Fecal coliform and *E. coli* in microplastic biofilms grown in wastewater and inactivation by peracetic acid

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Abstract

Microplastics (MP) have been proposed as a vector for pathogenic microorganisms in the freshwater environment. The objectives of this study were (1) to compare the fecal indicator growth in biofilms on MP and material control microparticles incubated in different wastewater fractions and (2) to compare MP biofilm, natural microparticle biofilm, and planktonic cell susceptibility to disinfection by peracetic acid (PAA). Biofilms were grown on high-density polyethylene, low-density polyethylene, polypropylene MP or wood chips (as a material control) and incubated in either wastewater influent or pre-disinfection secondary effluent. Reactors were disinfected with PAA, biofilms were dislodged, and fecal coliform and *E. coli* were cultivated. Fecal indicators were quantifiable in both MP and wood biofilms incubated in the wastewater influent but only on the wood biofilms incubated in secondary wastewater effluent. More fecal coliform grew in the wood biofilms than MP biofilms, and the biofilms grown on MP and woodchips were more resistant to disinfection than planktonic bacteria. Thus, it may be possible to refer to the disinfection literature for fecal indicators in biofilm on other particles to predict behavior on MP. Treatments that remove particles in general would help reduce the potential for fecal indicator bypass of disinfection.

Keywords: biofilm, peracetic acid, fecal indicators, microplastic, wastewater, disinfection

Introduction

Wastewater treatment plants (WWTPs) have been shown to be reasonably effective at removing microplastics (MPs), with removal efficiencies in the 75%-100% range for conventional WWTPs utilizing activated sludge processes and secondary clarification (Conley *et al.*, 2019; Sun *et al.*, 2019). Although most MPs are removed in wastewater treatment processes, some will escape to the environment in the effluent and the fate of MP captured in biosolids remains to demonstrated. Because biofilm is well known to be more resistant to disinfection than planktonic organisms (Bridier *et al.*, 2011; Kim *et al.*, 2008; Lee *et al.*, 2020), MP biofilms may allow for wastewater bacteria such as *E. coli* and other fecal indicator organisms to bypass disinfection at a WWTP, as these organisms are known to form biofilms on natural particles as well as manmade particles (Fux *et al.*, 2005; Miao *et al.*, 2019; Song *et al.*, 2020). This may explain why fecal microbes were observed in MP biofilms far from wastewater effluent outfalls (Rodrigues *et al.*, 2019; Silva *et al.*, 2019). Thus, there is some concern that MPs could serve as a vector for pathogenic microorganisms in the freshwater environment.

There have been investigations into freshwater MP biofilms with a focus on using sequencing techniques to describe the microbial ecology (Eckert *et al.*, 2018; Miao *et al.*, 2019; Parrish and Fahrenfeld, 2019), biodegradation potential of MP or adsorbed organic compounds (Paço *et al.*, 2017; Park and Kim, 2019; Porter et al. 2020), and the prevalence of antibiotic resistance genes and pathogens/pathogen marker genes (Parrish and Fahrenfeld, 2019; Rodrigues *et al.*, 2019; Viršek *et al.*, 2017). Fecal indicators such as *E.coli* have been cultivated from and pathogens such as *Vibrio* have been observed in MP biofilm found in the marine environment (Kirstein *et al.*, 2016; Quilliam *et al.*, 2014). Total Coliform (TC) and *E. coli* are commonly used to evaluate drinking, surface, and wastewater quality as indicators of pathogens (EPA, 2012; EPA *et al.*, 2011).However, to our knowledge, whether MP biofilm behave differently from other microparticle biofilms during disinfection has not received attention.

