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Title	Polyethylene microplastics adhere to Lemna minor (L.), yet have no effects on plant growth or feeding by Gammarus duebeni (Lillj.)
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Publication date	2019-06-24
Original Citation	Mateos-Cárdenas, A., Scott, D. T., Seitmaganbetova, G., Frank N.A.M, v. P., John, O. H. and Marcel A.K, J. (2019) 'Polyethylene microplastics adhere to Lemna minor (L.), yet have no effects on plant growth or feeding by Gammarus duebeni (Lillj.)', Science of The Total Environment, 689, pp. 413-421. doi: 10.1016/j.scitotenv.2019.06.359
Type of publication	Article (peer-reviewed)
Link to publisher's version	http://www.sciencedirect.com/science/article/pii/S0048969719329328 - 10.1016/j.scitotenv.2019.06.359
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Download date	2026-11-05 14:17:19
Item downloaded from	https://hdl.handle.net/10468/8346

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PII: S0048-9697(19)32932-8
DOI: https://doi.org/10.1016/j.scitotenv.2019.06.359
Reference: STOTEN 33013

To appear in: *Science of the Total Environment*

Received date: 18 April 2019
Revised date: 20 June 2019
Accepted date: 22 June 2019

Please cite this article as: M.-C. Alicia, D.T. Scott, S. Gulzara, et al., Polyethylene microplastics adhere to Lemna minor (L.), yet have no effects on plant growth or feeding by Gammarus duebeni (Lillj.), Science of the Total Environment, https://doi.org/10.1016/j.scitotenv.2019.06.359

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Polyethylene microplastics adhere to *Lemna minor* (L.), yet have no effects on plant growth or feeding by *Gammarus duebeni (*Lillj.)

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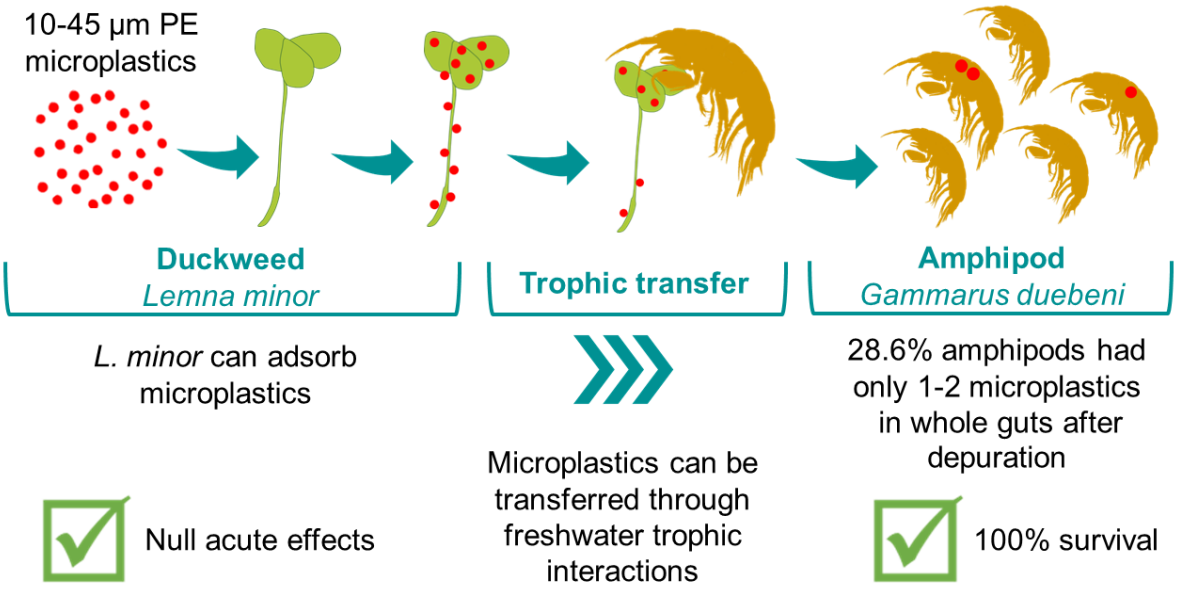
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Microplastics (1 – 1000 µm) are ubiquitous in the marine, freshwater and terrestrial environments. These microsized plastics are considered freshwater pollutants of emerging concern, although the impacts on organisms and ecosystems are not yet clear. In particular, effects of microplastics on freshwater aquatic plants and the fate of microplastics in the freshwater trophic chain remain largely unexplored. Here we demonstrate that 10-45 µm polyethylene (PE) microplastics can strongly adsorb to all surfaces of the duckweed species *Lemna minor*. Despite adsorbance of up to 7 PE microplastics per mm^2, seven day exposure experiments showed that photosynthetic efficiency and plant growth are not affected by microplastics. Rather, dense surface coverage suggests *L. minor* as a potential vector for the trophic transfer of microplastics. Here we show that the freshwater amphipod *Gammarus duebeni* can ingest 10-45 µm PE microplastics by feeding on contaminated *L. minor*.

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In this study, ingestion of microplastics had no apparent impact on amphipod mortality or mobility after 24 or 48 hour exposure. Yet, the feeding study showed that the fate of microplastics in the environment may be complex, involving both plant adsorbance and trophic transfer.



Keywords: Microplastics, Polyethylene, Freshwater, Aquatic plants, Freshwater macroinvertebrates, Ecotoxicology, Trophic transfer.

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Introduction

Microplastics are ubiquitous in the environment (Rochman, 2018). In fact, plastics are so widespread that they are now being considered as a stratigraphic marker for the Anthropocene (Waters et al., 2016). Microplastics are not just found near centres of human activity, but also in inaccessible locations such as the deep sea (Van Cauwenberghe et al., 2013) or the arctic (Cózar et al., 2017). Yet the quantification of microplastics present in the environment is still in its infancy, and is hampered by

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a lack of standardised protocols (Rodrigues et al., 2018). Microplastics were defined by Frias and Nash (2019) as “synthetic solid particle or polymeric matrix, with regular or irregular shape and with size ranging from 1 µm to 5 mm, of either primary or secondary manufacturing origin, which are insoluble in water”. Yet most protocols fail to monitor microplastics in the sub-200µm range. Furthermore, the size definition has been criticised as inconsistent (Hartmann et al., 2019). Hartmann et al., (2019) proposed characterising microplastics not just as a size range between 1 – 1000 µm, but also on solid state, shape, colour, origin and chemical composition. Furthermore, a microplastic is not just a particle made of inert plastic material, but a complex chemical cocktail of monomers, oligomers and additives such as plasticisers which are associated with the production of microplastics (Rochman et al., 2019).

There is an emerging knowledge base on the microplastics that are present in the marine environment (Setälä et al., 2018). However, studies of the presence, abundance and potential effects of microplastics in freshwater systems are still relatively scarce (Horton et al., 2017). This is striking as the freshwater environment has been identified as a route by which substantial amounts of plastics are transferred from terrestrial to marine environments (Derraik, 2002). Additionally, microplastics are now also considered “pollutants of emerging concern” in the freshwater environment itself (Dris et al., 2015; Eerkes-Medrano et al., 2015; Wagner and Lambert, 2018). Similar to what has been reported for the marine environment, microplastics in the freshwater environment occur in the water column (Horton et al., 2017) but have also been found ingested in fish and birds (O’Connor et al., 2019). Thus far, just one study did analyse environmental samples of microplastics within organisms at the lower levels of the freshwater trophic chain (Windsor et al., 2019). This study concluded that microplastics are ubiquitous in

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riverine macroinvertebrates and that all macroinvertebrates contained microplastics regardless of feeding type (i.e. filter feeders versus grazers). A pertinent question is whether these microplastics were ingested with feed as part of trophic interactions. So far, there are no studies on the association of microplastics with freshwater plants, nor on trophic transfer from plants to consumers, under natural conditions. Earlier studies demonstrated the association of microplastics with seagrass from natural environments (Goss et al., 2018), as well as with seaweed under laboratory conditions (Sundbaek et al., 2018).

