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

Neila Gross[1], Johnathan Muhvich[2], Carly Ching[2], Bridget Gomez[2], Yanina Nahum[3], and Muhammad H. Zaman[2, 3*]

Affiliations

[1]Department of Materials Science and Engineering, Boston University, Boston, MA 02215

[2]Department of Biomedical Engineering, Boston University, Boston, MA 02215

[3]Center on Forced Displacement, Boston University, 111 Cummington Mall, Boston, MA, 02215

*Corresponding author: ngross1@bu.edu

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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

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

119 **Methods**

120 Strains and culture conditions

121 *E. coli* MG1655 (ATCC 700926) was used in all experiments. All liquid cultures were grown in
122 Lysogeny Broth (Miller, LB) medium under shaking conditions at 180 rpm at 37 °C. MPs were
123 added first for a total of 48 hours to allow potential biofilm growth on the surface. Following the
124 initial MP exposure, subinhibitory levels of ampicillin (Sigma Aldrich, CAS No. 69-52-4),
125 ciprofloxacin (MP Biomedicals, CAS no. 86393-32-0), doxycycline (Sigma-Aldrich, CAS no.
126 24390-14-5), streptomycin (Sigma-Aldrich, CAS no. 3810-74-0) were added to the medium, as
127 indicated below.

128 Growth Curve Data

129 Spectrophotometer growth curves

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

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

146 CFU/mL growth

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

151 Antibiotic and MP susceptibility testing

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

160 Microplastic-antibiotic combination assays

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

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

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

177 Confocal Laser Scanning Microscopy (CLSM) image collection and analysis

178 Visualizing the cellular communities on the surface of the particles was done using confocal
179 microscopy. Using the cultures described in the susceptibility testing section, 500 µm diameter
180 polystyrene spheres were randomly selected out of each culture and dyed using the LIVE/DEAD
181 *Bac*Light Bacterial Viability Kit Protocol, per manufacturer instructions. Briefly, SYTO 9 was
182 used to analyze viable cells, as it can permeate all bacterial cell membranes. In contrast,
183 propidium iodide was used to count dead cells, as it can only enter cells with disrupted
184 membranes. Each plastic was put into a centrifuge tube with 1 mL of sterile water, and three µL
185 of staining solution (1.5 µL of each dye) was injected into the tube. Samples were incubated for
186 20 minutes at room temperature and protected from light.

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

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

199 Statistical Analysis

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

206 **Results**

207 **<u>Microplastics impact growth at high concentrations</u>**

208 Optical Density (OD) Readings

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

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

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

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

225 **Exposure to various polystyrene microplastic concentrations increases ciprofloxacin**
226 **resistance similarly.**

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

238 The concentrations of microplastics tested with the 500-um diameter polystyrene spheres were
239 40 MP/mL and 100 MP/mL. Conversely, the concentrations tested with the 10-um polystyrene

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

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

253 **Exposure to multiple different plastic compositions affects ciprofloxacin resistance.**

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

258 Previous studies have shown that bacteria have an affinity for plastic due to their high
259 hydrophobicity and oxidation, which can lead to easy adhesion.[31, 32] Of the three plastics used in
260 this study, polystyrene is the most hydrophilic, while polypropylene is the most hydrophobic.
261 We found that all three conditions with varying microplastic compositions had a significantly
262 higher MIC after ten days of exposure than the microplastic-free control exposed to just
263 subinhibitory levels of ciprofloxacin. While the control MIC fold change at day ten was
264 significantly lower than the samples exposed to the microplastics, the plastic compositions
265 themselves did not produce fold change values that were considered significantly different.
266 However, polystyrene/ciprofloxacin did reach a significantly different absolute MIC value
267 (0.00165 ± 0.000572 mg/mL) than polyethylene/ciprofloxacin (0.000743 ± 0.00036 mg/mL),
268 polypropylene/ciprofloxacin (0.000528 ± 0.000162 mg/mL) and the control of just ciprofloxacin
269 (0.00020625 ± 0.00004 mg/mL) (Figure 5, Table S3). Furthermore, the polystyrene
270 microplastics facilitated an absolute MIC higher than the ciprofloxacin-*E. coli* clinical breakpoint
271 (0.001 mg/mL).

