Watershed hydrology and salinity, but not nutrient chemistry, are associated with arid-land stream microbial diversity

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Abstract: Microbiota in streams drive many ecosystem functions, including whole-stream metabolism, nitrogen (N) cycling, and the production of basal resources that fuel stream food webs. Interactions between surface water and shallow, subsurface groundwater produce the oxygen and nutrient gradients that influence these microbially mediated biogeochemical functions. Microbial nutrient processing is often limited by nutrient availability, but we lack a clear understanding of the relationships between hydrology, water chemistry, microbial composition, and nutrient cycling. In this study we evaluated the prediction that the microbial (bacterial and archaeal) assemblage composition in surface and subsurface water (both within and among stream reaches) would be related to dissolved nutrient concentrations, surface–subsurface hydrologic connectivity, and reach-scale N cycling rates. To evaluate our predictions, we collected data on water chemistry, whole-stream hydrological connectivity, biogeochemical function, and surface and subsurface water microbial assemblage composition at 6 streams in the southwestern USA. We found no correlation between microbial assemblage composition and stream nutrient concentrations or cycling rates, but observed that subsurface waters in some streams had higher taxonomic richness than surface waters. Instead, differences in microbial assemblage composition among the study streams were correlated with both watershed size and stream water Br^- concentrations. These results suggest that the longer, deeper groundwater flowpaths in larger watersheds, which promote solute accumulation, influence streamwater microbial assemblage composition. These contrasting streamwater microbial assemblages were associated with different reach-scale sinks for N immobilization (fine benthic organic matter versus filamentous algae). Overall, results suggest that the catchment-scale history of water movement, and salinity, may affect both microbial diversity and the fate of N. The mechanisms by which stream microbial diversity influences ecosystem function require more attention. In particular, we need a better understanding of how microbial biogeography is influenced by the spatiotemporal heterogeneity of stream ecosystems.

Key words: arid-land streams, surface–subsurface interactions, nitrogen uptake, microbial diversity, bacteria, archaea, 16S rRNA gene, watershed, residence time

Streams are important hotspots of nutrient processing on the landscape. The combination of incoming nutrients and high benthic surface area means that a proportionately large amount of nitrogen (N) uptake and processing can occur in small streams (Mulholland et al. 2008). Nutrient inputs from the watershed drive biogeochemical processes (Hynes 1975), and dynamic hydrological conditions maintain heterogeneous gradients of oxygen (O), carbon (C), and N in streambed sediments, allowing a variety of microbially mediated biogeochemical transformations to proceed (Dahm et al. 1998, Malard et al. 2002). Specifically, microbial dissimilatory metabolic activities such as nitrification and denitrification vary along benthic subsurface flowpaths (Beaulieu et al. 2011), and control the amount and form of N in stream water. In the whole stream reach, microbial heterotrophs and plant and algal autotrophs as-

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similate and remove N from the surface water. Despite the high diversity of organisms involved in stream N cycling and the likelihood that populations with different nutritional demands or physiological constraints respond differentially to changes in the environment, stream biogeochemistry is often studied without considering the composition of organisms driving reach-scale function.

Dissolved N concentrations are the best predictor of N processing rates across streams that differ in watershed land-use activities and hydrological characteristics (Mulholland et al. 2008). Nutrient availability may also affect the niche differentiation and competitive interactions among N-cycling microbiota in diverse assemblages (including all bacteria and archaea), because some taxonomic groups are adapted to grow more efficiently at low nutrient concentrations and others to grow faster at high nutrient concentrations (Button 1985, Andrews and Harris 1986). Many studies have shown that microbial assemblage composition is related to N concentrations in natural stream waters (reviewed by Zeglin 2015). When nutrient loading effects are experimentally isolated from other environmental covariates, increases in aggregate microbial growth and activity are clearly linked to changes in assemblage composition. As nutrient loading increases, microbial taxa that tolerate high nutrient levels increasingly dominate and overall microbial diversity declines (Van Horn et al. 2011). However, differential microbial responses to nutrient additions can be muted when ambient nutrient limitation is low (Olapade and Leff 2006), and relationships between stream water nutrient concentration and microbial assemblage composition may be masked by other factors that influence microbial taxon distributions and relative abundances, such as metals contamination (Ancion et al. 2013). Further, differences in microbial assemblage composition caused by differences in resource availability do not necessarily translate to, or predict, differences in assemblage function, since functional redundancy among taxa or secondary limitation by other nutrients can mediate microbial activity (Comte and del Giorgio 2009, Baxter et al. 2012).

Within streams, most nutrient processing occurs in the benthos, and greater interaction of water with streambed sediments promotes increased nutrient removal (Valett et al. 1997, Zarnetske et al. 2011). As water moves through microbe-laden sediments, solutes in the water feed microbial metabolism, and metabolic products subsequently change the chemical environment to promote different microbial processes. Surface–subsurface hydrological connectivity within a stream reach should, therefore, promote heterogeneity in types and rates of microbial metabolism (Baker et al. 1999), including heterogeneity in N-cycling metabolic pathways (Jones and Holmes 1996). The diversity of microbial groups that drive benthic N cycling and N removal (denitrification) processes can be taxonomically resolved, but dissolved N concentrations may not be the primary physicochemical factor that affects microbial taxonomic diversity (Baxter et al. 2012). Along subsurface flow paths, differences in dissolved oxygen concentration or organic matter supply may be more strongly related to microbial assemblage structure than N concentrations (Findlay and Sinsabaugh 2003, Sliva and Williams 2005). Also, physical flow conditions can affect the architecture and taxonomic composition of biofilm assemblages (Besemer et al. 2007), and movement of cells out of soil pore waters (Crump et al. 2012) and from streambed sediments to the surface water (Fazi et al. 2008) can also affect streamwater microbial assemblage composition. Thus, reach-scale hydrological complexity is likely to promote microbial diversity (Lowell et al. 2009), but this relationship may or may not be directly linked to N uptake and processing rates.

The objectives of this study were to: 1) assess whether microbial assemblage composition differs between surface and subsurface flow environments, both within and among streams, 2) identify whether streamwater microbial assemblage composition is related to nutrient concentration and reach-scale hydrological connectivity, and 3) learn if streamwater microbial assemblage composition and physiochemical conditions are correlated with stream reach-scale N removal and denitrification. We predicted that microbial assemblage composition would differ between surface and subsurface waters consistently across streams, that microbial diversity would be lower in streams with higher inorganic N concentrations (due to competitive dominance of nutrient-loving taxa) and higher in streams with higher hydrological connectivity (due to greater benthic physicochemical heterogeneity), and that streams with greater reach-scale nitrate uptake and denitrification rates would have a microbial assemblage distinct from streams with lower nitrate uptake and denitrification rates.