There are some early indications that microplastics may affect aquatic plants under laboratory conditions. Microplastic had negative effects on the root length of *Lemna minor* (Kalčíková et al., 2017) and the shoot length of *Myriophyllum spicatum* (van Weert et al., 2019). Yet, there is no consensus on the impacts of microplastics on freshwater plants. There is more knowledge concerning the effects of microplastics on invertebrates, and particularly on the ecotoxicological model species *Daphnia magna.* Microplastics increased mortality of starved daphnids, whereas no effect was found on daphnids fed prior to exposure (Jemec et al., 2016). Aljaibachi and Callaghan, (2018) concluded that microplastics have no effect on *D. magna* if food is present. However, this contradicted an earlier study by Rehse et al., (2016) where a similar microplastic exposure caused short-term immobility in pre-fed daphnids. The ingestion of microplastics is related to species characteristics such as feeding type and morphology as well as microplastic availability (Scherer et al., 2017). In the case of the detritivore, shredder *Gammarus pulex*, microplastic exposure does not have a negative effect on the amphipod after either acute (Bruck and Ford, 2018) or chronic exposure (Weber et al., 2018). However, a study by Au et al., (2015) on the amphipod *Hyalella azteca* showed that plastic microfibres were significantly more

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toxic than plastic microbeads. This was attributed to a slower egestion rate of the microfibres. Lack of egestion is important, particularly in the context of trophic mobility. Bioaccumulation of microplastics has been hypothesised to increase as particle size decreases (Rist et al., 2017; Wagner et al., 2014). In fact, a study by Jeong et al., (2016) demonstrated that the rotifer *Brachionus koreanus* retained smaller microplastics longer than bigger sized ones. Trophic transfer of microplastics has been reported between marine species, under natural conditions (Nelms et al., 2018; Welden et al., 2018). Under laboratory conditions, the transfer of microplastics through the food chain has been observed from seaweed to periwinkles (Gutow et al., 2015), from brine shrimp to zebrafish (Batel et al., 2016) or from mussels to crabs (Farrell and Nelson, 2013; Santana et al., 2017; Watts et al., 2014). However, it is not clear whether such trophic transfer does occur in the freshwater environment, where the fate of microplastics may be different as a result of the lower density of water, the local lack of wave action and the different producer and consumer species present.

In this study the association between polyethylene microplastics and the freshwater primary producer species, *L. minor*, was investigated. Potential effects on plant growth and photosynthetic efficiency were assessed using seven day (acute toxicity) and thirty day (chronic toxicity) tests. Finally, the transfer of microplastics from *L. minor* to the grazer *Gammarus duebeni* was quantified. The results generate a baseline for the understanding of impacts of microplastics on the lower levels of the freshwater trophic food chain.

Materials and methods

Stock suspensions of microplastics

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The microplastic particles chosen for this study were polyethylene (PE) microspheres, in this study referred to as PE microplastics. PE microplastics were provided in dry hydrophobic powder form and were stained fluorescent red by Cospheric (Santa Barbara, CA, USA; Product reference UVPMS-BR-0.995). The characteristics of these microplastics were a diameter of 10-45 µm, a density of 0.985g/cm^3, and a peak of fluorescence at 605nm. The supplier estimated the concentration based on size range and density as a total of 1.28x10^8 microplastics per gram. A 20% w/v stock suspension of PE microplastics was prepared using 0.1% Tween 20 in distilled water, and in accordance with the supplier's Standard Operating Procedure. Tween 20 (Polyoxyethylene (20) sorbitan monolaurate) is a common non-ionic surfactant which is used to minimise surface tension and to improve wetting of hydrophobic materials (Kim and Hsieh, 2001).

A known volume (µL) of the stock suspension was then pipetted into each of 100 mL bioassays. The dry weight (mg) of the microplastics pipetted into the bioassays was measured. Knowing all these variables, the final concentration was estimated as 50,000 microplastics mL^{-1}. The concentration was not expressed in mass units (particle mass/medium volume) as this causes difficulties when comparing studies (O'Connor et al., 2019; Phuong et al., 2016).

The final concentration of 50,000 microplastics mL^{-1} in the bioassays was set following preliminary threshold exposure tests (data not shown). Microplastic sample processing, characterization and analysis is currently limited to available equipment and few studies capture and accurately detect particles <100 µm in size from environmental samples (Kershaw et al., 2019; Song et al., 2015). Current environmental concentrations of the microplastic type and size tested in this study

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are still unknown due to current methodological limitations (O'Connor et al., 2019; Weber et al., 2018).

Here we used a single concentration of microplastics which is expected to be higher than current environmental concentrations. The use of surfactants to prepare microplastic suspensions is widely followed by researchers, however surfactant controls are rarely included in studies (Pikuda et al., 2018). Here, Tween 20 controls were run to account for any potential surfactant toxicity. The final Tween 20 concentration in the bioassays was 0.0005%.

Test organisms

Lemna minor (lesser duckweed)

The aquatic plant *L. minor*, commonly named lesser duckweed, is a floating freshwater macrophyte that belongs to the family Lemnaceae. *L. minor* can be found in ponds and slow moving streams. Test guidelines for aquatic toxicity testing have been designed specifically for *Lemna spp.* (EPA, 2012; OECD, 2006). Indeed, *L. minor* has been used extensively in ecotoxicology testing to assess growth inhibition and photosynthetic efficiency of water contaminants (Ziegler et al., 2019). Additionally, a previous study by Lahive et al., (2015) demonstrated that zinc particles can be transferred through trophic transfer from *L. minor* to the grazer *Gammarus pulex*. The *L. minor-G. pulex* system was specifically developed as a model for trophic transfer studies.

Axenic specimens were obtained from laboratory stocks grown at University College Cork, School of Biological, Earth and Environmental Sciences. These stocks originated from a pond in the Blarney area, Co. Cork, Ireland. The specific *L. minor* strain is registered in the Rutgers Duckweed Stock Cooperative (RDSC) database as

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strain number 5500 “Blarney”. Plants were cultured on 100 mL half-strength Hutner's medium in 300 mL HDPE plastic magentas with punctured lids and cotton wool plugs under 16/8 h photoperiod (light intensity of 50 $\mu molm^{-2}s^{-1}$) at 21 ± 2°C. Magentas (Magenta GA-7 Plant Culture Boxes), commonly used in plant culture protocols, are clear containers made of polycarbonate with polypropylene lid. Only *L. minor* sterile stocks were grown and kept in these plastic containers to avoid our tests to be in contact with plastic material. The plastics used for the tests have a distinctive fluorescence label that facilitates differentiation from any potential plastic contaminant, including those from magentas.

***Gammarus duebeni* (freshwater amphipod)**

The amphipod *Gammarus duebeni* is a freshwater crustacean commonly found in southern Ireland. *G. duebeni* are benthic macroinvertebrate species that live in sediments and the water column of freshwater rivers and streams. These amphipods are shredders and detritivores that can feed on a wide range of plant and animal materials. Freshwater amphipods (*Gammarus spp.*) have been widely studied as ecotoxicological model species, as well as for microplastic toxicity tests (Au et al., 2015; Bruck and Ford, 2018; Dedourge-Geffard et al., 2009; Redondo-Hasselerharm et al., 2018; Scherer et al., 2017; Straub et al., 2017; Weber et al., 2018). *Gammarus spp.* are able to feed on *L. minor* colonies, as previously shown in a zinc trophic transfer laboratory study by Lahive et al., (2015).

G.duebeni adults were collected between March and November 2018 from two local streams in Co. Cork (Ireland; coordinates Stream #1: 51°57'15.9"N 8°48'31.0"W and Stream #2: 51°55'07.0"N 8°37'46.5"W). Amphipods were kick-sampled, collected and transported in bags filled with stream water and sorted in the laboratory

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immediately after arrival. Local stream water was collected from the same locations. Prior to any experimental work, amphipods were acclimatised for at least 48 hours in 5 litre tanks containing a 50/50 mixture from local stream water and aerated, de-chlorinated tap water at 19°C. Acclimation tanks were continuously aerated and covered with a lid and black cloth to avoid exposure to direct light. Only adults (mixed females and males) whose length was between 14 – 21 mm were selected for bioassays.

