Microplastic-specific biofilm growth determines the vertical transport of plastics in freshwater

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ABBREVIATIONS: *ATR, Attenuated total reflectance; FTIR, Fourier-transform infrared spectroscopy; MP, Microplastics; SD, Standard deviation; SOT, Settling onset time; SV, Sinking velocity; LDPE, Low-Density Polyethylene; PP, Polypropylene; PS, Polystyrene; PET, Polyethylene terephthalate; PVC, Polyvinyl chloride.*

ABSTRACT: Understanding the sinking behavior of microplastics in freshwater is essential for assessing their environmental impact, guiding research efforts, and formulating effective policies to mitigate plastic pollution. Sinking behavior is a complex process driven by plastic density, environmental factors and particle characteristics. Moreover, the growth of biological entities on the plastic surface can affect the total density of the microplastics and thus influence the sinking behavior. Yet, our understanding of these processes in freshwater is still limited. Our research thus focused on studying biofilm growth on microplastics in freshwater. Therefore, we evaluated biofilm growth on five different polymer types (both microplastic particles and plates) which were incubated in freshwater for 63 days in a controlled laboratory setting. Biofilm growth (mass-based) was used to compare biofilm growth between polymer types, surface roughness and study the changes over time. Understanding the temporal aspect of biofilm growth enabled us to refine calculations on the predicted effect of biofilm growth on the settling behavior in freshwater. The results showed that biofilm formation is polymer-specific but also affected by surface roughness, with a rougher surface promoting biofilm growth. For PET and PS, biofilm tended to grow exponentially during 63 days of incubation. Based on our calculations, biofilm growth did affect the sinking behavior differently based on the polymer type, size and density. Rivers can function as sinks for some particles such as large PET particles. Nevertheless, for others, the likelihood of settling within river systems appears limited, thereby increasing the probability of their transit to estuarine or oceanic environments under hydrometeorological influences. While the complexity of biofilm dynamics on plastic surfaces is not fully understood, our findings help to elucidate the effect of biofilms on the vertical behavior of microplastics in freshwater systems hereby offering knowledge to interpret observed patterns in environmental plastic concentrations.

KEYWORDS: Biofilm, sinking velocity, microplastics, settling onset time, freshwater.

1. Introduction

Currently, microplastic (MP) pollution is a major environmental concern due to their omnipresence, persistence, and possible adverse effects on the environment and human health (Trainic et al., 2020). While an increasing number of effect studies are reported in literature, exposure assessments and insights into environmental concentrations lag behind. One of the knowledge gaps is named the “missing plastic paradox”, referring to the discrepancy between estimated ocean plastic input and the concentrations found in the ocean surface layers (Isobe & Iwasaki, 2022; Koelmans et al., 2017). Different arguments to explain this paradox have been put forward, but the argument of the rivers being a large sink for plastics seems to largely explain the missing plastic paradox. Models predicted large amounts of plastics being retained in rivers and river sediments (Drummond et al., 2022; Newbould et al., 2021; Ryan & Perold, 2021; Tramoy et al., 2020; van Emmerik et al., 2022). Yet, the exact mass balances and the role of the rivers remain to be established.

One of the key processes vital to establishing the role of rivers in the missing plastic paradox is the sinking behavior of microplastic in freshwater. This is a complex process driven by plastic density but also dependent on both environmental characteristics (such as temperature and light intensity) and particle characteristics (such as size, shape and surface properties) (Chen et al., 2019; Kooi et al., 2017; Kowalski et al., 2016). Moreover, the growth of biological entities on the plastic surface, referred to as biofilm formation, can affect the total density of the microplastics and thus influence the sinking behavior (Kooi et al., 2017; Semcesen & Wells, 2021; Van Melkebeke et al., 2020). Although a number of studies have already documented the effect of biofilm growth on microplastic sinking (Onda & Sharief, 2021), quantification and temporal changes in biofilm have not often been studied for microplastics in freshwater environment. Biofilm growth is expected to be dependent on both microplastic and environmental parameters (Onda & Sharief, 2021). The main driver for biofilm growth on microplastics is the surface area of the particle. Furthermore, environmental factors such as algae presence and growth, temperature and light intensity do also affect the growth rate of the biofilm (Kooi et al., 2017). Plastic surface characteristics, possibly changed by weathering processes (Fotopoulou & Karapanagioti, 2015), influence the interaction between micro-organisms and microplastics and thus also determine the biofilm growth (Carson 2013; Fu2019).

