Sun et al., 2019; Wang and Wang, 2018).

Studies simultaneous investigating the chemicals toxicity in the presence of anthropogenic particles, like microplastic, and natural particles are rare (Alimi et al., 2017). The studies investigating the effect of chemical-pollution-combined particles mainly addressed one aspect, e.g. toxicity of one chemical-associated-particle (sediment: Landrum et al., 1994; Lotufo and Fleeger, 1997, microplastic: Browne et al., 2013; Kleinteich et al., 2018). The comparison of the PAH-toxicity when dissolved or sorbed to different particle types remains largely unattended (Alimi et al., 2017). Due to the sorption characteristics of microplastic (described by Wang and Wang, 2018) and the previous studies with contaminant-associated particles, the combined effect of PAH and microplastic particles in the same compartment has to be tested and compared with natural microparticles. Therefore, the main goal of the present study was to compare the modulation of sublethal PAH-effects on gammarids by the presence of anthropogenic and natural microparticles. A preceding acute toxicity test for exposure of phenanthrene through water was performed to determine the LC_{50} via this exposure route and to determine the concentration range for sublethal toxicity measurements. On the assumption that sublethal endpoints are more sensitive and an earlier response than lethality (Maltby et al., 2002), *G. roeseli* were exposed to a range of sublethal phenanthrene concentrations with and without combination of polyamide (PA) or sediment microparticles (SP). These tests were intended to show the change in effect strength of phenanthrene to the gammarids by the presence of natural and anthropogenic microparticles. The measured endpoints were mortality, swimming behavior and feeding.

2. Materials and methods

2.1. Sampling and acclimatization of test organisms

Because of its environmental relevance, G. *roeseli* was chosen as test species. They have a widespread distribution, form a majority of biomass in stream ecosystems and play an important role in the food web (Gerhardt et al., 2011; Kelly et al., 2002). Furthermore, a high sensitivity towards a wide range of pollutants has been reported (Brock and van Wijngaarden, 2012; Hunting et al., 2016; Zhai et al., 2018). *G. roeseli* were collected from the river Moosach in Freising, Germany (48°23′38.8″N 11°43′26.1″E) between May and June 2018 and size selected (9.6 ± 1 mm, n = 50) by sieve passage. Acclimatization occurred in a climate chamber with constant temperature of 13 °C ± 0.5 °C and a 16:8 h light:dark cycle. First, individuals were kept in aerated aquaria filled with a mix of 50% bank filtrate from the river Moosach and 50% artificial water (ISO 6341 2012) for at least three days. During this part of the acclimatization period, gammarids were fed *ad libitum* with modified DECOTABs (Kampfraath et al., 2012). 10% of cellulose was replaced with ground Tetraphyll (Tetra GmbH, Germany), which was added as a supplementary food source (DECOTAB size 1 cm Ø, 0.5 cm high, dry weight 30.8 ± 3.0 mg (n = 150)). The subsequent acclimatization phase in 100% artificial water (ISO 6341 2012) started three days before the test. Gammarids were not fed during this step (US EPA, 2016). Mortality during acclimatization did not exceed 5%.

2.2. Test substances

Phenanthrene was chosen as a standard model PAH. It is one of the smallest PAHs with three benzene rings and a known low toxicity to humans compared to other PAHs (GESTIS Substance Database, 2019; Samanta et al., 2002). Due to its well-described chemical and toxicological characteristics (Verbruggen and van Herwijnen, 2012) it is often used as standard PAH (Barata Martí et al., 2005; Verrhiest et al., 2001; Zhang et al., 1997). Water solubility is 1.15 mg/L at 25 °C, so stock solution was made with methanol (99.8%). The Log K_{OW} is 4.52 (safety data sheet, IFA substance data base) and K_{OC} is around $2.97^{*}10^{4}$ ml/g (Zhang et al., 2009). Phenanthrene for toxicity testing (Phe, CAS: 85-01-8) and phenanthrene-d10 (CAS: 1517-22-2) for chemical analysis was purchased from Sigma-Aldrich (Germany), and stored at 8 °C. Methanol for chemical analysis of phenanthrene concentration in the artificial medium was purchased from VWR chemicals (Germany). Polyamide (PA), which has a high market share (Scheibitz and Spies, 2016), was used as an exemplary anthropogenic particle, while sediment particles from the river Moosach were used as natural reference particles. Granulates of PA were purchased from Lanxess (Cologne, Germany) as "Durethan C38F" (ISO 16396-PA 6/66, E, S14-020). The microparticles were generated by centrifugal milling the PA granulates and sieving (Ultra Centrifugal Mill Type ZM 200, Retsch, Germany). Particles were 40–63 μm in size, had a fragmental shape and were stored at room temperature (Fig. S1 B). With respect to Hartmann et al. (2015), stock solutions were prepared in 10 ml methanol, resulting in a concentration of 2.5 mg/ml for each stock solution. PA is stable in 100% methanol according to Bürkle GmbH (2011). Sediment microparticles (SP) were obtained from wet-sieved and dried fine sediment samples from the river Moosach in Freising, Germany. Particles were 45–63 μm in size, had a fragmental shape and were stored at room temperature (Fig. S1 A). A gravimetric measurement with Element Analyser EA 4000 (Analytik Jena) revealed total organic carbon of 1.64% (balancing method), limit of determination was 0.1%.