METHODS

Study design

To address our objectives, we conducted a comparative study of 6 arid-land streams that spanned a range in ambient N concentration and hydrologic connectivity. We sampled water from surface and shallow subsurface habitats in each stream for microbial diversity analysis as well as a large amount of water chemistry and stream nutrient-cycling function data. To capture a range of nutrient inputs and streamwater nutrient concentrations, streams were located in watersheds with contrasting land use (Table 1). Three streams (Agua Fria [AF], Rio Salado [RS], Sycamore Creek [SC]) drained watersheds that were in near-natural condition (reference sites), and 3 streams (Bernalillo Drain [BD], San Pedro [SP], Rio Puerco [RP]) drained watersheds with active agriculture adjacent to the study reach (agricultural sites). All study streams were either spatially intermittent or temporally ephemeral, which is characteristic of streams in the desert southwest USA region. Discharge

Table 1. Microbial diversity metrics for each stream (mean ± SE). OTU = operational taxonomic unit; No. = number; NMDS = non-metric multidimensional scaling; * = $p < 0.05$; $^{+}$ = $p < 0.1$. Different letters indicate a significant difference in analysis of variance (ANOVA) groupings among streams ($p < 0.008$, Tukey post-hoc test).

	OTU* richness	Chao1* richness	Evenness (Equitability)	Shannon's *H*	No. OTUs in all samples	NMDS axis 1	NMDS axis 2
Agua Fria River, AZ (AF)	$1869^{a} \pm 588$	$2771^{a} \pm 987$	$0.663^{ab} \pm 0.045$	$6.99^{ab} \pm 0.75$	9353	$0.11^{ab} \pm 0.1$	$-0.09^{ab} \pm 0.1$
Bernalillo Drain, NM (BD)	$4388^{b} \pm 315$	$6812^{+b} \pm 660$	$0.822^{b} \pm 0.006$	$9.92^{b} \pm 0.09$	16,474	$-0.18^{a} \pm 0.03$	$0.04^{abc} \pm 0.01$
Rio Puerco, NM (RP)	$1685^{a} \pm 212$	$1986^{a} \pm 228$	$0.762^{b} \pm 0.050$	$8.15^{ab} \pm 0.64$	7455	$-0.11^{ab} \pm 0.02$	$0.24^{c} \pm 0.03$
Rio Salado, NM (RS)	$1826^{a} \pm 390$	$2359^{a} \pm 508$	$0.563^{a} \pm 0.045$	$6.07^{a} \pm 0.63$	11,141	$0.33^{b} \pm 0.05$	$-0.01^{ab} \pm 0.03$
Sycamore Creek, AZ (SC)	$3811^{b} \pm 243$	$5548^{b} \pm 403$	$0.721^{ab} \pm 0.028$	$8.58^{b} \pm 0.39$	20,819	$-0.16^{a} \pm 0.03$	$-0.19^{a} \pm 0.02$
San Pedro Creek, NM (SP)	$3343^{*ab} \pm 569$	$4358^{*ab} \pm 777$	$0.796^{b} \pm 0.023$	$9.17^{b} \pm 0.37$	14,242	$-0.20^{a} \pm 0.01$	$0.13^{bc} \pm 0.04$

was low in all streams during the baseflow sampling periods (Q = 2.5 – 23.5 L/s), and the basin areas above each study reach were highly variable in size (9.4×10^2 to 1.6×10^7 ha). All study streams were located in separate catchments, so there was no hydrologic connectivity between any of the study sites. This work was done in conjunction with the Lotic Intersite Nitrogen Experiment II (LINXII) project, a continental-scale investigation of nitrate dynamics in stream ecosystems (Mulholland et al. 2008). During the summers of 2003, 2004, and 2005, we collected surface and subsurface water samples for microbial diversity analysis from the study streams, concurrent with the larger LINXII experiments that measured reach-scale nitrate uptake and denitrification rates.

To address predictions, we measured assemblage diversity and the relative abundances of all archaeal and bacterial microbiota present in stream water samples. Unique genotypes (taxa) were identified based on variation in the sequence of the 16S rRNA gene (Hugenholtz et al. 1998). Using this approach, traditional diversity parameters can be calculated and ordination analysis can be conducted. We predicted that alpha diversity (local richness) and evenness would be lower in higher-nutrient waters and higher in streams with greater hydrological connectivity, surface and subsurface waters would differ in assemblage composition as discerned through ordination analysis, and variation in diversity and assemblage composition across streams would be correlated with reach-scale nutrient cycling rates.

Sampling approach

The reach length used to estimate whole-stream nitrate (NO_3^-) transformation was based upon previously measured NO_3^- uptake length—the estimated distance a molecule of dissolved NO_3^- travels before being removed from the water column (100–300 m for these streams). Depending on the reach length and width, 2 to 3 transects of 2 to 3 wells each were installed in each reach at a distance of 50 to 100 m apart (Fig. 1) to collect shallow subsurface water for microbial diversity comparison with surface water. We installed 4 to 9 screened PVC wells 30 cm below the streambed surface in each stream reach and allowed them to equilibrate for 24 h before we began each NO_3^- uptake experiment. To ensure well sterility we bleached and autoclaved each well prior to installation.

Our approach to estimate NO_3^- uptake and denitrification rates and identify sinks for NO_3^- within each stream reach has been used extensively elsewhere (Mulholland et al. 2008, 2009, Hall et al. 2009). Briefly, a stable isotopic tracer of ^{15}N-nitrate (as $K^{15}NO_3$) was steadily added to each stream reach over the course of 24 h. We collected stream water after 12 and 24 h, and biomass from all stream biotic compartments (e.g., plants, filamentous algae, fine benthic organic matter [FBOM, 0–5 cm]) after 48 h, to measure the amount of ^{15}N that remained in the stream reach as NO_3^-, ammonium (NH_4^+), dissolved gases, or within organic material. These data were used to quantify the amount of NO_3^- that was biologically transformed or assimilated during the 24-h experiment.

We collected surface-water and subsurface water samples for microbial analysis 24 h post initiation of the NO_3^- uptake experiment. Each sample for microbial analysis consisted of 10 to 20 mL of unfiltered water that we collected using aseptic techniques and preserved with a cell lysis buffer to prohibit microbial cell growth during sample transfer and storage (Mitchell and Takacs-Vesbach 2008). We used sterile syringes to collect surface-water samples and sterile tubing and either a hand pump or a GeoPump (Geotech, Denver,

