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Microplastics as hubs enriching antibiotic-resistant bacteria and pathogens in municipal activated sludge

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ABSTRACT

Microplastics can serve as carriers of antibiotic-resistant bacteria (ARB) and pathogens, representing a pressing concern to aquatic biota and human health. Activated sludge units at municipal wastewater treatment plants (WWTPs) are "hotspots" converging microplastics and antibiotics. In this batch study with activated sludge samples from three domestic WWTPs, we demonstrated both polyethylene (PE) and polystyrene (PS) microplastics can acclimate biofilms enriched with sulfonamide resistance genes (*sul1* and *sul2*) and the associated mobile genetic element (*intI1*) in comparison with fine sands as control particles. Absolute abundances of these genes were further elevated by 1.2∼4.5 fold when sulfamethoxazole was initially spiked as a representative sulfonamide. The combination of 16S rRNA amplicon sequencing and differential ranking analysis revealed that microplastics selectively promoted antibiotic-resistant and pathogenic taxa (e.g., *Raoultella ornithinolytica* and *Stenotrophomonas maltophilia*) with enrichment indices ranging from 1.6 to 3.3. Furthermore, heterotrophic *Novosphingobium* and filamentous *Flectobacillus* accounted for 14.6 % and 3.3 % on average in microplastic biofilms, respectively, which were up to 2.8 and 11.1 times higher than those in sand biofilms. Dominance of these bacterial species may contribute to initial biofilm formation that facilitates subsequent colonization and proliferation of ARB and pathogens, thus amplifying their risks in the receiving environments and beyond.

1. Introduction

Microplastics (<5 mm) in wastewater treatment plants (WWTPs) have received increasing attention, given their imminent threats to aquatic ecosystems and public health (Browne et al., 2011; Murphy et al., 2016; Sun et al., 2019). Microplastic particles in personal care and cosmetic products, such as toothpaste and facial wash, are washed down the drain, converging at WWTPs as contamination "hotspots" (Cheung and Fok, 2017; Fendall and Sewell, 2009). It is estimated that a median-sized WWTP with an average treatment capacity of 5×10^7 m^3/year can discharge up to 2×10^6 microplastic particles per day (Sun et al., 2019). These microplastics perpetually enter inland rivers, estuaries, and other receiving waters that eventually drain to oceans (Murphy et al., 2016; Gillings et al., 2015).

As a unique microhabitat, microplastics can promote the formation of biofilm, a slimy buildup of interactive and resistant microorganisms on the surface (Zettler et al., 2013; Bowley et al., 2020; Yang et al., 2020). Molecular analysis of microplastic biofilms discloses a broad spectrum of antibiotic resistance genes (ARGs), conferring sulfonamide (SA), beta-lactam, aminoglycoside, and other antimicrobial resistance (Wu et al., 2019a; Yang et al., 2019; Zhao et al., 2020). Furthermore, plastisphere promotes horizontal gene transfer (HGT) (Arias-Andres et al., 2019), raising the chance of transmitting ARGs to (opportunistic) pathogenic bacteria (e.g., *Pseudomonas* (Wu et al., 2019a) and *Vibrio* (Bowley et al., 2020) species) that co-reside on microplastics. Arias-Andres et al. reported that the HGT frequency of antibiotic resistance plasmids in the plastisphere was three orders of magnitude higher than that in the planktonic communities (Arias-Andres et al., 2018). Another study indicated a significant correlation between concentrations of microplastics and the class 1 integron-integrase (*intI1*) gene, known as a mobile genetic element (MGE) for its ability to mobilize ARGs (Eckert et al., 2018).

Most of the prior studies have been centered on plastisphere in the freshwater and ocean (Yang et al., 2020; Li et al., 2018a; Oberbeckmann and Labrenz, 2020). However, sparse is known regarding the formation and composition of biofilms attached to microplastics over the municipal activated sludge treatment process, in which antibiotics, antibiotic-resistant bacteria (ARB), and human pathogens frequently commingle (Ju et al., 2019; Li et al., 2015; Pruden et al., 2018). Several recent studies have focused on the impacts of microplastics on community structure and performance (e.g., nitrification) of activated sludges (Zhang and Chen, 2019; Wang et al., 2020a). In this study, we set up microcosms using three different municipal activated sludges and investigated the acclimated plastisphere regarding the shifting of ARG abundances and microbial