2.3. Bioassay

Toxicity determination was conducted in a two-step approach. As the main goal was to determine microparticle-induced changes in sublethal effects, an acute toxicity assay for phenanthrene was performed first to determine lethal thresholds for adult *G. roeseli*. Then, the main toxicity assay with sublethal concentrations was performed, based on the results from acute toxicity assay. Sublethal toxicity of Phe alone was compared with presence of polyamide (PA) or sediment microparticles (SP) and microparticles alone as described below.

The validity criteria and test conditions of all bioassays followed the Gammarid Amphipod Acute Toxicity Test (US EPA, 2016) with minor modifications in feeding protocol and test medium. Assays were conducted in 1 L appropriate aerated artificial water (ISO 6341 2012) in glass beakers under same conditions as acclimatization. Each beaker contained three glass stones as hiding places to reduce stress for the gammarids.

While gently mixing the stock suspension/solution to ensure a homogenous dispersion, Phe, PA and SP concentrations were pipetted directly into the medium. Following, five individuals were transferred into each prepared beaker and fed *ad libitum* with one modified DECOTAB. The parameters of the static assays were measured at the end of each treatment after 24 and 48 h with portable pH meter MultiLine® Multi 3630 IDS SET G (WTW, Germany). The exposure concentration was intentionally not maintained stable during the exposure period to show the reduction due to the adsorption behavior of the chemical, which is also a more environmentally realistic scenario.

2.3.1. Acute toxicity

Acute toxicity of phenanthrene was determined with a control, solvent control (methanol, 1 ml/L) and five concentrations of phenanthrene (nominal concentrations: 5, 50, 500, 1,000, 1500 μg/L, n = 3). Mortality was recorded after 24, 48, 72 and 90 h in the corresponding three replicates, which were then removed from the test. Therefore, the other treatments were not disturbed or stressed. Individual gammarids were classified as dead when they were not moving (including movement of legs), unbent or/and body color changed from white/grey to orange.

2.3.2. Sublethal toxicity

In order to determine the modulation of the sublethal phenanthrene toxicity in presence or absence of different microparticles, *G. roeseli* were exposed to eight sublethal exposure concentrations of phenanthrene in three exposure scenarios over 24 and 48 h: (1) phenanthrene alone (Phe), (2) phenanthrene and PA microparticles (Phe + PA), and (3) phenanthrene and natural microparticles (Phe + SP) in the water column. Nominal concentrations from 50 to 500 μg/L were chosen for the sublethal toxicity experiment in order to cover a range with expected sublethal effect concentrations. The nominal phenanthrene concentrations were: 50, 75, 100, 250, 315, 375, 435, and 500 μg/L. In consequence of the chemical analysis (2.4), the nominal concentrations were replaced by measured concentrations (Table 1) throughout the analyses and illustrations. Both, the sediment and PA microparticles were added at a concentration of 500 μg/L. Each treatment was replicated five times. Four treatments were added to serve as controls: medium only (control), medium with solvent methanol (solvent control) and two microparticle controls with SP and PA, respectively (SP control, PA control). Experimental setup was as described in chapter 2.3. After 24 and 48 h exposure, three endpoints were assessed including mortality, feeding rate and swimming behavior. The feeding rate, swimming behavior and chemical analysis (see 2.4) were determined for five test concentrations (50, 75, 100, 250 and 500 μg/L). Pooled samples for chemical analysis were taken after 0 and 48 h and stored at −20 °C until analysis.

2.3.2.1. Feeding rate

For feeding rate determination, DECOTAB leftovers were removed with a spoon at the end of the experiment (24 and 48 h) and transferred to small pre-dried aluminum dishes. The leftovers were dried for one day in a drying cabinet at 45 °C and then weighed with a Sartorius R200D Analytical Balance (Sartorius GmbH, Germany, 0.01 ± 0.02 mg). For the standard DECOTAB-weight, 150 freshly made DECOTABs were also dried for one day and weighed (30.8 ± 3.0 mg). To calculate feeding rate, leftover dry weight was subtracted from the standard DECOTAB dry weight. Estimated loss of weight was divided by the amount of living gammarids at the end of the exposure and calculated per day.

$$Feeding\ rate = \frac{DECOTAB\ standard\ dry\ weight\ [g] - DECOTAB\ leftover\ dry\ weight\ [g]}{living\ gammarids * test\ duration\ [d]} \quad (1)$$

2.3.2.2. Swimming behavior

A camera-based automated tracking system with the software Ethovision XT 9 (Noldus, Germany) was used for the measurement of velocity. Recording time was 10 min with a sample rate of 25 frames per second. The measurement setup was made of three cameras positioned 30 cm above 15 arenas. The five organisms of each beaker were transferred to five glass petri dishes (Ø 5.5 cm) filled with 10 ml of treatment water and placed under the camera in programmed arena position to ascertain individual swimming behavior. To avoid interfering influences of moving light or shadows a cardboard box was pulled over and a lightboard (M.Way, China) was placed under the setup. A ruler of 10 cm served as scale calibration standard in the videos. After automated tracking of *G. roeseli*, videos were manually checked for false detections and corrected if reasonable.

