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Headwater Stream Microbial Diversity and Function across Agricultural and Urban Land Use Gradients

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ABSTRACT Anthropogenic activity impacts stream ecosystems, resulting in a loss of diversity and ecosystem function; however, little is known about the response of aquatic microbial communities to changes in land use. Here, microbial communities were characterized in 82 headwater streams across a gradient of urban and agricultural land uses using 16S rRNA gene amplicon sequencing and compared to a rich data set of physicochemical variables and traditional benthic invertebrate indicators. Microbial diversity and community structures differed among watersheds with high agricultural, urban, and forested land uses, and community structure differed in streams classified as being in good, fair, poor, and very poor condition using benthic invertebrate indicators. Microbial community similarity decayed with geodesic distance across the study region but not with environmental distance. Stream community respiration rates ranged from 21.7 to 1,570 mg O_2 m^{-2} day^{-1} and 31.9 to 3,670 mg O_2 m^{-2} day^{-1} for water column and sediments, respectively, and correlated with nutrients associated with anthropogenic influence and microbial community structure. Nitrous oxide (N_2O) concentrations ranged from 0.22 to 4.41 μg N_2O $liter^{-1}$; N_2O concentration was negatively correlated with forested land use and was positively correlated with dissolved inorganic nitrogen concentrations. Our findings suggest that stream microbial communities are impacted by watershed land use and can potentially be used to assess ecosystem health.

IMPORTANCE Stream ecosystems are frequently impacted by changes in watershed land use, resulting in altered hydrology, increased pollutant and nutrient loads, and habitat degradation. Macroinvertebrates and fish are strongly affected by changes in stream conditions and are commonly used in biotic indices to assess ecosystem health. Similarly, microbes respond to environmental stressors, and changes in community composition alter key ecosystem processes. The response of microbes to habitat degradation and their role in global biogeochemical cycles provide an opportunity to use microbes as a monitoring tool. Here, we identify stream microbes that respond to watershed urbanization and agricultural development and demonstrate that microbial diversity and community structure can be used to assess stream conditions and ecosystem functioning.

KEYWORDS Benthic Index of Biotic Integrity, species-area curves, nitrous oxide, respiration, aquatic ecosystems, Chesapeake Bay, species-area relationships

Biodiversity is critical to ecosystem functioning and is threatened by anthropogenic activity (1). Microbes perform key ecosystem functions (2–4), necessitating a better understanding of how microbes and microbial diversity respond to environmental stressors (5–7). Streams are examples of threatened ecosystems, where watershed modification decreases stream integrity and water quality (8–11), altering macroinver-

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tebrate, fish, and microbial diversity (11–15). The use of macroinvertebrate and fish indices to assess stream conditions is fundamental to stream ecology (16) and depends on known relationships between stream integrity and community structure (17, 18). The Benthic Index of Biotic Integrity (B-IBI) is one such index, using the abundance and diversity of stream benthic macroinvertebrates to accurately distinguish degraded streams (B-IBI < 2.9) classified based on stream chemical and physical criteria (18). Biotic indices are calibrated to specific regions, as the distribution of stream macroinvertebrates is controlled by a combination of dispersal limitation and local environmental conditions (19). Despite their abundance and ecological importance, natural microbial communities, unlike macroinvertebrates, are not used in stream monitoring programs to assess stream conditions.

As with macroinvertebrates, dispersion and environmental selection control the spatial distribution of microbes along stream continuums (20). Dispersion, or the advection of microbes from the surrounding landscape, impacts headwater stream community composition, and with increasing stream order, environmental sorting becomes more important as stream residence times increase (20). Several studies have demonstrated the influence of the surrounding landscape on stream microbes, showing that watershed urbanization leads to shifts in bacterial communities (21–24). While alpha diversity generally remains constant (22–24), the abundances of taxa associated with anthropogenic activity and high-nutrient conditions increase in urbanized streams (22, 24). Similar to larger organisms, microbes respond to environmental disturbance and are strongly influenced by watershed land use (25, 26); therefore, their distribution may be used to further characterize stream conditions.

Microbes mediate important stream ecosystem functions, controlling the movement of carbon and nitrogen through freshwater ecosystems (2–4). Previous studies demonstrate the effects of urbanization on stream nutrient transformations, such as nitrogen uptake (27), nitrogen retention (28), and carbon processing (29, 30). Community respiration determines the fate of terrestrial carbon in headwater streams, where carbon is either lost as carbon dioxide during respiration or transported farther downstream (31). Community respiration is often used to assess ecosystem function (32), as rates are influenced by watershed land use (33–35), correlated with stream chemistry (30, 36), and sensitive to pollutants (37, 38). The effects of urbanization on stream dissolved organic matter quality (39) and respiration (33–35) have previously been demonstrated, and stream microbial community structure can potentially be used to monitor these ecosystem functions.

In addition to respiration, dissolved organic matter fuels stream denitrification and the microbial reduction of nitrate (NO_3^-) to nitrous oxide (N_2O) and dinitrogen (N_2) gases (40). Denitrification removes nitrogen from streams and is credited as the major source of the greenhouse gas N_2O (41, 42). Watershed land use and anthropogenic nitrogen loading alter rates of stream denitrification (43), increasing the amount of nitrogen transported downstream (44) and emissions of N_2O to the atmosphere (41). Urbanization has been linked to changes in denitrifier community composition (21, 22, 45, 46), and a previous study linked changes in denitrifier composition to changes in denitrification potential, and therefore nitrogen loss, in urban streams (21). However, it is less clear how changes in microbial community composition in response to land use modification alter N_2O production.

The goal of this study was to identify stream microbes that respond to watershed urbanization and agricultural development. These anthropogenic factors alter microbial diversity and community structure, which can be used to assess stream conditions and ecosystem functioning. We measured microbial diversity using 16S rRNA gene amplicon sequencing across 82 headwater streams within the Chesapeake Bay watershed in the state of Maryland in the spring and summer for 2 years. Measurements were collected in conjunction with stream physicochemical parameters and a macroinvertebrate indicator of stream health. Additionally, at a subset of streams, water column and sediment community respiration were measured using oxygen consumption methods, and N_2O concentrations were measured using gas chromatography. We deter-

mined how stream bacteria and archaea are distributed across gradients of watershed land use and stream conditions, assessed how changes in microbial community composition relate to benthic macroinvertebrate diversity and traditional indices of stream conditions, and determined how these changes influence stream function by relating microbial community composition to rates of microbial respiration and concentrations of N_2O.

RESULTS

Higher microbial alpha diversity in spring. Operational taxonomic unit (OTU) richness differed according to substrate (water and sediment) and ranged from 115 to 996 and 459 to 895 for water and sediment samples, respectively, with sediment richness being significantly higher than that of water (paired Wilcoxon, $P < 0.001$). Sediment Shannon diversity, at 6.2 ± 0.2 (mean $\pm$ standard deviation), was greater than water diversity, at 5.4 ± 1.1 (paired Wilcoxon, $P < 0.001$), and sediment communities were more even, at 0.94 ± 0.02, than were water communities, at 0.84 ± 0.11 (paired Wilcoxon, $P < 0.001$). Similarly, beta diversity (Bray-Curtis dissimilarity) differed by substrate (permutational multivariate analysis of variance [PERMANOVA], $R^2 = 0.11$, $P < 0.001$). Water communities had more *Actinobacteria*, *Bacteroidetes*, and *Proteobacteria*, while *Acidobacteria*, *Planctomycetes*, *Chloroflexi*, *Nitrospirae*, and *Verrucomicrobia* were more abundant in sediments (see Fig. S1 in the supplemental material).