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compositions in response to the exposure to spherical polyethylene (PE) and polystyrene (PS) microplastics. These two types of microplastics were selected considering their wide use in commercial products and prevalent detection in municipal wastewaters (Sun et al., 2019). For comparison, control treatments were prepared with fine sands as natural suspended particles abundant in the activated sludge tanks at WWTPs. Using the combination of quantitative PCR (qPCR), amplicon-based next-generation sequencing, and state-of-the-art bioinformatics approaches, enrichment of ARGs and microorganisms associated with microplastics were unraveled to discern their putative roles in the plastisphere with or without the co-existence of sulfamethoxazole (SMX), a model antibiotic for SAs. We focused on tracking the dynamics of genes (e.g., *sul1*, *sul2*, and *intI1*) and bacteria associated with SA resistance, because (1) SAs and SA resistance are frequently detected at high concentrations (10^4∼10^{11} copies of *sul* genes/gram dry sludge) (Li et al., 2015; Zhang et al., 2016) at WWTPs worldwide; (2) *intI1* is considered as a proxy of multiple ARGs (Gillings et al., 2015) conferring resistance to aminoglycoside (*aadA and aacA*), beta-lactam (*blaCTX* and *blaIMP*), and trimethoprim (*dfrA*) (Cantón and Coque, 2006; Li et al., 2012; Toleman et al., 2006), for instance, in addition to SAs (*sul1*), and plays a central role in ARG dissemination in municipal wastewaters (Ju et al., 2019); and (3) similar to many other micropollutants in WWTPs, SAs exhibit high adsorption potentials to microplastics (Xu et al., 2018; Guo et al., 2019), promoting the selective pressure to SA resistance in the plastisphere. This timely study provides an inaugural understanding of microplastics as hubs enriching ARB and pathogenic microorganisms during the conventional activated sludge treatment, posing substantial exposure risks if bypassing WWTPs.

2. Material and methods

2.1. Microparticle preparation

PE and PS spherical microplastics with diameters of 85∼106 μm (abbreviated as PE and PS, respectively) were purchased from Cospheric (Santa Barbara, CA). Sand (Sakrete Inc., OH) sieved with a stainless-steel mesh of a comparable size range (88∼105 μm) was used as a representative of natural materials. Sieved sand particles were washed three times with 30 % hydrogen peroxide to remove organic residues, neutralized with deionized water, and dried at 50 °C in an oven overnight. Prior to microcosm preparation, sand and PS were autoclaved at 120 °C for 20 min. Given its low melting point, PE was washed with sterile deionized water without autoclave. Sand and microplastic particles were visually examined using a white-field microscope to preclude microbial contamination. Hydrophobicity, specific surface area, and other parameters of microparticles were characterized with details in the Supplementary Data (SD).

2.2. Microcosm assays

Activated sludge samples were collected from aeration tanks of three WWTPs (designated as Sludge P, R, and L) located in northern New Jersey in June and October 2019. These WWTPs served resident populations ranging from 6.0×10^4 to 1.4×10^6, as well as a diversity of domestic industries. Thus, activated sludge samples from these three WWTPs were selected as seeding inocula for biological parallels to better represent sludge communities with varying constitutes and concentrations of ARB and pathogens. Sludge samples were washed three times with 40 mL of phosphate-buffered saline (20 mM sodium phosphate, pH 7.0). Flocs in sludge samples were eliminated by homogenization at 20,000 rpm for 10 min using VWR 200 homogenizer (Radnor, PA) and filtration through a 10-μm Whatman membrane. Filtered sludges were mixed with 360 mL of synthetic wastewater (Gao et al., 2018) and incubated at 30 °C while being shaken at 130 rpm for 24 h. Sludges were further diluted with synthetic wastewater to reach an OD_{600} of 0.1 prior to the microcosm preparation. Synthetic wastewater was used as the constituents (e.g., carbon sources, nutrients, and presence of micropollutants) and characteristics (e.g., pH and turbidity) varied greatly in wastewater samples collected from three WWTPs.

For each sludge sample, three treatments were prepared in 25-mL glass bottles containing 6 mg of one of the three microparticles (i.e., PE, PS, or sand) and 5 mL of the sludge culture. In addition, parallel treatments were spiked with SMX at an initial concentration of 100 μg/L, representing the relatively high contamination of SMX and other SAs detected in municipal and pharmaceutical wastewater (0.015∼1340 μg/L) (Deng et al., 2018). All treatments were conducted in triplicate. After incubation at 30 °C for 3 days (a typical solid retention time at WWTPs) (Riffat, 2012; Achermann et al., 2018), biofilm-attached microparticles were separated and harvested from the supernatant using density gradient centrifugation (Price, 2014) and stored at −20 °C before the DNA extraction. The extracted DNA of collected biofilms was further used for (1) qPCR for the enumeration of target ARGs and 16S rRNA genes for total biomass estimation and (2) 16S rRNA amplicon-based sequencing. Details regarding microparticle collection, DNA extraction, qPCR, and 16S rRNA sequencing are provided in the SD.