2.4. Chemical analysis

2.4.1. Instrumentation

Stir bars (Gerstel Twister®) coated with polydimethylsiloxane (PDMS, film thickness 0.5 mm, length 10 mm) were purchased from Gerstel GmbH & Co. KG (Germany). For incubation, 20 mL Headspace vials (Agilent, USA) with Sil/PTFE coated septa (Thermo Fisher, USA) were used. The Twisters were stirred on a multiple position magnetic stirrer (15 positions) from Thermo Fisher (USA). The thermal extraction/desorption-gas chromatography/mass spectrometry (TD-GC/MS) analysis was performed using a Gerstel

Table 1
Nominal and measured concentration of phenanthrene only and with microparticles after 0 and 48 h. * Label for samples not centrifuged, the other samples were centrifuged to remove microparticles. LOD for Phe was 2.5 * 10^{-3} μg/L.

Treatment	Nominal concentration	Measured concentration 0 h	Measured concentration 48 h
	[μg/L]	[μg/L] ± SD	
Control	< LOD	< LOD	< LOD
Solvent control	< LOD	< LOD	< LOD
SP control	< LOD	< LOD	< LOD
PA control	< LOD	< LOD	< LOD
Phe A	50	92.1 ± 0.1 *	19.9 ± 1.4 *
Phe B	75	78.9 ± 8.1 *	16.3 ± 0.5 *
Phe C	100	115.3 ± 2.2 *	21.1 ± 0.3 *
Phe D	250	329.3 ± 22.1 *	52.8 ± 10.6 *
Phe E	500	601.3 ± 42.8 *	141.8 ± 6.5 *
PA + Phe A	50	0.3 ± 0.4	0.0 ± 0.0
PA + Phe B	75	7.9 ± 5.9	2.2 ± 0.0
PA + Phe C	100	25.1 ± 3.3	4.6 ± 0.2
PA + Phe D	250	131.1 ± 3.4	28.3 ± 2.4
PA + Phe E	500	209.4 ± 5.3	34.1 ± 1
SP + Phe A	50	10.8 ± 0.3	0.9 ± 0.1
SP + Phe B	75	33.0 ± 0.6	1.5 ± 0.1
SP + Phe C	100	24.5 ± 0.6	4.3 ± 0.6
SP + Phe D	250	138.7 ± 3.4	27.0 ± 0.6
SP + Phe E	500	325.2 ± 10.1	24.4 ± 0.2

ThermalDesorptionUnit (TDU) 2 equipped with a Gerstel Multi-PurposeSampler (MPS) robotic ^{pro}, a Cooled Injections System (CIS 4) with C506 and an Agilent 7890B gas chromatograph equipped with an DB-5MS (Agilent) column combined with an Agilent 5977B MSD mass spectrometer.

2.4.2. Sample preparation and incubation

Standard solutions of phenanthrene and phenanthrene-d10 were dissolved in methanol. To obtain a calibration curve, the standard solutions were diluted in artificial water (ISO 6341 2012), adding 0.01 mg/L phenanthrene-d10 as an internal standard to a final volume of 10 ml. Samples containing sediment and microplastic particles were centrifuged before analysis. Water samples and 0.01 mg/L phenanthrene-d10 were diluted to a final volume of 10 ml in 20 ml headspace vials together with one stir bar and stirred at 24 °C, 1000 rpm for 60 min. The stir bar was removed, washed with LC/MS water, and dried with a lint-free tissue. Subsequently the stir bar was transferred into the thermodesorption tube. The limit of detection (LOD) for phenanthrene was 2.5 * 10^{-3} μg/L.

2.4.3. Chemical analysis

The concentration of phenanthrene was determined with stir bar sorptive extraction via TD-GC/MS. All measurements were conducted in triplicate. The TDU method was adopted from Ochiai et al. (2005). The TDU was set at an initial temperature of 20 °C (delay time 0.30 min; initial time 1 min) and a following ramp at 50 °C/min to an end temperature of 280 °C, hold time 1 min. The desorbed compounds were cryo-focused at −100 °C. Transfer from the stir bar to the cold trap was done in split-less mode. The CIS was programmed from −100 °C to 280 °C (held for 5 min) at 10 °C/s. Injection was done in a 1:100 split mode and the GC oven temperature gradient was as follows: 70 °C (2 min)/25 °C/min → 150 °C/3 °C/min → 200 °C/8 °C/min → 300 °C. Helium was used as carrier gas. The mass spectrometer was operated in the full scan mode (m/z range 40–550) with electron impact ionization (70V). Calibration concentrations were 50 μg/L to 0.05 μg/L.

