Microplastics as a novel substrate for antimicrobial resistance: Effects of concentration, composition, and size on *E. coli* multidrug resistance

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Word count: 2637

Abstract

Microplastics (MPs) have emerged as a significant environmental pollutant with profound implications for public health, particularly as novel substrates for antimicrobial resistance (AMR). Recently, studies have shown that microplastics might play an important role in the development of AMR due to their ability to accommodate not only microbial communities but also chemical contaminants and genetic material containing antibiotic-resistant genes through biofilm formation. This study investigated the effects of MP concentration, composition, and size on the development of multidrug resistance in *Escherichia coli* to elucidate the potential variables that impact AMR growth. Specifically, we exposed *E. coli* to subinhibitory levels of antibiotics and varying concentrations of different MP types, including polyethylene (PE), polystyrene (PS), and polypropylene (PP), across a range of sizes (3-10 µm, 10-50 µm, and 500 µm). Results indicated a direct correlation between MP presence and elevated levels of multidrug-resistant (MDR) *E. coli* strains. Notably, MPs exhibited a higher propensity for inducing resistance than control substrates such as glass, likely due to their hydrophobicity, greater adsorption capacities, and surface chemistries, which facilitate antibiotic binding. Furthermore, we observed that MPs not only fostered higher magnitudes of resistance at faster rates but also contributed to the formation of biofilms, which provide a protective niche for resistant bacteria. Our study underscores MPs urgent and multifaceted role in propagating antimicrobial resistance and highlights the immediate need for comprehensive environmental management strategies to mitigate the risk posed by microplastics.

Importance

Antimicrobial resistance is one of the world's most pressing global health crises, with an estimated 10 million deaths per year forecasted by 2050. With the pipeline of antibiotics running dry, it is imperative that mitigation strategies understand the mechanisms that drive the genesis of antimicrobial resistance to where it begins. One emerging dimension of antimicrobial resistance is the environment. This study highlights the relationship between a widespread environmental pollutant, microplastics, and the rise of drug-resistant bacteria found in the environment. [5] While it is known that microplastics facilitate resistance through several modes (biofilm formation, plastic adsorption rates, etc.), this study fills the knowledge gap on how different types of microplastics are contributing to antimicrobial resistance.

Introduction

Global plastic use and mismanaged disposal are significant environmental and public health concerns. Plastic use has increased twenty-fold since 1964, and prevailing estimates suggest global unmanaged trash will reach 155-265 megatons per year in 2060.[1] Not surprisingly, the detection of microplastics has significantly increased. Microplastics (MPs) are canonically insoluble synthetic particles or polymer matrices with regular or irregular shapes and sizes ranging from the micrometer to millimeter range.[2] Primary source microplastics include polyethylene, polypropylene, and polystyrene particles in cosmetic and medicinal products.[3] Several countries have banned the use of primary microplastics within certain industry segments; however,

microplastics are still generated through the degradation of existing plastics, creating contamination beyond legislation around new product formulations.[3] These degradation products, characterized as secondary microplastics, originate from physical, chemical, and biological processes, resulting in plastic debris fragmentation. Different sources of microplastics and their varying surface chemistry also cause them to occur in varying shapes and sizes, such as pellets, fibers, and fragments in environmental samples.[3] Depending on the conditions plastics are exposed to and the resulting change in surface properties, microplastics arise as a unique substrate that has proved to be very difficult to control.[25, 26]

Microplastics have infiltrated various ecosystems on the planet, ranging from submarine canyons[4] to the summit of Mount Everest.[5] Additionally, wastewater has become a significant reservoir for microplastics and other anthropological wastes. Despite global awareness, microplastics can persist through wastewater treatment plants, disseminating them into surrounding environments. Their persistence can be attributed to their small size, buoyancy, and hydrophobic properties, allowing them to adhere to organic matter and avoid sedimentation.[6] Consequently, treated wastewater effluents serve as a main source of microplastic pollution in aquatic environments where they accumulate in sediments and surface waters and interact with the organisms around them.[6] The repercussions of microplastics in wastewater are manifold, especially considering their impacts on human health.

Contemporaneously, increased rates of antimicrobial resistance (AMR)—the ability of microbes to protect themselves against antimicrobials such as antibiotics—have been observed in bacterial populations across the globe. AMR can be influenced by a multifaceted network of factors, including the overuse and misuse of antibiotics, poor sanitation and hygiene, and environmental contamination with antibiotic residues in wastewater.[27] Recent studies show that microplastics might play an important role in the development of AMR.[28, 29, 30] This is mainly due to their ability to accommodate not only microbial communities but also chemical contaminants and genetic material containing ARGs (antibiotic-resistant genes) through biofilm formation.[19] In these communities of bacteria that grow together colonizing microplastic surfaces, ARGs can be transferred to pathogenic bacteria through horizontal gene transfer, which includes conjugation, transformation, and transduction.[19] While it has been established that bacteria on the surface of microplastics host ARGs, there is limited knowledge on the mechanisms of AMR development as a function of microplastic properties (composition, size, concentration, etc.) as well as their interactions with various antibiotics.[8,11]

Understanding the interplay between AMR and microplastics is critical, especially in places with high infection rates and significant plastic waste, such as low-resource settings. The difficulty in treating infectious diseases in these areas, combined with inadequate wastewater treatment—which may result in higher concentrations of microplastics—may contribute to the observed increase in AMR cases among vulnerable populations. Therefore, understanding the fundamental interactions between microplastics and AMR development is imperative. This will ensure that

proper consideration is given to microplastics found in the environment, as well as critical information about how microplastics may affect the level of AMR in pathogenic bacteria. To date, few studies have looked at the effect of microplastic size, structure, and other features on the development of drug resistance in the presence of antibiotics. In this study, we investigate the impact of different microplastic characteristics, including microplastic concentration, surface composition (between plastics and other materials), size, and surface area, on the *in vitro* development of resistance to ampicillin, ciprofloxacin, doxycycline, and streptomycin in *E. coli*. These antibiotics are broad-spectrum, represent different antibiotic classes, interact with each other, and are readily found in wastewater systems.[12] More specifically, we probed if microplastic concentration (no. plastics/µL), size, surface area, and composition (between plastics types) had any impact on (1) bacterial growth alone, (2) antibiotic-specific AMR rates, and (3) multidrug resistance (MDR) rates between the four antibiotics. Our results identify that microplastics play an important and significant role in the development of AMR. Overall, our findings may provide context to wastewater surveillance data and provide insights into waste management and associated disease burdens.

Methods

Strains and culture conditions

E. coli MG1655 (ATCC 700926) was used in all experiments. All liquid cultures were grown in Lysogeny Broth (Miller, LB) medium under shaking conditions at 180 rpm at 37 °C. MPs were added first for a total of 48 hours to allow potential biofilm growth on the surface. Following the initial MP exposure, subinhibitory levels of ampicillin (Sigma Aldrich, CAS No. 69-52-4), ciprofloxacin (MP Biomedicals, CAS no. 86393-32-0), doxycycline (Sigma-Aldrich, CAS no. 24390-14-5), streptomycin (Sigma-Aldrich, CAS no. 3810-74-0) were added to the medium, as indicated below.

Growth Curve Data

Spectrophotometer growth curves

To determine what concentration of microplastics does not hinder the growth kinetics of the bacterial cells, we used a modified standard growth curve procedure from Brewster et al. with *E. coli* and varying concentrations of 10 µm diameter polystyrene spheres (Alpha Chemistry, Cat.No. ACM9003536-5).[13] Standard LB media was diluted with varying concentrations of the polystyrene microplastics and pipetted into a 96-well plate in 100 µL increments. 10 µL of WT *E. coli* MG1655 was then pipetted into their respective wells hosting the differing concentrations of MPs. Each well hosting *E. coli* had a respective well that had identical media to subtract from in the analysis. To monitor growth, O.D. 600 was measured every 5 minutes using a Spectra M5 plate reader with shaking between each measurement. Wells were seeded with exponential phase cells such that the starting O.D.> 600 of each well was ~0.08-0.09 after subtracting the initial

background readings the microplastics produced. We made the plate cover hydrophilic to avoid condensation at 37 degrees C.[20] Microwell plate covers were made hydrophilic by pouring 2 to 3 ml of 0.05% Triton X-100 in 20% ethanol into the cover and tilting several times to ensure even coverage of the inner surface. After 15–30 s, the treatment solution was poured off, and the cover was shaken to remove most of the remaining liquid. The cover was leaned against a vertical surface and allowed to air-dry.