Interannually, there was no difference in alpha diversity between samples collected in 2014 and 2015, and collection year only explained a small fraction of the variance in beta diversity between water and sediment communities (PERMANOVA, $R^2 = 0.01$, $P < 0.001$, and $R^2 = 0.01$, $P < 0.001$, respectively). Seasonally, water Shannon diversity was greater in spring than in summer (paired Wilcoxon, $P < 0.001$), a result of higher richness (paired Wilcoxon, $P < 0.001$) and evenness (paired Wilcoxon, $P < 0.001$) in spring. There was no seasonal difference in sediment Shannon diversity (paired Wilcoxon, $P = 0.49$), though sediment richness was greater in spring than in summer (paired Wilcoxon, $P = 0.01$), with no significant difference in evenness (paired Wilcoxon, $P = 0.13$). Additionally, there were small seasonal changes in water and sediment community structures (PERMANOVA, $R^2 = 0.05$, $P < 0.001$, and $R^2 = 0.02$, $P < 0.001$, respectively), with *Actinobacteria* being more abundant in summer water samples than in spring samples (Fig. S2).

Distance-decay relationships partially drive microbial diversity. Water sample alpha diversity metrics differed across the three geographic regions, while sediment diversity remained constant (Fig. 1). Streams on the Coastal Plain had lower Shannon diversity than did streams in the Piedmont and Highlands regions (Dunn's Kruskal-Wallis, $P < 0.001$ and $P < 0.001$, respectively). This was driven by both lower evenness (Dunn's Kruskal-Wallis, $P < 0.001$ and $P < 0.001$ in Piedmont and Highlands, respectively) and richness (Dunn's Kruskal-Wallis, $P < 0.001$ and $P < 0.001$ in Piedmont and Highlands, respectively) in Coastal Plain streams than that in the other regions. Regional differences in Bray-Curtis dissimilarity and taxon abundances were observed in water (PERMANOVA, $R^2 = 0.07$, $P < 0.001$; Fig. 2a and S3a) and sediment (PERMANOVA, $R^2 = 0.08$, $P < 0.001$; Fig. 2b and S3b) communities.

Partial Mantel tests detected correlations between water and sediment Bray-Curtis dissimilarity and geographic distance (the Euclidean distance between sampling locations) ($\rho = 0.26$, $P = 0.001$; and $\rho = 0.26$, $P = 0.001$, respectively), and no significant relationship was detected between Bray-Curtis dissimilarities and environmental distance and the Euclidean distance between streams based on the continuous environmental variables ($\rho = -0.01$, $P = 0.62$; and $\rho = 0.07$, $P = 0.07$, respectively). Positive distance-decay relationships were observed between the natural-logarithm-transformed least-squares linear regressions of water and sediment community similarity (Sørensen index) and geodesic distance. The absolute value of the regression coefficients (species-area Z-values) for water and sediment communities were 0.12 ($R^2 = 0.082$, $P < 0.001$) and 0.11 ($R^2 = 0.066$, $P < 0.001$) (Fig. 3) for water and sediment communities, respectively.

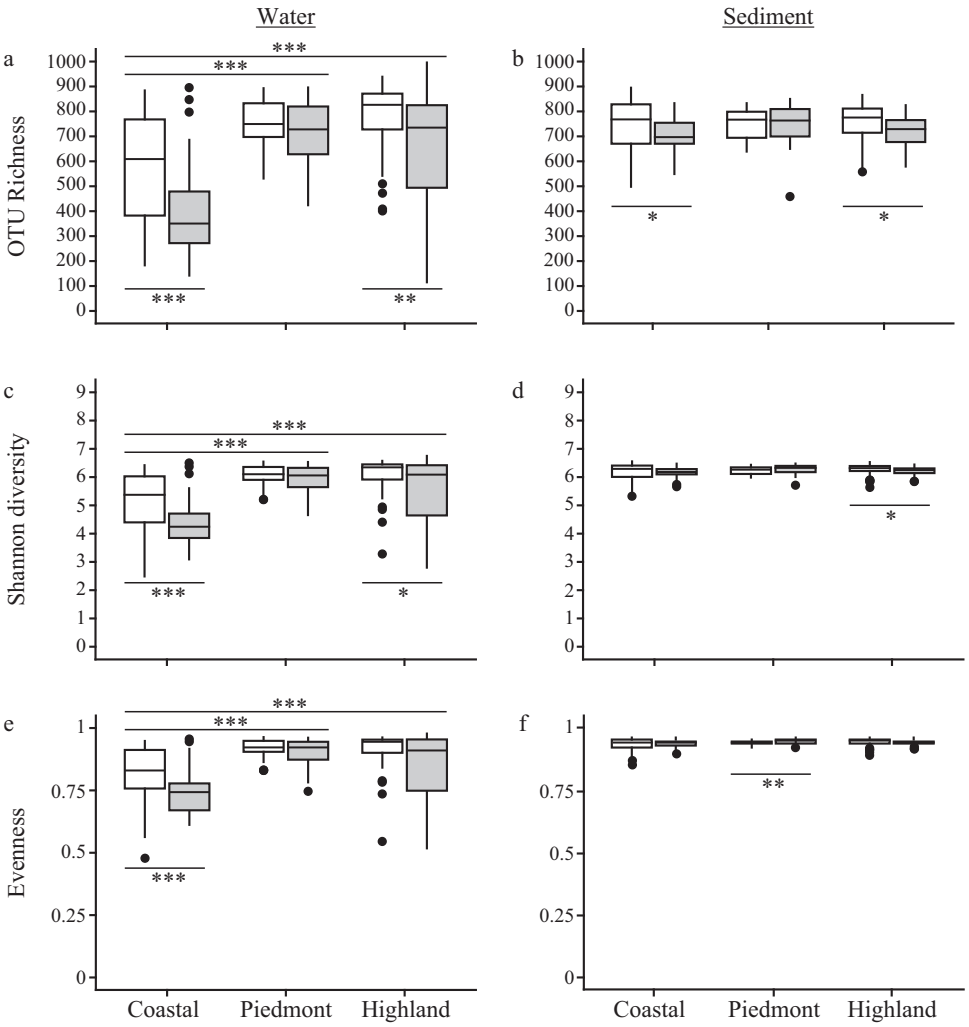

FIG 1 (a to f) Alpha-diversity metrics for water column (a, c, and e) and sediment (b, d, and f) samples in spring (white) and summer (gray) when grouped by geographic region. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, Dunn's test, with seasonal differences shown below and regional differences shown above.

The effect of land use on spatial scaling was examined by comparing the distance-decay relationships for streams in high-use urban, agricultural, and forested watersheds. Communities in streams with high urban and agricultural land use had significant distance-decay relationships in both water (urban, $R^2 = 0.102$, $P < 0.001$; agricultural, $R^2 = 0.100$, $P < 0.001$) and sediment (urban, $R^2 = 0.129$, $P < 0.001$; agricultural, $R^2 = 0.075$, $P < 0.001$) samples. No significant distance-decay relationship was observed for samples in highly forested watersheds. The species-area Z-values for highly urban (water, 0.08; sediment, 0.10) and agricultural (water, 0.07; sediment, 0.06) streams were lower than the Z-values when all streams were considered.

Microbial diversity relates to stream physicochemistry. Stream physicochemistry varied by geographic region (Table S2), land use (Table S3), and stream conditions (Table S4). N_2O concentrations ranged from 0.22 ± 0.00 to 4.41 ± 0.7 μg N_2O liter^{-1} (58 to 1,217% saturated; Table 1), with no difference in N_2O concentrations or saturation between individual streams sampled in 2014 and 2015 (paired t test, $P = 0.2$). N_2O concentration negatively correlated with percent forest cover and positively correlated with total nitrogen (TN), NO_2^-, and Br, NO_3^-, and NH_4^+ concentrations, and agricultural cover (Table S5). N_2O concentration did not correlate with water or sediment microbial diversity.