2.3. Microbial community analysis

16S rRNA sequence reads were processed using the QIIME2 pipeline (v2020.2.0) (Bolyen et al., 2019) with the Divisive Amplicon Denoising Algorithm 2 (DADA2) (Callahan et al., 2016) for sequence pairing, denoising, and chimera elimination. For the taxonomy assignment, operational taxonomic units (OTUs) were generated at 97 % of nucleotide sequence similarity and searched against the GenBank database using the NCBI BLASTN for top hits with the lowest e values (Sayers et al., 2020).

Differential ranking analysis was performed to identify taxa favored by microplastics (i.e., PE or PS) versus sand using Songbird (v1.0.3) (Morton et al., 2019). Feature ranking and log-fold change were subsequently visualized using Qurro (v0.7.3) (Fedarko et al., 2020). High-rank taxa with log-fold change greater than 1 were recognized as "microplastic-associated" since they are important contributors with significant increases in microplastic biofilms relative to the sand control. Ranking taxa on the basis of their log-fold changes mitigates compositional artifacts caused by the variance in total microbial loads among samples as the bias is uniformly distributed across the differential (Morton et al., 2019). Significant correlation between these high-rank taxa and their abundances in PE and PS biofilms was verified using two-way Student's *t*-test and Mann–Whitney *U* test based on Qurro-produced log-ratios, as well as the permuted *t*-test over 999 random permutations. Enrichment indices of microplastic-associated OTUs in PE and PS biofilms were estimated by normalization to the relative abundance of *Herbaspirillum Huttiense* as the reference taxon, given its even and ubiquitous distribution across all samples (Morton et al., 2019). Microbial ecology analyses are detailed in the SD.

3. Results and discussion

3.1. Microplastics enriched SA resistance genes and the associated MGE

After exposure to PE or PS, SA resistance genes (*sul1* and *sul2*) and the associated MGE (*intI1*) in microplastic-attached biofilms showed significantly greater ($p < 0.05$) or comparable abundances as compared with those detected in sand biofilms (Fig. 1a–c). For all sludge samples, exposure to PE resulted in significant increases of almost all three target genes (except *sul2* in Sludge P), implying the enrichment of SA-resistant bacteria on PE regardless of the sludge source. For instance, all three target genes were significantly enriched in PE biofilms cultured in Sludge R. Compared to the sand control, the abundance of *sul1, sul2,* and *intI1* genes increased by 1.7-, 30.1-, and 13.1-fold, respectively, in PE biofilms. In comparison with PE, PS had fewer target genes shown with significant