The process of biofilm growth over time and its subsequent effect on sinking behavior of microplastics have been limitedly studied. Based on a theoretical model developed by Kooi et al. (2017), all buoyant polymer types would show oscillating behavior (floating and sinking) in a marine environment due to algal biofilm growth and subsequent mortality (Kooi et al., 2017). Most microplastic research has focused on the marine environment until now, yet a comprehensive understanding of the vertical behavior of plastics in rivers is crucial considering their presumed role as sink in the missing plastic paradox. The study of Semcesen and Wells (2021) investigated the sinking behavior of various sizes of polypropylene (PP) microplastics after incubation in freshwater. And although both the study of Kooi (2017) and Semcesen and Wells (2021) provide valuable insights into the biofilm formation of microplastics and its effects on sinking behavior, they do not yet fully grasp the complexity of biofilm formation with possible differences in polymer types, surface characteristics and growth phases of the biofilm. Nonetheless, based on limited available information, such differences can be expected (Carson et al., 2013; Fu et al., 2019; Miao et al., 2021).

The goal of our current research was to study the biofilm formation on microplastics over time in freshwater environments, specifically in relation to the polymer type and surface characteristics. This information provides valuable insights on the fate and transport of microplastics in the freshwater environment. A mesocosm experiment simulating a freshwater environment was set up, to study the difference in biofilm growth on plastic plates and microplastic particles of different (both naturally buoyant and more denser) polymer types and with different surface roughness. Plastics were incubated for 63 days (nine weeks), and the microplastics were sampled weekly to study biofilm growth over time. These results allowed for a refined estimation of the effect of biofilm growth on the settling onset time (for naturally buoyant polymer types) and the sinking velocity (for denser polymer types) for varied sizes of microplastics. The elucidated effect of biofilm on the vertical behavior can provide valuable information for future studies and data that can be implemented in future modelling efforts, which can eventually help to identify hotspots, cold spots, transport routes, sources, and sinks of microplastics (Browne et al., 2011; Chubarenko et al., 2018). Increased understanding of the sinking behavior of microplastics in freshwater is essential for assessing their environmental impact, protecting ecosystems, maintaining water quality, safeguarding human health, guiding research efforts, and formulating effective policies to mitigate plastic pollution.

2. Materials and methods

2.1 Plastics

The experiments included five polymer types: LDPE, PP, PS, PET and PVC. For all tested polymer types, we obtained commercial microplastic particles (± 3 mm diameter) and plastic plates (57 x 28.5 x 2 mm) from Carat GmbH (Germany), except for the PVC plates that we bought in a local hardware store. Attenuated Total Reflectance – Fourier Transformation infrared spectroscopy (ATR-FTIR) confirmed the plastic polymer types. The PVC plates, although identified as PVC (using ATR-FTIR analysis), had a very low density of 0.630 g cm-3, which is not comparable to the PVC particles acquired via Carat (density 1.199 g cm-3). The plastics used were thoroughly characterized (FTIR-identification and size measurements) (Supplementary file 1).

2.2 Biofilm growth

For biofilm growth, the incubation in freshwater of the plastic plates and particles lasted for 63 days (nine weeks) (Figure 1). We weekly retrieved freshwater from the urban river Coupure located in Ghent, Belgium, to renew one third of the water in the experimental units. The experiment took place in spring (March to May 2022). Chlorophyll a (1.60 +/- 1.74 mg/L), pH (8.19 +/- 0.17), and conductivity (904.33 +/- 48.31 µS/cm) of the Coupure water (measured weekly before every renewal) remained stable over the experimental period, except for the Chlorophyll a concentration which increased over time (Supplementary file 2).

Before incubation, we weighted the MP particles and plates individually on an analytical balance (accuracy 0.01 mg; repeatability 0.015 mg) using pre-dried aluminum dishes (1 h, 60 °C). In addition, we collected images of the MP particles using a light microscope (Olympus SZX10, CellSens software) to estimate the surface area available per particle. We determined the two main dimensions (length and width) using ImageJ software, and estimated the third dimension based on 3D measurements of 25 separate particles of each polymer type and the ratio between length and width (Supplementary file 3).

The experimental setup of the incubation of the plates (n= 30; 6 replicates per polymer type) consisted of a glass aquarium (100 L) filled with freshwater from the river Coupure (Ghent, Belgium). Before incubation, we roughened the plates to increase the surface roughness. The plates were fully submerged (by weighing them down) to reduce the effect of buoyancy for the low density polymer types. The incubation of similar polymer types took place simultaneously. We incubated the particles (n = 720; 16 particles pooled per polymer type and per sampling point) individually (to increase the contact between particle and water) in freshwater (2 mL) retrieved from the urban river Coupure (Ghent, Belgium). The incubation was performed in standard laboratory well plates made of polystyrene plastic.

