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Environmental conditions affect the food quality of plastic associated biofilms for the benthic grazer *Physa fontinalis*

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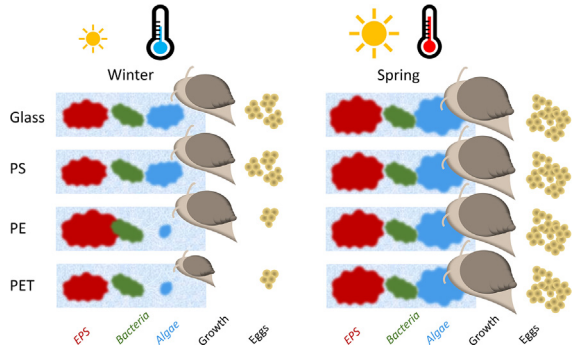
HIGHLIGHTS

- Plastic particles in freshwater affect the composition of their associated biofilms.
- Biofilms on PE and PET inhibit growth and reproduction in benthic grazers.
- Environmental conditions influence impact of plastic on biofilm formation.

GRAPHICAL ABSTRACT



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ABSTRACT

With an ever-increasing amount of plastic pollution in the various aquatic ecosystems around the world, the effects on organisms are still not fully understood. Most studies focus on direct effects posed by plastic intake or entanglement, but plastic debris can also affect primary production of biofilms and have an indirect impact on its consumers. This study investigates the primary production on three common plastic types in freshwater and its food quality for a benthic grazer. We hypothesized that different polymer types affect biofilm composition as well as the life parameters of its consumers. We incubated polyethylene (PE), polyethylene terephthalate (PET) and polystyrene (PS) as well as glass (control) in a productive freshwater creek for natural biofilm establishment. To account for changes in the environmental conditions, the experiment was conducted twice during winter and late spring, respectively. These biofilms were offered to the freshwater gastropod *Physa fontinalis* as sole food source. Growth and reproduction of the snails were measured to monitor sublethal effects. Additionally, biofilm composition was observed using confocal laser scanning microscopy (CLSM). In winter, snails feeding off PET and PE showed a significantly lower egg production and lower growth rates were observed on PET. No such effects occurred in spring. CLSM data revealed, that algal growth was significantly lower on PE and PET during the winter treatment compared to PS and glass. Since we could only find these effects during the colder and darker months (January–March), the microbial colonization on PE and PET was inhibited by the substrate under less favorable conditions of temperature and light. Hence, benign conditions may mask the adverse effects of microplastic on food webs. Our findings show that future studies on the plastisphere will need to consider such variations to further understand the influence of plastic pollution on primary production and higher trophic levels.

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1. Introduction

Plastic pollution is one of the most discussed environmental problems of the 21st century (Blettler et al., 2018, do Sul et al., 2014). Because of the sheer amount of plastic entering our ecosystems every year and their resulting abundance in sediments, it is even discussed to use plastic as a marker for the epoch of the Anthropocene (Bancone et al., 2020; Waters et al., 2016). However, its effects on the environment are not fully understood. There has been a steadily growing amount of studies focusing on different aspects of the problem, e.g. total plastic loads in different ecosystems (Eriksen et al., 2014) or plastic intake by organisms (Haegerbaeumer et al., 2019). (Micro)plastic pollution in the oceans still is the main focus of research and mainstream media, but since most marine plastic originates from land (Schmidt et al., 2017), freshwater systems, e.g. rivers (Klein et al., 2015), lakes (Eriksen et al., 2013) and reservoirs (Hübner et al., 2020), have gained growing attention in recent years. The effects of plastic exposure have been tested on various freshwater organisms, but the results show no clear trends on the toxicity of these pollutants. On the one hand no effects have been reported for some organisms and materials, while on the other hand effects on mortality, reproduction, growth and other life parameters have been shown for many others as reviewed by Haegerbaeumer et al. (2019). Most animals, e.g. fish (Collard et al., 2019), mollusks (Pedersen et al., 2020) and nematodes (Mueller et al., 2020), take up microplastics directly with their food or their filtering activity, however, plastic pollution might indirectly pose additional threats to aquatic biota.