L. minor **- Experimental design and endpoints**

All tests were carried out in sterile Pyrex borosilicate-glass crystallising dishes (125 mm diameter, 65 mm height and 500 mL capacity) with spout. Test dishes were covered with 150 mm diameter soda-lime watch glass dishes with fused edges (Merck, Germany) to avoid contamination. According to OECD Guideline 221 for testing with *Lemna sp*. (OECD, 2006), a total of nine fronds (three colonies of three fronds) were transferred to each test dish. Dishes were filled with a volume of 100 mL of sterile half-strength Hutner's medium containing 50,000 microplastics/mL for 3, 24, 72 or 168 hours/7 days. In case of short term (< 7 days) exposures, a “clean” control (Hutner's medium only) and a surfactant or “Tween” control (containing Hutner's medium and 0.0005%Tween 20) were included. Independent replicates (N = 8) were run for each time point. At the end of all exposures, fresh colonies were immersed in, and gently shaken in clean distilled water to remove any loose microplastics. Subsequently, colonies were carefully placed on a glass petri dish for inspection using a light microscope. All three major surfaces of a duckweed plant were separately inspected, i.e. the adaxial frond, the abaxial frond, and root(s).

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The number of PE microplastics adhering to *L. minor* tissues was visually counted using a Nikon E200 light microscope at magnifications of 4x and 10x. Subsequently, plant biomass was dried in a laboratory incubator (Ehret GmbH & Co KG, Germany) at 40ºC for 16 hours and rehydrated for three hours in separate petri dishes filled with 6 ml of distilled water. This process mimics the manipulation of biomass that is required for the trophic transfer feeding studies with *G. pulex* (Lahive et al., 2015). A count of microplastics adhering to rehydrated *L. minor* biomass provided an estimation of the number of PE microplastics fed to *G. duebeni*.

Relative Growth Rate (RGR)

The RGR based on biomass and on frond number was determined following seven days of growth (No, 2006). For RGR based on frond number, the initial (t = 0 days) and final (t = 7 days) number of fronds was recorded for all treatments. Next, for RGR based on biomass, colonies from each treatment vessel were harvested and surface water removed by gently covering them with absorbent paper. In the case of *L. minor* colonies grown in PE microplastic treatment, microplastics were fully removed from the colonies by gently immersing and shaking them in a 0.1% Tween 20 solution for 15 seconds. This technique was proven to remove microplastics consistently by observing the absence of microplastics on *L. minor* colonies using light microscopy. Next, fresh weight was measured using a Pioneer mass balance (Ohaus Corporation, USA) with an accuracy of 0.1 mg. The specific growth rates based on biomass and frond number were calculated according to (Connolly and Wayne, 2001):

$$RGR = \ln(Yf/Yi)/t$$

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Where *ln* is the natural logarithm, Y_f is the final fresh weight or frond number, Y_i is the initial fresh weight or frond number and *t* is the time of exposure.

Chlorophyll *a* Fluorescence

Chlorophyll *a* fluorometry was used as a sensitive assay to non-destructively monitor potential perturbations in growth and metabolism of plants (Baker and Rosenqvist, 2004). After 7-days growth, PE microplastics were removed from *L. minor* colonies using Tween 20 as previously detailed. Following this, plants were dark-adapted for 15 minutes, and chlorophyll *a* fluorescence was measured using a pulse amplitude modulated (PAM) imaging fluorometer (IMAGING-PAM M-Series, MAXI version) equipped with ImagingWin software (Heinz Walz GmbH PAM, Effeltrich, Germany). Measuring settings were set as follows; measuring light ML <1 $\mu molm^{-2}s^{-1}$; actinic light AL (30-40 $\mu molm^{-2}s^{-1}$ saturation pulse 1200 $\mu molm^{-2}s^{-1}$). Four parameters of interest, the maximal PS II quantum yield (Fv/Fm), the effective PS II quantum yield (Y(II)), the coefficient of non-photochemical quenching (qN) and the coefficient of photochemical quenching (qP), were calculated.

Root length

The length of *L. minor* roots was measured for all treatments at 24, 72 and 168 hours. Each colony was individually photographed, and root lengths and total frond area were calculated by image analysis using ImageJ software (Abràmoff et al., 2004).

Dry weight

Plant dry weight was measured in the long-term exposure experiment only. In long-term experiments *L. minor* colonies were left growing for 30 days without replacing the medium. Under these conditions, all plants would show a degree of deficiency

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stress. After the 30-day exposure, *L. minor* colonies were harvested and all PE microplastics were removed using Tween 20 as detailed previously. Then, the colonies were dried for three days at 60°C, and dry weights were determined using a Pioneer mass balance (Ohaus Corporation, USA).

Trophic transfer from *L. minor* to *G. duebeni:* experimental design and endpoints

G. duebeni adults (N=28) were individually placed in 100mL beakers filled with 100 mL previously aerated tap water. All beakers were shaded with aluminium foil to prevent direct light affecting the organisms. Amphipods were kept in absence of food for 24 hours prior to feeding to ensure starvation. After 24 hours of food deprivation, a single *Lemna minor* colony was fed to each individual for either 24 or 48 hours. "Control amphipods" (N=14) were fed clean duckweeds and "PE amphipods" (N=14) where fed duckweed previously grown for 72 hours on a suspension containing PE microplastics (50,000 microplastics mL^{-1}). Seven replicates with one individual per replicate were carried out per treatment for each time point. The number of replicates in this study is aligned with previous microplastic-amphipod ecotoxicological tests, where numbers range between three to ten replicates per treatment (Au et al., 2015; Blarer and Burkhardt-holm, 2016; Bruck and Ford, 2018; Redondo-Hasselerharm et al., 2018; Scherer et al., 2017; Weber et al., 2018).

Both clean and contaminated *L. minor* colonies had previously been dried as detailed before. The weight of each dried *L. minor* colony was recorded before and after (if not completely consumed) amphipod feeding using an Explorer mass balance with accuracy of 0.1mg (Ohaus Corporation, USA). This was done to track

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consumption and to estimate the numbers of microplastics consumed by each amphipod. Amphipod survival, moulting and fitness were recorded at 24 and 48 hours. A single clean *L. minor* colony was offered "*ad libitum*" per amphipod for another 24 hours at the end of each feeding period time to allow gut depuration. Amphipods were removed and immediately frozen and stored at -80ºC in a Forma 8800 Series Ultra-Low Temperature Freezer (Thermo Scientific). Whole *G. duebeni* guts were dissected and mounted on slides for microscopic examination (Blarer and Burkhardt-holm, 2016; Bruck and Ford, 2018). Presence or absence of PE microplastics was recorded under Green light (Filter cube N2.1, Excitation filter BP 515-560) using a Leica DFC490 fluorescence microscope.

Data analysis

Data are presented as mean ± standard error. Data were checked for normality with Shapiro-Wilk test and Q-Q plots. A one-way ANOVA with Tukey post hoc test were used for data following a normal distribution or a Kruskal-Wallis with Nemenyi post hoc test were used for non-normal data. A difference was deemed significant where $p \leq 0.05$. All statistical analyses and graphs were run using R software (version 1.1.383).