47 MPs are generally defined as anthropogenic polymer particles less than 5 mm in size and classified as
48 either primary or secondary (Kershaw and Rochman, 2015). Primary MPs are manufactured as such, and
49 secondary MPs are the result of environmental weathering of larger plastic items (Guerranti *et al.*, 2019;
50 Li *et al.*, 2018). Laboratory investigations of MP biofilms should seek to simulate environmental
51 conditions including the polymer types, size classes, morphologies, and textures of MPs observed in the
52 environment. A growing body of research into MPs observed in freshwater bodies is available (Li *et al.*,
53 2018; Meng *et al.*, 2020). Recent reviews indicated that polyethylene (PE), polypropylene (PP),
54 polyamide, polystyrene (PS), and polyester were the most commonly reported polymers in studies
55 reporting chemical identity of freshwater MP, with polyethylene being the most common (Enders *et al.*,
56 2015; Fahrenfeld *et al.*, 2019; Frère *et al.*, 2017; Kershaw and Rochman, 2015). Fragments, fibers, and
57 films were the most commonly reported MP morphologies in freshwater and the majority were secondary
58 in nature (Fahrenfeld *et al.*, 2019; Guerranti *et al.*, 2019; Li *et al.*, 2018), despite concerns over
59 microbeads previously added to personal care products, which were banned in the United States in 2015
60 and phased out by 2019 (Xanthos and Walker, 2017). These reports motivated the selection of MP
61 polymer types and morphologies selected for this study.

62 The objectives of this research were to (1) compare the prevalence of fecal indicator organisms (i.e., fecal
63 coliform and *E. coli*) in MP and natural microparticle biofilms and (2) evaluate the susceptibility of fecal
64 indicators in these biofilms to peracetic acid (PAA) disinfection compared to the planktonic fecal
65 indicator organisms. To achieve these goals, a bench-scale study was performed using high density PE
66 (HDPE), low density PE (LDPE), PP, PS, and wood chips, as a natural organic microparticle material
67 control. Particles were incubated in either municipal wastewater influent or pre-disinfection secondary
68 wastewater effluent and reactors were either disinfected with PAA or not treated. PAA is considered a
69 green disinfectant because it has not been reported to form regulated disinfectant by-products, and was
70 chosen due to its status as a disinfectant that will likely see increased use in the coming years (McFadden
71 *et al.*, 2017; Monarca *et al.*, 2002). PAA has a similar mechanism of disinfection to hypochlorite

(Koivunen and Heinonen-Tanski, 2005; McFadden *et al.*, 2017), as both oxidize cell membranes. The biofilm and planktonic cell fecal indicator concentrations were compared across materials, wastewater fractions (i.e., influent vs. pre-disinfection secondary effluent), and PAA treatment. Results presented here can provide insight into the role of microparticles as carriers of fecal indicator organisms and their susceptibility to disinfection.

Materials and Methods

MPs were either extracted from a personal care product or, to better simulate environmental MPs, generated from plastic materials labeled with the polymer composition. The plastic materials chosen included polymers commonly observed in freshwaters: HDPE, LDPE, PP, and PS. The polymer types were confirmed by Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR), as described below. MP fragments or films were created from the plastic materials by first cutting plastic items into small pieces with scissors, freezing with dry ice, and grinding with a coffee grinder (Bodum Inc., Triengen, Switzerland). The items cut and ground were an HDPE milk bottle to create fragments, a LDPE plastic shopping bag to create films, a polypropylene microcentrifuge tube to create fragments, and a polystyrene fork to create fragments. After grinding, materials were wet sieved to collect particles 500-2000 μm. Precautions were taken to prevent cross-contamination between polymer types: sieves were triple washed and checked for particles to ensure they were completely clean, and the grinder was thoroughly washed and compressed air was used to ensure no particles remained after drying. As a material control, wood chips were gathered from a Spanish Oak tree being cut on our campus and wet sieved to collect particles 500-2000 μm.

Reactors were prepared to simulate incubation of MP in different wastewater fractions: municipal wastewater influent or pre-disinfection secondary effluent. Five liters of secondary effluent were sourced from a conventional activated sludge wastewater treatment plant in NJ (grab samples, collected between 8-10 am) on April 22, April 24, and May 22, 2019 during regular flow conditions. The influent samples

were sourced from the same facility on January 29, February 3, and February 5, 2020. Samples were transported in a cooler back to the lab. Upon arrival in the lab, reactors were assembled and started immediately (less than 1 hour hold time). Aliquots of the wastewater samples were preserved by freezing at -20°C for chemical analysis, described below.

