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Environmental Pollution

Tyre particle exposure affects the health of two key estuarine invertebrates☆

SL Garrard [a,b,*], JI Spicer [a], RC Thompson [a]

[a] Marine Biology and Ecology Research Centre, School of Biological & Marine Science University of Plymouth, Drake Circus, Plymouth, PL4 8AA, UK
[b] Plymouth Marine Laboratory, Prospect Place, Plymouth, PL1 3DH, UK

ABSTRACT

Tyre wear particles may be the largest source of microplastic to the natural environment, yet information on their biological impacts is inadequate. Two key estuarine invertebrates; the clam *Scrobicularia plana* and the ragworm *Hediste diversicolor* were exposed to 10% tyre particles in sediment for three days. Both species consumed the particles, although *S. plana* consumed 25x more than *H. diversicolor* (967 compared with 35 particles.g^{-1} wet weight, respectively). We then investigated the impact of 21 days exposure to different concentrations of tyre particles in estuarine sediments (0.2, 1, and 5% dry weight sediment) on aspects of the health of *S. plana* and *H. diversicolor*. Reductions in feeding and burial rates were observed for *S. plana* but not *H. diversicolor*, whilst both species showed a decrease in protein content in response to the greatest tyre particle concentration (5%), linked to an 18% decrease in energy reserves for *H. diversicolor*. Five percent tyre particle exposure led to an increase in total glutathione in the tissues of *H. diversicolor*, whilst lipid peroxidation decreased in the digestive glands of *S. plana,* possibly due to an increase in cell turnover. This study found that *S. plana's* health was impacted at lower concentrations than *H. diversicolor*, likely due to its consumption of large quantities of sediment. At the high exposure concentration (5%), the health of both invertebrates was impacted. This study did not separate the effects caused by the microplastic particles versus the effects of the chemical additives leaching from these particles, but our results do indicate that future studies should investigate effects in isolation and in combination, to determine the main drivers of toxicity.

1. Introduction

Contamination of the environment by tyre wear particles is an emerging concern, with estimations that 0.2–5.5 kg of tyre tread is released into the environment *per capita* per year, with emissions greater in the USA and Europe than in developing countries (Baensch-Baltruschat et al., 2020). This estimate makes these particles the single largest source of microplastic pollution in the natural environment (Baensch-Baltruschat et al., 2020; Lassen et al., 2015; Sundt et al., 2014), with models predicting that 8–40% of all road-produced tyre wear enters surface waters (Baensch-Baltruschat et al., 2020; Lassen et al., 2015), yet only a small proportion (2%) is predicted to reach the sea (Unice et al., 2019), suggesting retention within river and estuarine sediments. Black fragments, accounting for 73% of microplastics found in two South Carolina estuarine sediments, were considered to be tyre wear, although this identification was not confirmed by chemical tests

(Gray et al., 2018). In Terra Nova, Antarctica, black particles identified as synthetic rubber made up 94% of the mass of plastic debris in marine sediments (Munari et al., 2017).

Research into the global distribution and dynamics of microplastics is ongoing, and empirical studies are aiding in our understanding of their distribution. Unlike other microplastics, empirical studies mapping tyre wear distribution in the environment are sparse (Parker-Jurd et al., 2021). This is primarily due to the difficulty in extracting and identifying environmental tyre wear particles using conventional microplastic methods such as density separation and FT-IR spectroscopy (Baensch-Baltruschat et al., 2020). Detecting chemical markers of car tyre pollution in environmental samples using pyrolysis-gas chromatography-mass spectroscopy (Py-GC-MS) has been hailed as a potentially effective method for quantifying tyre wear pollution, using markers such as Dipentene for natural rubber and 4-vinyl cyclohexane for synthetic rubber (Miller et al., 2022). Analysis of environmental

samples using pyrolysis-GC-MS indicates that road run-off, *via* surface water drainage, is likely to be the principal pathway of tyre wear pollution into estuarine and marine bodies (Parker-Jurd et al., 2021). Analysis of tyre wear pollution in river and estuarine sediments across the Seine (Europe), Chesapeake (USA) and Yoda-Lake Biwa (Japan) watersheds found tyre wear pollution in 97% of samples, with the highest concentrations occurring in the Seine watershed, exceeding 1% sediment dry mass in some samples (Unice et al., 2013).

Our understanding of the biological effects of tyre wear contamination is inadequate. Sedimentary exposure to tyre particles affects terrestrial organisms, inducing oxidative stress in the earthworm *Eisenia fetida* (Sheng et al., 2021) and reducing reproduction and survival in the springtail *Folosomia candida* (Selonen et al., 2021). Aquatic exposure affects growth and swimming behaviour in estuarine species; the springtail *Menidia beryllina* and the mysid shrimp *Americamysis bahia* (Siddiqui et al., 2022). As well as the physical effects caused by ingestion of particles, tyre wear particles can exert chemical effects through leaching of a cocktail of organic additives and metals into the surrounding water column and sediments (Halsband et al., 2020), or directly from the particles into the gastrointestinal fluids or organisms, as has been observed in fish (Masset et al., 2022; Masset et al., 2021). Leachates, with the particles removed, have proved lethal to marine copepods with an LC_{50} of 35 g.L^{-1} for *Calanus* sp. and <5 g.L^{-1} for *Acartia longiremis* (Halsband et al., 2020), whilst exposure to leachates of 30 g.L^{-1} tyre particles impaired swimming behaviour and caused oxidative stress in *Limnocalanus macrurus* (Lehtiniemi et al., 2021). A leachate concentration of 0.08 g.L^{-1} was associated with elevated markers of lipid peroxidation and membrane instability in the mussel *M. galloprovincialis* (Capolupo et al., 2021). This suggests that the chemical additives associated with tyre particles that leach into the water column, are toxic to aquatic animals.

Whilst our understanding of the impact of tyre wear exposure is still in its infancy, microplastics, in general, have received more attention. Microplastic exposure has been shown to cause an array of sublethal impacts on marine invertebrate health such as gut blockage (Welden and Cowie, 2016), DNA damage (Ribeiro et al., 2017), oxidative stress (Bour et al., 2018; Browne et al., 2013; Jeong et al., 2017; Ribeiro et al., 2017), inflammation (Wright et al., 2013a), and a reduction in energy reserves (Sussarellu et al., 2016; Watts et al., 2015; Welden and Cowie, 2016; Wright et al., 2013a). The degree of impact varies according to the size, polymer type, shape, concentration, and composition of chemical additives (de Sá et al., 2018; Jeong and Choi, 2020; Wright et al., 2013b).