Results

PE microplastics adhering to *L. minor*

Microplastics adhering to whole *L. minor* colonies

Multiple 10-45µm PE microplastics were found to be adhering to the surface of whole, exposed *L. minor* colonies (Fig. 1a). With increasing exposure time, there was a significant increase in the number of PE microplastics adhering to fresh and

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dried *L. minor* colonies (fresh: Kruskal Wallis, X^2 = 10.3, df = 3, p = 0.02, dried: Kruskal Wallis, X^2 = 11.2, df = 3, p = 0.01). The number of PE microplastics adhering to fresh colonies after 72 hours exposure was statistically significantly higher at $p < 0.05$ than the number of PE microplastics after 3 hours. For dried colonies, the number of PE microplastics after 72 hours exposure was statistically significantly higher at $p < 0.05$ when compared to both 3 and 24 hours.

After just 3-hour exposure, fresh *L. minor* single colonies had adsorbed an average of 16.8 ± 5.6 PE microplastics (means ± SE) per colony. The number of adhered PE microplastics increased significantly on fresh colonies overtime from 3 to 72 hours and decreased between 72 and 168 hours (Kruskal-Wallis, X^2 = 10.32, df = 3, p = 0.01). When *L. minor* biomass was first dried, the number of PE microplastics was significantly lower (Kruskal-Wallis, X^2 = 11.22, df = 3, p = 0.01). For both fresh and dried biomass, the highest number of adsorbed PE microplastics per colony was found after 72 hours exposure (fresh tissue: 126.13 ± 20.56, dry tissue: 42.22 ± 8.25).

Tissue specificity of microplastics adsorbance

A more detailed analysis of microplastic adsorption focussed on tissue specificity, comparing the adaxial frond surface, the abaxial frond surface and the roots of *L. minor* colonies (Fig. 1b). PE microplastics were present on all three *L. minor* surfaces of colonies exposed to microplastics. With increasing exposure time, there was a significant increase in the number of PE microplastics on abaxial fronds (Kruskal-Wallis, X^2 = 25.54, df = 3, p = $1.19e^{-05}$) and roots (Kruskal-Wallis, X^2= 9.89, df = 3, p = 0.02).

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The number of PE microplastics was also studied on fresh and dried tissues individually (Fig. 1b). The fresh abaxial frond surface showed a significant increase of PE microplastics up to 72 hours (Kruskal-Wallis, X^2=25.54, df = 3, p=1.19^{e-05}). A pairwise comparison Nemenyi post-hoc test showed differences in microplastic adsorption on fresh abaxial surfaces between 3h and 168h ($p < 0.05$), 3h and 72h ($p < 0.001$) and 24h and 72h ($p < 0.05$). Dried abaxial frond surface showed a significant increase of PE microplastics (Kruskal-Wallis, $X^2 = 14.55$, df = 3, $p = 0.00$). A pairwise comparison Nemenyi post-hoc test showed differences in adhering microplastics on dried abaxial surfaces at 3h compared to 72h exposure ($p < 0.01$).

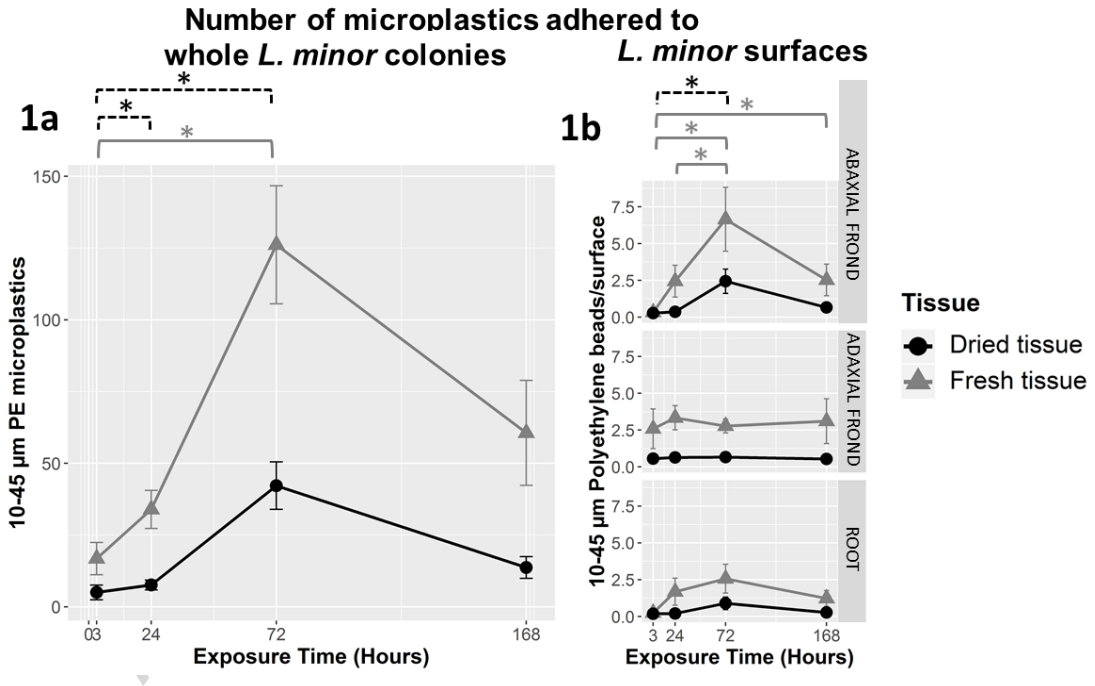


Figure 1. Adherence of 10-45µm polyethylene microplastics to *L. minor* as a function of exposure time. (**1a**) microplastics per colony (**1b**) microplastics per mm^2 abaxial frond surface, microplastics per mm^2 adaxial frond surface and microplastics per mm root length. Samples were either freshly harvested, or first dried. Independent replicates (N = 8) were run for each time point. The same colonies were used for measuring microplastics on fresh colonies, as well as dried colonies. Error

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bars represent standard error. Black dashed brackets show dried tissue significance, grey brackets show fresh tissue significance.

Impacts of microplastics on *L. minor* physiology

RGR

After 7 days growth, the mean (± SE) RGR for biomass accumulation is 0.22 ± 0.01 day^{-1} or 0.23 ± 0.01 day^{-1} for the clean control and the Tween treated sample, respectively (Fig. 2a). Colonies treated with PE microplastics showed an RGR of 0.23 ± 0.01 day^{-1} (Fig. 2a). The mean (± SE) for RGR based on Frond Number was 0.32 ± 0.01 day^{-1}, 0.33 ± 0.01 day^{-1} and 0.32 ± 0.01 day^{-1} for the clean control, Tween treatment and PE treatment, respectively (Fig. 2b).