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

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

275 We also studied the resistance development between polystyrene microplastic and glass spheres
276 in order to determine if plastic substrates had a specific effect or if any small particles would lead
277 to an increase in AMR. We tested 500-um diameter spheres at the same concentration of 40
278 MP/uL. The microparticles were exposed to subinhibitory levels of ciprofloxacin and compared
279 to a control, which only had the subinhibitory ciprofloxacin and no glass or plastic particles.
280 Interestingly, the glass facilitated a faster rate of resistance development but quickly stabilized at
281 a ciprofloxacin MIC of 0.00186 ± 0.00089 (Figure 6A, 6B, Table S.4). Conversely, the
282 polystyrene plastic spheres facilitated a statistically different MIC fold change and absolute MIC
283 value by the end of the ten-day study from its glass counterpart and antibiotic control. Both the
284 day 10 polystyrene and glass conditions crossed the ciprofloxacin-*E. coli* clinical breakpoint.
285 Notably, polystyrene surpassed the clinical breakpoint by 3X at 0.0037125 mg/mL (Table S.4)).
286 [21] The same experiment was performed with a smaller attachment surface of polystyrene 5 um-
287 diameter MPs and 3 um-diameter glass spheres (Figure 6C, 6D). The 10-um diameter particles
288 only yielded a significant difference from the antibiotic control in their ciprofloxacin absolute
289 MIC values (Figure 6C). However, when all sizes of particles (glass, MP 10-um diameter, and
290 glass, MP 500-um diameter) were compared, the 500 um-diameter MP significantly differed
291 from the glass particles of both sizes (Figure 6E), indicating that microplastics might be a unique
292 surface for bacteria to gain resistance.

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

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

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

303 **Exposure to microplastics of different sizes affects ciprofloxacin resistance development.**

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

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

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

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

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

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

344 After ten days of exposure to subinhibitory antibiotics, exposure to subinhibitory antibiotics was
345 halted, and bacteria were grown in antibiotic-free media for five days. The MIC was tested daily
346 to determine if resistance was stable (i.e., whether the mutations leading to resistance from day
347 10 of subinhibitory antibiotic exposure were stable). Results indicated that bacteria introduced to
348 microplastics were not only resistant past the clinical breakpoint[21] with the antibiotic they were

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

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

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

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

365 **Discussion and Conclusions**

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

375 Polystyrene, in particular, had the most significant impact on resistance development, which was
376 surprising given its relative hydrophilicity compared to polyethylene and polypropylene.
377 However, it is essential to note that studies have shown that *E. coli* prefers hydrophilic surfaces
378 over hydrophobic ones.[17] With this in mind, we expected glass beads of similar diameter to have
379 a higher adhesion rate and, therefore, a higher resistance to the tested antimicrobials. This,
380 however, was not the case, as polystyrene had a higher absolute MIC value and greater MIC fold
381 change over the glass condition. This indicates that plastics may be a unique substrate for
382 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.

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CRediT Statement:

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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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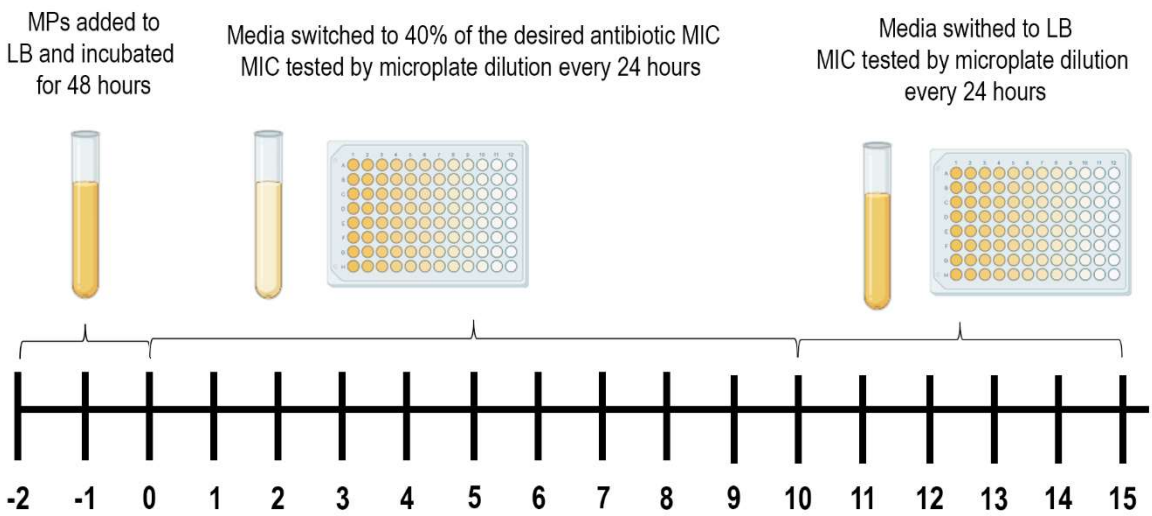
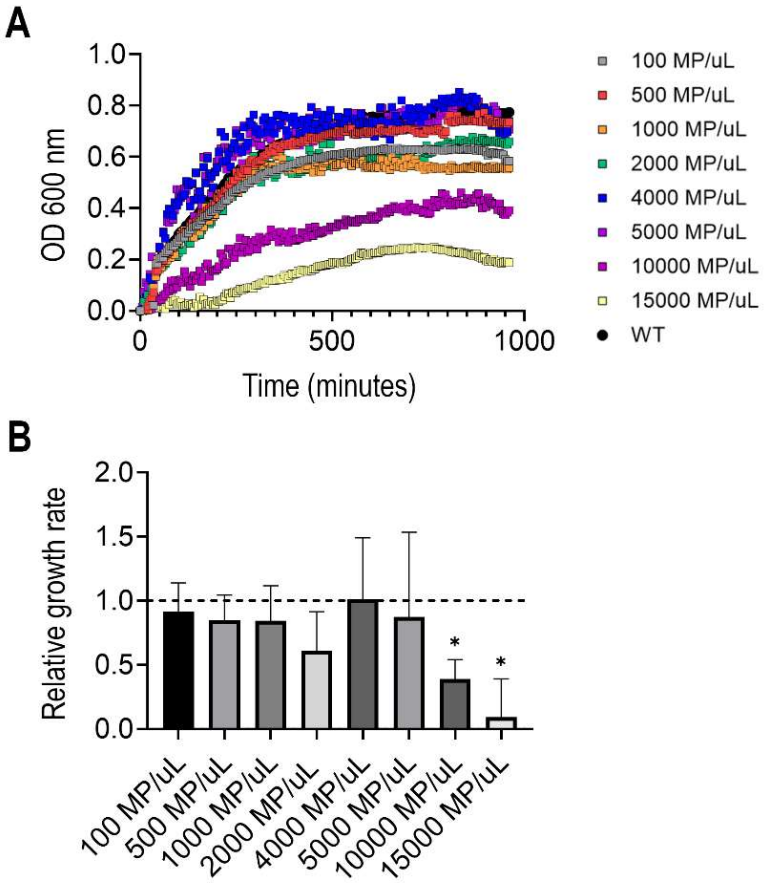
Figures:



