

Relationship between aquifer biofilms and unattached microbial indicators of urban groundwater contamination

Robert J. Scharping  | James R. Garey 

Department of Cell Biology, Microbiology, and Molecular Biology, University of South Florida, Tampa, FL, USA

Correspondence

James R. Garey, Department of Cell Biology, Microbiology, and Molecular Biology, University of South Florida, Tampa, FL, USA.
Email: garey@usf.edu

Present address

Robert J. Scharping, Department of Biology, Woods Hole Oceanographic Institution, Woods Hole, MA, USA and Woods Hole Coastal and Marine Science Center, U.S. Geological Survey, Woods Hole, MA, USA

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Abstract

Aquifers, springs and other groundwater-dependent ecosystems are threatened by urban land use, which causes water quality deterioration through nutrient loading, sewage infiltration, groundwater extraction and, along coasts, seawater intrusion. The presence of certain microbes in groundwater can indicate that an aquifer is anthropogenically contaminated. Interpretations made from observations of indicator microbes in groundwater are limited because the relationship between the presumably allochthonous indicator microbes and relevant autochthonous microbial communities has not been characterized. This study addressed whether autochthonous aquifer biofilms can influence the presence of presumed microbial indicators in groundwater, and simultaneously used microbial indicators to trace sources of urban contamination at a karst spring of conservation concern. These questions were approached using a 17-month time series analysis of attached biofilm and adjacent unattached bacteria in the submerged karst aquifer conduit associated with this spring. Environmental 16S rRNA gene sequencing was performed to characterize these communities, and community structure data were contextualized with groundwater geochemical and hydrogeological measurements. Linear regression models were developed to explain the relative abundance patterns of indicator microbes and other unattached microbes at this site. The results of this study suggest that dominant aquifer biofilms do not influence the presence of unattached microbial taxa that are presumed to be indicators of groundwater contamination, and generated new information about the origin of coliform bacteria at the study site. These results build confidence in the use of microbial indicators in groundwater-dependent ecosystem conservation strategies and inform future management plans for urban aquifers and springs worldwide.

KEYWORDS

conservation, groundwater-dependent ecosystems, indicator species, land use, microbial biodiversity, urbanization

1 | INTRODUCTION

1.1 | Conserving groundwater-dependent ecosystems in a changing world

Ecosystems that rely on a supply of groundwater to maintain their structure and function, termed groundwater-dependent ecosystems, are both ecologically important and sensitive to environmental changes. This ecosystem type, which includes aquifers, springs, wetlands and many surface water bodies, makes important contributions to the biodiversity of a region by providing habitats for endemic, relict and specialized obligate species (Humphreys, 2006; Kløve, Ala-aho, et al., 2011). Groundwater-dependent ecosystems also provide useful services to people. For example, they support globally significant sources of potable and irrigation water, act as filters for nutrient and toxin loads, generate nursery habitats for economically important game species and are at the core of many recreational activities and cultural sites (Kløve, Allan, et al., 2011; Stinnesbeck et al., 2018). However, because groundwater-dependent ecosystems tend to be isolated from similar ecosystem types, and because public awareness of groundwater is low, they are particularly vulnerable to anthropogenic disturbance (Jyvasjarvi et al., 2018; Kløve, Ala-aho, et al., 2011; Kløve, Allan, et al., 2011).

Human land use, and urbanization in particular, is a major threat to the structure and functioning of groundwater-dependent ecosystems (Green et al., 2011; Kløve et al., 2014). The world is rapidly becoming more urban, which puts stress on groundwater-dependent ecosystems. From 1950 to 2018, the global urban population increased from 0.8 billion to 4.2 billion, respectively, accounting for 30% and 55% of the entire population (United Nations, 2019). The world population became more urban than rural beginning in 2007, and this urban dominance is projected to increase through 2050, when 68% of the entire population is expected to be living in urban areas (United Nations, 2019). Groundwater response to urbanization is complex. While aquifer overexploitation and dewatering for infrastructure development lowers groundwater levels (Kløve, Allan, et al., 2011), intense urbanization is often associated with rising groundwater levels (Sharp, 2010). This is because many urban land uses increase aquifer recharge rates, such as the construction of impervious land cover which decreases evapotranspiration rates, overwatering of lawns and playing fields, implementation of aquifer storage strategies, introduction of imported water and leakage from water management systems (Sharp, 2010). Due to increases in aquifer recharge caused by urban land use,

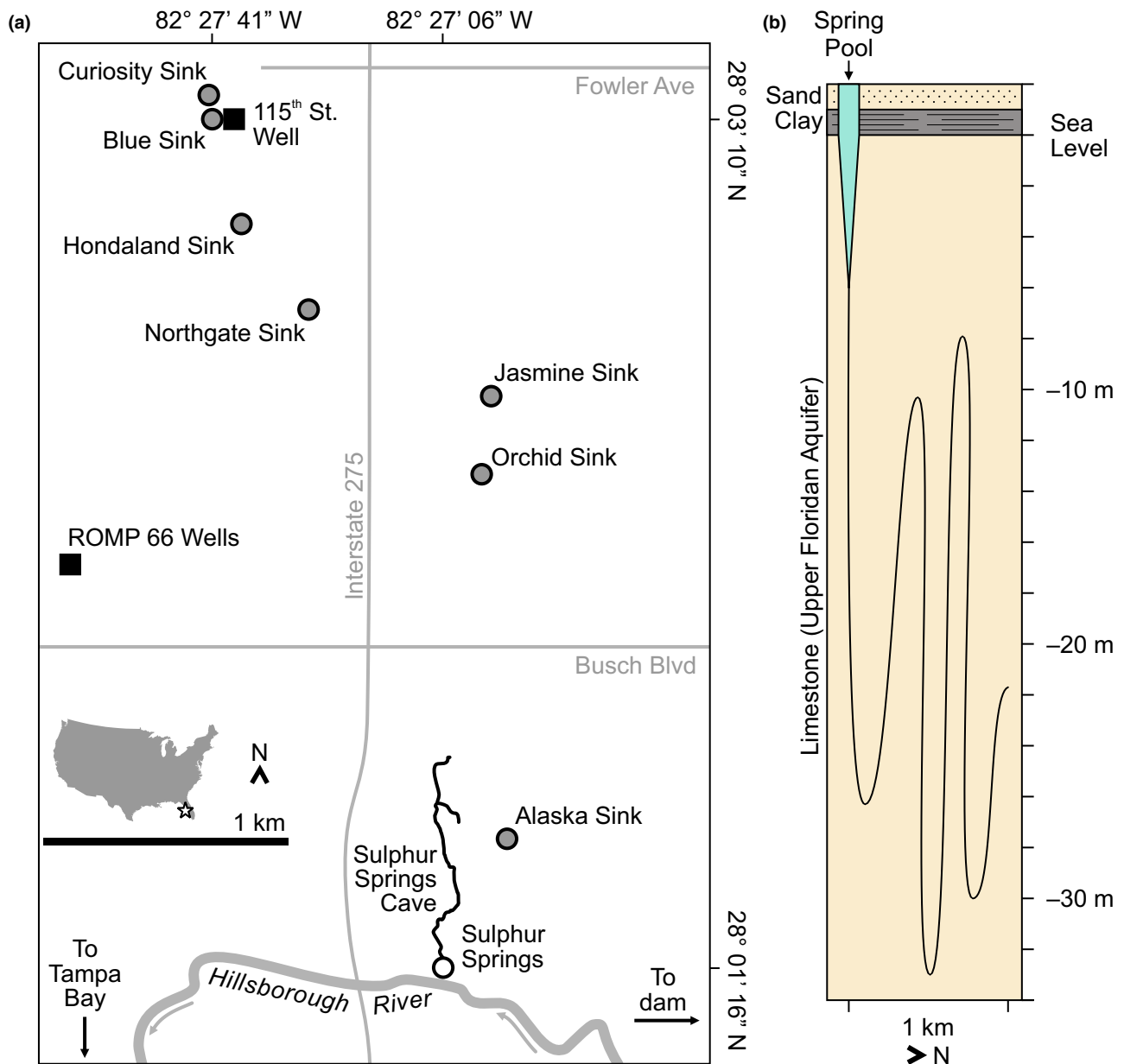
urban groundwater-dependent ecosystems are exposed to high loads of inorganic nutrients and infiltration of domestic sewage and industrial wastewater (Huang et al., 2020; Sharp, 2010). In addition, dense coastal populations often rely on aquifers as primary sources of drinking water. Seawater intrusion resulting from groundwater overuse can negatively impact groundwater-dependent ecosystems, especially if intruding seawater exhibits chemically reducing conditions (Danielopol et al., 2003; Kløve, Allan, et al., 2011; Michael et al., 2017).