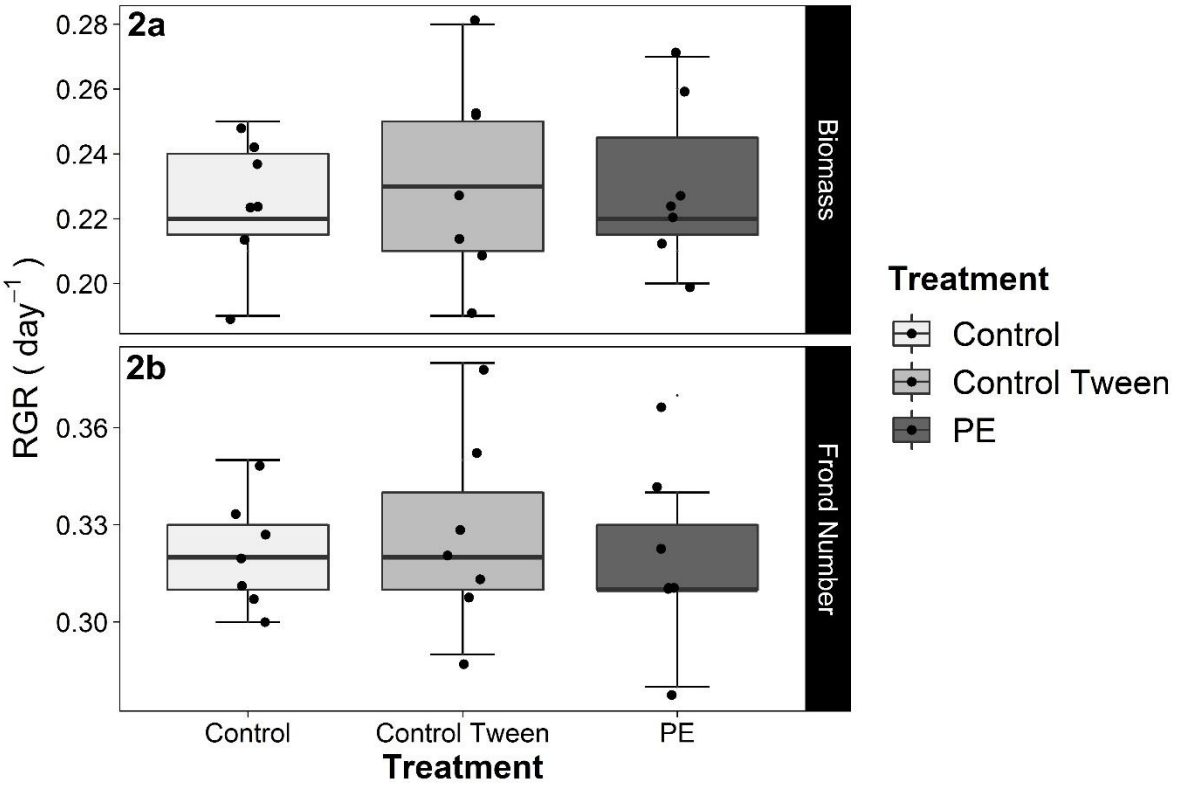


Figure 2. Relative Growth Rate of *Lemna minor* (RGR (day $^{-1}$) based on Biomass (**2a**) and Frond Number (**2b**) after a seven day growth test (N = 7). Boxplots midline show the median. Lower and higher limits of the boxes show first Q1 and third Q3 quartiles (25th and 75th percentile). Upper whisker

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shows Q3 + (1.5 x IQR) and lower whisker shows Q1 – (1.5 x IQR). IQR is the Interquartile Range. The scatter dots show N = 7 data points for each treatment and measurement.

There was no significant variation in *L. minor* biomass RGR between treatments (Fig. 2a, Kruskal-Wallis, $X^2 = 0.14$, df = 2, p = 0.93). Likewise, frond number RGR was not significantly different between treatments (Fig. 2b, Kruskal-Wallis, $X^2 = 0.26$, df = 2, p = 0.88).

Chlorophyll *a* Fluorometry

Values of Fv/Fm, Y(II), qP and qN indicate efficient photosynthetic energy conversion in control plants. None of the measured chlorophyll *a* fluorescence parameters showed any significant difference between either of the two controls and *L. minor* exposed to PE microplastics (Table 1). Thus, the overall photosynthetic efficiency of *L. minor* was not significantly affected by PE microplastics after a seven day exposure period.

Table 1. Commonly used fluorescence parameters to study plant stress. All parameters are recorded and calculated using ImagingWin Software. Photosynthetic activity of dark-adapted *L. minor* colonies (N = 3) was measured after 7 days growth with, or without microplastics. One-way ANOVA: FvFm: F = 0.02, df = 6, p = 0.98; Y(II): F = 0.14, df = 6, p = 0.87; qP: F = 0.12, df = 6, p = 0.89 and qN: F = 0.41, df = 6, p = 0.68.

Chlorophyll *a* Fluorescence parameters		**Treatments** (means ± SD)		
Photochemical quenching parameters		**Control**	**Control Tween**	**PE microplastics**
Fv/Fm	Maximum quantum yield of PSII	0.75 ± 0.02	0.74 ± 0.02	0.75 ± 0.02
Y(II)	Quantum yield of PSII	0.59 ± 0.02	0.60 ± 0.04	0.61 ± 0.06
qP	Coefficient of photochemical quenching	0.85 ± 0.01	0.86 ± 0.04	0.87 ± 0.07

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Non-photochemical quenching parameters				
qN	Coefficient of non photochemical quenching	0.28 ± 0.02	0.26 ± 0.02	0.25 ± 0.04

Root length

The length of *L. minor* roots is dependent on a wide variety of environmental conditions. Here, the root length of *L. minor* colonies exposed to PE microplastics for 24, 72 and 168 hours was analysed. Overall, root length increased with time (Kruskal-Wallis, X^2 = 33.05, df = 2, p-value = 6.657e-08). At 24 hours all roots had the same length, as expected as all plants were taken from the same starting culture at t=0h. A post hoc Nemenyi test showed that *L. minor* root length at 168 hours was significantly longer than at 24 hours (p value < 0.001) or 72 hours (p value < 0.001). Therefore, a further statistical analysis at each given time point was carried out to understand the potential effect of PE microplastics on root lengths (Fig. 3). In this particular case, data grouped per time point were normally distributed, hence a One-way ANOVA was selected. At 24 hours, the mean root length (± SE) of the clean control (0.96 ± 0.08) and Tween control (1.09 ± 0.06) were smaller than the length of roots of colonies exposed to PE microplastics (1.32 ± 0.16). However, the effect of microplastics on root length was not significant (One-way ANOVA, F = 2.89, df = 2, p = 0.09). Similarly, at 72 hours and at 168 hours the effect of PE microplastics on root length was not significant (One-way ANOVA, F = 1.84, df = 2, p = 0.19; One-way ANOVA, F = 0.22, df = 2, p = 0.80, respectively).

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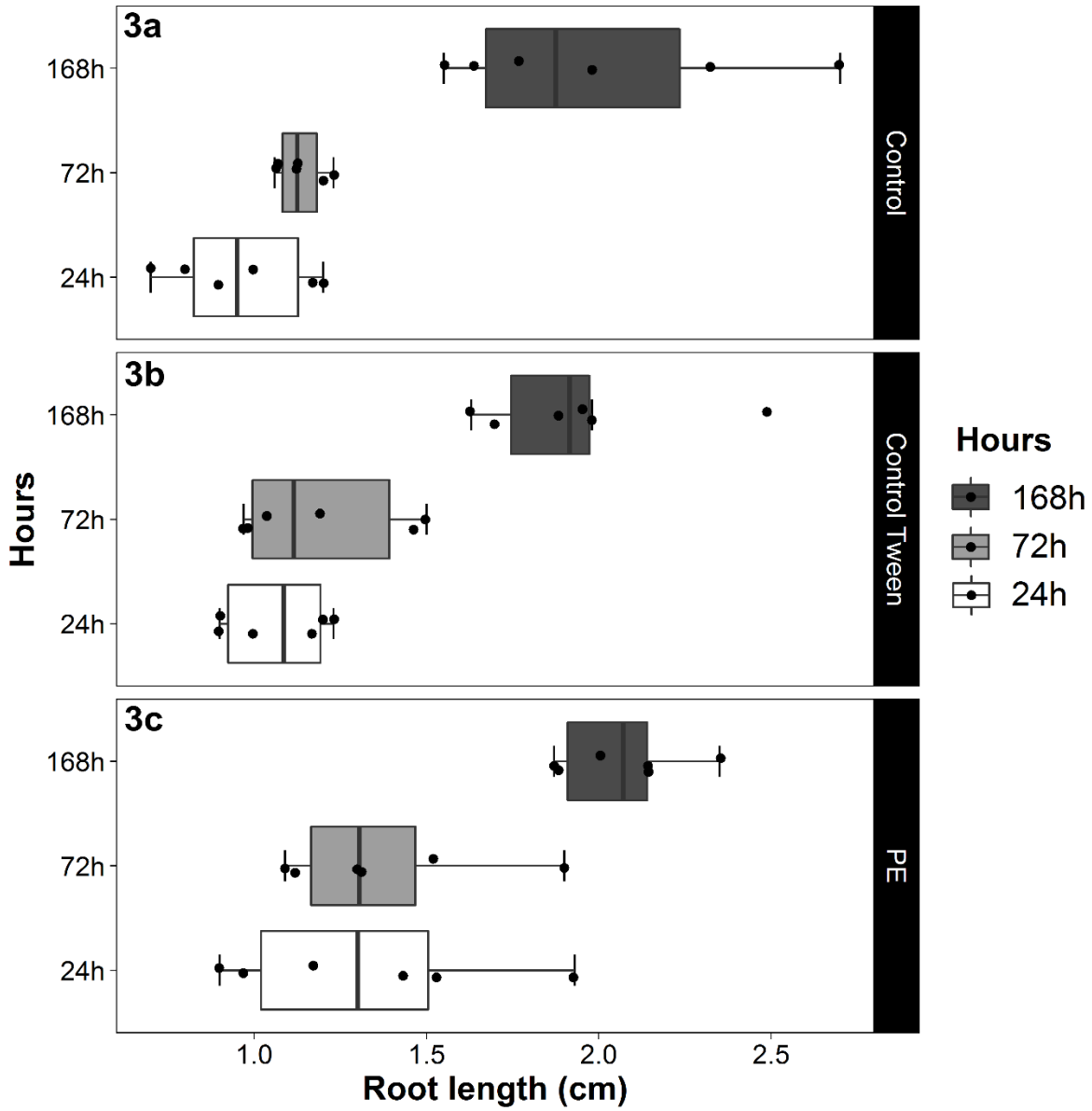