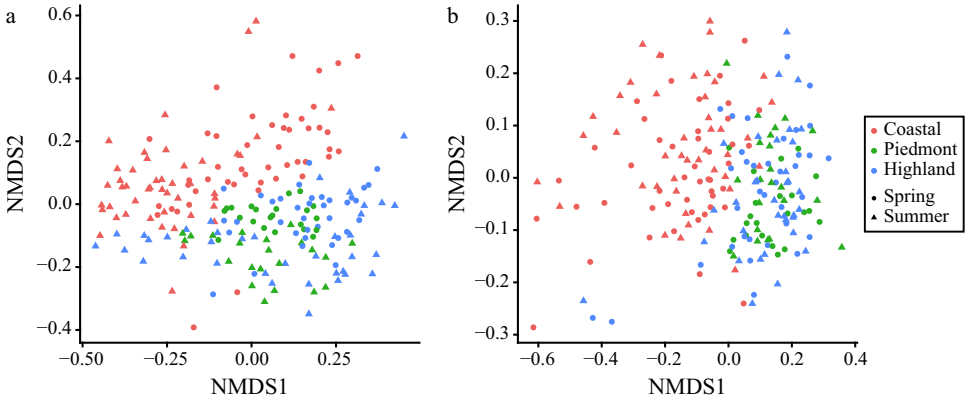

FIG 2 (a and b) Nonmetric multidimensional scaling (NMDS) plots of Bray-Curtis distances for all water column (a) (stress, 0.18; PERMANOVA, region, $R^2 = 0.07$, $P < 0.001$; season, $R^2 = 0.06$, $P < 0.001$) and sediment (b) (stress, 0.13; PERMANOVA, region, $R^2 = 0.08$, $P < 0.001$; season, $R^2 = 0.09$, $P < 0.001$) samples; symbol color indicates the geographic region, and symbol shape denotes the season.

Respiration rates in Coastal Plain streams ranged from 21.7 to 1,573 mg O_2 m^{-2} day^{-1} and 31.9 and 3,668 mg O_2 m^{-2} day^{-1} for water and sediments, respectively (Table 2). There was no significant difference in water rates measured in streams in both 2014 and 2015 (paired *t* test, $P = 0.9$), while sediment rates were higher in 2014 than those in 2015 (paired *t* test, $P = 0.04$). Water respiration rates most strongly negatively correlated with specific conductance, Cl^-, Ca, and urban cover and positively correlated with forest cover, embeddedness, and Shannon diversity (Table S6). Sediment respiration rates negatively correlated with Zn, total phosphorus (TP), and PO_4^{3-} concentrations (Table S7). Both water and sediment microbial community structures correlated with respiration rates (Mantel, $r = 0.51$, $P = 0.001$; and $r = 0.24$, $P = 0.04$, respectively).

Water Shannon diversity negatively correlated with several environmental variables, including embeddedness, Cu, thalweg depth, dissolved organic carbon (DOC), and TP and positively correlated with forest cover and stream velocity (Table S8). The best-fit stepwise multiple linear regression model explained 41% of the variance in water Shannon diversity and included pH, DOC, SO_4^{2-}, TP, forest cover, Mg, Cu, and thalweg depth as predictor variables. Sediment diversity negatively correlated with DOC, embeddedness, and forest cover, and positively correlated with pH, B-IBI, NO_3^-, and TN (Table S9). A stepwise linear regression model explained 22% of the variation in sediment Shannon diversity, with pH, DOC concentration, forest cover, Mg concentration, and thalweg depth being the most significant predictors. Similarly, Bray-Curtis

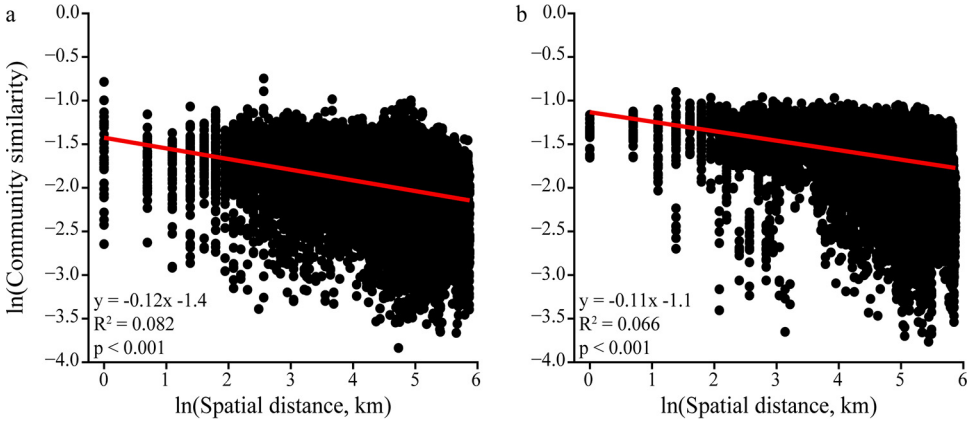

FIG 3 Relationship between microbial community composition (Sørensen index) and geodesic distance for water column (a) and sediment (b) samples. The model fitting is linear least-squares regression.

TABLE 1 Nitrous oxide concentrations and percent saturation relative to equilibrium at *in situ* temperature

	Concn and saturation data by yr			
	2014		2015	
Stream site	N_2O concn (mean ± SD) (μg liter^{-1})	% saturation	N_2O concn (mean ± SD) (μg liter^{-1})	% saturation
CORS102	1.16	290	1.06 ± 0.03	302
LMON302	0.87 ± 0.01	224		
LOCR102	1.68 ± 0.32	537	1.16 ± 0.04	353
MATT104	0.55 ± 0.01	140		
MATT115	1.17 ± 0.03	286		
MATT320	0.55 ± 0.00	152		
NASS108	0.22 ± 0.00	58	0.48 ± 0.04	120
NASS302	1.33 ± 0.01	394	0.74 ± 0.01	197
PAXL294	0.55 ± 0.01	142	0.65 ± 0.03	168
SEAS109	1.1 ± 0.03	263		
SEAS111	1.41 ± 0.01	385		
UMON134	0.55 ± 0.04	139		
UMON299	2.88 ± 0.10	711		
UPCK102	1.65 ± 0.01	398	1.11 ± 0.05	287
UPCK113	1.28 ± 0.00	316		
UPCR208S			0.76 ± 0.03	179
WIRH215	4.26 ± 0.15	1,217		
WIRH220	4.41 ± 0.07	1,116	4.13 ± 0.12	1,040

dissimilarity correlated with several environmental variables (Tables S10 and S11), including pH, DOC concentration, embeddedness, and Zn concentration. The measured physicochemical variables explained 7% of the variation in community structure according to constrained correspondence analysis in both stream water and sediment communities.

Strong associations between taxon abundance and stream physicochemistry were observed in water and sediment samples (Fig. 4). *Pedosphaerales* positively correlated with forest cover and negatively correlated with acid-neutralizing capacity (ANC) and urban cover (Fig. 4a). The order iii1-15 (*Acidobacteria*) strongly negatively correlated with TP and Zn concentrations. *Cenarchaeales* (*Thaumarchaeota*) negatively correlated with DOC and TP concentrations. The strongest sediment associations were positive correlations between *Pirellulales* (*Planctomycetes*) and ANC, Ca concentration, and conductivity, and the strongest negative associations were between *Acidobacteria-*

TABLE 2 Rates of water column and sediment respiration from streams on the Coastal Plain

	Respiration rates by yr (mean ± SD) (mg O_2 m^{-2} day^{-1})			
	2014		2015	
Stream site	Water	Sediment	Water	Sediment
CORS102	148.8	1,563.7	181.9 ± 51.7	204.7 ± 68.8
LMON302	37.5	788.2		
LOCR102	246.0	3,220.3	267.3 ± 79.6	31.9 ± 9.9
LOWI104	221.7	2,441.1		
MATT104	95.0	128.7		
MATT115	52.6	394.2		
MATT320	23.8	1,897.1		
NASS108	348.5	716.5		
NASS302	1,573.2	3,667.9	248.2 ± 31.8	96.3 ± 19.6
PAXL294	45.3	235.7	264.2 ± 49.9	498.2 ± 123.4
SEAS109	21.7	264.2		
SEAS111	35.9			
UMON134	452.6	1,741.3		
UPCK102	314.3			
UPCK113	112.3		77.2 ± 8.0	98.2 ± 18.8
UPCK208	79.0	889.1	67.3 ± 12.5	97.7 ± 18.8
WIRH215	56.5	1,124.4		
WIRH220	77.0	1,593.4	1,029.3 ± 633.9	357.6 ± 233.4

