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Biofouling impacts on polyethylene density and sinking in coastal waters: A macro/micro tipping point?

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ABSTRACT

Biofouling causing an increase in plastic density and sinking is one of the hypotheses to account for the unexpectedly low amount of buoyant plastic debris encountered at the ocean surface. Field surveys show that polyethylene and polypropylene, the two most abundant buoyant plastics, both occur below the surface and in sediments, and experimental studies confirm that biofouling can cause both of these plastics to sink. However, studies quantifying the actual density of fouled plastics are rare, despite the fact that density will determine the transport and eventual fate of plastic in the ocean. Here we investigated the role of microbial biofilms in sinking of polyethylene microplastic and quantified the density changes natural biofouling communities cause in the coastal waters of the North Sea. Molecular data confirmed the variety of bacteria and eukaryotes (including animals and other multicellular organisms) colonizing the plastic over time. Fouling communities increased the density of plastic and caused sinking, and the plastic remained negatively buoyant even during the winter with lower growth rates. Relative surface area alone, however, did not predict whether a plastic piece sank. Due to patchy colonization, fragmentation of sinking pieces may result in smaller pieces regaining buoyancy and returning to the surface. Our results suggest that primarily multicellular organisms cause sinking of plastic pieces with surface area to volume ratios (SA:V) below 100 (generally pieces above a couple hundred micrometers in size), and that this is a "tipping point" at which microbial biofilms become the key players causing sinking of smaller pieces with higher SA:V ratios, including most fibers that are too small for larger (multicellular) organisms to colonize.

1. Introduction

The observation that we can only account for a small percentage of the plastic waste that budget estimates predict has ended up in the ocean (Lebreton et al., 2019) has led to hypotheses about the fate of this "missing plastic". Many polymers are denser than seawater and will sink, but the two most produced plastic polymers are less dense than seawater so they should float: polyethylene (PE) with a density of 0.91 to 0.965 g • cm^{-3} and polypropylene (PP) with a density of 0.90 g • cm^{-3} (Andrady, 2017). Potential losses of the buoyant plastic include: (1) fragmentation into pieces small enough to pass through the nets that have been used in most surveys (generally 333–500 μm mesh); (2) biofouling increasing the density so that plastic sinks below the surface (Kooi et al., 2017; Ye and Andrady, 1991); (3) wind mixing (Kukulka et al., 2012; Reisser et al., 2015); and (4) ingestion and egestion of plastic as fecal pellets (Cole et al., 2016). The first two hypotheses of fragmentation and biofouling are related, in that smaller particles have a higher surface area to volume ratio (SA:V) so colonization by organisms denser than seawater will have more of an effect on smaller particles. Biofouling leading to sinking of otherwise buoyant low-density polyethylene (LDPE) was first reported over 45 years ago (Holmström, 1975), and described LDPE sheets covered with calcareous bryozoa and algae that were recovered from fish-

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ing trawls in water up to 400 m deep. Since then, there have been multiple reports of plastics that should be buoyant occurring below the surface or at the bottom (Bergmann et al., 2017; Gomiero et al., 2019; Peng et al., 2017). The change in plastic density due to fouling was first quantified by Ye and Andrady (1991) who reported that the PE and PP materials they tested in the subtropical waters of Biscayne Bay, USA became denser than seawater, reaching a specific gravity of 1.03 to 1.14 after 7–9 weeks. Since then, a number of authors have studied the role biofouling plays in altering the buoyancy of plastic in both marine and fresh waters, but most have only described whether the plastic sinks (Chen et al., 2019; Fazey and Ryan, 2016), or measured sinking rates (Kaiser et al., 2017; Miao et al., 2021). Other than Ye and Andrady's gravimetric measurements, there are very few data available regarding the actual density of fouled plastic, but density measurements are critical to determining the transport of plastic in aquatic systems. The sinking rate of particles depends on density, size, and shape (Kowalski et al., 2016), but the density of a piece of plastic marine debris (PMD) determines where it ultimately ends up, whether at the surface, on the bottom, or suspended at a level of equal density in the water column (Kooi et al., 2017).

The fouling community is diverse and includes organisms from unicellular bacteria and diatoms to animals such as barnacles. Ye and Andrady (1991) described but did not quantify the initial formation of a "transparent slimy biofilm on the surface" after a couple of days. Rummel et al. (2017) suggested that studying how biofilms influence vertical transport of microplastic should be a research priority to understand the fate and effects of microplastic in aquatic environments. The formation of a microbial biofilm on plastic, the so-called Plastisphere (Zettler et al., 2013), alters the physical, chemical, and biological characteristics of plastic in aquatic environments, and Wright et al. (2020) point out that the Plastisphere forms the "interface" and thus drives interactions between the plastic and the environment. In this study our goal was to quantify how biofouling by microbial biofilms alone, as well as by natural fouling communities including multicellular algae and metazoans, affects the density of PE pieces in a coastal environment. Our hypotheses were: (1) The SA:V of a piece of plastic would determine when and if a piece of plastic sinks; and, (2) During early spring bloom conditions, rapid growth by attached microbes such as diatoms could increase the density of microplastic particles enough to cause them to sink, resulting in seasonal pulses of microplastic from surface waters to the benthos.

2. Materials and methods

2.1. Polymer properties

There are many formulations of PE with varying additives, crystallinity, and surface texture, among other characteristics. In this study we used defined PE polymers of uniform composition provided by the American Chemistry Council (ACC). Three formulations of PE (Table S1) of 6 different thicknesses (0.025, 0.051, 0.076, 0.102, 0.152, and 0.203 mm) were sterilized by immersing in 70% ethanol for 10 min, then rinsing in 0.2 µm sterile-filtered laboratory grade distilled/deionized water before being used for experiments. The different thicknesses provided a range of SA:V ratios ranging from 10 to 80 using 5 × 5 mm pieces.