CFU/mL growth

Wild-type E. coli MG1655 was cultured at 37C in 4mL of the media of interest with varying microplastic concentrations (100, 500, 1000, 2000, 4000, 5000, 10000, and 15000 plastics/uL) in culture tubes and sampled at 0, 1, and 24 hours. These samples were plated in triplicate on LB agar to determine the CFU/mL at the time point of interest.

Antibiotic and MP susceptibility testing

To determine the initial Minimum Inhibitory Concentration (MIC) of bacterial cells post-microplastic exposure, cells were outgrown in drug-free media until substantial biofilm growth was detected (~48 hours) using confocal microscopy (procedure below). Microparticles (plastics and glass) were vortexed and spun down to release the biofilm on the surface. The supernatant was subsequently used in a standard broth microdilution MIC in a 96-well plate using LB media. The MIC was determined via a microplate reader (Spectra M5, 600 nm) to determine where growth stalled. The initial MIC was then taken and used below for the subinhibitory exposure throughout the ten-day experiment.

Microplastic-antibiotic combination assays

After the initial growth period and MIC testing as described above, saturated liquid cultures were passaged once a day into fresh amended media–4 mL of LB broth, 40% of the initial MIC of a singular antibiotic, and their respective growth conditions (differing concentration, size, composition of plastics or glass)--via 1:100 dilution in until day ten. Every two days, all groups were tested for susceptibility to the antibiotic they were grown up in via standard broth microdilution assays described above. As an exposure-based control, cells in drug-free media with the same variable of microplastics (concentration, size, composition), cells with no adhesion sites (no microplastics or glass spheres) but with drug (40% MIC), as well cells in drug-free and particle free media were also tested. The wild type, no antibiotic or particle exposure cells would serve as a control for the impact of microplastics and subinhibitory concentrations of antibiotics on resistance development. A schematic of the experiment is displayed below in Figure 1.

----------Insert Figure 1----------

In addition to testing antibiotic resistance in the antibiotic the cells were grown in, resistance development was also tested using the same standard broth microdilution assay described above. This protocol was amended for the three different antibiotics used in the study. The schematic of these assays can be seen in Figure 1.

Confocal Laser Scanning Microscopy (CLSM) image collection and analysis

Visualizing the cellular communities on the surface of the particles was done using confocal microscopy. Using the cultures described in the susceptibility testing section, 500 µm diameter polystyrene spheres were randomly selected out of each culture and dyed using the LIVE/DEAD *Bac*Light Bacterial Viability Kit Protocol, per manufacturer instructions. Briefly, SYTO 9 was used to analyze viable cells, as it can permeate all bacterial cell membranes. In contrast, propidium iodide was used to count dead cells, as it can only enter cells with disrupted membranes. Each plastic was put into a centrifuge tube with 1 mL of sterile water, and three µL of staining solution (1.5 µL of each dye) was injected into the tube. Samples were incubated for 20 minutes at room temperature and protected from light.

Stained samples were imaged with an inverted CLSM (Olympus DSU spinning disk confocal) using a 10X oil immersion objective. Samples individually stained with propidium iodide and SYTO 9 were first analyzed separately to ensure clear signals without overlap. The particles were removed from the dye and suspended in a costar clear round bottom 96 well plate to preserve the MP shape while imaging. After finding the sample and adjusting brightness parameters, z-stacks with an optimized step size were taken for each sample to obtain a 3D visualization of biofilm viability, starting at the base of the spherical particle where growth started and finishing at the top of the sphere where the growth ended.

Z-stacks were analyzed using FIJI.[14] 3-D renderings were made by taking the .oir file raw data from the microscope and merging the red and green channels. Following the merge, the channels were elucidated by merging the stacks via image -> stacks -> z project with a “Max Intensity” projection type.

Statistical Analysis

The significance of MIC fold changes was determined using an ordinary one-way ANOVA, assuming a Gaussian distribution of the residuals and equal standard deviations. Each variable was compared to the mean of a control, which varied depending on the study. Multiple comparisons were corrected using the Dunnett test, with each P value adjusted accordingly. Finally, the residuals were tested for clustering or heteroscedasticity using the Brown-Forsythe and Bartlett’s tests.

Results

Microplastics impact growth at high concentrations

Optical Density (OD) Readings

Eight different concentrations of microplastics were grown with *E. coli* for 18 hours to determine whether microplastics influence bacterial growth. The microplastic concentrations for this study were chosen to allow the comparison of conditions independent of growth to remove growth as an effector of resistance dynamics. The microplastics used were 10-um diameter polystyrene spheres at concentrations of 100 MP/µL, 500 MP/µL, 1,000 MP/µL, 2,000 MP/µL, 4,000 MP/µL, 5,000 MP/µL, 10,000 MP/µL, and 15,000 MP/µL and OD readings were taken every five minutes to quantify growth (Fig. 2A). Of these concentrations two (10,000, and 15,000 MP/uL) exhibited a significantly different normalized OD reading relative to the growth without plastics (WT). (Fig. 2A).

----------Insert Figure 2----------

In addition to OD readings, the viability of the bacteria grown with microplastics was tested by measuring the colony-forming units per mL (CFU/mL). At one hour of growth, there was no significant difference between the growth of the solutions; however, at 24 hours, three microplastic solutions (5,000, 10,000, and 15,000 MP/uL) showed a significantly higher number of CFU/mL compared to the WT (Figure 3A, 3B).

----------Insert Figure 3----------

Exposure to various polystyrene microplastic concentrations increases ciprofloxacin resistance similarly.

In order to study the effect of antibiotic residues—thought to be another component that leads to resistance—we combined antibiotic residues and microplastics in our study to mimic real-world conditions.[5] Microplastic concentration's (number of plastics/uL) effect on the development of ciprofloxacin resistance was investigated under subinhibitory ciprofloxacin conditions. The ciprofloxacin MIC for *E. coli* was determined to be between 0.0375 and 0.01875 ug/mL, which is consistent with the values in the literature.[2] To empirically understand whether the concentration of microplastics affected ciprofloxacin resistance development and magnitude of resistance, we exposed the bacteria to subinhibitory levels of ciprofloxacin (0.0075 ug/mL, 40% of the initial MIC detailed above) and different concentrations of polystyrene microplastics. Resistance was determined in MIC fold changes relative to the control (WT *E. coli* grown without microplastics or subinhibitory levels of antibiotics).

The concentrations of microplastics tested with the 500-um diameter polystyrene spheres were 40 MP/mL and 100 MP/mL. Conversely, the concentrations tested with the 10-um polystyrene

diameter beads were 1,000 MP/uL, 500 MP/uL, 100 MP/uL, and 10 MP/uL. There was no significant difference in ciprofloxacin minimum inhibitory concentration (MIC) fold changes between the different concentrations of microplastics (Figure 4B, D); however, there was a significant difference in ciprofloxacin MIC fold changes between each concentration of microplastic and the microplastic-free counterpart (bacteria with subinhibitory levels of ciprofloxacin but no microplastics) as well as the WT (no microplastics or antibiotics introduced during growth) (Figure 4A, C). The absolute MIC values increased up to 0.003713 ± 0.00066 mg/mL of ciprofloxacin—over three times the Clinical & Laboratory Standards Institute (CLSI) defined 0.001 mg/mL clinical breakpoint—for the larger diameter beads and 0.002508 ± 0.004 mg/mL of ciprofloxacin for the smaller beads (Table S.1 and S.2).[34] Following these results, for the 10 um diameter polystyrene MPs, we chose 100 MP/uL, as well as 40 MP/mL for the 500-um diameter polystyrene MP, as a baseline concentration in the future studies.