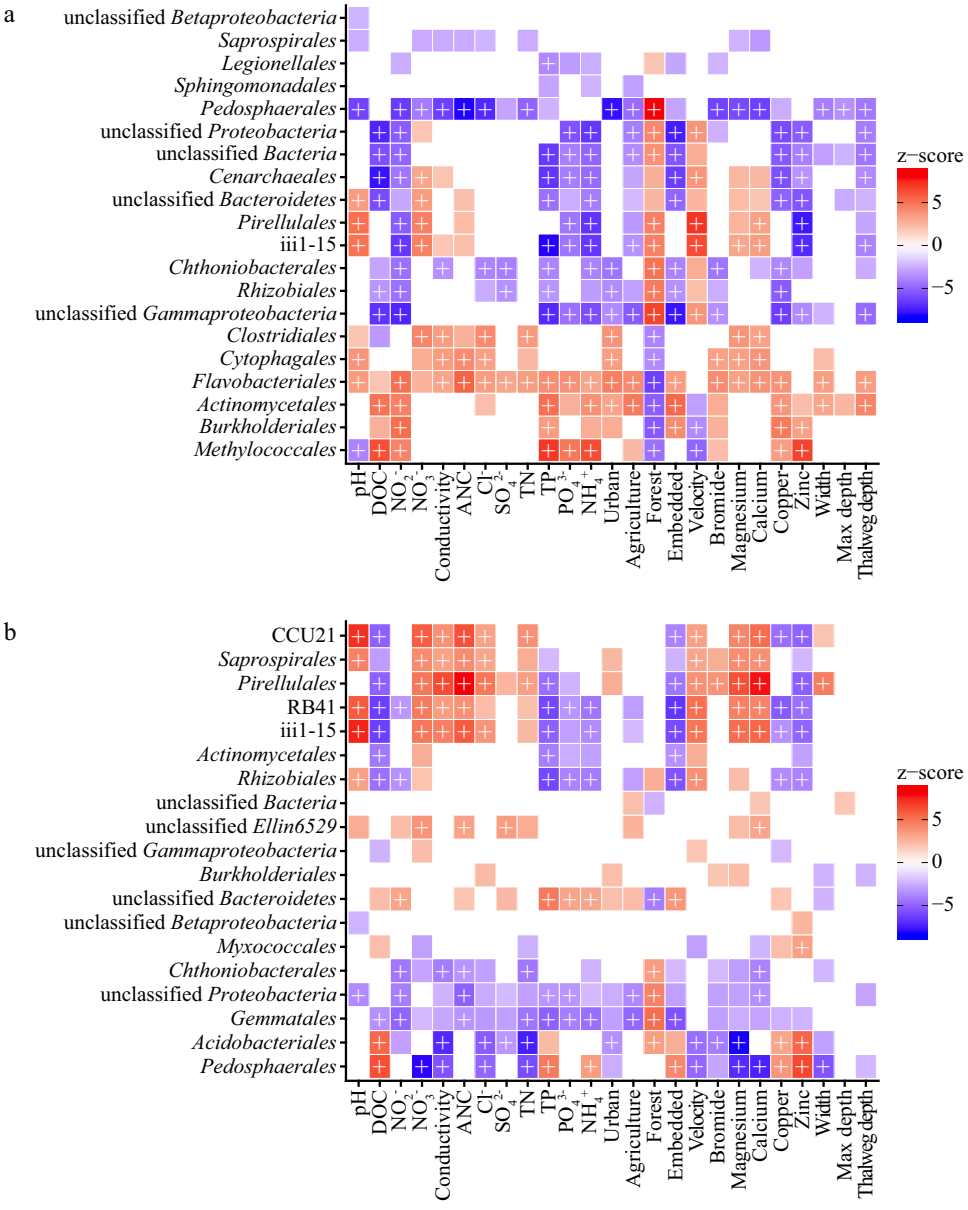

FIG 4 Relationship between the most abundant water column (a) and sediment (b) taxa at the order rank level and environmental variables. Color indicates the associated Z-score. Correlations with a |Z-score| of <1.96 are not shown, and + symbols denote |Z-scores| of >3. Max, maximum.

les and Mg and TN concentrations and between *Pedosphaerales* with NO_2^- and Mg concentrations (Fig. 4b).

Microbial communities vary according to watershed land use and stream conditions. Stream water alpha and beta diversity differed in watersheds with high agricultural, urban, and forested land use. Sediment community structure also differed according to land use, with no change in alpha diversity. Forested streams had higher water Shannon diversity, richness, and evenness than that of agricultural (Kruskal-Wallis, $P < 0.001$) and urban (Kruskal-Wallis, $P < 0.001$) streams (Fig. 5). Similarly, community structure differed in both water (PERMANOVA, $R^2 = 0.11$, $P < 0.001$) and sediment (PERMANOVA, $R^2 = 0.07$, $P < 0.001$) communities. Taxa were identified that were more abundant and pervasive in streams in watersheds with high forested, agricultural, and urban land use (Tables 3 and S12). Forested streams had more taxa in the phyla *Verrucomicrobia*, *Planctomycetes*, and *Acidobacteria*. Agricul-

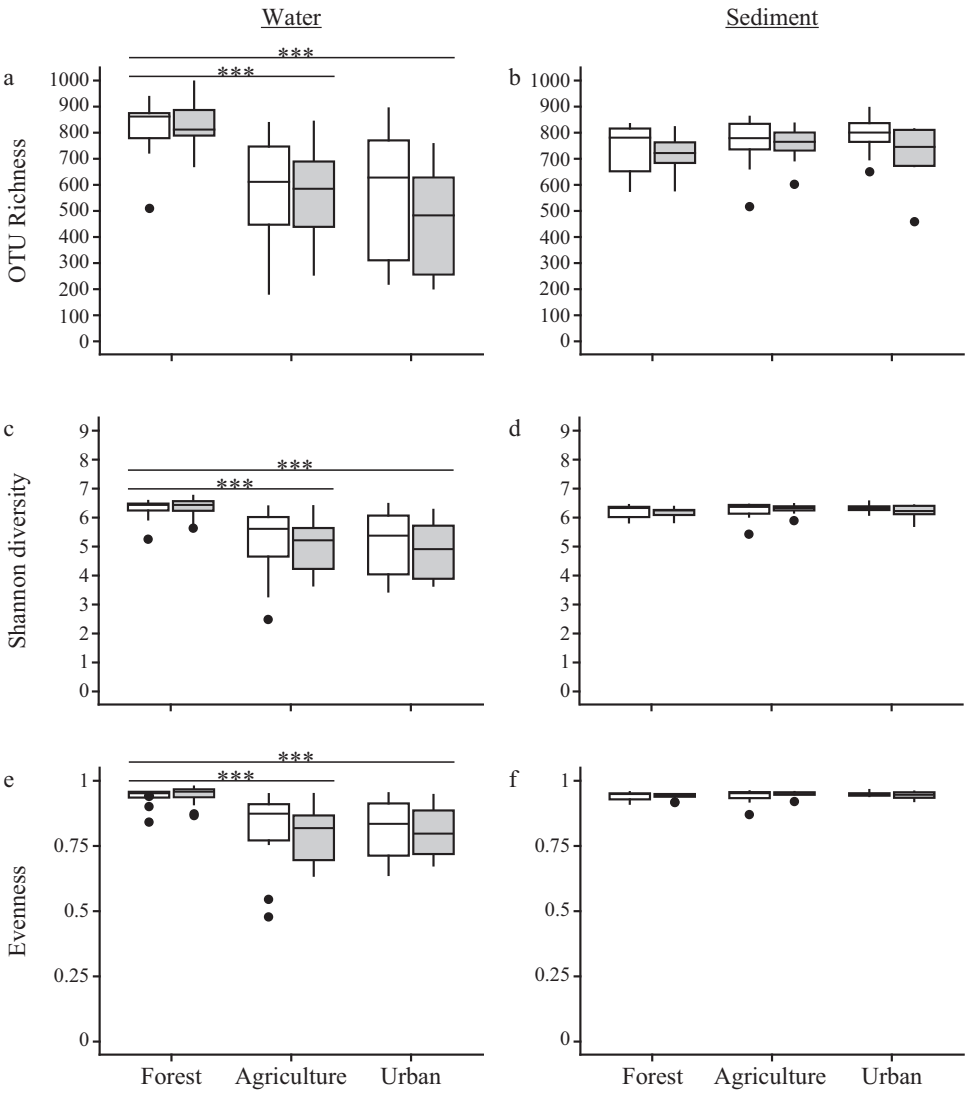

FIG 5 (a to f) Alpha diversity metrics for water column (a, c, and e) and sediment (b, d, and f) samples in spring (white) and summer (gray) from streams in watersheds with highly forested (>90%), agricultural (>50%), and urban (>50%) land use. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, Dunn's test, with seasonal differences shown below and land use differences shown above.