The biofilm experiment was performed on three different sampling dates (triplicate) for each wastewater fraction with two reactors for each particle type on each date to allow for analysis of paired PAA and non-PAA treated reactors. For the secondary effluent experiment, reactors (500 mL glass beakers) contained 200 mL of wastewater and a 0.21 mL volume of MP (LDPE, HDPE, PP) or wood chips (Fig. S1). The number of microparticles in the selected volume were counted to normalize results with respect to concentration. For the influent experiment, reactors were prepared with LDPE and wood in the same manner. LDPE was chosen for the influent experiment as it is extremely common in environmental samples (Kershaw and Rochman, 2015; Stanton *et al.*, 2020) and to reduce the size of the experimental matrix. As an inoculum control, separate reactors were prepared using DI water instead of wastewater to incubate MP (LDPE) and wood particles. All reactors were covered with sterile aluminum foil and incubated at room temperature for 24 hours while being stirred at 120 rpm with stir bars to simulate the shear expected in turbulent flow (Halász *et al.*, 2007). After 24 hours, one of the duplicate reactors for each experimental condition was disinfected using PAA at a nominal concentration of 2 mg/L and a contact time of 25 minutes. This nominal CT was selected based on previously reported PAA disinfection studies reviewed by Kitis (Kitis, 2004). CT is the product of the concentration of disinfectant and the contact time, and is used to calculate the effective dose of disinfectant. The PAA concentration was measured, as described below, immediately after dosing and at the end of the 25 minute contact time to allow for calculation of the actual CT value achieved. Then, the reactors were quenched with catalase and 1 mL of 100 mg/L sodium thiosulfate (Fiorentino *et al.*, 2015; Formisano *et al.*, 2016) and 1 mL of freshly prepared bovine catalase (Wagner *et al.*, 2002) at a concentration of 100 mg/L to remove disinfectant residual. PAA concentration was measured again following the quench to confirm that no

disinfectant remained after quench. The other duplicate reactor for each experiment condition did not receive PAA treatment.

To study the fecal indicator concentrations in the MP and wood microparticle biofilms separately from the planktonic bacteria, the particles were collected on sterile 63 µm stainless steel mesh (TWP Inc., Berkley, CA), rinsed with PBS to remove any loosely attached microbes, and the filtrate reserved. The microparticles were placed into microcentrifuge tubes with 1.5 mL of phosphate buffered saline (PBS). The particles were then vortexed (Vortex-Geni 2, Scientific Industries, Inc., Bohemia, NY) on the maximum speed setting of 10 for two minutes to dislodge the biofilm. This setting was chosen based upon results of a preliminary experiment where MPs were incubated in wastewater, described below. Serial dilutions of the dislodged cells in the PBS supernatant (10^{-2}, 10^{-3}, 10^{-4}) were used for quantification of total coliform and *Escherichia coli* using EPA Method 1604 (EPA, 2002). These fecal indicators were chosen because they are of regulatory interest in drinking, surface, and wastewater. Samples of the reactor filtrate were diluted (10^{-2}, 10^{-3}, 10^{-4}) to quantify planktonic cells. All plates were incubated at 36°C for 24 hours and photographed for counting under visible light to quantify total coliform and UV light to quantify *E. coli*. ImageJ software was used to assist the counting of the plates.

A preliminary biofilm dislodging study was performed to choose an appropriate amount of time and intensity of vortexing required to remove the biofilm from the particles without reducing fecal indicator cell viability. Briefly, PE from a personal care product and PS particles generated as described above were incubated for 24 hours in grab samples of pre-disinfection secondary effluent collected from the same WWTP on March 3rd, 2019. Particles were collected on stainless steel mesh and transferred into microcentrifuge tubes with PBS, as described above. The microcentrifuge tubes were vortexed at either high (speed setting 10) or low speed (speed setting 7), for a time of either one or two minutes resulting in four experimental conditions for each particle type (i.e., high speed 1 min, low speed 1 min, high speed 2 min, low speed 2 min) (Arias-Andres *et al.*, 2018; Masangkay *et al.*, 2020). Fecal coliform were quantified as described above.

Chemical analyses

Aliquots of the wastewater samples were analyzed for basic water quality parameters including pH, chemical oxygen demand (COD), and conductivity. pH and conductivity were measured with a field meter (Orion Star A329, Thermo Scientific). COD was measured using Hach Method 8000 with Hach COD vials (20–1500 mg L−1 range) and a DR2700 spectrophotometer (Hach, Loveland, CO). PAA residual was measured using a commercial kit (Peracetic Acid Vacu-Vials, CHEMetrics, Midland, VA).