2.5. Statistical analyses

Statistical analyses were conducted with RStudio (RStudio, 2015). Data were tested for normality using the Shapiro-Wilk test and homogeneity of variance with the Levene's Test. Due to low replicate number, non-normality and heterogeneity of variance, mortality was tested with exact Fisher-test (EF) for significance of deviation between the treatments to estimate whether the observed mortality is independent of the treatment and/or the microparticles. When EF showed a significant p-value (<0.05) pairwise Wilcoxon test with Benjamini-Hochberg correction (pW-BH, Benjamini et al., 1998) was used as post hoc test to identify those treatments significantly different to the control. After this, log-logistic regression (LL.2.5, LL.3, LL.4 and LL.5) was conducted to identify the best model fit. The models were compared with Likelihood Ratio-Test (LRT) and Akaike-Information-criterion (AIC). For swimming behavior, influence of beaker was tested first with ANOVA. For this, the model 'y = ß$_{00}$ + ß$_1$ * Treatment * beaker' was chosen because of assumed interaction of beaker and phenanthrene concentration. Since the model resulted in normal distribution and homogeneity of variance, ANOVA could be used. ANOVA showed just in one case out of five significant influence from beaker on the effect, which lead to the further exclusion of beaker effects. Due to resulting non-normality and heterogeneity, Kruskal-Wallis-test (KW) was conducted to test differences between the three exposure scenarios, followed by pairwise Wilcoxon test (pW). One-way ANOVA by Welch (AW) was used to test significant difference between the treatments. As post-hoc test pW-BH was used. Furthermore, linear regression was used to fit a model between concentration and velocity. Feeding rate was analysed with KW to test for differences between the three exposure scenarios. Additionally, AW was used to test for significant differences in the exposure scenarios, followed by the post-hoc test pW-BH. Furthermore, linear regression was used to fit a model between concentration and feeding rate. Data from GC/MS were acquired with MassHunter Workstation Software (Ver.B.08.000 from Agilent Technologies). Ion chromatograms were extracted for m/z 178.2 for phenanthrene and 188.2 for phenanthrene-d10. Integration was conducted automatically, and software supported. Data analysis was conducted with Microsoft Excel 2016.

3. Results

3.1. Water chemistry

Physicochemical parameters in medium measured at start and

termination of the exposure period were within the acceptable range for the test organism for all experiments and treatments (US EPA, 2016). The measured mean values (±standard deviation) were: Temperature 12.7 ± 0.6 °C; oxygen 101% ± 1.5%; pH 7.6 ± 0.2, and conductivity 677 ± 13 μS/cm.

3.2. Acute toxicity

The preceding test for acute toxicity was based on nominal test concentrations and revealed an increasing mortality with increasing phenanthrene concentration at all four timepoints (24, 48, 72 and 90 h). Phenanthrene revealed very similar effect responses, with low mortality at 5 (<10%) and 50 μg/L (7% and 13%), followed by a strong increase to more than 60% up to nearly 100% in the concentrations 500, 1000 and 1500 μg/L. LC_{50} was reached at a nominal phenanthrene concentration of 471.9 μg/L for 24 h, 441.1 μg/L for 48 h, 125.9 μg/L for 72 h and 83.6 μg/L for 90 h (Fig. S2 and Table S1).

3.3. Chemical analysis

The chemical analysis of the water samples from the sublethal toxicity assay indicated a discrepancy between predicted nominal and measured concentrations at the beginning of the experiment, with around 20% more phenanthrene in the treatment with Phe (Table 1). Phenanthrene concentration in all control treatments was below limit of detection ($2.5 * 10^{-3}$ μg/L). Exposure scenarios of Phe with microparticles showed a reduction of phenanthrene in the water column by 60% in the highest concentrations and 99% in the lowest concentrations compared to the Phe in samples from the start of the test. Compared to exposure scenario with Phe, concentration was constantly (0 and 48 h) more than 50% lower (mean 24% ± 18%) with microparticles in the water column. Phenanthrene concentration reduction was similar in plastic and sediment particle treatments. After 48 h, the measured concentration of phenanthrene revealed a decrease down to around 20% ± 3% of the measured initial concentrations in the Phe exposures and 17% ± 10% (Phe + PA) and 11% ± 7% (Phe + SP) of initial concentrations with microparticles.

3.4. Sublethal toxicity

Based on the LC_{50} of the preceding acute toxicity test, nominal concentrations with maximum 500 μg/L were chosen for the subsequent sublethal toxicity assay. Whole analysis was conducted with the measured concentrations of Phe after 0 h (Table 1).

3.4.1. Mortality

Phe exposure to expected sublethal concentrations resulted in a low mortality of *G. roeseli* not exceeding 20%, except for a phenanthrene concentration of 601 μg/L after 48 h (84%, pW, $p = 0.01$). Exposure scenarios with microparticles alone did not result in any significant differences in mortality of *G. roeseli* (pW, $p > 0.7$) irrespective of microparticle species (PA or SP) and exposure time (24 h and 48 h, Fig. 1). The mortality of G. *roeseli* exposed to Phe with additional microparticles (Phe + PA and Phe + SP) did not significantly differ neither to the Phe exposure nor among each other (EF, $p > 0.2$). The combined exposure resulted in lowered mortalities < 20% after 24 h (EF, $p > 0.1$). Only after 48 h at the highest concentration a significantly increased mortality occurred (Phe + SP, 36%, pW, $p < 0.05$). Though, models show clearly lower courses in mortality with microparticles added, especially for 48 h.