2.2. Microbial biofilms

We tested 25 individual unialgal cultures to determine whether they would attach to plastic and could cause sinking of the plastic films (Table S2). Note that our cultures were not axenic, so the density changes we report are for a biofilm consisting of a single photosynthetic alga, as well as any associated bacteria. Cultures were grown in f/2 medium + silicate (Guillard and Ryther, 1962) made with sterilized Wadden Sea water. Approximately 1 ml of culture was inoculated into a 125-ml flask containing 50 ml of f/2 and 6–10 squares of polymer that had been sterilized by immersion in 70% ethanol for 10 min, then rinsed in sterile laboratory grade water. For each polymer and thickness, 3 replicate flasks were incubated on an orbital shaker (60 rpm) at 20 °C, 16:8 hour light:dark cycle, at an illumination of 50 $\mu mol \bullet m^{-2} \bullet s^{-1}$. Control flasks were included for each polymer and thickness, containing f/2 culture medium and plastic, but no microalgae. Flasks were checked starting during the lag phase, through exponential growth, and into senescence; daily for the first week, then twice a week for at least 6 weeks. At each time point, each culture flask was examined to determine whether plastic had visible biofilms, had sunk below the surface film, or had sunk to the bottom of the flask. As soon as any piece of plastic was observed below the surface, it was tested in a density column. Cultures to check the density of diatom biofilms alone (without plastic) were grown under the same culture conditions as the flasks above, but in flat polystyrene tissue culture flasks. One week after the cultures were inoculated and had formed a visible biofilm on the bottom of the flask, a cell scraper was used to remove the biofilm. Ten milliliters of this slurry were transferred to a 15-ml centrifuge tube and centrifuged gently (1000 rpm for 2 min) in a clinical centrifuge with a swing-out rotor. A wide-bore pipet was used to gently transfer a portion of the biofilm pellet from the bottom of the tube to central portion of the surface of a thawed Nycodenz density gradient (see Section 2.4 below).

2.3. Natural biofilms

Individual plastic strips 5 cm wide by 10 cm long of each PE polymer, high density PE (HDPE), low density PE (LDPE), and linear low density PE (LLDPE) of each thickness (0.025, 0.051, 0.076, 0.102, 0.152, and 0.203 mm) were placed in a 25 × 25 cm bag of fiberglass window screening (mesh opening 1.0 × 1.7 mm) to keep them from escaping and to protect them from macro-grazers. We wanted to monitor the development of the biofilm and fouling community without it being removed by fish, crabs, sea stars, etc. in this shallow productive system. The plastic pieces in the mesh bag were immersed in seawater at the NIOZ jetty (N 53.002×4.789 E) in the Wadden Sea, a shallow tidal inlet of the North Sea (Fig. 1a), by attaching it with stainless steel clips to a vertical anchor line secured at the top to a platform over the water and at the bottom to a steel weight resting on the sea floor. This kept the samples approximately 1.5 m off the bottom, and 0.5–2 m below the surface (tidal range ~1.5 m). Samples were deployed in May 2019 and sampled at 14, 28, 42, 56, 152, 259, and 335 days. At each sampling the mesh bags were placed in a clean bucket of whole seawater from the site and taken to the laboratory. In the lab 0.5 cm was cut from the bottom of each strip with sterile scissors and rinsed gently in 0.2-µm filtered seawater from the site to remove non-attached organisms. At each sampling, the remaining experimental plastic strips were transferred to a clean mesh bag to reduce external fouling, kept continuously immersed in seawater, and re-submerged at the jetty anchoring system within 30 min, after removing any large invertebrates (bivalves, tunicates) that had settled as larvae. Back in the lab, the 0.5 × 5 cm strip was cut into sections approximately 5 × 5 mm with sterile scissors and some pieces were used to determine density immediately (see below). Other pieces were preserved for DNA analyses in Gentra Puregene Tissue cell lysis solution (QIAGEN Benelux B.V., Venlo, The Netherlands) and frozen at −20 °C. For scanning electron microscopy (SEM), plastic was fixed in 4% paraformaldehyde at 5 °C for 2–18 h, then transferred to a 50:50 solution of phosphate buffered saline (PBS) and ethanol and stored at −20 °C.

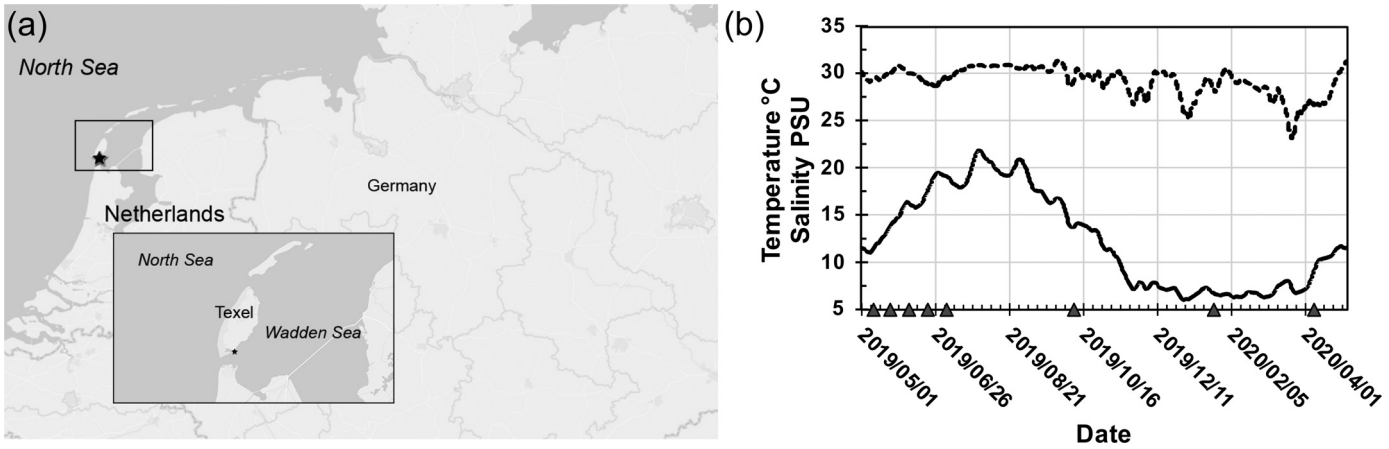

Fig. 1. a) Geographic location of experiment off the island of Texel in the Wadden Sea; b) Temperature (solid line) and salinity (dashed line) at the site, using a 6-day moving average, with sampling time points indicated by triangles on bottom axis.