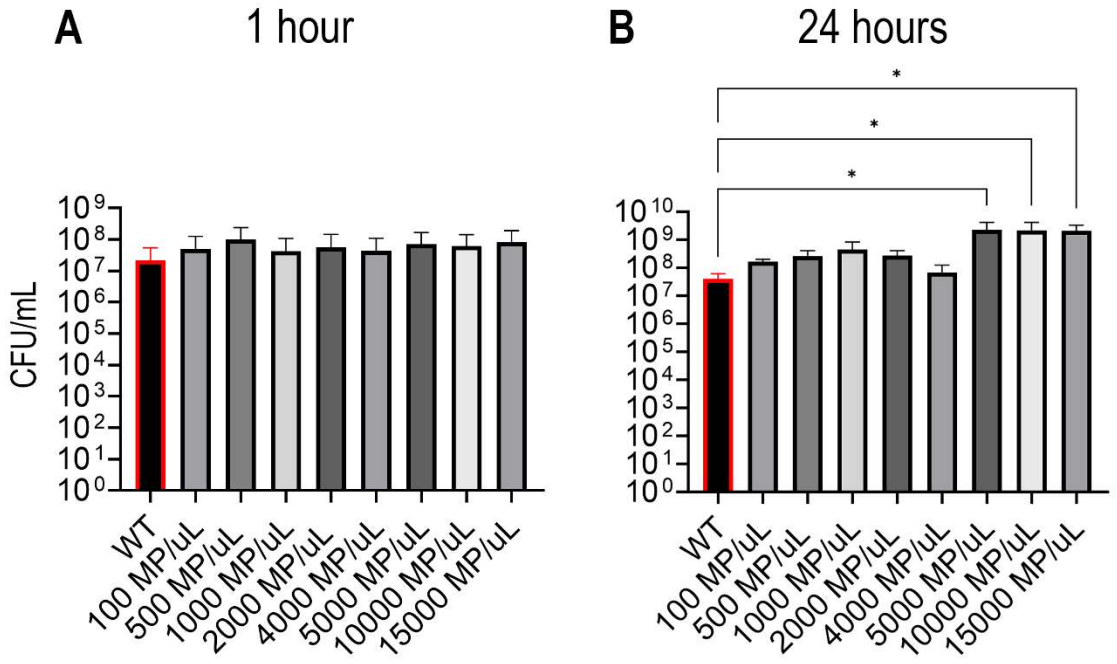
535

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



539

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