MP particles were analyzed via ATR-FTIR on a Bruker ALPHA. Spectra were collected in transmittance mode. Spectra were compared to the Bruker polymer library and siMPle (Primpke *et al.*, 2018) to confirm their polymer identity.

Statistical Analysis

The fecal indicator concentration data were analyzed using PERMANOVA, via the adonis2 function in the R package vegan, for the initial biofilm dislodging study to the compare impact of vortex speed and time and for the subsequent experiments to compare between particle type and PAA treatment for a given matrix (i.e., biofilm or filtrate). Log removals of fecal indicators were compared between matrix and material using PERMANOVA, as well. Results for total coliform and *E. coli* from each experiment (i.e., influent and pre-disinfection secondary effluent) were analyzed separately. The *E. coli* data were Box-Cox transformed because more than 20% of samples resulted in too few colonies to count. The coefficients of the transformation were obtained with the boxcoxfit function in R. Then the data were analyzed in the same manner as the total coliform data with the adonis2 function. After confirming normality of the PAA concentration data with a Shapiro test, these data were compared between reactors fed with wastewater influent with different particle types (i.e., LDPE vs. wood) with a Welch two sample t-test. The number of particles per reactor were compared across particle type with a Kruskal test followed by a pairwise t-test with a Holm correction for multiple comparisons.

Results

The substrates for biofilm growth were generated for the LDPE, HDPE, and PP MP or collected for the wood microparticles used as a material control in this study (Fig. 1). An equal volume of these microparticles was added to each reactor but because of their differences in morphology, and therefore packing, this resulted in a range of particle concentrations of each per reactor (LDPE: 132±13 particles/reactor, HDPE: 146±9 particles/reactor, PP: 326±33 particles/reactor, wood: 348±252 particles/reactor). There was a significant difference between the number of particles in the HDPE and PP reactors ($p=0.037$, pairwise.t.test) and no significant difference between particle types in other reactors (all $p > 0.05$, pairwise.t.test). Fecal indicators grown on these particles as biofilms are presented on a per-volume of particles basis and described on a per-particle basis to control for the differences in particles per reactor. The materials used to create the MPs were each labeled with their composition and confirmed by FTIR. Spectra are shown as Fig. S2 and matched the expected polymers in the siMPle library with 69.1% match for LDPE, 96.4% for HDPE, 98.6% for PP, and 85.4% match for wood (poplar was the greatest hit, although the wood used was from a Spanish Oak tree). These expected matches corresponded to the highest scores for HDPE and PP, second highest for LDPE (HDPE was the highest match), and third highest for the wood (other fibers had higher scores).

To compare the prevalence of fecal coliform and *E. coli* in microplastic biofilms and material controls, the biofilms needed to be dislodged from the particle surfaces after removal from the reactors. Options for vortex speed and duration were tested in the preliminary study. The resulting total coliform concentrations were analyzed via PERMANOVA, which indicated that there was not a significant difference in the number of viable dislodged CFUs between vortexing at high or low speed (10 or 7, respectively, $p=0.16$) or between vortexing for one or two minutes ($p=0.65$, Fig. S3). Going forward, a vortexing time of two minutes at high speed was chosen to dislodge the biofilms.

Fecal indicator growth on microplastics

193 Batch reactors with either wastewater influent or pre-disinfection secondary effluent were used to grow
194 biofilm on up to four different types of 500-2000 µm particles. For reactors with wastewater influent,
195 both fecal coliform and *E. coli* were quantifiable in biofilms grown on LDPE and wood as well as in the
196 reactor filtrate (Fig. 2, 3). The biofilm total coliform concentrations grown in wastewater influent were
197 significantly higher for wood than the LDPE particles on a CFU per mL of particles basis (p=0.002,
198 PERMANOVA, Fig. 2a) and on a CFU per particle basis (p=0.014, PERMANOVA, Fig. 2c). However,
199 there was no significant difference in filtrate total coliform concentration between the reactors incubated
200 with the different particle types (p=0.071, PERMANOVA, Fig. 2b). Similar observations were made for
201 *E. coli:* wood biofilm had higher concentrations compared to LDPE on a CFU per mL of particles basis
202 (p=0.005, Fig. 3a) and on a CFU per particle basis (p = 0.019, Fig. 3c) and no difference in concentrations
203 between the reactors incubated with the different particle types for filtrate (p=0.821, Fig. 3b). The
204 wastewater influent chemical quality parameters across the three reactors were consistent for COD
205 (459±96 mg/L), TSS (290±86 mg/L), pH (7.3±0.02), and conductivity (865±48 µS/cm) (Table S1).