2.4. Density

To determine density, we used gradients produced using Nycodenz (5- (N-2, 3-dihydroxypropylacetamido)−2, 4, 6-tri-iodo-N, N'-bis (2, 3 dihydroxypropyl) isophthalamide); (ProteoGenix, Schiltigheim, France). When frozen at −20 °C and then allowed to thaw at room temperature, Nycodenz solutions self-form a density gradient (Murayama et al., 2001). We were particularly interested in quantifying densities when plastic started to sink in seawater, so we chose a 20% Nycodenz solution to produce a gradient that ranged from approximately 1.0068–1.3523 g • cm^{-3} and that is linear between 1.02 g • cm^{-3} (approximate density of seawater) and 1.15 g • cm^{-3}. Because we were determining the density of live biofilms, we dissolved the Nycodenz in 1 x PBS (phosphate buffered saline) solution. We froze 40 ml of the 20% Nycodenz solution in 50-ml centrifuge tubes, removing the number needed the morning of a sampling date and allowing it to thaw slowly at laboratory temperature (~ 20 °C) without being disturbed. Once the Nycodenz thawed and came to room temperature, we gently added our sample (plastic or diatom biofilm) to the surface, along with a mix of three color-coded density standard beads from Cospheric (www.cospheric.com): Green: 1.02 g • cm^{-3}, Blue: 1.13 g • cm^{-3}, and Red: 1.20 g • cm^{-3}. Plastic samples were added in the center of the tube, and a small drop of density beads were added around the edges to avoid the beads interfering with the biofilms. Tubes were then centrifuged at 3000 rpm for 30 min in a temperature-controlled centrifuge with swing-out rotors at 20 °C. After centrifugation, the distance from the surface of the Nycodenz to the center of each sample and to each band of beads was measured to the nearest mm. Linear regression of the depth vs. density of the standard beads provided a calibration curve for each tube, that was used to calculate the density of cultures and plastic samples with biofilms between 1.02 and 1.20 g • cm^{-3}. Outside of this range, the slope of the Nycodenz density gradient changes, so for plastic pieces that settled outside of our density bead range, we report only ordinal densities (details below and in Supplementary Material).

2.5. Microscopy

Light micrographs of live phytoplankton on plastic were collected on a Zeiss Axio Observer (Zeiss, Breda, Netherlands). Plastic samples for SEM were stored in PBS: ethanol (50:50) at −20 °C until analysis, then processed and imaged according to a protocol that has proven successful for Plastisphere samples (Zettler et al., 2013). Samples were dehydrated on ice through an ethanol series: 10 min each in 70%, 85%, 95%, followed by 3 × 15 min in 100% ethanol. After dehydration, samples were immediately critical point dried in a Samdri 780A (Tousimis, Rockville, MD), then sputter coated with 5–10 nm of platinum using a Leica EM MED020 (Leica Microsystems, Inc. Buffalo Grove, IL). Imaging was done on a Zeiss Supra 40VP SEM (Carl Zeiss Microscopy, Thornwood, NY).

2.6. Community characterization (see supplementary material for more details)

We extracted total genomic DNA using the Gentra Puregene Tissue DNA Isolation kit (QIAGEN Benelux B.V., Venlo, The Netherlands) following manufacturer's instructions with modifications. PCR amplifications employed the Needham-Fuhrman approach to amplifying all three domains of life (Bacteria, Archaea, and Eukarya) simultaneously. These target the V4-V5 region using the primer combination 515F-Y/926R: 5′-GTGYCAGCMGCCGCGGTAA-3′ and 5′-CCGYCAATTYMTTTRAGTTT-3′ (Needham and Fuhrman, 2016), and have been shown to provide good results for bacteria and archaea (Parada et al., 2016), as well as eukaryotes (Abdala Asbun et al. 2020; Vaulot et al., 2021). All 72 samples that represented the initial colonization community until the plastic started to sink (t1 (2 weeks) through t4 (8 weeks)) were extracted, amplified, and purified. At each of these timepoints, we sequenced 18 pieces of plastic (6 of each polymer and 3 of each thickness). Of these 72 samples, 70 were successfully sequenced at the USeq Sequencing facility at the University of Utrecht on an Illumina MiSeq Sequencing Platform (Illumina, San Diego, CA). Community abundance matrices of ASVs (amplicon sequence variants) were obtained after running the CASCABEL pipeline (Abdala Asbun et al., 2020) after removing mitochondrial and chloroplast sequences. We used the phyloseq package for data analysis and visualization, keeping only the most abundant taxa, those observed at least 500 times for bacteria and 100 times for eukaryotes. The data for this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB45258 (https://www.ebi.ac.uk/ena/browser/view/PRJEB45258).

3. Results

3.1. Environmental conditions

Changes in salinity and temperature over the course of the experiment are shown in Fig. 1b, along with sampling dates. Additional environmental parameters for each sampling date are shown in Table S3. Our experiment was designed to capture the spring

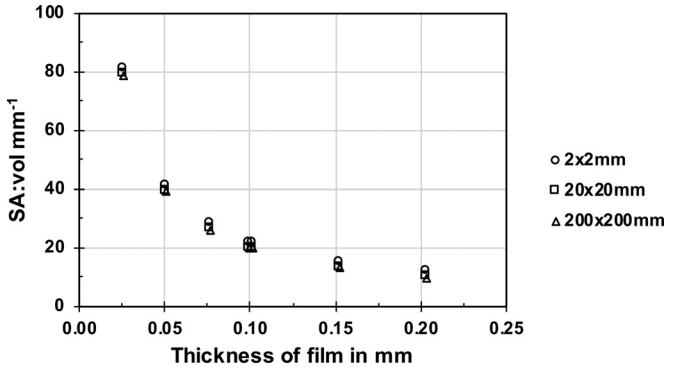

Fig. 2. Calculated surface area to volume ratios for plastic films used in this study. For each thickness, the SA:V ratio of pieces of 3 sizes are plotted, showing that the length and width of a piece of film have relatively little impact on the SA:V of thin films.