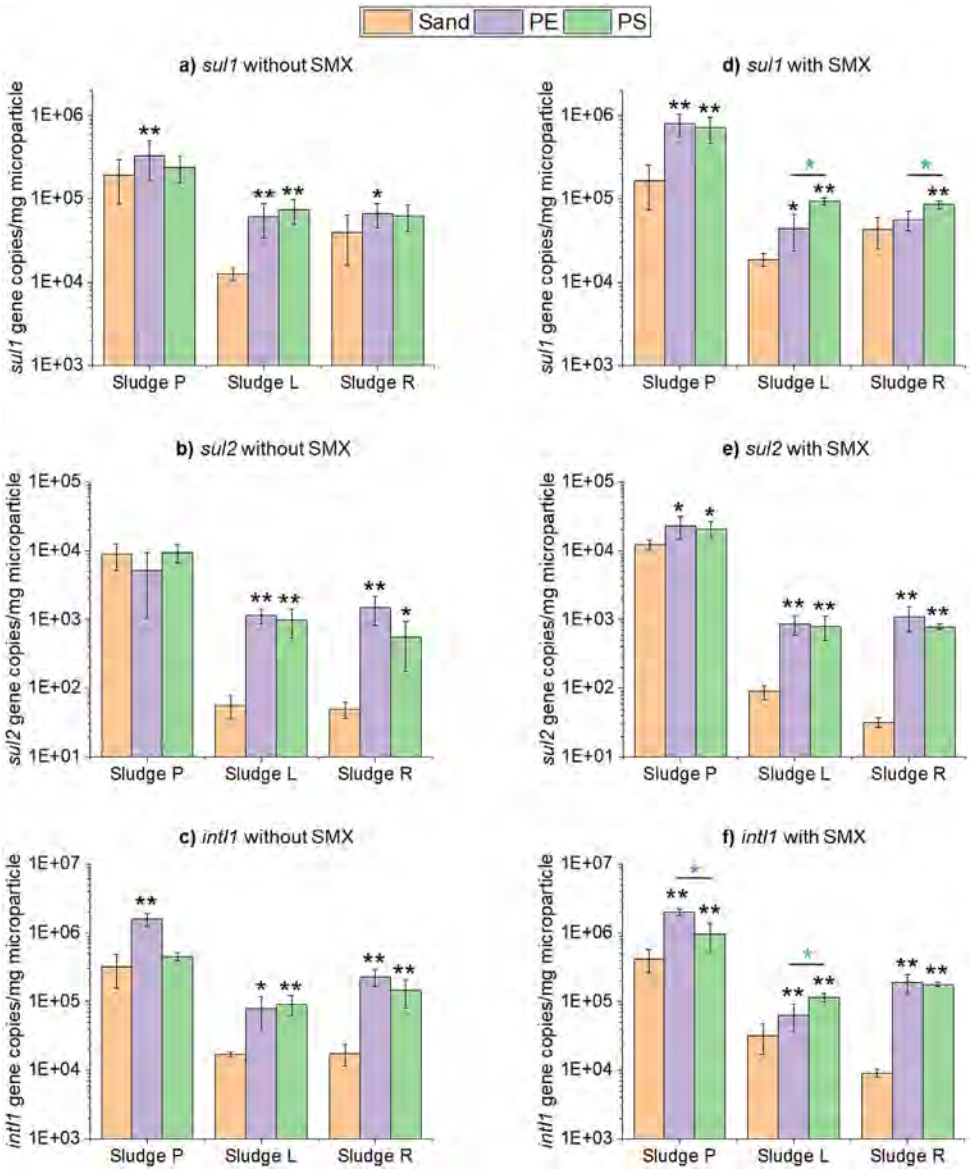

Fig. 1. Absolute abundance of *sul1*, *sul2*, and *intI1* in microparticle biofilms cultivated in different activated sludge samples with or without the presence of SMX (100 μg/L). Significant differences between gene abundances among microplastic and sand biofilms were indicated with asterisks in black, purple, and green when compared to sand, PE, and PS (*, $p < 0.05$; **, $p < 0.01$) based on the two-way Student's *t*-test.

increase relevant to the sand control. None of these three target genes were significantly enriched in PS biofilms cultured in Sludge P. In contrast, all three target genes were significantly enriched in PS biofilms cultured in Sludge L. Compared to the sand control, the abundance of *sul1, sul2,* and *intI1* genes increased by 5.8-, 17.2-, and 5.4-fold, respectively. Though with variance, PE is likely more potent in enriching these genes relevant to SA resistance than PS.

When SMX co-existed with microparticles, almost all *sul1, sul2,* and *intI1* genes in PE and PS biofilms were significantly enriched for all three sludge samples compared to the sand control (Fig. 1d–f). Interestingly, with the presence of SMX, PS appeared to be more proficient in enriching these genes relevant to SA resistance than PE in general. For all three sludge samples, significantly higher abundances of *sul1, sul2,* and *intI1* genes (1.7∼24.6 fold) were detected in PS biofilms than the sand biofilms. In Sludge P, the SMX addition also increased all three target gene abundances by 2.1–4.8 times in PS biofilms as compared to those without SMX (Fig. 1). Further, *sul1* in Sludge R and L and *intI1* in Sludge L were significantly higher ($p < 0.05$) in PS biofilms than those in PE biofilms. These observations were probably due to the higher adsorption of SMX by PS (0.35 μg/mg PS vs 0.17 μg/mg PE, Fig. S1), eliciting greater selective pressure of SA resistance in the plastisphere (Sun et al., 2018). Our findings echoed previous reports demonstrating microplastics as a potential vector of a greater diversity of ARGs, encompassing those three genes that were monitored in this study (Wu et al., 2019a; Parrish and Fahrenfeld, 2019; Wang et al., 2020b). In different ecosystems embracing river, estuary and marine waters, *sul* genes were often detected at high abundances in microplastic biofilms, ranging from 2 times to several orders of magnitude higher than other frequently detected ARGs, such as *tetA, ermE,* and *qnrS* (Wang et al., 2020b; Lu et al., 2019; Wang et al., 2020c).