----------Insert Figure 4----------

Exposure to multiple different plastic compositions affects ciprofloxacin resistance.

Next, we sought to determine if plastic composition affected the development and magnitude of ciprofloxacin resistance. This experimental design tested and compared the three most common plastic types–polystyrene, polyethylene, and polypropylene–under exposure to subinhibitory levels of ciprofloxacin.[24]

Previous studies have shown that bacteria have an affinity for plastic due to their high hydrophobicity and oxidation, which can lead to easy adhesion.[31, 32] Of the three plastics used in this study, polystyrene is the most hydrophilic, while polypropylene is the most hydrophobic. We found that all three conditions with varying microplastic compositions had a significantly higher MIC after ten days of exposure than the microplastic-free control exposed to just subinhibitory levels of ciprofloxacin. While the control MIC fold change at day ten was significantly lower than the samples exposed to the microplastics, the plastic compositions themselves did not produce fold change values that were considered significantly different. However, polystyrene/ciprofloxacin did reach a significantly different absolute MIC value (0.00165 ± 0.000572 mg/mL) than polyethylene/ciprofloxacin (0.000743 ± 0.00036 mg/mL), polypropylene/ciprofloxacin (0.000528 ± 0.000162 mg/mL) and the control of just ciprofloxacin (0.00020625 ± 0.00004 mg/mL) (Figure 5, Table S3). Furthermore, the polystyrene microplastics facilitated an absolute MIC higher than the ciprofloxacin-*E. coli* clinical breakpoint (0.001 mg/mL).

----------Insert Figure 5----------

Enhanced Antimicrobial Resistance on Microplastic Substrates Compared to Glass in Biofilm Formation Studies

We also studied the resistance development between polystyrene microplastic and glass spheres in order to determine if plastic substrates had a specific effect or if any small particles would lead to an increase in AMR. We tested 500-um diameter spheres at the same concentration of 40 MP/uL. The microparticles were exposed to subinhibitory levels of ciprofloxacin and compared to a control, which only had the subinhibitory ciprofloxacin and no glass or plastic particles. Interestingly, the glass facilitated a faster rate of resistance development but quickly stabilized at a ciprofloxacin MIC of 0.00186 ± 0.00089 (Figure 6A, 6B, Table S.4). Conversely, the polystyrene plastic spheres facilitated a statistically different MIC fold change and absolute MIC value by the end of the ten-day study from its glass counterpart and antibiotic control. Both the day 10 polystyrene and glass conditions crossed the ciprofloxacin-*E. coli* clinical breakpoint. Notably, polystyrene surpassed the clinical breakpoint by 3X at 0.0037125 mg/mL (Table S.4)). [21] The same experiment was performed with a smaller attachment surface of polystyrene 5 um-diameter MPs and 3 um-diameter glass spheres (Figure 6C, 6D). The 10-um diameter particles only yielded a significant difference from the antibiotic control in their ciprofloxacin absolute MIC values (Figure 6C). However, when all sizes of particles (glass, MP 10-um diameter, and glass, MP 500-um diameter) were compared, the 500 um-diameter MP significantly differed from the glass particles of both sizes (Figure 6E), indicating that microplastics might be a unique surface for bacteria to gain resistance.

----------Insert Figure 6----------

To understand how bacteria are interacting with the microplastics and the glass spheres qualitatively, we used confocal microscopy to view the surface of the microparticles. The glass and polystyrene spheres were dyed on day 10 of subinhibitory ciprofloxacin exposure and compared to each other (Figure 7). Green pixels indicate viable growth (e.g., live cells). In contrast, red indicates nonviable growth (dead cells). The glass (Figure 7A) depicts two spheres, each having a significantly decreased amount of growth on the surface of the sphere, and most of the growth exhibited was colonized by dead cells. Conversely, the polystyrene condition had mostly live cells, and bacteria colonized the whole surface.

----------Insert Figure 7----------

Exposure to microplastics of different sizes affects ciprofloxacin resistance development.

MIC variation based on the size and surface area of microplastics was investigated following the results in the above section. The sizes of polystyrene microplastics investigated were 5 um (100 MP/uL), 10 um (100 MP/uL), and 500 um (40 MP/uL) diameter spheres. All three size variations were subjected to subinhibitory ciprofloxacin along with a control that had no plastic exposure, and the ciprofloxacin MIC was tested. Consistent with the confocal microscopy (Figure 7), results showed that the difference between the microplastics themselves had no significant statistical difference. Still, *E. coli* grown in subinhibitory antibiotics with 500 um diameter microplastics had larger ciprofloxacin MIC fold changes, with the 500 um beads consistently

surpassing 3X the ciprofloxacin-*E. coli* clinical breakpoint (Figure 8). Moreover, the other two groups (10 um and 5 um diameter) also surpassed the ciprofloxacin clinical breakpoint. While the results showed no significant difference between the different surface areas of the beads, the cells grown with microplastics and antibiotics again consistently had higher absolute MIC values and larger MIC fold changes compared to the antibiotic-grown control (Figure 8A, 8B).

----------Insert Figure 8----------

The presence of microplastics and subsequent biofilm impacts multidrug resistance (MDR)

Next, we sought to determine if our observations extended beyond ciprofloxacin; furthermore, we determined if cells exposed to microplastics and subinhibitory levels of a given antibiotic become resistant to other classes of antibiotics, rendering the bacteria multidrug-resistant (i.e., were the effect we observed specific to ciprofloxacin or broad to multiple classes of antibiotics). The four antibiotics we tested were ampicillin (Beta-lactam antibiotic), ciprofloxacin (Fluoroquinolone), doxycycline (Tetracycline), and streptomycin (Aminoglycoside), all commonly found within the environment and exhibit bactericidal behavior.[12,15] We measured changes in MIC in cells that were grown with a.) microplastics (500 um diameter polystyrene spheres) AND subinhibitory levels of a *singular* antibiotic or b.) just subinhibitory levels of a *singular* antibiotic.

Our results suggest that the addition of microplastics led to an increase in AMR for nearly all antibiotics. In the first four rows of Figure 9A, we show the fold change of antibiotics relative to WT for bacteria grown in either antibiotics alone or antibiotics and microplastics. In the last row of Figure 9A, we show the level of antibiotic resistance for bacteria grown solely with microplastics or WT. In each case where bacteria were grown and tested in the same antibiotic, the addition of microplastics to antibiotics in the media led to an increase of at least 5 times more antibiotic resistance compared to antibiotics alone (Figure 9B). Interestingly, bacteria grown in ciprofloxacin with MPs generally had higher levels of multidrug resistance (up to 171 times that of the control grown in the antibiotic) (Figure 9B, Table S.5). The other antibiotics with MPs led to more resistance to ciprofloxacin of up to 75-fold higher than the antibiotic control (Table S.5). Additionally, bacteria grown in streptomycin with MPs developed extremely high levels of resistance to ciprofloxacin, doxycycline, and streptomycin (Table S.5). The addition of microplastics alone also led to an increase in antibiotic resistance compared to WT for all antibiotics tested, though generally not as much as either antibiotics alone or antibiotics with microplastics combined (Figure 9A).