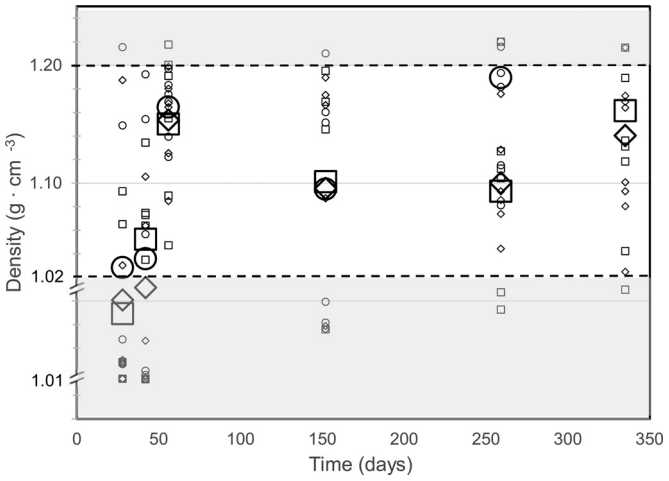

Fig. 3. Density of individual plastic pieces of each polymer at each time point (small symbols; HDPE circles, LDPE squares, LLDPE diamonds). Averages for each resin type are shown as larger open symbols of the same shape. The dashed line at 1.02 g • cm^{-3} represents the approximate density of seawater, so pieces below the dotted line will float, and those above it will sink. Note that our density gradients were only linear between our 1.02 and 1.20 g • cm^{-3} beads (dotted lines), so density values in this region are quantitative. In the shaded areas above and below this region, the location of the plotted piece of plastic gives an indication of the relative, but not absolute density.

bloom as water temperatures rose and then continued sampling at lower frequency throughout an annual cycle. Temperature and salinity vary considerably in this intertidal coastal area with significant freshwater input.

3.2. Density change due to microbial biofilms alone

The SA:V ratio of the six thicknesses of plastic we used ranged from about 10 to 80 (Fig. 2). We used pieces approximately 5 × 5 mm to simplify handling and to conform to the generally accepted definition of microplastic but note that the SA:V ratio is determined mostly by the thickness of the piece of plastic rather than the other dimensions.

Unialgal cultures including dinoflagellates, cyanobacteria, and diatoms attached readily to the surface of HDPE, LDPE, and LLDPE pieces of all thicknesses (Fig. S1a, b). In all of our tests we observed only seven individual pieces of plastic that sank due to colonization by unialgal cultures alone: One with diatom WH23, and two each with cyanobacterium *Phormidium* sp., cyanobacterium *Spirulina* sp., and dinoflagellate *Prorocentrum lima*. Six of these were pieces with high SA:V ratios of 80, and one with SA:V ratio of 40. In all cases, this occurred during late exponential or stationary phase, and was due to large clumps of cells extending beyond the plastic surface (e.g. Fig. S1a, b). These were fragile enough that they usually broke off during the centrifugation in the density gradients, and the plastic became buoyant again. In our experiments, microbes attached to the actual surface of the plastic did not generally provide sufficient ballasting to cause plastic to sink. The pieces of plastic were initially quite hydrophobic and floated at or on the surface film of the seawater. All cultures attached to plastic and appeared to decrease the hydrophobicity of the plastic within a week, allowing even the thickest (0.203 mm) pieces to slip below the surface film (Fig. S1c). Plastic strips in the control flasks without microalgae or bacteria remained on the surface film throughout the experiment. Of the cultured microbes that caused our 5 × 5 mm pieces of plastic to sink, diatoms attached most consistently to the plastic surfaces and formed robust biofilms. Based on this, and because diatoms have relatively dense silicon dioxide frustules (shells) and are some of the most commonly reported early colonizers, we also determined the density of biofilms formed by three diatoms isolated from surfaces in coastal waters for calculating whether microbes alone might be able to cause sinking of plastic with even higher SA:V (isolate names, source, and density are shown in Table 1).

3.3. Density change due to natural fouling communities in the Wadden Sea

The attached communities that developed on plastic during the *in-situ* colonization experiment caused two of the pieces with the highest SA:V ratio to move down in the density gradient after just 2 weeks (Table S5), but none exceeded the density of seawater until week 4 (Fig. 3). As soon as pieces started moving down in the density gradients, we knew their density was increasing. However, because our first density calibration bead was 1.02 g • cm^{-3}, we could not quantify the exact density until a piece exceeded that value and became denser than seawater. These pieces that were increasing in density but would still float in seawater are plotted ordinally without a scale between 1.01 g • cm^{-3} (floating on top of the density column) and 1.02 g • cm^{-3} in Fig. 3. Likewise, samples with densities above our densest standard of 1.20 g • cm^{-3} are plotted without a scale above 1.20 g • cm^{-3}. Generally speaking, pieces with equal fouling coverage that had higher SA:V increased in density more quickly (Fig. S2), but the patchy distribution of fouling often obscured this pattern (Fig. S3).

By week 6 the average density of all three polymers was greater than seawater, but even after that there were generally a minority of pieces at each sampling that were buoyant (Fig. 3 and Table S5). HDPE is the densest polymer of this group, and it remained densest for the first 28 days, but after that there was no clear pattern in density differences among the three polymers. Note that at the final time point 7, all but five plastic strips had been lost due to a storm, including all HDPE so only LDPE and LLDPE are shown.

3.4. Community composition

After two weeks plastic strips were coated with a thin slimy biofilm, with occasional worm tubes and small colonies of bryozoa. As time went on, bryozoan colonies continued to spread and visual and SEM surveys (Fig. 4) demonstrated a wide spectrum of other invertebrates colonizing the plastic surface including bivalves, echinoderms, colonial hydroids, barnacles, tunicates and worms.