Figure 3. Root length of *L. minor* after 24, 72 and 168 hour exposure to PE microplastics (N = 6). Root lengths shown are grouped by the treatment *L. minor* colonies were exposed to: Control (**3a**), Control Tween (**3b**) and PE microplastics (**3c**). Boxplots midline show the median. Lower and higher limits of the boxes show first Q1 and third Q3 quartiles (25th and 75th percentile). Upper whisker shows Q3 + (1.5 x IQR) and lower whisker shows Q1 – (1.5 x IQR). IQR is the Interquartile Range. The scatter dots show N = 6 data points for each treatment and measurement.

30 day chronic exposure to PE microplastics

Exposure periods longer than circa ten days result in depletion of the medium, and plant nutrient deficiencies. As a result the RGR and photosynthetic performance all

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showed low values (data not shown). Therefore, the measurement of dry weight was selected as the most suitable for assessing *L. minor* growth in long term exposure experiments (Fig. 4). Dry weight data were normally distributed (N = 10). Overall, the dry weight of *L. minor* has a tendency to be lower for plants exposed to PE microplastics. However, a One-Way ANOVA showed that the difference in dry weight between the different treatments at thirty days was not significant (One-way ANOVA, F = 2.71, df = 2, p = 0.12).

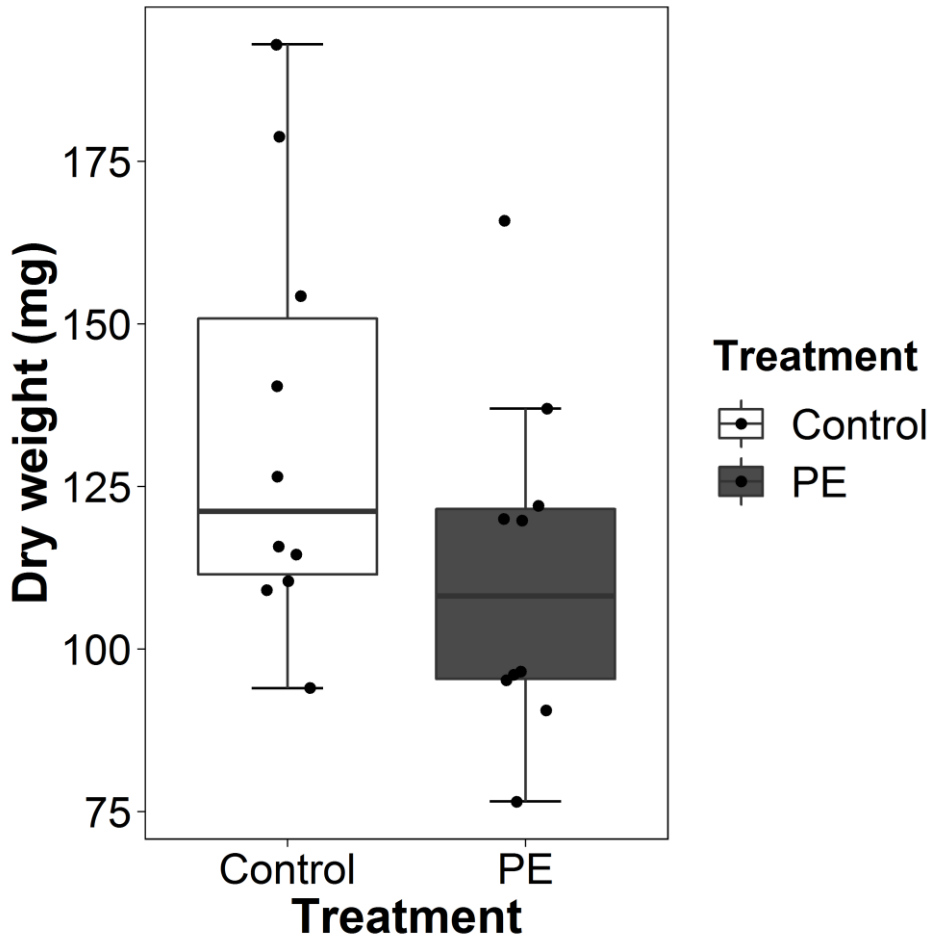


Figure 4. *L. minor* dry weight (mg) after 30 days growth (N =10). Boxplots midline show the median. Lower and higher limits of the boxes show first Q1 and third Q3 quartiles (25th and 75th percentile). Upper whisker shows Q3 + (1.5 x IQR) and lower whisker shows Q1 – (1.5 x IQR). IQR is the Interquartile Range. The scatter dots show N = 10 data points for each treatment and measurement.

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Trophic transfer of microplastics from *L. minor* to *G. duebeni*

Duckweed biomass (mg) consumed by amphipod

A total of 28 adult *G. duebeni* were fed either a clean *L. minor* colony or one previously grown for 72 hours on a suspension containing PE microplastics. *G. duebeni* individuals (N = 28) were fed for 24 hours (N=14) or 48 hours (N=14). After feeding, all amphipods were individually transferred for a 24 hour depuration period.

G. duebeni survival was found to be 100% (N = 28) across all treatments and feeding times. The mean (± SE) of biomass consumed (mg) at 24 hours (Fig. 5a) was higher for amphipods feeding on PE contaminated *L. minor* (0.46 mg ± 0.07) compared to those feeding on clean control biomass (0.29 mg ± 0.06). However, this was not statistically significant (One-way ANOVA, $F = 3.37$, $df = 12$, $p = 0.09$). In the case of *G. duebeni* feeding on *L. minor* for 48 hours (Fig. 5b), 0.40 ± 0.05 mg PE contaminated biomass was consumed per amphipod. In comparison, 0.49 ± 0.07 mg of clean biomass was consumed. Furthermore, amphipods consumed significantly more clean duckweed biomass when exposed for 48 hours compared to 24 hours (One-way ANOVA, $F = 4.70$, $df = 12$, $p = 0.05$). No such trend was observed for amphipods feeding PE-treated duckweeds for 24 and 48 hours (One-way ANOVA, $F = 0.41$, $df = 12$, $p = 0.53$).