The incubation conditions were a 12 h:12 h dark : light cycle and the plastics were kept in a temperature-controlled room (T: 15 °C). We renewed the water (1/2 to 1/3 of the volume) every week.

For the microplastic particles, we gathered the samples every week to follow the biofilm growth over time (Figure 1). At every sampling point, we collected 16 particles per polymer type from the wells, gently dabbed them off with tissue paper to remove most of the water and placed them in one pre-dried aluminum dish (1 h, 60 °C). To determine the plate and biofilm's fresh weight, we weighted the dishes containing the plates on an analytical balance. Subsequently, the aluminum dishes dried in the oven (60 °C) for 24 hours. After that, the dishes reached room temperature in an exicator and we weighed them again on an analytical balance for the determination of the dry weight of the plates with biofilm, according to Wilson et al (2017). After 63 days of incubation, we collected the final set of particles and removed all plates from the aquaria and weighted them as described before. The mass of biofilm was normalized for the surface area and expressed as fresh weight per surface area.

2.3 Surface roughness of plastics

Scanning electron microscopy images were collected from the used materials (sanded plates, virgin particles). Of each sample, we collected three SEM images. We analyzed surface roughness in triplicate per image using ImageJ2 software and the SurfCharJ plugin. The measure for surface roughness is the root mean square deviation (Rq) calculated from the surface plot as described by Chinga et al. (2007).

2.4 Data processing

2.4.1 Biofilm growth

To measure the effect of polymer type and surface roughness on biofilm growth, we calculated the fresh biofilm mass per surface area for all plates and pellets, based on the assumption of a 90 % water content of the biofilm. After normality and homogeneity evaluation, we performed a non-parametric Kruskal-Wallis test to study differences in biofilm mass between polymer types and different surface roughness. The post-hoc analyses consisted of a pairwise Wilcox test.

To study the evolution of biofilm growth over time (t, days), we fitted a log-linear model to the biofilm masses of all sampling time points to study exponential growth. When the exponential growth model showed a good fit, the growth function was extracted.

$mass_{bf} = C_1 \ e^{(C_2 \ t)}$ (Eq. 1)

with C_1 and C_2 as the coefficients of the polymer-specific exponential growth curve of the biofilm which are used in further predictions on the effect of biofilm formation on the vertical behavior of microplastics (section 2.5).

2.5 Estimated effect of biofilm formation on vertical behavior

Based on the data of the two experiments (plates and particles) on the growth of the biofilm, we extrapolated the effect of the biofilm growth on the vertical behavior for varied sizes of plastics, considering the importance of the available surface area for biofilm growth. The effect of increased roughness on the increased available surface area was not taken into account.

For this extrapolation, some assumptions were made. First, in the biofilm, a 90% water content is assumed (Schmitt & Flemming, 1999). Second, since no biofilm density for freshwater algae species was found, a default biofilm density for marine species of 1388 kg/m³ was used (Kooi et al., 2017). Importantly, reported densities are very variable ranging between 1030 to 4350 mg/mL, as mentioned by Amaral-Zettler et al. (Amaral-Zettler et al., 2021). It might be that the actual density is lower ranging between 1100 and 1180 kg/m³ (Amaral-Zettler et al., 2021; Van Melkebeke et al., 2020) which could have a minor impact on the calculations performed in this paper. The third assumption is that the biofilm is assumed to be homogeneously distributed across the total particle's surface with a uniform thickness. Fourth, the average plastic particle is assumed to be spherical (Kooi et al., 2017). Finally, the exponential growth is assumed to start at seven days (based on experimental data), the growth between 0 and 7 days is unknown. Biofilm mass at the start (T0) is set to zero.

For the naturally buoyant polymers (LDPE, PP), we calculated the effect of the biofilm formation on the settling onset time for various sizes of microplastics. The settling onset time (SOT) is the time needed for the density of the particles (and attached biofilm) to be equal to the density of the water, which equals the timepoint when a particle can start sinking (Kooi et al., 2017). The formula used to calculate the SOT was:

$$SOT = \frac{\ln\left(\frac{\rho_{bf} V_{pl}}{C_1 \times 4 \times \pi \times r_{pl}^2} \times \frac{\rho_{pl} - \rho_{wat}}{\rho_{wat} - \rho_{bf}} + 1\right)}{C_2} \text{ (Eq. 2)}$$

In which ρ_{bf}, ρ_{wat} and ρ_{pl} are the densities of the biofilm (1388 kg/m^3), water (999.19 kg/m^3), and plastic (Supplementary file 1), respectively. V_{pl} is the volume of the plastic particle and r_{pl} is the corresponding radius. The SOT was calculated for several sizes of plastics with r_{pl} ranging between 10 and 0.00001 mm. C_1 and C_2 are the coefficients of the polymer-specific exponential growth curve of the biofilm (derived from equation 1).