The use of indicator species is a key component of conservation plans for ecosystems impacted by anthropogenic stressors (Fleishman & Murphy, 2009; Landres et al., 1988) and is particularly useful in ecological assessments of urban aquifers. The presence of certain microbes, such as faecal coliform bacteria, in groundwater can indicate the presence of contaminants associated with urban land use (Jiang et al., 2018; Wicki et al., 2015). While indicator microbes alert resource managers to anthropogenic groundwater contamination, knowledge gaps persist concerning the relationship between presumably allochthonous indicators and relevant autochthonous aquifer microbial communities (Epting et al., 2018; Page et al., 2017). These knowledge gaps limit the ability of managers and scientists to interpret the presence or absence of indicator microbes in groundwater-dependent ecosystems.

The autochthonous communities of aquifer environments are dominated by microbes living attached to surfaces, and these attached cells often form biofilms. Current estimates of attached:unattached ratios for microbial communities dwelling in the continental subsurface vary by five orders of magnitude (Magnabosco et al., 2018), but typically fall within the range of 100:1–1,000:1 (Flemming & Wuertz, 2019). Furthermore, attached microbes that form biofilms account for 20%–80% of cells in these environments (Flemming & Wuertz, 2019). To verify whether certain groundwater-borne microbes are indeed indicators of anthropogenic aquifer contamination, the community dynamics of autochthonous subsurface biofilms must be studied concurrently with those of unattached microbes that are presumed to be allochthonous.

The current study addresses whether autochthonous aquifer biofilms can influence the presence of presumed microbial indicator taxa in groundwater. This question was approached through a time series analysis of two microbial communities in a submerged karst aquifer conduit, (a) biofilm attached to the conduit wall and (b) the unattached bacteria present in the adjacent water column. It was hypothesized that the community structure dynamics of the biofilm would be reflected in those of the unattached bacterial community.

FIGURE 1 The study area in metropolitan Tampa, Florida, USA. Panel a: The location of Sulphur Springs (open circle) and sinkholes that exhibit hydraulic connections to Sulphur Springs (grey circles). Also shown are the two groundwater well sites used in the study (black squares). Grey arrows indicate direction of Hillsborough River flow. Panel b: Approximate geologic profile of Sulphur Springs Pool and Cave based on the lithology of a nearby well (O'Sullivan & Werner, 1991), the elevation of the spring pool and a diver-collected depth profile of the cave (Scharping et al., 2018). Thickness of the sand/clay layer represents that of the immediate vicinity of Sulphur Springs Pool, and this layer grows thicker northward of the spring (Stewart & Mills, 1984). The black curved line generalizes the extent of Sulphur Springs Cave. Panel c: The impounded Sulphur Springs Pool, looking south towards the Hillsborough River. The entrance to Sulphur Springs Cave is in the middle of this pool, ~8 m beneath the water's surface. The scum line seen around the walls of the pool is revealed when groundwater extraction from the pool lowers its water level. A pump intake pipe with a diameter of ~1 m is located below the water's surface



In concert with this aim, taxa related to the commonly used microbial indicator group, coliform bacteria, were used to trace sources of urban contamination at Sulphur Springs, a karst spring of conservation concern in metropolitan Tampa, Florida, USA. The relative abundances of other unattached microbial taxa were investigated in an attempt to trace different sources of groundwater that feed Sulphur Springs. Increased understanding of the contamination and water sources of Sulphur Springs will inform future ecosystem management plans for this site and for springs in other urban watersheds, especially in karst landscapes. About 16.5% of the global population lives on karst landscapes, and karst aquifers supply ~9% of the global population with freshwater (Goldscheider et al., 2020; Stevanovic, 2019). Next-generation DNA sequencing was used to characterize the biofilm and unattached bacterial communities collected inside the cave underlying Sulphur Springs throughout a ~1.5-year period. These data were contextualized with hydrological and physicochemical measurements, and with a literature review of previous studies of Sulphur Springs urban karst hydrogeology and coliform contamination dynamics.

1.2 | Sulphur Springs urban karst hydrogeology

Sulphur Springs Cave is an underwater cave system part of the limestone karst topography of Tampa, Florida, USA (Figure 1a,b). The cave discharges water via a submerged artesian spring and fills the impounded Sulphur Springs Pool (Figure 1c). Sulphur Springs is one of the main tributaries of the Hillsborough River, which is itself the largest tributary of Tampa Bay (Moyer et al., 2015). The Hillsborough River is dammed upstream of Sulphur Springs to store public water supply in the Hillsborough River Reservoir (SWFWMD, 2015). During dry seasons, local resource managers frequently extract spring water from Sulphur Springs Pool (Scharping et al., 2018), and the extracted water is pumped to the base of the Hillsborough River Dam to maintain flows and low-salinity habitat in estuarine portions of the river when releases from the reservoir are insufficient to do so (SWFWMD, 2015).

The limestone in which Sulphur Springs Cave is developed hosts the confined Upper Floridan Aquifer, which is locally overlain by a clay confining unit and an unconfined, sandy surficial aquifer (Figure 1b; Stewart & Mills, 1984). The top of the confined aquifer in this area is typically 10–15 m below land surface (Stewart & Mills, 1984), and most of the ~1 km of Sulphur Springs Cave passageway lies at depths ~10–30 m below the potentiometric surface of this aquifer (Figure 1b). The confined aquifer surrounding Sulphur Springs Cave contains freshwater, but fractures in the cave floor provide pathways for deep-sourced saline water to enter the cave (Scharping et al., 2018). Near these fracture-fed saltwater vents, vertically intruding saline water and slightly brackish cave water remain density stratified, and this phenomenon produces a karst subterranean estuary in Sulphur Springs Cave. The salinity of Sulphur Springs water has increased from fresh to ~5,000 $\mu\text{S}/\text{cm}$ between 1946 and 2017 as a result of seawater intrusion through these

fractures, which has been exacerbated by spring water extraction from Sulphur Springs Pool (Scharping et al., 2018). Groundwater flow in the Sulphur Springs area is generally from north to south and is accelerated by the presence of fractures and karst solution features, including a series of sinkholes within ~4 km north of Sulphur Springs (Figure 1a).

