Environmental Microbiome

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Microbial composition on microplastics mediated by stream impairment

Anne L. Gilewski[1,2*], Saurav Shrestha[1], Sharon N. Kahara[1] and Nikolas M. Stasulli[1]

Abstract

Background Studies into biofilm interactions with microplastic polymers in marine environments are widespread in the literature. Increasing evidence suggests that lotic microplastics are a significant contributor and may accumulate harmful or pathogenic organisms, thereby contributing to the degradation of marine ecosystems where they meet riverine systems. Suboptimal water quality of these riverine systems may influence these biomes. This project compared the microbial diversity of biofilms that developed on microplastics to natural stone substrates in an impaired and unimpaired section of the Quinnipiac River Watershed. In this project, the influence of impairment was studied based on microbial diversity via 16S rRNA gene sequencing while monitoring total colony and fecal coliform colony counts using standard water sampling methods.

Results Total coliform colony counts were greater in the impaired Quinnipiac River site than in the unimpaired Honeypot Brook tributary and on the microplastic substrate than the stone substrate. Sequenced features to the class level were dominated by *Alphaproteobacteria, Betaproteobacteria,* and *Gammaproteobacteria,* comprising 75% of the community biome. Simpson's Diversity indices indicated that within the two substrates, there was little variation between the communities. However, it was noted that microplastic alpha diversity trended slightly lower than the stone. Further analysis of common aquatic enteropathogens showed that the genus *Citrobacter* was significantly more abundant on the microplastics at both locations.

Conclusions Our results indicate impaired waterbodies with a microplastic burden may retain greater fecal coliform bacterial loads than unimpaired waterbodies. Increased microplastic loads in compromised lotic systems may have an additive impact. Water quality remediation and careful monitoring are recommended to reduce this effect. Comparing this study with environmental community analysis could provide valuable insight into preferential surface attachment of bacteria onto microplastic.

Keywords Microplastic, Biofilm, 16S rRNA gene, Freshwater, Pollution, Coliform, Riverine, Stream, Biome, Impairment

Introduction

A growing body of research in the last decade has focused on the impact of freshwater microplastic burden to elucidate the contribution lotic system pathways have on the inland and marine environments [5, 19, 41]. Freshwater abundance is similar to that found in marine waterbodies [20]. An estimated 80% of microplastics are introduced into marine systems from land, enhanced by river transport [37]. Significant sources of microplastic input arise from ground runoff and wastewater treatment plants (WWTPs) [9, 16, 24, 26].

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When a microplastic particle enters an environment, surface colonization by pioneer organisms begins immediately upon discharge [40]. The subsequent secretion of extracellular polymeric substances by bacteria that have adhered to the microplastics form a distinct biome that alters the topography and density of the microparticle as it ages [13, 17, 21, 40]. Like marine plastics, freshwater particle biomes preferentially select from the surrounding environment due to the novel substrate surface compared to natural substrates [26, 50]. Specific environmental conditions, such as nutrient levels, salinity, pH [32], geography and seasonality [23, 51], and the substrate surface itself [40], are also integral in the selection for organisms that promote microplastic primary or secondary succession. Knowledge gaps exist regarding identifying a core microorganismal community and the relevant increase or decrease in microplastic microbiome biodiversity versus naturally found substrates [1, 5].

The Quinnipiac River Watershed encompasses 430 square kilometers (km^2) of eight sub-watersheds that drain into a 38-mile urban river artery. The watershed originates in wetland Deadwood Swamp at the border of Plainville and Farmville, Connecticut, and terminates in New Haven Harbor [7]. Point and non-point sources of pollution have historically been an issue. As of the 2010 State of the Watershed report, 75 miles of rivers and streams in the watershed were impacted by enterobacterial pollution, with 20.8 miles affected by polychlorinated biphenyl (PCB) compounds [7]. The presence of known endocrine disrupters has been found in this waterway since 2022 [10]. Microplastic pollution is persistent in the watershed. A 2020 Quinnipiac River Fund Final Report reported that the river discharges 272 million microplastics annually at the Meriden wastewater treatment plant (WWTP) and 72 million particles from the North Haven plant, with the greatest concentrations seen in decreasing temperatures [6].

Colonization within wastewater treatment systems before discharge can protect the developing biomass as these organisms are not subjected to natural grazers [9, 16]. Human-associated pathogenic microorganism attachment has also been demonstrated to preferentially select microplastics after secondary and tertiary wastewater treatment by offering a favorable, low-biodegradable surface compared to organic particles [18]. Microplastics may have an additive effect in impaired waterbodies, increasing their potential for sequestration of harmful or pathogenic organisms [43].

This project aimed to examine microbial community differences in an anthropogenically-introduced substrate with that of stone typically found in the environment and the influence of waterbody impairment. We hypothesized that the polypropylene microplastic would form a distinct biofilm different from that of the stone substrate at the impaired sampling site compared to the non-impaired site, relative to the Cheshire, Connecticut WWTP, thus increasing the role of microbiome development in polluted waters. We sought first to identify any preferential coliform colony accumulation on the microplastic substrate over the stone through selective culturing and, second, assign microbial phylogeny to describe the substrate communities based on alpha and beta diversity indices.