This study aimed to better understand the risks associated with tyre wear contamination in the benthic estuarine environment, by focussing on the effects of sedimentary tyre particle exposure on aspects of the function of two key estuarine invertebrates; the clam *Scrobicularia plana* and the ragworm *Hediste diversicolor*. These species were chosen as they play key roles in the structure and functioning of estuarine sediments (Boldina-Cosqueric et al., 2010; Moreira et al., 2006; Tankoua et al., 2013), and are an important food source for higher trophic levels (Cardoso et al., 2009). Both species are potentially exposed to sedimentary reservoirs of tyre particles in contaminated areas through feeding, or to leachates through absorbance across the surface of their skin. Furthermore, both are recognised as good test organisms for biomonitoring purposes (Berthet et al., 2011; Solé et al., 2009), with behavioural, biochemical and genetic effects observed as a result of pollution exposure (Buffet et al., 2011; Buffet et al., 2014).

Our objectives were two-fold: to determine whether (1) these animals consumed tyre particles mixed in the sediment and (2) increasing concentrations of tyre particle exposure were associated with negative effects on their health. Organism health was assessed by utilising a suite of behavioural (feeding and burial rates), physiological (condition index) and biochemical (energy reserves and oxidative stress) markers to incorporate indices of health at differing levels of biological organisation.

2. Material and methods

2.1. Tyre particle production and sediment characterization

Tyre particles were produced from four used car tyres (see Table S1 for details), with at least 5 mm tread remaining, by gentle continuous rotation of tyres against a 20" disc sander (VSM KP510e), to mimic friction caused by tyre tread against the road. To obtain microplastic particles, equal weight proportions of each tyre were ground for short bursts in a coffee grinder (RHoet, 25,000 rpm) with liquid nitrogen added to prevent burning (Redondo-Hasselerharm et al., 2018), before finally being sieved (250 μm mesh). Tyre particle size distribution was assessed using a Malvern Mastersizer 2000 (Fig. S1).

Observation of the tyre particles under high power magnification (Leica S9E stereomicroscope) revealed small quantities of aluminium oxide from the sander disc. Whilst this was a small portion (0.3% by weight of the tyre particles), in order to eliminate the possibility that any of the effects observed resulted from the presence of sanding disc grit, we included an additional control adding the equivalent concentration of sanding disc grit expected in the highest tyre particle treatment.

2.2. Animal collection and acclimation

S. plana, *H. diversicolor* and sediment were collected during low tide from an intertidal mudflat on the estuarine section of the River Yealm Devon, UK (4°03′28.36″ W, 50°32′24.51″ N), in April 2021. *S. plana* and *H. diversicolor* were removed from the sediment using a pitchfork, placed inside a bucket containing a layer of mud and returned to the laboratory within 4 h. Several buckets of surface sediments (top 2 cm) were collected to ensure an adequate supply of organic material for test organisms to use as a food source for the duration of the experiment. This site was chosen because of the relatively low levels of anthropogenic disturbance, indicated by the absence of urbanisation within a primarily agricultural catchment.

Similar sized *S. plana* (3.5 ± 0.5 cm shell width) and *H. diversicolor* (body weight 0.45 ± 0.1 g) were selected for use in experiments. They were maintained in two aquaria (vol. = 12 L) each filled with continually aerated, filtered (2 μm) sea water (Temperature = 13 °C, Salinity = 25), at a stocking density of 10 and 12 per litre respectively, and left for 3 days to acclimate to laboratory conditions and to allow gut depuration to ensure equal levels of hunger before use in experiments.

Sediments were prepared as follows: sediment was collected from the environment and sieved (2 mm) to remove macrofauna and debris then left to settle overnight to retain smaller sediment fractions and organic material. Triplicate sediment samples were dried at T = 105 °C for 24 h in an oven for wet: dry weight ratio and particle size characterised using a Malvern Mastersizer 2000.

Sediment had a grain size range of between 1 and 2000 μm and a mean size of 45 (±0.2) μm. The organic content of the sediment was 9.0 ± 0.2%. The mean size of tyre particles was larger than the sediment, but sizes overlapped (Fig. S1), with a size range of between 8 and 500 μm and mean size of 136 (±5) μm. Whilst particles were sieved through a 250 μm mesh, the Mastersizer measures the longest length, and some particles fell into the 250–500 μm size category. This is comparable to tyre wear particles produced under realistic conditions in a road simulator laboratory, which have a size of 4–350 μm with a mode of 100 μm (Kreider et al., 2010). Based on calculating particle volume (mL) and mass (g.mL-1), the average number of tyre particles per gram was 4.0 (±1.2) million.

2.3. Tyre particle ingestion

In a preliminary experiment to verify that tyre particles were ingested by both *S. plana* and *H. diversicolor*, individuals (n = 5 in each case) were exposed to 10% tyre particles.kg^{-1} sediment dry weight (dw) in individual Pyrex beakers (vol. = 1 L) containing sea water (T = 13 °C,

S = 25). After placement on the sediment surface, all individuals burrowed overnight and were considered healthy. After 3 days, individuals were removed and euthanised in liquid N_2, before being digested using 10% KOH (Fisher Chemicals) at T = 50 °C for 48 h and then vacuum filtered (2.7 μm glass microfibre filter paper (Whatman GF/D). The resultant digestate was then examined under a microscope (35x magnification, Leica EZ4) and visible particles were enumerated using the criteria of Knight et al. (2020): that the particle 1) was black, 2) did not crumble when compressed with a needle, and 3) returned to its original shape after compression. Agglomerations of particles were separated with the needle. To test the efficacy of this KOH digestion method for retrieval and quantification of tyre particles, mantle tissue of *S. plana* was spiked with 10 tyre particles before digestion. All 10 particles were recovered.