Sinkholes and fractures in the Sulphur Springs area breach the clay confining unit and connect the underlying confined aquifer with (a) surface water bodies, (b) urban stormwater run-off and (c) the surficial aquifer. Curiosity Sink and Blue Sink (Figure 1a) historically acted as swallets for Curiosity Creek, a short, intermittent, sinking stream that drains a swampy area north of the Sulphur Springs area. Flooding in the 1970s filled in Curiosity Sink and Blue Sink with sediment and debris, which reduced the connectivity between these sinks and the confined aquifer, and reduced the efficiency with which these sinks drain Curiosity Creek (MWH, 2009; SWFWMD, 2009). This in-filling has blocked previously established dye trace connections between these two sinks and Sulphur Springs (Menke et al., 1961; MWH, 2009). However, dye tracing conducted postblockage showed that dye injected into Blue Sink can be detected in nearby wells (Wallace, 1993), and, even more recently, pumping-induced drawdown of Blue Sink water levels generated a depression cone in the potentiometric surface of the underlying confined aquifer (SWFWMD, 2009). This implies that while the rapid, direct connection between Blue Sink and Sulphur Springs Cave may be blocked, hydraulic communication between Blue Sink and the confined aquifer still exists, likely via primary (bedrock matrix) porosity. Importantly, the zone of influence that Blue Sink has on the confined aquifer intercepts the Hondaland Sink area (Figure 1a; SWFWMD, 2009), a series of sinkholes that have maintained hydraulic connectivity with Sulphur Springs (MWH, 2009).

Pump tests and dye tracing have confirmed that rapid hydraulic connections remain intact between Sulphur Springs and other major sinkholes in the area (Figure 1a), including those that receive urban stormwater run-off (MWH, 2009). Tracer dye injected into Jasmine Sink or Orchid Sink takes ~24 hr to travel to Sulphur Springs, and dye injected into Alaska Sink takes ~6 hr to arrive at Sulphur Springs (MWH, 2009). Stormwater run-off from large commercial and residential areas is directed into Jasmine Sink and Orchid Sink, and Alaska Sink receives a run-off from a small residential area (Stewart & Mills, 1984; Wallace, 1993).

A final way that sinkholes and fractures in the Sulphur Springs area contribute to the rapid recharge of the local confined aquifer is by connecting it to the overlying surficial aquifer (Menke et al., 1961). These features contribute to leakiness in the confining unit that divides the two aquifers. Where the surficial aquifer water table is lower than the potentiometric surface level of the underlying confined aquifer, water may move upward through fractures and sinkholes from the confined aquifer into the surficial aquifer. When the confined aquifer potentiometric surface level is lower than the water table in the overlying surficial aquifer, groundwater can move downwards from the surficial aquifer into the confined aquifer. Under the latter hydraulic condition, urban contaminants residing in

the surficial aquifer could enter the confined Sulphur Springs system. Extracting water from Sulphur Springs Pool lowers water levels in the pool and reduces potentiometric surface levels in the confined aquifer nearby (MWH, 2009), and this activity could facilitate downwards leakage from the surficial aquifer. The sinkholes in the Sulphur Springs area can act as sinks (i.e. siphons) or springs (Wallace, 1993), a phenomenon that illustrates the dynamic relationships between sinkhole water levels, surficial aquifer water table levels and confined aquifer potentiometric surface levels.

1.3 | Using microbial indicators in Sulphur Springs

Sulphur Springs has been monitored for many decades (Menke et al., 1961; Rosenau et al., 1977; Scharping et al., 2018; Stewart & Mills, 1984; Wallace, 1993), and several studies have used coliform bacteria as indicators of the impact of urbanization on Sulphur Springs. Coliform counts have been measured at Sulphur Springs to indicate the presence of bacteria that are dangerous for human consumption and contact and also to trace sources of urban stormwater run-off (MWH, 2009; Stewart & Mills, 1984; Wallace, 1993). These studies generally associated high coliform counts at Sulphur Springs with rainfall events strong enough to bring stormwater into the confined aquifer through sinkholes. However, measurements collected simultaneously at the sinkholes and Sulphur Springs show that coliform counts usually increase by an order of magnitude between the points where stormwater enters and exits the confined aquifer (MWH, 2009). This suggests that stormwater flushing through sinkholes is not itself the main source of coliform contamination at Sulphur Springs.

Sewage systems are possible alternative sources of Sulphur Springs coliform contamination. Major sewer line junctions are present in the surficial aquifer directly overlying Sulphur Springs Cave (Stewart & Mills, 1984), and domestic septic tanks are used in portions of the Sulphur Springs area that not serviced by the sewer system (e.g. the areas north and west of Blue Sink). High coliform counts at Sulphur Springs have been associated with periods of spring pool pumping (Stewart & Mills, 1984), an activity which lowers confined aquifer potentiometric surface levels throughout the Sulphur Springs area (MWH, 2009). Pumping of the Sulphur Springs Pool thus increases the likelihood of downwards leakage from surficial aquifer sewage systems into the confined aquifer.

Few studies have examined the role of microbes other than coliform indicators in the Sulphur Springs ecosystem (Garman, 2010; Scharping et al., 2018), but to properly contextualize the abundance of indicator microbes collected in Sulphur Springs water, the autochthonous biofilm communities of Sulphur Springs Cave require characterization. Two conspicuous biofilm types inhabit Sulphur Springs Cave (Scharping et al., 2018). Brown-coloured biofilm grows in thick mats on every solid surface in the cave except for areas near fracture-fed saltwater vents, where delicate, filamentous, white biofilm grows instead (Scharping et al., 2018). These biofilms also differ from one another in their microscopic

structure, and it is hypothesized that the white biofilm is dominated by sulphur-oxidizing bacteria (Scharping et al., 2018). In the present study, these biofilms were sampled to contextualize a time series analysis of the unattached bacterial community collected from inside Sulphur Springs Cave. Brown biofilm made up the overwhelming bulk of the visible Sulphur Springs microbial community, so samples of this biofilm were collected in a time series complementary to that of the adjacent unattached bacteria. White biofilm samples were collected on one date in this time series to better understand the microbial communities living in Sulphur Springs saltwater vent habitats.

2 | MATERIALS AND METHODS

2.1 | Microbial community sample collection, processing and DNA sequencing

Microbial community samples were collected from Sulphur Springs Cave by closed-circuit SCUBA divers under the auspices of the University of South Florida research diving programme. Samples of brown biofilm and unattached bacterial communities were collected from Sulphur Springs Cave at ~125 m linear penetration into the cave, at a depth ~28 m below the potentiometric surface. These samples were collected on 11 dates from 13 February 2017 to 06 July 2018 (Figures 2, 3). White biofilm samples were collected from a fracture-fed saltwater vent ~45 m linear penetration into the cave, ~23 m below potentiometric surface level on 29 March 2018.