For the denser polymers (PET and PS), we calculated the effect of the biofilm formation on the sinking velocity (SV) using Equation 3 (Kooi et al., 2017). For the calculations, we calculated a water density (ρ_{wat}) of 999.19 kg/m^3 and a dynamic viscosity (v_{wat}) of 0.0012 kg/m*s based on the experimental temperature (13.4°C) and the measured conductivity of the Coupure water (904 ± 48.31 µS/cm) (Kooi et al., 2017; Sharqawy et al., 2010). The kinematic viscosity (μ_{wat}) was subsequently calculated as the ratio of the dynamic viscosity and the density of the water resulting in a μ_{wat} of 1.19 x 10-6 m²/s. g was set at 9.8 m/s², and the dimensionless settling velocity ω_* was calculated as described in Kooi et al. (2017).

$$SV = -\sqrt[3]{\frac{\rho_{tot} - \rho_{wat}}{\rho_{wat}} \times g \times \omega_* \times \mu_{wat}} \text{ (Eq. 3)}$$

To calculate the total density of the biofouled plastic (ρ_{tot}), equation 4 was used (Kooi et al., 2017), in which the $mass_{tot}$ was measured, and the density of the biofilm (ρ_{bf}) was set at 1388 kg/m^3, according to Kooi et al. (2017). The volume of the virgin plastic (V_{pl}) was calculated from the density of the plastic (Supplementary file 1) and the measured mass of the virgin plastic ($mass_{pl}$).

$$\rho_{tot} = \frac{mass_{tot}}{V_{tot}} = \frac{mass_{tot}}{\frac{mass_{tot} - mass_{pl}}{\rho_{bf}} + V_{pl}} \text{ (Eq. 4)}$$

We calculated the SV for three sizes of plastics with r_{pl} set at 10, 1 and 0.1 mm. In the calculations, changing particle (plastic and biofilm) diameter due to the biofilm was considered by including the thickness of the biofilm according to Kooi et al. (2017):

$$Thickness_{bf} = \sqrt[3]{V_{tot}\frac{3}{4\pi}} - r_{pl} \text{ (Eq. 5)}$$

3. Results and discussion

Our study dives into microplastic sinking behavior, focusing specifically on (1) biofilm growth and its influencing characteristics, and (2) the temporal evolution of biofilm development. Our observations highlight an interplay between these factors and the sinking behavior of microplastics. Biofilm growth, a dynamic process, profoundly affects the sinking behavior of microplastics in freshwater environments. Moreover, understanding the temporal aspects of biofilm growth refines predictive models, enabling more accurate assessments of microplastic behavior in freshwater ecosystems. Including these multifaceted influences on microplastic sinking, our study advances the understanding of microplastic fate and provides invaluable insights into their ultimate deposition and accumulation in sediments in aquatic freshwater systems. This knowledge can contribute to the formulation of a more comprehensive and effective approach targeting mitigation strategies and regulatory measures.

3.1 Characteristics that can affect biofilm growth

After 63 days of incubation in freshwater, biofilm had formed on the surfaces of the plates (n=30), with an average of 8.37 ± 7.22 µg per mm². Notably, differences on the plates (Figure 2) revealed polymer-dependent biofilm masses, ranging from lowest masses found on PS (2.96 ± 1.91 µg/mm²) to highest biofilm mass on PVC (18.37 ± 10.08 µg/mm²). PS exhibited a significantly lower biofilm mass than PVC (p=0.0065) and PET (p=0.0433). Trends were evident between PS and LDPE or PP (p=0.0812; p=0.0617), though not statistically significant. PVC displayed the highest biofilm content, significantly surpassing PS, PET (p=0.0093), and LDPE (p=0.0065).

Regarding microplastic particles (n = 80, groups of 16 particles), an average biofilm of 0.97 ± 0.67 µg per mm² was recorded after a 63-day incubation. Polymer-specific biofilm growth was again observed (Figure 2), highlighted PET particles exhibiting the highest growth with 1.83 ± 0.14 µg/mm², while LDPE particles showed the lowest at 0.24 ± 0.02 µg/mm². The biofilm masses were significantly different between the polymer types ($p<0.001$).