544

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

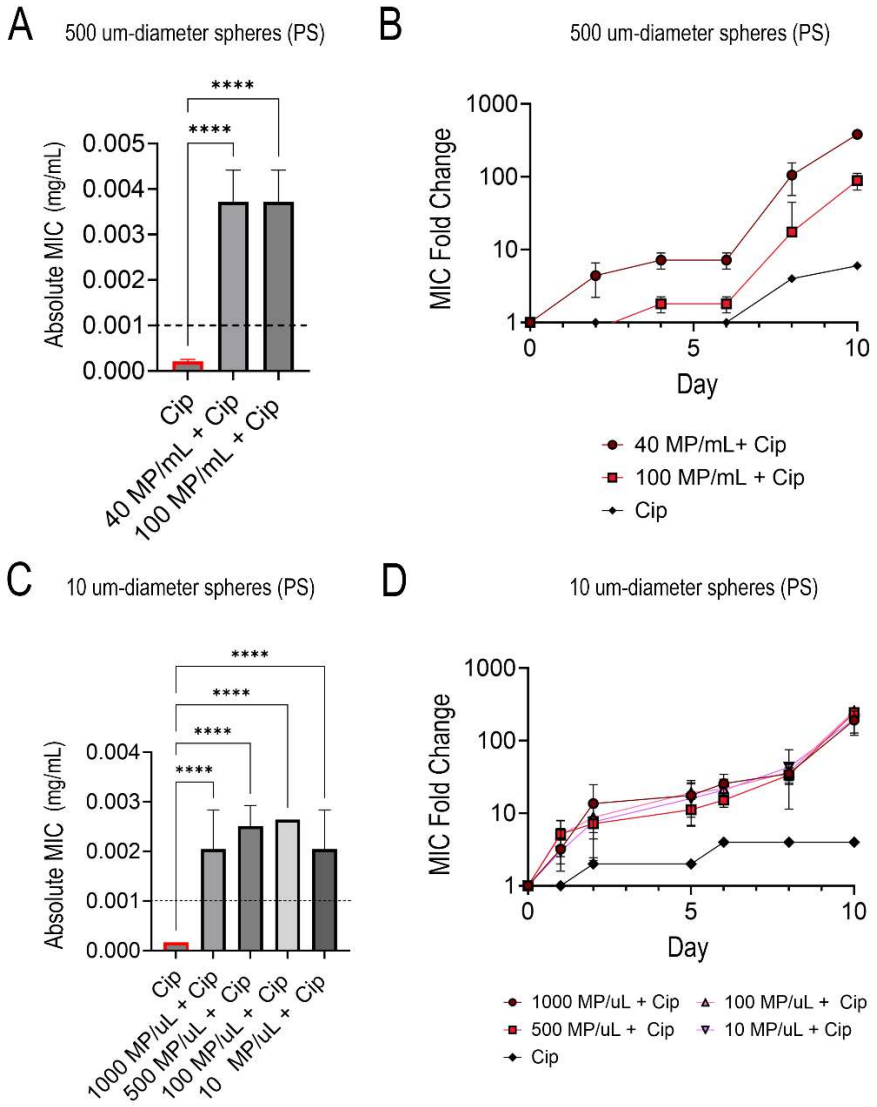
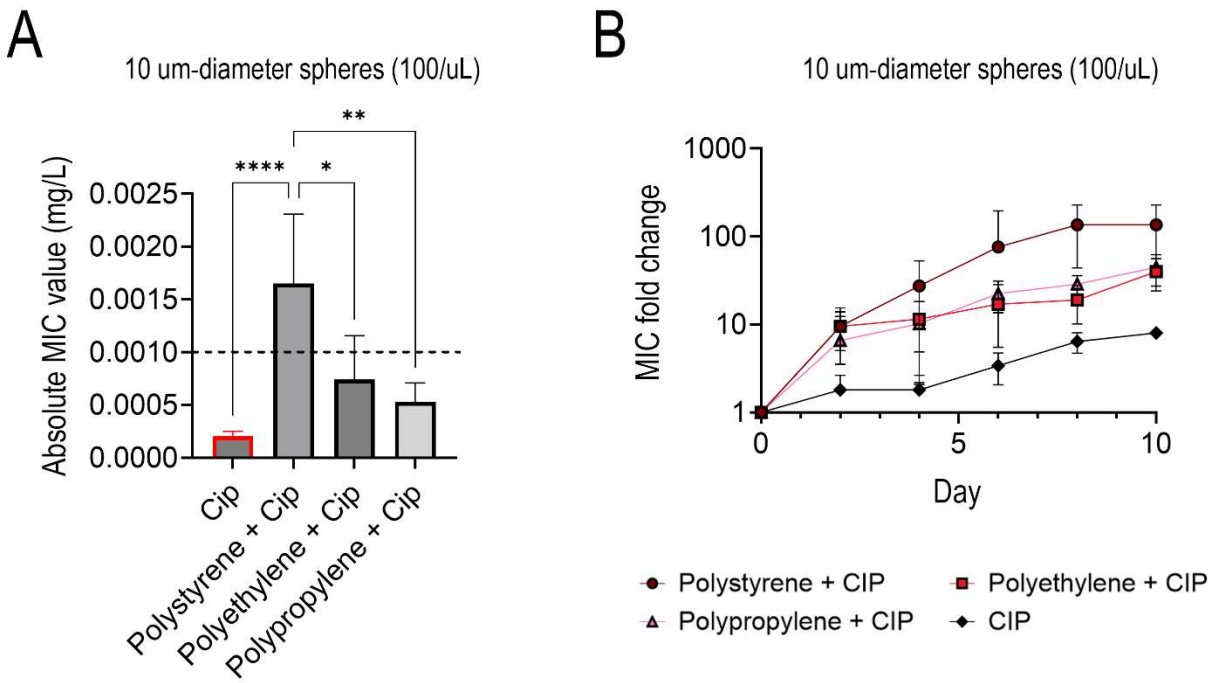


