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Stream Biofilms in Western Tennessee Provide Insight into Land Use History and Ecosystem Function

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Abstract

Many streams in western Tennessee (United States) are heavily modified, altering stream morphology and microbial community dynamics. Given the interest in restoring degraded streams, integration of microbial communities and processes is needed to inform restoration efforts. We investigated community assembly dynamics of biofilm microbial communities in a degraded and undisturbed stream within the same watershed; the degraded stream is scheduled to be restored. We established biofilm sampling plots, which included the factors light exposure, length of biofilm development, and temporal variation. We conducted metabarcoding targeting bacteria and eukaryotes, investigating community dynamics of 13 functional or taxonomic groups. We also collected stream chemical data including pH, dissolved oxygen, oxygen reduction potential, total dissolved solids, K^+, NO_3^-, electrical conductivity, and temperature. We observed greater diversity and higher abundances in the more natural stream, but these patterns are taxonomically context-dependent. Using a community assembly analytical framework, we identified strong temporal effects of community structure and stream chemistry. These dynamics also highlighted biomarkers that are potential indicators of stream conditions. This work highlights the need to integrate aquatic microbial communities into conversations around stream restoration.

Keywords: metabarcoding; biofilms; microbial assembly; river restoration; physiochemistry; West Tennessee

1. Introduction

Global waterways support human populations as well as ecosystems but are complex landscapes, and the needs of humans and the environment must be balanced. The ecosystem services these waterways provide include not only drinking water for humans and wildlife, but also the movement and cycling of nutrients throughout the ecosystem [1,2]. Rivers and streams are often modified, and these modifications can have lasting environmental impacts through sediment movement and nutrient runoff, reductions in water flow, and diminished biodiversity [3]. Historically, waterways in western Tennessee (USA) were dominated by bottomland forests and floodplains. However, most of these rivers were heavily modified and channelized in the 1950s and 1960s for agricultural uses or flood prevention purposes [4]. These modifications, however, may also alter the diffusion of nutrients and pollutants in these waterways [5]. This may result in altered nutrient accessibility within streams, which may limit growth and biodiversity of microorganisms and alter competition and/or successional dynamics [6], which may limit plant and animal

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populations through trophic interactions [7]. Rivers in western Tennessee are a biodiversity hotspot in the United States, and modifications may threaten this biodiversity. The largest watershed in western Tennessee, the Hatchie River watershed, supports high levels of biodiversity, comprising many endemic and endangered taxa, with over 100 fish species and 35 freshwater mussel species represented [8,9]. While plant and animal diversity in the Hatchie River watershed are well studied, data on microbial systems, including micro-invertebrates, which serve as a base for aquatic food webs, are poorly understood.

Biofilms are dynamic communities of microbes that can form interconnected networks [10] and consist of not only microbes, but an amalgamation of a matrix of extracellular polymeric substances (EPS), which are composed of polysaccharides, proteins, lipids, nucleic acids, amino acids, and humic substances, among other components [11,12]. Biofilms readily form on surfaces when microorganisms begin excreting extracellular polysaccharides [13]. Biofilm communities are dynamic and are influenced by environmental changes, e.g., nutrient availability, but how these interactions impact these communities is still poorly resolved [14]. Biofilms may promote the arrival of new phenotypes that are adapted to the changing biofilm landscape [15]. Further, biofilms serve important biological roles, including aiding carbon, phosphorus, and nitrogen acquisition [16]. Consequently, biofilms have been suggested as a way to assess ecosystem health [17–19].

Stream degradation and how this impacts abiotic and biotic factors is a complex problem that requires a holistic framework [20]. The Stream Functions Pyramid model [21] has been proposed as a way to interpret interacting factors to understand their respective roles in shaping stream ecosystems. This model has five hierarchical layers, including hydrology, hydraulics, geomorphology, physicochemical, and biological. Incorporation of community assembly models into biofilm development may provide insight into the drivers of early and late-stage biofilm establishment, which may help inform stream restoration by providing insight into community dynamics and environmental processes [22,23].

There have been several investigations into the ecological impacts of stream degradation; however, research in the Mid-South region of the USA is lacking. Studies from Europe [24] and elsewhere within the United States (e.g., West Virginia, [25]) demonstrate the impacts of land use types on biofilms and stream microbiology. However, there is a lack of local context applicable to the unique physio-geography of the Mid-South region of the United States. This region has distinct agricultural zones and floodplains, as well as is influenced by the New Madrid Seismic Zone [4,26]. Rivers in western Tennessee have a history of modifications and channelization, and studies investigating how these alterations impact biofilm communities can be used to inform future restoration efforts and improve the biodiversity within this region.

We set up a field experiment to examine what shapes and drives stream biofilm communities by comparing a degraded stream with a natural one. In particular, we tested how successional age, weekly sampling, and light availability affect the composition of stream biofilms. Using a staggered sampling design, we evaluated the environmental, temporal, successional, and chemical factors that influence stream biofilm community structure and the processes governing community assembly. Finally, we contrasted the drivers of bacterial and eukaryotic biofilm communities, which were categorized into 13 distinct taxonomic and/or functional groups. We investigated (1) if there are fundamental microbial and physicochemical differences between disturbed and natural streams, (2) how these community differences manifest across 13 different microbial taxonomic or functional groups across streams, successional time, and sampling event, and (3) if community assembly mechanisms differ between these 13 groups and our experimental design. Further, we aimed to identify key taxa that may be biomarkers for stream conditions within the region. We expected to see differences not only in stream chemistry between stream types, but

also differences between and within the microbial communities across streams. We further predicted that community composition and assembly processes would vary along gradients of successional age, sampling week, and light availability, with the strength and direction of these patterns differing among taxonomic and/or functional groups. We hypothesized that natural streams would support greater community diversity and richness than streams experiencing greater levels of disturbance or degradation, as different taxonomic groups are expected to vary in their tolerance and responses to environmental stress and disturbance.

2. Materials and Methods

2.1. Site Description

This experiment was conducted within two streams in Haywood County, Tennessee, United States. Middle Muddy Creek is a channelized low-volume first-order stream that is directly influenced by agricultural practices (soybean, corn, and hay historically) and drains into the Hatchie River. Middle Muddy Creek was acquired by the West Tennessee River Basin Authority in 2021 for restoration purposes. Bear Creek is entirely within the Hatchie National Wildlife Refuge (NWR) and is a tributary to the Hatchie River. Bear Creek was formerly channelized but has been allowed to re-establish as a natural stream since the refuge was founded in 1964. Bear Creek was selected as a 'natural' control site for comparison to Middle Muddy Creek, as this is the most similar site within the same watershed and county that is undisturbed in recent history. Three plots were established (Table 1, Figure 1) in 2023 within each creek to assess pre-restoration community dynamics across the height of the growing season (Hatchie NWR permit #42525-07052003).