Materials and methods

Site selection

Study locations were chosen based on water quality classification and human use criteria outlined in the Quinnipiac River Watershed 2012 water quality assessment (QRWA, 2013). Sites were either 'fully impaired' or 'unimpaired' and supported one or more of the following categories: recreational activities, aquatic life, or fish consumption. Honeypot Brook (Cheshire Park, 1000 Highland Avenue, Cheshire, CT 06140) was the unimpaired deployment site. The Quinnipiac River (Quinnipiac Park River Walk and Canoe Launch/Treatment Plant, 1325 Cheshire Street, Cheshire, CT 06140) served as the impaired deployment site (Fig. 1).

Cage deployment and biome development

To test differences in biofilm development between microplastic and stone substrates, 10 individual replicate biomes were created by suspending substrate-filled, 3-inch, household tea infusers (Thunder Group®, City of Industry, CA 91748) wired shut with enameled floral wire (Hillman™, Tempe, Arizona, 85284). The infusers were suspended in two rows of 5, secured with a 150-pound strength fishing line (Reaction Tackle, Big Bend, Wisconsin, 53103). The cage from which they were suspended was constructed from schedule 40 PVC1/2″–1″ piping assembled in a cube conformation and sand-weighted to rest on the sediment bed. This approach was similar to one that Magadini et al. [22] employed, allowing constant water flow-through and contact with the substrates (Fig. 2).

Commercially made 3 mm polypropylene plastic pellets (Polly Plastics™ Polypropylene beads, Midland, Michigan, 48642) were used as the 'microplastic substrate'. The natural stone substrate was collected from the unimpaired Honeypot Brook location and sifted through stainless steel 4 mm and 2 mm field sieves to capture a 3 mm size comparable to the plastic pellets. Both microplastic and stone substrates were soaked in 70% isopropyl alcohol (Fisher Chemical, Fair Lawn, New Jersey, 07410) for 1 h and dried thoroughly to ensure the initial substrate surface was disinfected. Each tea infuser was wet-weighed using

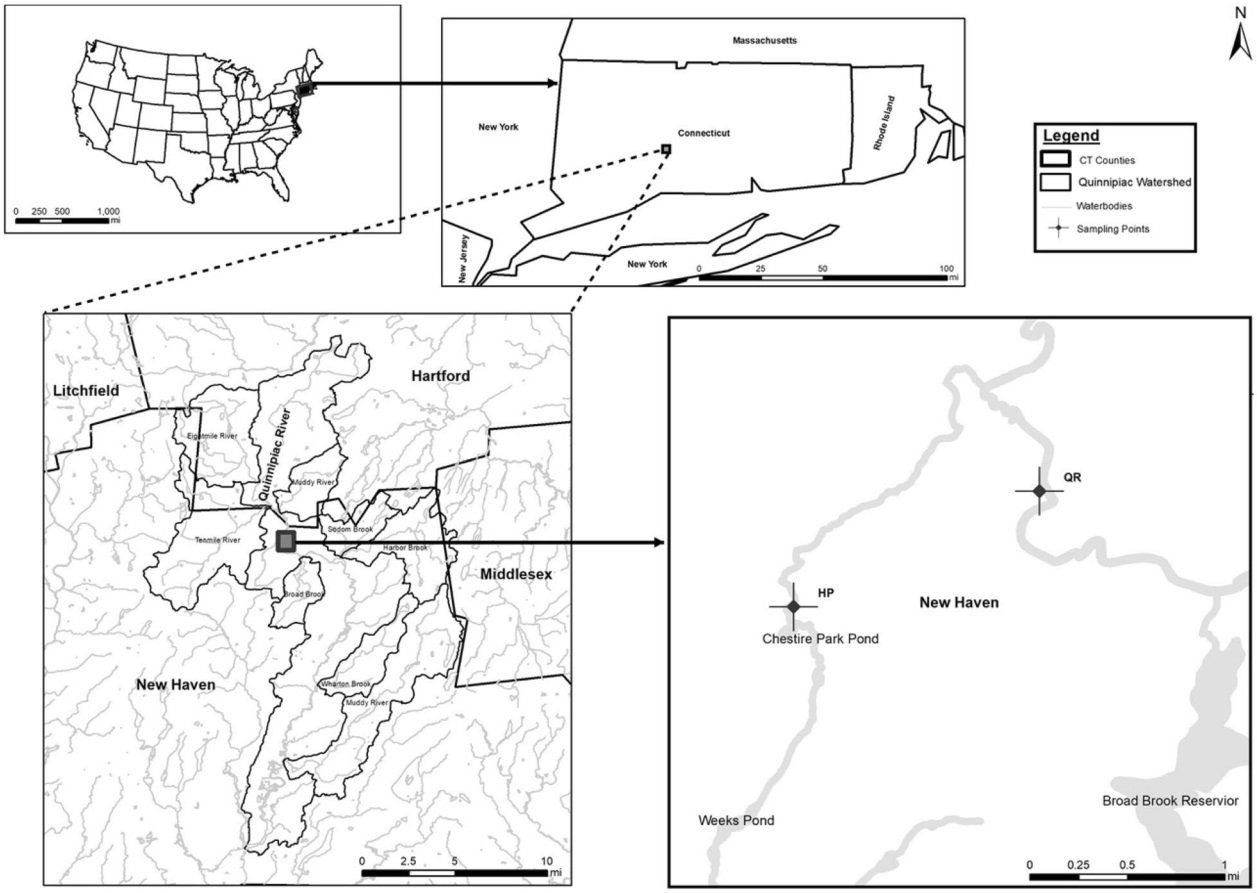

Fig. 1 Map of study area location of the unimpaired Honeypot Brook (HP) and impaired Quinnipiac River (QR) locations. Image: S. Shrestha

Fig. 2 Cage construction using schedule 1/2–1″ 40 PVC piping threaded with 3-in stainless steel tea infusers on 150-pound fishing line. Photo: A. Gilewski

sterile, deionized water on an analytical balance (Mettler Toledo, Cole-Palmer®, Vernon Hills, Illinois 60061) and then dried for 10 s. Two tablespoons of stone or microplastic substrate were added, dipped, and allowed to drip dry for 10 s. The resulting wet weight of the infuser and substrate was recorded. Five infusers of each substrate (10 total) were held as non-deployed controls.