Importantly, the results of the PVC particles were excluded for further analysis due to measured negative biofilm mass values (-2.68 ± 0.45 µg per mm²). This anomaly is expected to be attributed to the drying process, affecting the mass of the particles and hindering relevant insights on biofilm growth rates.

Based on the results of the biofilm growth after a 63-day incubation of both pellets and plates, some interesting observations can be made to explain the observed differences in biofilm growth. First, the polymer-specific biofilm growth is apparent in results of biofilm masses observed on the plates and is comparable to conclusions of previous research (Kaiser et al., 2017). More specifically, the study of Miao et al. (2021) on biofilm colonization on plastics in freshwater environments, also showed generally higher biofilm growth on PVC compared to PP and PET, although some location-specific effects were observed. The polymer-specific growth of biofilm has also been described earlier in the marine environment, whereas in contrast to our results, PS seemed to be the most susceptible to biofilm growth (Li et al., 2019). However, as different microbial communities and differences in salinity gradients can lead to differences in biofilm growth (Li et al., 2019). Polymer-dependency was also observed in the microplastic particles, although in a different matter. For example, PS showed the lowest biofilm growth on the plates, the PS particles showed the second highest biofilm mass after the 63 days of incubation, which is more in line with previous observations in the marine environment (Li et al., 2019). A possible explanation for this difference could be the buoyant behavior of the LDPE and PP polymers due to their low density and the experimental setup. The experiment using the particles was performed by incubating the particles individually in wells. In contrast to the experiments using the plates, the particles were not forced to be submerged by weighing them down. Due to the buoyant behavior, it could be assumed that the particles were not fully exposed to the incubating water all the time and the biofilm would grow at the air-water interface. Moreover, during the weekly change of the water, the particles can be turned, and therefore, the growth of a biofilm might be slowed down or even reversed by exposure to air. Although this might have hampered the growth of a biofilm, it can be argued that this is, nonetheless, a relevant situation resembling field conditions. This is supported by field data which generally report a biofilm coverage of less than 50% of the particle (Amaral-Zettler et al., 2021; Fazey & Ryan, 2016). Secondly, when comparing the biofilm growth on the particles to that of the plates (of the same polymer composition), it is noticeable that the amount of biofilm on the particles (normalized for surface area) is lower compared to that of the plastic plates (Table 1). As an example, biofilm growth of PET particles was 1.83 ± 0.14 $\mu g/mm^2$ and that of PET plates was 6.21 ± 1.70 $\mu g/mm^2$. These results suggest that both polymer type and surface roughness could impact biofilm growth. The plates were sanded before incubation resulting in a higher surface roughness compared to the microplastic particles (Supplementary file 4), which could explain the differences in biofilm masses observed between particles and plates (Table 1). Increased irregularities on the surface of plastics could increase the attachment of microorganisms and thus promote biofilm formation, definitely in the first stages of biofilm formation (Carson et al., 2013; Fu et al., 2019). If surface roughness is analyzed between the different polymer types, no significant difference is observed between the different polymers ($p > 0.05$, Supplementary file 4) therefore, surface roughness cannot explain all observed differences in biofilm growth.

In summary, based on the results, it is clear that the observed differences can be explained by a combination of polymer-specific characteristics (e.g. crystallinity, hydrophobicity, surface charge), surface roughness and buoyance of the pristine particle in freshwater.

3.2 Biofilm growth over time

The growth of biofilm on the particles was followed over time by weekly sampling. During the 63-day incubation, the amount of biofilm grew exponentially on the PET and PS particles while the LDPE and PP particles showed an oscillating biofilm mass (Figure 3). For both PET and PS, the log-linear model fit was significant (p_{PET}= 0.003994; p_{PS} = 0.05099) while for LDPE and PP no exponential growth could be confirmed (p_{LDPE}= 0.1854; p_{PP} = 0.4124) (Supplementary file 5). The exponential growth phase of PET and PS started around 34 days (five weeks) of incubation.

Based on the biofilm growth on the polymer plates, we would, however, expect similar growth of biofilm for LDPE and PP compared to PET (Figure 2). This unexpected biofilm growth on the LDPE and PP particles could be again explained by the buoyant behavior of both polymers resulting in fragmentary biofilm growth. As this is expected to happen in the environment as well with buoyant particles, it was hypothesized that the biofilm on buoyant polymer particles will not follow exponential growth and might be extremely challenging to model. In contrast, the results of the study of Rozman et al. (2023) showed an exponential growth of biofilm on small PE microplastics at the air-water interface. The discrepancy between both studies cannot be explained and more research is thus warranted.