tural streams had more taxa in the phyla *Bacteroidetes*, *Firmicutes*, and *Chloroflexi*, while urban streams had several abundant taxa in the *Proteobacteria*, *Firmicutes*, and *Chloroflexi* phyla.

Benthic macroinvertebrate Shannon diversity weakly correlated with water and sediment microbial Shannon diversity ($\rho = 0.17$, $P = 0.008$; and $\rho = 0.15$, $P = 0.02$, respectively), and B-IBI scores weakly correlated with sediment microbial Shannon diversity (Spearman, $\rho = 0.15$, $P = 0.03$). Additionally, benthic macroinvertebrate Bray-Curtis dissimilarities correlated with sediment and water microbial Bray-Curtis dissimilarities (Mantel, $\rho = 0.37$, $P = 0.001$; and $\rho = 0.35$, $P = 0.001$, respectively), and B-IBI scores weakly correlated with water (Mantel, $\rho = 0.07$, $P = 0.02$) and sediment microbial community structure (Mantel, $\rho = 0.13$, $P = 0.002$). Microbial community structure was only slightly different in streams classified as in good, fair, poor, and very poor condition using the B-IBI (PERMANOVA, water, $R^2 = 0.02$, $P = 0.006$; sediment, $R^2 = 0.03$, $P < 0.001$). *Hydrogenophaga* spp. (*Burkholderiales*), unclassified *Deltaproteobacteria* in the order *Desulfobacterales*, and heteroC45 (*Chthoniobacterales*), a *Verrucomicrobia* bacterium, were all more abundant and pervasive in streams in very poor condition than in streams in good and fair condition (Table 4).

TABLE 3 Microbial OTUs indicative of streams in highly forested, agricultural, and urban watersheds[a]

Group	Substrate	Taxonomy (domain/phylum/class/order/family/genus)	Indicator *r*	*P* value	A[b]	B[c]
Agriculture	Water	*Bacteria/Bacteroidetes/Flavobacteriia/Flavobacteriales/Cryomorphaceae/Fluviicola*	0.77	0.03	0.61	0.95
	Water	*Bacteria/Cyanobacteria/*4C0d-2/YS2/unclassified/unclassified	0.70	0.006	0.54	0.90
	Water	*Bacteria/Proteobacteria/Epsilonproteobacteria/Campylobacterales/Campylobacteraceae/Sulfurospirillum*	0.63	0.021	0.84	0.48
	Water	*Bacteria/*OP3/BD4-9/unclassified/unclassified/unclassified	0.60	0.028	0.58	0.62
	Water	*Bacteria/Acidobacteria/*BPC102/MVS-40/unclassified/unclassified	0.60	0.017	0.57	0.62
	Sediment	*Bacteria/Firmicutes/Bacilli/Bacillales/Bacillaceae/Bacillus*	0.80	0.001	0.68	0.95
	Sediment	*Bacteria/Chloroflexi/Anaerolineae/*GCA004/unclassified/unclassified	0.78	0.002	0.62	1.00
	Sediment	*Bacteria/Proteobacteria/Gammaproteobacteria/Methylococcales/Crenotrichaceae/Crenothrix*	0.75	0.002	0.59	0.95
	Sediment	*Bacteria/Verrucomicrobia/Verrucomicrobiae/Verrucomicrobiales/Verrucomicrobiaceae/Prosthecobacter*	0.74	0.003	0.67	0.81
	Sediment	*Bacteria/Verrucomicrobia/Verrucomicrobiae/Verrucomicrobiales/Verrucomicrobiaceae/Luteolibacter*	0.73	0.010	0.57	0.95
Forest	Water	*Bacteria/Verrucomicrobia/Spartobacteria/Chthoniobacterales/Chthoniobacteraceae/*DA101	0.91	0.001	0.83	1.00
	Water	*Bacteria/Planctomycetes/Planctomycetia/Gemmatales/Gemmatacea/*unclassified	0.84	0.001	0.71	1.00
	Water	*Bacteria/Planctomycetes/Phycisphaerae/*WD2101/unclassified/unclassified	0.84	0.001	0.70	1.00
	Water	*Bacteria/Elusimicrobia/Elusimicrobia/*FAC88/unclassified/unclassified	0.84	0.001	0.76	0.92
	Water	*Bacteria/Verrucomicrobia/Pedosphaerae/Pedosphaerales/*auto67_4W/unclassified	0.84	0.001	0.70	1.00
	Sediment	*Bacteria/Proteobacteria/Alphaproteobacteria/Rhizobiales/Hyphomicrobiaceae/*unclassified	0.80	0.001	0.67	0.96
	Sediment	*Bacteria/Acidobacteria/Chloracidobacteria/*PK29/unclassified/unclassified	0.77	0.001	0.63	0.92
	Sediment	*Bacteria/Verrucomicrobia/Spartobacteria/Chthoniobacterales/Chthoniobacteraceae/*DA101	0.73	0.001	0.53	1.00
	Sediment	*Bacteria/Planctomycetes/Planctomycetia/Gemmatales/Gemmatacea/*unclassified	0.72	0.001	0.52	1.00
	Sediment	*Bacteria/Acidobacteria/Acidobacteriia/Acidobacteriales/Koribacteraceae/*unclassified	0.71	0.049	0.59	0.85
Urban	Water	*Bacteria/Proteobacteria/Betaproteobacteria/Burkholderiales/Comamonadaceae/Hydrogenophaga*	0.83	0.001	0.86	0.81
	Water	*Bacteria/Proteobacteria/Alphaproteobacteria/Sphingomonadales/Sphingomonadaceae/Novosphingobium*	0.78	0.001	0.61	1.00
	Water	*Bacteria/Proteobacteria/Betaproteobacteria/Burkholderiales/Alcaligenaceae/*unclassified	0.78	0.001	0.81	0.75
	Water	*Bacteria/Proteobacteria/Betaproteobacteria/Burkholderiales/Comamonadaceae/Rhodoferax*	0.74	0.021	0.55	1.00
	Water	*Bacteria/Bacteroidetes/Saprospirae/Saprospirales/Chitinophagacea/Sediminibacterium*	0.72	0.010	0.56	0.94
	Sediment	*Bacteria/Proteobacteria/Deltaproteobacteria/Desulfuromonadales/Geobacteraceae/Geobacter*	0.74	0.004	0.55	1.00
	Sediment	*Bacteria/Firmicutes/Clostridia/*OPB54/*unclassified/unclassified*	0.62	0.017	0.69	0.56
	Sediment	*Bacteria/Chloroflexi/Anaerolineae/*CFB-26/unclassified/unclassified	0.61	0.037	0.53	0.69
	Sediment	*Bacteria/Proteobacteria/Deltaproteobacteria/Syntrophobacterales/Syntrophaceae/Desulfobacca*	0.57	0.034	0.58	0.56
	Sediment	*Bacteria/Firmicutes/Clostridia/Clostridiales/*unclassified/unclassified	0.56	0.013	0.63	0.50

[a]High defined as >90% for forest, >50% for agricultural, and >50% for urban watersheds.
[b]A, mean relative abundance of the OTU in each group compared to all groups.
[c]B, relative frequency of each OTU belonging to each group.