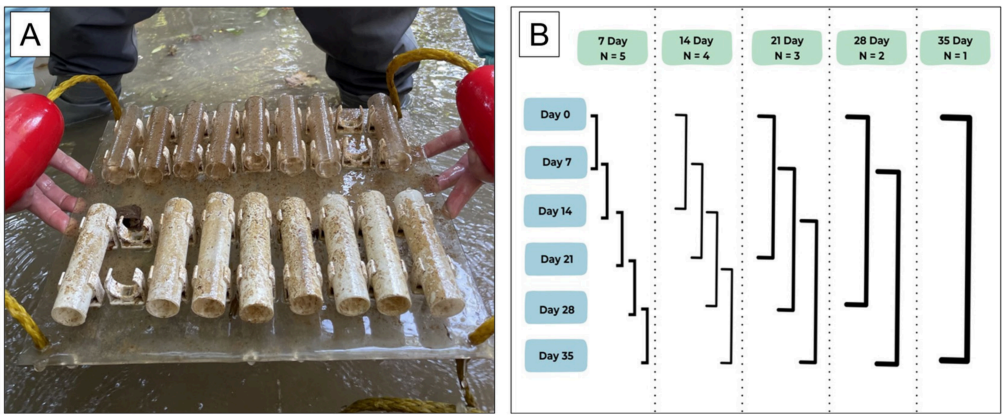


Figure 1. (**A**) Photo of sampling apparatuses used (photograph by Shawn Brown); (**B**) diagram of the staggered temporal experimental design used in this study. Opaque and clear pipes were immersed in the streams for varying lengths of growth across the 35-day sampling period (see Table S1 for additional details).

Table 1. Location of established plots, including GPS coordinates and altitude (meters above sea level). Sampling apparatuses were deployed on 7 August 2023, with a terminal sampling date of 11 September 2023.

Plot ID	GPS Coordinates	Altitude (m asl)
Middle Muddy Creek 1	35.407958 N, 89.421577 W	116
Middle Muddy Creek 2	35.407897 N, 89.419303 W	107
Middle Muddy Creek 3	35.407888 N, 89.415352 W	99
Bear Creek 1	35.512727 N, 89.266250 W	100
Bear Creek 2	35.512815 N, 89.266350 W	105
Bear Creek 3	35.512952 N, 89.266492 W	98

2.2. Sampling Design, Collection, and Physicochemical Data

At each plot (Table 1), a biofilm collection apparatus was positioned to allow for biofilm development. These apparatuses consisted of transparent, high-density polyethylene (HDPE) sheets (30 cm × 30 cm, 0.5 cm thick) with pipe clamps attached to secure acrylic pipes (2.22 cm inner diameter, 15 cm length; McMaster-Carr, Elmhurst, IL, USA). This sheet was attached to cement blocks with 1 m of polypropylene rope with plastic floats attached, allowing for pipes to remain suspended ~15 cm below the water surface. Acrylic pipes were selected for this work because, per the manufacturer, they are 92% UV transparent, which suggests minimal disruptions of autotrophic growth. These pipes were either left transparent or coated with white acrylic spray paint to preclude light availability within the pipes, allowing for differentiation between autotrophic-dominated and heterotrophic-dominated biofilms (Figure 1).

Pipes were deployed on 7 August 2023 and were sampled every seven days over 35 days to assess biofilm development. We selected this deployment date to capture most of the algal and cyanobacterial activity, as in this region these are generally at their peak in late August or early September. The staggered temporal sampling design (Figure 1, Table S1) allowed us to assess successional dynamics (7-, 14-, 21-, 28-, or 35 days of growth), sampling event to assess effects associated with temporal dynamics (sampled on days 7, 14, 21, 28, and 35), and pipe opacity to examine autotrophic or heterotrophic dominated communities (opaque, clear). In all, this resulted in a total of 180 experimental units (30 per plot). Due to flooding, the Bear Creek plots were inaccessible for two of the sampling events (August 14 and 21). During each sampling event (weekly), pipes were collected in the field; conventionally sterile (new) nitrile gloves and plastic zip-lock bags were used for this work. Pipes were frozen within three hours of collection at −20 °C until processing.

During each sampling event, we measured several water-quality parameters within each plot, including temperature (°C), pH, total dissolved solids (TDS; ppm), electrical conductivity (EC; mS/m), and oxidation–reduction potential (ORP; mV), using a Rowyet 7-in-1 Water Quality Meter (Rowyet, Dongguan City, China). Nitrate (NO_3^-) and potassium (K^+) concentrations were quantified in ppm with HORIBA LAQUAtwin Compact Nitrate and Potassium Meters (HORIBA Advanced Techno Co., Kyoto, Japan). Dissolved oxygen (DO; %) was assessed using a Hanna HI 9829 Multiparameter Waterproof Meter (Hanna Instruments, Woonsocket, RI, USA). Prior to each sampling event, all meters were calibrated based on best practices of the manufacturer with appropriate reagents. Water depth and stream width were measured at Middle Muddy Creek plots only, as Bear Creek was too wide to measure.

2.3. DNA Extractions and Metabarcoding

Prior to processing, pipes were thawed, and their external surfaces were carefully cleaned with microwave-sterilized paper towels to remove biofilms and other debris. This step was performed to minimize the potential for DNA originating from material on the exterior of the pipes and to ensure that subsequent analyses primarily represented microbial growth associated with the interior surfaces. Each pipe was transferred to a sterile 50 mL centrifuge tube containing 30 mL of sterile 1× phosphate-buffered saline (PBS; Thermo Fisher Scientific, Waltham, MA, USA). Biofilms were detached from the interior pipe surfaces using a sterile, single-use stiff-bristled brush [27] with manual agitation for 2 min. Samples were subsequently sonicated (CPX2800; Branson Ultrasonics, Danbury, CT, USA) for 10 min at maximum oscillation to further dislodge biofilm material [28]. The pipes were then removed, and the remaining suspension was centrifuged at 5000× *g* for 10 min at 4 °C (Legend X1R; Thermo Fisher Scientific, Waltham, MA, USA). The supernatant was discarded, and the resulting pellet was transferred to a DNeasy PowerBiofilm extraction

tube (QIAGEN, Germantown, MD, USA). DNA was extracted according to the manufacturer's standard protocol and quantified using a NanoPhotometer N60 (Implen, München, Germany). Bacterial and eukaryotic communities were characterized using a two-step metabarcoding approach [29]. Bacterial communities were assessed by amplifying the V4 region of the 16S rDNA gene using the primer pair nexF-NNN-515F.Apprill and nexR-NNN-806R.Parada, while the 18S rDNA region was targeted for eukaryotic community characterization using nexF-NNN-TAReuk454FWD and nexR-NNN-TAReukREV3 [30–32]. The primers incorporated Nextera sequencing primer sequences (nexF and nexR) and three ambiguous nucleotide positions (NNN) to improve primer annealing efficiency. Primary PCRs were performed in 25 µL reaction volumes containing 2.5 µL of template DNA, 12.5 µL of Phusion Green Hot Start II Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), 2.5 µL each of forward and reverse primers (1 nM), and 5 µL of molecular-grade water. For bacterial amplification, PCR conditions consisted of an initial denaturation at 98 °C for 30 s, followed by 25 cycles of 98 °C for 20 s, 56 °C for 30 s, and 72 °C for 60 s, with a final extension at 72 °C for 10 min. Eukaryotic amplifications began with denaturation at 98 °C for 30 s, followed by 10 cycles of 98 °C for 10 s, 57 °C for 30 s, and 72 °C for 40 s. This was followed by five cycles at each of five progressively increasing annealing temperatures (45, 46, 47, 48, and 49 °C), resulting in 35 total cycles, and a final extension at 72 °C for 10 min. A second PCR was performed using 25 µL reactions containing 12.5 µL of Phusion Hot Start II Master Mix, 2.5 µL of the first-round PCR product, 2.5 µL of second-round primers (0.5 nM), and 7.5 µL of molecular-grade water. The second-round primers contained sample-specific molecular identifiers (MIDs) and Illumina linker sequences [33]. Amplification consisted of an initial denaturation at 98 °C for 30 s, followed by eight cycles of 98 °C for 20 s, 50 °C for 30 s, and 72 °C for 60 s, with a final extension at 72 °C for 10 min. PCR products were evaluated by electrophoresis on 1.5% agarose gels to verify successful amplification. Individual libraries were subsequently purified and normalized using the Just-a-Plate 96 PCR Normalization and Purification Kit (Charm Biotech, Cape Girardeau, MO, USA). Molecular-grade water was included as a negative control during both DNA extraction and library preparation. No visible amplification or contamination was detected in the negative controls during gel electrophoresis. Following normalization, bacterial and eukaryotic libraries were pooled separately into two composite libraries and subjected to an additional purification using the Zymo DNA Clean & Concentrator-5 kit (Zymo Research, Tustin, CA, USA). Libraries were sequenced using an Illumina MiSeq platform with 300 bp paired-end sequencing at the Integrated Genomics Facility at Kansas State University (Manhattan, KS, USA).