Table 1
Source and density of biofilms formed by selected diatom cultures on plastic.

Culture	Source	Average Density (g • cm^{-3})
WH23	*Navicula* sp. isolated from PE in Woods Hole, USA	1.17
A6	isolated from an antifouling painted surface in seawater at Texel, The Netherlands	1.15
A8	isolated from *Ulva* seaweed at Texel, The Netherlands	1.18

*A6 and A8 courtesy of Dr. Louis Peperzak.

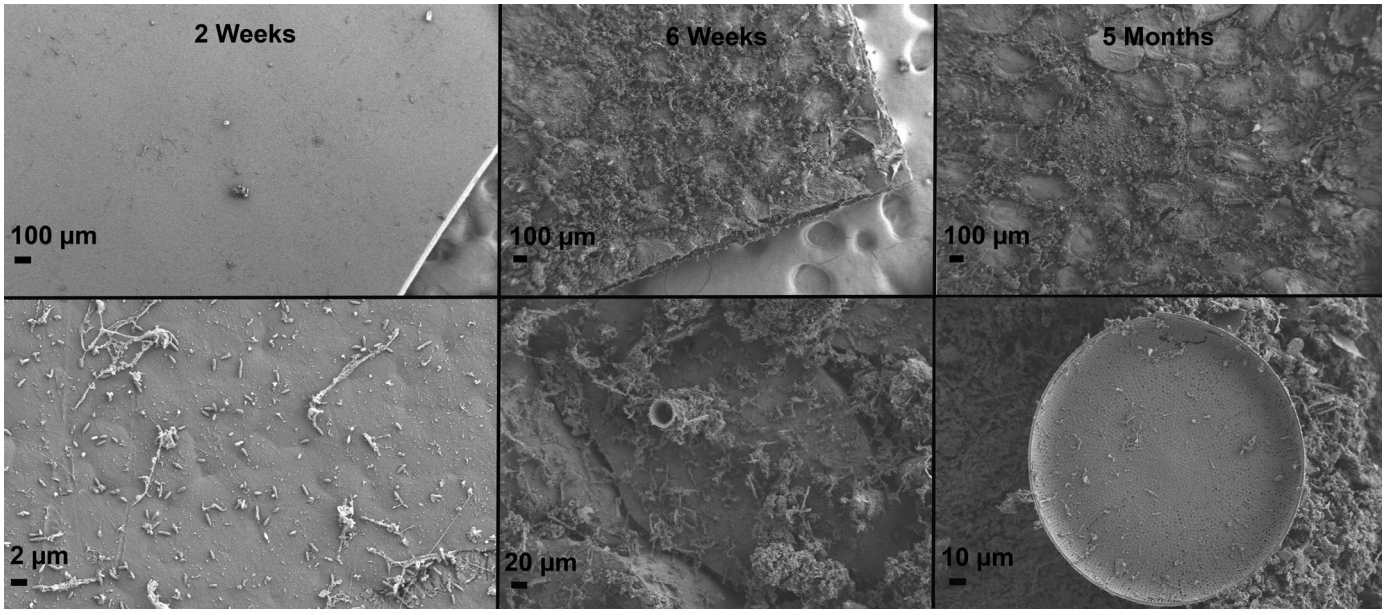

Fig. 4. Scanning electron micrographs of HDPE 0.10 mm thick over time. Top row are low power micrographs to show general appearance of surface; bottom row shows same pieces at higher power to see individual micro-organisms.

Amplicon sequencing of DNA extracted from the plastic samples revealed the complex communities that developed on the plastic and how they changed over time (Fig. 5). For eukaryotes at week 2 (t1), ciliates, echinoderms, annelids, and bryozoa had colonized the plastic, with some variation depending on the polymer, but by week 4 (t2), bryozoa became the dominant organisms on all substrates in this system. Bacterial communities were dominated by Proteobacteria and Bacteroidota throughout the experiment, with some changes over time among the less dominant groups including increases in Firmicutes and Planctomycetota. Photosynthetic cyanobacteria were present at all time points but seldom made up more than 5% of the population.

4. Discussion

4.1. Density changes due to microbial biofilms

A number of studies have documented increases in weight and sinking due to biofilm development and biofouling (reviewed in Onda and Sharief, 2021), but far fewer quantify density changes. The impact of microbial biofilms compared to macroalgae and invertebrates is also not clear, despite the often-stated opinion that (microbial) biofilms increase weight of plastic and cause sinking. In some cases, biofilms may even cause the density of plastic particles to decrease (Miao et al., 2021). Measured densities of microbial cells and biofilms reported in the literature are few and vary widely. Van Ierland and Peperzak, 1983 used density gradient centrifugation to measure the density of nine diatom species in natural marine plankton samples from the North Sea, ranging from 1.03 to 1.23 g • cm^{-3}. In a theoretical study, Kooi et al. (2017) used a median biofilm density of 1.38 g • cm^{-3}; this was calculated from estimated wet weight and volumes reported by Fisher et al. (1983), but these yield density estimates from 0.53 to 4.35 g • cm^{-3}, so they are not very realistic (the density of granite stone is only 2.75 g • cm^{-3}). Van Melkebeke et al. (2020) used more realistic values of 1.1 ± 0.1 g • cm^{-3}, but these were based on calculations, not measurements. We measured the density of biofilms created by three types of diatoms that were isolated from PE and other surfaces in marine systems and found these ranged from 1.15 to 1.18 g • cm^{-3} (Table 1). Diatoms are consistently reported as early and dominant colonizers of PMD (Bravo et al., 2011; Carson et al., 2013; Eich et al., 2015; Zhao et al., 2020), and the silicon dioxide frustule (density ~2.6 g • cm^{-3}) of these protists can make them substantially denser than seawater. Consequently, diatoms might be expected to contribute to sinking of PMD. However, our laboratory experiments with diatoms and other attached phytoplankton showed that although they appear to decrease the hydrophobicity and cause plastic to sink below the surface film (Fig. S1c), microbial biofilms (consisting of unialgal cultures along with associated bacteria, EPS, and adhered particles) alone rarely caused sinking of microplastic. Some of those that did sink had relatively loosely attached clumps of cells, so despite the fact that they sank in our experimental containers that were oscillated to simulate movement at sea, in the density column many of the clumps of cells detached and the polymer became buoyant again. Sinking due to microbial biofilms alone may be more prevalent in freshwaters with a density around 1 g • cm^{-3} (Chen et al., 2019).

