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# Connecting Riparian Phyllospheres to Aquatic Microbial Communities in a Freshwater Stream System

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**Abstract:** The role that aquatic aerosols might play in inter-ecosystem exchanges in freshwater riparian environments has largely been understudied. In these environments, where freshwater streams are used both as drinking water and for treated waste disposal, water features like waterfalls, downed trees, and increased streamflow can serve as bioaerosol producers. Such water features could have an important role in the bacterial colonization of surrounding surfaces, including the riparian phyllosphere. In this study, we explore the influence of a freshwater stream's bacterial community composition and micropollution on riparian maple leaves exposed to bioaerosols produced from that stream. Using culture-based and non-culture-based techniques, we compared phylloplane microbial communities in riparian zones, adjacent non-riparian forested zones, and the surface waters of the stream. In this system, riparian zone maple leaf surfaces had higher bacterial counts than non-riparian zone trees. Using metagenomic profiling of the 16S rRNA gene, we found that, while microbial communities on leaves in both the riparian zone and forested sites were diverse, riparian zone bacterial communities were significantly more diverse. In addition, we found that riparian leaf bacterial communities shared more amplicon sequence variants (ASVs) with stream bacterial communities than forest leaves, indicating that the riparian zone phyllosphere is likely influenced by bioaerosols produced from water surfaces.

**Keywords:** phyllosphere; phylloplane; aerosol; riparian; freshwater; *Hymenobacter*; *Sphingomonas*; *Oxalobacter*; *Acer rubrum*



**Citation:** Dueker, M.E.; Lansbury, B.; Perron, G.G. Connecting Riparian Phyllospheres to Aquatic Microbial Communities in a Freshwater Stream System. *Aerobiology* **2024**, *2*, 59–71. https://doi.org/10.3390/aerobiology2030005

Received: 31 May 2024
Revised: 13 August 2024
Accepted: 26 August 2024
Published: 29 August 2024



## 1. Introduction

The phyllosphere, or the above-ground external surfaces of plants, is thought to be the largest microbial habitat on earth [1]. Phyllosphere microbial communities (PMCs) heavily influence plant fitness and ecosystem function, and they serve as a major source of bioaerosols globally [2]. PMCs are a diverse mixture of fungi, bacteria, archaea, algae, and viral particles, with bacterial communities often dominating [3,4]. Understanding the assembly and structure of the PMC is therefore vital for the effective management of food safety and security, appreciating the local- and ecosystem-level functions, and predicting the impacts of climate change both locally and globally [5]. Despite these realities, key questions remain unanswered regarding environmental impacts on the sources for and functions of PMCs [6].

Bioaerosols are a dominant source for phyllosphere colonization [7], representing all major earth biomes, including marine, soil, plant, and animal sources [8]. Land-based bioaerosol sources are better studied, but water surfaces are also a major source of bioaerosols [9], through the action of bubbles bursting at marine and freshwater surfaces and launching microscopic droplets into the air [10–17]. Bubbles are introduced in both marine and freshwater environments through wind–wave interactions [18–20] wave–shore

interactions [21]; human recreation (e.g., boating, swimming); and water features creating whitewater, including art installations, fountains, downed trees, waterfalls, and gas evolution, among others. Bioaerosols created from water surfaces can be important mechanisms for the transfer/transport of viable microbes and nutrients to land-based surfaces and ecosystems [9,18–20].

It follows then that water systems would be an important influence on the microbial colonization of adjacent phyllospheres, particularly in riparian zones. In the United States, freshwater systems are vulnerable to treated and untreated sewage discharge due to failing infrastructure, with an estimated 1.2 trillion gallons of untreated wastewaters entering rivers annually [22]. The connection between water and air quality through microbial aerosols has been demonstrated mostly in marine environments, including coastal oceans [19,23–25], estuarine coastlines [20,26], and polluted shipping canals [27,28]. As these studies continue, it is clear that what is in the water (including chemical and biological pollution) is also transferred to the air, where it travels inland to deposit on terrestrial surfaces. Studies of this phenomenon and its implications for freshwater systems, however, have not been conducted, particularly as it relates to the environmental distribution of water-borne sewage-associated microbes and micropollution (genetic material, antibiotic resistance gene (ARG) cassettes). The connection between riparian PMCs and water quality, until our study, has been largely unknown.

The microbial content of ambient aerosols varies based on seasonal and geospatial influences [29,30]. Dispersal and delivery of bioaerosols to leaf surfaces are controlled by geographical proximity to bioaerosol sources [31,32], meteorological conditions like wind speed, wind direction, and precipitation [1], and leaf topography [33]. Once aerosolized microbes deposit on the leaves of plants and trees, they become either short-term or long-term epiphytic residents [30,34], according to host and established PMC responses to these new microbes [35,36]. Recent studies have demonstrated that, despite the geospatial variability in microbial content of aerosols, trees appear to have species-specific core microbial communities [3]. The influence and source for microbes not included in that core microbiome, and their interactions with that core microbiome, are not well understood.