Sequence Processing and Analysis

More than 6.3 million bacterial and eukaryotic sequence reads were obtained and processed separately using mothur v1.48.0 [34], following procedures largely based on [35]. Primer sequences were removed using cutadapt v4.7 [36], after which bacterial and eukaryotic sequences were independently aligned to the SILVA reference alignment (v138.1) [37]. Quality filtering included removal of sequences containing ambiguous bases, homopolymers exceeding 10 nucleotides, off-target sequences, and putative chimeric sequences. Following quality control, more than 3 million eukaryotic and 2.7 million bacterial sequences were retained for downstream analyses. Sequences were clustered into operational taxonomic units (OTUs) using the greedy clustering algorithm implemented in VSEARCH [38], with a 97% sequence-similarity threshold. The resulting OTU tables were subsequently used to calculate community diversity estimates and conduct downstream community analyses.

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Bacterial diversity was estimated by subsampling at a depth of 974 sequences per sample with 1000 iterations, and the mean values were used for analyses. Taxonomic identification of OTUs was assigned using naïve Bayesian classification [39] against the RDP database (v19). To evaluate community structure, Bray–Curtis dissimilarity values were calculated using the same iterative approach as above. Visualization of communities was performed using Non-Metric Multidimensional Scaling (NMDS) ordination using the R package *vegan*, and these were visualized using *ggplot2* [40,41].

Eukaryotic reads were processed (as above). Since this universal eukaryotic primer pair amplifies taxa across Eukarya, we separated major eukaryotic groups for analyses [42]. From the resultant eukaryotic OTUs and taxonomy information (from naïve Bayesian classification using the SILVA database), we parsed these OTUs into taxonomic or functional categories, and we only included groups that contained at least 50 OTUs. This resulted in 12 taxonomic or functional groups for eukaryotes. These included Chlorophytes (algae), Non-Chlorophyte algae, Annelids, Arthropods, Cercozoans, Ciliophorans, Unknown Eukaryotes (unable to be reliably classified), Remnant Eukaryotes (that were taxonomically identified but at an insufficient depth for a separate category), Fungi, Mollusca, Platyhelminthes, and Rotifera. After parsing alpha diversity metrics were estimated as above; however, there was not a fixed subsampling depth due to having split them into smaller taxonomic groups, thus groups were subsampled to a depth to include at least 90% of samples for each eukaryotic group to maintain comparability of alpha diversity metrics across samples (Chlorophytes—50 sequences per sample, Non-Chlorophyte algae—50 sequences per sample, Annelids—40 sequences per sample, Arthropods—40 sequences per sample, Cercozoans—50 sequences per sample, Ciliophorans—50 sequences per sample, Unknown Eukaryotes—50 sequences per sample, Remnant Eukaryotes—50 sequences per sample, Fungi—40 sequences per sample, Mollusca—40 sequences per sample, Platyhelminthes—40 sequences per sample, and Rotifera—50 sequences per sample).

All sequence data are accessioned at NCBI in SRA under the BioProject PRJNA1504259 and BioSamples SAMN62049918-SAMN62050183.

2.5. Statistical Analyses

We tested if stream chemistry differed between our creeks by performing a nested MANOVA (individual samples nested within streams) in base R. This tests variations between the two streams, and then identifies which variables drive observed differences [43]. This was visualized using the R package *candisc* [44,45], which uses a Canonical Discriminant Analysis structure.

We tested if alpha diversity differed across our experimental design (for all 13 groups) using a permutation-based ANOVA (analysis of variance) in the R package *permuco* [46] using 1000 permutations, with stream, pipe type, length of growth, and sampling event, along with all possible interactions as factors. Where tests were significant, a Tukey HSD test was used to determine which factors differed [47] and used partial eta squared (η_p^2) values to understand effect sizes (>0.06 equates to a medium effect size and >0.14 equates to a large effect size [48,49]). We visualized broad taxonomic differences across streams by generating stacked histograms of relative abundances for each taxonomic category. Using these data, we tested if taxonomic composition differed between streams, length of growth, and sampling event using χ^2 tests in R [50].

We tested if communities differed for each of the 13 groups across the experimental design using Type II PERMANOVAs (stream, pipe type, length of growth, and sampling event, along with all possible interactions based on Bray–Curtis values from above) using the R package *vegan* [40] (999 permutations). Where PERMANOVAs were significant, Tukey HSD tests were conducted in the R package *emmeans* (v.1.11.2-8, [51]), and the effect size (η_p^2) was calculated as above.

Potential biomarker OTUs, OTUs that were significantly more abundant (enriched) with a particular experimental factor, were identified. OTU data often suffer from sparsity and zero-inflation; to counteract this, we used the R package *POSTm* [52] for each of the 13 groups. This is a phylogeny-guided method that extends kernel-based approaches to the OTU-level while connecting OTUs based upon abundances as well as abundances of phylogenetically close neighbors. Maximum likelihood phylogenetic trees were inferred using *FastTree2* [53] on *MAFFT*-aligned OTU sequences, and trees and associated community matrices were used in these *POSTm* models. This results in POST, SO *p*-values (single-OTU test), and a Best C-value (the c value corresponding to the minimum POST *p*-value) [52]; the Benjamini–Hochberg procedure was used to control for multiple comparisons. Biomarker identification using *POSTm* identifies OTUs that are differentially abundant with treatments, not which treatment they are more abundant in. To determine this, directionality of significant biomarkers was determined based on average relative abundances. These putative biomarkers were further queried to attempt to identify them taxonomically, as the SILVA database has only limited representation. We took representative OTU sequences of these *POSTm* biomarkers and used BLASTn against GenBank to try to identify these taxa more specifically.

We additionally examined the ecological mechanisms underlying community assembly and evaluated whether the relative importance of these processes varied across the sampling framework. Community assembly was assessed using the R package *iCAMP* [54], which applies phylogenetic bin-based null model analyses to estimate the relative contributions of stochastic and deterministic processes to community structure. This approach further resolves community assembly into five specific ecological processes: Homogeneous Selection (HoS), Heterogeneous Selection (HeS), Homogenizing Dispersal (HD), Dispersal Limitation (DL), and Drift (DR).

Community composition data and the corresponding phylogenetic trees generated using *FastTree2*, as described above, were used to identify phylogenetic bins based on the relatedness of taxa within clades. These phylogenetic groups were subsequently evaluated using the *iCAMP* null models to determine the dominant assembly process associated with each bin. The resulting estimates were summarized as stacked histograms for each of the 13 taxonomic groups, allowing patterns in the relative contributions of assembly processes to be visualized across the experimental sampling network.