The qPCR results also revealed that the abundances of *sul1* and *intI1* were one to two orders of magnitude greater than *sul2* (Fig. 1). A positive correlation was further evident between *sul1* and *intI1* genes (Spearman's $r = 0.92, p = 4.57 \times 10^{-8}$), suggesting their co-occurrence in our samples (Fig. S2). Such correlation was also reported in several previous studies (Zhang et al., 2016; Le et al., 2016), supporting the frequent localization of *sul1* immediately downstream of *intI1* (Gillings et al., 2015). Though not directly detected, many other ARGs (e.g., *aadA, blaCTX*, and *dfrA*)

(Cantón and Coque, 2006; Toleman et al., 2006) are likely carried by the highly abundant *intI1* in PE and PS biofilms. The enrichment of *intI1* genes also suggests a greater HGT rate in microplastic biofilms relative to sand biofilms.

3.2. Microplastics selected for specific taxa conferring antibiotic resistance and pathogenicity

Differential ranking revealed 8 taxa (or OTUs) significantly enriched on microplastics with their relative abundances 1.2∼11.1 times (enrichment indices) greater in PE or PS biofilms than sand biofilms (Figs. 2 and 3). These taxa were representatives of high-rank microorganisms in PE (Set 1 in Fig. 2a) and PS (Set 3 in Fig. 2b) with log-ratios that were not only above 1, but also significantly greater than those in sand biofilms (*t*-test, $p < 0.01$; Mann–Whitney *U* test, $p < 0.05$) (Fig. 2c and d). Permutation t-tests based on the natural log-ratios of these microbial sets in PE and PS vs sand biofilms resulted in t-values of 45.38 and 48.89 ($p = 0.02$), respectively. These results validated that the selection of these 8 taxa was not random, and their enrichment was tightly associated with PE and PS. Notably, two emerging human pathogens, *Raoultella ornithinolytica* and *Stenotrophomonas maltophilia*, were identified with PE/sand enrichment indices of 3.3 and 2.9 and PS/sand enrichment indices of 3.2 and 1.6, respectively (Fig. 3). Undesirable exposure to these two species may cause adverse health effects spanning from respiratory infection to bacteremia (Seng et al., 2016; Brooke, 2012). Several *R. ornithinolytica* isolates (e.g., Ro24724) from infected patients conferred resistance to SMX and other antimicrobial drugs. Whole-genome sequencing of Ro24724 revealed the existence of *sul1*, *sul2*, and *intI1* genes on its megaplasmid pRo24724 (NZ_CP021328) (Seng et al., 2016; Zheng et al., 2015). Interestingly, the Spearman analysis revealed positive correlations between the absolute abundance of *R. ornithinolytica* and *sul2*/*intI1* genes (FDR corrected $p < 0.05$, Spearman's r = 0.91 and 0.84, respectively) in L and R samples, supporting their roles in conferring SA resistance in plastisphere. In addition, *S. maltophilia* often carries *sul1* and *sul2* genes on the class 1 integron and a plasmid, respectively, contributing to its emergence as a global health issue (Brooke, 2012).

Some other microplastics-associated taxa (e.g., *Acinetobacter parvus* and *Sphingobacterium multivorum*) may confer resistance to an extended spectrum of antibiotics beyond SAs (Fig. 3). *A. parvus* species exhibited resistance to carbapenems and colistin (the drug of last resort against carbapenem-resistant Gram-negative bacteria) (Choi et al., 2012). *S. multivorum* can produce metallo-β-lactamase and serine β-lactamases, inactivating carbapenems and cephalosporins, respectively, via hydrolysis (Blahová et al., 1997). In addition to their selected existence in plastisphere revealed in this study, these bacteria are ubiquitous in the environment and can cause bacteremia and healthcare-associated infections, particularly in immunocompromised patients (Barahona and Slim, 2015; Munoz-Price and Weinstein, 2008).

3.3. Microplastics promoted biofilm formation

Certain microplastic-associated taxa are also key players responsible for the development of biofilms. Particularly, *Novosphingobium pokkalii* accounted for nearly 16 % in PE and 14 % in PS biofilms, which were ∼3 times greater than sand biofilms (Fig. 3). Members of the *Novosphingobium* genus are reputed for their ability to degrade a wide range of aromatic compounds (e.g., polychlorophenol and hexachlorocyclohexane) (Krishnan et al., 2017) and even plastic polymers (e.g., polyvinyl alcohol [PVA]) (Pathak, 2017). A recent study attributed