On each sampling date, triplicate brown biofilm samples were collected using sterile plastic syringes or jars prefilled with sterile nanopure water to prevent implosion at depth. On the same dates, unattached bacterial community samples were collected in triplicate sterile 1-L plastic bottles, which were also prefilled with sterile nanopure water. Triplicate white biofilm samples were collected using sterile plastic syringes prefilled with sterile nanopure water. Sampling jars and bottles were purged underwater with helium gas before biofilm or groundwater was collected. Samples were kept on ice and in the dark during transport back to the laboratory, where they were immediately vacuum-filtered onto 0.2- μ m pore size Millipore filters. Using sterile techniques, biofilm residue was scraped from filters into microcentrifuge tubes then dried in a vacuum centrifuge, and unattached bacteria filters were transferred into conical vials. Samples were kept at -20°C until genomic DNA extraction using Qiagen DNeasy PowerSoil kits. Extracted DNA was stored at -80°C until sequencing.

Paired-end 300-bp Illumina MiSeq sequencing analysis of biofilm and unattached bacteria DNA was performed at an external facility (Applied Biological Materials Inc.) using Earth Microbiome Project universal primers (515F/806R), which target the V4 region of the prokaryotic 16S ribosomal RNA gene. Sequencing error rates were <0.2%. Sequence reads are available at GenBank/DDBJ/EMBL under the BioProject PRJNA517336. Quality control of raw sequence reads was performed using program MOTHUR v1.42.3 (Schloss et al., 2009).

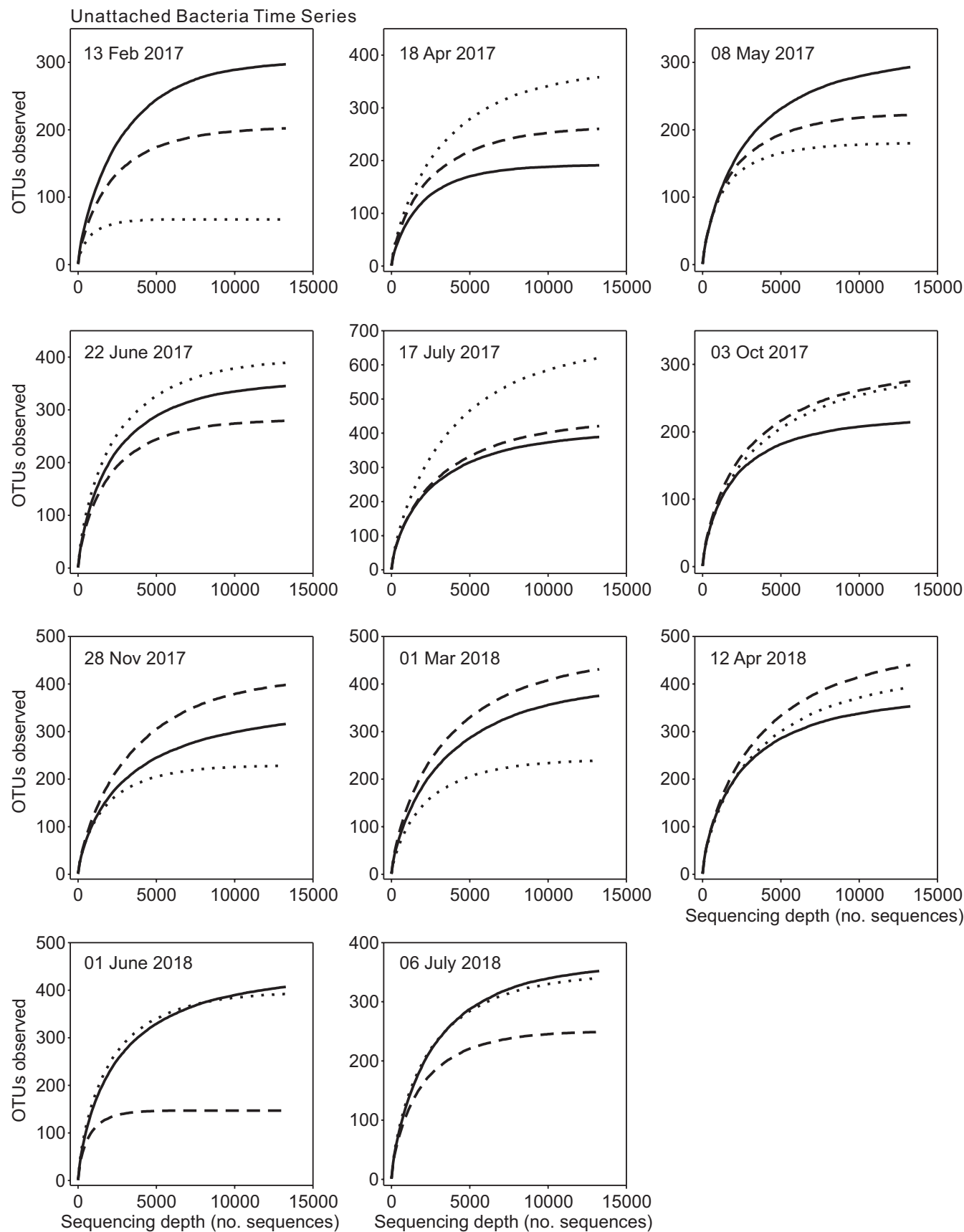


FIGURE 2 Rarefaction curves for unattached bacterial community samples collected from the Sulphur Springs Cave water column. Different lines on a given plot represent replicates