Each tea infuser was notch-coded with a wire cutter according to sample site, substrate, and replicate number. Each set was identified on the cage using colored zip-ties. Two frames per site were tethered with zip-ties and submerged in the water column. The weighted frames were tied to reinforcing bars at the waterbody bank and identified with signage.

Weekly visits were made during deployment to ensure cages remained submerged and intact. Ten replicates from each substrate were harvested on days 30, 60, and 90 from May to August 2023 at each site. The samples were removed from the launch frame using nitrile gloves (Medline FitGuard® Touch, Northfield, Illinois, 60093), drip-dried for 10 s, and individually weighed in clean weigh boats on a field analytical scale (OHAUS® Navigator™, Parsippany, New Jersey, 07054). Samples were placed in a Whirl-Pak sample bag (Whirl-Pak®, Fort Atkinson, Wisconsin, 53538) and transferred to ice in a

cooler before transport to the University of New Haven for biofilm harvest.

At the Day 60 harvest date, it was noted that the sample cage at the unimpaired Honeypot Brook location was removed from the brook. A previous visit a week prior confirmed the position, and it was unclear how long the cage had been out of the water. The cage was replaced in the brook and allowed to soak for several minutes to rehydrate potentially desiccated biomass. The extent of biofilm disruption due to remaining out of the brook is unknown, though sequencing data did not indicate any significant reduction in feature counts.

It is also suspected that by Day 90, the accumulated detritus on the exterior of the infuser may have reduced flow-through to the substrate itself but would have affected both substrates similarly.

Water quality and sample collection

Site water collection followed Method A, as described by the Environmental Protection Agency's protocol for proper sampling (EPA, 2012). Three in-water meter readings were collected downstream of the deployed apparatus. Dissolved oxygen (DO) in milligrams/liter (mg/L) and salinity in parts per thousand (ppt) were measured via YSI Conductivity, Optical Dissolved Oxygen meters (YSI Pro30 Conductivity Meter; YSI ProODO Optical Dissolved Oxygen Instrument, YSI Inc., Yellow Springs, Ohio 45387). pH was measured with an Orion Star handheld meter (Thermo-Fisher Scientific™ Orion Star A121, Waltham, Massachusetts, 02451).

A total of 18 water samples (6 per site×3 sampling periods) were taken and processed for nitrate (YSI Nitratest, YPM163) in parts per million (ppm) and phosphate (YSI Phosphate LR, YPM177) in ppm analysis using a YSI EcoSense® 9500 Photometer (YSI Inc., Yellow Springs, OH 45387). Results were recorded in Microsoft Excel (Microsoft® Excel for Mac, Microsoft Corporation, version 16.82).

Selective media preparation and plating

Replicate plates of Brilliance™ *E. coli*/fecal coliform selective media (Brilliance™ agar CM0956, Thermo-Fisher™ Scientific, Waltham, Massachusetts, 02451) were prepared using sterilized nanopure water (Barnstead E-Pure Water Purification System, Thermo-Fisher™ Scientific, Waltham, Massachusetts, 02451) per manufacturer's instructions. To control fungal overgrowth, the media was treated with Amphotericin-B (A4888-1G, Sigma® Life Sciences, St. Louis, Missouri, 63103) prepared as 0.1 g in 10 mL dimethylsulfoxide (BP231, Fisher Bioreagents, Thermo-Fisher™ Scientific, Waltham, Massachusetts, 02451), filtered sterilized through a 0.22 µm (µm) pore size filter (Millex®, Duluth, Georgia, 30097), then added as 1 mL/1 L of liquid media. Approximately 25 mL of sterilized media was added to Petri plates (Fisherbrand™, Thermo-Fisher™ Scientific, Waltham, Massachusetts, 02451) and dried under a laminar flow hood for 24 h. Several plates were reserved for quality-control testing with known Gram-positive and Gram-negative bacteria: *Lactococcus lactis*, *Staphylococcus aureus*, *Clostridium sporogenes*, *Escherichia coli*, *Klebsiella pneumoniae*, *Alcaligenes faecalis*, *Salmonella typhimurium*, and *Enterobacter aerogenes*. The plates were incubated at 30 °C for 24 h. No growth was observed on the Gram-positive streaked plates, which is expected of this media per manufacturer instructions. *E. coli* was confirmed to have dark purple colonies, while *K. pneumoniae* and *E. aerogenes* grew bright pink colonies; both results confirmed that the media was prepared correctly with appropriate chromatic changes.