Wright et al. (2020) mention the lack of experimental measurements of the impact of microbial biofilms on plastic density. They also calculated the thickness of biofilms of various densities (1.1–1.5 g • cm^{-3}) necessary to make PP and PE spheres of different diameters sink. Our experimental results support their model prediction that microbial biofilms 10 µm thick with a den-

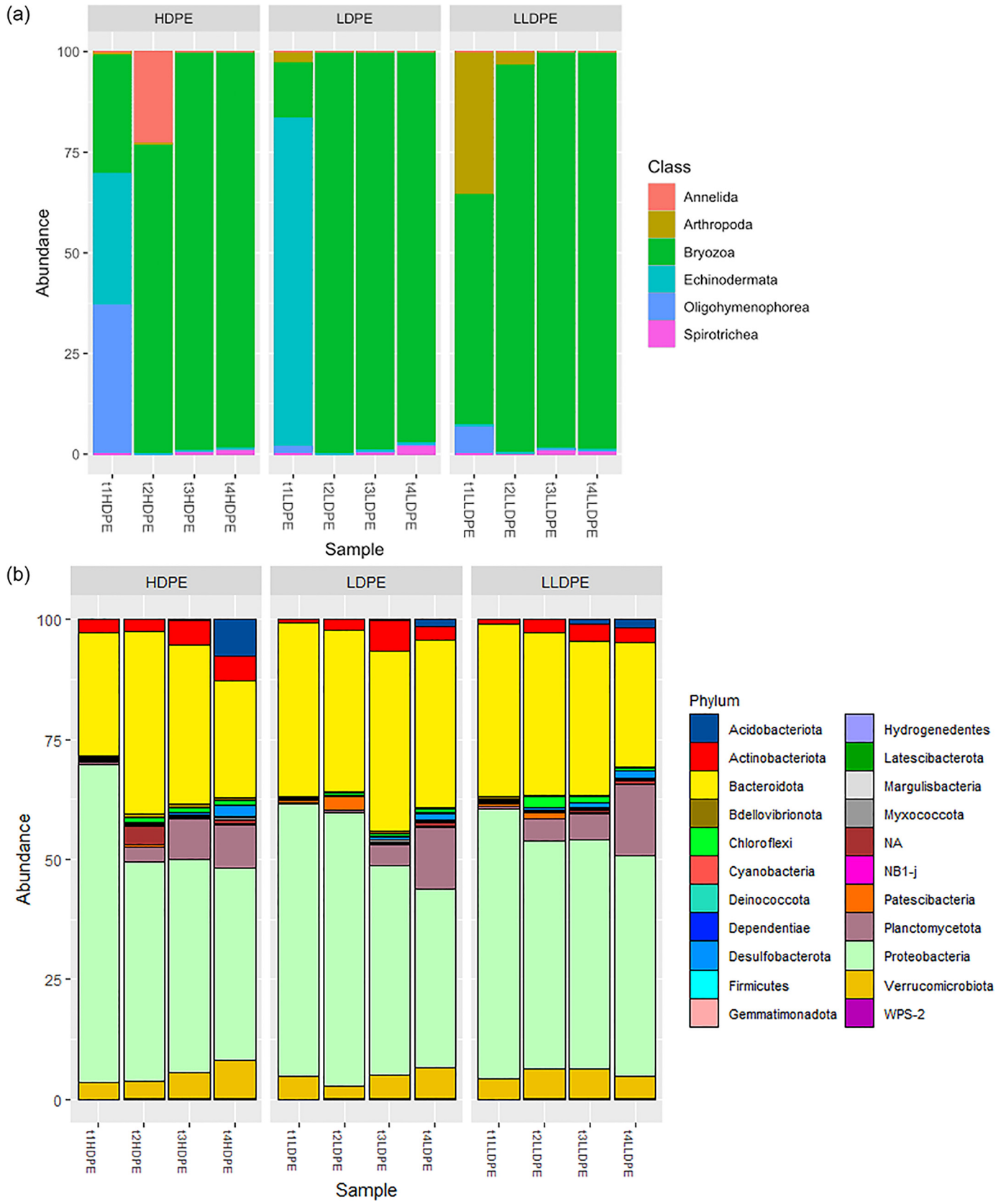

Fig. 5. Relative abundance of sequences for most abundant taxa over time (those observed at least 500 times for bacteria and 100 times for eukaryotes), shown separately for HDPE, LDPE, and LLDPE for t1 (2 weeks), t2 (4 weeks), t3 (6 weeks) and t4 (8 weeks). a) Eukaryotes; b) Bacteria.