2.4. Experimental exposure to different concentrations of tyre particles

To expose individuals of both species to different concentrations of tyre particles, the following procedure was carried out. Replicate microcosms were constructed using transparent plastic aquaria (vol. = 3L) each containing 800 g wet weight (ww) of sieved sediment (n = 30; 2 species x 5 treatments x 3 replicates). Sediments were spiked with one of four concentrations of tyre particles: 0 (control and sanding grit control), 0.2, 1 and 5% tyre particles. kg^{-1} sediment^{dw} of sediment. These concentrations were chosen as analysis of sediments around the Seine watershed suggests sedimentary concentrations regularly exceed 0.2%, with some exceeding 1% (Unice et al., 2013).

Given the tendency of tyre particles to clump and float in sea water, tyre particles were first added to solutions of filtered (2 μm) sea water (vol. = 40 mL) with added surfactant (0.1% v/v, Tween-20, Sigma-Aldrich, USA) and vortexed for 2 min (Ziajahromi et al., 2018). The resultant suspension was transferred to a Pyrex beaker (vol. = 3 L) containing 800 g of sediment. Sediment and water were mixed *via* vigorous stirring with a fork for 5 min to homogenise before the resultant mixture was added to each mesocosm. If sediment colour (and therefore tyre particle distribution) did not appear homogenous, the mixture was stirred again. Sediment was overlayed with filtered (2 μm) sea water (S = 25) at a ratio of 3:1 (v:v), maintained at T = 13 °C, and continually aerated using an airstone linked to a compressed air supply.

After sediment settled overnight, six *S. plana* were added to half the microcosms and eight *H. diversicolor* to the other half. All individuals burrowed within 4 h. A water change (50%) was conducted twice a week and water quality parameters (temperature, pH, salinity, O$_2$, ammonia) were monitored regularly (Table S2). After 21 days, the experiment was terminated and individuals were removed from the sediment.

2.5. Burrowing assay

After exposure, burrowing by both *S. plana* and *H. diversicolor* was investigated using the method of Bonnard et al. (2009) and Buffet et al. (2011). Burrowing rate was deemed a suitable endpoint, as copper exposure affected the burrowing behaviour of *S. plana* (Buffet et al., 2011) and microplastic exposure led to reduced feeding/burrowing in the lugworm *Arenicola marina* (Wright et al., 2013a). Microcosms for burrowing assays consisted of plastic aquaria (vol. = 3 L for *S. plana* and 1 L for *H. diversicolor*, n = 10 in each case) each containing sieved sediment (collected from the sampling site) filled to a depth of 5 cm and overlain with filtered (2 μm) sea water (S = 25) to a further 5 cm depth.

Individuals (n = 10, ie 3–4 per replicate) were removed from their holding tanks and placed on top of the sediment. The number of individual *S. plana* that remained unburrowed was recorded every 5 min (1st hour of exposure), 10 min (2nd hour), 20 min (3rd and 4th hour) and 30 min (5th - 6th hour). The number of unburrowed *H. diversicolor* was recorded every 2 min over a period of 6 min.

2.6. Feeding rate assay

The clearance rate of *S. plana* was determined using the modified method of Buffet et al. (2013). *S. plana* were removed from the sediment and placed in beakers (vol. = 1 L) containing filtered (2 μm) sea water (S = 25, T = 13 °C) overnight. Individuals (n = 10, 3–4 per replicate) were transferred to smaller glass beakers (vol. = 350 mL) each containing 300 mL filtered (0.45 μm) sea water (S = 25, T = 13 °C) and placed on a magnetised stirrer plate. Individuals were allowed to settle for 30 min, prior to the addition of *Isochrysis* algal suspension (vol. = 500 μl; concentration = ~1–1.5 × 10^4 cells.mL^{-1}, Cellpharm Ltd., Malvern, UK). A water sample (vol. = 20 mL) was removed immediately upon addition of the algal cells (T0), and a second sample was removed at 60 min (T1). Algal concentration in both samples was determined using a particle count and size analyser (Beckman Z2 Coulter, Wycombe, UK). The clearance rate was calculated using the equation:

$$Cr = \frac{V[\ln(C0) - \ln(C1)]}{T} \tag{1}$$

where V is the volume of seawater (mL), C0 is the cell density (cells. mL^{-1}) at T0 and C1 is the cell density (cells.mL^{-1}) at T1. T is the time (in hours) between T0 and T1.

The feeding rate of *H. diversicolor* was determined using the method of Moreira et al. (2005), but using decapsulated *Artemia* cysts (Waterlife Research Inc., London. UK) rather than larvae. Decapsulated *Artemia* cysts are a known laboratory food source for *H. diversicolor* (Costa et al., 2000), and are easily enumerated under low power magnification. Individual *H. diversicolor* (n = 10) were transferred to Pyrex beakers (vol. = 300 mL) containing 100 mL of filtered, seawater (S = 25) and 100 decapsulated *Artemia* cysts. *H. diversicolor* were left to feed for 1 h before being carefully removed and rinsed with deionised water over the beaker to prevent any loss of uneaten cysts. Contents of each beaker were poured into grided Petri dishes and manually counted under a microscope (10X magnification, Leica EZ4). The feeding rate is expressed as no.eggs.h^{-1}.

2.7. Condition index

Condition index is used as a measure of bivalve health and therefore only measured for *S. plana*. After behavioural assays were completed, individuals (n = 16, 5–6 individuals per replicate) were carefully removed from their shells using a scalpel. Both shell and soft tissue were wiped with absorbent paper and weighed to the nearest mg (Denver Instrument TP303). The condition index of *S. plana* was calculated by dividing soft tissue wet weight by shell dry weight (Zeng and Yang, 2021). After weighing, soft tissues were stored individually at T = −80 °C awaiting biochemical analysis.

2.8. Energy reserves

Energy reserves (carbohydrates, lipids and proteins) were quantified using whole body tissues of both *S. plana* and *H. diversicolor* (8 and 10 replicates per treatment respectively). See supplementary material for a full description of assay protocols. Tissues were homogenised in buffer (5:1, 50 mM Hepes, adjusted to pH 7.50) using a pestle and mortar, centrifuged (1500 rpm, T = 4 °C) for 5 min and the supernatant stored individually at T = −80 °C prior to analysis. Protein content was determined using the Biuret assay (Robinson and Hogden, 1940), carbohydrate content based on the anthrone reagent protocol (Van Handel, 1967), and lipids based on the vanillin assay procedure (Van Handel and Day, 1988).