Accumulated biomass harvest

The non-deployed control replicate and deployed replicate infusers were emptied within the Whirl-Pak bag, rinsed with the open tea infuser with 10 mL of sterile saline, and agitated for 30 s. A 1 mL aliquot of this 'biomass solution' was used for tenfold serial dilutions and plating. From each dilution, 200 µL were plated on duplicate plates as detailed in similar water quality testing methodologies [2, 14], then agitated for six-quarter turns using 8–10 2 mm autoclaved borosilicate glass beads. The plates were incubated at 30 °C (VWR™, Radnor, PA 19087) for a minimum of 12 h and up to 24 h for color development. Plates with heavy overgrowth were labeled 'Too Numerous to Count' and not included in the final dataset. Purple and pink colonies were counted using a wide-field stereo microscope. Results were recorded on an Excel spreadsheet.

Substrate DNA extraction

Each replicate's remaining 9 mL of biomass solution was centrifuged for 15 min at 10,000×*g* to pellet the sample (Eppendorf® Centrifuge 5804 R, Enfield, Connecticut 06082). The resultant supernatant was removed, and the pellet was placed in a −80 C freezer until the following day. After thawing at room temperature, DNA was extracted using the Qiagen© DNeasy PowerSoil® Pro Kit (Kit 384, Qiagen© LLC, Germantown, Maryland 20,874) per manufacturer instructions with the modification of using 250 µl (µL) of the pelleted biomass solution in place of 250 µL of soil. One µL of the extracted DNA was analyzed for purity via A_{260}/A_{280} absorbance ratio using a Nanodrop™ One^c Microvolume UV–Vis spectrophotometer (Thermo-Fisher Scientific™, Waltham,

Massachusetts, 02451). The extracted DNA samples were banked in a freezer at − 20 °C until the study period concluded.

Library preparation and real-time basecalling

Extracted DNA samples were thawed in batches according to the harvest date and randomly selected for processing. Genomic libraries were generated using the Oxford® Nanopore 16S rRNA sequencing kit using Kit 9 chemistry (SQK-16S024, Oxford Nanopore Technologies, Oxford Science Park, UK, OX4 4DQ) per the manufacturer instructions using all recommended consumables and reagents. DNA was amplified via a Bio-Rad T100™ thermocycler (Bio-Rad, Hercules, California, 94547) per protocol cycling conditions. DNA quantification of the eluted samples was performed using a Qubit 4 fluorometer (Invitrogen by Thermo-Fisher Scientific™, Waltham, Massachusetts, 02451) with the dsDNA HS Assay Kit (Ref. Q32851, Invitrogen by Thermo-Fisher Scientific™, Life Technologies Corporation, Eugene, Oregon, 97402). Samples with less than 200 nanograms (ng) of library DNA were repeated under a new barcode array. Up to 24 barcoded samples from the same plate were pooled and frozen at − 20 °C.

Real-time basecalling and demultiplexing of each pool were performed on the MinION Mk1c sequencing unit (MC-115173, Oxford Nanopore Technologies, Oxford Science Park, UK, OX4 4DQ) using the FLO-MIN106 flow cell (R9.4.1, Oxford Nanopore Technologies, Oxford Science Park, UK, OX4 4DQ). The minimum desired target was 15,000 reads per barcode with a total pool read of 5 million. Sequencing runs for each pool were approximately 22–24 h. Barcodes created in processing error, repeated or had less than 200 ng of genetic material have been excluded. Pass/fail basecall data per pool can be found in Table S1. Concatenated fastq files and study metadata can be found on the NCBI repository under BioProject ID PRJNA1096657. All relevant analysis and plotting scripts can be found on GitHub.com, annegilewski/freshwater-microplastics.

Downstream analysis was performed utilizing the MetONTIIME pipeline [25] developed for long-read analysis in the QIIME 2 [4] environment with the Docker (version 4.27.1) [27] container interface. Taxonomic identification was assigned using the BioProject 33175 (NCBI, PRJNA3317, 2008) reference database with the VSEARCH [38] classifier. The maximum number of reads per sample was set at 15,000 with a base pair minimum of 1000 and a maximum of 2000 to ensure coverage of the 16S region. Confidence in the feature identity to define the phylum taxonomic level is 80%, for genus 95%, and for species 97%. [48]. De Novo clustering was set at 90% to identify the Family taxonomic level. The minimum consensus for a match was set at 70%, with a minimum alignment identity of 80%. The maximum taxonomic level for identity was set to 6 (the level of genus). The complete table of the modified MetONTIIME pipeline parameters is in Table S2. Due to the size of the fastq output files, pooled barcodes were batched up to six at a time. Absolute frequency feature tables were separated by taxonomic level 2–6 corresponding to Phylum through Genus. These tables were then merged and filtered in the QIIME2 command line. Features that contained > 10 frequency hits across samples and were present in > 3 samples were retained for analysis. All plots were prepared in R (version 4.2.2) [36] and RStudio (version 2022.7.2.576) [39] using the following packages: *dplyr* [45], *tidyr* [44], *ggplot2* [47], *ggpubr* [15], and *stringr* [46].

Results

The total number of samples for this study was 140: 120 deployed samples, 10 non-deployed substrate controls, and 10 empty infuser controls. Following the sequencing data filtration described above, the final dataset represented 108 16S rRNA gene-sequenced microbiome samples. For further analysis, any rare features that appeared in fewer than 3 samples with fewer than 10 total reads were removed to mitigate potential amplification and sequencing errors.