After ten days of exposure to subinhibitory antibiotics, exposure to subinhibitory antibiotics was halted, and bacteria were grown in antibiotic-free media for five days. The MIC was tested daily to determine if resistance was stable (i.e., whether the mutations leading to resistance from day 10 of subinhibitory antibiotic exposure were stable). Results indicated that bacteria introduced to microplastics were not only resistant past the clinical breakpoint[21] with the antibiotic they were

grown in but to three other families of antibiotics. Furthermore, the bacteria passed the clinical breakpoint of resistance with the other antibiotics without having ever been introduced to them. This result was consistently found in all four families of antibiotics we tested (ampicillin, ciprofloxacin, doxycycline, and streptomycin).

----------Insert Figure 9----------

Resistance stability was tested following the ten-day frame in which bacteria were passaged in their respective media. Of the sixteen conditions, 81.25% (13 out of 16) of the bacteria grown in microplastics and subinhibitory antibiotics retained the day ten resistance to their respective antibiotic or *gained* resistance (Figure 10). The conditions to lose their resistance were bacteria grown in strep-tested in amp, grown in strep-tested in strep, and grown in dox-tested in amp. Conversely, 43.75% (7 out of 16) of the bacteria grown in just microplastics retained or grew in resistance to their respective antibiotics, indicating a relatively unstable mechanism of antibiotic resistance. Surprisingly, only 18.75% (3 out of 16) of the bacteria grown in the subinhibitory antibiotics condition retained or grew in resistance. Of the three conditions that expressed a stable resistance, two were grown in doxycycline (Figure 10).

----------Insert Figure 10----------

Discussion and Conclusions

Microplastics are significant environmental pollutants with critical implications for public health, particularly in the context of increasing AMR. Understanding how microplastics affect the development of AMR is a crucial step to better understand how environmental factors shape AMR in bacteria. This will only become more important as both microplastics and AMR become more prevalent. Our study showed that microplastics significantly impact the antimicrobial resistance rate of development and its magnitude. More specifically, microplastics facilitated post-breakpoint multidrug resistance to four distinct families of antibiotics (Figure 9A, 9B). Breakpoints are an integral part of modern microbiology practice and define susceptibility and resistance to antibacterials in the clinical setting (e.g., related to human health).[16]

Polystyrene, in particular, had the most significant impact on resistance development, which was surprising given its relative hydrophilicity compared to polyethylene and polypropylene. However, it is essential to note that studies have shown that *E. coli* prefers hydrophilic surfaces over hydrophobic ones.[17] With this in mind, we expected glass beads of similar diameter to have a higher adhesion rate and, therefore, a higher resistance to the tested antimicrobials. This, however, was not the case, as polystyrene had a higher absolute MIC value and greater MIC fold change over the glass condition. This indicates that plastics may be a unique substrate for bacteria to develop and maintain resistance to.

While the complete mechanism is not yet known for antimicrobial resistance on microplastics, the current prevailing theory indicates that biofilm formation upon the plastics allows for greater horizontal gene transfer and, thus, higher resistance rates.[29] As depicted in the CLSM images in Figure 7, we observed a dense biofilm that spans the entire surface of the microplastic, compared to the glass, which hosts clusters of biofilm not covering the entire surface and with a large portion of nonviable cells post-subinhibitory antibiotic exposure. This would also explain the higher MIC values shown in Figure 6 as the microplastic far surpasses the magnitude of the MIC fold change as well as the MIC value of the drug needed (0.00384 ug/mL (MP) vs. 0.00186 ug/mL (Glass)). Further research is needed to investigate the cellular-level interactions and material properties of plastics that contribute to such high resistance rates.

It is well known that biofilms play a crucial role in the spread of antimicrobial resistance. Bacteria within biofilms produce persister cells that are metabolically inert, which is one mechanism for evading antibiotics.[22] These cells can survive even in high concentrations of antibiotics.[22] Furthermore, current research suggests that biofilms act as refugia of MDR plasmids by retaining them, even in the absence of antibiotics.[23.] This would support our results with respect to the microplastic properties we investigated. First, microplastic concentration was not factored into different resistance rates. Instead, size and composition had more of an impact on the rate of resistance development and magnitude of resistance. A larger-sized microplastic would have a larger biofilm and, therefore, a greater capacity to develop resistance. The plastic composition can also play a role in both bacterial attachment and biofilm growth. Microplastics are known to serve as electron donors for bacterial biofilms to feed on, inducing a faster or easier attachment of the bacteria to the surface and promoting bacterial growth and colonization.[31, 35] This may explain the higher concentration of biofilm on polystyrene MP compared to glass (Figure 6), which in turn would explain polystyrene's larger resistance load.

We believe that the potential ramifications of extensively multidrug-resistant bacteria facilitated by the addition of microplastics in the clinical setting are significant. Moreover, we found bacteria exhibiting this behavior within ten days of subinhibitory antibiotics and microplastic exposure. The rate of AMR development and the surpassing of clinical breakpoints in both single and multidrug tests highlight a need to monitor microplastics and antibiotic levels in the environment. This is especially true in areas with inadequate waste disposal and substandard public health infrastructure, such as low-and middle-income countries (LMICs) and vulnerable populations.[18] Future studies in this area should focus on the disparities of wastewater treatment in LMICs and high-income countries and how the different environmental factors shape AMR development. Additionally, wastewater and environmental monitoring should also include the presence of microplastics, as they have the potential to exacerbate AMR outbreaks. Our work can inform the ongoing development of AMR surveillance strategies, helping to predict and prevent future outbreaks.

Acknowledgments

Thank you to Matt Lim for guidance at the confocal microscope and Boston University's shared CORE facility for the usage of their instruments.

Funding

This study was made possible by NSF GRFP grant number 2023354788 and supported by funds from the College of Engineering and Center on Forced Displacement at Boston University. The funders had no role in study design, data collection and interpretation, or the discussion to submit the work for publication.

CRediT Statement:

NG: Conceptualization, Methodology, Investigation, Writing-Original Draft, Writing- Review and Editing
JM: Investigation, Writing-Review and Editing
CC: Conceptualization, Writing-Review and Editing
BG: Investigation, Writing-Review and Editing
YN: Writing- Review and Editing
MHZ: Writing-Review and Editing

Competing Interests

Authors have no competing interests to declare.

Data Sharing

All data used for this study has been included in the manuscript or supplementary material.

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Figures:

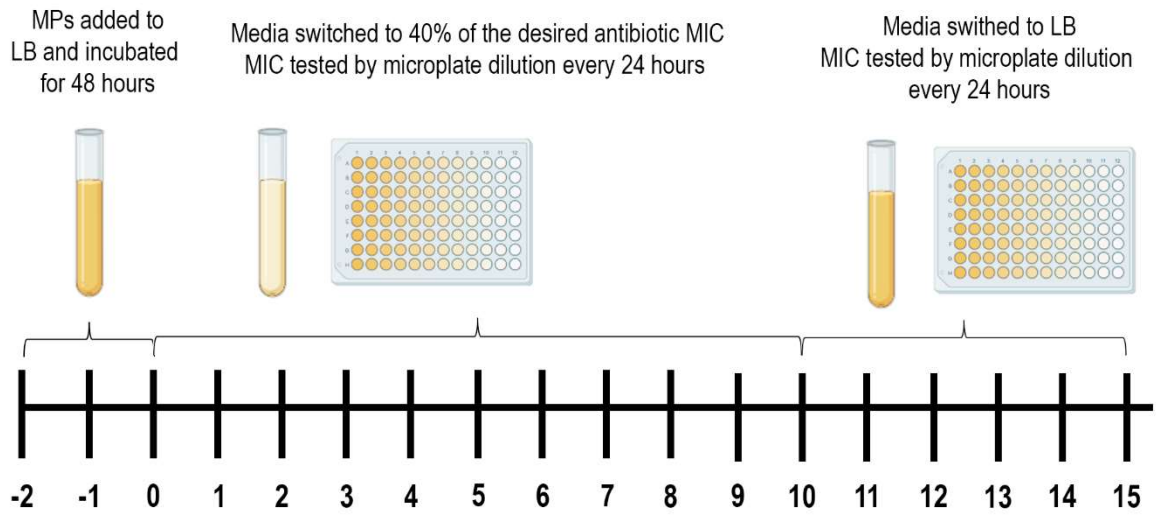


Figure 1. *Experimental schematic. In all experiments, cells were grown with microplastics two days prior to subinhibitory antibiotic exposure. Following day zero, cell MIC was tested in a standard microdilution plate in the antibiotic of interest.*