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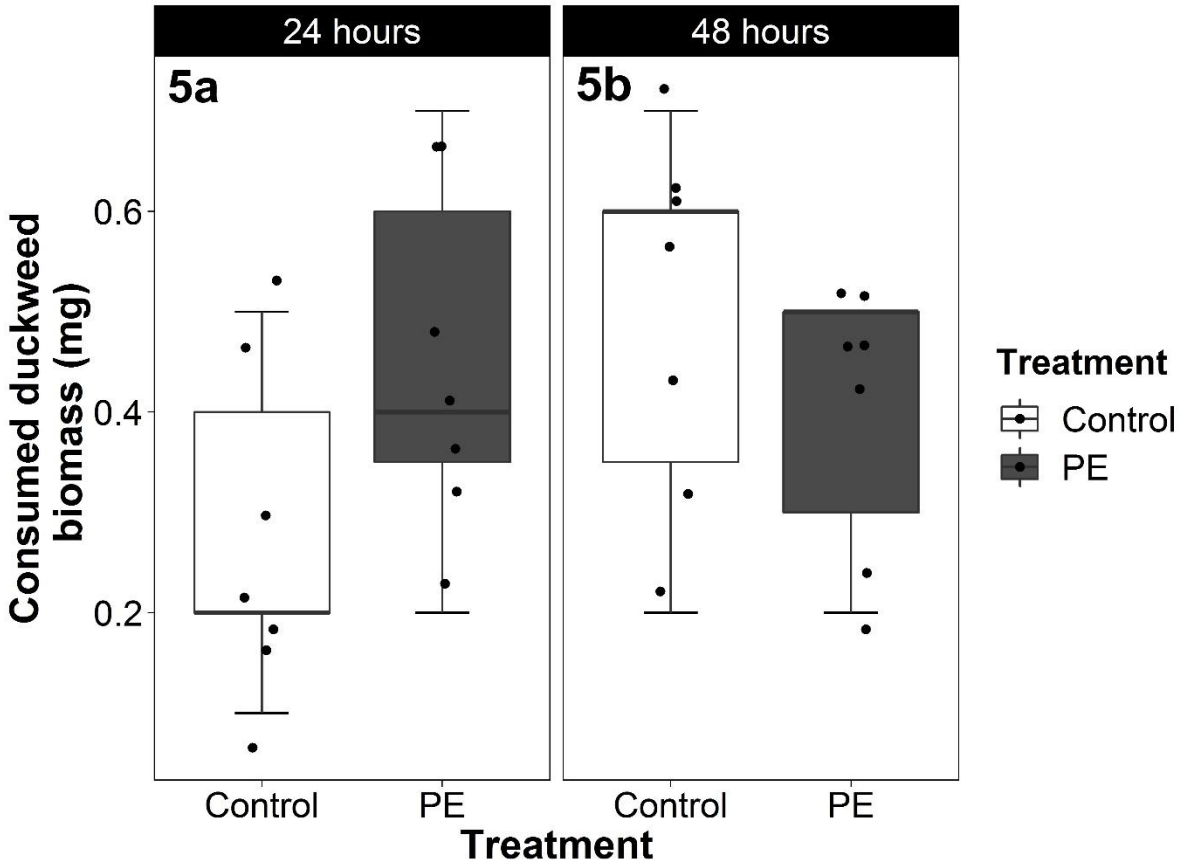


Figure 5. Total duckweed mass (mg) consumed per amphipod at 24 hours (**5a**) and at 48 hours (**5b**) before gut depuration period. Boxplots midline show the median. Lower and higher limits of the boxes show first Q1 and third Q3 quartiles (25th and 75th percentile). Upper whisker shows Q3 + (1.5 x IQR) and lower whisker shows Q1 – (1.5 x IQR). IQR is the Interquartile Range. The scatter dots show N = 7 data points for each treatment and measurement.

G. duebeni gut contents

Out of the total number of whole guts across the two PE microplastic exposure times (N =14), only 28.57% (N = 4) contained 1-2 microplastics after a 24 hour depuration period (Table 2). *G. duebeni* guts of organisms exposed to clean controls were microplastic free.

Table 2. Number of PE microplastics (10-45 µm diameter) in *G. duebeni* guts fed clean or PE contaminated duckweed (42 PE/duckweed colony) for 24 hours (N =

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14) or 48 hours (N = 14)

Feeding time	Treatment	Total dissected *G. duebeni* guts	Number of microplastics (MPs) in *G. duebeni* guts		
			No MPs	One MP	Two MPs
24 hours	Control	7	7	-	-
	PE	7	5	1	1
48 hours	Control	7	7	-	
	PE	7	5	1	1

Discussion

Adsorption of microplastics by *L. minor*

Here we show that polyethylene microplastics adhere to all *L. minor* colonies grown in the presence of microplastics. High numbers of PE microplastics adhered to the abaxial frond surface, which was in direct contact with the medium in which microplastics were suspended. Interestingly, small numbers of microplastics were also found on the adaxial frond surface, an observation that could relate to either incidental waves and/or the binding affinity between microplastics and frond surface. Under the experimental exposure of this study, highest numbers of microplastics were found at 72 hours incubation, after which numbers adhered slightly decreased. This decrease may well be a result of a combination of rapid plant growth, together with lower numbers of available microplastics due to clumping of particles in the medium. Previously, Goss et al. (2018) reported the presence of microplastics on the blades of the seagrass *Thalassia testudinum* collected in the natural environment. At this stage, it is not clear whether adherence of microplastics to plant surfaces is a common phenomenon. Also, the actual physicochemical interaction by which

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microplastics adhere to plant surfaces is not known. However, in the case of 10-45 um PE microplastics, hydrophobic interactions are probably of importance as microplastics can be washed off plant surfaces using a high concentration of surfactant (data not shown). Adsorption of microplastics onto primary producers may depend on hydrophobic or hydrophilic attractions or hydrogen bond formation between microplastic and plants surfaces, and is likely to depend on the microplastic characteristics and plant species properties such as surface morphology (Bhattacharya et al., 2010;Gutow et al., 2016). Adsorption of microplastics to surfaces is not limited to plants. For example, Bhattacharya et al. (2010) showed the adsorption of positively charged polystyrene (PS) nanoplastics to two freshwater microalgae (*Chlorella sp*. and *Scenedesmus sp.).* Similarly, the brown algae *Fucus vesiculosus* adsorbed polyacryl wool fibres and PS microbeads and fragments (Gutow et al., 2015). The number of microplastics adhering to *F. vesiculosus* surfaces was directly related to the plastic concentration in the suspension. The factors contributing to microplastic adsorption by *F. vesiculosus* could be their phaeophycean hairs and the release of alginate, which has gelatinous properties, when the algae is cut (Sundbaek et al., 2018).

If adsorption to plant and algal structures is common, then this will impact on the environmental fate of microplastics. In terms of the former, plants are well known for their capability to adsorb pollutants. For example, trees can adsorb large amounts of particulate matter on leaves, contributing to the improvement of air quality (Nowak et al., 2006). *L. minor* has previously been studied because of its capability to adsorb nanoparticles, which has potential for phytoremediation (Ekperusi et al., 2019). *L. minor* has been shown to effectively remove pollutants such as CuO, colloidal solutions of metal nanoparticles, silver nanoparticles or zinc oxide nanoparticles

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(Yue et al., 2018; Olkhovych et al., 2016; Ortaç et al., 2014, Fikirdeşici-Ergen and Üçüncü et al., 2014, respectively). Similar to the adherence of these particles to plants, adherence of microplastics can have substantial consequences for the environmental fate of microplastics.

Microplastics have no short term effect on *L. minor* physiology

We found that a substantial number of microplastics covered surfaces of *L. minor* fronds (Fig. 1). Yet, the presence of PE microplastics had no effect on *L. minor* growth nor photosynthetic efficiency following seven days microplastic exposure. Detailed analysis of photosynthetic performance also did not reveal any evidence of a negative impact of microplastic exposure. Finally, longer term exposure studies (30 days) showed no conclusive evidence of an impact of microplastics on the growth of *L. minor*. These results agree with those from Kalčíková *et al.* (2017). These authors found no effects of PE microplastics ranging in 30-600 µm in size on *L. minor* frond number and photosynthetic pigment concentration after seven days exposure at concentrations of 0, 10, 50 and 100 mg/L (concentration in our study was 36 mg/L). Kalčíková et al. (2017) did, however, find that *L. minor* had shorter roots when exposed to microplastics. This was not observed in this study. In fact, there was a non-significant tendency for longer roots in PE-exposed plants. Root length is a highly sensitive parameter, and a range of environmental factors can cause a change in root length. For example, both drought and a lack of nutrients tends to cause root elongation (Cairns et al., 2011; Farooq et al., 2009). Root damage has also been observed, potentially caused by sharp 30-600 µm PE microplastics (Kalčíková et al., 2017). Root growth can also be considered in the context of the hormonally controlled root-shoot ratio of plants. PS microplastics reduced the shoot to root ratio (S:R) of the freshwater macrophytes *Myriophyllum spicatum* (van Weert

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et al., 2019). However, this effect was accompanied by a decrease in RGR. Thus, although the current study found no evidence for impacts of PE microplastics on plant growth and photosynthesis, impacts on plant long-term developmental processes remain to be analysed.