Plastic debris in aquatic environments, like any other submerged solid surface, can serve as substratum for microbial communities of algae, bacteria and fungi (Hoellein et al., 2014). These microorganisms form consortia of cells, that are connected by a matrix of extracellular polymeric substances (EPS) and physiologically differ from planktonic individuals of the same species (O'Toole and Kolter, 1998). Biofilms play an important role in freshwater ecosystems as they serve as a basis of the benthic food web and are consumed by various invertebrates and fish (Hall and Meyer, 1998). Analyses of plastic associated biofilms indicate an influence of the artificial substrates on the microbial communities (McCormick et al., 2014; Ogonowski et al., 2018) but the so called "plastisphere" remains a highly controversial topic and requires ongoing research, since an impact of plastic pollution on primary production might have severe consequences for the aquatic food chain.

Vosshage et al. (2018) observed an influence of polymethyl methacrylate (PMMA) and polycarbonate (PC) on the nutritional value of biofilms for the pulmonate *Radix balthica*. The snails feeding on those biofilms showed a decrease in growth, caused by inhibited consumption (on PC) and an altered biofilm composition (on PMMA). This indicates that plastic pollution might already affect benthic food webs at their microbial base. However, as these experiments were conducted under optimal growth conditions for the establishment of biofilms (high light availability, warm temperatures and sufficient nutrient supply), information on other or less favorable environmental growth conditions are lacking. Further studies on this topic are virtually absent (Rummel et al., 2017).

This study investigates the effects of plastic on a benthic grazer as well as its food source, covering two trophic levels. The snail *Physa fontinalis* (L.; Gastropoda: Pulmonata) is commonly found among submerged vegetation in most slow-flowing streams and lakes all over Europe. They largely feed on biofilms growing on stones and plants (de Wit, 1955). We chose *P. fontinalis* as a model organism for benthic grazers due to its abundance and broad distribution (Leung et al., 2004).

The biofilm composition on three common plastic types and a control were analyzed. Additionally, we investigated the sublethal effects of these biofilms on the snail *P. fontinalis*, by monitoring its growth, reproduction and feces production. To account for variations in the environmental parameters temperature and light during biofilm formation, we conducted the same experiment twice during winter (lower light availability and lower temperatures) and late spring (higher light availability and higher temperatures). We hypothesized that 1) plastic influences the microbial communities growing on it, 2) there is an effect of plastic-associated biofilms on the nutritional value for the benthic grazer *P. fontinalis*, manifested in its growth and reproduction, and 3) such effects are subject to the respective environmental conditions.

2. Material and methods

2.1. Biofilm cultivation

We chose polyethylene terephthalate (PET), polystyrene (PS) and high density polyethylene (HDPE, further referred to as PE) as plastic substrates since these materials are among the most abundant plastic types found in the environment (Imhof et al., 2016). They were obtained from a supplier of materials for research and development (Goodfellow Ltd.) and cut into slides of 26 × 76 × 1 mm. Polymer types were initially verified via Fourier transform infrared with Attenuated total reflection analysis (ATR-FTIR; Agilent Cary 670).

Standard glass microscopy slides (VWR, Radnor, PA, United States) of the same size were used as control. The biofilms were cultivated in a eutrophic, slow-flowing creek in the Rieselfelder of Muenster, Northwest Germany, which is connected to the outflow of a wastewater treatment plant (WWTP) in 2 km distance. This WWTP purifies 60,000 m^3 municipal wastewater per day via tertiary treatment processes (Hübner et al., 2020), resulting in a constant water and nutrient supply and temperatures suitable for cultivation, especially in winter (12 ± 2 °C). In spring the temperature rose up to 20 °C (17 ± 3 °C). Daily water temperature data were provided by the civil engineering office of the city of Muenster, in addition to measuring on sampling days. To account for variations in light availability, mean daily shortwave radiation data was obtained from a permanent weather station located in close proximity to the sampling site (see Supplementary data, Fig. 1).