Figure 4. *Absolute ciprofloxacin MIC values for 500-um diameter polystyrene MPs at two different MP concentrations with the dashed line indicating the ciprofloxacin clinical breakpoint concentration (A), time series fold change of 500-um diameter polystyrene MPs at different MP concentrations and the 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)*



557

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

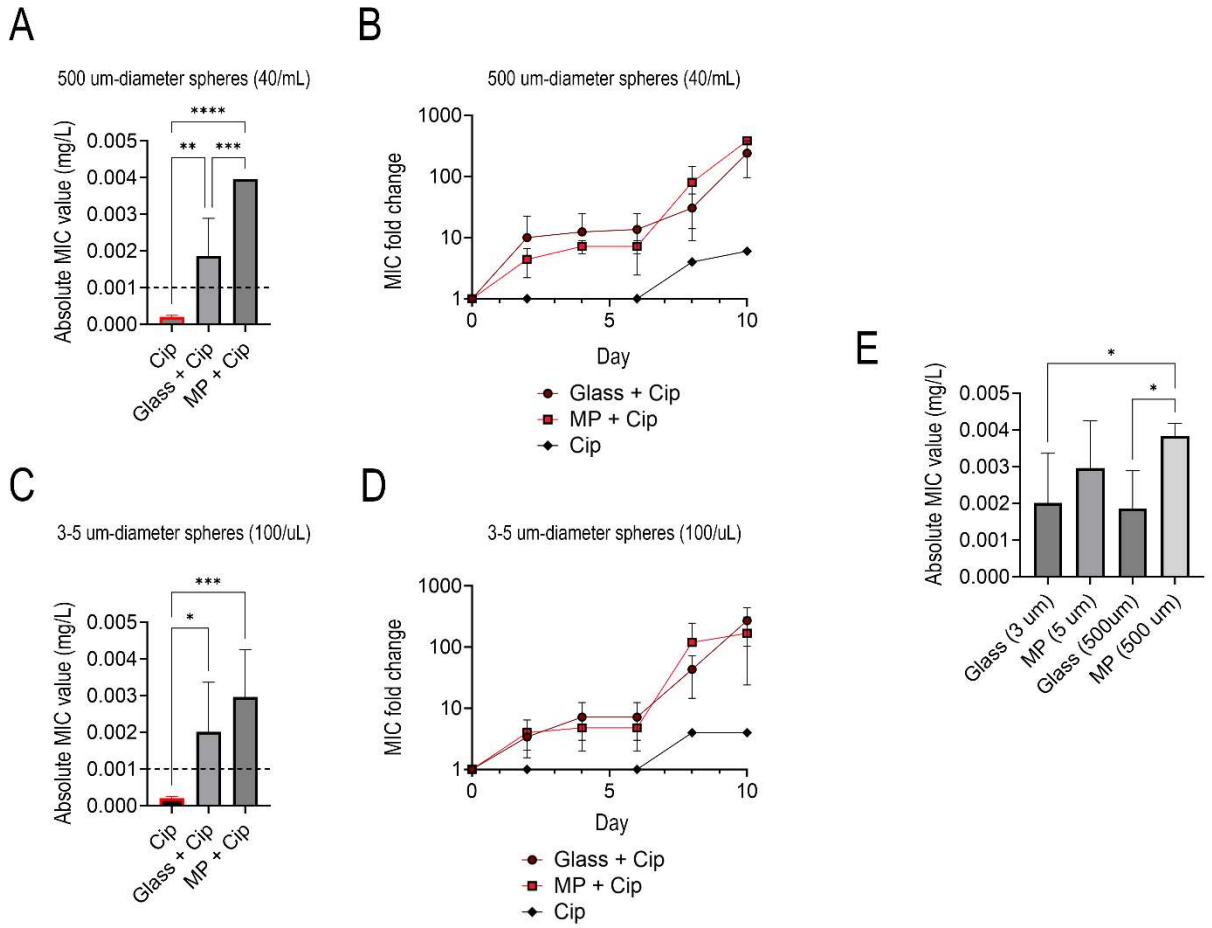
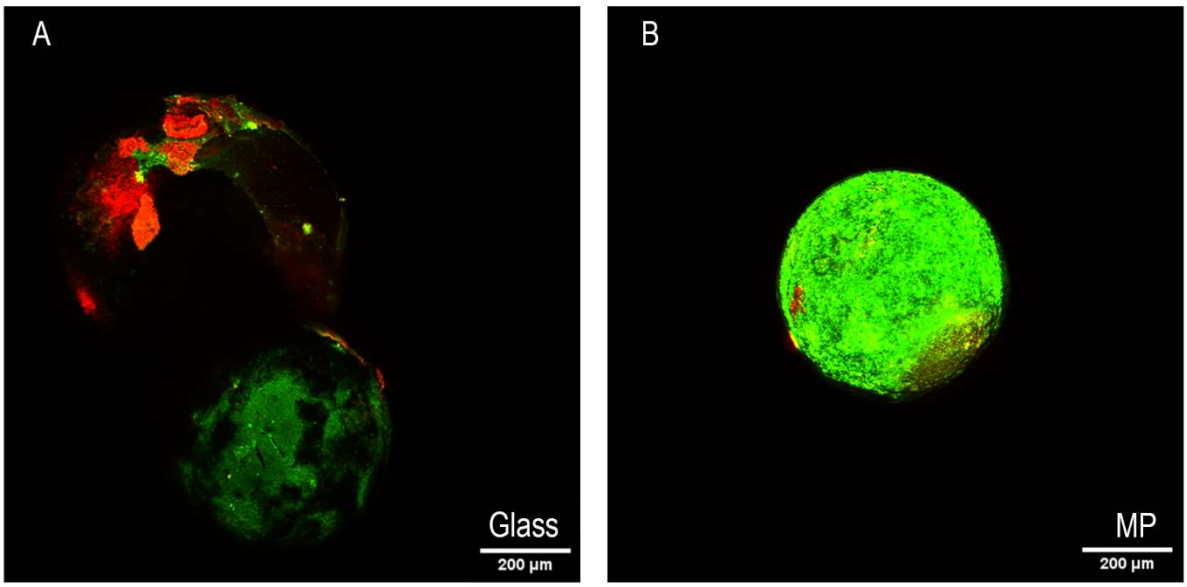
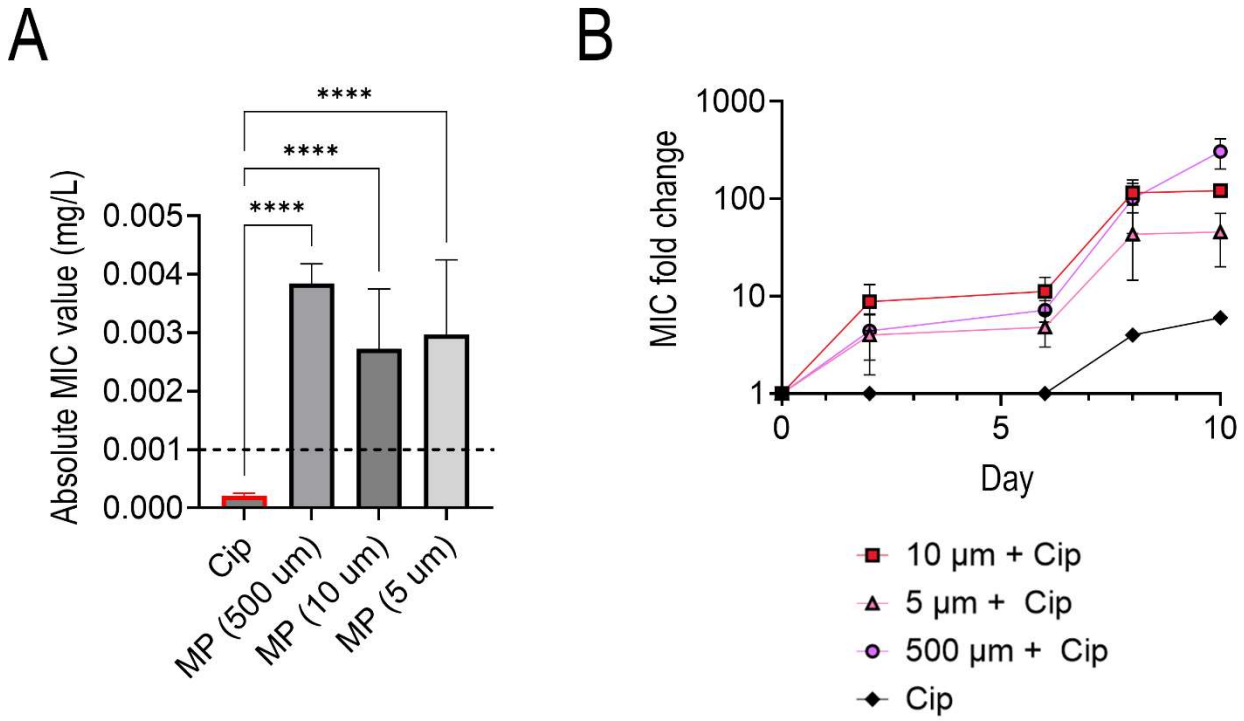