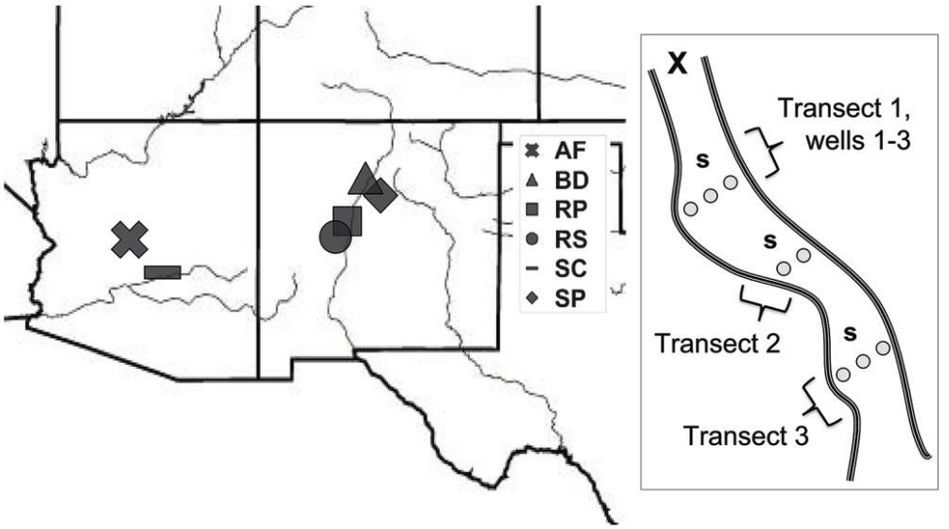

Figure 1. Locations of the 6 study streams in New Mexico and Arizona, USA: Agua Fria River, AZ (AF); Bernalillo Drain, NM (BD); Rio Puerco, NM (RP); Rio Salado, NM (RS); Sycamore Creek, AZ (SC); and San Pedro Creek, NM (SP). Inset shows a generic schematic of the model well and surface-water sampling scheme in each stream reach: X is the experimental nitrate injection point, S shows surface-water grab sample locations, and gray circles are shallow subsurface well sample locations at 3 transects spaced longitudinally through each experimental reach. Exact sampling locations and numbers varied by site due to differences in stream morphology.

Colorado) to collect subsurface water samples. All water samples were collected into sterile centrifuge tubes, preserved with an equal volume of lysis buffer, transported to the laboratory on ice, and stored at −80°C until further analysis. After samples were collected for microbial DNA analysis, water was collected for chemical analysis. Specifically, the concentration of the conservative tracer Br^- in subsurface water was measured and used to calculate the proportion of surface water reaching the subsurface at each well point (Crenshaw et al. 2010).

Water chemistry, nutrient cycling, and transient storage analysis

We filtered surface-water samples for dissolved solute analysis (N ions, phosphorus [P], bromide [Br^-]) through 0.45-μm GF/F filters in the field. In the lab, a Dionex Ion Chromatograph (Thermo Fisher Scientific, Waltham, Massachusetts) was used to measure NO_3^--N and bromide (Br^-) concentrations, and automated phenate colorimetry (Technicon AutoAnalyzer; Seal Analytical, Mequon, Wisconsin) was used to measure NH_4^+-N concentrations. Dissolved organic C (DOC) and total soluble N (TSN) concentrations were measured on a Shimadzu TOC/TN analyzer (Shimadzu, Kyoto, Japan). We report DON as (TSN – [NO_3^--N + NH_4^+-N]). We used an ascorbic acid-molybdenum blue colorimetric assay to measure soluble reactive P (SRP). DO concentration and temperature were measured in situ with a DataSonde probe (Hydromet, Loveland, Colorado). $^{15}NO_3^-$-N and $^{15}NH_4^+$-N concentrations were obtained by reducing (in the case of $^{15}NO_3^-$-N) and concentrating dissolved solutes, diffusing the ^{15}N onto an acidified filter as NH_3 gas, and analyzing the ^{15}N content of the filter, a method modified from (Sigman et al. 1997). Isotopic analyses were conducted on a Europa Integra mass spectrometer (Crewe, England) at the Stable Isotope Laboratory at the University of California, Davis.

To determine NO_3^- transformation parameters, we calculated the distance specific whole stream NO_3^- uptake rate (k_{NO3}, μg N/m) as the negative slope of the decay curve of $^{15}NO_3^-$-N flux downstream from the addition point, then derived the vertical velocity (V_{fden}, cm/s) and areal specific rate (u_{den}, μg N/m^{2}s) of NO_3^- uptake within each reach to compare nitrate removal among streams with different widths and discharge (Newbold et al. 1981, Stream Solute Workshop 1990, Mulholland et al. 2004). We used analogous metrics for gaseous ^{15}N measurements to calculate the denitrification parameters k_{den}, V_{fden}, and u_{den} (Mulholland et al. 2009). We also estimated the proportion of nitrate added to each stream reach that was assimilated into different compartments of biomass from mass–balance calculations comparing the amount of NO_3^--N added to the amount recovered in each biomass sample, scaled by the areal coverage of stream benthos by each compartment type (Mulholland et al. 2000, Tank et al. 2017).

We used the OTIS-P model (Runkel 1998) to estimate 2 parameters associated with transient storage and hydrological connectivity at each study reach: the transient-storage zone cross-sectional area in m^2 for the study reach (A_s) and the hydrologic retention factor (F_{med}^{200}), a metric representing the fraction of median water travel time

through a reach due to transient storage, which is better suited for comparison among streams with different flows and channel dimensions (Runkel 1998). This model assumes a stream is comprised of 2 flow zones: a main channel where solute dynamics are governed by advection and dispersion, and a transient storage zone where water is retained for a longer duration (e.g., in eddy pools or shallow subsurface flow). The model describes the exchange between the main channel cross-sectional area and transient storage zone (A_s) as a mass transfer coefficient. The empirical data used to estimate these parameters are the changes over time in the concentration of a conservative (nonbiologically reactive) solute in stream water after a known amount is added. For this study, we added Br^- to each stream concurrent with the NO_3^- uptake experiments, and used Br^- concentration dynamics to calculate these reach-scale hydrologic connectivity parameters. Bromide was used instead of the more commonly used conservative tracer chloride, because chloride concentrations in some study streams were excessively high. Bromide and chloride concentrations both are associated with overall stream water salinity levels.

Bacterial and archaeal 16S rRNA gene sequence library preparation and analysis

We used a modified phenol-chloroform method to extract genomic DNA from the microbial cells present in 300- to 1000-μL aliquots of unfiltered water, each subsampled from the environmental samples that were collected and preserved in the field. In this standard protocol, cells were first lysed by incubating the sample with 0.5 volume (relative to the exact starting volume) of 1% CTAB at 60°C for 1 h and then with a final concentration of 2% sodium dodecyl sulfate (SDS) at 60°C for 1 h, periodically inverting the incubation tubes to mix. Then, DNA was isolated by removing other molecules in the sample with an equal volume of phenol : chloroform : isoamyl alcohol (24 : 24 : 1) solution followed by 2 extractions with an equal volume of chloroform. Finally, DNA was precipitated by adding 2× the original volume of 95% ethanol and 0.1 volume of 3M sodium acetate. The DNA was then cleaned with 70% ethanol and reconstituted in 10 mM TRIS (pH 8.0). This primary DNA extract was archived at −80°C until further preparation for microbial gene sequencing.

To estimate the microbial diversity and composition in each sample, we used universal ribosomal RNA gene primers (515F/806R) and the standard Earth Microbiome Project's protocols (Caporaso et al. 2012) to prepare a library of 16S rRNA gene amplicons for Illumina sequencing (Illumina, San Diego, California). These primers are 'universal' in that they target regions of the ribosomal small subunit gene that are conserved across the domains Bacteria and Archaea, and also cover a variable region within this gene that provides sequence information to taxonomically resolve the diverse microbial (bacterial and archaeal) genotypes. Polymerase chain reaction (PCR) of each sample was run in triplicate, using uniquely barcoded primers for each sample, then combined into one tube, cleaned, and submitted for sequencing at the Kansas State Integrated Genomic Facility. Sequencing was done with an Illumina MiSeq with 2 × 150 paired end cycles, with a 15% PhiX spike.