PVC frames were used to hold 100 slides at once in a 45° angle position, closely under the water surface and facing south. The slides were cultivated for 21 days per batch and stored after collection in dark boxes in filtered creek water at 4 °C until further use. Fresh slides were cultivated after each sampling date to maintain a constant supply. The incubation periods lasted from January–March (winter) and April–June (spring), respectively.

2.2. Exposure in grazing experiment

The test organisms were obtained from a commercial distributor for aquarist supplies who kept the snails in quarantine to exclude diseases and parasites. The acclimatization as well as all experiments were conducted in a climate-controlled room at 20 °C with a 16:8 light cycle. Acclimatization lasted for two weeks in glass containers gradually filled with reconstituted water (Osterauer et al., 2010). Tetra Min fish flakes (Tetra) were fed ad libitum.

The grazing experiments in winter and spring lasted for six and eight weeks, respectively. Prior to the experiments, the shell of each individual was measured from the outer lip to the apex. In winter the average size was 5.5 mm (±1.5 mm), while the snails obtained in spring were smaller and averaged at 3.5 mm (±1.5 mm). 1 L glass beakers with 500 mL of reconstituted water were used as containers. Three individuals of *P. fontinalis* were randomly placed into each beaker, with eight replicates per substrate, i.e. 96 individuals per trial in total. Dead individuals were removed immediately. One slide with biofilm was placed into each beaker and exchanged twice a week. 250 mL of water were exchanged weekly and evaporated water was substituted with deionized water. Each individual was measured weekly using a digital caliper as well as a digital microscope (VHX-5000, Keyence Corp.). Egg packages were removed and counted weekly to monitor reproduction. Feces were collected from each beaker with a pipette twice a week, dried at 60 °C and weighed weekly to monitor the amount of digested biofilm.

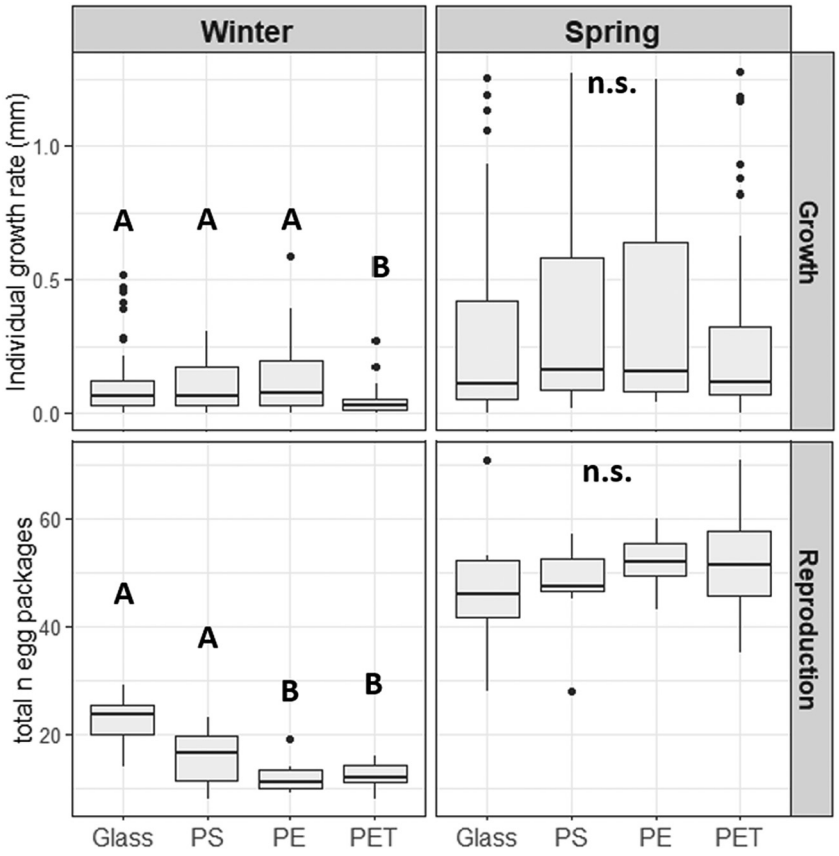

Fig. 1. Individual growth rate per week (upper layer) and total number of egg packages per week (lower layer) in winter and spring on Glass, PS (polystyrene), PE (polyethylene), and PET (polyethylene terephthalate) ($n = 96$ per trial). Significant differences from the control are indicated by different letters ($p \leq 0.01$, two-sided t-test; n.s. = not significant).