To evaluate whether community assembly processes differed across the sampling framework, Type II PERMANOVA analyses were conducted independently for each of

the 13 groups across all phylogenetic bins using the *RVAidememorie* R package [55]. When an ecological assembly process showed a significant association with a sampling factor, that factor was considered to influence community assembly. When multiple ecological processes were significantly associated with a given factor, the factor was classified as having a mixed influence on community assembly.

3. Results

3.1. Chemical Variables

MANOVA results indicated significant differences in water chemistry among streams and sampling events, whereas differences among plots were not significant. Stream identity had a strong effect on overall chemical composition ($F_{1,17}$ = 44.747, $p < 0.0001$), while plot effects were not significant ($F_{1,17}$ = 2.181, p = 0.1233). Sampling event also significantly affected chemical variables ($F_{4,17}$ = 2.622, p = 0.0010; Table S2). The two streams exhibited distinct chemical profiles. Bear Creek generally had higher dissolved oxygen concentrations, slightly warmer water temperatures, and somewhat higher pH values. In contrast, Middle Muddy Creek exhibited higher total dissolved solids (TDS), electrical conductivity (EC), nitrate (NO_3^-), and potassium (K^+) concentrations, as well as a marginally higher oxidation–reduction potential (ORP) (Figure S1).

3.2. Alpha Diversity Estimates

Observed OTU richness and diversity were generally greater in Bear Creek than in Middle Muddy Creek, with a few exceptions among the microinvertebrate groups. These patterns were largely associated with interactions between biofilm growth duration and sampling period (Figure S2; Tables S3 and S4). OTU richness was higher in Bear Creek for bacteria and seven of the 12 eukaryotic categories examined, including Chlorophytes, Arthropods, Cercozoans, Ciliophorans, Unknown Eukaryotes, Mollusks, and Platyhelminthes. Richness also increased with biofilm growth duration across eight of the 13 taxonomic categories, including Chlorophytes, Non-Chlorophyte algae, Annelids, Cercozoans, Eukaryotic Remnants and Unknowns, Fungi, and Rotifers. This increase in richness with growth duration was generally more pronounced in Bear Creek. Diversity followed similar patterns, with higher values in Bear Creek for bacteria and six eukaryotic categories: Chlorophytes, Arthropods, Cercozoans, Ciliophorans, Eukaryotic Remnants, and Platyhelminthes. Diversity also increased with biofilm growth duration across eight of the 13 taxonomic categories, including Non-Chlorophyte algae, Cercozoans, Ciliophorans, Eukaryotic Remnants, Platyhelminthes, and Rotifers.

Temporal sampling also influenced community richness and diversity. Six of the 13 taxonomic categories exhibited differences in OTU richness among sampling events, including Chlorophytes, Non-Chlorophyte algae, Cercozoans, Unknown Eukaryotes, Platyhelminthes, and Rotifers. In general, later sampling events, corresponding to later periods in the season, were associated with greater OTU richness. This temporal increase was particularly evident in Bear Creek for algae, Annelids, Arthropods, Cercozoans, Eukaryotic Remnants, and Unknown Eukaryotes, although similar temporal trends were also observed in Middle Muddy Creek. Sampling period likewise affected community diversity, with later sampling events generally supporting more diverse communities in both streams, while overall diversity remained higher in Bear Creek (Table S3).

3.3. Taxonomic Identifications

The broad taxonomic compositional structure of bacteria differs between Middle Muddy Creek and Bear Creek (χ^2 = 42.68, $p < 0.001$; Figure 2). There are increases in phyla diversity based on read abundances broadly in Bear Creek, driven by increased

abundances of poorly identified taxa at the expense of the relative abundances of the bacterial phyla Verrucomicrobia, Planctomycetes, as well as Acidobacteria. Increased taxonomic compositional diversity was also observed in Bear Creek as compared to Middle Muddy Creek for eukaryotic taxa (χ^2 = 157,445, $p < 0.001$; Figure 3).

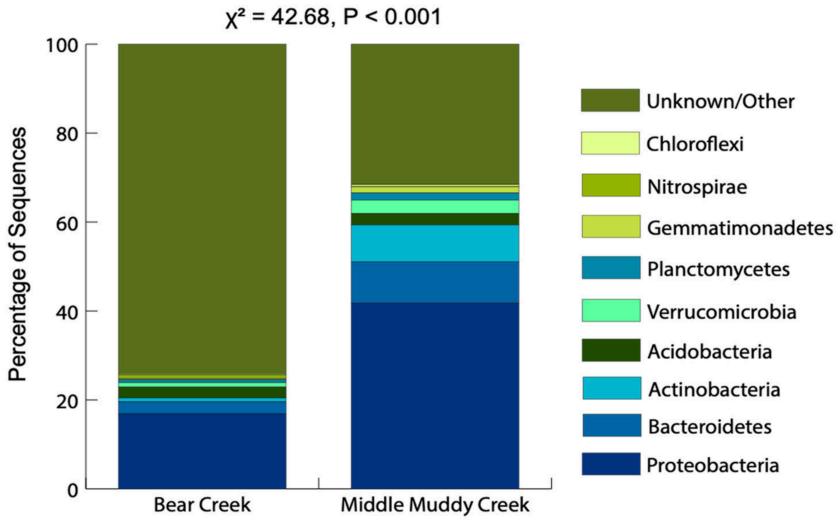


Figure 2. Taxonomic composition was greater in Bear Creek compared to Middle Muddy Creek. Presented are stacked histograms and associated χ^2 test statistic and *p*-value, illustrating the percentage of sequences composed of the top nine most abundant bacterial phyla, with an additional category for the unknown and remaining phyla not listed.

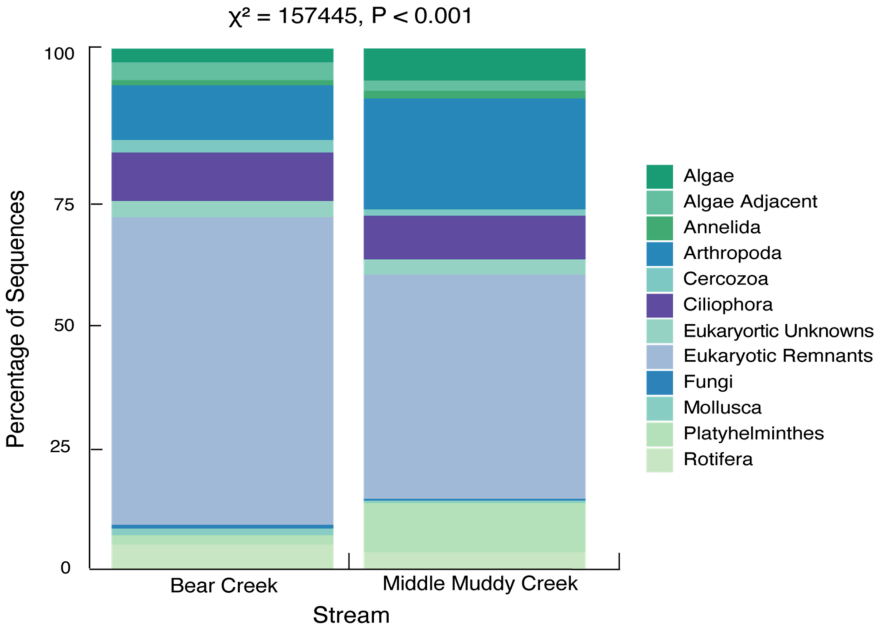


Figure 3. Eukaryotic taxonomic composition was greater in Bear Creek compared to Middle Muddy Creek. Presented are stacked histograms, and associated χ^2 test statistic and *p*-value, illustrating the relative abundance of detected sequences across 12 eukaryotic taxonomic or functional groups.