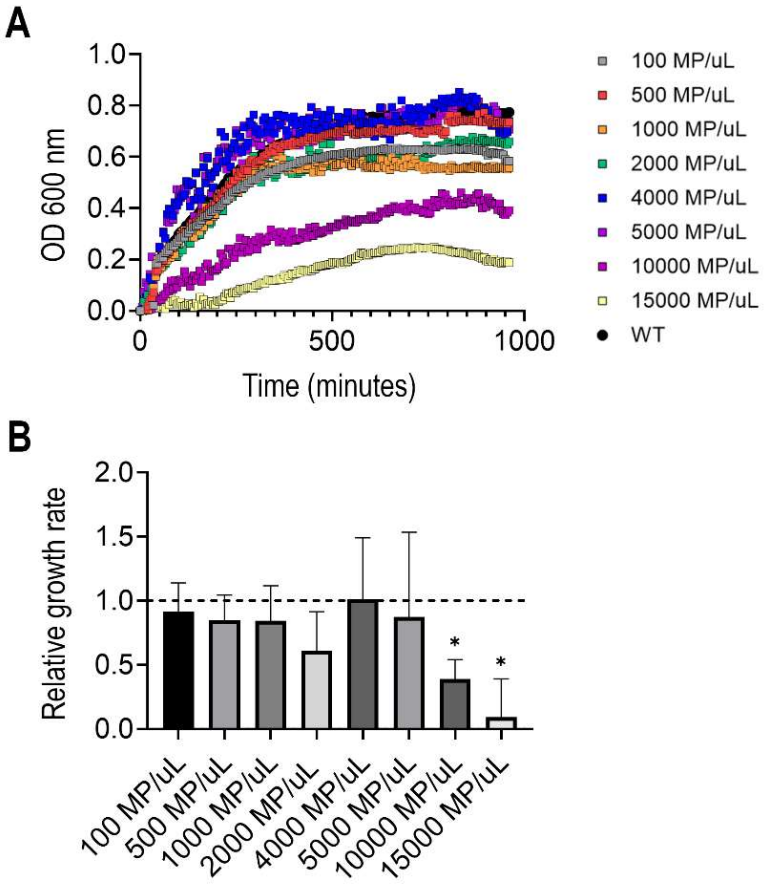


Figure 2. *Growth curve OD readings with various MP concentrations (A) and the relative growth rate to the WT, indicated by the dashed line (B). Error bars represent the standard deviation of a population of >8. * indicates a significant difference from the growth rate to the WT.*

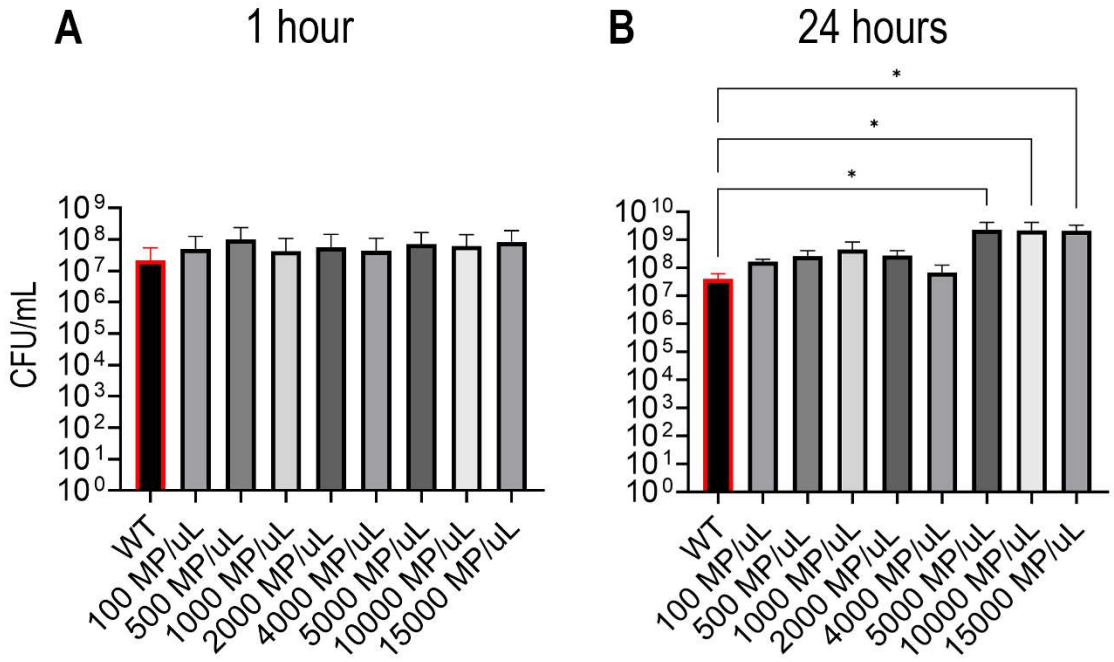


Figure 3*. CFU/mL readings after 1 hour of growth (A) and 24 hours of growth (B) in various microplastic concentrations. Red border highlights the control, * represents a statistical significance from the control.*