DISCUSSION

The aims of this study were to understand how stream bacteria and archaea are distributed across gradients of watershed land use and water quality, to assess how changes in microbial community composition relate to benthic macroinvertebrate diversity, and to discern how changes in stream conditions alter stream ecosystem processes, as reflected in community respiration and N_2O concentrations. Bacterial and archaeal diversity significantly differed across the geographic regions of Maryland (Fig. 1 and 2), demonstrating the influence of the surrounding landscape on headwater stream microbial communities. Regional alluvium composition likely influenced stream alpha diversity, causing lower alpha diversity in Coastal Plain streams (Fig. 1). Sediments on the Coastal Plain of the eastern United States are composed of gravel, sand, silt, and clay (47), making streams more embedded (Table S2). Embeddedness was the envi-

TABLE 4 Microbial OTUs indicative of stream conditions according to the B-IBI

Group[a]	Substrate	Taxonomy (domain/phylum/class/order/family/genus)	Indicator *r*	*P* value	A[b]	B[c]
Good	Sediment	*Archaea/*unclassified/unclassified/unclassified/unclassified/unclassified	0.59	0.04	0.58	0.60
Poor	Water	*Bacteria/Proteobacteria/Gammaproteobacteria/Methylococcales/Crenotrichaceae/Crenothrix*	0.71	0.03	0.58	0.86
	Water	*Bacteria/Proteobacteria/Betaproteobacteria/Gallionellales/Gallionellaceae/Gallionella*	0.53	0.04	0.53	0.54
Very poor	Water	*Bacteria/Proteobacteria/Betaproteobacteria/Burkholderiales/Comamonadaceae/Hydrogenophaga*	0.62	0.01	0.72	0.54
	Water	*Bacteria/Proteobacteria/Deltaproteobacteria/Desulfobacterales/Desulfobulbaceae/*unclassified	0.60	0.00	0.53	0.69
	Water	*Bacteria/Verrucomicrobia/Spartobacteria/Chthoniobacterale/Chthoniobacteraceae/*heteroC45_4W	0.59	0.00	0.56	0.62
	Water	*Bacteria/Proteobacteria/Alphaproteobacteria/Rhizobiales/Beijerinckiaceae/*unclassified	0.57	0.041	0.60	0.54
	Water	*Bacteria/Proteobacteria/Gammaproteobacteria/Alteromonadales/Chromatiaceae/Rheinheimera*	0.54	0.005	0.63	0.46
	Water	*Bacteria/Proteobacteria/Alphaproteobacteria/Rhodospirillales/Rhodospirillaceae/Magnetospirillum*	0.50	0.003	0.68	0.38

[a]No indicative OTUs were identified from streams in fair condition.
[b]A, mean relative abundance of the OTU in each group compared to all groups.
[c]B, relative frequency of each OTU belonging to each group.

ronmental factor that most strongly negatively correlated with Shannon diversity (Table S8), and homogeneous fine sediments have been shown to have lower diversity than that of sites with riffles, shallow turbulent sections (48). Similarly, community structure varied across the geographic regions (Fig. 2) and strongly correlated with DOC concentration, pH, and embeddedness (Table S10 and S11), all of which significantly differentiate Coastal Plain streams from the other regions (Table S2). This finding is in agreement with those from previous studies, demonstrating the strong influence of DOC concentration and pH on freshwater communities (20, 49, 50).

Despite the strong influence of stream chemistry on microbial communities (20, 49, 50), in this study, geodesic distance explained more of the variation in community composition than environmental distance. Partial Mantel test results indicated that community structure was correlated with geographic distance rather than the measured environmental variables. Geographic distance is likely a strong controlling factor in structuring headwater stream communities because there are regional differences in landscape, and stream microbes are locally seeded from the surrounding soil (20, 25, 26). Alpha diversity was greatest in spring, when water flow through the landscape is greatest and, therefore, when advection of microbes from the surrounding landscape is greatest to headwater streams. While seasonal changes in microbial diversity during fall and winter are unknown, the higher diversity in spring than in summer was likely due to higher terrestrial inputs in spring, further demonstrating the influence of landscape on stream microbial communities.

Distance-decay relationships were also observed between water column and sediment community similarity and geodesic distance (Fig. 3), further highlighting the finding that headwater stream microbes display geographic distribution patterns. Alternatively, the distance-decay relationship could be a result of spatial differences in unmeasured environmental variables. Microbial distance-decay relationships have been observed previously in streams (51, 52). Z-values represent the rate at which species similarity decreases with increasing distance; in this study, Z-values (0.12 and 0.11) are similar to microbial values from soil, salt marshes, and lakes (53–56) but lower than regional differences observed in salt marshes (57), suggesting different dispersal limitations across regional scales. In contrast to highly urban and agricultural streams, community dissimilarity in highly forested streams did not increase with distance. Neither geographic distance nor environmental distance correlated with community structure, implying that highly forested streams have a similar terrestrial microbial source.

Microbial diversity differed in streams in watersheds with high urban, agricultural, and forested land use. In contrast to previous studies, degraded streams had lower alpha diversity than that of forested streams (Fig. 5) (22–24), likely due to elevated pollution and habitat loss. Several abundant and pervasive taxa found in urban and agricultural streams (Table 3) are often associated with high-nutrient and low-oxygen environments. Members of the order *Burkholderiales* (families *Alcaligenaceae* and *Comamonadaceae*) were abundant in urban streams and correlated strongly with several anthropogenic nutrients (Fig. 4a). *Comamonadaceae* are often associated with high-nutrient conditions and are ubiquitous in many environments, including aquatic, soil, activated sludge, and wastewater (24, 58). *Comamonadaceae* have previously been associated with urban streams (24) and have been found to have the highest number of urban-tolerant taxa (23, 24). *Sulfurospirillum* spp., in the order *Campylobacterales*, were abundant in highly agricultural streams and are often associated with microaerophilic polluted habits, commonly growing on arsenate or selenate using NO_3^- and sulfur compounds as electron acceptors (59, 60). In contrast, an unclassified and potentially phototrophic member of *Acidobacteria* and *Hyphomicrobiaceae* (*Rhizobiales*) were more abundant in forested streams. These taxa are often associated with low-nutrient conditions and were previously identified as indicators of forested streams (21, 22) and shown to decrease in abundance with increasing watershed urbanization (23).

Only weak associations were detected between sediment and water microbial community composition and B-IBI scores. This is in contrast to findings of Simonin

et al., who found that stream microbial community structure correlates with a macroinvertebrate biotic index in North Carolina (23). Simonin et al. identified concurrent changes in microbial taxa and environmental conditions associated with the biotic index, finding a higher number of negatively responding taxa ($n = 68$; taxa at higher abundance in streams in good condition) than positive responding taxa ($n = 8$; taxa at higher abundance in streams in fair and poor condition) (23). Here, only one taxon (an unclassified *Archaea*) was more abundant and pervasive in streams in good condition, while several taxa were found to be abundant and pervasive in streams in very poor condition (Table 4). *Hydrogenophaga* spp. (*Burkholderiales*) and an unclassified member of *Desulfobacterales*, commonly associated with anaerobic, reducing, and contaminated environments (58, 61), were both more abundant in streams in very poor condition (Table 4). Forested water communities were more even than agricultural and urban communities, suggesting that certain taxa increase in abundance disproportionality in degraded streams, which is likely why the indicator analysis identified more taxa in streams in very poor condition. The findings here suggest that land use cover and stream chemistry are better predictors of headwater stream microbial community composition than are macroinvertebrate indices of stream conditions.