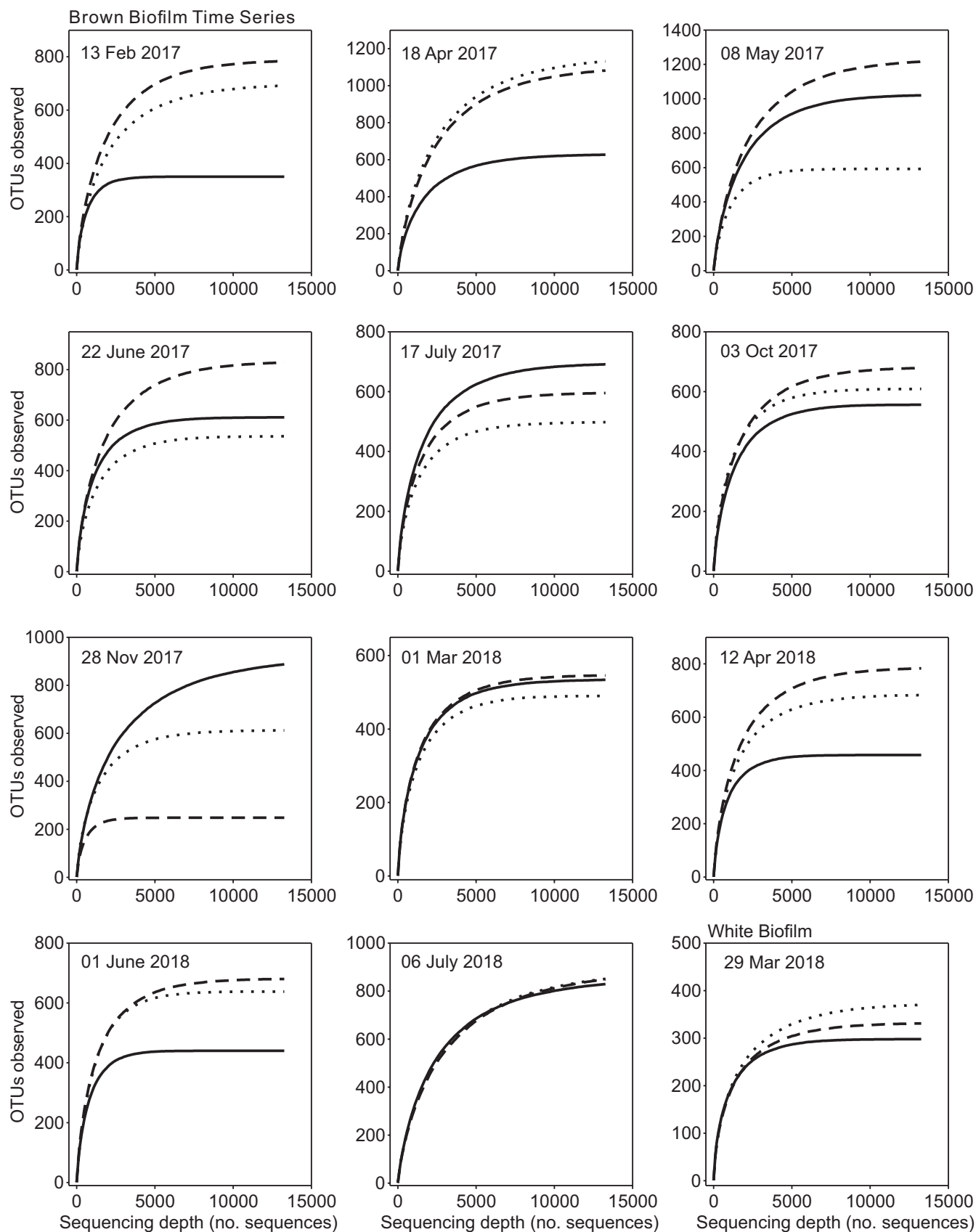


FIGURE 3 Rarefaction curves for the brown and white biofilm samples collected from Sulphur Springs Cave. Different lines on a given plot represent replicates

MOTHUR software was also used to group sequences into operational taxonomic units (OTUs) based on sequences having 97% similarity (OptiClust algorithm; Westcott & Schloss, 2017). To limit the influence of sequencing artefacts on our analyses, rare OTUs ($n \leq 10$ sequences) were removed (Brown et al., 2015), and then, each sample was subsampled to 13,273 sequences (i.e. the sequencing depth of the sample which exhibited the lowest sequence count). Rarefaction curves generated from each replicate showed that this procedure produced sequencing depths that adequately captured the diversity of the communities (Figures 2, 3). Taxonomic identifications of OTUs were assigned using the SILVA v132 database. Program ESTIMATES v9.1.0 was used to calculate Shannon diversity index and evenness values based on OTU abundances (Colwell, 2013), and program R v3.6.2 was used to summarize community composition at higher taxonomic levels with treemap plots (R Development Core Team, 2008).

2.2 | Physicochemical and hydrological measurements

Physicochemical and hydrological measurements were used to explain patterns in Sulphur Springs Cave unattached bacterial community structure. Water samples for analyses of total-P and ammonium-N concentrations were collected from the Sulphur Springs Cave water column on dates corresponding to the microbial community time series. These samples were collected ~125 m linear penetration into the cave in triplicate acid-washed glass jars prefilled with nanopure water. The jars were purged with helium gas to facilitate underwater sample collection. Samples were kept on ice in the dark during transport to the laboratory, where they were immediately analysed using a DR 3900 spectrophotometer (Hach). Analytical precision was $\pm 0.7 \mu\text{M-P}$ for the total-P test (HACH method 8190, acid persulphate digestion) and $\pm 1.3 \mu\text{M-N}$ for the ammonium-N test (HACH method 10205, salicylate reaction). Ammonium-N concentration was not measured on biological sampling date 28 Nov 2017. Specific conductance was measured in situ using a Hydrolab DS5X multiprobe (OTT HydroMet), calibrated with a $10,000 \mu\text{S/cm}$ KCl standard solution and bracketed by a $200 \mu\text{S/cm}$ KCl standard solution (RICCA Chemical Co.).

To characterize the saltwater vent habitat colonized by the white biofilm described above, water samples for general nutrient analysis were collected from inside the saltwater vent and from the Sulphur Springs Cave water column just upstream of the vent. These samples were collected on 14 Nov 2018. Water samples for sulphide-S (precision $\pm 0.5 \mu\text{M-S}$, HACH method 8131, N,N-dimethyl-p-phenylenediamine sulphate reaction), sulphate-S (precision $\pm 104 \mu\text{M-S}$, HACH method 8051, barium reaction), ammonium-N (precision $\pm 1.3 \mu\text{M-N}$, Hach method 10205, salicylate reaction), nitrite-N (precision $\pm 0.22 \mu\text{M-N}$, HACH method 10207, aromatic amine reaction), nitrate-N (precision $\pm 3.8 \mu\text{M-N}$, HACH method 10206, 2,6-dimethylphenol reaction) and total-P (precision $\pm 0.7 \mu\text{M-P}$, HACH method 8190, acid persulphate digestion) analyses were collected in triplicate from both locations

in acid-washed plastic syringes that were prefilled with nanopure water. Specific conductance was measured as described above.

Measurements of water levels throughout the Sulphur Springs area were used to complement biological and physicochemical samples collected from Sulphur Springs Cave. Daily mean Sulphur Springs Pool levels were recorded and published online by the U.S. Geological Survey (waterdata.usgs.gov; station number 02306000). Daily mean water levels in the three wells (ROMP 66 surficial aquifer, ROMP 66 confined aquifer and 115th Street confined aquifer; Figure 1a) and one sinkhole (Blue Sink; Figure 1a) used in this study were recorded and published online by the Southwest Florida Water Management District (watermatters.org; district site ID numbers 19238, 18670, 670753 and 670721, respectively). Lithologies of these wells were published by the Southwest Florida Water Management District (watermatters.org; SWFWMD, 2009).

2.3 | Statistical analysis and explanation of linear regression model variables

Relationships between the Sulphur Springs Cave biofilm and unattached bacterial communities were analysed using program PRIMER v7/PERMANOVA+ (Clarke & Gorley, 2015). To compare the overall structures of the biofilm ($n = 11$ dates) and unattached ($n = 11$ dates) communities, a single resemblance matrix was generated using proportionate relative abundance data from each sample. This approach accounted for differences in sequencing depth between the community types. This matrix was analysed using ANOSIM, PERMDISP and PERMANOVA permutational tests (see Anderson and Walsh (2013) for discussion about these tests). Results of these tests were visualized through nonmetric multidimensional scaling in PRIMER. To compare patterns in the structural dynamics of the two communities throughout the study period, two separate resemblance matrices were generated (one for each community type using raw relative abundance data), and a two-way RELATE test was performed (i.e. PRIMER's version of a Mantel matrix correlation test). This test grouped replicates in each matrix by date ($n = 3$ field replicates per date) before calculating Spearman correlation coefficients. All resemblance matrices were generated using Bray–Curtis (dis)similarity.