206 For reactors with pre-disinfection secondary effluent, both fecal indicators were quantifiable in the reactor
207 filtrate and biofilms grown on the wood chips, but too few to count (not countable, NC) for all
208 microplastic biofilm in that matrix (i.e., HDPE, LDPE, and PP). The limit of detection was 1 CFU/mL for
209 the filtrate and 4.8 CFU/mL of particles for the biofilm. While not quantifiable, fecal indicators were
210 observed in the HDPE, LDPE, and PP biofilm and filtrate. When quantifiable, wood microparticles in the
211 pre-disinfection secondary effluent had biofilm total coliform concentrations of 21000±13000 CFU/mL of
212 particle and 6200±1700 CFU/100 mL in the filtrate. The secondary effluent across the three sampling
213 dates was consistent in chemical composition, with COD (25±4 mg/l), TSS (5±1 mg/l) and pH (7.1±.05)
214 and conductivity (852±25 µS/cm).

215 ***PAA disinfection***

To determine the susceptibility to PAA disinfection of fecal indicator organisms in particle biofilms compared to the planktonic fecal indicator organisms, half of the reactors were treated with a PAA dose of 2 mg/L. For the reactors with wastewater influent, the PAA concentration measured immediately after dosing was 1.80±0.05 mg/L and 1.70±0.02 mg/L for the LDPE and wood reactors, respectively. After 25 min of exposure the final PAA concentrations for the LDPE reactors was 1.33±0.05 mg/L and for the wood reactors was 1.25±0.03 mg/L. Thus, the CT for the LDPE reactors was 33.1±1.3mg/(L·min) and the wood chip reactors was 31.2±0.8 mg/(L·min) (Table S2). None of these values significantly differed by particle type (all $p>0.05$, Welch two sample t-test). After quenching the reactors, the residual PAA concentration was below detection in all reactors. Following quenching, biofilm and filtrate samples were collected for cultivation of fecal indicators.

For the reactors with wastewater influent, there was no significant difference in biofilm total coliform concentrations between PAA treated and untreated reactors ($p=0.34$, all by PERMANOVA) or as a function of treatment and material (i.e., LDPE vs. wood, $p=0.17$). In contrast, PAA treatment resulted in a significant decrease in total coliform concentrations observed in the filtrate of treated reactors compared to nontreated reactors ($p=0.018$). Next, the log-removals of TC were compared (Fig. 2d). There was no significant difference in log-removal by material ($p=0.19$), matrix ($p=0.58$), nor was interaction between these variables observed ($p=0.54$). The PAA treatment did not significantly reduce the *E. coli* concentration for the biofilm or filtrate in untreated compared to treated reactors (both $p>0.14$, Fig. 3d). Likewise, there was also no significant difference in log-removal of *E. coli* when comparing the plastic and wood reactors ($p=0.31$) or when comparing biofilm and filtrate ($p=0.88$).

For the reactors with pre-disinfection secondary effluent, total coliform and *E. coli* were not quantifiable (NC) on all but10 plates out of 24. There was an average log inactivation of 0.5 for the wood biofilm, and no quantifiable results for the treated wood filtrate. The untreated wood filtrate had an average concentration of 6200±1700 CFU/100 mL. Based on the detection limit of 1 CFU/mL for filtrate, log inactivation of the wood filtrate can be estimated at 3.8.