3.4. Community Structure

Sampling event (sampling week) significantly drove community structures for all 13 categories, with communities also differing between streams for all categories, with bacterial community separation being exceptionally pronounced ($F_{1,100}$ = 24.2731, p = 0.001, η_p^2 = 0.1953; Figures 4 and S3). Several significant interaction terms were also observed (Tables 2 and S5) and are context-dependent.

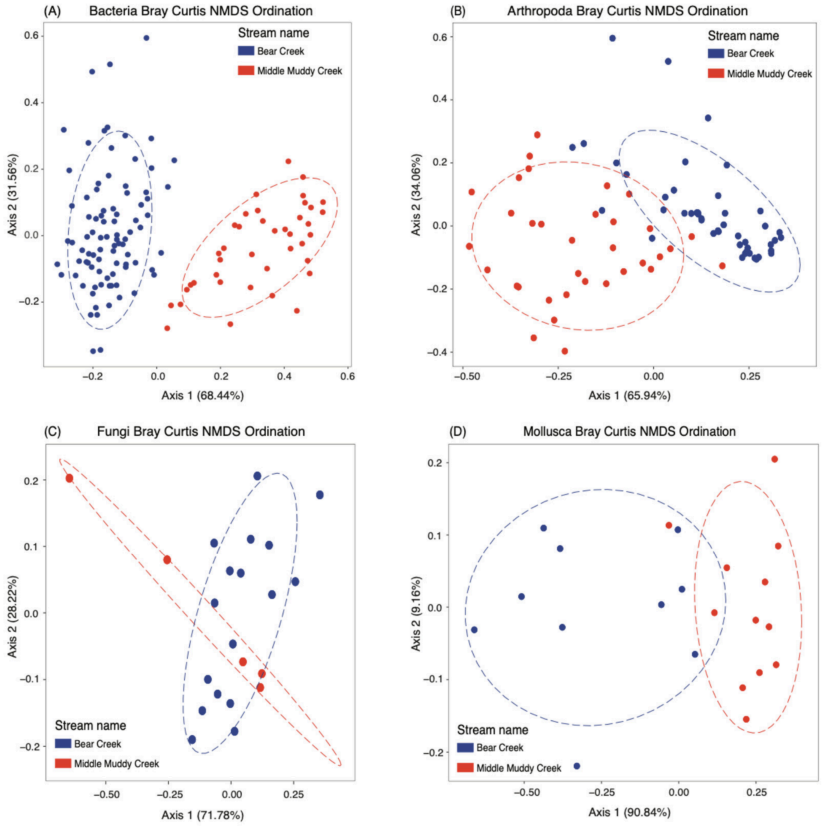


Figure 4. Visualizations of Non-Metric Multidimensional Scaling of community structural differences between streams and select eukaryotic taxonomic categories that featured the greatest effect sizes for streams from Type II PERMANOVAs (Table 2). The dissimilarities between streams were based upon a Bray–Curtis Dissimilarity for bacteria (**A**), arthropod (**B**), fungi (**C**), and mollusks (**D**) communities. Included are 95% community ellipses.

Table 2. Results of Type II PERMANOVAs for all 13 groups for each main treatment effect (full results are presented in Table S5). Presented are associated pseudo-F test statistics, R^2 values, *p*-values, and effect size values (partial eta-squared). Significant factors are bolded and italicized.

Taxonomic Group	Stream				Pipe Type			
	F Statistic	R^2	*p*-Value	η_p^2	F Statistic	R^2	*p*-Value	η_p^2
Bacteria	***24.2731***	***0.1434***	***<0.001***	0.1953	0.8415	0.0050	0.668	0.0083
Chlorophytes	***7.7519***	***0.0606***	***0.001***	0.0948	1.0093	0.0079	0.415	0.0135
Non-Chlorophytes (Algae)	***10.9709***	***0.0819***	***0.001***	0.1355	***4.4076***	***0.0164***	***0.001***	0.0305
Annelida	***5.8072***	***0.0246***	***0.001***	0.0565	***4.1733***	***0.0177***	***0.003***	0.0412
Arthropoda	***42.4815***	***0.1497***	***<0.001***	0.2449	***2.3050***	***0.0081***	***0.027***	0.0173
Cercozoa	***22.0993***	***0.0550***	***0.001***	0.0884	***3.1135***	***0.0077***	***0.001***	0.0135
Ciliophora	***12.3145***	***0.0392***	***0.001***	0.0749	***2.3840***	***0.0076***	***0.002***	0.0154
Eukaryotes Unknown	***26.1623***	***0.0655***	***<0.001***	0.1046	***3.9270***	***0.0098***	***0.001***	0.0172
Eukaryotes Remnants	***24.2056***	***0.0696***	***0.001***	0.1089	***3.7484***	***0.0108***	***0.001***	0.0186
Fungi	***21.3047***	***0.1049***	***0.001***	0.4073	***4.7533***	***0.0234***	***0.001***	0.1329
Mollusca	***17.2984***	***0.1494***	***0.001***	0.3657	0.9789	0.0085	0.442	0.0316
Platyhelminthes	***15.1233***	***0.0549***	***0.001***	0.0933	***1.9921***	***0.0072***	***0.03***	0.0134
Rotifera	***35.0872***	***0.1173***	***<0.001***	0.2100	***2.6834***	***0.0090***	***0.006***	0.0199

Table 2. *Cont.*

Taxonomic Group	Length of Growth				Sampling Event			
	F Statistic	R^2	*p*-Value	η_p^2	F Statistic	R^2	*p*-Value	η_p^2
Bacteria	***5.2898***	***0.0312***	***0.001***	0.0502	***3.8423***	***0.0908***	***0.001***	0.1332
Chlorophytes	***1.8036***	***0.0705***	***0.001***	0.1086	***2.2410***	***0.0525***	***0.001***	0.0833
Non-Chlorophytes (Algae)	***4.2633***	***0.0636***	***0.001***	0.1086	***4.7761***	***0.0535***	***0.001***	0.0928
Annelida	***6.8833***	***0.1458***	***0.001***	0.2619	***5.7469***	***0.0730***	***0.001***	0.1509
Arthropoda	***3.0100***	***0.0530***	***0.001***	0.1030	***4.6118***	***0.0650***	***0.001***	0.1234
Cercozoa	***4.9734***	***0.0619***	***0.001***	0.0983	***6.9549***	***0.0692***	***0.001***	0.1087
Ciliophora	***3.8749***	***0.0616***	***0.001***	0.1131	***7.8757***	***0.1002***	***0.001***	0.1717
Eukaryotes Unknown	***7.1455***	***0.0895***	***0.001***	0.1376	***6.3089***	***0.0632***	***0.001***	0.1013
Eukaryotes Remnants	***4.6806***	***0.0673***	***0.001***	0.1057	***6.0144***	***0.0692***	***0.001***	0.1083
Fungi	***8.1692***	***0.2010***	***0.001***	0.5685	***11.5572***	***0.1707***	***0.001***	0.5280
Mollusca	***7.1636***	***0.3093***	***0.001***	0.5442	***4.5649***	***0.1183***	***0.001***	0.3134
Platyhelminthes	***2.8178***	***0.0512***	***0.001***	0.0875	***5.7119***	***0.0830***	***0.001***	0.1345
Rotifera	***4.7933***	***0.0801***	***0.001***	0.1537	***5.3663***	***0.0717***	***0.001***	0.1399

3.5. OTU Enrichment Across Sampling Design

Twenty-three significant biomarker taxa from the Bacteria, Algae, Fungi, and Mollusca taxonomic groups were identified after correction for multiple comparisons. These candidate site-associated OTUs may be reflective of changes between streams, potentially suggesting they may serve as a marker of stream health and condition (Figure 4; Bacteria, Fungi, and Mollusca), but additional work is required to validate this across western Tennessee. Of these, 13 were enriched in Middle Muddy Creek: five bacteria, three fungi, and five mollusks. We present SILVA-based identification here based on naïve Bayesian classification, as we had good bootstrap support for these identifications, but BLAStn-based identities are also presented (Tables S6–S8).