We used the QIIME 1 software package (http://qiime.org/) to quality-filter, join, and demultiplex raw Illumina sequence data (Caporaso et al. 2010). We subsequently used QIIME's open-reference workflow to assign these data to operational taxonomic units (OTUs) based on 97% DNA sequence similarity, a convention used to define shallow lineages for microbial diversity and assemblage composition analysis (Schloss and Handelsman 2005). A representative sequence for each OTU was selected and aligned to the GreenGenes 16S rRNA gene reference database (version 13.8; http://qiime.org/home_static/dataFiles.html), and the Ribosomal Database Project (RDP) classifier was used to place this sequence in a hierarchical taxonomy (Cole et al. 2009). The remaining non-aligned OTUs, chimeric sequences (identified with ChimeraSlayer; https://sourceforge.net/projects/microbiomeutil/files/), and OTUs with 2 or fewer reads were removed from further analysis. The resulting quality-filtered dataset included 3,790,008 sequence reads of ~250 basepairs in length, and because each sample was represented by a different number of sequences, the dataset was filtered to include an equal number of randomly selected reads (30,600) per sample ($n = 56$) for normalized calculation of diversity metrics. The final dataset included 1,713,600 high-quality reads and 45,807 total OTUs, and associated sequence data are available at the National Center for Biotechnology Information, Sequence Read Archive database under accession number SRP140667.

To evaluate our predictions, we calculated several microbial diversity and assemblage composition metrics. Rarefied data of 30,600 reads per sample were used to generate diversity parameters, because samples included different numbers of reads and the number of unique OTUs detected in any sample increases with the number of sequence reads (Schloss and Handelsman 2006). Total detected OTU richness, evenness (as equitability), and diversity (as Shannon's H) were calculated, and the Chao1 index was used to estimate the total richness of OTUs in each sample because samples seldom contain all of the taxa present at a site (Chao and Lee 1992, Schloss and Handelsman 2006). As a coarse categorization of assemblage composition, the proportional number of sequences within each sample that affiliated with all major prokaryotic Phyla and Subphyla were calculated and reported as % relative abundance. Beta-diversity, as the difference in relative sequence abundance of all OTUs between 2 samples, was described

in a Bray–Curtis distance matrix of all samples (both within and among streams). Finally, the total number of unique OTUs across all samples from each stream was tallied using the 'compute_core_microbiome' function to estimate gamma diversity. These metrics were generated with QIIME 1 (Caporaso et al. 2010) and then exported for further analysis in R (R Development Core Team 2010).

We used non-metric multidimensional ordination analysis (NMDS) (R *vegan* package; *metaMDS* and *monoMDS* functions) to model the differences among all samples in the relative proportions of OTUs. Ten iterations of the *metaMDS* command were run, all of which gave similar solutions. We selected the model with the lowest stress value (0.18). We also used 2-way permutational analysis of variance (PERMANOVA and *adonis* functions in the R *vegan* package; Oksanen et al. 2007) to assess how much variation in OTU composition among samples was associated with stream and type of hydrologic habitat (surface vs subsurface) within streams. We used NMDS axes 1 and 2 values for each sample to quantify how OTU-level assemblage composition varied among samples. Two-way ANOVA was used to evaluate whether collection stream or surface vs subsurface habitat affected microbial diversity, and to learn if samples were significantly grouped along the ordination gradients of assemblage composition.

We also used ANOVA to determine if discharge and nutrient chemistry (surface water habitats) and % surface water in wells (subsurface habitats) varied among streams. If an ANOVA showed that these attributes varied with stream, Tukey's post-hoc analysis was used to show which streams differed from one another. We used Pearson's correlation coefficients to quantify relationships between reach-scale hydrological connectivity, nutrient chemistry, nutrient cycling parameters, and whole-stream average microbial diversity metrics. Some variables were $\log_{10}(x)$-transformed before ANOVA or correlation analysis to fit assumptions of normality (Br^- and NO_3^- concentrations, watershed area, u_{NO3}, u_{den}, v_{fNO3}, v_{fden}, and all relative abundance values). The ANOVAs, post-hoc tests, and the correlation analyses were conducted in R. A value of $\alpha = 0.5$ was used as the significance threshold for statistical tests, except for (sub)Phylum level assemblage composition data, which included 22 multiple comparisons from the same set of taxon features, so an α-value of $0.5/22 = 0.00227$ was used to adjust for multiple comparisons.

RESULTS

Site physicochemistry, hydrology, and nutrient cycling attributes

The study streams spanned a substantial range in environmental conditions (nutrient concentrations, hydrological connectivity, and nutrient processing rates), gradients which were expected to be correlated with microbial diversity (Table S1). Among all 6 study streams, ammonium-N was consistently low (2–4 μg/L), but background concentrations of nitrate-N ranged from 1 to 297 μg/L, DON concentrations ranged from 44 to 222 μg /L, and DOC concentrations ranged from 0.9 to 3.8 mg/L. The % of surface water found in shallow subsurface wells ranged from 39.8 to 75.0%, and the area of the transient storage zone relative to total flow area (A_s/A) and the fraction of median water travel time due to transient storage (F_{MED}^{200}) varied 200 and 117×, respectively, among streams (A_s/A: 0.0039–0.78, F_{MED}^{200}: 0.0012–0.14). Also, nitrate-N uptake and denitrification rates and velocities and differed 8 to 4623× among streams. Concentrations of the salinity proxy Bromide varied by almost 30× among streams (35–1026 μg/L).

Microbial diversity

Sample OTU richness, Chao1 estimated total richness, evenness, and Shannon's diversity per sample all varied significantly among streams ($p < 0.0001$, Table 1). BD and SC had the highest, whereas AF, RP, and RS had the lowest sample and Chao1 estimated richness (Table 1, Fig. 2). RS had the lowest, and BD, RP, SC, and SP had the highest, Shannon's diversity and OTU evenness (Table 1). Only OTU richness and Chao1 estimated richness differed between surface and subsurface waters, as indicated by a stream by subsurface interaction ($p < 0.05$, Table 1, Fig. 2). Richness was significantly higher in subsurface waters than surface waters at SP, and marginally so at BD (Table 1, Fig. 2). The total number of OTUs in a sample ranged from 7455 in RP waters to 20,819 in SC waters.