While there has been some study of the contribution of the phyllosphere to soil microbes, and discussion of the eventual impact on water microbes as water travels from land to waterways, the contribution of aquatic microbiomes to land-based microbiomes, including the phyllosphere, is still poorly understood. Studies of the movement of soil microbes from riparian zones into waterways have been conducted, e.g., [37], but they do not include the phyllosphere. Dodds et al. [38] studied the potential transfer of PMCs to waterways through run-off but did not consider the role that waterways themselves have in influencing PMCs to begin with.

The influence of air pollution on PMC structure and function has been studied, demonstrating a relationship between proximity to traffic and other forms of air pollution and the degradation of PMC function [39–41]. Anthropogenic impacts on PMCs in urban arenas have included a reduction in alphaproteobacteria in urban environments [42] but a higher diversity of microbes than non-urban PMCs overall. Leaves and trees are known to be effective filters of air pollution and are sometimes even used to clean air [43]. Interestingly, there are many studies outlining how riparian zones and the phyllosphere in particular protect waterways from pesticide applications [44], but no studies to date have examined the role the riparian zone may play in protecting inland ecosystems from the pollution present in the water itself.

Here, we explore the potential for riparian phyllospheres to intercept microbial aerosols contributed by the rural/suburban freshwater systems known to contain treated and untreated sewage inputs. We compared *Acer rubrum* (red maple) PMCs in a freshwater stream riparian zone adjacent to the aerosol-creating water features (a historical dam spillway and a waterfall) to *A. rubrum* PMCs in the same forest system but located well above the freshwater stream. Due to proximity to the waterway and the aerosols created by the distinct water features, we expected to find a clear difference in PMC diversity and

structure between the riparian and further inland forest *A. rubrum* PMCs. Furthermore, we hypothesized that riparian zone phyllospheres would include a detectable influence from the adjacent water surface, including shared aquatic microbial species, sewage indicators, and micropollution.

## 2. Materials and Methods

### *2.1. Study Sites*

As previously described in de Santana et al. [45], this study was conducted on the Saw Kill, a 23.0 km freshwater tributary of the Hudson River, located in the upper Hudson Valley, during the summer of 2015. The Saw Kill watershed drains 57 $km^2$, including forest, wetland, agricultural, and suburban developed land. There are 2 permitted sewage outfalls (NYSPDES permit #'s NY0271420, NY0031925) on the Saw Kill, and one permitted drinking water facility (130,000 gallons per day) between the two sewage outfalls.

Four phyllosphere sampling sites (two riparian sites (adjacent to de Santana et al. stream sampling sites), and two forest sites > 65 m upslope of the water sampling sites) were chosen to represent the "riparian" and inland "forest" sites (Figure 1). Because of their presence in both the riparian and forest sites, and to control for host-specific differences in PMC bacterial communities, we sampled only from *Acer rubrum* (red maple) at each site. Specifically, three *A. rubrum* at each site were marked prior to the sampling campaign to ensure consistent collection from the same trees throughout (Figure 1). The riparian trees were exposed to the constant production of aquatic aerosol through a dam spillway and a waterfall. Preliminary aerosol monitoring conducted during a clear, windless day demonstrated a significant difference in aerosol particle number and size distribution between the forest and riparian tree sites (Supplemental Figure S1), with the riparian zone containing significantly higher aerosol particles in the range known to harbor bacterial aerosols [46]. All sampling was conducted between 6/22/15 and 7/22/15.