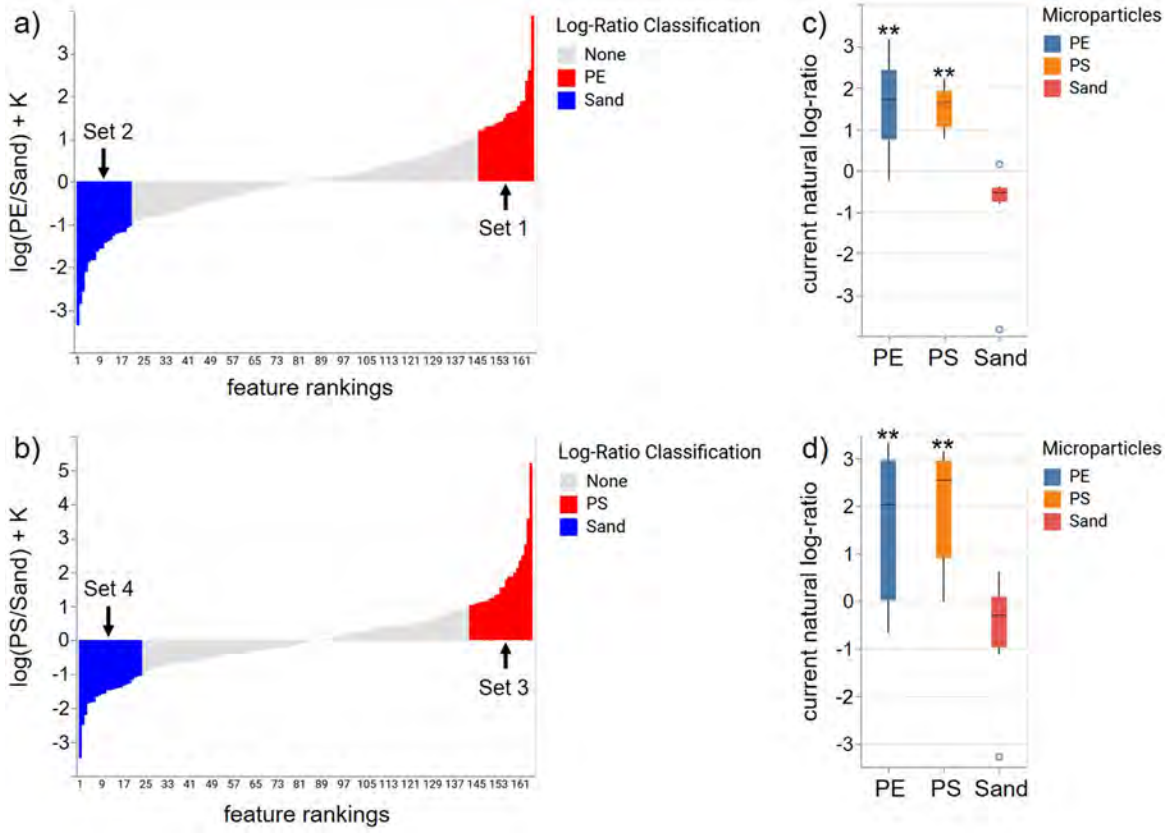

Fig. 2. Differential ranking of taxa associated with microparticle components: a) PE vs sand and b) PS vs sand. The y-axis represents the log-fold change that is known up to some bias constant K, and the x-axis numerically orders the rank of each taxon in the analysis. c-d) Qurro-produced log ratios were significantly different among PE, PS, and sand biofilms based on selected taxon sets determined by the differential ranking analysis. Significant differences between microplastic and sand samples were tested using two-way Student's *t*-test (**, $p < 0.01$).