3.4.2. Feeding rate

Feeding rate was independent from microparticle exposure scenario, i.e. neither Phe nor the combination with SP or PA after 24 and 48 h influenced the gammarids' feeding (KW, $\chi^2 = 0.72$ for 24 h/0.68 for 48 h, $df = 2$, $p > 0.5$). Control feeding rates varied from 0.88 to 1.40 mg/gammarid*day within 24 h and 0.65–1.15 mg/gammarid*day within 48 h. Also, feeding rates within the treatments varied randomly (Fig. S3). In Phe exposures, there was no change in feeding rate with increasing phenanthrene concentration compared to the controls (AW, F(6,11.12) 24 h, F(6,12.13) 48 h, $p > 0.5$). Variation of feeding rates occurred only for Gammarids exposed to Phe + SP after 24 h (AW, F(7, 13.15), $p < 0.001$) at concentrations 80, 92 with higher feeding rate and 329 μg/L with lower feeding rate (pW, $p < 0.05$) and after 48 h at the highest concentration resulting in lower feeding rate (pW, $p = 0.28$).

3.4.3. Swimming behavior

Swimming behavior did not change after exposure to microparticles compared to controls and was on average 1.5 ± 0.7 cm/s and 1.8 ± 0.7 cm/s after 24 and 48 h (pW, $p > 0.5$ (PA) and $p > 0.4$ (SP)). Effect of phenanthrene on velocity after 24 and 48 h was significantly different in the presence of microparticles (KW, $\chi^2 = 59.8$ (24 h)/35.0 (48 h), $df = 2$, $p < 0.001$). Swimming velocity during exposure with Phe was lower than in exposures with Phe and microparticles (pW, $p < 0.001$) after 24 and 48 h. Phe with microparticle exposures (Phe + PA and Phe + SP) resulted in similar decreasing velocity (pW, $p = 0.77$ for 24 h, 0.93 for 48 h). Swimming velocity of *G. roeseli* decreased significantly with increasing phenanthrene concentration for the Phe exposure (Fig. 2, AW, F(6, 40.7/16.1), $p < 0.001$). At lowest concentration (80 μg/L), Phe resulted in decreased velocity of 0.3 cm/s slower than the control after 24 h (pW, $p = 0.018$) and 0.6 cm/s slower after 48 h (pW, $p < 0.001$). Velocity reduction coincided with increasing concentration. At highest phenanthrene concentration, gammarids swam with a speed of 0.27 (24 h) and 0.35 cm/s (48 h), which equals a reduction by 0.84 and 1.16 cm/s from control to 601 μg/L. The same negative trend occurred in exposures with Phe + PA (AW, F(7, 40.7/33.0), $p < 0.001$) and Phe + SP (AW, F(7, 78.3/14.3), $p < 0.001$). Although velocity was reduced even at lowest concentrations for both exposure scenarios and timepoints, significant difference was detected from 115 μg/L for Phe + PA (pW, $p < 0.01$ for 24 and 48 h) and from 92 μg/L for Phe + SP (pW, $p < 0.01$ for 24 and 48 h). Gammarids exposed for 24 h to phenanthrene and microparticle swam 0.21 (Phe + PA) and 0.17 (Phe + SP) cm/s at 601 μg/L, which is slightly slower than in the Phe exposure, while exposure over 48 h resulted in a comparable or faster speed in highest concentration (0.33 (PA) and 0.48 (SP)). Velocity reduction followed a linear relationship (Fig. 2).

4. Discussion

Against the common hypothesis, the measured influence of microparticles on the ecotoxicity of phenanthrene was reducing, ruling out their role as vectors. Furthermore, the potential of plastic particles acting as a more effective sorbent and carrier than natural microparticles has been relativized for polyamide and phenanthrene, because they did not modulate effects different to naturally occurring inorganic sediment particles based on effects on gammarids. Instead, the presence of microparticles revealed a reduction of mortality as well as changes in sublethal endpoints such as swimming behavior. This impact can be explained by adsorption of phenanthrene to the microparticles, resulting in a reduced bioavailability of phenanthrene.

Acute toxicity determination revealed delayed rapid mortality due the baseline toxicity of the hydrophobic chemical (Mayer and Reichenberg, 2006). Phe exposure resulted in mortality of adult *G. roeseli* following a sigmoidal dose-response curve over the