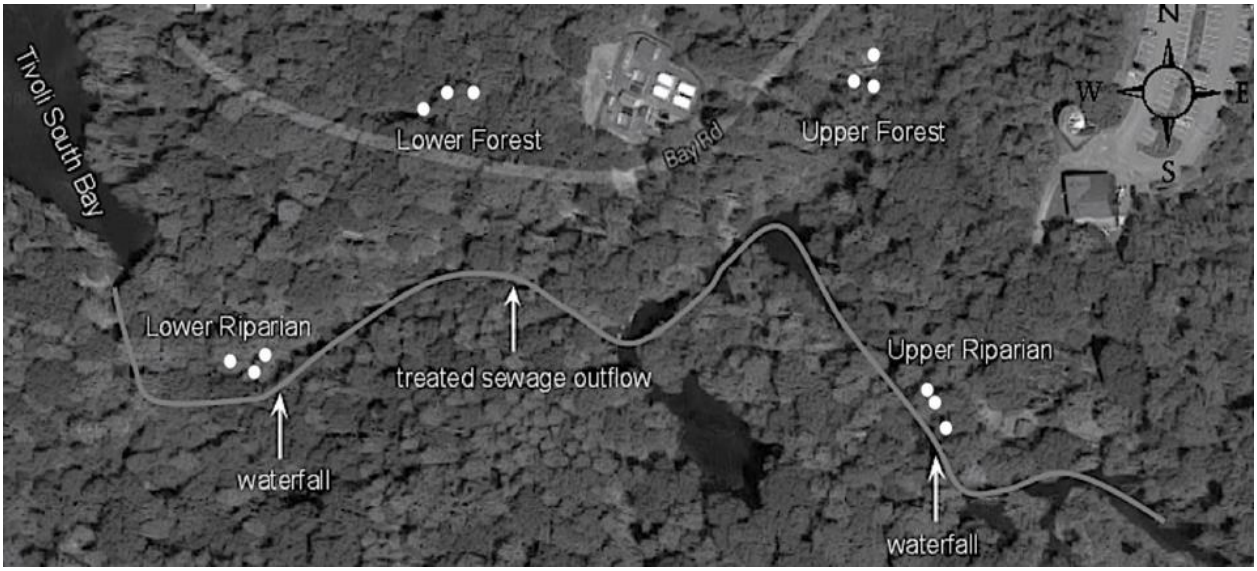


**Figure 1.** Study sites near the Saw Kill (solid line, flow is right to left). Locations of *A. rubrum* used for leaf sampling denoted by white circles. Aerosol-creating waterfalls upstream of both riparian sites noted, along with the location of the outflow for the Bard College wastewater treatment plant.

### *2.2. Surface Water and Phyllosphere Sampling*

Surface water samples were collected as per de Santana et al. [45] in sterile and acid-washed 2 L Nalgene bottles from the mid-channel, within 0.5 m of the stream surface, at each site. All samples were placed on ice and transported to the laboratory for analysis within 2 h of sampling. A list of all water samples, along with their physical characterization

(i.e., GPS coordinates, date, temperature, salinity, conductance, particulate), is available in de Santana et al. [45].

We used methods similar to those employed by Laforest-Lapointe et al. [24] and Tang et al. [47] to sample the *A. rubrum* leaf PMCs. Tree leaves collected during the sampling campaign (mid-summer) were fully mature but not senescing, minimizing leaf age influence on PMCs. For each sampling event, three leaves were randomly collected with sterilized forceps from each tree at 250–300 cm above ground-level. Two leaves from each tree were immediately placed in a sterile 50 mL falcon tube to be later washed for use in culturable bacteria counts and bacterial DNA extraction, and one leaf was placed in a sterile, clear Nasco Whirl-Pak® bag for processing in the lab.

The leaf "wash", representing both adaxial and abaxial leaf PMC, was obtained as follows: 15 mL of endotoxin-free Hyclone Hypure™ Cell Culture Grade water was pipetted into each of the falcon tubes containing two leaves from each tree. The tubes were then vortexed at maximum speed for 5 min and then centrifuged at 1300 rpm for 3 min. Supernatant from each was then transferred into a new sterile tube, with wash from all three trees per site pooled for downstream assays.

### *2.3. Bacterial Analyses*

#### 2.3.1. Culture-Based Methods

Leaf surface area (LSA) was estimated by outlining sterile bagged leaves on grid paper using a light table to minimize disturbance of the attached microbial communities. These leaves (one per tree) were then "printed" onto LB agar media with 0.004 g Amphotericin B (LB/AmB), in order to deter fungal growth [48]. Specifically, the adaxial side of each leaf was pressed gently onto the agar surface using a sterilized stirring rod, and then removed and discarded [49]. Bacterial colonies were counted after 96 h of growth in the dark and at room temperature (22–25 °C). Concentrations of culturable bacteria on the leaf surfaces and in the water samples were also determined by spreading 100 µL of pooled leaf "wash" onto agar plates with the same media described above [19]. These plates were incubated along with the leaf-print plates.

#### 2.3.2. Culture-Independent Methods

For each water sample, we filtered 750 mL of surface water through a 0.22 µm Sterivex filter. We also filtered the remaining pooled leaf wash (~14.9 mL) from each site through a 0.22 µm Sterivex filter. A filter of only sterile, endotoxin-free water was also made to serve as a control for extraction methodology and sequencing methodology. We then extracted total DNA from these filters using the PowerWater DNA Isolation kit (MoBio Laboratories Carlsbad, CA, USA), now available as the DNeasy PowerWater DNA Isolation Kit (QIAGEN, Hilden, Germany). DNA concentrations in the extractions were measured using a Thermo Fisher Nanodrop 2000 (Thermo Scientific, Willmington, DE, USA). All extractions of water and leaf filters contained more DNA than the control filter.

We quantified the presence of the 16s rRNA gene and *IntI1* (ARG indicator) genes in the water and leaf extractions using quantitative PCR (qPCR), following the procedure described in de Santana et al. [45]. In summary, we processed each sample in triplicate using the primers described in Gaze et al. [50] and the PowerUp SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). Samples were then run using the Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA), with an internal standard curve constructed from a serial dilution of the *Escherichia coli* strain SK4903. Data files are available in de Santana et al. [45].

To characterize the overall bacteria community structure in each sample, we amplified the V4 region of our 16S rRNA gene amplicon sequencing library using primers 515F and 806R, as outlined in the Earth Microbiome Project [51]. Samples were sequenced on the Illumina Miseq platform using 250 bp paired ends by the Wright Labs (Huntingdon, PA, USA) and are described in de Santana et al. [45]. Taxonomy was established using different

curated databases and taxonomic assignment algorithms (Table S2). Raw reads are/will be publicly available on the Sequence Reads Archives of the NCBI (accession number: TBD).