Water sampling

All samples taken during the study period were "dry", meaning there was less than 0.1″–2.0″ of precipitation in the previous 96 h (EPA, 2008). Average temperatures at both sites were identical, with comparable salinity values. The pH remained static at the unimpaired Honeypot Brook site (HP) (~ 6.6), while the impaired Quinnipiac River site (QR) experienced a peak in July (~ 7.1). Dissolved oxygen was also consistently lower at the impaired site than unimpaired, with the lowest value (~ 85%) at the Day 60 sampling point. Nitrate and phosphate of both sites were within a similar range, except for a possible phosphate spike at the unimpaired site at Day 60. Four of the six samples tested out of range (> 4.0 ppm), possibly due to poor water column mixing at those sample sites or from an incidental pulse dose from a fertilization application from the surrounding neighborhood. A summary table of the averaged water quality sampling data can be found in Table S3.

Microbiome community assemblage

Taxonomic distribution by class was visualized for the top 10 most abundant organisms per sample by percent relative abundance for site and substrate. (Fig. 3). Among the 87 classes of bacteria present, 70% of the reads were

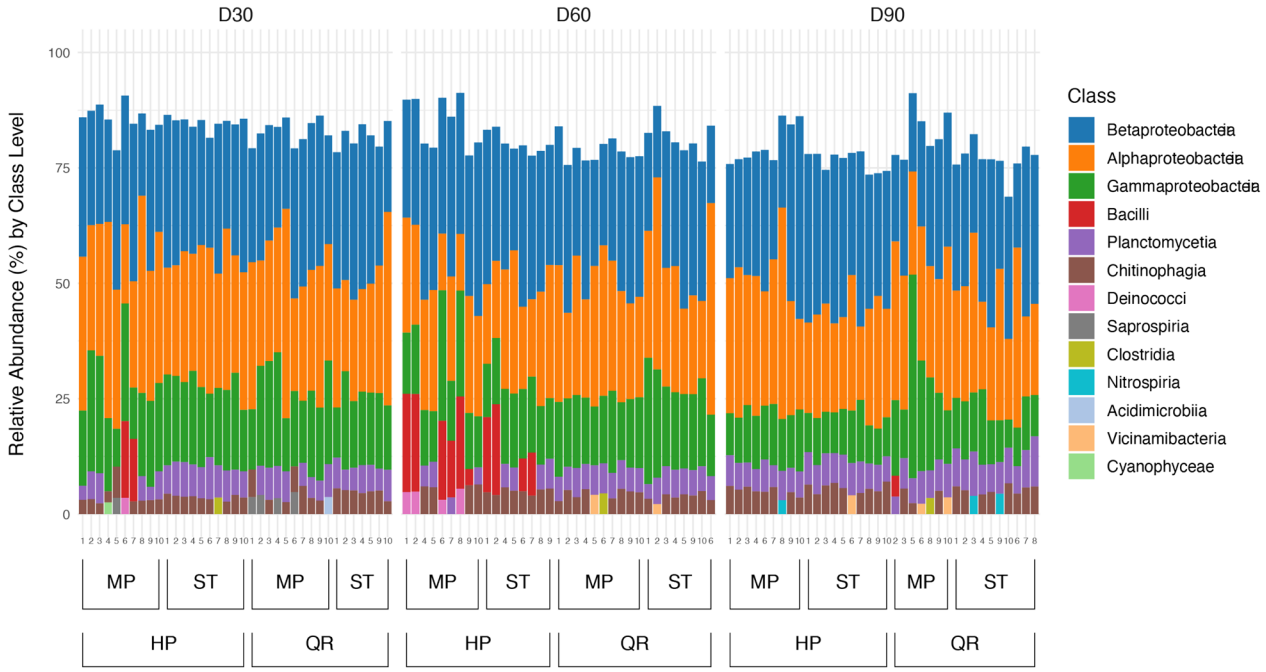

Fig. 3 Total relative abundance (percent) of top classes on microplastic (MP) and stone (ST) substrate at the impaired (QR) and impaired (HP) sites over the sampling period. Each bar represents one replicate sample, n = 108

represented by the *Pseudomonadota* phyla: *Alphaproteobacteria* (μ = 26.3%), *Betaproteobacteria* (μ = 28.4%), and *Gammaproteobacteria* (μ = 15.3%).

Other noted findings are the rise of *Bacilli* and subsequent decrease in *Planctomycetia* in twelve samples (Day 30, n = 2) and (Day 60, n = 10) at the unimpaired location on both microplastic (n = 8) and stone (n = 4). Additionally, *Clostridia* was found in three samples (2 microplastic and 1 stone) and present at each sampling point.

Alpha and beta diversity

Standard Simpson's diversity indices were created in the QIIME2 command line for the family taxonomic level. Using a jitter plot to visualize sample clustering on the microplastic and stone substrates with respect to the site, the unimpaired site microplastic samples demonstrated a broader range of dissimilarity than the impaired site microplastic samples. Stone sample indices were more tightly clustered than microplastics', suggesting more similar richness and abundance, and had a mean diversity index that trended higher (Fig. S1). A Bray–Curtis dissimilarity analysis, also at the family taxonomic level visualizing the combination of site and substrate, showed minimal separation, suggesting similar communities across samples (Fig. S2). PERMANOVA analyses of site, substrate, and site + substrate interaction confirmed no significant trending (analysis not shown). However, when the collection date was included, we noted that the temporal succession did play a significant role ($p = 0.008$) in diversity between groups.