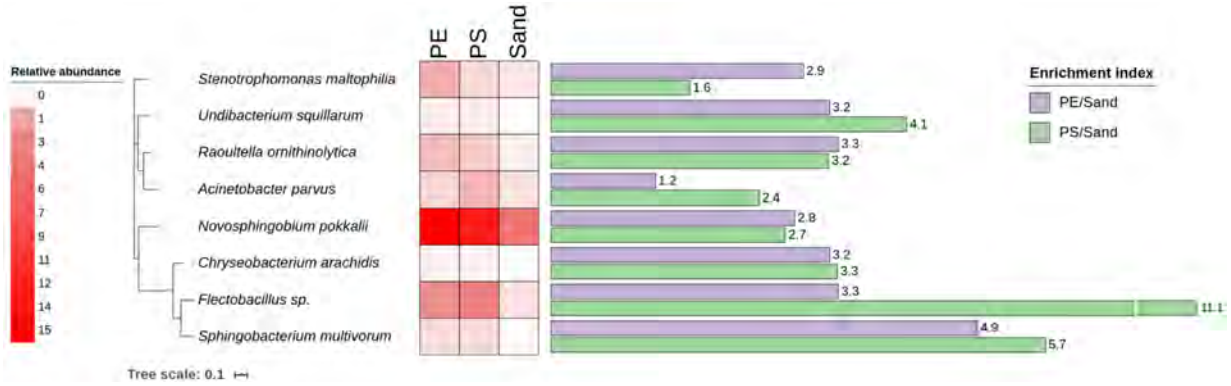

Fig. 3. Phylogenies and enrichment indices of microplastic-associated taxa. The phylogenetic tree of microplastic-associated OTUs was constructed using a maximum likelihood algorithm with 500 bootstrap replicates. The heatmap demonstrates relative abundances of selected OTUs in PE, PS, and sand. The bar chart depicts the enrichment indices of microplastic-associated OTUs in PE and PS biofilms to those in sand biofilms with *Herbaspirillum Huttiense* as the reference taxon.

Novosphingobium to the observed biodeterioration of amorphous regions on PE as evident by the increase in the microplastic crystallinity (McGivney et al., 2020). Thus, it is plausible to postulate *N. pokkalii* as a primary colonizer on microplastics in the activated sludge treatment process (Foulon et al., 2016). In addition, *Flectobacillus* sp. was also significantly enriched in microplastic biofilms, up to an 11-fold increase as compared to the sand control (Fig. 3). As filamentous bacteria (Justice et al., 2008), *Flectobacillus* can stretch over 10 μm in length and secrete acidic polysaccharide and other adhesive substances that build bridges between non-attached surfaces (Jahnke et al., 2016), thus promoting aggregation of suspended particles, such as the microplastics in our systems. Further, the Spearman analysis revealed positive correlations between the absolute abundance of these two dominant species (*N. pokkalli* and *Flectocillus* sp.) and the total biofilm biomass enumerated as the 16S rRNA gene abundance (FDR corrected $p < 0.05$, Spearman's r = 0.70 and 0.54, respectively) (Fig. S4), corroborating their putative roles in biofilm formation.

Collectively, the enrichment of *N. pokkalii, Flectobacillus* sp., and other bacteria that promote biofilm formation on microplastics is probably conducive to the higher total biomass as evident by both microscopic imaging (Fig. S5) and 16S rRNA gene enumeration (Fig. 4) that revealed an approximately 4.5-fold increase in microplastic biofilms as compared to those on sand. Plastisphere with high cell density can serve as a protected breeding ground facilitating the proliferation of ARB and dissemination of ARGs via HGT in long term once microplastics enter the environment. PICRUSt2 results also revealed the enrichment of genes responsible for extracellular substance secretion in microplastic biofilms.

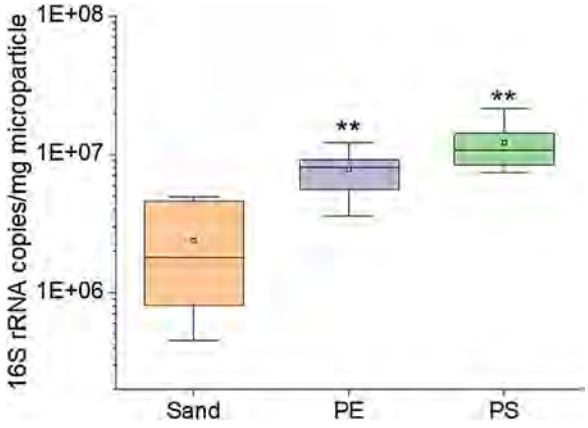

Fig. 4. Absolute abundance of 16S rRNA gene copies in biofilms acclimated on three types of microparticles. Significant differences between microplastic and sand samples were tested using two-way Mann–Whitney *U*-test (**, $p < 0.01$).

For instance, the *wza* gene encodes the outer membrane protein in charge of exporting and depositing capsular polysaccharides to the cell surface (Cuthbertson et al., 2009). Relative abundance of this *wza* gene was significantly greater in microplastic biofilms than sand biofilms (Welch's *t*-test, FDR corrected $p = 4.77 \times 10^{-3}$) (Fig. S6). *Wza*-coding protein is an essential component of the Wzx/Wzy-dependent pathway, which was considered as a prevalent polysaccharide biosynthesis/transport system in Proteobacteria (Cuthbertson et al., 2009), a dominant phylum in all biofilm samples in our investigation.

