Effects of plastic polymer composition on early microbial association in a freshwater environment

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Abstract
Plastic polymers are seen by many as inert materials in the environment. The anthropogenic use of plastics has led to their abundance in every ecosystem. Although their prevalence in terrestrial and marine environments has been reported by others, their impact and abundance in freshwater environments is not as widely understood. It is known that microbes can affect plastics by modifying the buoyancy, and it is also quite likely that their actions can alter the surface chemistry, thus leading to enhanced or diminished interactions with other organisms and the environment.

Six different plastic polymers (high density polyethylene, HDPE; low density polyethylene, LDPE; polyethylene terephthalate, PET; polypropylene, PP; polystyrene, PS; and polyvinyl chloride, PVC) were used as substrates to analyze early colonization events in biofilm formation in the Niagara River. The communities that developed on the surfaces of the plastics were analyzed at one and seven days.

The Proteobacteria, Bacteriodetes, Actinobacteria, Cyanobacteria and Firmicutes were the major bacterial phyla noted with the Proteobacteria being the predominant bacterial group for each sample. The data suggests that 1, initial colonization happens quickly; 2, there are distinct differences of the microbial inhabitants from one plastic to another and 3, the composition of the members on each plastic changes over the course of the experiment. The data would imply that the surface chemistry of the plastic does have an impact on the taxa that reside in the biofilm and that it is not merely a surface that is indiscriminately being inhabited by the microbes.

Keywords: biofilm, plastic polymers, operational taxonomic units (otu), Niagara River.

Introduction
The ubiquity of bacteria is a well-established fact and these free-living organisms are often found to be involved in complex consortia known as biofilms. Recent attention has focused on the microbiome (1-3), those microbial inhabitants of the human body, and it has been recognized that these biofilms in and on us are a complex ecosystem (4, 5).

There has been scant attention paid to the bacterial communities found in the environment. However recent advances in sequencing techniques (6) have allowed for a much better perspective on the diversity and abundance of microorganisms in an environment (7). What has become evident from the studies is the complex interplay of microbes in every ecosystem (8). Biofilms are not homogenous layers of bacteria like sedimentary rock, but rather a complex consortium, vastly interdependent and strongly mediated by chemical signaling.

Bacterial biofilms consist of a microbial community attached to a surface through an extracellular matrix (9) or via some other type of interaction with its substrate. Life in a biofilm has some distinct advantages: namely, access to products and by-products of other microbes; protection from antibiotics, other toxins, and toxic compounds in the surrounding environment; localization of excreted materials such as enzymes; and shelter from predation (10). Such close association also allows for easy genetic exchange and

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chemical signaling with agents valuable for quorum sensing.

Biofilm development follows a set of steps that have been described earlier by Kolter's lab (11). The typical series of steps begins with planktonic cells attaching to a substrate through a loose or non-specific connection. Next, an initial expansion of the cells leads to the formation of micro-colonies. These initial inhabitants begin to produce an extracellular polymer surface and then, often, a 3-D matrix is formed as the biofilm matures. Cell-cell communication begins and chemical signaling is observed in many forms, not the least of which is through quorum sensing. Cells may leave the biofilm and re-enter the planktonic lifestyle as well, and one should view a biofilm as a dynamic consortium of cells.

The composition of a biofilm may be impacted by a number of external phenomena such as predation, viral infection, water flow, and interactions with other surfaces. Rivers are a dynamic system where conditions are constantly changing, often increasing the frequency of those phenomena. Biofilms in such environments are confronted with a unique set of variables. For instance, there is a flux of nutrients across the substrata. This interface between the biofilm and the water is part of a greater nutrient spiral, dictated by the upstream nutrient flux. Generally microbial communities have the greatest access to soluble inorganic compounds (12). The stream side location of industrial plants, sewage treatment, and other facilities which add chemicals or dissolved organic material (DOM) to the water column can directly affect the ecological community of a waterway.

It is logical to conclude that organisms living in the water would be coated with biofilms and there are many interesting examples of the advantages of these bacterial denizens, such as those found associated with the Hawaiian squid *Euprymna scolopes* (13), octopi (14), coral (15), sponges (16), and fish (17).

In addition to the biotic substrates, several investigators have begun to address the microbial biofilms found in marine environments on abiotic surfaces (18, 19). One of the most common xenobiotic materials found in the oceans is plastic. The fate of plastics in the oceans is one that has only recently been elaborated (20, 21). Large accumulations of debris, either washed in from shore (22), dumped as trash from ocean-side communities (23), or lost material due to shipwrecks or natural disasters, can, and frequently does, end up in gyres where massive agglomerations of anthropogenic wastes can be found (24).