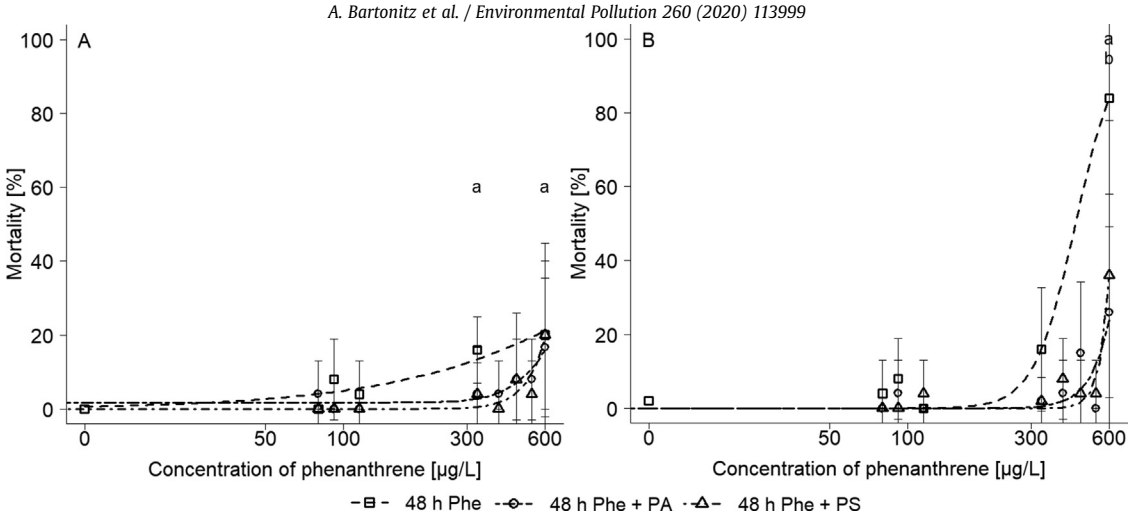

Fig. 1. Mean mortality of *G. roeseli* after 24 (A) and 48 h (B) exposure to phenanthrene alone (Phe) or in combination with PA or SP of 40–63 µm. Mortality was counted for dead (including eaten) gammarids and calculated per beaker (n = 5). Concentration range of phenanthrene (measured concentration 80–600 µg/L) was pre-selected from the acute toxicity test for sublethal effect concentrations. Dose-response-curves for exposure scenarios are shown by lines. Labels a (Phe), b (Phe + PA) and c (Phe + SP) mark significant differences to solvent control. Statistical information for regression are given in SI (Table S2). Error bars represent standard deviation.

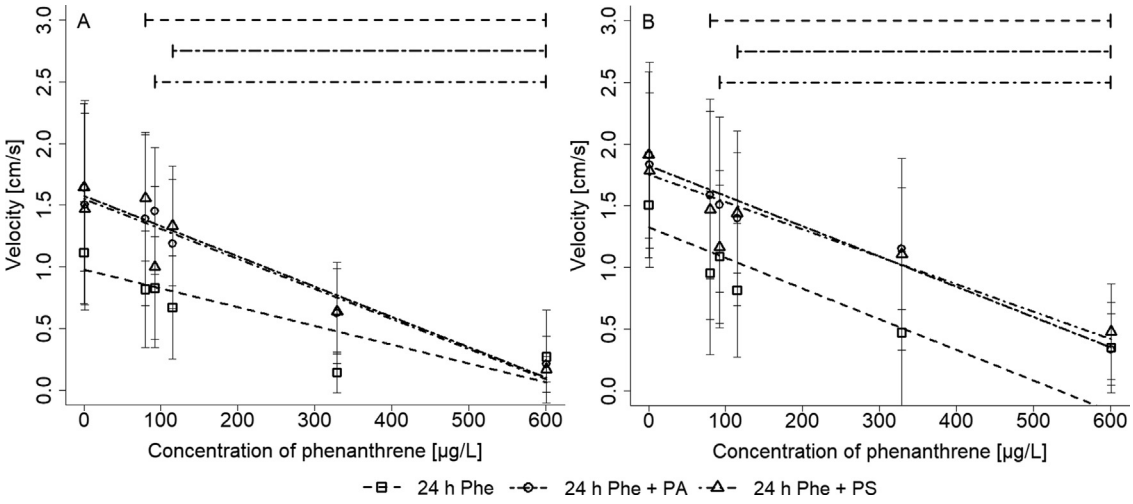

Fig. 2. Mean Velocity of *G. roeseli* after 24 (A) and 48 h (B) exposure to Phe and in combination with PA or SP. Error bars represent standard deviation. Linear regression for exposure scenarios are shown by lines. Horizontal Lines for Phe (dashed), Phe + PA (twodash) and Phe + SP (dotdash) mark significant differences to control. Velocity of individual *G. roeseli* was measured with Ethovision XT 9. Dead gammarids were excluded. Statistical information for regression are given in SI (Table S3).

course for 24, 48, 72 and 90 h. Acute toxicity thresholds (LC_{50}) of phenanthrene were 471.9 µg/L after 24 h and 441.1 µg/L after 48 h and further decreased over time. Thereby, *G. roeseli* was more sensitive to phenanthrene compared to other crustaceans like *Daphnia magna* (24 h LC_{50}: 678.4 µg/L - 853.7 µg/L (Verrhiest et al., 2001)) or copepods (*Oithona davisae,* 24 h LC_{50} at 440.2 µg/L – 604.2 µg/L (Barata Martí et al., 2005)). The sudden and strong increase in mortality after a steady low mortality can be explained by a critical concentration of the hydrophobic chemical that is reached in the lipid membranes (Mayer and Reichenberg, 2006; Mayer and Holmstrup, 2008). Phenanthrene might be tolerated by adult *G. roeseli* until a certain threshold is exceeded. Additionally, phenanthrene is hydrophobic with a log K_{OW} of 4.16–4.67 and most likely adsorbed to surrounding compartments such as food, particles or the organism (Teuten et al., 2007). As a consequence, there was after some time less phenanthrene available to the test organisms than at the beginning of the experiment, but at higher concentrations effective doses of phenanthrene remained. Also Lee et al. (2014) concluded a reduction of dissolved chemicals concentration and bioavailability due to sorption kinetics for hydrophobic chemicals and microplastics, but with the condition of a sufficient microplastic concentration. According to Bakir et al. (2014a) the plastic particle - water equilibrium of phenanthrene and polyethylene or polyvinylchloride can be reached within 24 h at a temperature of 18 °C. Therefore, this sorption is likely to happen in a relevant timescale. This is further supported by the measured phenanthrene concentrations in the sublethal toxicity experiment which were reduced remarkably after 48 h. It is thus likely that organisms not affected in the first hours of the exposure might not suffer from the bound phenanthrene in low concentrations but from remaining phenanthrene in higher concentrations.