Plated media

Nine of the ten non-deployed substrates showed no coliform growth after 24 h. The tenth plate had minimal colony growth after this time but was attributed to processing contamination versus growth on the virgin substrate. As such, we were confident in the absence of coliforms on the stone and microplastic substrates before starting the study period. At the Day 30 and Day 60 harvests, we noted significant issues with selective media counting owing to overgrowth at 1:1, 1:10, and 1:100 dilutions, particularly in the pink non-*E. coli* coliform colonies. Day 90 samples were diluted to 1:1000 and provided the most accurate counting data.

Day 90 samples were used to represent the succession of bacterial communities (n = 80) for the final analysis of fecal coliform growth. Testing with Shapiro-Wilks analysis confirmed the data set was non-normally distributed for the total coliform colonies ($W = 0.84$, $p = 9.53e-08$). Mann–Whitney U testing revealed significantly greater total colony counts at the impaired site compared to the unimpaired site ($W = 583$, $p = 0.037$). Microplastics also had more total colony counts than stone substrate ($W = 1038$, $p = 0.022$). A Poisson generalized linear model was applied using the *lme4* package [3] for mixed-effect

modeling, using site and substrate as predictors and total colonies per gram of substrate as the response. Four different a priori models were created. Akaike information criterion (AIC) was then used to rank the models, resulting in the selection of the site + substrate model. Model goodness-of-fit was evaluated with a likelihood ratio test (LRT) against the null model. All models were determined to be sound (Table S4). Further assessment of confidence intervals was set at 95%, indicating that none of the top-ranked models overlapped 0 (Table S5). The resulting box plot (Fig. 4) showed a significant increase ($p < 0.01$) in coliform counts on the microplastic substrate at the impaired site over the microplastics at the unimpaired site. Further, an additional significance ($p < 0.05$) towards colonies on the microplastic versus stone within the impaired site was also noted. No significance was seen between the substrates at the unimpaired site.

Further exploration of the colony distribution in the 16S sequencing data was performed on the Day 90 subset by filtering the *Enterobacteriaceae* genus, along with other genera known for having pathogenic organisms, such as *Vibrio*, *Aeromonas*, *Salmonella*, *Shigella*, *Clostridium*, and *Legionella,* by percent relative abundance (Fig. S3). Mann–Whitney U statistical analysis ($\alpha < 0.05$) was performed to determine the significance of the median difference of these organisms based on substrate type. The results were visualized via a Cleveland Dot plot in R. *Aeromonas* and *Citrobacter* counts were significantly more abundant ($p < 0.05$) on the microplastic whilst *Clostridium* and *Legionella* were more significant on the stone. *Enterobacter* also demonstrated greater relative abundance on the microplastic but not significantly so (Fig. 5).

The results indicate that the microbiome communities had similar composition and class taxon accumulation across the study period, with minimal differences in alpha and beta diversity. Total plated coliform counts were greater at the impaired (QR) and microplastic (MP) substrates. 16S rRNA gene sequencing of genera of human disease concern indicates that both substrates have the potential to harbor distinct pathogens at either site.