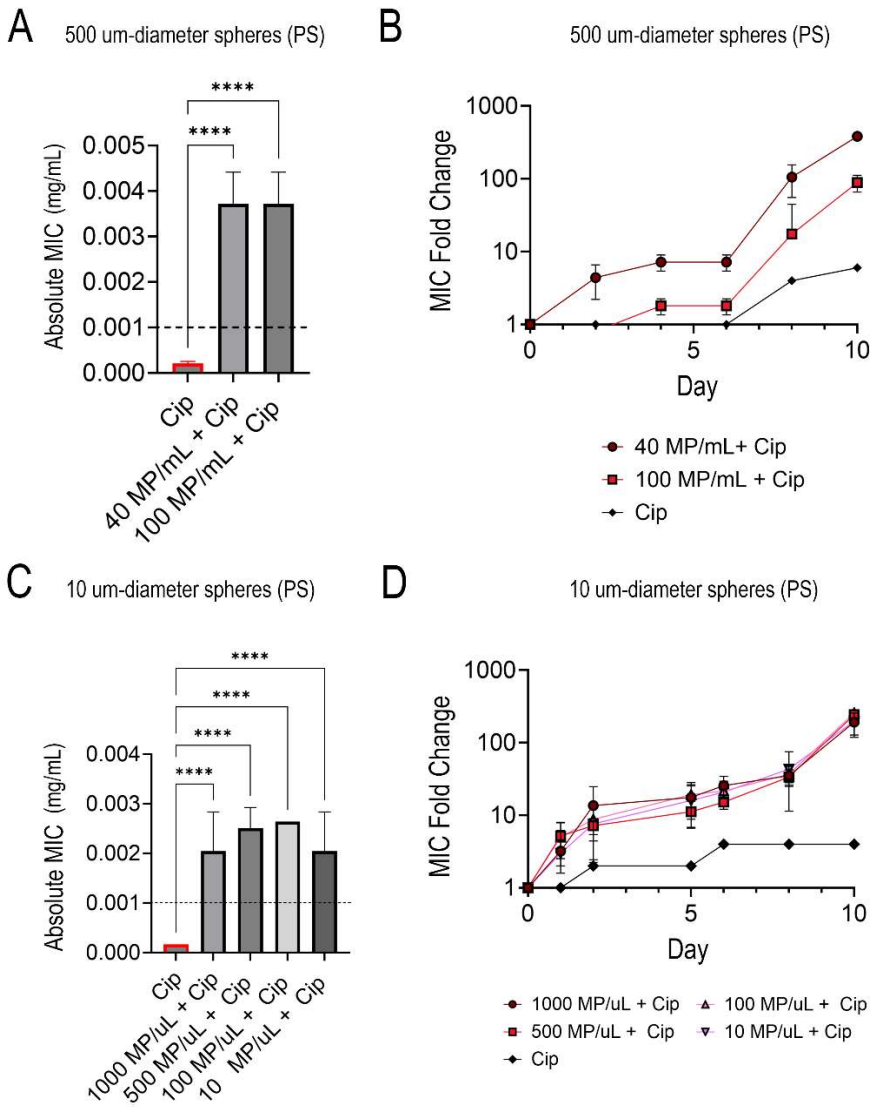


Figure 4. *Absolute ciprofloxacin MIC values for 500-um diameter polystyrene MPs at two different MP concentrations with the dashed line indicating the ciprofloxacin clinical breakpoint concentration (A), time series fold change of 500-um diameter polystyrene MPs at different MP concentrations and the subinhibitory antibiotic control (Cip) relative to the WT (B), Absolute ciprofloxacin MIC values for 10-um diameter polystyrene MPs at four different MP concentrations with the dashed line indicating the ciprofloxacin clinical breakpoint concentration (C)), time series fold change of 10-um diameter polystyrene MPs at different MP concentrations and the subinhibitory antibiotic control (Cip) relative to the WT (D)*

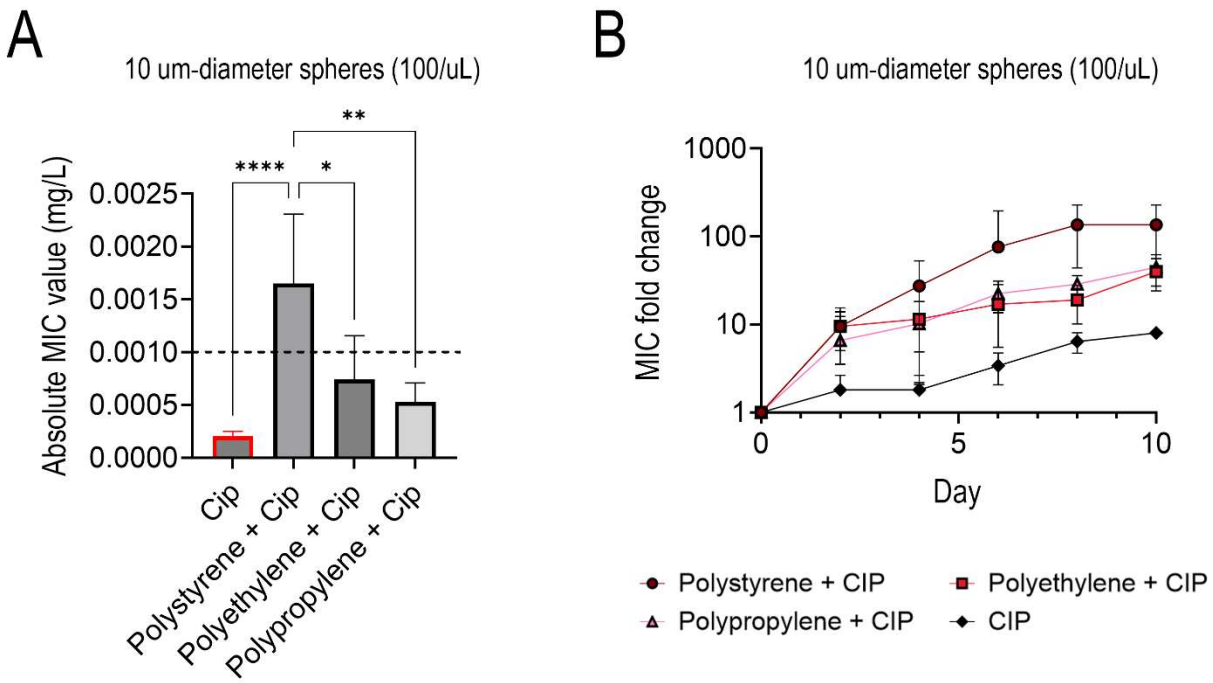
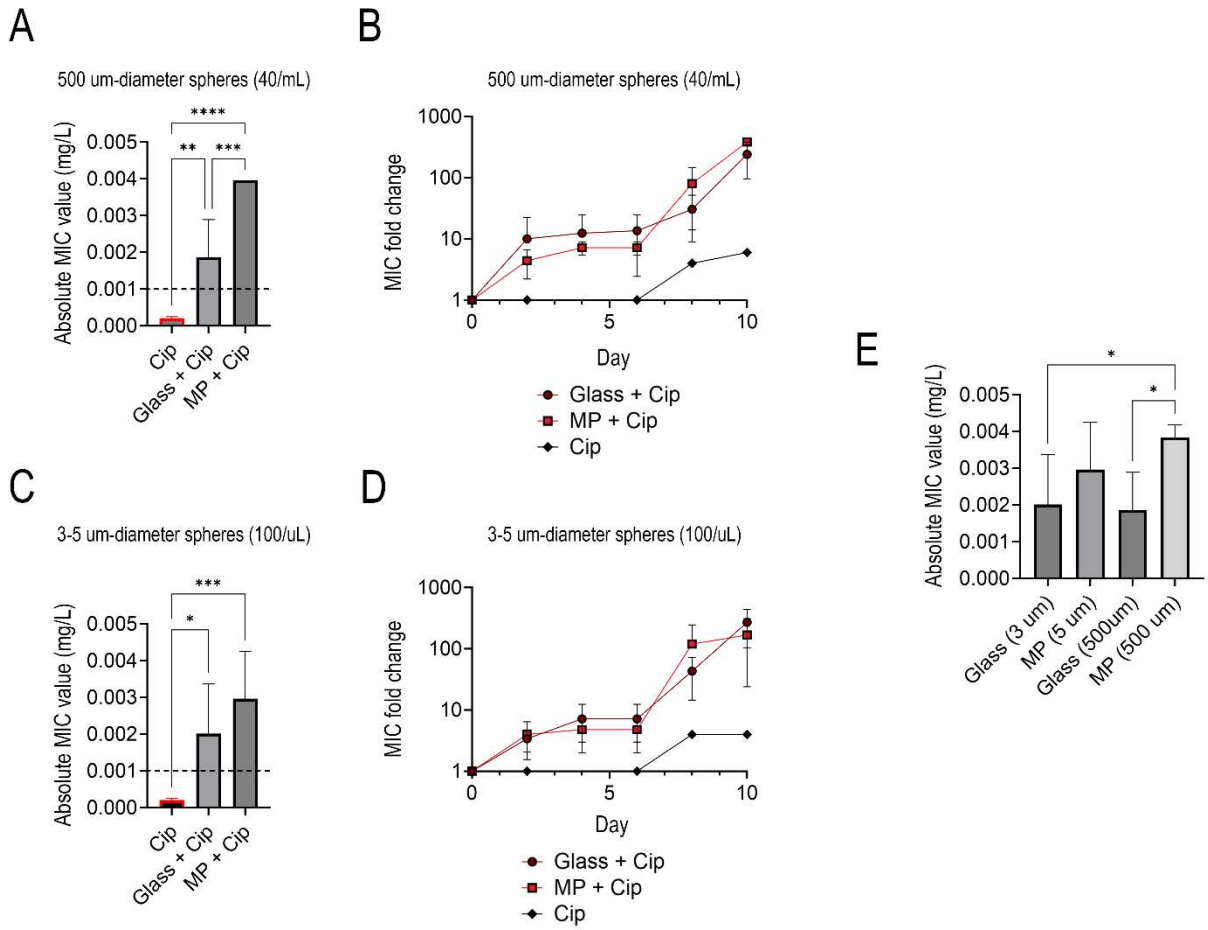
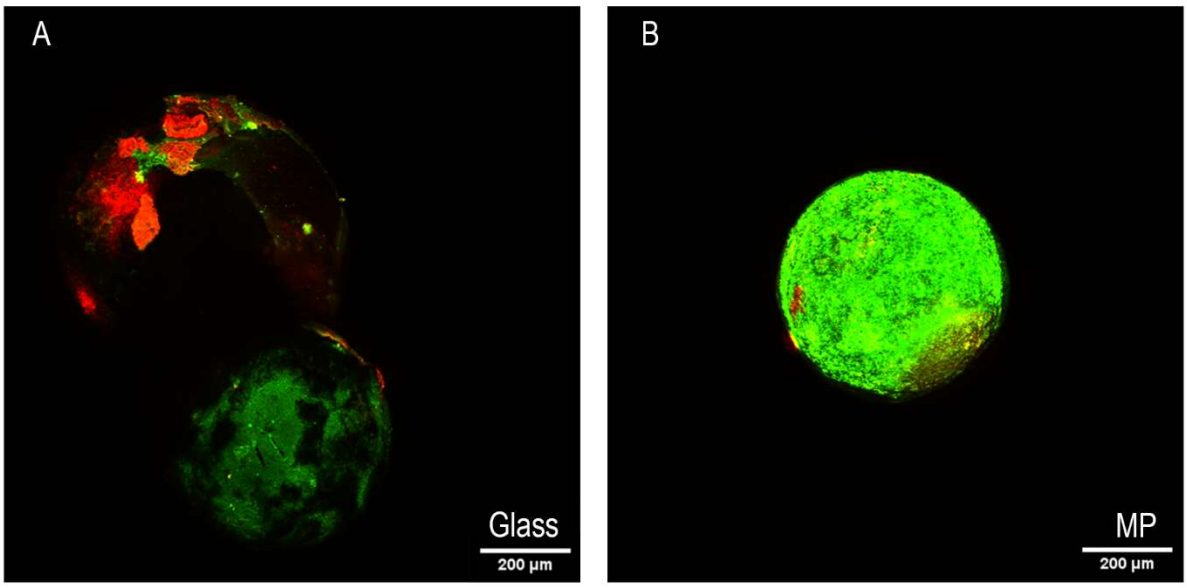