Surface hydrophobicity of microplastics is a governing factor that facilitates initial attachment of bacterial cells and subsequent development of biofilms of greater biomass in comparison with sand (Fig. 4) (Zettler et al., 2013; Yang et al., 2020; Wang et al., 2020d). Average water contact angles were measured for PE, PS, and sand particles as 119.7, 87.9, and 52.1 θ°, respectively (Table S2). The higher average contact angle, the more hydrophobic particle surface. Thus, the hydrophobicity of microparticles in this study can be ranked as PE > PS >> sand. Though sand had a much greater surface area (2.78 m^2/g) that was almost three orders of magnitude greater than the two microplastic particles (0.0030 m^2/g for PE and 0.0031 m^2/g for PS), significantly lower total biomass was acclimated onto sand (Fig. 4). These results supported that surface hydrophobicity of microparticles played a more prominent role in biofilm formation than their surface area and other characteristics (e.g., density) (Table S2). It is plausible that bacteria with hydrophobic cell surfaces tend to attach onto a non-polar surface (O'Toole et al., 2000; Krasowska and Sigler, 2014). This was evident by a recent study that reported bacterial density on PE and PS was 1∼3 orders of magnitude greater than that on glass when incubated in treated wastewater and river water (Parrish and Fahrenfeld, 2019).

3.4. Environmental implications and limitations

Overall, our study provides a new perspective of plastisphere in activated sludge treatment systems that have been prevailingly employed at municipal WWTPs worldwide. Microplastics are proved as unique microhabitats that enrich ARB and pathogens from activated sludges. Although our study focused on the assessment of SA resistance genes, a high abundance of *intI1* reflects the co-existence and high mobility of many other ARGs that are frequently carried by this mobile element. Notably, this study demonstrated microplastics themselves can enrich ARGs directly from activated sludges compared to sand as the suspended natural particle. Acclimation and propagation of ARB can be worsened by the co-existence of corresponding antibiotics. Though the present microbial community study may be limited by a moderate sample size of 18, the statistical power was enhanced by the use of the state-of-the-art differential ranking analysis, enabling the identification of taxa with unique linkages with microplastics. In general, primary colonizers (e.g., *Novosphingobium pokkalii*) resided and proliferated on microplastics,

facilitating biofilm formation and further attachment of antibiotic-resistant and pathogenic bacteria which were significantly enriched on microplastics (e.g., *Raoultella ornithinolytica* and *Stenotrophomonas maltophilia*) or occurred at remarkably high abundance among all particles (e.g. *Klebsiella quasipneumoniae*) (see SD).

This inaugural study also raises urgent calls for further investigation on plastisphere in activated sludge units and subsequent wastewater treatment facilities (e.g., secondary clarifiers and disinfection reactors). The critical role of *Novosphingobium* in biofilm formation on microplastics observed from our study and their ubiquitous distribution in global activated sludges (Wu et al., 2019b) (see SD) underscore the fundamental understanding of their prominent contribution in sludge-derived plastisphere and their possible ability to accelerate microplastic biodeterioration. Note that fate and transport of microplastics along the wastewater treatment train can be greatly influenced by the prosperity of the attached biofilms and the integrity and aggregation of the microplastic particles. In conventional aeration tanks, biofouling can lower the buoyancy of microplastics. These biofilm-carrying microplastics are likely to encounter submerging – resurfacing cycles due to the detachment and regrowth/attraction of microorganisms in the following wastewater processes (Rummel et al., 2017). Previous research demonstrated that persistence of primary colonizers on microplastics can facilitate the attachment of secondary colonizers. For example, *Vibrio crassostrea*, a human pathogen, was frequently detected in microplastic biofilms and considered as a potential secondary colonizer since its colonization was promoted when microplastics were precolonized with marine bacterial assemblages (Foulon et al., 2016). Further, owing to the protection by the EPS matrix, disinfectants and the other natural agents become less efficient in attacking and destroying hazardous detriments (e.g., ARGs, ARB, and pathogens) in biofilms. For example, a 600-fold increase of chlorine dosage was required for a 4-log reduction in *Staphylococcus aureus* in biofilm as compared with its planktonic state (Davies, 2003). Thus, through the voyage of microplastics, ARB and pathogens may be more tolerant of disinfectants and have a higher chance to bypass the wastewater treatment processes and contaminate receiving ecosystems, particularly those that are remote and vulnerable. To gain a better understanding of these microplastics-associated taxa and further validate our findings, future studies engaging metagenomics and transcriptomics are critical to uncovering the prevalence and expression of a broad spectrum of ARGs and other molecular processes relevant to biofilm formation and the dissemination of virulent factors in the activated sludge-derived plastisphere. Enrichment and isolation, as well as single cell analysis (Li et al., 2018b), are also underscored, warranting the comprehensive characterization of microplastic-associated taxa in genetic, physiological, and ecological facets.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.hazl.2021.100014.

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