sity of 1.1 g • cm^{-3} could cause PE spheres smaller than 100 µm in diameter to sink. Our WH23 cells isolated from PE in Woods Hole, USA measure 12.5 × 4.5 × 3 µm (L x W x H), with a density: 1.17 g • cm^{-3}. A biofilm layer 10 µm thick would accommodate three layers of these 3- µm thick diatoms, and if they covered 100% of a 100 µm PE sphere, the combined density of the PE and diatoms would be 1.035 g • cm^{-3}, denser than seawater. While theoretically possible, 100% coverage of a plastic piece by a three-cell thick biofilm of diatoms is unlikely. Also a 100- µm sphere has a SA:V ratio of 60, and in our experiments films with a higher SA:V of 80 did not sink even when heavily coated with a diatom biofilm, so as other authors have mentioned, shape is important as well as density. However, diatoms can almost cover a piece of plastic recently immersed in seawater (Fig. S4), so calculations using a slightly more realistic scenario of a layer of diatoms one cell thick covering 100% of the surface suggest diatoms could cause the sinking of a PE sphere of 40 µm diameter with a SA:V ratio of 150. At this scale, it starts to beg the question of whether the particle is plastic covered with microbes, or a microbial/TEP aggregate with included plastic pieces. Under some bloom conditions, diatoms can cover almost 100% of the plastic surface (Fig. S4), but field data suggest coverage is almost never this high (Dussud et al., 2018). Our own observations, based on SEM imaging of the biofilms on hundreds of pieces of plastic collected in the Pacific and Atlantic (Amaral-Zettler et al., 2015), as well as in the Mediterranean (Amaral-Zettler et al., 2021) also show that natural biofilms on plastic seldom cover more than 50% of the surface of a piece of plastic. However, they are often greater than 1 cell thick and include dead cells and diatom frustules that can extend the microbial biofilm up to a thickness of 50 µm. These results, based on actual measured values for biofilm density, coverage, and thickness, suggest that microbial biofilms alone can cause plastic to sink, but generally only on items with SA:V ratios above 100 such as thin films and fragments and filaments with diameters less than 50 µm. For larger fragments, the most important role of microbial biofilms causing plastic to sink may be by decreasing hydrophobicity and providing chemical cues that encourage the settlement and facilitate adhesion of invertebrates (Zardus et al., 2008). We observed that pieces originally floating on top of the surface film eventually sank below the surface, even though they were still buoyant (Fig. S1c). Lobelle and Cunliffe (2011) noted that this behavior correlated with quantitative decreases in hydrophobicity of PE food bags. This may be the first stage of sinking because it increases the surface area available for settlement of additional organisms (Bravo et al., 2011). Many of the taxa encountered on beached debris by Winston et al. (1997) are calcifying, and these can increase the density of plastic quickly.

4.2. Surface area to volume considerations

Ryan (2015) pointed out the importance of SA:V ratios of different shapes, and observed fewer thin items further from shore in the South Atlantic. Subsequently Fazey and Ryan (2016) used HDPE and LDPE sheets of thicknesses ranging from 0.1 to 4 mm to show in field experiments that smaller pieces (down to 5 mm) and pieces with higher SA:V ratio sink faster when colonized. They quantified coverage by macro-colonizers and concluded that biofouling may account for a substantial portion of the "missing" surface plastic. We confirmed their finding that for a given size and shape, pieces with high SA:V ratios generally sink sooner (Figs. S2, S3). Van Melkebeke et al. (2020) also provided analyses of sinking rates and calculations showing that films are far more likely than other shapes of plastic to develop a biofilm sufficient to cause sinking of buoyant plastics. Based on this, we concentrated on thin films as the items most likely to sink quickly due to biofouling. For films the thickness dominates their SA:V (Fig. 2), while the length and width determine the SA and therefore who can colonize a given piece. For example, a piece of film that is 0.1 mm thick and 2 × 2 mm has a SA:V ratio of 22, while a piece of the same film that is 200×200 mm has a SA:V ratio of 20.02. This 10% difference in SA:V is minor relative to the 10,000 x difference in the SA of those same pieces (8.8 mm^2 vs. 80,080 mm^2), and this huge difference will influence what and how many organisms can colonize the plastic. The much larger piece has a marginally lower SA:V, but it is more likely to develop a fouling community of invertebrates and other multicellular organisms that will make it sink. This also means that depending on shape, "macroplastic" films many cm in size have higher SA:V ratios than "microplastic" fragments and are more likely to sink.

4.3. Density changes due to mixed biofilms of microbes and metazoans

In our field experiments, the fouling community caused the density of plastic to increase rapidly during the spring bloom, and by week 6 most pieces of plastic were sinking, as the average density for all three polymers was greater than that of seawater (Fig. 3). The timing and average densities during our experiment are very similar to those reported in the pioneering work by Ye and Andrady (1991), but in their case the early colonization leading to sinking was dominated by algae, while ours was due primarily to bryozoa (Figs. 4, 5). Several factors affect our results, including constraining the plastic strips in a mesh bag as opposed to experimentally tethered or naturally free-floating plastic. We did this to contain the plastic in this high energy tidal environment with significant wind exposure, as well as to exclude macro grazers to keep them from "resetting" our biofilms. The mesh bags undoubtedly change the hydrodynamics and access to the plastic surface by settling propagules. The colonization by microbes should not have been strongly affected, and in addition to bryozoans we saw bivalves, tunicates, echinoderms, and crustaceans settling on the plastic. However, we saw very little macroalgae, though these have been primary colonizers in other studies mentioned above, and our setup in mesh bags, as in all experiments, undoubtedly exerted some filtering influence on the resulting communities.

Our molecular results (Fig. 5) provided more details on other taxa, including microbes, that colonized the plastic. After two weeks the plastic visually appeared mostly bare but already had a diverse community of bacteria and microbial eukaryotes. By four weeks small colonies of bryozoa were already visible, and molecular results confirmed they were the dominant colonizer, but that other animal groups including annelids, arthropods, and echinoderms had also started to settle, along with numerous bacteria and protists, including ciliates (Fig. 5a, b). The Plastisphere community is dynamic and changes seasonally and geographically (Amaral-Zettler et al., 2020). Photosynthetic cyanobacteria were present at all time points (Fig. 5b), but the communities at this location during the almost yearlong experiment were dominated by heterotrophs. Diatoms and other phototrophic protists were present, but seldom seen in the SEM images and contributed only a small proportion of the sequences analyzed so they were below the cutoff of 100 occurrences across all samples we chose for plotting. The primary colonizers at different times and in different locations will almost certainly vary, but it appears that at least in most coastal areas, there are macro-organisms that will colonize plastic and cause it to sink, including algae (Ye and Andrady, 1991), bivalves (Kaiser et al., 2017), and bryozoa (this study). How commonly fouling causes the sinking of plastic in the oligotrophic open ocean is unknown, but the most common taxa reported from pelagic PMD generally include a number of calcifying organisms including barnacles, bryozoa, and shelled mollusks (Goldstein et al., 2014; Gregory, 2009; Kiessling et al., 2015).