Figure 5. *Absolute ciprofloxacin MIC values for 10-um diameter polystyrene, polyethylene, and polypropylene MPs with the dashed line indicating the ciprofloxacin clinical breakpoint concentration (A), time series fold change of 10-um diameter polystyrene, polyethylene, polypropylene MPs, and the subinhibitory antibiotic control relative to the WT (B)*



562

563 ***Figure 6.*** *Absolute ciprofloxacin MIC values for 500-um diameter and 10-um diameter*
564 *polystyrene and glass spheres at 40 particles/mL (6A) and 100 particles/uL (6C), respectively,*
565 *with the dashed line indicating the ciprofloxacin clinical breakpoint concentration. Time series*
566 *fold change of the 500-um (6B) and 10-um (6D) diameter polystyrene, glass spheres, and the*
567 *subinhibitory antibiotic control relative to the WT. Absolute MIC value of all glass and MP*
568 *samples (6E).*



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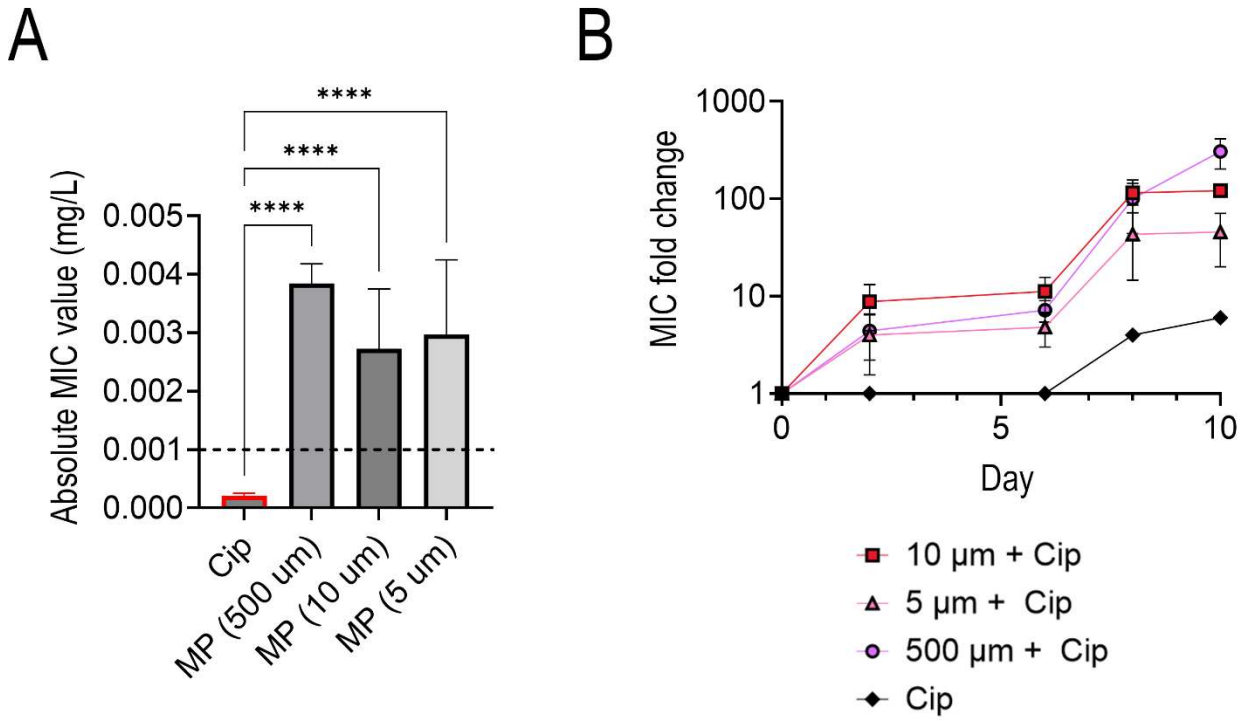
570 ***Figure 7.*** *CLSM image of 500-um diameter glass (left) and polystyrene (right) spheres at day 10*
571 *of subinhibitory ciprofloxacin exposure.*