Multivariate linear regression models were developed to determine how well the relative abundances of major taxa in the unattached bacterial community were explained by environmental changes. Program R v3.6.2 was used to generate these models. Triplicates of unattached Enterobacteriales, Betaproteobacteriales and Campylobacteriales proportionate relative abundance data were averaged for each date ($n = 11$) and used as regressors for three respective models. Predictors for the Enterobacteriales and Betaproteobacteriales models included water level difference between the ROMP 66 Wells, water level difference between Blue Sink and the 115th Street Well, and water level in the Sulphur Springs Pool. Predictors for the Campylobacteriales model were total-P concentration, ammonium-N concentration and specific conductance measured in Sulphur Springs Cave.

Hydrological predictor variables used in the Enterobacteriales and Betaproteobacteriales models described the potential for groundwater to travel through the confining unit (via drainage structures, such as cracks, joints, fractures, etc.) that separates the surficial and confined aquifers in the Sulphur Springs area. The difference between water levels in the ROMP 66 surficial aquifer and the ROMP 66 confined aquifer wells was used to determine the direction of potential confining unit leakage for the general study area (Figure 1a). The difference between water levels in Blue Sink and the 115th Street confined aquifer well (located on the bank of Blue Sink; Figure 1a) was used to determine whether Blue Sink was injecting surficial aquifer water and/or surface water into the underlying confined aquifer, or whether injection from Blue Sink would be hydraulically dammed by high confined aquifer head. This measurement was also assumed to be a proxy of whether the same conditions were occurring in other area sinkholes that receive urban stormwater run-off (i.e. Jasmine Sink and Orchid Sink; Figure 1a). Positive values measured at either site indicated the potential for surficial aquifer water and/or surface water to flow into the confined aquifer. Water levels in Sulphur Springs Pool drop rapidly when spring water is being extracted and pumped to the Hillsborough River (Scharping et al., 2018), so Sulphur Springs Pool levels were used as a measure of pumping activity and of confined aquifer potentiometric surface levels in the immediate area. Lower Sulphur Springs Pool levels indicated the increased potential for groundwater in the surficial aquifer water to leak into the underlying confined aquifer and ultimately into Sulphur Springs Cave.

3 | RESULTS

3.1 | Analysis of unattached and biofilm communities

The Sulphur Springs Cave unattached bacterial community was dominated by three orders, Enterobacteriales, Betaproteobacteriales and Campylobacteriales (Figure 4). These orders collectively accounted for 76%–94% of the unattached community during study period, with a mean combined relative abundance of $87 \pm 5\%$ for the entire period ($n = 11$ dates). While Betaproteobacteriales and Campylobacteriales remained consistently abundant in the Sulphur Springs Cave water column, Enterobacteriales relative abundance varied widely among sampling dates (Figure 4). Betaproteobacteriales and Campylobacteriales, respectively, exhibited mean relative abundances of $39 \pm 10\%$ and $32 \pm 8\%$ and ranges of 24%–51% and 14%–43% during the study period ($n = 11$). The mean Enterobacteriales relative abundance during the study period was $15 \pm 16\%$, with a range of 0.02%–50% ($n = 11$). Changes in the relative abundances of these orders did not follow a clear seasonal pattern (Figure 4).

The 3 orders of bacteria that comprised the majority of the unattached community were themselves represented by only a few taxa (Figure 5). Unidentified *Enterobacteriaceae*

made up $100 \pm 0.01\%$ (range 99.97%–100%) of the unattached Enterobacteriales ($n = 11$). The unattached Betaproteobacteriales were represented primarily by unclassified members of that order, which accounted for $89 \pm 6\%$ (range 81%–96%) of the total Betaproteobacteriales abundance ($n = 11$). Finally, the genus *Sulfurimonas* made up $96 \pm 1\%$ (range 93%–98%) of unattached Campylobacteriales abundance. Overall, the unattached bacterial community was represented by 313 ± 79 OTUs and exhibited a Shannon diversity index value of 2.70 ± 0.39 and an evenness value of 0.47 ± 0.05 ($n = 11$; Table 1).

The brown biofilm community sampled on the same dates as the unattached bacterial community was more diverse and evenly represented than the unattached community (Table 1). The brown biofilm was represented by 686 ± 151 OTUs and exhibited a Shannon diversity index value of 5.22 ± 0.40 and an evenness value of 0.81 ± 0.06 ($n = 11$ dates; Table 1). A comparison of the treemap plots representing the structure of each community type illustrates the higher evenness values of the brown biofilm community compared to that of the unattached community (Figure 5). The higher diversity and evenness of the brown biofilm made this community more difficult to broadly characterize than the unattached community (Table 1), but the main taxonomic groups that represented the brown biofilm were the order Methylospirales (average relative abundance $16 \pm 6\%$, range 4%–24%, $n = 11$), the phylum Planctomycetes ($14 \pm 3\%$, 9%–21%, $n = 11$), the class Anaerolineae ($9 \pm 4\%$, 4%–18%, $n = 11$) and unclassified Bacteria ($12 \pm 3\%$, 8%–17%, $n = 11$). The orders that dominated the unattached community contributed to only a small proportion of the brown biofilm community (Enterobacteriales, $3 \pm 10\%$, 0.00%–33%; Betaproteobacteriales, $5 \pm 2\%$, 2%–7%; Campylobacteriales, $2 \pm 3\%$, 0.22%–10%; $n = 11$).

White biofilm growing at a fracture-fed saltwater vent was collected on a single date within the study period and was not analysed according to the unattached bacteria/brown biofilm time series.

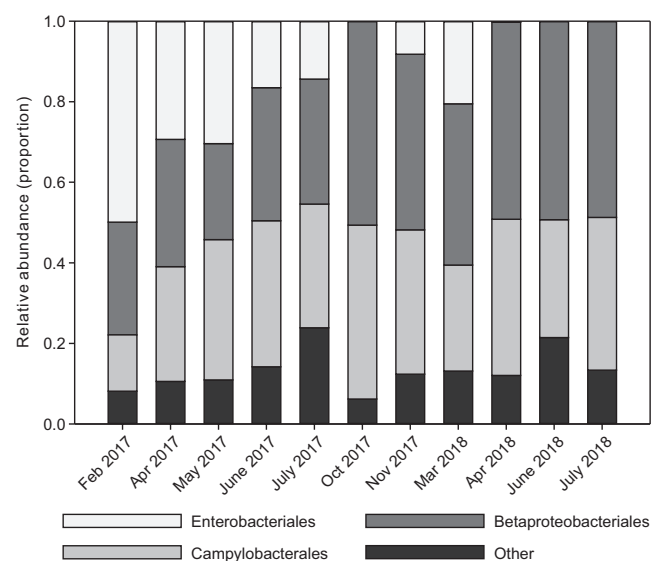


FIGURE 4 Relative abundances of orders in the Sulphur Springs Cave unattached bacterial community throughout the time series