Discussion

Biofilm growth

Fecal indicator organisms were observed in the biofilms of all of the microparticles incubated in wastewater influent but only with countable concentrations for wood microparticles incubated in pre-disinfection secondary effluent. Thus, PE was not a more attractive substrate for fecal coliform and *E. coli* than the natural substrate when incubated in wastewater influent. In fact, the highest concentrations of total coliform and *E. coli* were observed in reactors containing wood particles. Correcting for the differences in particle concentration did not change this observation, which is expected due to the fact that particle counts were not significantly different between MP and wood reactors. The higher *E. coli* and total coliform concentrations in wood biofilm and filtrate is not surprising, as wood has several properties that make it a good substrate for biofilm compared to MP. LDPE is resistant to being metabolized by microorganisms due to its long chain structure (Hadad *et al.*, 2005; Mehmood *et al.*, 2016) whereas wood contains carbohydrates that can serve as a nutrient source bioavailable to microorganisms (Broda and Popescu, 2019; Sailer *et al.*, 2010). In addition, the wood chips have a rough and complex microstructure as well as increased wettability over LDPE, which could enhance biofilm attachment (Farber *et al.*, 2019; Hou *et al.*, 2011). Comparisons for biofilm formation by polymer type were explored only with the pre-disinfection secondary effluent which did not show significant growth, therefore reporting conclusions about the biofilm formation (and disinfection) by different MP types is not possible here. Previous studies have demonstrated that biofilm microbial communities are affected by substrate morphology, size class, and material (Miao et al., 2019; Parrish and Fahrenfeld, 2019; Quilliam et al., 2014).

The findings of this study are in agreement with another recent study of biofilm formation in freshwater that reported *E. coli* is more likely to colonize wood than PE particles (Song *et al.*, 2020). Other recent studies that have demonstrated that fecal indicator organisms prefer natural substrates such as stone, wood, and seaweed to MP (Miao *et al.*, 2019; Quilliam *et al.*, 2014). Fecal indicators are used as

surrogates for monitoring for pathogens and there is a growing body of research to understand the possibility of MPs harboring pathogenic organisms and therefore serving as vectors for harmful microbes to be transported far from their sources (Silva *et al.*, 2019). Potentially pathogenic microbial species such as *Vibrio* and *Pseudomonas* were identified in biofilms colonizing microplastic particles found in marine waters and freshwater, respectively (Kirstein *et al.*, 2016; McCormick *et al.*, 2014; Parrish and Fahrenfeld, 2019). It is worth noting that some of the studies reporting potential pathogens in microplastic biofilm relied upon amplicon sequencing techniques, which may not be able to accurately identify microbes at the species level nor does it capture information about viability.

Comparing the two wastewater fractions, as expected, particles incubated in wastewater influent grew more fecal indicators in the biofilm: fecal indicators were only quantifiable on the wood microparticles incubated in pre-disinfection secondary wastewater effluent. Coliform and *E. coli* were also observed in biofilms dislodged from the LDPE films, but below quantification. Neither indicator organism was quantifiable on the PP and HDPE fragments. This observation may be due to the low amount of available nutrients in the secondary effluent. Note, the COD in secondary effluent was 5% of that in the influent, indicating less carbon which the wood microparticles could potentially provide. Or, again, the difference may be due to the differences in surface texture between the wood and MP particles.

It is important to note that the MPs created for this study by cutting and grinding virgin plastic may not have the same surface texture of environmental secondary microplastic. In the environment, PE and other polymers degrade primarily via UV radiation, with heat and water both accelerating the breakdown of the molecular structure (Briassoulis *et al.*, 2004; Gewert *et al.*, 2015; Gulmine *et al.*, 2003). UV radiation from the sun initiates chain scission and brittle failure, which causes microplastic particles to slough off of larger plastic debris (Cai *et al.*, 2018; Lambert and Wagner, 2016). Microplastics that have been degraded in the laboratory with UV irradiation have been reported to have increased surface roughness (Cai *et al.*, 2018; Gulmine *et al.*, 2003; Hiejima *et al.*, 2018). Therefore, future studies may seek to use

LDPE microplastics that have been artificially weathered in a chamber similar to Brandon et al. (Brandon *et al.*, 2016) to serve as an improved representation of environmental microplastics.