It has been recognized that these plastics may act as concentrators of persistent pollutants in the environment, as well as other hazardous organic pollutants (25). Physical and chemical properties of the various types of plastic can influence this binding and sorption. Similarly, those same qualities may influence microbial adhesion, community composition, and physiology.

The processes of biofilm formation and the lack of understanding of microbial ecology on unique substrates inspired this study. There is a demand for 245 million tons of plastic per year, 75-80 million of which is used for packaging (26). As evidenced by garbage patches in ocean gyres (24, 27) or a short walk through the city or along the beach, that demand of plastic often becomes part of the ecosystem. This study intends to statistically compare microbial communities found on six different plastic types in common use: polyethylene terephthalate, PET; low-density polyethylene, LDPE; high-density polyethylene, HDPE; polystyrene, PS; polyvinyl chloride, PVC; and polypropylene, PP. Though a preliminary endeavor, this paper provides a baseline to further study the aforementioned parameters of lotic systems and microbial ecology on an ever-accumulating substrate. Like it or not, plastic in the environment can no longer be considered as a simple pollutant but must be studied as its own ecosystem.

**Material and Methods**

**Sample collection.**

Plastic disks of 12.56 cm diameter and 2.4 grams were suspended onto stainless steel cables in the lower Niagara River in Lewiston, NY, (43.172196N, -79.049780W). After an incubation period of either 1 or 7 days the plastic disks were cut free from the cable using sterile clippers. Each disk was individually placed into sterile, 1 quart, polyethylene terephthalate bags then stored on ice for transport to the lab.

The DNA from each disk was extracted using the PowerBiofilm DNA isolation kit from MO BIO laboratories Inc. To extract the biofilm from each disk 350 µl of solution BF1 was added to each bag. Individual plastic disks were scrubbed for 10 minutes with a sterile nylon bristle toothbrush. The resultant liquid was then processed per the manufacturer’s instructions. DNA integrity was verified via PCR analysis using bacterial universal primers 27F, 5’-AGAGTTTGATCMTGGCTCAG-3’ and 907R, 5’-CCGTCAATTCTCCTTTRAGTTT-3’.

**DNA sequencing.**

DNA sequencing was performed by Molecular Research (MRDNA, Shallowater, TX) using standard 27F-519R primers (28). A single-step 30 cycle PCR using HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA) was used under the following conditions: 94°C for 3 minutes, followed by 28 cycles of 94°C for
30 seconds; 53°C for 40 seconds and 72°C for 1 minute; after which a final elongation step at 72°C for 5 minutes was performed. Samples were sequenced utilizing Roche 454 FLX titanium instruments and reagents and following manufacturer’s guidelines.

Data processing.
Sequences with short reads (less than 200 bp) or ambiguous base calls were removed. Remaining sequences were denoised and chimeras were removed. Operational taxonomic units (OTUs) were defined after removal of singleton sequences, clustering at 3% divergence (97% similarity). OTUs were then taxonomically classified using BLASTN against a curated GreenGenes database (29) and compiled into each taxonomic level.

Data analysis
Each sampling period potentially represents different successional stages in biofilm development, with 1 day representing a period of random attachment of bacteria to plastic substrates and 7 day representing a more mature biofilm community. We accounted for this distinction by separately examining sampling period data in all analyses. Dominance and similarity (using Jaccard’s index) of bacterial phyla, orders, genera, and species were determined using OTU counts by sampling period. Genera OTUs were further used to calculate the Shannon-Wiener index, Simpson’s index (1-D), and Chao1 species pool estimates for each plastic by sampling period. We used non-metric multidimensional scaling (NMDS) as well as cluster analysis to compare bacterial species community composition among plastics. All analyses were conducted in the R software environment version 3.1.1 (30). Diversity calculations, DCA, and PROTEST (31) were additionally performed using functions provided in the Vegan package version 2.0-10 (32) for R.

1 Results
OTU Dominance
A total of 126,502 OTUs were detected across plastic groups and sampling periods. The 1 day period had nearly twice as many total OTUs as the 7 day period, with 83,026 (mean 13,837.7 ± 865.5 s.e.) in the former and 43,476 (mean 7,246 ± 1,287.1 s.e.) in the latter. A range of 1,415-16,005 (mean 10,541.8 ± 1,238.7 s.e.) OTUs was observed among plastics separated by sampling period with the minimum observed from PS at 7 day and the maximum from LDPE at 1 day (Table 1).