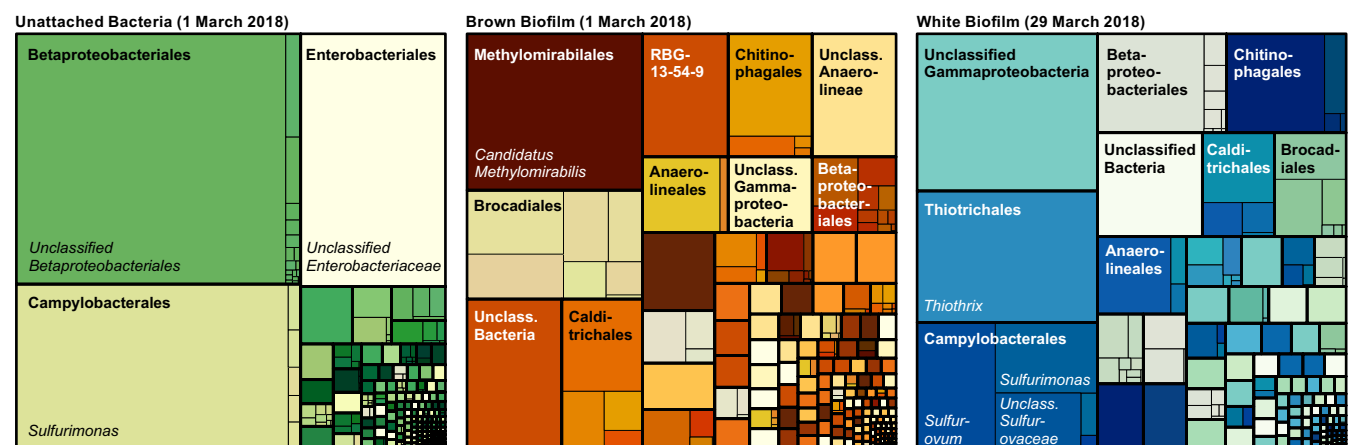


FIGURE 5 Treemap plots summarizing Sulphur Springs Cave unattached bacterial and brown biofilm community structures on 1 March 2018, as well as the white biofilm community structure on 29 March 2018. Thick lines and bold text represent the relative abundances of different orders, while thin lines and italicized text represent the relative abundances of different genera

Community	<i>n</i>	Number of OTUs grand mean	Shannon diversity grand mean	Evenness grand mean
Unattached	11	313 ± 79	2.70 ± 0.39	0.47 ± 0.05
Brown Biofilm	11	686 ± 151	5.22 ± 0.40	0.81 ± 0.06
White Biofilm	1	333	4.22	0.73

Abbreviation: OTUs, operational taxonomic units.

TABLE 1 Diversity metrics of the Sulphur Springs Cave bacterial communities

TABLE 2 Physicochemistry upstream of and inside the sampled Sulphur Springs Cave saltwater vent

Site	Specific Conductance (μS/cm)	Sulphide-S (μM-S)	Sulphate-S (μM-S)	Ammonium-N (μM-N)	Nitrite-N (μM-N)	Nitrate-N (μM-N)	Total-P (μM-P)
Cave conduit	4,700	<0.2 ^a	3,500 ± 170	<1 ^a	3.0 ± 0.0	21 ± 0.2	5.8 ± 0.3
Saltwater vent	12,400	24 ± 0.6	7,100 ± 82	19 ± 0.2	<1 ^a	<16 ^a	5.8 ± 0.1

^aMeasurements were below analytical range.

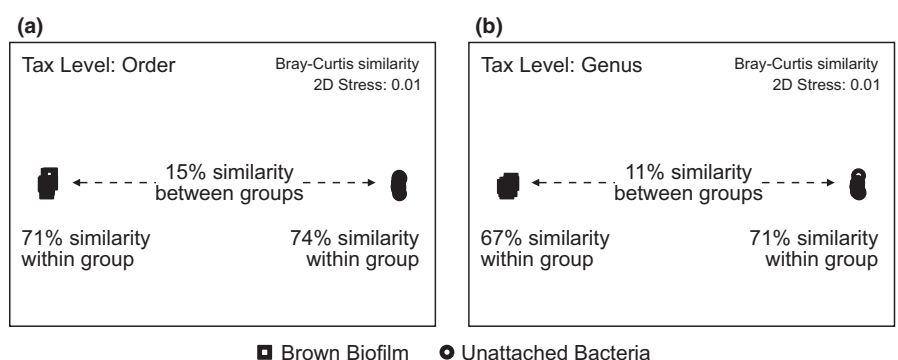


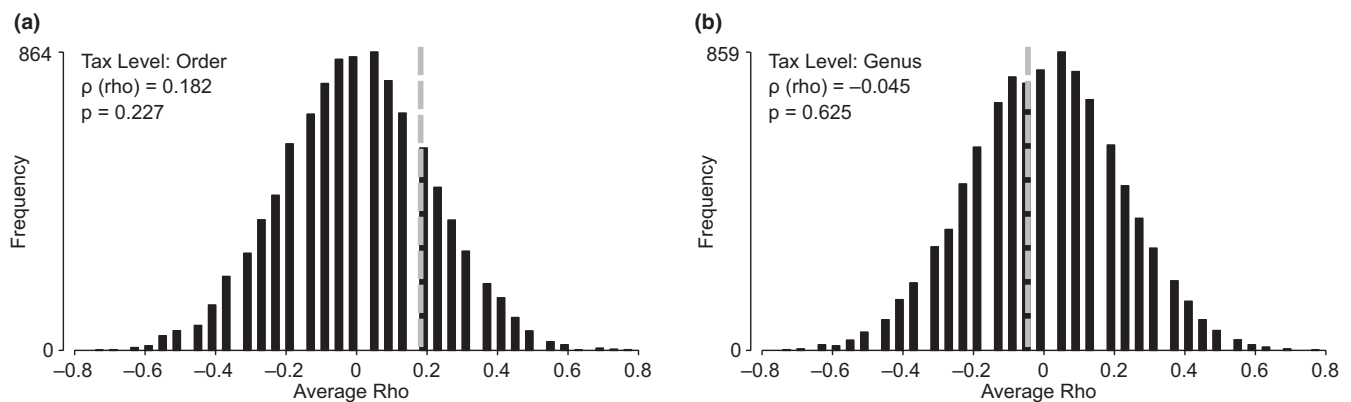
FIGURE 6 Nonmetric multidimensional scaling plots summarizing differences between the overall community structures of the unattached bacterial and biofilm communities in Sulphur Springs Cave at the order (panel a) and genus (panel b) levels

The white biofilm was represented by 333 OTUs and exhibited a Shannon diversity index value of 4.22 and an evenness value of 0.73 (Table 1). The identifiable orders with the largest relative abundances in this community were Thiobacteriales (13.3% of the community) and Campylobacteriales (12.8% of the community). White biofilm Thiobacteriales was entirely represented by the sulphur-oxidizing genus *Thiothrix*, and 98% of the white biofilm Campylobacteriales was accounted for by the sulphur-oxidizing genera *Sulfurimonas* and

Sulfurovum and by unidentified members of the family *Sulfurovaceae* (Figure 5). Another major component of the white biofilm community was a group of unidentified Gammaproteobacteria, which accounted for 16% of the community (Figure 5). The orders Enterobacteriales and Betaproteobacteriales, respectively, made up 0.00% and 7% of the white biofilm community. The saltwater vent habitat of the white biofilm exhibited a density stratified redox gradient, with high concentrations of sulphide and ammonium issuing from the vent