### *2.4. Bioinformatic and Statistical Analyses*

Regional meteorological parameters, as captured by the WeatherUnderground (https://www.wunderground.com/, URL accessed 31 October 2015) sites in our sampling region, were compared to the PMC measurements and evaluated through correlation analyses (Pearson's product–moment correlation). Culture-based bacterial measurements and qPCR results were pooled by sample site type (riparian vs. forest) and the differences were determined using a *t*-test. All statistical analyses were performed using R version 4.3.3 [52].

All sequencing data used in this study are reported under BioProject accession number PRJNA1137380 in the NCBI database. As described in de Santana et al. [45], raw Illumina sequences were filtered and trimmed with Trimmomatic, ver. 0.39,21 using the following parameters: ILLUMINACLIP:TruSeq3-PE.fa:2:30:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 M INLEN:100. Subsequent steps were performed using QIIME2, ver 2021.222. DADA2 [53] was used to denoise the paired reads (using the following parameters: --p-trunc-len-f 250, --p-trunc-len-r 233) with the median non-chimeric read count per sample estimated as 21,741 reads. Alpha- and beta-diversity analyses were performed on rarefied and non-rarefied amplicon sequence variant (ASV) lists using phyloseq [54]. Because they did not show differences in trends, we are providing the non-rarefied results in this paper, according to McMurdie et al. [55]. Analysis of sewage-associated bacterial genera in PMCs was conducted according to Dueker et al. [26], and a phylogenetic tree (collapsed by genera) was created using the plot_tree function in phyloseq.

## 3. Results

### *3.1. Environmental Conditions and Quantification of Leaf PMCs*

We compared PMC density by estimating the total CFU counts on the leaf surface through the leaf prints. While the adaxial leaf surface area did not differ significantly between the riparian and forest sites, we found that adaxial CFU density varied significantly between the two ecosystems ($t = -4.3$; $DF = 21.11$; $p < 0.001$; Figure 2A). The riparian phyllosphere had, on average, more than three times the abundance of bacterial CFUs per $cm^2$ as the forest phyllospheres. Interestingly, we found that CFUs grown from leaf washes (which would include abaxial communities) did not differ significantly between the forest and riparian leaves ($t = 0.29$; $DF = 30.8$; $p = 0.77$; Figure 2B). These results suggest there might be an additional CFU source for the adaxial side of leaves in the riparian zone.

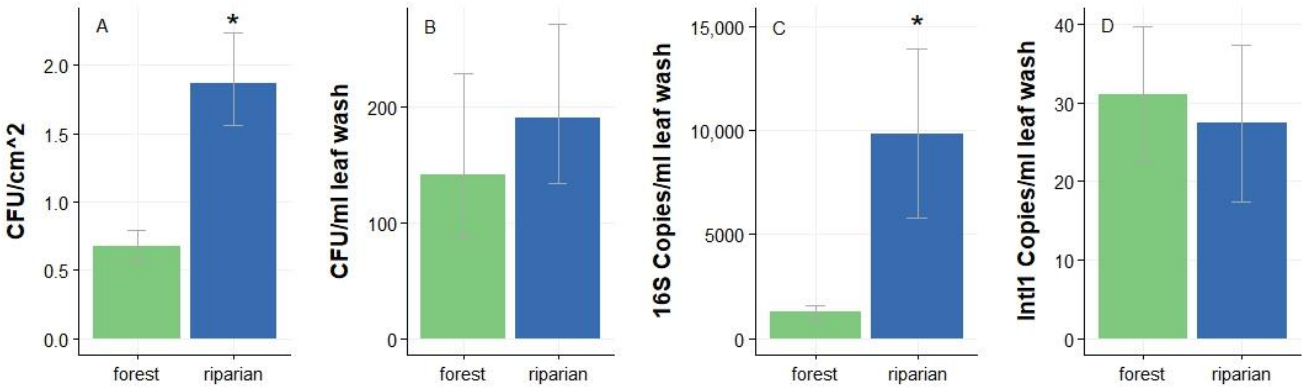


**Figure 2.** Culture-based phyllosphere bacteria counts: (**A**) geometric mean and standard error of culturable bacteria grown from leaf prints and normalized by leaf surface area (riparian $n = 60$, forest $n = 60$), (**B**) geometric mean and standard error of culturable bacteria grown from leaf wash (riparian $n = 20$, forest $n = 20$), and culture-independent (qPCR) abundances of (**C**) 16S gene copies (riparian $n = 20$, forest $n = 20$), and (**D**) ARG indicator IntI1 copies per ml leaf wash (riparian $n = 20$, forest $n = 20$). Statistically significant differences denoted by an asterisk (*).