Figure 6. *Absolute ciprofloxacin MIC values for 500-um diameter and 10-um diameter polystyrene and glass spheres at 40 particles/mL (6A) and 100 particles/uL (6C), respectively, with the dashed line indicating the ciprofloxacin clinical breakpoint concentration. Time series fold change of the 500-um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



569

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



572

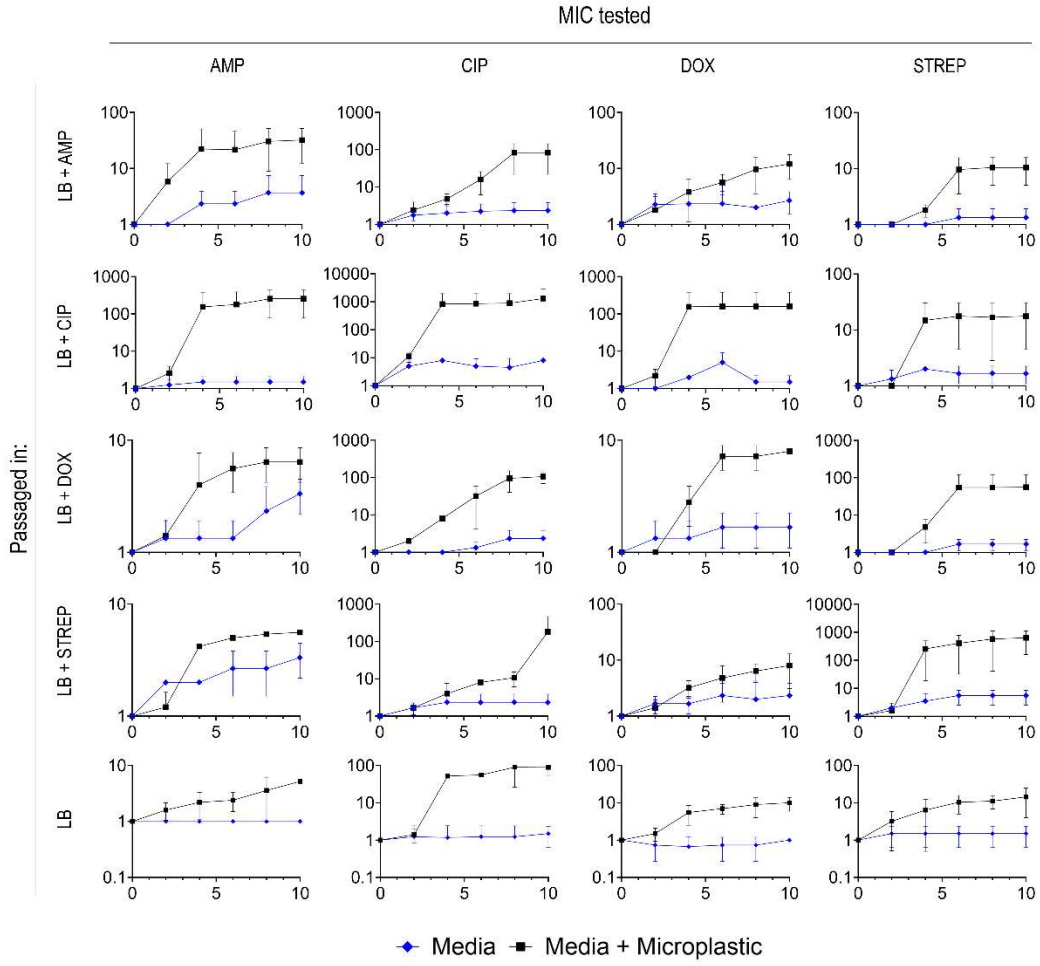
573 ***Figure 8.*** *Absolute ciprofloxacin MIC values for 5, 10, and 500-um diameter polystyrene MPs*
574 *with the dashed line indicating the ciprofloxacin clinical breakpoint concentration (A), time*