The strongest OTU-specific biomarkers associated with Middle Muddy Creek differed among taxonomic groups. Among bacteria, the two most abundant biomarkers were OTU000006 and OTU000030, both of which were putatively identified as *Azotobacter vinelandii* (Pseudomonadaceae). For fungi, OTU000287 and OTU000121 were the most abundant site-specific biomarkers, with both receiving their best taxonomic assignment as uncultured Cryptomycota species. The two most abundant mollusk biomarkers were OTU000175, putatively identified as *Gibbosula polysticta* (Margaritiferidae), and OTU000214, potentially identified as *Biomphalaria glabrata* (Planorbidae). However, because mollusk sequence representation in global genetic reference databases is limited, these taxonomic assignments should be interpreted cautiously.

Bear Creek was characterized by 10 OTU-specific biomarkers, with biomarkers detected exclusively within the bacterial and Mollusca groups. The two most abundant bacterial biomarkers, OTU000103 and OTU000150, could not be assigned beyond the domain-level classification of Bacteria. Among Mollusca, OTU000044 was potentially identified as *Corbicula fluminea* (Cyrenidae), while OTU000061 received a putative identification as *Biomphalaria glabrata* (Planorbidae). Complete *POSTm* results are provided in Tables S6–S8.

3.6. Community Assembly Processes

Investigation of community assembly processes of the 13 groups across our experimental design identified differences between the assembly dynamics that demonstrate that eukaryotic communities are largely driven by stochasticity and bacterial communities are deterministically driven (Figures 5 and S4; Tables 3 and S9). The following eukaryotic groups were significant for stochastic or, in rare cases, deterministic assembly processes: Chlorophytes were predominantly driven by the stochastic process of Homogenizing Dispersal; non-chlorophytic algae assembly was driven by the stochastic process of Dispersal Limitation as well as the deterministic process of Heterogeneous Selection. Annelids were largely driven by the deterministic process of Heterogeneous Selection, and Arthropods were driven by the deterministic process of Dispersal Limitation as well as the stochastic process of Homogenizing Dispersal. Cercozoans were driven by deterministic and stochastic processes (Heterogeneous Selection—deterministic, Dispersal Limitation—stochastic); Ciliophorans were driven by stochastic processes (Homogenizing Dispersal and Dispersal Limitation). Overall, several bacterial communities (33.33%) were driven by mixed assembly processes (Dispersal Limitation & Heterogeneous Selection), with the rest (66.67%) being driven strictly by deterministic Heterogeneous Selection (Table 3).

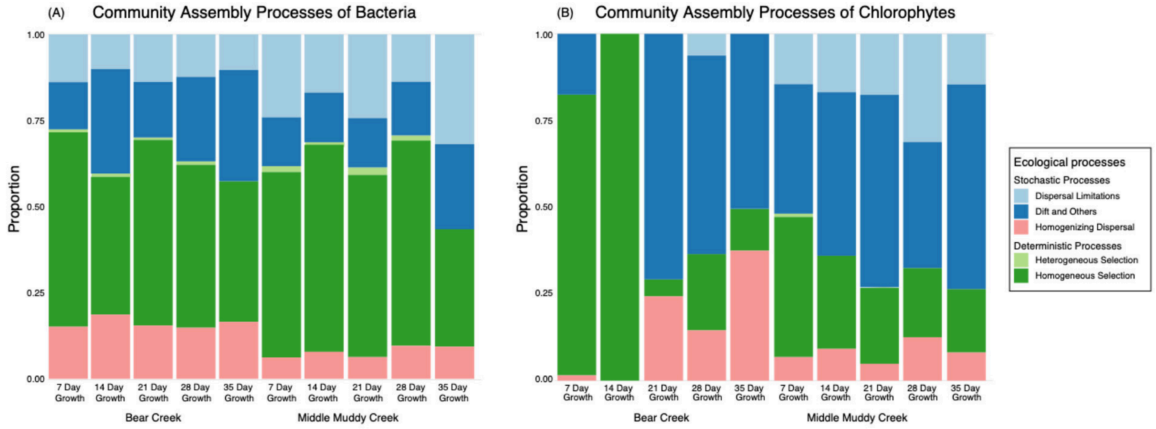


Figure 5. Stacked histograms of iCAMP-identified assembly processes across varying lengths of growth between both streams for bacteria (**A**) and chlorophytes (**B**) indicate changing drivers of assembly dynamics over time.

Table 3. Table of assembly processes driving each of the 13 groups, where PERMANOVA tests were significant. Processes marked with an 'X' indicate that it was determined to at least partially drive the assembly for a particular treatment; if both processes were filled, then it was considered a mixed influence. If no processes were significant, they were denoted with a hyphen.

Taxonomic Group	Stream		Length of Growth		Sampling Event	
	Deterministic	Stochastic	Deterministic	Stochastic	Deterministic	Stochastic
Bacteria	X	X	X	-	X	-
Chlorophyta	-	X	-	-	-	-
Non-Chlorophytes	X	-	X	-	-	X
Annelida	-	-	-	-	X	
Arthropoda	-	X	-	X	-	X
Cercozoa	X	-	-	X	-	X
Ciliophora	-	X	-	X	-	X

Table 3. *Cont.*

Taxonomic Group	Stream		Length of Growth		Sampling Event	
	Deterministic	Stochastic	Deterministic	Stochastic	Deterministic	Stochastic
Eukaryotes Unknown	-	-	-	X	-	X
Eukaryotes Remnants	-	-	-	X	-	X
Fungi	-	-	-	-	-	-
Mollusca	-	-	-	-	-	-
Platyhelminthes	-	-	-	-	-	-
Rotifera	-	-	-	-	-	-

4. Discussion

This study examined fundamental differences in biofilm microbial communities between disturbed and natural streams in western Tennessee, United States. Drivers of community structure, biomarker taxa, and community assembly processes for 13 taxonomic or functional groups across succession age (length of growth), temporal sampling event, and pipe opacity were investigated. In one of the most comprehensive examinations of the drivers and taxonomic patterns of stream microbial dynamics in the USA, we identified major differences between streams and taxon-specific differences in community dynamics and assembly processes. This represents one of the first attempts to assess differences between the heterotrophic and autotrophic-dominated aquatic communities via biofilm formation (*but see* [19]). Previous work highlighted the influences of stream morphology and hydrology on biofilm accumulation and diversity, yet there is a shortage of information on how interactions between these factors may influence stream ecology [56,57]. It is critical to understand these interactions and stream microbial dynamics to inform future restoration efforts.