We then investigated bacterial load in leaf wash using qPCR to account for the large proportion of bacteria that cannot grow on agar or in normal laboratory conditions. The abundance of 16S rRNA genes was significantly higher in the riparian phyllosphere than in the forest phyllospheres ($t = -2.17$; $DF = 22.1$; $p = 0.04$; Figure 2C). Interestingly, we found the presence of ARG indicator *IntI1* on both the riparian and forest leaves (Figure 2D), with forest leaves appearing to harbor higher numbers, but the difference was not significant ($t = 0.27$, $DF = 25.76$, $p = 0.79$).

Over the course of the sampling campaign, air temperature ranged from 76 to 93 deg F, and relative humidity (RH) ranged from 57 to 77% in the sampling region (Supplemental Table S1). There were scattered rain events throughout that time period, resulting in half of the sampling dates with little to no rain, and half of the sampling dates having 0.13–1.08 inches of rainfall within 24 h before sample collection. Correlation testing confirmed that this environmental variability was not driving culture-based and culture-independent PMC measurements, with the single possible exception of air temperature and leaf print bacterial counts ($r = 0.35$, $p = 0.03$).

### *3.2. Comparing Bacterial Communities in Riparian and Forest Ecosystems*

#### 3.2.1. Riparian Leaf PMCs Show Higher Level of Diversity

From 39 leaf wash samples, including abaxial and adaxial surfaces, we found a total of 3975 amplicon sequence variants (ASVs) or bacterial types (Supplemental Table S2). Overall, we identified 25 different phyla, 147 orders, and 582 genera among the bacterial ASVs (Supplemental Table S3). While a full list of taxonomic groups is available in Supplemental Table S3, the most common bacterial phyla were Proteobacteria (74.2 ± 2.9%) followed by Bacteroidota (14.4 ± 0.85%), Actinobacteriota (4.78 ± 0.81%), and Firmicutes (3.44 ± 1.2%) (Supplemental Figures S3 and S4). Beijerinckiaceae (14.4 ± 1.0%) was the most frequent bacterial family, followed by Hymenobacteraceae (10.1 ± 1.1%), Comamonadaceae (9.78 ± 3.4%), Oxalobacteraceae (8.88 ± 2.0%), Enterobacteriaceae (7.51 ± 7.1%), and Sphingomonadaceae (6.08 ± 1.1%) (Supplemental Figure S4).

On average, we found that the riparian bacterial communities harbored a higher number of ASVs, (136.33 ± 54.86) than bacterial communities found on the forest leaf surfaces (105.22 ± 35.32) ($F_{(1,34)} = 5.39$; $p = 0.03$). We found a similar difference when considering the Chao1 index, which predicts the number of bacterial types using sampling depth; the number of predicted ASV was once again higher in riparian microbial communities ($F_{(1,34)} = 4.69$; $p = 0.04$; Figure 3A).

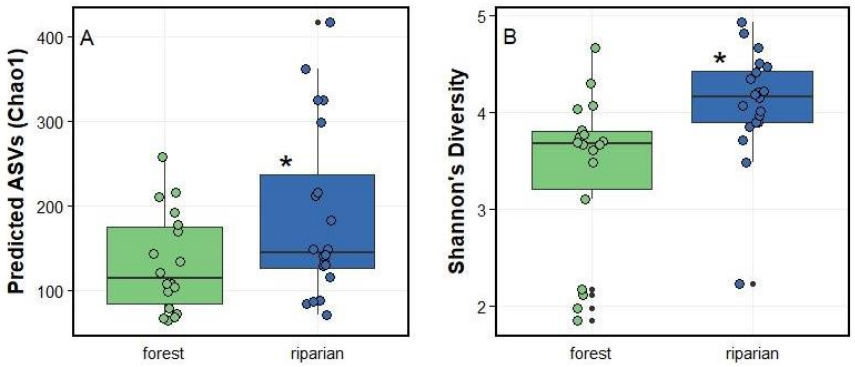


**Figure 3.** Alpha-diversity comparisons between forest leaf PMC samples ($n = 18$) and riparian leaf PMC samples ($n = 20$): (**A**) predicted ASV's (Chao1 index) and (**B**) Shannon Diversity index calculated from non-rarefied samples. Boxes and lines denote data range and mean, and black points represent outliers. Green and dark blue points denote by-sample index value. Statistically significant difference denoted with an asterisk (*).

To account for possible differences in evenness between bacterial types, we also compared diversity using Shannon Diversity index. We again found a higher diversity in riparian leaf PMC ($F_{(1,34)} = 8.56$; $p = 0.006$; Figure 3B). The latter indicates that the riparian

ecosystems host a larger number of bacterial types, and that these bacterial types are more equally distributed.