3.4 Effect of biofilm growth on vertical behavior

Having conducted a thorough analysis of biofilm growth, tracking its progression over time, and assessing the influencing parameters, it is relevant to use this information when predicting the behavior of particles in the freshwater environment. Our approach involves considering various polymers, taking into account observed discrepancies in biofilm growth on different polymer surfaces. Additionally, we factor in the size of particles, recognizing its role in determining the available surface area for plastic and subsequent biofilm growth. Notably, we cannot consider surface roughness due to a lack of available information on the expected roughness of particles in the freshwater environment. Fotopoulou and Karapanagioti (Fotopoulou & Karapanagioti, 2015) did observe an increased surface roughness in pellets in the coastal environmental compared to virgin macroplastics from local manufacturers, however, the roughness was not quantified and could not be linked to the age of the particles. In the absence of specific data on surface roughness in the freshwater environment, we extrapolate insights from the data gathered on biofilm formation on microplastic particles, assuming a smooth surface and linked poorer attachment of microorganisms (Carson et al., 2013), potentially resulting in an underestimation of biofilm formation.

In the following calculations, we also considered the distinct behaviors of plastics, acknowledging that buoyant plastics may exhibit different effects compared to denser polymers that tend to sink. For buoyant microplastics, the growth of a biofilm could increase the total density of the particles and thus induce sinking behavior, therefore the effect of biofilm growth on the settling onset time (SOT) was calculated. For sinking polymer types, the growth of a biofilm is believed to affect the terminal sinking velocity (SV) of a particle.

3.4.1 Estimated effect on sinking behavior for buoyant polymers

The results of these calculations indicate that the SOT is dependent on both the size and the density of the polymer particle. Importantly, due to insufficient data on smaller particles, predicting their behavior introduces more uncertainty compared to larger particles (1mm). Smaller microplastics exhibit a quicker initiation of settling compared to larger microplastics, a finding consistent with reported literature (Amaral-Zettler et al., 2021; Fazey & Ryan, 2016). Previous measurements showed that only a 10µm thick biofilm would already be able to cause sinking of PE sphere of 100 µm, assuming a biofilm density of 1.1 g/cm^3 (Amaral-Zettler et al., 2021). For larger particles, the settling process, based on microorganisms, can encompass two or three months. The settling of larger particles is expected to be impacted more by attachment of larger organisms such as bryozoa (Amaral-Zettler et al., 2021), which were not introduced in the current experimental setup. In the marine environment, a biofilm based on microorganism attachment has been reported not to be sufficient for the larger particles to cause sinking (Amaral-Zettler et al., 2021), however, based differences in density of freshwater, this could happen in freshwater. The data of the current experiment does question the settlement of the larger particles in an environmentally relevant time frame (taking into account the transport of the particles). Higher chances are that they will be transported in the upper water layers to the estuaries influenced by hydrometeorological influences (van Emmerik et al., 2022). When they arrive there, the attached microbial biofilm could reduce the hydrophobicity and provide chemical signals that enhance invertebrate settlement and induce sinking in estuaria or coastal regions (Amaral-Zettler et al., 2021; Lobelle & Cunliffe, 2011).

Additionally, denser polymer types tend to start settling sooner compared to less dense polymer types, although differences are small. A denser particle requires less biofilm mass to surpass water density, aligning with the conclusions drawn by Kooi et al. (2017). For instance, LDPE (920 kg/m^3), denser compared to PP (906 kg/m^3), appears to settle slightly earlier compared to PP, while assuming equal sizes (and thus the same surface) (Figure 4). Importantly, as we were not able to fit an exponential growth model to the biofilm growth on LDPE and PP particles, an exponential biofilm growth was nonetheless assumed for LDPE and PP based on the similarities with PET in biofilm growth on the plates (Figure 2). However, it must be kept in mind that it is possible that the growth of biofilm on buoyant polymers does not follow an exponential growth curve, resulting in challenges to predict the SOT and increasing uncertainty in the subsequent results.

In contrast to the results of the theoretical model of Kooi et al. (2017), we do not observe a plateau in SOT for larger particles. This plateau was explained by the authors to be a contribution of the radius and surface-to-volume ratio and linked to collision frequency between particles and algae. The experimental results of Fazey and Ryan (2016) also do not indicate the presence of such a plateau phase. The predicted SOT is higher compared to the experimental data gathered by Fazey and Ryan (2016) (Figure 4), although the main trend is followed. It is noteworthy that the data by Fayez and Ryan (2016) originates from a marine environment, potentially accounting for variations in SOT when compared to the freshwater environment in our study, as suggested in Supplementary file 6 (Li et al., 2019). Further validation of our calculations through comparison with additional experimental data would be valuable in reinforcing the robustness of our findings.