Our samples were held at constant depth and protected from macro grazers by a mesh bag, so we did not observe defouling and resurfacing of submerged samples as proposed by Ye and Andrady (1991). At our experimental site we saw very little algal growth, so lack of light as they sank would not affect our pieces as much. The primary causes of the density increase in our experiment was encrustation by carbonate producing bryozoa, and their ballasting shells would not necessarily be removed even if they are grazed or die as the plastic sinks. In our experiment once it became denser than seawater, the plastic remained negatively buoyant throughout the year, including during winter when growth rates are lower. We have also commonly observed in SEM images that the lower frustule of diatoms remains attached to plastic after the diatom is grazed, so these would continue to ballast the plastic. In addition, Winston et al. (1997) in their survey of encrusting organisms on plastic, found that some were found only on buoyant plastic, some were found only on non-buoyant plastic, but most were found on both buoyant and non-buoyant plastic, so sinking alone will not always lead to loss of the ballasting organisms. Cozar et al. (2014) suggested that in the deep-sea carbonate ballast may dissolve at depth due to increasing acidity below the CCD, but this mechanism does not exist in coastal waters, and at least some calcium carbonate encrusting organisms persist when buoyant plastic such as PE sinks (Gundogdu et al., 2017). In shallow freshwaters, loading of the biofilm with mineral particles caused the sinking of experimental plastic pieces (Chen et al., 2019), and this may also play a role in shallow, turbid coastal marine environments such as the Wadden Sea. Kooi et al. (2017) predicted vertical oscillations based on photosynthetic algae alone, but the plastisphere has many heterotrophs and animals that are not immediately impacted by the loss of light at night or as they sink into the twilight zone. In fact, if the phototrophs start dying, this could lead to an increase in heterotrophs that can utilize the POM and DOM released from the dying cells.

As pointed out by Gerritse et al. (2020), several studies have remarked on the paucity of floating plastic particles smaller than 1 mm (Cozar et al., 2014; Eriksen et al., 2014; Kooi et al., 2017) supporting the hypothesis that smaller particles with higher SA:V ratio may be removed more rapidly due to ballasting. While in general this is true (Fig. S2), during our experiment, smaller sections cut from larger pieces that sank sometimes became buoyant again (those plotted below dotted line in Fig. 3). Ye and Andrady (1991) mentioned the non-uniformity of fouling, as did Fazey and Ryan (2016), and in our experiment the uneven distribution of bryozoa and other larger organisms meant that sometimes a subsample had insufficient ballasting to sink (Table S5). So, although fragmentation increases SA:V ratio, it will not always increase sinking, and may actually lead to pieces of plastic on the bottom rising back to the surface.

4.4. Suggestion of a SA:V "tipping point" for metazoan vs. microbe mediated sinking

Our results suggest that for microplastic in the mm-size range and above, fouling by biofilms consisting only of microbes with their associated EPS and bacteria alone is seldom sufficient to cause sinking, but the diverse community of invertebrates in coastal regions that settle after the microbes are established can cause sinking of microplastic, as well as thin plastic films of almost any size. During the spring bloom, this may lead to a pulse of buoyant plastic to the benthos. Zhao et al. (2020) calculated that the microbial carbon on the floating plastic in the World Ocean represents approximately 1% of the total carbon in surface microlayer of the ocean, so sinking due to biofouling may be moving a portion of this C to the deep ocean continually, representing an important source of C to the benthic community. Calculations based on our measured diatom densities and dimensions provide additional evidence that pieces of plastic much smaller than those we used may sink from the surface during the spring bloom, but this remains to be demonstrated. However, many of the calcifying invertebrates such as barnacles, mollusks, and bryozoa that cause most of the density increase cannot mature on fragments of plastic smaller than a couple of hundred micrometers square. Microbes such as diatoms on the other hand can attach to plastic pieces down to 10′s of micrometers in size. Kaiser et al. (2017) found that even pieces of PE as small as 1 mm could be colonized by multiple mussels that caused them to sink. They suggested there is a lower limit to the size of particles that can be sunk by attached invertebrates and point out the lack of data regarding the impact of fouling on sub-mm size plastics that are more readily incorporated into food webs. Limited data on the density of bacterial cells suggest they range from 1.11 – 1.18 (Lewis et al., 2014). This study did not quantify density changes due to heterotrophic bacteria alone, as our objective was primarily to investigate the impact of biofilms on plastic in illuminated surface waters where phototrophs typically flourish. However, bacteria can attach to plastic particles as small as 5 μm (Yi et al., 2021), and probably play a larger role in altering the density of smaller pieces of plastic, especially on those transported below the euphotic zone where biofilms will be dominated by heterotrophs. Our data support this size (SA:V) "tipping point", and we suggest it is at a SA:V ratio of approximately 100, with sinking of larger pieces of plastic with SA:V below this dominated by invertebrates, but for smaller pieces of plastic with SA:V above 100, including small fibers, sinking may be mediated by microbial biofilms or incorporation into aggregates (Zhao et al., 2018). The exact SA:V for the proposed tipping point will vary depending on the polymer, geographic region, and season due to differences in polymer density, microbial and larval populations, and environmentally constrained growth rates.

5. Conclusions

Biofilm formation and fouling organisms will vary from system to system, but this work provides a range of experimentally determined results that start to constrain realistic changes in density due to biofouling of plastic in coastal environments. These data are critical to help modeling efforts that inform plastic litter budgets predicting the fate of plastic pollution in the environment. Our results confirm that fouling by multicellular organisms can cause buoyant plastic to sink in coastal environments, but that except for pieces with SA:V above 100, microbial biofilms alone seldom cause sinking. As plastic pieces become smaller, at some point they are too small for multicellular organisms to attach. Therefore, future work should quantify the role of bacteria, archaea, and eukaryotic protists in density changes at these smaller scales.

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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Supplementary materials

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