The concentrations of protein, carbohydrate and lipid were each transformed into energy equivalents using Gnaiger's enthalpies of combustion (protein 24 J mg^{-1}, lipid 39.5 J mg^{-1}, and carbohydrates 17.5 J mg^{-1}) (Gnaiger, 1983). The total of each of the fractions provided

the total available energy per gram wet weight of each individual.

2.9. Oxidative stress

To assess oxidative damage, lipid peroxidation was measured using the thiobarbituric acid reactive substance (TBARS) assay, using malon-dialdehyde (MDA) as a marker (Aguilar Diaz De Leon and Borges, 2020). Antioxidant defences were quantified by measuring total glutathione (GSH), a key compound involved in protecting cells from oxidative damage (Forman et al., 2009). See supplementary material for a full description of assay protocols. The digestive gland and gills of *S. plana* (8 replicates per treatment) and whole body tissues for *H. diversicolor* (10 replicates per treatment) were carefully blotted dry with absorbent paper, weighed and individually frozen in liquid N_2 and stored at T = −80 °C for subsequent analysis. The digestive gland and gills were selected as these are the main organs for toxicant metabolism and exposure (Beyer et al., 2017; Capolupo et al., 2021; Livingstone, 1991).

Tissues were homogenised in buffer (3:1, 50 mM HEPES, 1 mM EDTA, 1 mM DTT, adjusted to pH 7.50) using a Cat X520D with a T6 shaft (medium speed, Bennett & Co., Weston-Super-Mare). Homogenates were centrifuged for 5 min (13,000 rpm at T = 4 °C) and the supernatant was carefully drawn off. Protein and TBARS assays were run immediately. Lipid peroxidation was calculated by measuring the formation of thiobarbituric acid reactive substances (TBARS), quantified by reference to MDA absorbance (Camejo et al., 1998). Quantification of GSH was based on the cyclic reduction assay, using the recycling reduction of reduced glutathione with Ellman's reagent (DTNB) in the presence of excess glutathione reductase (Owens and Belcher, 1965).

2.10. Data analysis

All data were tested for normality with the Shapiro-Wilkes test and visually inspected for homogeneity of variance, to ensure they conformed to the assumptions of ANOVA. To test for differences between controls to determine whether sanding grit caused any effects, a two-tailed Student's t-test was employed (Table S3). If p > 0.05, data were pooled. If differences occurred, both controls were included in the analysis. Tyre particle exposure effects were tested using a two-way nested analysis of variance (ANOVA) with Treatment (n = 4) as a fixed factor and Aquarium (n = 3) as a random factor. Pairwise comparisons were made by comparison of estimated marginal means. Where data did not conform to the assumptions of ANOVA after transformation, a log-likelihood ratio tested the significance of the random factor (aquarium). In each case, the random factor was not significant (p > 0.05), and was omitted from the analysis, and tyre particle exposure effects were tested using a Kruskal-Wallis test. To test for the effect of tyre particle exposure on burial speeds, a quasibinomial GLM using a link logit function compared the slopes between treatments, due to the sigmoid distribution of *S. plana* data. To get all combinations of slopes for pairwise comparisons, levels of the factor were reordered and the quasibinomial GLM rerun. All analyses were performed using SPSS v25, except for the quasibinomial GLM, which was analysed using R (R Core Team, 2020).

3. Results

3.1. 3.1 Tyre particle uptake

A preliminary experiment in which both species were exposed to 10% sedimentary tyre particles for 3 days confirmed that these particles were ingested by both species. Ingestion was considerably higher for *S. plana* which consumed more than 25 times more particles than H. diversicolor (Fig. S2). S. plana ingested a mean of 967 particles. g −1 ww (range = 424–1250), or 584 particles. individ-1 (range = 151–1341), whilst *H. diversicolor* ingested 35 particles. g-1 ww (range = 2 = 55) or 17 ± 6 particles. individ-1 (range = 2–33).

3.2. Experimental exposure to varying concentrations of tyre particles

No mortality was observed for *H. diversicolor*, whilst low mortality (<10%) was observed for *S. plana* during the experimental period (21 days). Mortality was low (<10%) in all treatments, suggesting a three-week exposure to these concentrations of tyre particles was adequate for investigating sub-lethal effects.

Visual analysis of the intestine of both species at the end of the experiment showed tyre particles appeared to aggregate in the intestine of *S. plana* in response to sedimentary exposure, with concentrations appearing higher in the intestines than in the sediment (See Fig. S3 and S4). This was not observed for *H. diversicolor*. Whilst the intestine of *S. plana* contained large quantities of sediment, the intestines of *H. diversicolor* showed little, if any (See Fig. S5), which was consistent with the results of our preliminary ingestion experiment, suggesting that *S. plana* will consume larger quantities of tyre particle contaminated sediment. No tyre particles were observed in the intestines of control individuals (Fig. S3), suggesting low/no tyre wear contamination at the collection site on the Yealm Estuary.

3.3. Energy reserves

3.3.1. Lipid and carbohydrate

There was no significant difference in tissue lipid or carbohydrate concentration between treatments for either *S. plana* (F_3 = 1.579, p = 0.287 and F_3 = 2.119, p = 0.195) or *H. diversicolor* (F_3 = 0.758, p = 0.557 and F_3 = 1.982, p = 0.216). Mean concentration of carbohydrate across all treatments was 7.2 (range = 4.0–10.6) mg carbohydrate.g ww^{-1} for *S. plana* and 26.3 (range = 10.0–42.7) mg carbohydrate.g^{-1} ww for *H. diversicolor*. The mean concentration of lipids across all treatments was 8.4 (range = 5.5–11.3) mg lipid.g^{-1} ww for *S. plana* and 12.8 (range = 8.3–20.9) mg lipid.g^{-1} ww for *H. diversicolor*.

3.4. Protein

There was a significant effect of tyre particle exposure on the protein concentration of *S. plana* (Fig. 1a; F_3 = 7.151, p = 0.019), although this effect was not dose dependant. There appeared to be a difference (8% increase) between the control and 0.2% tyre particle concentration but this was not significant (p = 0.187). Protein content of *S. plana* decreased by 11% in sediments spiked with 5% tyre particles compared with controls (p = 0.029). There was also by 19% reduction as a result of exposure to 5% tyre particle exposure compared with the 0.2% tyre particle exposure (p = 0.002). In *H. diversicolor* protein content appeared to decrease in a dose-dependent manner in response to tyre particle exposure (Fig. 1c; F_3 = 5.596, p = 0.035), although the only statistical difference was between the control and the 5% tyre particle concentration (21% decrease, p = 0.009).