Variation in bacterial and archaeal assemblage composition

The NMDS ordination showed greater separation in OTU relative abundance among different streams than be-

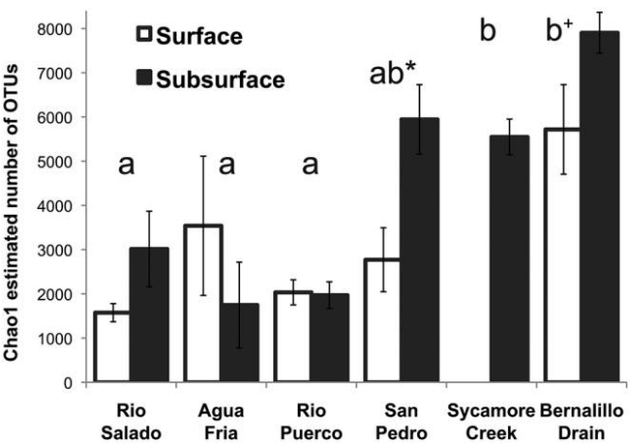

Figure 2. Chao1 estimated bacterial and archaeal 16S rRNA gene richness (mean ± SE) in surface and subsurface waters of the 6 study streams using ANOVA post-hoc significant (Tukey comparisons, $p < 0.01$) differences among sites. Sites with the same letter are not significantly different. * = $p < 0.05$; $^+$ = $p < 0.1$.

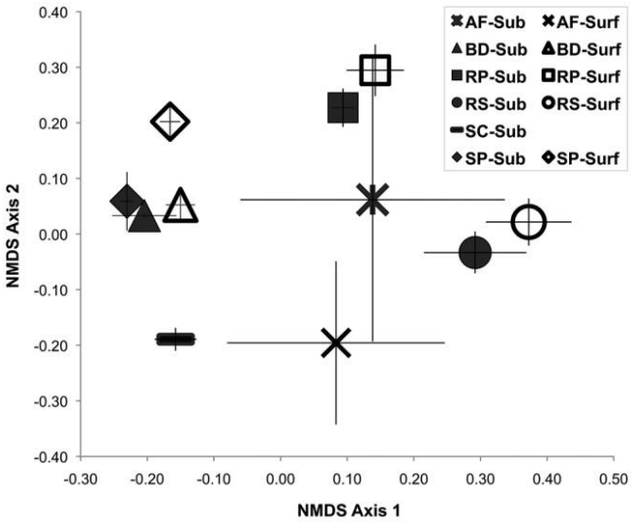

Figure 3. Non-metric multidimensional scaling (NMDS) ordination plot showing the locations (means ± SE) of surface and subsurface samples for the 6 study streams in ordination space based on Bray–Curtis distances between samples in operational taxonomic unit relative abundances.

tween surface and subsurface waters (Fig. 3). PERMANOVA showed that 34% of the variation in microbial assemblage composition was associated with stream ($p < 0.001$), whereas only 2% of the variation in assemblage composition was associated with surface and subsurface waters ($p = 0.029$). Based on 1-way ANOVA of NMDS Axis 1 scores, BD, SC and SP were similar to one another, RS was unique from the other 5 streams, and RP and AF were intermediate between RS and the other three streams (Table 1, Fig. 3). Based on NMDS Axis 2, both RP and SC were unique from the other four streams, which were intermediate between RP and SC (Table 1, Fig. 3). There was no significant effect of surface vs subsurface water grouping based on NMDS Axis 2 scores.

Correlation analysis showed that a number of diversity metrics and phylum-level relative abundances covaried with NMDS Axis 1 and 2 scores (Table 2). Microbial assemblages with higher richness, evenness, diversity, and relative abundances of OTUs affiliated with Verrucomicrobia, Planctomycetes, and Proteobacteria characterized samples with negative Axis 1 values, and samples with higher relative abundances of OTUs affiliated with Cyanobacteria and Chloroflexi tended to have positive Axis 1 values (Table 2, Fig. 3). Fewer variables were correlated with NMDS Axis 2, but samples with a higher relative abundance of OTUs affiliated with Cyanobacteria tended to fall on the negative end of Axis 2, whereas samples with greater abundance of Alpha- and Betaproteobacteria tended to fall on the positive end of this axis. Correspondingly, 2-way ANOVA of the relative abundance of OTUs affiliated with individual major bacterial and archaeal (sub)Phyla showed numerous significant differences among sites (Fig. 4, Table S2). For example, BD had the highest relative abundance of Acidobacteria and Verrucomicrobia, RS had the highest relative abundance of Cyanobacteria and Chloroflexi, and RP and RS had the highest relative abundance of Crenarchaea. Within streams, significant differences in coarse taxonomic affiliations occurred between surface and subsurface waters only for Chloroplast-associated sequences (Table S2), which were more abundant in surface than subsurface waters (3.5 ± 2.5% > 1.5 ± 1.1%).

Table 2. Non-metric multidimensional scaling (NMDS) ordination axis correlations (Pearson's *r*) with bacterial and archaeal subphylum relative abundance and diversity metrics. Only the groups showing significant correlations with either axis are shown. * = $p < 0.002$. See Table 1 for definitions of abbreviations and metrics.

	Axis 1		Axis 2	
Metric	*r*	*p*	*r*	*p*
OTU richness	−0.74	<0.0001*	−0.19	0.171
Shannon's *H*	−0.73	<0.0001*	0.12	0.401
Chao1 richness	−0.72	<0.0001*	−0.21	0.129
Verrucomicrobia	−0.70	<0.0001*	−0.05	0.722
Equitability	−0.65	<0.0001*	0.26	0.057
Proteobacteria (Other)	−0.64	<0.0001*	0.06	0.675
Betaproteobacteria	−0.61	<0.0001*	0.54	<0.0001*
Planctomycetes	−0.58	<0.0001*	−0.03	0.853
Alphaproteobacteria	−0.58	<0.0001*	0.51	<0.0001*
Gammaproteobacteria	−0.52	<0.0001*	−0.28	0.036
Cyanobacteria	0.56	<0.0001*	−0.46	0.0004*
Chloroflexi	0.74	<0.0001*	−0.27	0.046