3.2.2. Bacterial Communities in Riparian Ecosystems Are Distinct from Forest Ecosystems

Due to the nonparametric nature of our dataset, we conducted a non-metric multi-dimensional scaling analysis using the Bray–Curtis dissimilarity matrix, which roughly identifies the proportion of similarities between each community pair. As expected, we found that PMCs differed significantly between the riparian and forest leaves ($F_{(1,36)}$ = 2.38; $R^2$ = 0.06; $p$ = 0.004). We also compared the microbial communities according to their phylogenetic overlap. This measure, which is weighted to account for the abundance of each ASV in addition to its phylogenetic distance, is predicted to be a better reflection of the functional diversity present in the population. Close phylogenetic matches are predicted to have more similar metabolism and ecological functions. The non-metric multidimensional scaling analysis on weighted UniFrac scores found a statistical difference between the forest and riparian leaf PMCs ($F_{(1,35)}$ = 3.10; $R^2$ = 0.08; $p$ = 0.01). Then, we repeated the analysis with unweighted UniFrac and found again that the riparian and forest PMCs differed significantly ($F_{(1,35)}$ = 1.43; $R^2$ = 0.04; $p$ = 0.04), confirming that the leaf PMCs in the riparian and forest areas were fundamentally different at the composition level and likely at the functional level as well.

Given the significant difference in overall population structure between the forest and riparian leaf PMCs, we identified which ASVs differed between the riparian and forest communities. Overall, we found 186 ASVs that changed in relative abundance between the forest and riparian areas (DESeq2; adj-$p \leq 0.05$), including 161 ASVs that were higher in abundance in the riparian area. While the most important observed differences were the over-abundance of *Hymenobacter* sp. and *Sphingomonas* sp. in the riparian PMCs, we also found an increased abundance of wastewater indicators and potential human pathogens in the riparian area, including *Clostridium* sp., *Enterococcus* sp., *Klebsiella* sp., *Mycoplasma* sp., and *Yersinia* sp. (Supplemental Figure S6).

Interestingly, using methods as per Dueker et al. [26] to identify bacterial genera known to be primarily associated with sewage, we found that 80% of riparian bacterial samples ($n$ = 20) and 78% of forest bacterial samples ($n$ = 18) harbored sewage-related bacterial genera (Figure 4). While the mean relative abundance of these sewage-associated bacteria was 1.7% in the riparian PMC samples and 0.4% in the forest PMC samples, this difference was only marginally significant ($t$ = −1.76; $DF$ = 20.63; $p$ = 0.09).

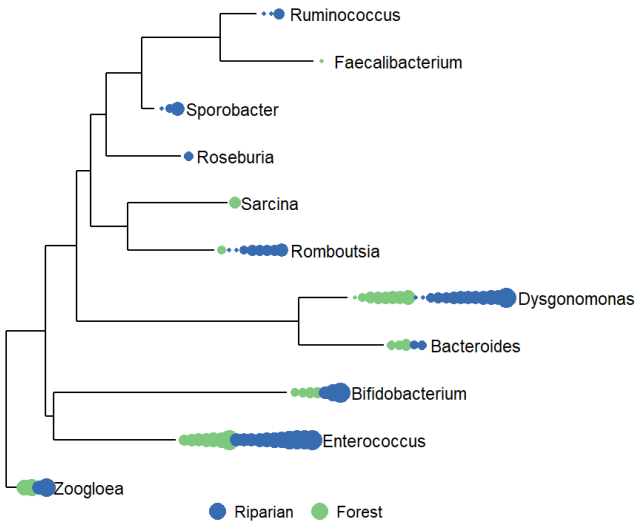


**Figure 4.** Phylogenetic tree demonstrating the by-sample abundances of ASV's identified as sewage-related found on riparian (dark blue), and forest (green) phyllospheres. Each point represents a sample, point size relates to # of ASVs, ranging from 1 to 125.

### *3.3. Water–Phyllosphere Connections*

Finally, we assessed a possible connection between the phyllospheres and the water ecosystem located near the riparian trees sampled. To do so, we compared the bacterial community composition identified in the riparian and forest ecosystems to the bacterial community composition isolated from the Saw Kill stream (Figure 5). We found that only 87 ASVs were shared across all ecosystems, which accounted for 6.0%, 8.6%, and 2.4% of all ASVs in riparian, forest, and stream microbiomes, respectively (Supplemental Table S4). We also found that the riparian and forest bacterial communities shared 310 ASVs (Supplemental Table S5) (Figure 4), which accounts for 21.5% and 30.8% of all ASVs found in the riparian and forest communities, respectively. The latter also represents the highest proportion of shared ASVs between the two ecosystems. Interestingly, we found that the riparian microbiomes shared 90 ASVs (Supplemental Table S6) with the stream microbiome, while the forest communities shared only 25 ASVs (Supplemental Table S7) with the stream microbiome. Using the non-parametric Fisher's Exact test, we found that the riparian bacterial communities share a significantly higher proportion of ASVs with the water surface bacteria communities than the forest bacterial communities do (Fisher's Exact test: adj-$p < 0.01$).

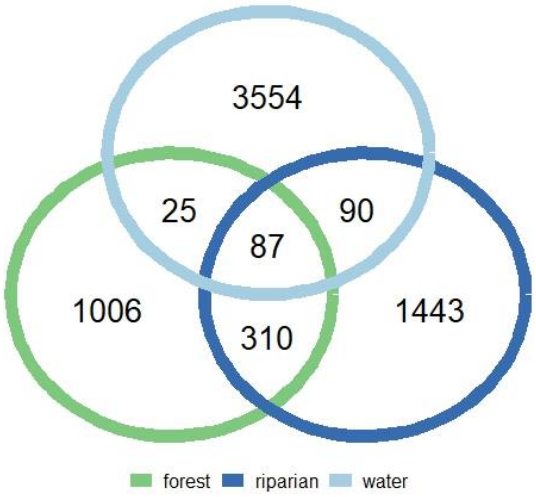


**Figure 5.** Venn diagram demonstrating the number of shared ASVs between forest (green), riparian (dark blue), and water (light blue) microbial communities.