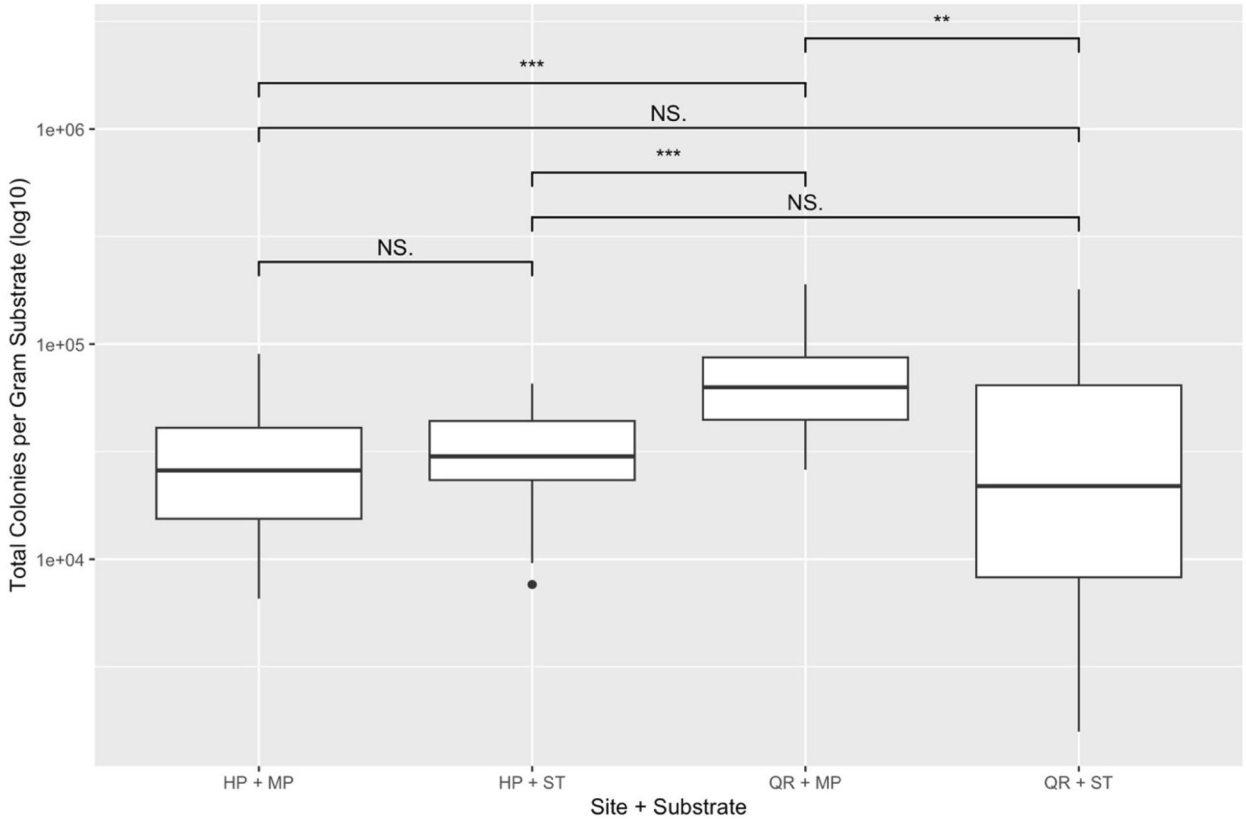

Fig. 4 Total coliform colonies taken from Day 90 samples and diluted to 1:1000. QR = impaired, HP = unimpaired, MP = microplastic, ST = stone. Median (middle horizontal bar in box), interquartile range, minimum, maximum, and outliers are represented. n = 80. *** ≤ 0.01, ** ≤ 0.05, NS = not significant

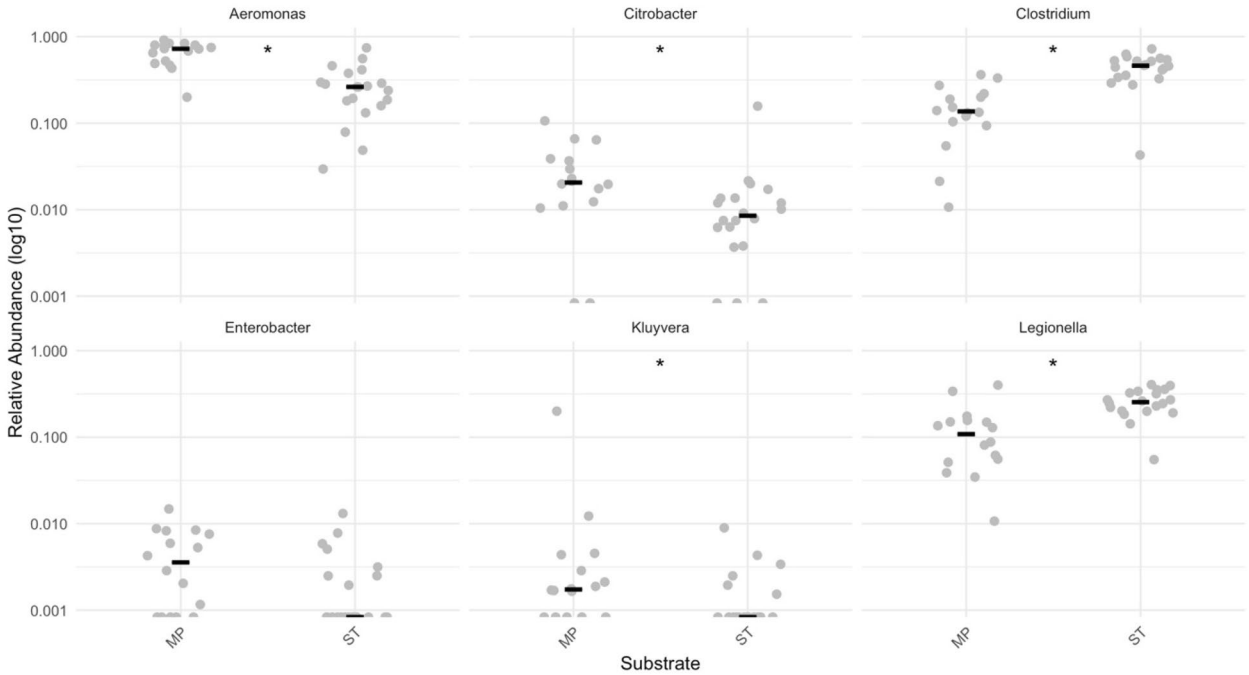

Fig. 5 Representative abundances (log10) of select genera on microplastic (MP) and stone (ST). n = 38, * = $p < 0.05$

Discussion

His study aimed to analyze the differences, if any, between the microbial communities colonizing anthropogenic polypropylene microplastic and natural stone substrates over 90 days in a freshwater riverine system. We also evaluated the influence of site impairment as a co-factor to determine if water body health impacts microbiome development. Research into freshwater riverine systems is significant due to their proximity to urban areas and the services provided through transport, wastewater discharge, and maritime or recreational activity [26, 49]. By comparing biofilm assemblage in an impacted riverine section with an unimpacted section, we sought to test the potential disparate effect of water quality and the influence of microplastic burden that could be present in a freshwater urban and non-urban riverine system. We acknowledge that this study was not a full replication as only one site was used for the impaired and unimpaired locations; however, these results indicate that further within-condition replication is warranted in a future study.