One major finding of this work, though perhaps not surprising, is the major differences between biofilms between Middle Muddy Creek, which is heavily modified and has large agricultural influences, and Bear Creek, which is an unmanaged and natural stream. Most waterways within western Tennessee have been channelized to meet agricultural, forestry, or urban expansion needs [3,4]. These modifications result in flow energy changes, and this, alongside sporadic and intense flooding, has contributed to streambank degradation seen throughout this region [58]. This has resulted in instabilities in bank morphology and surface water disconnection in Middle Muddy Creek. During periods of reduced precipitation, surface flow was intermittent within Middle Muddy Creek; in contrast, Bear Creek was always flowing. This surface disconnection can lead to microbial communities being readily disrupted, restarting successional trajectories, which may result in a more conserved gene pool, as well as altered competition dynamics between species with limited nutrient availability [59–61], which helps to explain the broad community differences seen here. This intermittent flow in Middle Muddy Creek may also result in frequent functional disruptions, resulting in unique chemical attributes as well as fostering stress-tolerant oligotrophic phyla that persist under influxes of abiotic and biotic stressors [61,62]. Middle Muddy Creek contains high abundances of stress-tolerant bacterial taxa such as 'Proteobacteria' and Planctomycetes, including the *POSTm*-enriched Planctomycetes biomarkers OTU000027, OTU000081, OTU000092, OTU000124, and OTU000273, and the 'Proteobacteria' biomarkers OTU000065, OTU000180, OTU000165, OTU000275, and OTU000310, which may also be linked to stress tolerance [63]. It should be noted that recent taxonomic changes have eliminated the phylum name Proteobacteria and have been redescribed as Pseudomonadota [64], but 'Proteobacteria' was retained here as this aligns with the RDP database

used for taxonomic identification. Many species of Planctomycetes are resistant to nitrate, nitrite, and ammonium fluxes [65], which is further supported by chemical measurements observed here. During our sampling, Bear Creek had no disruptions in flow and even had a slow-moving bank overflow flooding event. In contrast, Middle Muddy Creek's flooding events are more sporadic and typical of bank-full events (i.e., flash flooding events with high energy flow). As a result, the extreme variations in flow and depth may have caused persistent system instability, leading to lower stress-tolerant species persistence within the stream [66–68]. Additionally, the chemistry of Middle Muddy Creek may have contributed to the increased abundance of Verrucomicrobia, which may have the capacity for degradation and metabolism of complex polysaccharides and residual agricultural organic matter [69,70], which may partially explain their enrichment in Middle Muddy Creek. An increase in Acidobacteria also corresponds with chemical data; the previous literature indicates that their presence often coincides with high stress and poor nutrient environments, as seen in Middle Muddy Creek [71]. The genus *Azotobacter* is a well-studied nitrogen-fixing bacterium that, unlike most diazotrophs, is an obligate aerobe. Additionally, some taxa within *Azotobacter* have also been shown to produce diverse iron-scavenging siderophores that may aid in survival in environments of high stress [72]. It should be noted that generally *Azotobacter* are found in terrestrial systems, so their enrichment in Middle Muddy Creek may not be biological in nature even though they were found in biofilms, but rather due to soil runoff from degraded banks and/or flash flooding events; more work is required to verify if these are active in this creek. The fungal phylum Cryptomycota, of which several biomarker OTUs within Middle Muddy Creek belong, is *incertae sedis* within the Kingdom Fungi and largely serves roles in cycling energy through the systems they inhabit, as well as acting as parasites and competitors to other microbes [73]. *Biomphalaria glabrata*, a mollusk which was identified as a biomarker taxon from Middle Muddy Creek, is a model in physiological studies due to its high sensitivity to aquatic biotic and abiotic stressors, which is reflective of the stream conditions found in Middle Muddy Creek [74]. All these potential biomarkers and their unique responses to the conditions of a more degraded stream highlight the need to understand the microbial ecology of a 'natural' stream to aid restoration plans, as this can give managers a target for restoration monitoring success.

The successional patterns observed over increasing biofilm growth duration were generally consistent with previous studies. Among the eukaryotic communities, Chlorophytes appeared to function as early-colonizing or pioneer taxa, representing an average of 43.02% of the taxa identified through the *POSTm* analysis. As succession progressed, biomarker communities became more taxonomically diverse and increasingly included taxa that could not be confidently assigned to a specific taxonomic group. In particular, 51.33% of the Unknown Eukaryote biomarker taxa were associated with mid-stage succession, increasing to 60.43% during late-stage succession [75]. These findings highlight the need for expanded biodiversity surveys and DNA barcoding efforts in understudied ecosystems and geographic regions, including streams throughout the Mid-South United States. Increasing genetic reference data from this region would improve the taxonomic resolution of future community assessments.

Bacterial communities displayed a similar successional pattern to that reported in previous studies, with Proteobacteria consistently representing a dominant component across all stages of biofilm development. Their relative representation was greatest during mid-stage succession, when Proteobacteria accounted for approximately 50.17% of sequences, followed by a decline during late-stage succession (Table S10) [76,77]. These results are consistent with previous observations that Proteobacteria commonly dominate microbial communities in stream ecosystems.

Although pipe opacity has been shown to influence biofilm community structure [28], the magnitude and mechanisms of this effect remain poorly understood. In the present study, significant differences between autotroph- and heterotroph-dominated treatments were detected for both bacterial and Chlorophyte communities. Several factors related to the sampling environment and characteristics of stream biofilms may explain these patterns. Stream flow is known to influence microbial community development, and despite the limited light availability within opaque pipes, algae and bacteria transported within the water column may readily attach to developing biofilms. Consequently, colonization of the experimental pipes may occur regardless of the photosynthetic conditions within the pipe environment [78,79]. Further, because the pipes were relatively short (15 cm), incident light may have reached portions of the pipe interior at different times of day, and light reflected from the surrounding stream environment may have entered the pipes. This possibility may have been particularly relevant given the relatively high concentrations of total dissolved solids in the streams, potentially contributing to greater similarity between communities developing in opaque and transparent pipes. Another explanation is that light availability may alter biofilm community structure and function without necessarily producing corresponding differences in overall diversity. Previous work examining biofilms across different light intensities found that changes in light availability affected biofilm structure and ecological function, including processes occurring within streams, but did not necessarily alter community diversification [80]. Collectively, these findings are consistent with both the present results and previous research indicating that light availability may exert a weaker influence on stream biofilm communities than factors such as stream location and the duration of exposure to the stream environment.

These differences in stream functionality impacted microbial community structure as well as community assembly and biofilm establishment mechanisms. In previous studies, it was found that deterministic processes largely shape assembly processes [81], regardless of season, but successional age may influence the strength of these processes. In low-diversity bacterial communities, it has been suggested that deterministic processes drive community assembly [18,82]. Bacterial communities have been described as largely assembling via deterministic processes, whereas stochastic processes largely dominate eukaryotic assembly dynamics [83], but much less is known about assembly dynamics for eukaryotic groups within streams and how these assembly dynamics may differ across taxonomic and/or functional groups. In this study, it was seen that bacterial biofilms were driven by deterministic processes, with Heterogeneous Selection and Homogeneous Selection largely driving community assembly. Heterogeneous Selection is selection for a variety of differences or traits, which facilitate community divergence, highlighting which species are undergoing selection for community establishment [84]. This suggests that bacterial taxa that can persist in varying environmental conditions and nutrient fluxes are more stress-resistant [66,85] and play an outsized role in community structure and function.