Data on feces mass is shown only for the winter trial. The large amount of biofilm in the spring trial made a separation between feces and biofilm, that was scraped off but not digested by the snails, prone to errors.

2.3. Confocal laser scanning microscopy

An analysis of the biofilms via Confocal laser scanning microscopy (CLSM) was applied after the experiment, following the method used in Vosshage et al. (2018). Briefly, three slides per substratum and testing period were fixed in 3.5% paraformaldehyde after sampling and observed using a confocal microscope (TCS SP5X, Leica, Wetzlar, Germany), controlled by the software LASAF 2.7.3.0723 (Leica). To stain bacteria and the glycoconjugates of the extracellular polymeric substances (EPS), the flourochromes SYBR®Green (ThermoFisher Scientific Inc.; Waltham, USA) and the lectin AAL-Alexa568 (Vector Laboratories, Burlingame, USA) were applied, respectively, directly before analysis. Chlorophyll A in algae does not need staining to record emissions. Laser excitations were at 490, 561 and 633 nm. Emission signals were recorded from 480 to 500 nm (reflection), 510–580 nm (SybrGreen), 590–650 nm (AAL-Alexa568) and 650–720 nm (chlorophyll A). Samples were examined using a 25 × NA0.95 water immersible lens at zoom 2. Each slide was scanned five times at random locations, with each scan measuring 310 × 310 μm. Scan steps in z-direction were either 1 or 2 μm, depending on biofilm thickness. Volumes of the biofilm components were quantified via digital image analysis using JImageAnalyzer 1.4 (Staudt et al., 2004).

2.4. Data analysis

Data on shell growth was converted into individual growth rate per day and reproduction was shown as weekly number of egg packages per individual. Differences within both parameters were analyzed with a two-sided t-test. Normality and homogeneity of variances were confirmed by using the Shapiro-Wilk-Normality-Test and the Brown-Forsythe Levene Test, respectively.

Due to the overall heterogeneity of the biofilms, the threshold for significance in volumes was set at $p = 0.01$. Biofilm volumes per μm^2 were calculated from pixel data and compared with an analysis of variance (ANOVA). The necessary assumptions of normality and homogeneity of variances were confirmed as described above. Before the statistical analysis, all biofilm data was log10- transformed to achieve normal distribution.

All calculations were performed with the software RStudio.

3. Results

3.1. Sublethal effects on P. fontinalis

In winter, *P. fontinalis* feeding off PET and PE showed a significantly lower egg production than individuals feeding off PS and glass ($p < 0.01$; Fig. 1). PET egg production was half the number of egg packages produced compared to the control group. Growth rates were significantly lower of snails grazing on PET biofilms compared to other substrates ($p \leq 0.01$; Fig. 1). This is supported by the significantly lower amount of feces dry mass that was produced in the PET treatment (Fig. 2).

However, these different effects of polymer types were only found in the winter trial but not in the spring trial. During the warmer months no effects of the different polymer types on the life parameters tested in *P. fontinalis* were identified, neither in reproduction nor in growth. Furthermore, both total growth and reproduction was higher in the spring trial on all substrates compared to the trial conducted in winter.