Trophic transfer of microplastics in the freshwater food chain

Windsor et al. (2019) reported the presence of microplastics in half of the freshwater detritivores and filter feeder species sampled across all riverine sites tested. This finding demonstrates that microplastics are entering lower levels of freshwater food webs. Yet, data on the presence of microplastics in freshwater macroinvertebrates are scarce, as most studies have focused on fish and birds (O'Connor et al., 2019).

The transport of microplastics between trophic levels of the freshwater food chain has not yet been recorded. However, trophic transfer of microplastics has been observed between marine prey-predator species (Welden *et al.,* 2018; Nelms *et al.,* 2018) under natural conditions. In addition to this, the transfer of microplastics through marine food webs has been tested under laboratory conditions. Gutow *et al.*, (2015) showed that the periwinkle *Littorina littorea* did consume microplastics adhered to the brown seaweed *Fucus vesiculosus,* which had previously been exposed to microplastics. The absence of microplastics in the periwinkle midgut gland and the presence in the faecal pellets indicated that microplastics did pass through the gut. Other laboratory studies have observed trophic transfer of microplastics from brown seaweeds to periwinkles (Gutow et al., 2015), from brine shrimp to zebrafish (Batel et al., 2016) or from mussels to crabs (Farrell and Nelson, 2013; Santana et al., 2017; Watts et al., 2014). Additionally, multilevel food webs

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have showed that nanoplastics can also be transferred through more than two trophic levels (Cedervall et al., 2012; Chae et al., 2018).

The transfer of microplastics via the freshwater food chain is still not well understood. Bruck and Ford (2018) used an artificial powdered seaweed feed containing a controlled concentration of microplastics in their tests. Other studies have provided either an uncontaminated food source (Blarer and Burkhardt-holm, 2016; Bruck and Ford, 2018; Redondo-Hasselerharm et al., 2018; Weber et al., 2018), food during the egestion period only (Scherer et al., 2017) or no food at all (Redondo-Hasselerharm et al., 2018). Here, we used a more environmentally relevant approach to explore whether microplastics can transfer through feeding from lower trophic levels (macrophytes) to higher levels (macroinvertebrate) by feeding dead *L. minor* biomass to the co-occuring detritivore *G. pulex* (Lahive et al., 2015).

In this study, we show that *L. minor* can adsorb an average of 42 PE microplastics per single dried colony of 0.98 mg at 72 hours, which was selected for the feeding test. These microplastics are potentially available for trophic transfer to *G. duebeni*. An average of 0.46 mg of PE contaminated duckweed was consumed by amphipods after 24 hours feeding (N = 7). Assuming a homogeneous distribution of PE microplastics across a *L. minor* colony, it can be concluded that amphipod may have ingested 20 PE microplastics. At 48 hours, *G. duebeni* consumed 0.40 mg of PE duckweeds on average (N = 7), and this corresponds to 17 PE microplastics. Although there was a slight decrease of biomass consumed per amphipod at 48 hours compared to 24 hours, this was not significant. No conclusions can be drawn concerning a potential effect on the intake of PE contaminated duckweed at longer exposure times. It was found that very small numbers (one or two) of microplastics ended up in the gut of 28.6% (4 out of 14) *G. duebeni.* The finding that low numbers

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of microplastics are present in the gut of *G. duebeni*, agrees with Bruck and Ford, (2018) who found that only 8% of the exposed amphipods had one microplastic in the gut. Low numbers of microplastics in the gut may indicate that amphipods are able to selectively feed (Arsuffi and Suberkropp, 1989) excluding microplastics. Another factor could be a rapid excretion of microplastics by *G. duebeni.* Au et al. (2015) showed that amphipods are able to completely egest 10-27 µm polyethylene microplastics at longer exposures. This study cannot distinguish between these two scenarios.

Microplastics do not affect *G. duebeni* in short term exposure studies

The effects of microplastics can be linked to exposure characteristics such as particle shape, dose or time. But the effects also depend on various aspects related to the trophic ecology of the species studied. Feeding type and morphology, play a role in the species sensitivity to microplastics (Chae et al., 2018; Horton et al., 2017; Scherer et al., 2017). We found 100% survival rates after a 24 or 48 hour microplastic trophic transfer study using *G. duebeni*. Likewise, moulting activity was not affected by the presence of microplastics on food. These findings were expected as recent literature shows no negative effects of pristine microbeads and fragments on amphipod survival, not even following long exposures (Au et al., 2015; Bruck and Ford, 2018; Redondo-Hasselerharm et al., 2018; Weber et al., 2018). However, some studies have shown effects of microplastics on invertebrates, indicating that such effects occur under specific conditions. For example, Blarer and Burkhardt-holm (2016) found that a four week chronic exposure of amphipods to polyamide fibres had an impact on food assimilation, but during the second week only. Chronic

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exposure of *Daphnia magna* to nanoplastics decreased population growth and body size of individuals and produced malformations on neonates (Besseling et al., 2014). Plastic fragments and fibres had a greater effect on *D. magna* than natural particles and plastic microbeads (Ogonowski et al., 2016; Ziajahromi et al., 2017). Some studies argue that the impacts of microplastics depend on environmental factors such as food to plastic ratio. For example, the food:plastic ratio provided to *D. magna* during microplastic exposures determined the biological effect, rather than the presence of microplastics per sé (Aljaibachi and Callaghan, 2018; Rehse et al., 2016). Thus, although no negative impacts of microplastics were found in this study, it would be premature to exclude such effects.

Conclusion

This study demonstrates that the aquatic plant *L. minor* can rapidly adsorb 10-45 μm polyethylene microplastics. Microplastics were present on all *L. minor* colonies exposed to microplastics from 3 h to 168 h exposures. The highest number of adsorbed microplastics per colony was found at 72 h. Accumulation of microplastics was greater on those *L. minor* surfaces in direct contact with the suspension. No impact was found on *L. minor* RGR, chlorophyll *a* fluorescence and root length at seven days. A 30 days exposure showed no conclusive evidence of microplastic effects on the growth of *L. minor*. This study also shows that microplastics can be transferred through the freshwater food chain, from the primary producer *L. minor* to the consumer *G. duebeni*. Microplastics were present at low numbers in the guts of *G. duebeni* after <48 hour exposures. Microplastics did not affect *G. duebeni* survival at short exposures.

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Acknowledgments

The authors would like to thank the Irish Environmental Protection Agency (EPA) for funding the research project entitled “Impacts of Microplastics in the Irish Freshwater Environment (IMP)” project no. 2016-W-MS-23 as part of their EPA research programme 2014-2020.

Author contributions

AMC and MJ conceived and planned the study aims, AMC designed the methodology, AMC, DTS and GS carried out the experimental work, AMC carried out statistical analysis and drafted the manuscript. MJ, FVP and JO supervised the project.

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Highlights

- Polyethylene microplastics strongly adsorb to *L. minor*
- Acute exposure to microplastics had no effect on *L. minor* photosynthesis and growth
- Microplastics can be transferred from *L. minor* to the macroinvertebrate *G. duebeni*
- Ingestion of microplastics had no apparent impact on *G. duebeni*

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