Disinfection

Results of the disinfection study demonstrate that biofilm microbes were more resistant to disinfection than planktonic microbes, but that fecal indicators in MP biofilm did not have different log- inactivation compared to wood microparticle biofilms. The first observation was expected: biofilms are generally considered to be more difficult to disinfect than planktonic organisms (Bridier *et al.*, 2011; Kim *et al.*, 2008; Lee *et al.*, 2020), and that is due to a few important factors. First, a disinfectant will act on the surface of a biofilm and may not penetrate enough to reach microbes living closer to the substrate thus allowing them to escape disinfection (Bridier *et al.*, 2011). Second, the extracellular matrix (ECM) can protect biofilm organisms from direct action of disinfectant on their cell membranes (Fux *et al.*, 2005). Disinfectant consumed oxidizing the ECM will not be available to oxidize the cell membranes of the target microbes. This lowers the effectiveness of a given concentration of disinfectant (Stewart, 2015). The levels of PAA inactivation observed for fecal indicators in the pre-disinfection secondary effluent were similar to the results from particles incubated in the wastewater influent. Following PAA disinfection, fecal indicators could not be quantified for the wood filtrate, suggesting again a greater susceptibility of planktonic cells to disinfection.

The fact that MP and wood biofilm were equally resistant to disinfection (i.e., had similar log-inactivations) indicates that understanding the relative concentration of MP compared to other buoyant microparticles in WW effluent would help indicate which particle type is contributing most to the bypassing of disinfection by biofilm fecal indicator organisms. This is significant because it highlights the importance of optimizing wastewater treatment processes for the removal of neutrally buoyant particles such as MP and/or removing biofilms during disinfection. While WWTP's are not thought to be the only source of MP in the freshwater environment (Fahrenfeld *et al.*, 2019), they are not 100 percent effective at

313 removing MP, allowing a path for pathogenic organisms from wastewater to bypass disinfection
314 processes. Any differences in the fate of these different buoyant microparticles following release in
315 effluent will have an impact on the ultimate hazard or lack thereof with respect to their fecal indicator
316 loads.

317 **Conclusion**

318 Microplastic biofilms did not prove to be more resistant to disinfection than natural substrate (i.e., wood
319 chips). However, biofilms dislodged from wood microparticles grew the most fecal coliform and *E. coli*
320 of the substrates studied, likely due to surface texture and availability of nutrients. The biofilms were
321 more resistant to disinfection than planktonic bacteria, as expected. Given that the MP biofilms behaved
322 similarly to other microparticles with regard to disinfection, one may rely on the literature for disinfection
323 of biofilm fecal indicators on other particles when predicting MP behavior. While it has been suggested
324 that MP is potentially more difficult to remove than naturally occurring particles due to the neutral
325 buoyancy of MP in contrast to the positive buoyancy of wood and negative buoyancy of sediment
326 particles. (Ngo *et al.*, 2019), WWTPs have been found to be 70-100% effective at removing MP from
327 wastewater.(Conley *et al.*, 2019; Sun *et al.*, 2019). Nonetheless, wastewater treatment processes that in
328 general remove particulates that carry harder to disinfect biofilms will reduce the loading of fecal
329 microbes to effluent receiving water bodies.

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The data that support the findings of this study are available from the corresponding author upon reasonable request

Figure Captions

Fig. 1 Images of the 500-2000µm particles used in the reactors. (A) LDPE, (B) HDPE, (C) PP, (D) wood.

Fig. 2 a. Log total coliform (TC) CFU for the dilodged biofilm (TC/mL of particles) and b. filtrate (TC/100 mL filtrate) grown in wastewater influent. c. Log total coliform (TC) CFU for the dilodged biofilm on a per-particle basis (TC/100 particles). Results are shown for reactors with microplastic (LDPE) or control microparticles (wood chips) with peracetic acid (PAA) disinfection and without (no).d. Log removal of TC for both matrices (biofilm and filtrate) and particle types (LDPE or wood). N=3. In the box and whisker plots, the boxes represent the 25%, median, and 75% of data and the dots represent outliers.

Fig. 3 a. Log *E. coli* CFU for the dilodged biofilm (EC/mL of particles) and b. filtrate (EC/100 mL filtrate) grown in wastewater influent. c. Log *E. coli* CFU for the dilodged biofilm on a per-particle basis (EC/100 particles). Results are shown for reactors with microplastic (LDPE) or control microparticles (wood chips) with peracetic acid (PAA) disinfection and without (no).d. Log removal of *E. coli* for both matrices (biofilm and filtrate) and particle types (LDPE or wood). N=3. In the box and whisker plots, the boxes represent the 25%, median, and 75% of data and the dots represent outliers.

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