3.5. Energy content

The total energy content (J.g^{-1} ww) of *S. plana* decreased by 6% and 11% in the 1 and 5% tyre particle exposure respectively, with the greatest energy content in the 0.2% exposure (Fig. 1b) seemingly driven by changes in protein content. However none of the differences were statistically different (F_3 = 3.906, p = 0.070).

Exposure to tyre particles in the sediment had a significant negative effect on the energy content of *H. diversicolor* (Fig. 1d; F_3 = 4.790, p = 0.048), with an 18% decrease in mean energetic content between controls and those exposed to 5% tyre particles (p = 0.009). This decrease in total energy was driven by the reduction in protein concentration.

3.6. Oxidative stress

Whilst initially it was suggested that lipid peroxidation should increase as a result of tyre particle exposure, lipid peroxidation decreased
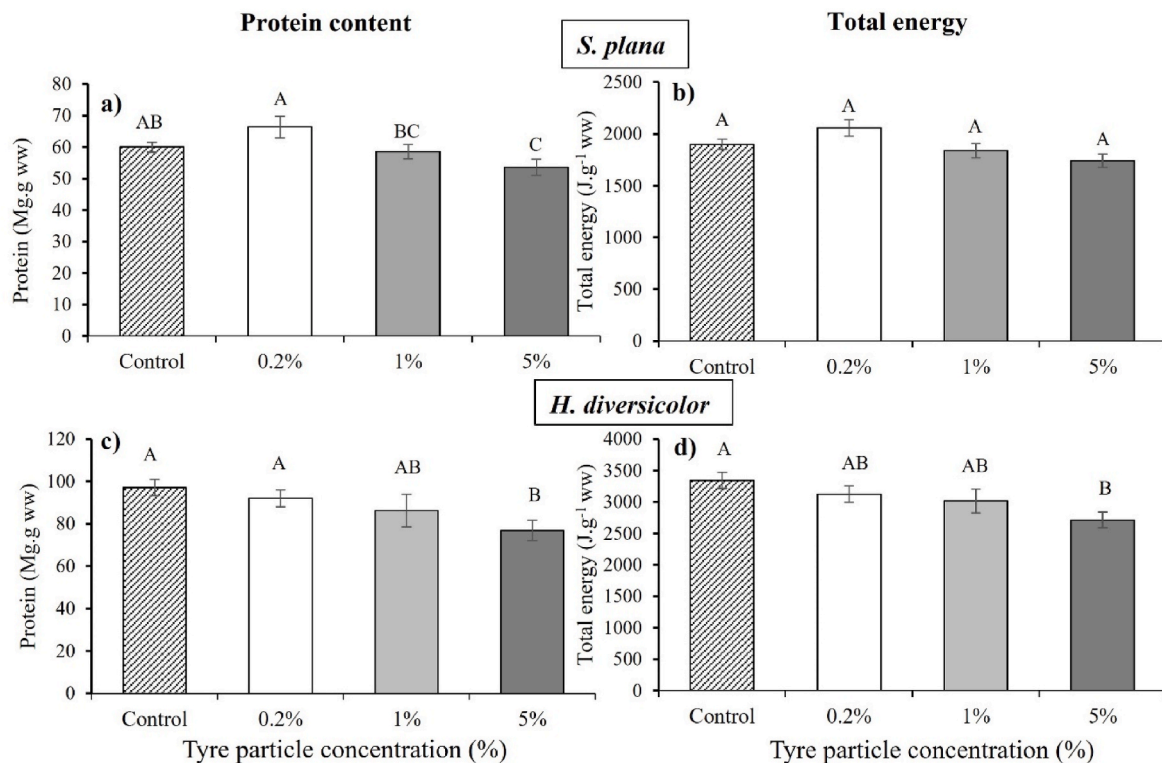
Fig. 1. The effect of exposure to 4 different concentrations of tyre particles (Control, 0.2, 1 and 5%) on the energetic resources and content of *S. plana* (a, b) and *H. diversicolor* (c, d). Measurements of soft tissue wet weight (ww) concentrations of protein (a and b) and total energy (c and d) are shown (n = 8 for *S. plana* and n = 10 for *H. diversicolor*). Data are presented as mean (±SE) and significant effects of tyre particle exposure detected using a nested ANOVA followed by estimated marginal means pairwise comparisons. Treatments denoted by different letters indicate a significant difference at the 0.05 confidence level.

in the *S. plana* digestive gland in a dose-dependent manner in response to tyre particle exposure (Fig. 2a $F_3 = 3.515$, p = 0.026). Compared to the control, concentration of malondialdehyde (a marker of lipid peroxidation) decreased by 15% at 1% tyre particle exposure and by 42% in response to 5% tyre particle exposure (p = 0.043 and 0.005 respectively). There was no effect of tyre particle exposure on lipid peroxidation levels in the gills of *S. plana* (Fig. 2c; $F_3 = 1.505$, p = 0.277) or the whole body tissues of *H. diversicolor* (Fig. 2e; $F_3 = 1.259$, p = 0.350). Interestingly, there was a 71% increase in lipid peroxidation between the control and the secondary sanding grit control, although this was not significant (p = 0.095), and values were pooled as per the described methods.

There was a significant dose-dependent increase in the concentration of total glutathione (GSH; an antioxidant defence) in *H. diversicolor* tissues in response to tyre particle exposure (Fig. 2f; $F_3 = 15.207$, p = 0.004). Compared to the control, concentrations of GSH increased by 90% at 5% tyre particle exposure (p = 0.007) with levels higher in individuals exposed to 5% tyre particle compared with controls and 0.2% tyre particles (p = 0.040). No significant difference in GSH concentrations in *S. plana* digestive gland (Fig. 2b) or gill (Fig. 2d) were detected as a result of tyre particle exposure (Kruskal-Wallis H = 4.643, *df* = 3, p = 0.200, and H = 0.351, *df* = 3, p = 0.775 respectively).