3.4.2 Estimated effect on sinking behavior for sinking polymers

Based on the calculated SV, a difference can be observed between the effect of biofilm on PET and PS on the longer term. In the case of PET, the SV shows a small decrease over 100 days due to biofilm growth (Figure 5). This aligns with the calculated density changes based on the experimental data (Supplementary file 7). In contrast, for PS, a polymer type with a lower density than PET, the biofilm formation appears to exert a more pronounced effect on SV over time, linked to the exponential growth of the biofilm (Figure 5). Unfortunately, no information is available on the growth rate of the biofilm on PVC (see section 3.2), therefore, the effect of biofilm growth on the SV of PVC is cannot be calculated.

As not much research has been done so far on the impact of biofilm formation on the settlement of different polymer types in freshwater, it is challenging to compare our results. Lee et al. (2022) studied the effect of biofilm formation and the settling behavior in wastewater treatment plants. Here it was also shown that the biofilm formation had a higher impact on the settling velocity of PS compared to that of PET, corroborating our results albeit in a different environment. Notably, the limited effect of biofilm formation on SV for PET contradicts the findings of Miao et al. (2021). However, it's crucial to note that Miao et al.'s study involved biofilm growth in the field under diverse environmental conditions and at different locations, potentially influencing biofilm attachment and subsequent growth, thus resulting in varying impacts on sinking behavior compared to controlled laboratory environments. Moreover, incubation in the field would allow for attachment of multicellular organisms which are more likely to change the density of PET profoundly and affect its sinking velocity (Amaral-Zettler et al., 2021; Kaiser et al., 2017; Onda & Sharief, 2021). This urges for more research and stresses the importance of environmental factors (Miao et al., 2021).

3.5 Environmental implications and future research needs

Our results shed a light on fate of microplastics in a river considering the multitude of different plastic particles present in the environment. These results underscore the need to focus more on the heterogeneity of plastic pollution instead of treating plastics as one group of pollutants (Hartmann et al., 2019). According to our findings coupled with existing knowledge, the tested polymers can primarily be categorized in three groups based on their assumed sinking behavior in freshwater.

The first group are the particles that will remain buoyant, unaffected by biofilm growth in an environmental relevant time frame (before reaching estuaria). The behavior of these particles is expected to be mainly dictated by hydrometeorological influences such as water flow, wind and rain. Consequently, they have a higher likelihood of being transported towards estuarine or oceanic environments, unless trapped by vegetation on the river banks (Ghinassi et al., 2023; van Emmerik et al., 2022). Examples of particles in this group are larger LDPE and PP particles where, based on our results, the settling onset time is assumed to be prolonged, possibly allowing these particles to reach estuaries before significant settling occurs unless they are retained within river compartments (e.g. riverbanks, vegetation; Ghinassi et al., 2023; van Emmerik et al., 2022). As mentioned before, decreased hydrophobicity due to microbial biofilm formation could stimulate attachment of multicellular organisms, eventually still resulting in settling of these particles (Amaral-Zettler et al., 2021). The second group of particles contain PS particles along with smaller LDPE and PP particles. Our study suggests that their settlement along the river is influenced by biofilm formation. Notably, biofilm growth on PS has a substantial impact on their location in the water column. Larger PS particles (10 mm) will slowly settle along a river resulting in higher concentrations of PS in the sediment and lower concentrations in the surface waters. Moreover, the more slow settling could increase interactions with the suspended sediment particles resulting in aggregation and increased settlement (Serra & Colomer, 2023). Our data hereby supports the observation of PS in the sediment (e.g. Bonyadi et al., 2022; Everaert et al., 2022), which could not directly be explained merely by the density of PS. The third group consists of the larger PET, of which the sinking velocity is merely influenced by density of the polymer. The impact of biofilm formation on the sinking velocity of PET is relatively minor.

Our works shows that rivers can serve as sinks for certain particles, while for others, the likelihood of settling within river systems appears relatively restricted, increasing the probability of their transportation to estuarine or oceanic environments through currents or entrapment by other compartments such as vegetation (van Emmerik et al., 2022). This evidently is still a very broad generalization and further mechanistic insights are needed and there are a few points that need to be considered in future research. First, biofilm growth is expected to be a dynamic and complex process which is challenging to represent using a laboratory setup. As mentioned before, the buoyant behavior of some polymer types could result in deviations from an exponential growth curve and not a full coverage of the particle (Amaral-Zettler et al., 2021; Fazey & Ryan, 2016). Moreover, the biofilm is not expected to grow indefinitely, expecting rather a logistic growth curve. A maximum possible thickness of 500 µm was reported for a biofilm in seawater (Van Melkebeke et al., 2020). Furthermore, various defouling processes are expected to happen in the environment including grazing, algae mortality due to loss of photosynthetic potential (darker environment), inter- or intra species competition or erase-and-restart scenarios (Berezina et al., 2021; De Tender et al., 2017; Fazey & Ryan, 2016; Kooi et al., 2017), which could cause resurfacing of submerged particles (Ye & Andrady, 1991). Future research should therefore focus to gather knowledge on the complexity of the biofilm process by e.g. including longer incubation times and comparing biofilm growth on floating and submerged particles.