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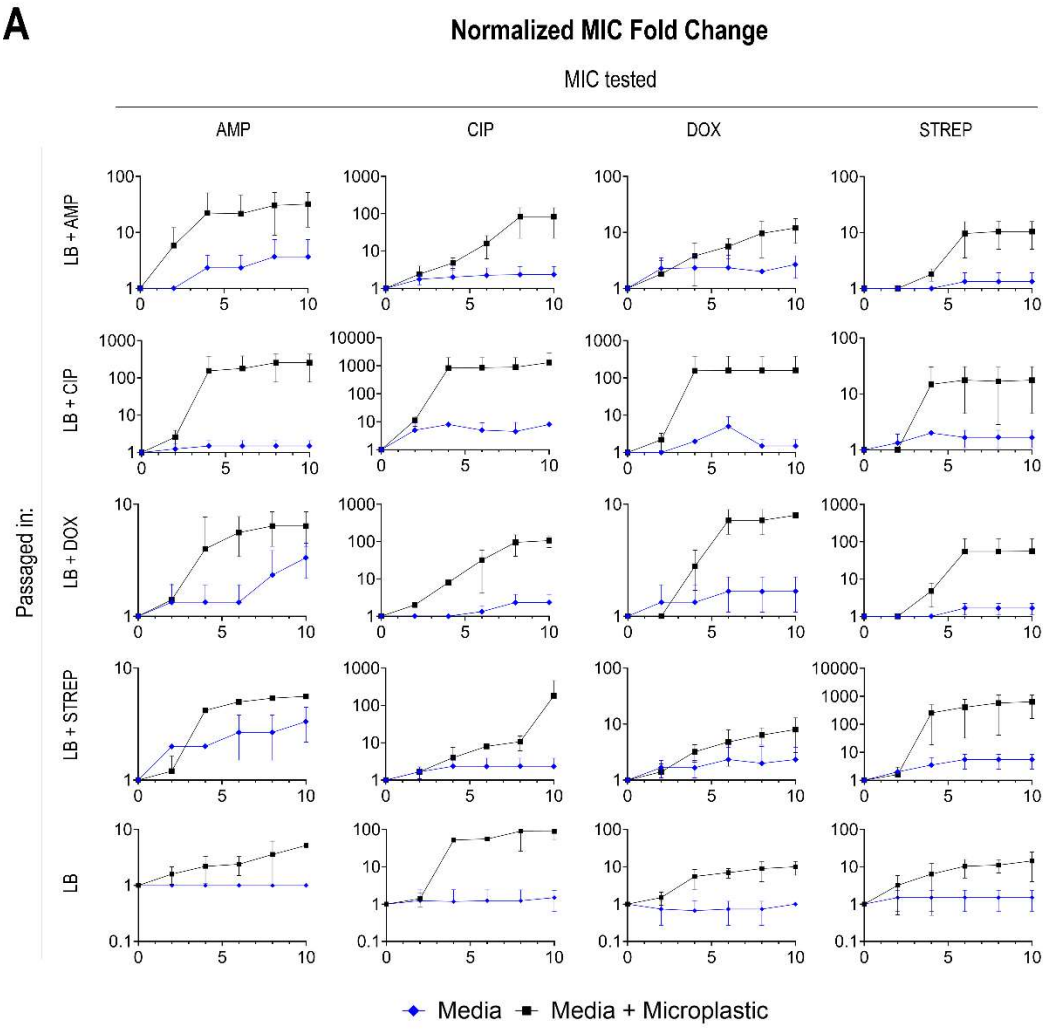
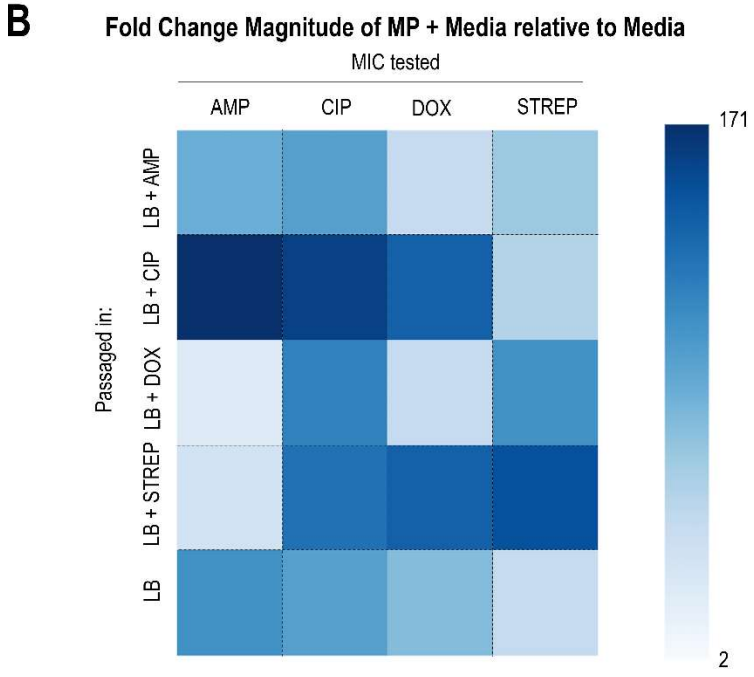
573 ***Figure 8.*** *Absolute ciprofloxacin MIC values for 5, 10, and 500-um diameter polystyrene MPs*
574 *with the dashed line indicating the ciprofloxacin clinical breakpoint concentration (A), time*

575 *series fold change of 5, 10, and 500-um diameter polystyrene MPs, and the subinhibitory*
576 *antibiotic control relative to the WT (B)*

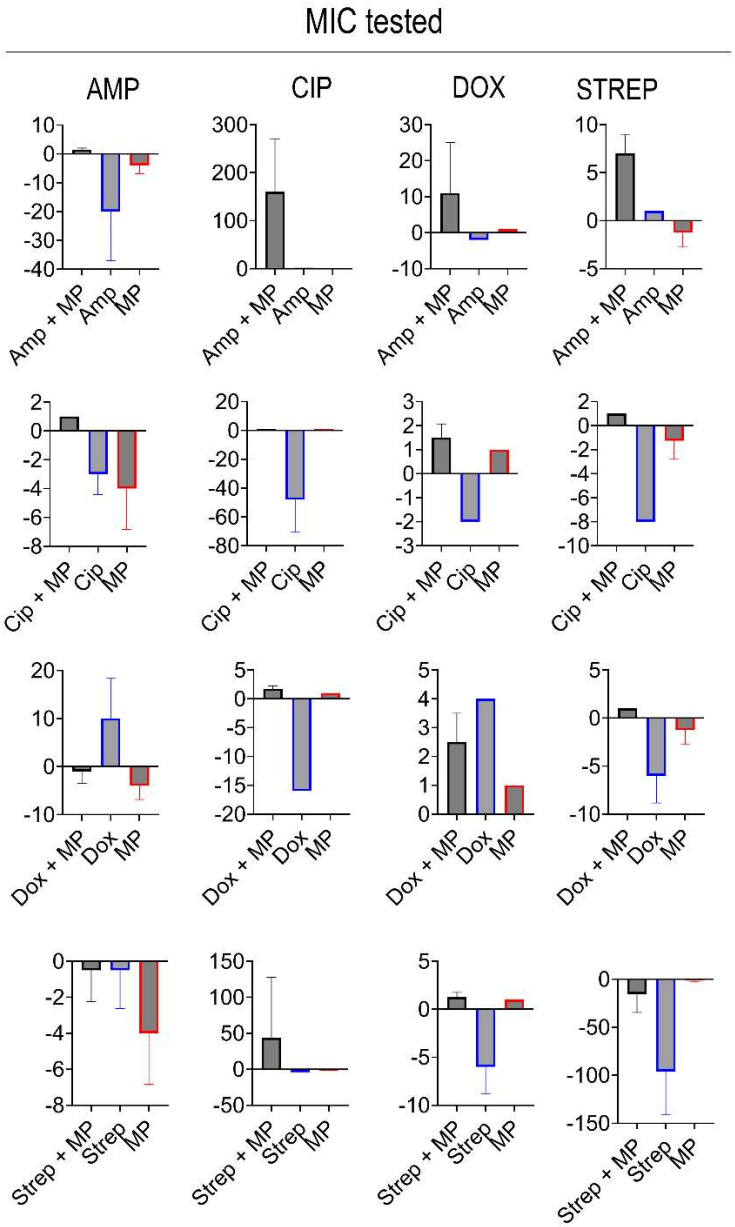
577





579 ***Figure 9.*** *Normalized 10-day time (x-axis) series fold change (y-axis) of 500-um diameter*
580 *polystyrene MPs, grown up in one of four antibiotics (ampicillin, ciprofloxacin, doxycycline, and*
581 *streptomycin) and then tested for the MIC in all four antibiotics (A), heatmap depicting the*
582 *magnitude of the fold change between microplastic + media and media (e.g. microplastic + LB*
583 *+ ampicillin relative to LB + ampicillin).*

584



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586 ***Figure 10.*** *Five-day resistance stability was measured in fold change (y-axis) relative to day 10*
587 *of the MDR study above. The bacteria grown with subinhibitory antibiotics + MP (black),*
588 *bacteria + subinhibitory antibiotics (blue), and bacteria + MPs (red) (x-axis) are shown.*