## 4. Discussion

Here, we investigated the possible link between the bacterial communities found on the phyllospheres of trees and those found in the surface waters of a freshwater stream system. Overall, we found that the leaves of *A. rubrum* near the stream (and a water feature creating aquatic aerosols) sustained higher numbers of culturable and total bacteria than *A. rubrum* in the surrounding forest. These results suggest that there exists a separate bacterial source for riparian leaf PMCs and confirms the utility of employing both traditional culture-based methods and culture-independent techniques in studying leaf PMCs.

Additionally, we found that the riparian leaf bacterial communities were compositionally different and more diverse than the communities isolated from forest leaves. Crucially, we also found that the riparian PMCs shared a significantly higher proportion of bacterial types with surface water bacteria communities than did the forest sites. These results, while limited to *A. rubrum*, suggest that there exists a strong link between riparian leaf PMCs and the adjacent surface water bacterial content in freshwater ecosystems.

Studies of temperate forest leaf PMCs, and *A. rubrum* in particular, are sparse. To our knowledge, there has not been a previous study of riparian leaf PMCs for *A. rubrum*. However, the PMCs in our study are similar in structure at the phylum and class levels to previous studies of temperate forest PMCs, with proteobacteria (specifically Alphapro-

teobacteria and Gammaproteobacteria) dominating [42,56,57]. Furthermore, the PMCs in our study had prominent representation of the bacterial families previously noted as core PMC constituents in two temperate forests (including *A. rubrum*) in Québec, Canada. These included Beijerinckiaceae, Enterobacteriacea, Oxalobacteraceae, and Hymenobactereacea [56,57].

A separate study observing leaf PMCs across an urban gradient (including *A. rubrum*) used several sampling sites on the shore of the Lachine Canal in Montréal, Canada [42]. In this study, the *A rubrum* PMC diversity increased with urban intensity, and leaf bacterial communities of *A. rubrum* were more impacted by increased urbanization than the other trees sampled (including hackberry (*Celtis occidentalis*), white ash (*Fraxinus americana*), sugar maple (*Acer saccharum*), and white spruce (*Picea glauca*) [42]. While the trees we sampled were located in a managed forest/stream area, the riparian zone trees were along a stream impacted by urban/suburban upstream activities [45].

Given the difference in the overall population structure we found between the riparian and forest *A. rubrum*, we then investigated what ASVs differ between riparian and forest communities. Our results suggest that the bacterial communities in each zone differ both at a broad phylogenetic level and at a finer scale, indicating an important difference in terms of community composition and likely at the functional level as well.

When looking at specific changes in ASVs, we found that the riparian communities had a higher number of bacteria taxa and unique bacteria taxa not found in the forest ecosystem. We also found that the riparian communities were more evenly distributed, measured as a higher Shannon Diversity Index. Interestingly, Laforest-Lapointe et al. [56] found that Québec forest *A. rubrum* communities had a mean Shannon Diversity Index of $3.7 \pm 0.06$; however, in their subsequent study of urban exposure effects, near-shore *A. rubrum* leaf PMC Shannon Diversity Indices increased with urban exposure [42], ranging from 3.75 at low urban intensity to ~4.3 at medium urban intensity. Here, we found that the riparian zone *A. rubrum* supported a significantly higher mean Shannon Diversity Index, i.e., 4.1, than the forest zone, i.e., 3.4, which is similar to that which was described by Laforest-Lapointe. Further study is needed to understand the drivers of this difference, but proximity to water aerosol sources and what they represent (e.g., urban influence through upstream sewage and runoff) may have significant impacts on *A. rubrum* PMCs.

Forest aerosols are known to contain bacteria commonly found on PMCs, confirming air to leaf bidirectional exchanges at forest ground-level [58]. A recent study of forest aerosol bacteria at ground-level in summer outlined the dominance of bacterial phyla similar to those found on this study's PMCs, including Gammaproteobacteria, Alphaproteobacteria, Firmicutes, Actinobacteria, and Bacteroidota [58]. In both the Québec forest PMCs and our study's PMCs, the bacterial families were dominated by bacterial genera commonly detected in aerosols, including *Hymenobacter* [59–62], *Sphingomonas* [62,63], and *Oxalobacter* [62].