Twenty-six bacterial phyla were detected from plastic-associated bacterial communities, with 97% of the OTUs attributed to two dominant phyla: Proteobacteria (82%) and Bacteroidetes (15%). Proteobacteria was also the dominant phylum and accounted for 63-90% of phyla reads in all the plastic samples. Phyla richness was 62% similar (Jaccard coefficient of 0.62) between 1 day and 7 day samples. Reads were further classified down to order, and large differences in bacterial communities associated with different plastics during each sampling period were observed (Fig 1). A total of 90 orders were detected, with 78% similarity in order richness between 1 day and 7 day samples (Jaccard coefficient 0.78). While 81% of all reads were assigned to Burkholderiales (48%), Flavobacteriales (12%), Myxococcales (11%), and Oceanospirillales (10%), these orders varied in dominance among plastics and between sampling periods (Fig 1) We detected 374 genera when further classifying the data. Genera richness from 1 day and 7 day samples was 66% similar (Jaccard coefficient 0.66). Five dominant genera accounted for at least ten percent of all OTUs: an uncharacterized Comamonadaceae genus (21%), Sphaerotilus (12%), Flavobacterium (11%), an uncharacterized Nannocystineae genus (11%), and Balneatix (10%). A total of 501 species were detected, but only 32% of these could be matched to known species in the GreenGenes 16s rRNA database. Species richness of sampling periods were 65% similar (Jaccard coefficient 0.65), however OTUs that make up a small percent of the population (<50) were observed for most species in both the 1 day (86%) and 7 day (83%) periods.

Table 1 provides the total observed OTUs, observed genera, Chao1 genus pool size estimates, and diversity metrics for each plastic type for 1 day and 7 day sampling periods. The total number of observed genera increases from day 1 to day 7 for all plastic types except for LDPE and PS. Showing a similar trend, there is an increase in genera diversity over the sampling period for all plastic types except for PS and LDPE, which only show a moderate increase relative to the other plastics (Table 1). It is also interesting to note that the Chao1 estimates for LDPE and PS are much closer to the observed number than they are for any other plastic (Table 1). Given the nature of the calculation, this would indicate that the majority of DNA reads for these plastics exceed 1 or 2 reads per genera, possibly suggesting a more mature, stable, and exclusive community.

Figs 2 and 3 graphically depict the changes in the community at the genus level between each plastic over the sampling period. Fig 2 shows the percent composition of the 200 most abundant genera for each plastic type, labeled and ordered based on those with the greatest contribution on day 1. Each color represents
Table 1: Diversity-Related calculations and metrics for bacterial communities of biofilms developing on six plastics. The six plastics abbreviated as follows: HDPE (High-density polyethylene), LDPE (Low-density polyethylene), PET (Polyethylene terephthalate), PP (Polypropylene), PS (Polystyrene), and PVC (Polyvinyl chloride). Values are provided for both 1-day and 7-day sampling periods.

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<th>Shannon Index</th>
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2 Discussion

This study observed the formation of biofilms over a rather short period of time compared to the entire lifespan of plastic in the environment. However, within that time, the variable nature of the initial colonization is quite apparent. Genera diversity within the biofilm community increased from Day 1 to Day 7 for all plastic types except PS (Table 1). This increase in diversity was most likely the product of rapid succession, with the total observable OTUs being nearly halved on all plastics except PP, and a numeric increase in observable genera on all plastics except LDPE and PS (Table 1). The Chao1 estimates displayed in Table 1 indicate that the number of singletons and doubletons observed on LDPE and PS is far lower than the other plastic types indicating a settling or maturation of the biofilm. This could also explain the distancing in the nMDS plots of Fig 4c.

The maturation of the biofilm is most likely the result of interspecies interactions and the development of microenvironments on the plastic, this is illustrated in Fig 4. Generally speaking, the plastics shared a more similar community composition at 1 Day vs. 30% at 7 Days). At Day 1 Comamonadaceae has the highest relative abundance across all plastic types except PVC where it has the second highest abundance. However, by Day 7 Comamonadaceae has a much smaller relative abundance whereas other genera (such as Flavobacterium, Balneatrich, and Nannocystineae) have become predominant members of the community. Similarly, Aeromonas is virtually non-existent on any plastic on Day 1, but by Day 7 it’s relative abundance on LDPE is greater than that of Comamonadaceae. This trend repeats with many of the genera observed.

The obvious question of interest is what role the different plastics themselves play in directing community development. By treating the individual plastics
as specific sites, it is possible to compare how similar the individuals of each community are to one another. Clearly shown in Fig 4, given the same distribution of bacteria in the water, all six plastics developed unique communities where changes at the species level seem to decrease, but the number of genera go up significantly (Table 1). This indicates to some extent, without negating the idea, that the interactions between the biofilm community and the plastics are not so specific such that a single, unique species is capable of exploiting a unique niche, but instead that during early colonization (<1 week) many genera of bacteria with the same ‘game-plan’ and tool set of genes are able to thrive.