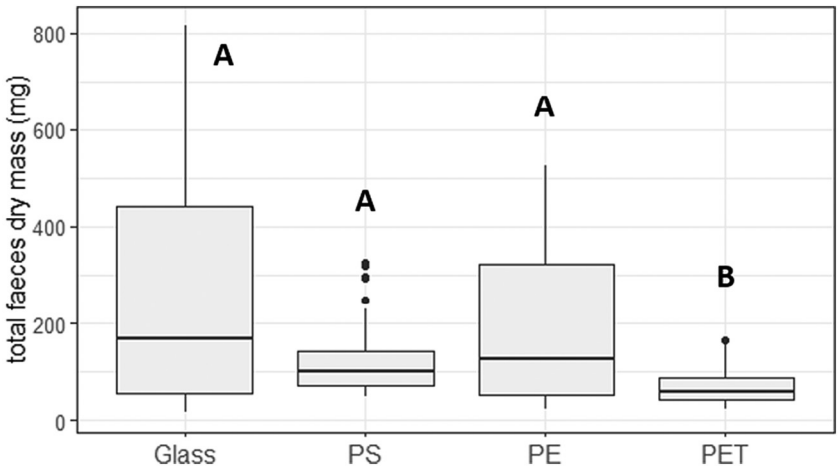

Fig. 2. Total feces dry mass per week in winter on Glass, PS (polystyrene), PE (polyethylene), and PET (polyethylene terephthalate). Significant differences from the control are indicated by different letters ($p \leq 0.01$, two-sided *t*-test; n.s. = not significant).

There was no difference in mortality, which stayed low (<10%) in all trials and treatments.

3.2. Biofilm composition

The data on the composition of the biofilms obtained through CLSM confirm the pattern observed in the grazing experiments. Biofilms grown in spring show a greater variation in their composition within the substrates rather than among the substrates, with no significant differences among the plastic types (Fig. 3). In spring, the largest area is covered by EPS, followed by algae and bacteria.

In winter, the composition of the biofilms significantly differed among substrates. The growth of algae was significantly lower on PE and PET ($p \leq 0.01$), with only a fraction of the volumes detected on glass and PS. In contrast, the production of EPS glycoconjugates is significantly higher on PE than on the control ($p < 0.01$). Bacteria volumes did not differ among the substrates.

Apart from differences among substrates, algal volume was generally lower during the winter trial than in spring. In contrast, bacteria and EPS were in the same range during both seasons. Exemplary images are shown in the supplementary data (Fig. 2).

4. Discussion

While neither growth rates nor reproduction of *P. fontinalis* was affected by polymer type in the spring trial, reproduction of *P. fontinalis* was significantly lower in winter when feeding on biofilms grown on PET and PE and the snails also grew significantly less in the PET treatment. Lower growth and reproduction rates on PET may be attributed to lower feeding rates and lower amounts of exploitable biomass in biofilms grown on PET as the amount of feces was significantly lower than in the other treatments. Furthermore, in winter biofilms on PE and PET contained significantly less algae than biofilms on PS or glass. A low amount of algae probably resulted in a restricted nutrient supply compared to glass associated biofilms. Low food quality of periphyton, has been shown to negatively affect the growth and reproduction of their consumers (Stelzer and Lamberti, 2002; Auld and Henkel, 2014).

The biofilms used were cultivated under natural light exposure, which increased up to trifold towards the second trial conducted in spring and was overall low in winter. Ohta et al. (2011) studied the influence of light intensity on the food quality of periphyton and consequently on the growth rates and reproduction of the freshwater snail *Gyraulus chinensis*. They identified C:P ratios as explanatory variable for snail growth, which is dependent on light. Under lower light conditions, C:P ratios were higher leading to lower growth rates of the snails compared to intermediate light levels. Although the experiments of Ohta et al. (2011) were conducted under oligotrophic conditions, Similar mechanisms might have caused the different effects on *P. fontinalis* - in the current study. Hence, higher light availability may have also resulted in lower C:P ratios in biofilms, consequently leading to better growth rates of *P. fontinalis*.

Biofilm compositions in spring generally showed proportions which have been previously described in natural freshwaters (Neu and Lawrence, 1997), with high volumes of EPS followed by lower volumes of algae and bacteria. The high variation within the substrates, especially in spring, was expected due to environmental biofilm heterogeneity (Singer et al., 2010). Natural biofilm formation starts with an initial coverage of the substrate by a conditioning film, consisting of a layer of macromolecules (Yang et al., 2016). This conditioning film is the foundation for the maturing biofilm, which consequently is less influenced by the substrate in later stages of its development (Oberbeckmann et al., 2014). This might contradict the assumption that plastic-associated biofilms develop a structure and composition, that is distinct from natural substrates. However, multiple studies in different aquatic environments could find distinct microbial communities on plastic substrates, termed the "Plastisphere" (Zettler et al., 2013; Wright et al., 2021) but the underlying mechanisms of these distinctions are still controversial.