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

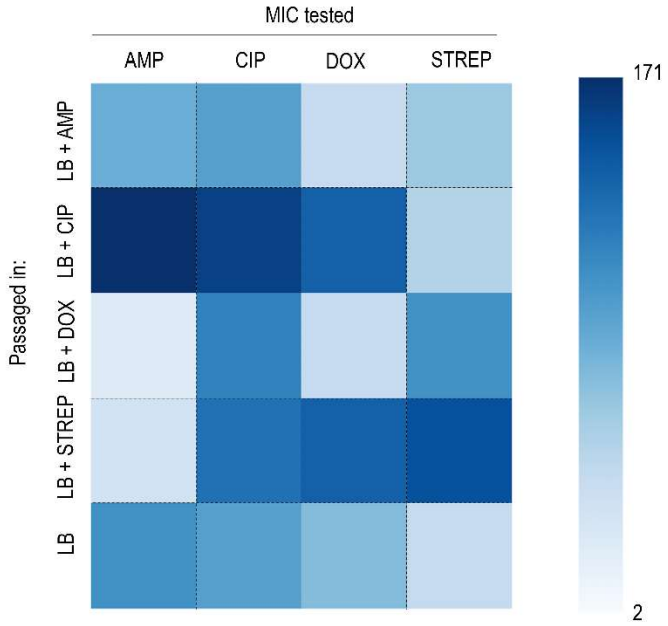
A

Normalized MIC Fold Change



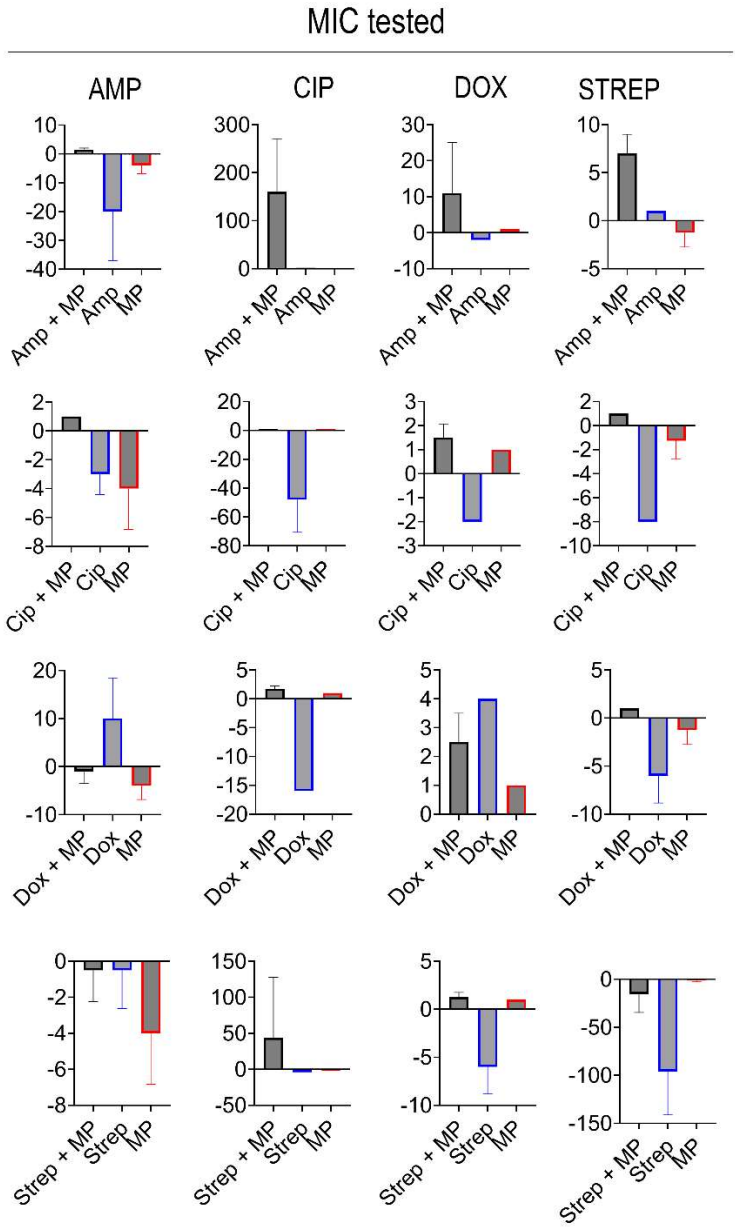
B

Fold Change Magnitude of MP + Media relative to Media



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



585

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.*