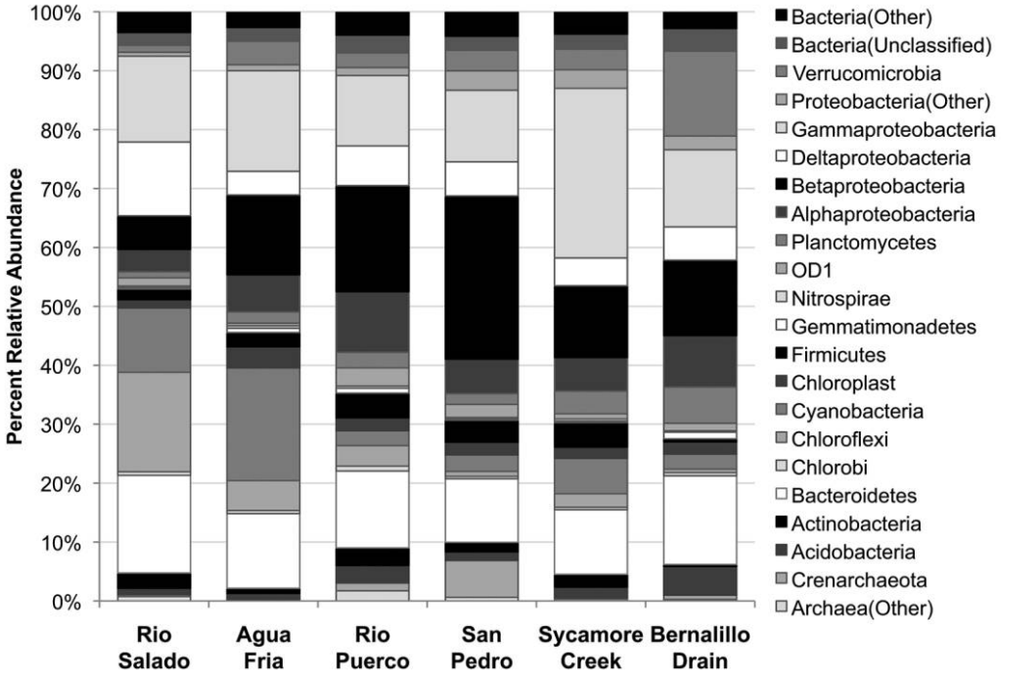

Figure 4. Mean relative abundances of all OTUs aggregated to major bacterial and archaeal Phyla or Subphyla at each study stream.

Relationships between microbial diversity & stream reach-scale attributes

Few correlations between whole-stream microbial diversity metrics and reach-scale hydrological and biogeochemical factors were significant (Table 3). We did not observe ($p > 0.05$, Fig. 5A–D) the expected relationships between microbial diversity and either dissolved N concentrations (nitrate, ammonium or organic N) or hydrological attributes (the proportional size of the transient storage zone [A_s/A]), the fraction of median water travel time due to transient storage (F_{MED}^{200}), or the % of shallow groundwater derived from surface water [%SW]). There were also no significant relationships between reach-scale biological N-cycling parameters (nitrate uptake rate, nitrate uptake velocity, denitrification uptake rate or velocity) and microbial diversity. In addition, neither stream water pH, whole-stream respiration, nor primary production was correlated with microbial diversity ($p > 0.05$, Table 3). The α-diversity parameters of sample richness and Chao1 estimated OTU richness, however, were both negatively correlated with watershed size, negatively associated with bromide concentration, negatively associated with the % of nitrate uptake into fine benthic organic matter (FBOM), and positively associated with the % of nitrate uptake into filamentous algae ($p < 0.05$, Table 3, Fig. 5E–H). The % of nitrate uptake into filamentous algae vs FBOM was also correlated with microbial gamma diversity (total unique OTUs across all samples from each stream) and the primary gradient in microbial assemblage composition (NMDS Axis 1) ($p < 0.05$, Table 3). These latter results show an association between low microbial diversity, a unique microbial assemblage composition, and substantial nitrate uptake into FBOM in 3 of the study streams (RS, AF, RP), and inversely, an association between high microbial diversity, a different microbial assemblage composition, and substantial nitrate uptake into filamentous algae in the other 3 study streams (SP, SC, BD).

DISCUSSION

Our goal was to characterize and quantify the microbial assemblage composition and diversity present in both surface and subsurface water of six streams that differed in a variety of characteristics. Specifically, we wanted to learn the extent to which microbial diversity and assemblage composition was related to stream nutrient availability, nutrient cycling, hydrology, and a variety of abiotic parameters such as salinity, pH, and watershed size. We observed large differences in microbial diversity and assemblage composition among streams, with among-stream microbial assemblage composition much greater than within-stream variation. The streams also covered a substantial range in nutrient concentrations and nutrient cycling rates, with levels characteristic of N-limited arid-land streams (Grimm 1987, Mulholland et al. 2009). Hydrological connectivity ranged from low levels similar to narrow, channelized agricultural streams (Sheibley et al. 2014) to higher levels more similar to free flowing arid-land streams (Valett et al. 1997, Harvey et al. 2003), with an accordingly variable amount of surface–subsurface exchange within reaches (Dent et al. 2007, Crenshaw et al.

Table 3. Correlations (Pearson's *r*) between microbial diversity metrics and stream reach-scale biogeochemical and hydrological attributes. * denotes a significant correlation ($p < 0.05$); $^{+}$ denotes a marginally significant ($p < 0.1$) correlation. See Table 1 for definitions of abbreviations and metrics.

	OTU richness	Chao1 OTU richness	Evenness (equitability)	Shannon's H	OTUs in all samples	NMDS Axis 1	NMDS Axis 2
Nutrient concentrations							
NO_3-N (μg/L)	−0.30	−0.18	0.24	−0.28	−0.45	−0.52	−0.062
NH_4-N (μg/L)	0.30	0.24	0.01	0.39	0.24	−0.54	0.22
DON (μg/L)	−0.34	−0.35	0.16	0.03	−0.55	0.25	0.71
Hydrological and geochemical characteristics							
As/A	0.22	0.21	−0.21	−0.05	0.58	−0.34	-0.83^{+}
FMED200	−0.03	−0.05	−0.70	0.54	0.43	0.20	−0.79
% SW	−0.00	−0.08	−0.62	0.11	−0.16	0.13	0.05
pH	−0.32	−0.33	−0.18	−0.21	−0.49	0.53	0.63
Br^{-} (μg/L)	-0.80^{+}	-0.79^{+}	−0.29	−0.48	-0.77^{+}	0.69	0.52
Watershed area (ha)	−0.88*	−0.91*	−0.57	−0.72	−0.63	0.71	0.21
Reach-scale nutrient cycling parameters							
u_{den} (μg N m^{-2} s^{-1})	−0.38	−0.35	−0.03	−0.19	−0.30	0.00	−0.18
u_{NO3} (μg N m^{-2} s^{-1})	0.25, 0.64	0.31	0.03	0.12	0.42	−0.10	−0.46
V_{fden} (cm/s)	−0.40	−0.38	0.08	−0.12	−0.53	0.013	0.14
V_{fNO3} (cm/s)	0.17	0.29	0.30	0.26	−0.01	−0.18	−0.19
ER (g O_2 m^{-2} d^{-1})	0.38	0.37	0.84*	0.75^{+}	0.22	−0.71	0.32
GPP (g O_2 $m^{-2}d^{-1}$)	0.64	0.71	0.35	0.48	0.34	−0.36	−0.12
% NO_3-N to FBOM	−0.93*	−0.90*	−0.65	-0.79^{+}	−0.84*	0.94*	0.33
% NO_3-N to filamentous algae	0.97*	0.93*	0.70	0.85*	0.84*	−0.93*	−0.19

2010). However, we did not find support for the prediction that streams with high inorganic nutrient concentrations would have low diversity, or that different nutrient removal rates would correspond with distinct microbial assemblages, and we found only limited support for the prediction that microbial assemblages would differ between surface and subsurface waters or be related to reach-scale hydrological connectivity. Instead, we found evidence that either salinity (indicated by bromide concentration) or other factors associated with watershed area might influence microbial diversity, composition and function.