3.7. Burrowing rate, feeding rate and condition index

There was a significant effect of tyre particle exposure on the clearance rate of *S. plana* (Fig. 3a; $F_3 = 6.457$, p = 0.001), although this was not dose-dependent. Clearance rate decreased by 52% in the 0.2% tyre particle treatments (p = 0.007) and by 42% in the 1% tyre particle treatment (p = 0.048) compared to the control. In response to 5% tyre particles, the clearance rate increased and was 22% higher than the control, although this increase was not significant (p = 0.880). There

was no difference in the feeding rates of *H. diversicolor* (Fig. 3c, $F_3 = 0.935$, p = 0.431) or the condition index of *S. plana* ($F_3 = 0.774$, p = 0.512) between treatments.

Burial rates were compared using a quasibinomial GLM with a link logit function. Rates of burrowing were significantly reduced in *S. plana* exposed to all concentrations of tyre particles (Fig. 3b; quasibinomial GLM; $X_2 = 74.109$, *df* = 3, p < 0.001). This was the only parameter measured in this experiment in which the control and sanding grit significantly differed, with *S. plana* in the sanding grit burrowing faster than those in the control (p < 0.001). The burrowing speed of *S. plana* exposed to all tyre particle concentrations was slower than the control (p < 0.05) and the sanding grit control (p < 0.001), although the decrease observed in the 0.2% exposure was largely driven by the failure of one individual to burrow (Fig. 3b). Other than this one, all individuals burrowed within 200 min (Fig. 3b).

All *H. diversicolor* burrowed within 6 min of placement in aquaria, and burial speeds were not affected by tyre particle exposure (Fig. 3d, quasibinomial GLM; $X_2 = 2.780$, *df* = 3, p = 0.595).

4. Discussion

4.1. Tyre particle ingestion

S. plana is a deposit and filter-feeding bivalve, consuming sediment from around its burrow using its siphon to extract organic matter and microbes, and filtering microalgae from the water column when the tide is high (Hughes, 1969). In contrast, *H. diversicolor* is omnivorous, an active predator or scavenger, who can obtain nourishment from filter feeding and feeding on the uppermost sedimentary layer (Nielsen et al., 1995). Despite their different lifestyles, both species were impacted as a result of exposure to tyre particles. Infaunal deposit-feeding bivalves such as *S. plana* have been shown to contain higher concentrations of
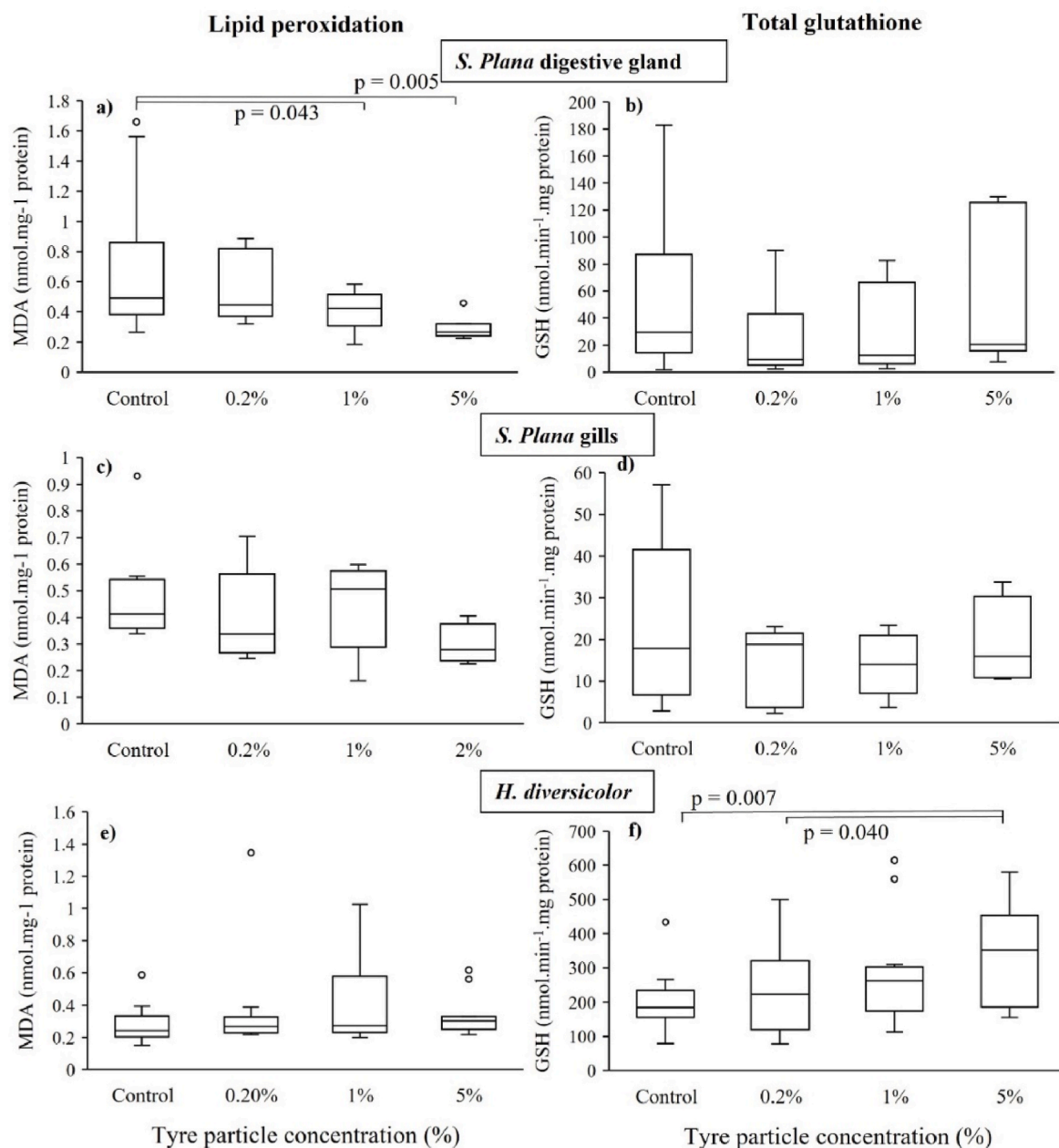
Fig. 2. Exposure to 4 different concentrations of tyre particles (Control, 0.2, 1 and 5%) on lipid peroxidation determined by the concentration of malondialdehyde (MDA; a, c, e) and total glutathione (GSH; d,e, f) in *S. plana* digestive gland (a, b) and *S. plana* gills (c, d), or in the tissues of *H diversicolor* (d, e) n = 10 for *H. diversicolor*, n = 8 for *S. plana* digestive gland and n = 4 for *S. plana* gills (2 gills pooled per sample). Data are presented in boxplots, showing data distribution, and significant effects of tyre particle exposure detected using a nested ANOVA followed by estimated marginal means pairwise comparisons for figures a, c, e and f and Kruskal-Wallis tests for figures b and d. Horizontal bars indicate a significant difference at the 0.05 confidence level.