Oberbeckmann et al. (2017) found that plastic specific microbial communities can only develop under certain conditions, i.e. high salinity and low nutrition, which triggers a more specialized biofilm formation (Stanley and Lazazzera, 2004). Respective disadvantageous conditions might have been present during the winter period in this study. Especially light exposure was restricted in the first period, due to short days and cloud coverage. This would also primarily affect photoautotrophic organisms and explain the low detection levels of chlorophyll A (algae) in our CLSM data. An incubation of 21 days might have been too short a time to develop a mature biofilm under these conditions, in contrast to the warmer months, which is further underlined by the lower total biofilm volumes on all substrates incubated in winter. The respective substrates therefore had more impact on the microbial communities in winter than in spring. This effect might have not been detectable in a later stage of maturity. In comparison to the control, algal volumes on PE and PET were significantly lower, while PE showed higher volumes of EPS. A similar trend is visible on the other plastic substrates. Such an increased release of EPS might be an indicator for stressful conditions for primary producers (Scott et al., 2014), hereby caused by environmental factors in winter combined with the plastic substrates.

Additional external stressors might have been present during cultivation in this natural waterbody. Lower algal volumes and varying

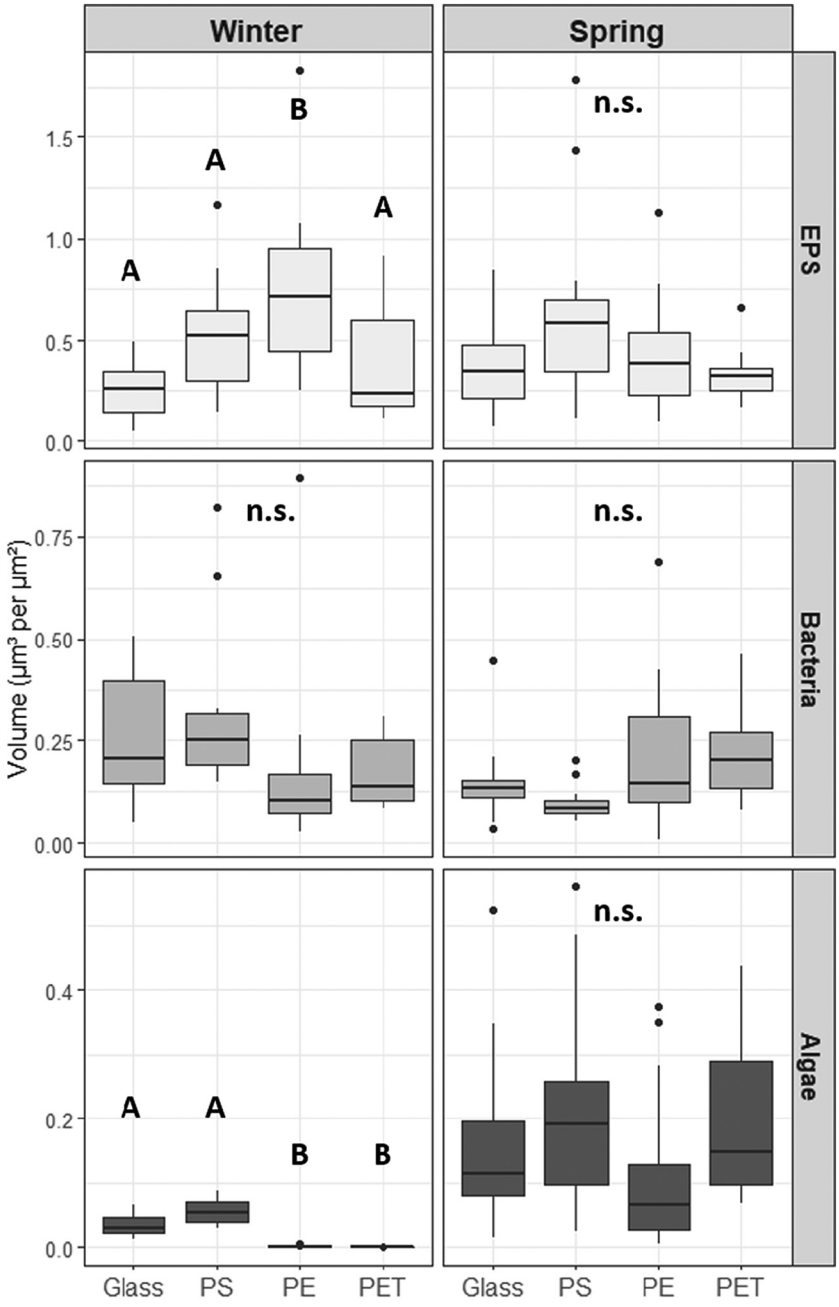

Fig. 3. Volumes of biofilm components in winter and spring on glass, PS (polystyrene), PE (polyethylene), and PET (polyethylene terephthalate). Different letters indicate significant differences ($p \leq 0.01$, ANOVA; n.s. = no significance). Note the different scales on the y axis.

effects on growth and reproduction may also result from potential adsorbance of different pollutants frequently found in freshwater, such as pharmaceuticals, pesticides or metals. In the water where the slides for biofilm colonization were cultivated, different herbicides (terbutylazine and isoproturon) were detected (Gitter et al., 2012). Terbuylazine and other herbicides were shown to better adsorb to PE than to PS (Wang et al., 2020), while PET was not tested in this study. However, PET was shown to adsorb glyphosate and mercury (Magrì et al., 2021). In addition, higher concentrations of metals (such as Pb > Cd > Zn > Cu > Co > Ni) sorb to PE than other polymer types, i.e. PS (Li et al., 2019; O'Connor et al., 2016). Metals like Pb (Woolery and Lewin, 1976; Silverberg et al., 1977), and Cd (Hart and Scaife, 1977) were proven to affect growth rates of algae. In general, adsorbance of