There was no support for the prediction that inorganic N cycling and microbial diversity were related. Neither N concentrations nor nitrate uptake or transformation rates correlated with α-, β-, or γ-diversity (Table 3, Fig. 5A, B). Lotic microbial diversity is often related to nutrient availability, but other site-specific environmental factors (e.g. organic matter availability, temperature, hydrology) can exert stronger physiological constraints on microbial niche differentiation (Zeglin 2015). In general, microbially mediated processes with greater substrate specificity are predicted to show stronger links between microbial diversity and function, as opposed to processes with higher functional redundancy (Findlay 2010) such as nitrate assimilation and denitrification. A weak correlation between NMDS Axis 2 and nitrate concentration (Table 3) was driven by high nitrate concentration at SP, and while this weak correlation does not represent a coherent pattern among all sites, it is notably associated with the highest relative abundance of Crenarchaeota (Table S2), a group which contains the globally dominant types of ammonia-oxidizers, which are metabolically specialized chemolithoautotrophs (Leininger et al. 2006, Prosser and Nichol 2008). It is also possible that had we measured the abundance or diversity of genes specifically indicative of denitrifiers, or attributes of the benthic microbiota, we may have detected relationships with stream water nitrate concentration and processing rates. We focused on water-associated microbiota to directly pair microbial and water chemistry analysis. However, the heterogeneous physicochemical characteristics of surfaces comprise different microbial habitats than water, and support biofilms that can affect stream biogeochemistry (Battin et al. 2016), so benthic biofilm analysis may have shown higher correlations with reach-scale functions. Overall, however, no aspect of microbial assemblage composition that we measured was significantly or predictably related to dissolved nutrient concentrations or reach-scale nutrient cycling rates across all streams.

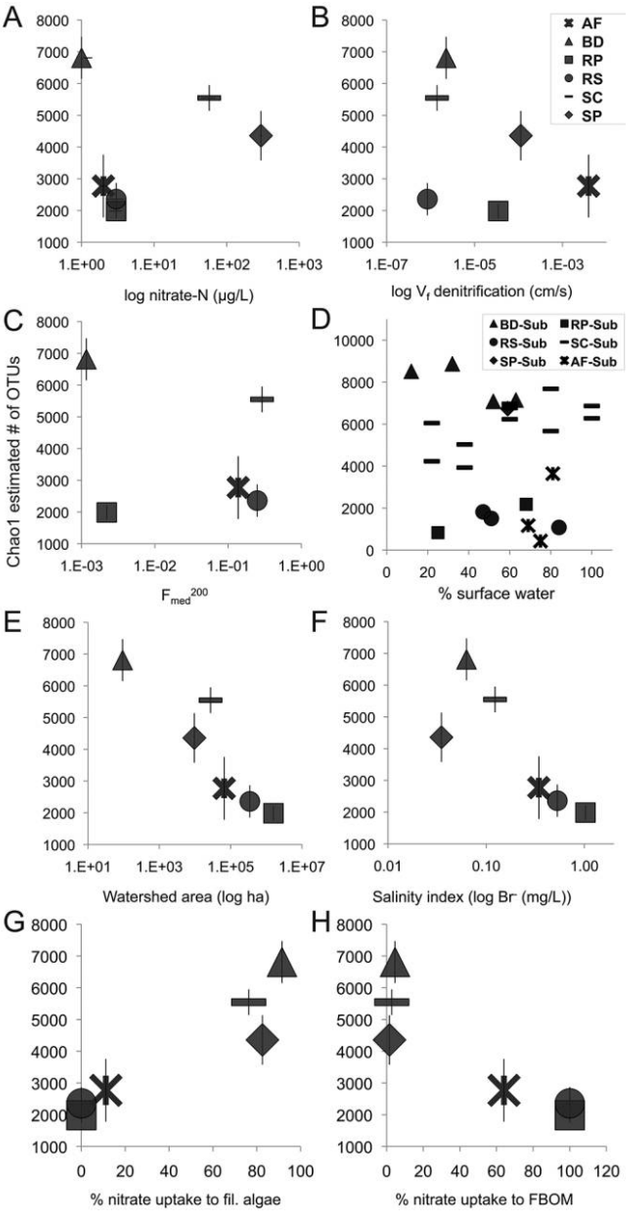

Figure 5. Cross-site correlations between microbial richness (mean ± SE) and whole-stream nutrient and hydrological parameters.

There was limited support for the prediction that within-reach hydrological factors and microbial diversity would be related. Microbial diversity parameters were not correlated with reach-scale hydrological connectivity indices nor to the amount of surface water within each subsurface well sampled (Table 3, Fig. 5C, D). Still, a small but detectable portion of the assemblage difference among all samples collected (2.3%) was attributable to flow path (surface vs subsurface) (Fig. 3). In some streams, a clear difference in microbial assemblages has been observed between surface and subsurface waters (Beier et al. 2008, Danczak et al. 2016), but these differences can be contingent on site conditions. For example, different microbial assemblages were observed between surface and subsurface waters in low permeability but not high permeability streambeds (Nogaro et al. 2013). The microbial assemblage composition in stream subsurface waters is also known to be sensitive to temporal environmental changes (Feris et al. 2003, Febria et al. 2012) and can differ between up- or downwelling locations within bed-scale flowpaths (Lowell et al. 2009). We did not address either of these two aspects of spatiotemporal heterogeneity in our study, but they may have contributed to the heterogeneity in microbial diversity we observed (Fig. 3). Two of our study streams (SP and BD) had higher microbial richness in subsurface than surface waters (Fig. 2), indicating that some factor promotes greater diversity of microbial taxa in subsurface water habitats at those sites; however, these 2 streams were not similar in their surface/subsurface water mixing levels, reach-scale connectivity, or bed texture (Table S1). Instead, these 2 sites were most similar in their overall microbial assemblage composition (Fig. 3), suggesting a more complex or different relationship between hydrology and microbial diversity than we originally anticipated.

Instead of nutrient availability, nutrient cycling, or reach-scale hydrology, the substantial differences in microbial assemblage composition and diversity among the 6 study streams (Figs 2–4) were more strongly related to watershed area upstream of the sampling reach and stream-water bromide concentrations (Table 3, Fig. 5E, F). Two factors, salinity and water residence time, might explain these patterns. First, total salinity levels are a fundamental constraint on cellular physiology and, therefore, a primary driver of microbial niche differentiation (Lozupone and Knight 2007, Martiny et al. 2015). Prior work at Rio Salado (RS), the site at 1 extreme of the observed microbial assemblage gradient and a high-bromide stream (Figs 3, 4, 5F), showed that high-salinity (measured as conductivity) saturated sediments contained lower diversity bacterial assemblages that were characterized by putatively halotolerant lineages, whereas low-salinity, unsaturated sediments contained higher diversity bacterial assemblages characterized by more generalist, soil-associated lineages (Zeglin et al. 2011). Second, greater residence time within a drainage basin is associated with larger watershed area, and residence time can directly affect microbial assemblage composition, as it is more likely that certain taxa will become competitively dominant over longer periods of stability. In lake waters, surface stream waters, and ground waters, longer residence times have been associated with significantly different, more putatively habitat-specialist dominated, microbial assemblages (Lindstrom and Bergstrom 2004, Ben Maamar et al. 2015, Ruiz-González et al. 2015). Thus, stream reaches draining larger watershed areas are more likely to

carry a different and less diverse water microbiota (Besemer et al. 2013), as we also observed in our data.