In agreement with the idea that structure determines function, in this study, water community respiration correlated with microbial community composition. Similarly, previous studies report that changes in community metabolism, specifically, the degradation of organic matter, are related to shifts in community composition and diversity (62–67). In contrast, other studies report that respiration depends on substrate availability rather than community composition due to functional redundancy (68), finding no connection between stream bacterial diversity and the activity of enzymes associated with carbon cycling (69). The weaker correlation between community respiration and community composition in sediments compared to water samples could be due to a high level of functional redundancy within sediment communities; if dominated by generalists, shifts in community composition would likely not significantly affect rates of respiration (70).

Degraded streams had lower rates of community respiration than forested streams, as evidenced by the positive correlation between water respiration and forest cover and the negative correlation between respiration and urban cover (Table S4). Rates of community respiration also negatively correlated with several physicochemical variables (Table S6 and S7), including conductivity, Cl^-, Ca, Mg, pH, SO_4^{2-}, and ANC. All signatures of anthropogenic influence, ANC, Cl^- and Zn concentrations, and pH, were found to previously correlate with benthic stream respiration across the Highlands, Piedmont, and Coastal Plain regions of the eastern United States (30), and Zn is a common urban pollutant (33, 71–73). These results suggest that environmental conditions associated with land use drive differences in community respiration, altering carbon processing in headwater streams.

In addition to altering carbon transformations, watershed modification affected stream nitrogen processing. Agricultural streams had higher N_2O concentrations than those of forested streams (Table S5), with higher N_2O concentrations being associated with elevated TN, NO_2^-, NO_3^-, and NH_4^+ concentrations (Table S5). The N_2O concentrations measured in this study, at 0.22 to 4.41 μg N_2O liter^{-1} (58 to 1,217% saturated), were comparable to the values reported for agricultural streams in Illinois (74, 75) but lower than values published for agricultural drainage waters in Scotland, UK (76) and higher than values reported for other forested and agricultural streams (42, 76–78). N_2O production is known to vary by land use, with higher production from denitrification in streams in agricultural and urban basins (41, 76, 79), and changes in community composition have been shown to influence denitrification rates in agricultural (80) and urban streams (21). However, Audet et al. found no difference in N_2O concentrations measured in forested and agricultural streams in Sweden (76). Stream N_2O concentrations are often correlated with dissolved nitrogen concentrations (41, 76, 79, 81); however, variability in this relationship is often observed between sites (74, 75, 77). In this study, N_2O concentrations were not correlated with microbial community compo-

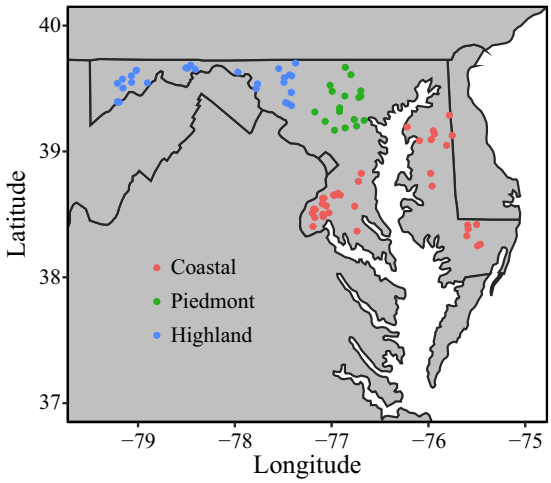

FIG 6 Map of Maryland indicating headwater stream sampling locations. Symbol colors indicate the geographic region.

sition, but rather, N_2O production was likely elevated in streams indirectly due to high rates of denitrification in response to NO_3^- pollution.

We demonstrate that headwater stream microbial communities and ecosystem processes, such as microbial carbon and nitrogen transformations, respond to gradients in land use and stream conditions. Regional differences in stream microbial communities and the observed distance-decay relationships are further evidence that stream communities are seeded from the surrounding landscape. Across geographic regions, microbial community composition varied in streams with high urban, agricultural, and forested land use, and changes in microbial diversity and land use correlated with stream community respiration, linking changes in biodiversity to changes in ecosystem function. Our results suggest that certain microbial groups respond to land use similarly across ecosystems, making them potential candidate taxa to be used in the development of a microbial index of stream conditions.

MATERIALS AND METHODS

Sample collection and physicochemistry. Stream sediment and water column samples were collected across three general geographic regions (Coastal Plain, Highlands, and Piedmont) in Maryland under baseflow conditions during spring (March and April) and summer (June to September) of 2014 and 2015 (Fig. 6). In 2014, 82 headwater streams (Table S1) were sampled for microbial diversity (Coastal Plain, $n = 36$; Highlands, $n = 29$; Piedmont, $n = 17$), and in 2015, 23 streams across the three regions (Coastal Plain, $n = 10$; Highlands, $n = 8$; Piedmont, $n = 5$) were resampled to assess temporal variability. Community respiration and N_2O samples were collected at a subset of sites (Tables 1 and 2) based on proximity to the laboratory to ensure proper temperature and light-controlled incubations. Sampling sites were colocated and collected in parallel with Maryland Biological Stream Survey (MBSS) sampling, a Maryland Department of Natural Resources (DNR) monitoring program that assesses the condition of wadeable streams via physicochemical and biological variables.

One water sample for bacterial and archaeal diversity was collected from each stream in a 500-ml sterile bottle by submerging the bottle into the stream water. Water samples ($n = 210$) were refrigerated until they were filtered on to 0.22-μm pore size, 47-mm-diameter polyethersulfone filters (Mo Bio, Carlsbad, CA, USA) and stored at −80°C. Three sediment samples ($n = 630$) were collected within pools from each stream by inserting the open plunger end of a sterile 5-ml syringe to a depth of 1 cm, and the cores were stored in Whirl-Pak bags at −80°C until extraction.

Benthic invertebrate samples were collected to calculate a Benthic Index of Biotic Integrity (B-IBI) (17), which is a legal biocriterion in the state. Covariates used as predictors of stream quality in our analyses were provided by the Maryland DNR Monitoring and Non-tidal Assessment Division (https://dnr.maryland.gov/streams/Pages/default.aspx), including watershed land use (urban, agricultural, and forested), substrate embeddedness, average thalweg depth, maximum depth, average stream width, average velocity, pH, specific conductance, acid-neutralizing capacity (ANC), and dissolved organic carbon (DOC), chloride (Cl^-), sulfate (SO_4^{2-}), total nitrogen (TN), total phosphorus (TP), orthophosphate (PO_4^{3-}), ammonium (NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-), magnesium (Mg), calcium (Ca), bromide (Br), zinc (Zn), and copper (Cu) content. Land use data, benthic macroinvertebrate samples, and water chemistry samples were collected in spring of each year, while substrate embeddedness, average

thalweg depth, maximum depth, average stream width, and average velocity were measured each summer.

Nitrous oxide concentrations. Nitrous oxide samples were collected from a subset of Coastal Plain and Highlands streams (Table 1) in summer 2014 and summer 2015. Samples were collected in triplicate in 160-ml glass serum vials by inserting silicon tubing into the bottom of the vial and then inverting and submerging the vial into the stream water with the other end of the tube venting to the atmosphere. Samples were preserved with 100 μl of a saturated mercuric chloride ($HgCl_2$) solution, sealed with gray butyl septa and aluminum crimp tops, and stored at room temperature until analysis.

Nitrous oxide concentrations were measured using a headspace equilibration method, as described by Laperriere et al. (82). Each headspace was overpressurized with an addition of 2.5 or 5 ml of ultrahigh-purity (UHP) N_2 and equilibrated with the underlying stream water by gentle shaking at room temperature for at least 2 h. Subsamples from each headspace were analyzed using an SRI Greenhouse gas monitoring gas chromatograph (GC) equipped with an electron capture detector (ECD), dual HayeSep D packed columns, and a 1-ml sample loop (SRI Instruments, Torrance, CA, USA). The carrier gas was UHP N_2, and the sample loop and column oven were heated to 60°C and 100°C, respectively. Two certified standards, 0.1 ppm and 1 ppm N_2O, from Matheson Tri-Gas were used for daily calibration. The N_2O concentrations (C_{N_2O}) from the original stream sample were calculated according to Walter et al. (83), using the following equation:

$$C_{N_2O} = \frac{\left(F \times PV_w + \frac{xP}{RT}V_h\right)}{V_w}$$

where F is equal to $\ln F = A_1 + A_2(100/T) + A_3\ln(T/100) + A_4(T/100)^2 + S[B_1 + B_2(T/100) + B_3(T/100)^2]$, S is salinity, T is the equilibration temperature (K), A and B are constants from Weiss and Price (84), x is the dry gas mole fraction of N_2O in the headspace, P is atmospheric pressure, V_w is the water volume, V_h is the headspace volume, and R is the gas constant (in liters atm K^{-1} mol^{-1}). The equilibrium N_2O concentration with the atmosphere at *in situ* temperature was calculated using the Weiss and Price (84) solubility equations, using an atmospheric mole fraction of 328 ppb (https://www.esrl.noaa.gov/gmd/hats/data.html).