Occurrences of mixed influences of stochastic and deterministic processes in the establishment of eukaryotic communities might be expected. For many groups, assembly patterns within streams have, to our knowledge, never been investigated. Major groups such as Chlorophyte and Non-Chlorophyte algae, Annelids, Arthropoda, and Ciliophora are commonly described in the literature in the construction of eukaryotic microbial communities; however, Cercozoans and Rotifers are less well represented in the literature. For example, Cercozoans and Rotifers are major predators of bacteria and play large roles in nutrient cycling, and incorporating these groups into our understanding of stream ecology advances our understanding of these streams and provides new avenues of future investigations. Because these are poorly studied, this can lead to key biogeochemical processes being underrepresented, leading to downstream complications in the quantification of

biodiversity [86–89]. For example, the detection and abundance of Rotifers may not solely be an indicator of biodiversity with more 'rare' taxa represented, but it could instead serve as a marker for declines in environmental health [90].

In early successional communities, characterized by relatively short periods of biofilm development, stochastic processes are expected to play a greater role in determining community structure. Within stream ecosystems, stochasticity may be driven by variable flow conditions, precipitation, flooding, and other episodic disturbances that can alter community composition by disrupting competitive interactions or influencing which taxa are able to persist following disturbance events [91,92]. As communities become more established over time, deterministic processes may increasingly influence community assembly. Under an environmental filtering framework, for example, abiotic conditions such as pH, temperature, and other physicochemical characteristics can impose selective pressures that favor particular taxa, resulting in a greater deterministic influence on community composition [93–95].

This pattern may be particularly relevant to Bear Creek, a relatively natural stream where episodic environmental disturbances, such as flooding and drought, may exert less severe or persistent effects on microbial communities. Reduced disturbance may allow established taxa to persist and interact over longer periods, providing greater opportunity for environmental filtering and other deterministic processes to shape community assembly.

Community assembly processes vary across time, in addition to streams, but these patterns differ between eukaryotic taxonomic groups. As time proceeds, diversity and assembly processes shift. As communities develop, they are heavily influenced by stochastic processes, but once established, deterministic processes may drive these communities [96,97]. These patterns, however, likely depend on taxonomy and organism size [98], reproductive strategies, and/or resting state or potential to produce cysts. In larger body microbes, there may be decreased colonization and diversification, and thus there is a greater probability for stochastic events to cause major changes within community structures, and the reverse is true for smaller groups [98]. However, the lack of detailed studies related to assembly processes in many of these eukaryotic groups makes comparisons of our results difficult. Further studies of these groups from an ecological perspective should be conducted to better understand how assembly processes may interact with ecosystem functionality. Additionally, the scarcity of certain taxonomic groups may also influence the processes driving community structure, with rarer taxa being predominantly driven by stochastic processes whereas more abundant taxa being driven by determinism [99,100].

The consequences of anthropogenic activities on waterways have been well documented, and as such, there are set practices that have demonstrated effectiveness in restoring degraded streams. One method to combat the morphological changes is to introduce a natural-channel design, which could range from introducing new vegetation along the waterway to physically connecting the stream back to the floodplain, which the stream was originally part of [101,102]. Even though microorganisms play an outsized role in stream health, they are rarely considered in restoration decisions [103]. This work clearly demonstrates that lotic microbes differ in community organization and function between the more natural and disturbed streams, and restoration efforts should be conducted in a way to increase habitat diversity for key microorganisms to thrive.

5. Conclusions

We investigated biofilm community dynamics in a disturbed stream as compared to a natural stream in western Tennessee, United States. In doing so, we contrasted differences in microbial community and assembly responses across 13 different microbial taxonomic and functional groups with successional age, light exposure, and sampling event. We find

that the disturbed stream has decreased diversity and less stable community assembly dynamics. We also describe for the first time community assembly processes for several ecologically important but understudied eukaryotic groups in stream systems. Taken together, we provide valuable data on the differences between disturbed and natural streams that should be part of the conversations going forward on stream restoration plans.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/environments13090492/s1, Table S1: Tabulation of the numbers of samples for each treatment combination, as well as a summation of samples numbers per main factor; Table S2: Results of the nested MANOVA analysis conducted on physiochemical measures for both streams, their plots, and the sampling event; Table S3: The experimental factors influencing species diversity suggest that stream and length of growth have the most significant overall effect upon several of the 13 taxonomic groups. Table of the results of the ANOVA analysis upon Simpson's diversity metric and the ηp2 values for significant factors; Table S4: The experimental factors influencing alpha diversity metrics suggest that stream and sampling event have the most significant overall effect upon several of the 13 taxonomic groups. Table of the results of the ANOVA analysis upon the richness metric and the ηp2 values for significant factors; Table S5: Table of the Type II PERMANOVA results and interactions for all 13 taxonomic groups for each treatment and their interaction terms. Presented are the pseudo-F-statistic, R2 value, *p*-value and partial eta square effect size. Significant terms are bolded and italicized; Table S6: Table of the POSTm-enriched OTUs for all 13 of the taxonomic groups differentiated by stream, with their potential taxonomic ID indicated. IDs were derived from NCBI's BLASTn, with the highest percent identity used as the potential identity. Accession numbers for best BLASTn match are included. MC = Middle Muddy Creek, BC = Bear Creek; Table S7: Table of the POSTm-enriched OTUs for all 13 of the taxonomic groups differentiated by length of growth (LG), with their potential taxonomic ID indicated. IDs were derived from NCBI's BLASTn, with the highest percent identity used as the potential identity. Accession numbers for potential identities are included; Table S8: Table of the POSTm-enriched OTUs for all 13 of the taxonomic groups differentiated by sampling event (SE), with their potential taxonomic ID indicated. IDs were derived from NCBI's BLASTn, with the highest percent identity used as the potential identity. Accession numbers for potential identities are included; Table S9: Table of the assessment Type II PERMANOVA results for all 13 taxonomic groups for each treatment for the different ecological processes. Significant terms were equated to that term being the dominating process—if there were more than one process occurring, then it was deemed a mixed influence. If no processes were deemed significant, they were denoted with NA. To determine percentages, there were two assigned ecological processes for each treatment; each of the ecological processes for each taxonomic group would be equivalent to 33.33%; Table S10: Table of the successional stage assessment of bacterial phyla across lengths of growth in this study and their percent composition across time; Table S11: Table of the SRA Accession IDs for sequences; Figure S1: Results of candisc visualization examining stream physiochemistry between Bear Creek and Middle Muddy Creek, suggesting chemical differences between streams (left) and vectors of specific variables identifying which streams they are enriched in (right); Figure S2: Observed species richness for all 13 taxonomic groups examined across both streams for varying sampling events. The overall trends highlight greater richness within Bear Creek (more natural stream), which coincides with previous literature and test statistics; Figure S3: Graphs of the Non-Metric Multidimensional Scale Models of the eukaryotic taxonomic group community structural differences between the two study streams, which featured the greatest effect size for the streams from their Type II PERMANOVA. The dissimilarities between the two streams were based upon a Bray-Curtis Dissimilarity Index for eukaryotic communities; Figure S4: iCAMP ecological process proportions across varying lengths of growth between both study streams for the remaining taxonomic groups not presented in the main manuscript. Due to poor sample representation of some groups for samples, no data can be presented for these events.

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Data Availability Statement: Sequences are available under SRA at NCBI for BioProject accession number PRJNA1504259 (Table S11), with additional data and sequence information available upon request.

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