pollutants than other infaunal invertebrates (Langston et al., 1987). This was the case in this study, with *S. plana* ingesting more than 25x the number of tyre particles compared to *H. diversicolor*. The size of the tested tyre particles overlapped with natural sediment sizes and was similar in size to tyre particles produced in a road simulator laboratory (4–350 μm; Kreider et al., 2010). The number of particles in the intestine of *S. plana* suggests that these manufactured particles also overlap with the size of sediment that this species ingests.

4.2. Burrowing and feeding rates

Ingestion rates may help explain why feeding and burial rates only decreased for *S. plana*, with ingestion leading to false satiety and

lethargy. The lethargy observed may be linked to the reduction in feeding rate, as observed in *A. marina* (Wright et al., 2013a). Exposure to copper has been shown to lead to reduced burial rates in *S. plana* (Buffet et al., 2011), and TBT and copper to reduced burial rates in juvenile *S. plana* (Ruiz et al., 1994). At the greatest concentration, *S. plana* clearance rate increased to higher than the control. Unlike in other exposure studies, *S. plana* was removed from a microplastic contaminated environment into an uncontaminated environment, and this increase may be an attempt to clear its gut of tyre wear particles.

A reduction in feeding is a sensitive measure of sublethal effects of pollution in some estuarine species (Moreira et al., 2006; Soares et al., 2005), and has been recorded in other marine invertebrate microplastic exposure studies (Besseling et al., 2013; Cole et al., 2015; Watts et al.,
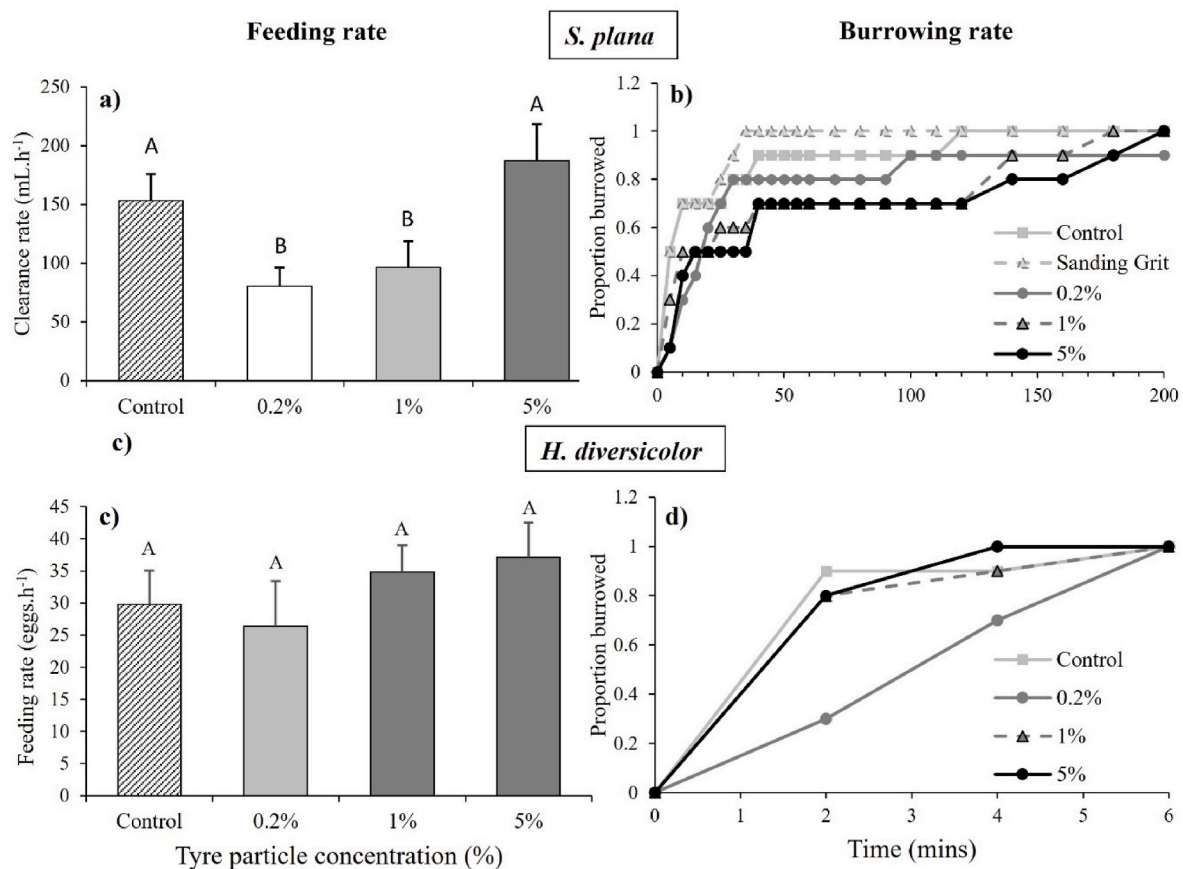
Fig. 3. The effect of tyre particle exposure on *S. plana* and *H. diversicolor*. a) Mean clearance rate (+SE) *S. plana* after 21 days of sedimentary exposure to increasing concentrations of tyre particles. Treatments denoted by the same letters do not differ significantly ($p < 0.05$). b) Line graph of proportion of buried *S. plana* ($n = 10$) through time (mins) after sedimentary exposure to different treatments; control, 0.2%, 1%, and 5% sedimentary concentration of tyre particles. c) Mean feeding rate (+SE) of *H. diversicolor* after 21 days of sedimentary exposure increasing concentrations of tyre particles. d) Line graph of proportion of buried *H. diversicolor* ($n = 10$) through time (mins) after sedimentary exposure to different treatments; control, 0.2%, 1%, and 5% sedimentary concentration of tyre particles.