The measurement of sublethal endpoints such as swimming behavior during the exposure of *G. roeseli* with sublethal phenanthrene concentrations proved to be highly sensitive, even at the lowest concentrations. The most important observation was the reducing toxic effect of both used microparticle types to phenanthrene, which follows a strong interaction of the contaminant with the present natural and anthropogenic microparticles. This effect is

most likely due to a reduced bioavailability of adsorbed phenanthrene. Concerning mortality and swimming behavior, another observation was that the degree of reduction was independent of added particle type. As supported by chemical analysis of the artificial water with and without particles, these outcomes can be explained by a fast and, most important, similar adsorption to both particle types. Also, with regard to the sublethal toxicity assay and as initially intended, mortality was very low in all exposure scenarios with phenanthrene (including cannibalism). Due to the low mortality, no change in mortality due to microparticles was observed. This is comparable to results of exposure with phenanthrene in combination with sediment on *Diporeia* spp., in which a low mortality around 12% ± 3% at a phenanthrene concentration of 110.5 μg/g sediment was found (Landrum et al., 1994). Moreover, when exposed to sediment-associated phenanthrene (max. conc. 333 mg/kg) for ten days (Gust, 2006), *Hyalella azteca* also showed low mortality (5% ± 8%). Therefore, focus in this assay was on the sublethal endpoints swimming behavior and feeding. The responses by sublethal endpoints are more sensitive compared to mortality and made an earlier detection of adverse effects possible. The exposure to polyamide and sediment particles in the used particle concentration without phenanthrene did not induce any adverse responses on the tested endpoints, as expected. Both, polyamide and sediment particles are very likely non-toxic, but it is possible that the potential toxicity of the plastic particles is not severe enough to be detected using these endpoints on the organism level. The molecular level could be a more sensitive level than organism level (Blarer and Burkhardt-Holm, 2016; Karami et al., 2016). Pplastic particle toxicity has been reported in other species exposed to a comparable size-class and concentration as used in this study. Karami et al. (2016) for instance reported damage on inner organs in *Clarias gariepinus* after exposure to < 60 μm sized low-density polyethylene particles in concentrations of 50 and 500 μg/L. Also Blarer and Burkhardt-Holm (2016) did not observe any impact on feeding rate and wet weight change, but in assimilation efficiency for *Gammarus fossarum* after application of polyamide fibres (2680 fibres/beaker) and polystyrene beads (12,500 beads/ml). This is in line with other studies not finding effects of plastic microparticles even at very high concentrations and relevant particle sizes according to endpoints from molecular to organism level (reviewed by Burns and Boxall, 2018). Consequently, it can be anticipated that there was no measurable effect from 40 to 63 μm sized polyamid microparticles as from sediments for *G. roeseli* at the tested concentration. Effect size of phenanthrene is lowered in the presence of particles due to adsorption to the particles. In this context, a strong influence of microparticles on phenanthrene-exposed gammarids' swimming velocity has been shown for 24 and 48 h. When exposed to phenanthrene and microparticles, gammarids swam in mean 0.5 cm/s faster than when exposed to Phe. Apart from this, feeding was not influenced by microparticles. Reduction of swimming velocity does not correspond with results reported by Ma et al. (2016) with *Daphnia magna* tested for 10 days with 0.05–1.2 mg/L phenanthrene. They found no impact at a concentration of 500 mg/L particles (10 μm) on the phenanthrene-induced effect. Though, phenanthrene-loaded low-density polyethylene particles < 60 μm resulted in higher molecular stress response than only phenanthrene exposure on catfish (Karami et al., 2016). The highly sensitive changes on the molecular level can thereby be seen as early warning signals, but cannot be interpreted regarding their adverse effects without a linkage to phenotypic consequences (Beggel et al., 2011; Beggel et al., 2012). Hence, rather than an undetected counteracting positive effect induced by the particles, because none was found in the particle control, this result indicates a decreasing bioavailability of phenanthrene due to adsorption to the microparticles. Consequentially, as the chemical potential of phenanthrene is decreased when adsorbed to microparticles, the measured lowered effect is most likely caused by the remaining dissolved chemical or from the chemical in the food (Koelmans et al., 2016; Kwon et al., 2017).