The sites selected for this study were purposefully chosen based on their waterbody health status to analyze the impact of the location with regard to coliform accumulation. The impaired Quinnipiac River location is at the site of a wastewater treatment plant (Quinnipiac Recreation Area/Treatment Plant, Cheshire, CT) along the main river artery. An additional plant, Water Pollution Control, Southington, CT, is approximately 3 miles upstream. Conversely, the unimpaired Honeypot Brook location is a tributary of the artery fed by surface and groundwater. We found no significant differences in site location for temperature and salinity of the water quality indicators that influence biome development [23, 32]. Of the other parameters, dissolved oxygen was lower during the study period, and an elevated Day 60 pH level was noted at the impaired site. However, with limited data, we cannot speculate on seasonal influence as our study was conducted over the summer months, but future directions could compare winter and summer sampling periods.

Microbiome analysis from 16S rRNA gene sequencing showed that three *Proteobacteria* classes (*Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*) were abundant across site and substrate. Wu et al. [50] found similar percentages (60–77%) when comparing lab-cultured riverine microplastic and stone biofilms. Members of the phyla *Alphaproteobacteria* and *Gammaproteobacteria,* along with *Cyanobacteria,* have been shown to establish the microbiome early in the colonization process in riverine and marine environments [8, 26, 49].

The microplastic substrate at the impaired site showed statistically higher median total coliform counts than any other combination of site and substrate (Fig. 4). Based on the generalized linear model analyses, both site and substrate appear to have the most significant additive

influence on coliform adhesion, suggesting a co-influential action [9, 34].

The alpha diversity indices (Fig. S1) showed lower diversity in microplastic substrates than in stone, aligning with similar studies looking at artificial and natural substrates. Lower diversity can be attributed to critical factors in microplastic microbiome selectivity, including the polymer type and pioneer colonization [26, 28, 30].

This particular section of the main Quinnipiac River artery and other tributaries within the watershed are unsuitable for recreation, fishing, or sustaining aquatic life (QRWA, 2013). Based on plated results, we did confirm the presence of fecal coliforms in higher abundance on the microplastic substrate at the impaired (QR) site. However, these data only present a generalized picture of waterbody health. Sequence analysis of Day 90 genera from the *Enterobacteriaceae* class confirmed that the genera *Escherichia* were not a significant driver of biomass accumulation (Fig. S3). Interestingly, non-*Escherichia* genera were more abundant, suggesting that expanding analyses to all species in the *Enterobacteriaceae* genera is warranted.

Additionally, the results showed significantly increased relative abundance for several other genera known to harbor species considered human pathogens (Fig. 5). Wastewater-enriched systems host a wide diversity of organisms, many of which may survive treatment processes [26, 33, 42]. Our study does support the application of 16S rRNA gene analysis in freshwater riverine systems to provide greater resolution of microplastic microbiome attachment. Beyond *E. coli,* other enteropathogens may be in higher abundance, thus necessitating the inclusion of metagenomic sequencing in bacterial water quality monitoring.

Future directions for this study include additional exploration of community richness and evenness over time, which could be an interesting focus. As Qiang et al. [34] described, the dominant taxon was distinct from primary colonizers in the first 18 days of lab incubation, with a leveling off from days 18–31. Though outside of the scope of our analyses, a similar observation using Pielou's evenness suggested significant change within groups between D30 and D60 ($p = 6.13e07$) (Fig. S4). Indeed, this was underscored in our beta diversity analysis, wherein the collection date influenced clustering between site and substrate pairing.

Examination of successional growth over an extended study period may provide more information into early and later biofilm development and the influence of seasonal variability. Comparing environmental communities from water samples [23, 34] with that of the adhered community to the microplastic substrate would be another approach to analyze the incident of preferential selection.

The limitation of selective media use for coliform monitoring is that the methodology indicates the presence or absence of *E. coli* and non-*E. coli* organisms. While we did not see a significant abundance of *E. coli* in the substrate microbiomes (Fig. S3), it was evident that the latter was more problematic and too general to determine what fraction of these organisms may be pathogens. Although more costly, using 16S rRNA gene sequencing elucidated the specific genera included in this category. The microbiomes of the microplastic and stone substrate were not overwhelmingly distinct at the class level as first hypothesized; however, there appears to be support that an impaired waterbody with microplastics may suffer from an additive impact of their presence. That is, the discharge of these particles in riverine systems alone does not address the issue's totality; the system's condition must also be considered.

Microplastic biomes present a complicated environmental issue that has the potential to be more impactful in impaired urban riverine ecosystems. The results of this study demonstrated a paired interaction of site and microplastic substrate concerning coliform attachment and adhesion of known potentially pathogenic organisms. Given the importance of these waterways for communities, particularly those near polluting sources, improving water quality should be a top priority.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40793-025-00685-7.

Additional file 1

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Author contributions
ALG designed and implemented the project and was the manuscript's first author. SS performed field and laboratory analysis as a research assistant and prepared Fig. 1 for this manuscript. SNK advised ALG on field protocols and was a major contributor to writing the manuscript. NMS advised ALG on laboratory protocols and data analysis and was a major contributor to writing the manuscript. All authors have read and approved the final manuscript.

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Availability of data and materials
The datasets generated and/or analyzed during the current study are available in the NCBI BioProject ID repository, PRJNA1096657. Additional scripts can be found on GitHub.com, annegilewski/freshwater-microplastics.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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