We also found that *A. rubrum* PMCs in our study include water pollution-associated bacteria. Aerosolization of water pollution into coastal air has been documented in polluted urban environments, with sewage-associated bacteria increasing in aerosols according to source proximity [23,26–28]. It is likely that a similar phenomenon occurs in freshwater stream systems as well. Freshwater systems often receive treated and untreated sewage inputs, creating a possible source for aerosolized sewage-related pollution. We did find an increased abundance (albeit with marginal significance) of bacteria associated with wastewater and infectious disease in riparian leaf PMCs. These bacteria included *Ruminococcus* sp., *Sporobacter* sp., *Roseburia* sp., *Romboutsia* sp., and *Dysgonomonas* sp. The riparian leaf PMCs also had higher numbers of *Enterococcus* sp. (sewage-indicating bacteria also detected in the stream using culture-based techniques [45]) than the forest leaf PMCs.

While we did not determine the sources for the PMC bacteria in this study, this finding indicates a detectable link between what is on the riparian leaves and what is in the adjacent waterways, which could occur either through bioaerosols originating from the waterway impacting on the leaves, or the leaves providing these bacteria to the stream through runoff

or re-aerosolization through wind and rain interactions. While more work is needed to explicitly determine the mechanisms of connection, our study does establish the possibility for many pathways to exist.

Finally, the fact that sewage bacteria and the micropollutant and ARG indicator *IntI1* [64] were present in a majority of water [45] and phyllosphere samples demonstrates that the *A. rubrum* phyllosphere could act as an extra-enteric reservoir for sewage-associated bacteria and ARGs, raising possible health concerns. On the other hand, our findings suggest that riparian zone vegetation could perhaps mitigate the spatial transport of aerosolized water bacteria, which is a previously unappreciated role for riparian zones. Essentially, riparian zones may not only protect waterways from external pollutants (pesticides, herbicides, etc. [44]), but they may also be filtering and/or capturing aerosolized pollution from contaminated waterways before moving into interior terrestrial environments.

## 5. Conclusions

The goal of our study was to detect and quantify the microbial exchange between seemingly disconnected microbiomes (water surfaces and leaf surfaces) in a small freshwater system. Our analyses, using culture-based and non-culture-based techniques, demonstrated a clear connection between the stream microbiome and riparian *A. rubrum* phyllospheres. In this system, the riparian zone maple leaf surfaces had higher bacterial counts than the non-riparian zone trees. Using metagenomic profiling of the 16S rRNA gene, we found that, while microbial communities on leaves in both the riparian zone and forested sites were diverse, the riparian zone bacterial communities were significantly more diverse. In addition, we found that riparian leaf bacterial communities shared more amplicon sequence variants (ASVs) with stream bacterial communities than forest leaves.

Indicators of sewage pollution, including sewage-associated bacteria and genetic materials associated with sewage (and antibiotic resistance), were detected in both the riparian zone and forest zone *A. rubrum* PMCs. The presence of these bacteria and antibiotic resistance indicators on the trees in our study implies the possibility that phyllospheres can serve as extra-enteric habitats for sewage-associated bacteria. To our knowledge, the connection between the riparian PMCs and water quality, until our study, was largely unknown. Our findings highlight the necessity for the future study of the movement of water pollution (including viruses, bacteria, and genetic micropollution) from polluted waterways into adjacent areas, as well as the subsequent role that the phyllospheres may play in filtering these bioaerosols from the air.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/aerobiology2030005/s1: Figure S1: Aerosol particle size distributions; Figure S2: Relative abundance of bacterial phyla in riparian and forest PMC's; Figure S3: Relative abundance of bacterial classes in riparian and forest PMC's; Figure S4: Relative abundance of bacterial families in riparian and forest PMC's; Figure S5: Compositional similarities between microbial communities (PCoA); Figure S6. Genus-level differences between riparian and forest PMC communities (Deseq2); Table S1. Mean regional meteorological conditions by sample date; Table S2. Breakdown of ASVs by sample; Table S3. ASV taxonomy to genus level; Table S4. ASVs shared between riparian and forest and water microbiomes; Table S5. ASVs shared between riparian and forest microbiomes; Table S6. ASVs shared between riparian and water microbiomes; Table S7. ASVs shared between forest and water microbiomes.

**Author Contributions:** Conceptualization, M.E.D., B.L. and G.G.P.; methodology, M.E.D., B.L. and G.G.P.; resources, M.E.D. and G.G.P.; data curation, M.E.D., B.L. and G.G.P.; writing—original draft preparation, M.E.D., B.L. and G.G.P.; writing—review and editing, M.E.D. and G.G.P.; funding acquisition, M.E.D. and B.L. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by the Hudson River Foundation Polgar Fund.

**Institutional Review Board Statement:** Not applicable.

**Data Availability Statement:** All data are accessible through the article, with further detail provided in Supplemental Materials. All bacterial sequences are available under BioProject accession number PRJNA1137380 in the NCBI database In addition, all stream water data are available through a Scientific Data paper by de Santana et al. [45].

**Acknowledgments:** The authors would like to thank the Bard Summer Research Institute for supporting this project as well as the Bard Biology Program, the Center for Environmental Sciences and Humanities. In addition, the authors thank Daniella Azulai and Haley Goss-Holmes for assistance in the field and Dylan Dahan for help with the qPCR analyses.

**Conflicts of Interest:** The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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