Furthermore, among our 6 study sites, the reaches draining larger watersheds with presumably longer water residence times also had higher bromide concentrations. In arid and semi-arid landscapes, surface flow is ephemeral and quick, but subsurface groundwater flow is slow (Fan 2015). Salinity, therefore, accumulates as groundwater moves through catchments following long subsurface flow paths, before reaching a point in the landscape where surface flow emerges (Fan 2015, Crossey et al. 2016). So, long groundwater residence times and high salinity may together select against transient taxa in favor of taxa that grow and reproduce better in increasingly saline waters, thereby changing assemblage composition and reducing microbial diversity. In other stream and river networks, the total richness and relative proportion of soil porewater associated microbial taxa is greater upstream, in smaller watersheds where water has traveled a shorter distance since leaving the shallow surface soil (Crump et al. 2012, Besemer et al. 2013, Ruiz-González et al. 2015, Savio et al. 2015). In the smallest watershed and only 1st-order stream in our study, BD, microbial assemblages contained a significantly higher proportion of the phyla Acidobacteria and Verrucomicrobia (Table S2), groups that are characteristic of soil habitats (Lauber et al. 2009, Bergmann et al. 2011). This site also had the highest total microbial richness, and a significantly higher richness in subsurface waters than surface waters (Fig. 2, Table 1), patterns that could also be attributed to a greater input of transient cells leaching from adjacent soils. In contrast, divergent microbial assemblage composition and low diversity at the 3 sites draining large watersheds, RP, RS and AF (Figs 3, 5) suggested competitive dominance by specialist taxa. For example, members of phyla Alphaproteobacteria, Chloroflexi, and Cyanobacteria were most abundant at these three sites, respectively (Table 3).

In mesic watersheds, evidence for selection for 'aqueous specialist' taxa in waters with longer lotic residence time is accumulating, in that members of phylum Actinobacteria tend to be more dominant lower in the watershed, possibly indicating competitive dominance of taxa with a more planktonic lifestyle (Crump et al. 2012, Read et al. 2015, Ruiz-González et al. 2015, Savio et al. 2015, Niño-García et al. 2016). In arid landscapes, where water often spends more time below- than aboveground as it moves downslope (Fan 2015), taxa that thrive best in the physicochemical conditions of the catchment's soil, aquifer, or parent material may become dominant (Ben Maamar et al. 2015, Crossey et al. 2016). Lotic microbial assemblage composition and function can also be affected by basin-scale geochemistry, geomorphology, or sediment texture (Mosher and Findlay 2011, Larouche et al. 2012, Tatariw et al. 2013). Our study was not designed to evaluate the turnover of microbial assemblage composition through arid river networks in contrasting basins, but we found evidence that suggests watershed-scale hydrogeology could be an important mechanism determining arid stream water microbial assemblage composition. Our results further suggest that a relationship between salinity and diversity also might be important at regional scales, particularly in arid regions where saline springs are common.

Our data did not show any direct relationships between microbial diversity and stream reach-scale N-cycling function, but did reveal an unexpected association between stream microbial diversity and the fate of nitrate within each study reach. In streams with high microbial diversity, 76 to 92% of nitrate uptake was attributable to filamentous algal biomass, whereas at sites with low microbial diversity, 64 to 100% of nitrate uptake was attributable to the fine benthic organic matter (Fig. 5G–H). This pattern could be reflective of the effects of salinity on the physiological limits of biological functioning, which can influence both microbial diversity (Fig. 5F) and algal growth (Hart et al. 1991, Nielsen et al. 2003, Velasco et al. 2006). Higher salinity may have constrained algal production and thus promoted the importance of microbial production and N uptake in stream benthic sediments. Often, greater net N retention in small streams is associated with proportionally greater autotrophic production and greater uptake into autotrophic biomass (Grimm et al. 2005, Hall et al. 2009, Tank et al. 2017), thus mechanisms that cause a reduction in autotrophic N uptake (such as high salinity) may impair this important ecosystem service. It is possible that salinity at levels high enough to limit the diversity of lotic bacteria and archaea (as indicated by > 130 μg/L Br^-) may also limit the proliferation of filamentous algae to a point where the fate of nitrate is affected. More specifically, in our study, the 2 streams with intermediate salinity (RS and AF: 340–530 μg/L Br^-) also had the highest stream ecosystem respiration (ER, 12–23 g O_2 m^{-2} d^{-1}) and proportional magnitude of ecosystem respiration over gross primary production (ER:GPP, 3.1–6.6×); but in the stream with highest salinity (RP: > 1000 μg/L Br^-) both GPP and ER were lowest (1.6 and 0.9, respectively; Table S1). If this observation is representative of other arid-land streams, then intermediately high salinity may favor the metabolism of heterotrophic over autotrophic organisms, and very high salinity may impair biological processes overall.

Our results suggest that watershed hydrology and stream water salinity could affect the assemblage composition and proportional reach-scale functional importance of streamwater bacteria and archaea. One implication of greater N immobilization in benthic vs filamentous biomass is the decreased likelihood that spates would cause large-scale transport and remineralization of the N contained in autotrophic biomass (Grimm 1987), i.e., the streambed could be more N retentive in moderately saline arid-land stream reaches. Also, beyond taxonomic diversity, the metabolic diversity of benthic and subsurface bacterial and archaea in saline streams is likely to be affected by unique ground water geo-

chemistry, so the availability of chemically-reduced iron and sulfur compounds, or chemolithoautotrophic uptake, may affect the fate of N (Burgin and Hamilton 2007). Similarly, certain N-cycling functional groups, such as nitrifiers, may be differentially affected by salinity (Bernhard et al. 2007). Our study cannot identify the specific causal mechanisms affecting microbial diversity among these arid-land streams, but the relationship between salinity and microbial assemblage composition was clear, and may be related to the longer time scales of water movement through larger catchments that promote accumulation of solutes and selection for specialized microbial taxa. In future work, it will be important to quantify the independent and interactive effects of water residence time and salinity on stream biodiversity and function. In both mesic and arid regions, higher streamwater salinity is often caused by shorter-term environmental phenomena, such as impervious surface runoff, discharge of industrial or mining effluent, or expansion of crop irrigation (Cañedo-Argüelles et al. 2013, Kaushal et al. 2018). In cases where streamwater microbes have experienced different lengths of time to acclimate or turn over in response to altered geochemical conditions, responses in diversity and function may be different, or thresholds for loss of stream function may be lower, than those observed in our study. Overall, this study highlights how information on microbial assemblage composition can shed light on the relative importance of hydrological and biogeochemical factors at different scales as drivers of lotic diversity and function.

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