different pollutants was high for PE and higher if compared to PS. Hence, adsorbance of herbicides or metals may lead to a lower growth rate of algae on PE than on PS. However, less studies examined the adsorbance of pollutants to PET, in particular in comparison to other polymer types. Rochman et al. (2013) showed that even if concentrations of PAHs and PCBs adsorbed to HDPE, LDPE, and PP were consistently much higher than concentrations adsorbed to PET and PVC, still a reasonable amount of these pollutants were found on PET. Hence, pollutants might be an additional stress factor which inhibits algal growth rates on PET and PE and thus leads to lower growth and reproduction rates in *P. fontinalis*.

5. Conclusion

In conclusion, the fact that *P. fontinalis* does not reject the plastic-associated biofilms might lead to an ecological trap in areas highly polluted by plastic debris. The snails that feed off biofilms of PE and

PET regularly, might suffer from long term effects in their populations, due to a reduction of their growth and reproduction. These effects need to further be studied in multigenerational experiments. It is further necessary to verify, whether *P. fontinalis* would chose those plastic-associated biofilms when given another option.

Further, natural biofilms are highly heterogenous and the strong influence of the cultivation parameters makes a controlled experimental design very difficult. A future establishment of lab-grown plastic associated biofilms could verify the underlying mechanisms which affect the microbial communities and consequently its consumers. However, the influence of seasonal variations and environmental conditions on biofilm growth and composition should not be disregarded in future studies to further understand the impact of plastic pollution on primary production. Environmental conditions such as light availability and temperature may alter the effects of plastic on primary production and higher trophic levels. Also, the effects of additional stressors like pollutants that might be anhanced by adsorbtion on plastics should be further studied. Benign conditions during cultivation may mask the adverse effects of microplastic on biofilms and food webs, leading to an underestimation of ecological threats.

CRediT authorship contribution statement

Diana N. Michler-Kozma: Conceptualization, Methodology, Validation, Visualization, Formal analysis, Writing – original draft, Writing – review & editing. **Thomas R. Neu:** Supervision, Methodology, Writing – review & editing. **Friederike Gabel:** Funding acquisition, Conceptualization, Supervision, Writing – review & editing.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.151663.

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