Secondly, the characteristics causing the polymer-specificity, e.g. crystallinity, additives, surface hydrophobicity, remain unknown and could not be pinpointed due to low variability between the materials used in the current experiment. Additives such as plasticizers (Amobonye et al., 2021) are suggested to have an impact on biofilm growth. Additives present in the polymers were mostly unknown or not present (PE, PS, PP). For PET, catalyst leftover (Sb_2O_3) was reported to be present. For PVC particles, softeners were added and for the PVC plates, additives are unknown. Therefore, additives are in this case not expected to affect the biofilm growth and observed effects. Crystallinity and surface hydrophobicity could impact attachment of microorganisms and could explain the observed differences (Amobonye et al., 2021; Tokiwa et al., 2009). The shape of a particle can also affect biofilm attachment and subsequent effects on sinking behavior as already studied (Amaral-Zettler et al., 2021; Van Melkebeke et al., 2020)

Finally, the weathering status could influence the biofilm formation directly (Gewert et al., 2015) and indirectly by changing plastic characteristics (e.g. surface roughness and hydrophobicity) (Fotopoulou & Karapanagioti, 2015; Lambert & Wagner, 2016). In the future, more research should focus on the complex interaction between plastic characteristics, weathering and biofilm growth to assess their joint effects on vertical transport and predict the fate and impact of plastics in the environment.

Conclusion

In conclusion, our results highlight that both polymer density and biofilm formation can affect the settling behavior of a polymer particle. The biofilm formation itself is influenced by the polymer (e.g. based on density), surface roughness and size. By considering the temporal aspect of biofilm growth, predictive calculations enabled more accurate and polymer-specific assessments of microplastic distribution in freshwater environments. The calculations revealed that the rivers can indeed function as sinks for some particles such as large PET particles. Nevertheless, for others, the likelihood of settling within river systems appears limited, thereby increasing the probability of their transit to estuarine or oceanic environments by the currents. Although we are far from grasping the full complexity of biofilm dynamics on plastics surfaces, the knowledge gathered in this study can help to explain observed patterns in environmental concentrations of plastic pollution and increase the understanding of the sinking behavior of microplastics in freshwater.

ASSOCIATED CONTENT

Supporting Information

Detailed information (.docx) on the used plastic materials (S.1), characteristics of the freshwater (S.2), measured length and width and calculated length/width ratio (S.3), analysis of the surface roughness (S.4) exponential growth curves (S.5), the effect of environmental conditions on sinking velocity (S.6) and the calculated differences in sinking velocity (S.7) are provided in the supporting information.

FIGURE CAPTIONS

Figure 1: Overview of the experimental design of the incubation of plastic plates and pellets in freshwater from Coupure, a river in Belgium, for 9 weeks (63 days). Water was renewed weekly (1/3 renewal). For plastic plates, we collected the samples after 9 weeks (63 days). For microplastic pellets, we collected the samples weekly. Biofilm mass was measured. The illustration was created with BioRender.com.

Figure 2: Fresh biofilm mass (normalized for surface) in plates (6 replicates) and particles (16 replicates) incubated with freshwater for 63 days. Differences are observed between particles and plates and between polymer-types. The average biofilm mass per surface ($\mu g/mm^2$) ± SD is indicated above each boxplot.

Figure 3: Growth of biofilm during 63 days of incubation in freshwater. Fresh biofilm mass per surface vs. incubation time in days is depicted. The datapoints are fitted with a smoothed curve using the loess smoothing method.

Figure 4. Settling onset time (days) in correspondence with the available surface of the polymer particle. The results of the sinking experiments of Fazey and Ryan (2016) are added as grey dots.

Figure 5. Calculated effect of biofilm growth on sinking velocity (mm/s) over time for polyethylene terephthalate (PET) and polystyrene (PS) particles with different radii (10, 1 and 0.1 mm). The model estimated the sinking velocity at different timepoints and the trends is shown with the line graphs.

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COMPETING INTERESTS

The authors declare there are no competing interests.

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