Several studies dealt with the distribution of PAHs and found concentrations ranging from mg adsorbed to sediment and μg to ng dissolved in water (Kafilzadeh, 2015; Luo et al., 2008; Rabodonirina et al., 2015). Thus, the tendency of PAHs to adsorb to plastic has already been proven (Alimi et al., 2017; Bakir et al., 2014a; Wang and Wang, 2018) and sorption equilibrium of phenanthrene to polyethylene was within 24 h (Bakir et al., 2014a). This is also confirmed by the analytically determined concentrations of the phenanthrene test solutions in this study. The artificial medium clearly exhibited a fast decline of phenanthrene concentration within 48 h to 20% of initial concentration with phenanthrene only and a mere fraction with microparticles rapidly after test start. More studies with varying particle species and sizes need to be conducted to verify if the particle type is an important factor for sorption and resulting effect modulation. Nevertheless, as stated by Bakir et al. (2014a) and shown by Rochman et al. (2017) or Lee et al. (2014), it depends on both polymer and pollutant properties. Therefore, the chemical potential and toxic effect of a contaminant is modulated by the presence of microparticles in the aquatic environment and this possibly depends on the respective particles surface quality, internal diffusion and chemical properties.

An open question is whether a toxic phenanthrene concentration is also reached in the organism if the substance is transported by ingested phenanthrene-loaded particles to the organism. The study by Bakir et al. (2016) indicates to some degree that the ingestion of loaded plastic is a negligible additional pathway compared to chemical uptake from the surrounding compartment or food. Ingestion and egestion of particles via food in μm size by *G. roeseli* is very fast and nearly in equilibrium with 6 and 8 ± 5 particles per minute, while uptake from surrounding medium was lower (unpublished data). Tracking of fluorescent particles showed egestion rates within 24 h comparable to Straub et al. (2017). Whilst sorption equilibrium of phenanthrene to polyvinyl chloride and polyethylene is reached within 24 h, desorption is slower with 5 days to reach equilibrium in seawater again (Bakir et al., 2014b). Although desorption of phenanthrene is faster under simulated gut conditions (Bakir et al., 2014a), it is likely that egestion of phenanthrene-associated particles is too fast for phenanthrene to desorb in an appreciable amount or even reach equilibrium to cause significant effects (Mohamed Nor and Koelmans, 2019). Even the assumed faster desorption from microplastic (Wang and Wang, 2018) does not intervene. Additionally, uptake of particles via the water is very low compared to ingestion via food, which lowers the possibility of phenanthrene uptake with particles again. Even if the particles are ingested, the release of the hydrophobic contaminant is very unlikely, intensified by the plastics characteristics like size and intraparticle diffusion (Seidensticker et al., 2019). Due to minimal ingestion and slow desorption kinetics, it can be concluded that suspended particles are negligible carriers for sorbed chemicals compared to the surrounding compartment. The used amount of solved phenanthrene and suspended polyamide particles in medium was higher and that of sediment particles lower than the known concentrations in freshwater. Consequently, especially environmental concentrations of phenanthrene, around 3–45 ng/L (Kafilzadeh, 2015; Luo et al., 2008), are most likely not leading to acute mortality or change in behavior of *Gammarus* spp. Partly, particle concentrations could represent a scenario where sediment and sand become a rare source and plastic particle concentration in environment rises as discussed by Koelmans et al. (2013) or Enders et al. (2015).

In view of environmental relevance, this study focused more on effect and modulation detection than environmental concentrations. Due to the aim of the study to see modulated effects, the higher concentrations were needed. Thus, with respect to their environmental occurrence, it seems that the impact of microplastic compared to natural microparticles is approximately equivalent. In cases like the observed, where the chemical adsorbs to microplastic and sediments to a similar extend, the higher bioavailability due to higher abundance of sediments makes sediment much more hazardous. There is an uneven higher probability for sediment to be ingested and release the chemical than for microplastic. Further, the adsorption and desorption of chemicals highly depends on the environmental parameters like abundance of organic matter (Seidensticker at al. 2017), pH or salinity (Wang et al., 2018). Also, after ingestion, the potential of the plastic particle to act as a vector and release the chemical highly depends on particle and chemical characteristics (Seidensticker et al., 2019). This indicates possible different behavior of the chemical in the environment than in static laboratory experiments and it has to be mentioned that the effects and modulation by microparticles could differ with the varying combinations. However, many recent model-based studies evaluated that the role of microplastic as a vector for anthropogenic pollutants not added to plastics is a common misconception (Mohamed Nor and Koelmans 2019; Koelmans et al., 2019). Nevertheless, it is necessary to examine a broad empirical area for chemical and (plastic) particle toxicity, as it is rising in the last years, to generate a stronger basis for risk assessment and regulation.

CRediT authorship contribution statement

Astrid Bartonitz: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing - original draft, Writing - review & editing. **Ihuoma N. Anyanwu:** Conceptualization, Investigation, Methodology, Writing - review & editing. **Juergen Geist:** Conceptualization, Supervision, Resources, Writing - review & editing. **Hannes K. Imhof:** Conceptualization, Formal analysis, Supervision, Writing - review & editing. **Julia Reichel:** Methodology, Investigation, Writing - review & editing. **Johanna Graßmann:** Methodology, Supervision, Writing - review & editing. **Joerg E. Drewes:** Resources, Supervision, Writing - review & editing. **Sebastian Beggel:** Conceptualization, Formal analysis, Methodology, Supervision, Writing - review & editing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2020.113999.

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