2015; Welden and Cowie, 2016; Wright et al., 2013a). Tyre particle/microplastic ingestion may cause physical gut blockage, false satiety, and tissue damage (Cole et al., 2015; Welden and Cowie, 2016; Wright et al., 2013a), alongside the leaching of chemical additives which may have further negative impacts (Jang et al., 2021).

4.3. Energy reserves

Whilst behavioural changes were only recorded for *S. plana*, both species exhibited a significant reduction in protein content as a result of exposure to 5% sedimentary tyre particles, which translated into a reduction in energy reserves for *H. diversicolor*. A reduction in the protein content of marine invertebrates has been recorded in other sedimentary microplastic exposure experiments; Bour et al. (2018) found a significant decrease in protein content in the burrowing bivalves *Abra nitida* and *Ennucula tenuis* in response to exposure to large (125–500 μm) PE microparticles, leading to a significant decrease in energy reserves in *E. tenuis*, whilst Shang et al. (2021) found exposure to microplastics resulted in a reduction in all cellular energy stores (proteins, lipids and carbohydrates) in the mussel *Mytilus unguiculatus* (previously *M. coruscus*) in response to 2 μm diameter polystyrene spheres.

Protein may serve as a metabolic reserve in marine invertebrates (Bhat and Wagh, 1992), particularly during periods of low food supply or stress (Verslycke and Janssen, 2002). A reduction in protein content has been observed as an early result of toxicant-induced stress in the freshwater amphipod *Gammarus pseudolimnaeus* (Graney and Giesy, 1986) and in response to exposure to silver nitrate ($AgNO_3$) in the

earthworm Enchytraeus crypticus (Gomes et al., 2015).

4.4. Oxidative stress

A decrease in lipid peroxidation was observed in the digestive gland of *S. plana* as a result of exposure to 5% tyre particles. Exposure to PAHs, PCBs and heavy metals has shown to lead to alterations in cell structure in the digestive gland of molluscs (Cajaraville et al., 1990; Lowe and Clarke, 1989), which is likely caused by the coupling of digestive cell loss with basophilic cell hypertrophy (Zaldibar et al., 2007). The decrease in lipid peroxidation, coupled with GSH levels remaining stable, may suggest that exposure to these particles or their leachates led to an increase in cell turnover in the digestive gland. Tyre particles leach a cocktail of trace metals and organic pollutants (Halsband et al., 2020), which may cause digestive cell loss. In this present study, we were not able to discriminate between any effects of leachates as compared with the tyre particles themselves, although studies by Masset et al. (2021, 2022) found that heavy metals and organic compounds leach from the tyre particles into the gastrointestinal fluids of fish, backing up this theory.

Exposure to oxidants can lead to increased production of cellular glutathione to protect against oxidative damage (Forman et al., 2009). An increase in GSH levels was observed for *H. diversicolor* at the highest tyre particle exposure (5%), suggesting upregulation of oxidative defences. This increase in total glutathione has also been observed in the oligochaete *Lumbriculus variegatus* in response to microplastic exposure (Silva et al., 2021).

4.5. Environmental relevance

Analysis of sediments around the Seine watershed suggests sedimentary concentrations regularly exceed 0.2%, with some exceeding 1% (Unice et al., 2013), whilst sediments in gully pots in Norway contained up to 15% tyre wear particles (Mengistu et al., 2021). Burial and feeding rates in *S. plana* were affected at the lowest concentration (0.2%), suggesting that tyre wear pollution may pose significant threats at current environmentally relevant concentrations. Effects of tyre particle exposure on protein reserves and markers of oxidative stress were observed in both species, although only at the highest concentration of tyre particles tested (5%). This concentration is higher than concentrations occurring in estuarine sediments, although the experimental exposure period was relatively short (21 days) considering the lifespans of *H. diversicolor* (one year) and *S. plana* (four years), and long-term chronic exposure to tyre tread may impact energy reserves at lower concentrations.

S. plana and *H diversicolor* are important components of estuarine mudflats throughout Europe, and any reduction in their health is likely to lead to a potential loss of functioning of estuarine ecosystems. A meta-analysis of microplastic concentrations in differing trophic groups found that microplastics bioaccumulate up the food chain (Miller et al., 2020). *S. plana* is an important prey item for (often migratory) wading birds, fish and crabs (Hughes, 1970; Moreira, 1994; Wanink and Zwarts, 2001; Wouters and Cabral, 2009), and as such, the accumulation of tyre particles in the intestines of *S. plana* may lead to subsequent negative impacts on these animals.

In this study, tyre wear particles were produced from multiple tyres using friction against a surface to emulate the production of tyre wear in the environment. The experiment included a secondary control, which was contaminated with sanding disc grit, to ensure that any effects weren't caused by the contamination. For all measured endpoints, except *S. plana* burrowing rates, there was no significant difference observed between the two controls (Table S3). The burrowing rate of *S. plana* increased in response to sanding grit contamination, therefore strengthening the argument that burrowing rates decreased in response to tyre wear exposure.

Environmental tyre wear particles will form heteroagglomerates with road dust and other particles (Klöckner et al., 2020), differing from laboratory-produced particles (Baensch-Baltruschat et al., 2020). Agglomeration with other contaminants in road dust, such as brake pads or paint chips could increase their toxicity. Alternatively, toxicity may decrease with time in the environment, with impacts from leachates only occurring in the days after initial release. Whilst tyre and road wear particles may differ from laboratory-produced tyre particles, early evidence from this study suggests that high concentrations of tyre pollution could impact the health of the fauna within estuarine sediments, and therefore warrants further investigation.

4.6. Future directions

Whilst the importance of sedimentary exposure experiments is recognised (Moreira et al., 2005), such experiments are rare in marine microplastic research (Bour et al., 2018). As sedimentary pollution may be a key route of exposure to microplastics for many marine organisms, and benthic species, particularly deposit feeders may be particularly vulnerable, it is suggested that sedimentary toxicity studies become more commonplace for microplastic research in general. This study did not attempt to separate the effects caused by the microplastic particles versus the effects of the chemical additives leaching from these particles, but our results do indicate that future studies should investigate effects in isolation and in combination, to determine the main drivers of toxicity.

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Author statement

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Declaration of competing interest

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2022.120244.

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