Community respiration rates. Sediment and water respiration rates were measured in a subset of Coastal Plain streams (Table 2) using two O_2 consumption methods. In 2014, O_2 consumption was measured using a membrane inlet mass spectrometer (MIMS), following Kana et al. (85), and in 2015 using a Fibox 3 fiber optic oxygen meter (PreSens, Regensburg, Germany). In 2014, water incubations were conducted in 12-ml Exetainer vials (Labco, Lampeter, Wales, UK). For each stream, 9 water samples were collected by inserting a piece of tubing into the bottom of the vial and inverting and submerging the vial into the stream, with the other end of the tube venting to the atmosphere. Three vials were sacrificially killed with a concentrated $HgCl_2$ solution at three time points, with the first time point immediately after collection and the remaining time points every 4 to 6 h. Vials were transported in a dark cooler back to the laboratory, where they were incubated in the dark at *in situ* temperature for the remainder of the incubation. Sediment incubations were conducted in 160-ml serum vials with butyl septa and aluminum crimp tops. Modified from the above-described collection procedure, sediment was collected by inserting the plunger end of a sterile 30-ml syringe into the sediment and collecting 5 to 10 ml of sediment. The sediment was placed into each vial and topped with stream water.

In 2015, water and sediment incubations were conducted in 60-ml glass biological demand (BOD) bottles with ground glass stoppers. Each BOD bottle contained a PSt3 oxygen sensor (PreSens, Regensburg, Germany). Five replicates were collected, with one killed control from each stream using the methods described above. Once the bottles were full, a thin layer of stream water was added to the top of each stopper to reduce gas exchange with the atmosphere. The bottles were stored in a cooler and transported back to the laboratory, where they were incubated in the dark at *in situ* temperature. O_2 consumption was measured using a Fibox 3 fiber optic oxygen meter (PreSens) every hour until the killed control bottle equilibrated and thereafter every 3 to 6 h for up to 24 h. All respiration rates were calculated using linear least-squares models with the function lm in the R package stats v. 3.5.0 (86). Rates in 2014 were calculated by fitting a model through all 9 data points, while rates from 2015 are the mean of models from each of the replicate bottles. Water respiration rates were subtracted from sediment rates to isolate O_2 consumption in the sediments.

16S rRNA gene sequencing and processing. Water and sediment DNA were extracted using a PowerSoil-HTP 96-well soil DNA isolation kit (Mo Bio, Carlsbad, CA, USA), with modifications. For water samples, half of the filter was extracted, and filters were suspended in 925 μl of PowerSoil-HTP bead solution and 75 μl of solution C1 and vortexed for 10 min. Samples were digested with 20 μl of a 20 mg ml^{-1} proteinase K solution for 30 min at 56°C and then centrifuged for 1 min at 3,000 $\times$ *g*. The sediment samples were also digested with proteinase K; additionally, samples were bead beaten at 20 Hz for 20 min on a Qiagen TissueLyser. The PowerSoil-HTP 96-well soil DNA isolation kit protocol was followed for the remainder of the extractions.

16S rRNA gene amplicons were prepared using the standard Illumina protocol (San Diego, CA, USA) with primers 515F and 806R. After amplicon PCR, the three sediment core samples from each site were pooled prior to PCR cleanup. Following the second PCR cleanup, DNA was quantified using a Qubit double-stranded DNA (dsDNA) high-sensitivity kit. Illumina MiSeq 2 $\times$ 150-bp (samples collected in 2014) and 2 $\times$ 250-bp (samples collected in 2015) sequencing was conducted at the

University of Maryland Center for Environmental Science Institute of Marine and Environmental Technology.

Amplicon data were analyzed using the mothur software package v. 1.31.2 (87). The samples sequenced in 2014 did not have adequate read overlap to merge the reads, and for this reason, only the forward reads were used for all analyses. The mothur standard pipeline was followed, with modifications (87). Prior to quality screening, the samples contained 70,189,751 forward reads, with a median read length of 151 bp. Modified from the standard pipeline, sequences with primer mismatches were removed, and reads were trimmed using an average quality score cutoff of 35 over a 50-bp sliding window. After screening for sequencing and PCR error, 19,731,498 sequences remained, with a median read length of 132 bp. Sequences were aligned with SILVA (v. 119) and classified using Greengenes (v. 13.8.99). Sequences were binned based on taxonomy prior to clustering into operational taxonomic units (OTUs) at a 97% identity level, with a mean sample coverage of 61%. All samples were rarefied to 1,344 sequences, and after pooling, trimming, and rarefaction, 210 water and 204 sediment samples remained.

Statistical analyses. All statistical analyses were computed in R (v. 3.5.0) (86). OTU richness, Shannon diversity, and Pielou's evenness were used to estimate microbial alpha diversity and were calculated using the R package phyloseq (v. 1.24.2) (88). Benthic macroinvertebrate Shannon diversity was calculated using the function diversity in the vegan package. Linear least-squares models and stepwise linear regression models for Shannon diversity and the physicochemical data were fit using lm in the stats package. All colinear variables (Pearson's $|r| \geq 0.7$) were removed prior to stepwise linear regression analysis.

Beta diversity was quantified using Bray-Curtis dissimilarity and Sørensen indices and was calculated, with singletons removed using vegdist in the vegan package (v. 2.5.2) (89). Community similarity was visualized using nonmetric multidimensional scaling (NMDS), which was calculated using metaMDS in the vegan package. Correlations between Bray-Curtis dissimilarity and the physicochemistry were calculated using the mantel function in vegan. Similarly, the relationships between Bray-Curtis dissimilarity, Euclidean geographic distance, and environmental variables were examined using the mantel and partial.mantel functions in vegan. The geodesic distance between two sites was calculated using gdist in the lmap package. Distance-decay was calculated using the natural-logarithm-transformed linear least-squares regressions of water and sediment community similarity (Sørensen index) and geodesic distance. The absolute values of the regression coefficients equal the exponent Z in the taxon-area relationship $S = cA^z$, where A is area, S is the number of species, and c is a constant. Correlations between taxon abundance and environmental data were quantified using Spearman correlations with the function "associate" in the microbiome package (v. 1.1.10013; http://microbiome.github.io/). Correlations were calculated at the order rank level because a higher proportion of sequences were classified using Greengenes (v. 13.8.99) at the order rank level (73%) than the proportion classified at the family (57%) and genus (32%) rank levels. The resulting P values were converted to Z-scores, and only correlations with a |Z-score| of ≥1.96 are reported. Indicator taxon analysis was used to identify microbial taxa that were both abundant and pervasive in streams of a particular land use and stream condition. Indicator taxa were identified using multipatt in indicspecies (v.1.7.6), according to Dufrêne and Legendre (90) for highly urban (>50%), agricultural (>50%), and forested (>90%) streams, as well as streams classified by B-IBI scores as good (4 to 5), fair (3 to 3.9), poor (2 to 2.9), and very poor (1 to 1.9). An indicator was considered significant with an indicator statistic r of >0.5, specificity value A of >0.5, fidelity value B of >0.1, and P value of <0.05.

Data availability. Sequences are accessible from NCBI under BioProject accession number PRJNA545742.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.4 MB.

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We declare no conflicts of interest.

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