It must be noted that the plastics are not merely surfaces that act as generic catch-basins for organ-
isms willing to adopt a sessile lifestyle. It is quite likely that there is a large dynamic process occurring at the microbial level. Varying rates of maturation of the biofilms could be occurring which could explain some of the dissimilarity noted. Some researchers saw the predominance of early pioneer species being reduced and consequently replaced over time with more species diversity, which they proposed helped to increase the community stability (33). Previous investigations on the succession of bacterial communities on several artificial surfaces and also noted a large change in the composition of the communities on each surface when comparing early to later points in time (34). Those results are consistent with the findings of this research. Paradoxically, others have suggested via modeling techniques that there should be an initial high diversity in a biofilm that decreases with time, to which this report and others have found the opposite (35). Jackson proposed that the initial species richness would decline as some of the organisms would be less able to survive in a biofilm due to poor nutrient and oxygen availability and waste build-up (35).

The order of attachment of particular microbes may play a role on the composition of the biofilm. In a re-
Figure 3: Heatmap Showing the Dominance (Relative Abundance) of the Twenty Most Abundant Bacterial Genera of Biofilms Developing on Six Plastics. The plastic types are abbreviated as follows: HDPE (high-density polyethylene), LDPE (low-density polyethylene), PET (polyethylene terephthalate), PP (polypropylene), PS (polystyrene), and PVC (polyvinyl chloride). Values are provided for both 1 day and 7 day sampling periods.
Figure 4: Non-Metric Multidimensional Scaling (nMDS) Plots for Both 1 Day (a), 7 Day (b), and 1 Day vs 7 Day (c) Sampling Periods with Accompanying Dendrogram. Plot points represent six plastics: HDPE (high-density polyethylene), LDPE (low-density polyethylene), PET (polyethylene terephthalate), PP (polypropylene), PS (polystyrene), and PVC (polyvinyl chloride). Similarity matrices calculating using Bray-Curtis dissimilarity for nMDS plots and dendrograms.
view of the literature it has been noted that, as diversity increases, the stability of a community increases in a changing environment and that resilience arises in a microcosm containing a diverse community (36). One possible explanation for this study’s results is that the early colonizers are quite selective to the different surface chemistries of the plastics. This specificity of early colonizers based on substrate was seen by others (37). Later colonizers essentially interact with and bind onto the biofilm already produced by the earlier bacteria and consequently do not always interact with the plastic surface. Therefore, it is not unusual to assume that the late colonizers would be more similar from sample to sample compared to the early colonizers unless the early community produce some unusual compounds that has a large impact on who else can attach in the biofilm. Such were the conclusions from previous experimentation regarding long-term succession in biofilms (38). From our observations, the time table in which this occurs will vary based on environmental conditions and initial bacteria population. Despite the rapidness of substrate colonization observed in this study, one week may still be in the early colonization phase where the above described interactions are not as pronounced.

The composition of the bacterial community has been shown to be affected by protozoal grazing (39–42) and therefore it is reasonable to assume that the biofilm is in a constant dynamic state. It should also be noted that plastics may act as concentrators of persistent organic pollutants in the environment as well (25). The physical and chemical properties of various types of plastic can influence this binding and sorption. This preferential binding of certain organic compounds could also affect the microbes that make up the biofilm and thus alter the lifespan and final story of these pollutants.

Another consideration is the validity of any molecular data that relies on an amplification step as the use of any primer pair set with PCR has its limitations(43). It has been previously noted that many sequence reads were of low abundance and that some of this bacterial richness was lost with certain primer pairs when sampling aquatic microbial communities(43). All of the factors that microbiologists contend with (mutation, genetic drift, gene flow, immigration and emigration, selection) also play a role in community dynamics at the microscopic scale. Some unique circumstances exist as well, with horizontal gene transfer being one area that plays an important role in microbes. Numerous metrics are available to describe the diversity found within or among communities. While some find that certain microbial communities are more phylogenetically related than expected (44), others find that in a small number of cases the microbes in a biofilm are more distantly related from a phylogenetic perspective (45). The study of marine microbial communities has also led to the concept of networks of interacting microbes whose functional ability is determined by the sum total of biological activities present within the community (46). An argument could thus be made that the sum total of genes and hence richness of the community in terms of metabolic potential is increased with the addition of more diverse members, which would lead to community stability. This richness could be found with deep sequencing of a particular environment. It is also likely that the phenotypic make-up of a community may be quite flexible and that there may be much more unexpressed potential in any population than we recognize. Modern sequencing methods appear to bear this out. Temporal and spatial considerations are special challenges at the microscopic scale, as it requires much finer discrimination to demarcate the contribution of the members of a community at any time or place. Much remains to be learned regarding the colonization of xenobiotic materials in the environment.

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