TABLE 3 Results of permutational tests comparing unattached and biofilm community structures

Taxonomic level	Test	Test statistic	Significance level	Number of permutations
Order	PERMDISP	$F(1, 20) = 0.259$	$p = .644$	9,999
Order	ANOSIM	$R = 1.00$	$p < .001$	9,999
Order	PERMANOVA (main)	$F(1, 20) = 82.7$	$p < .001$	9,999
Order	PERMANOVA (pairwise)	$t(20) = 9.10$	$p < .001$	9,999
Genus	PERMDISP	$F(1, 20) = 0.611$	$p = .471$	9,999
Genus	ANOSIM	$R = 1.00$	$p < .001$	9,999
Genus	PERMANOVA (main)	$F(1, 20) = 71.1$	$p < .001$	9,999
Genus	PERMANOVA (pairwise)	$t(20) = 8.43$	$p < .001$	9,999

**FIGURE 7** Histograms representing the results of two-way RELATE tests. These tests showed that the community dynamics of the unattached bacteria and brown biofilm were not correlated with each other at either the order (panel a) or genus (panel b) level. Vertical dashed bars indicate matrix correlation coefficients (Spearman's rho) estimated by the tests. Vertical solid bars represent the frequency with which a permutational analysis estimated a particular correlation coefficient after randomizing sample labels**TABLE 4** Results of two-way RELATE tests comparing patterns in unattached and biofilm community structures

Taxonomic level	Spearman correlation	Significance level	Number of permutations
Order	Average Rho = 0.182	$p = .227$	9,999
Genus	Average Rho = -0.045	$p = .625$	9,999

compared to ambient concentrations of those nutrients collected upstream (Table 2). This environment, where reducing vent water interacted with oxidizing conduit water (Table 2), was an ideal habitat for the sulphur-oxidizing taxa which best characterized the white biofilm.

The dominant members of the unattached community did not reflect those of the brown biofilm community (e.g. Figure 5). The overall structures of the brown biofilm and unattached communities were significantly different from each other at both the order and genus levels (Figure 6, Table 3). Comparisons of the Bray–Curtis resemblance matrices generated separately for the two communities showed that patterns of change in the brown biofilm community structure did not correlate to patterns of change in the unattached community at either the level of order or genus (Figure 7, Table 4). Based on these results, it is unlikely that the members of the unattached community were supplied to the Sulphur Springs Cave water column by the brown biofilm community.

3.2 | Analysis of hydrological predictors used in linear regression models

Groundwater and surface water levels measured throughout the Sulphur Springs area were used in linear regression models to explain unattached Enterobacteriales and Betaproteobacteriales relative abundances. The two wells at the ROMP 66 station (Figure 1a) were, respectively, screened in the surficial aquifer and the underlying confined aquifer (Figure 8a). Blue Sink (Figures 1, 8b) breached an underlying confining unit and thus connected the confined aquifer to the surficial aquifer and to urban run-off entering the sinkhole (Figure 8a). On the bank of Blue Sink, the 115th Street Well was screened in the confined aquifer (Figures 1, 8a). During the study period, Sulphur Springs Pool water levels dropped rapidly in response to spring water extraction activity (Figure 9).

The potential for confining unit leakage was determined through comparisons of water levels in the Sulphur Springs area.

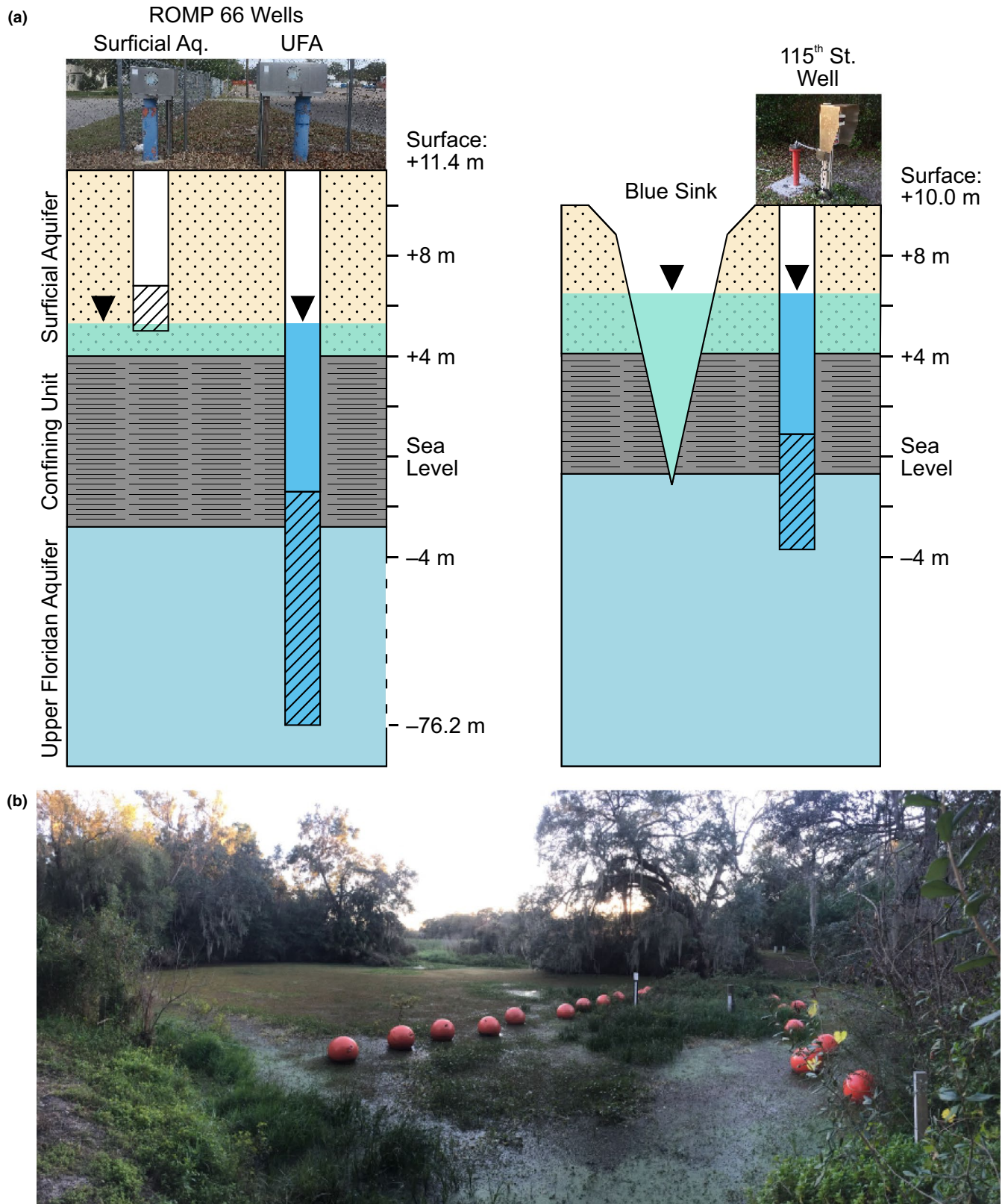
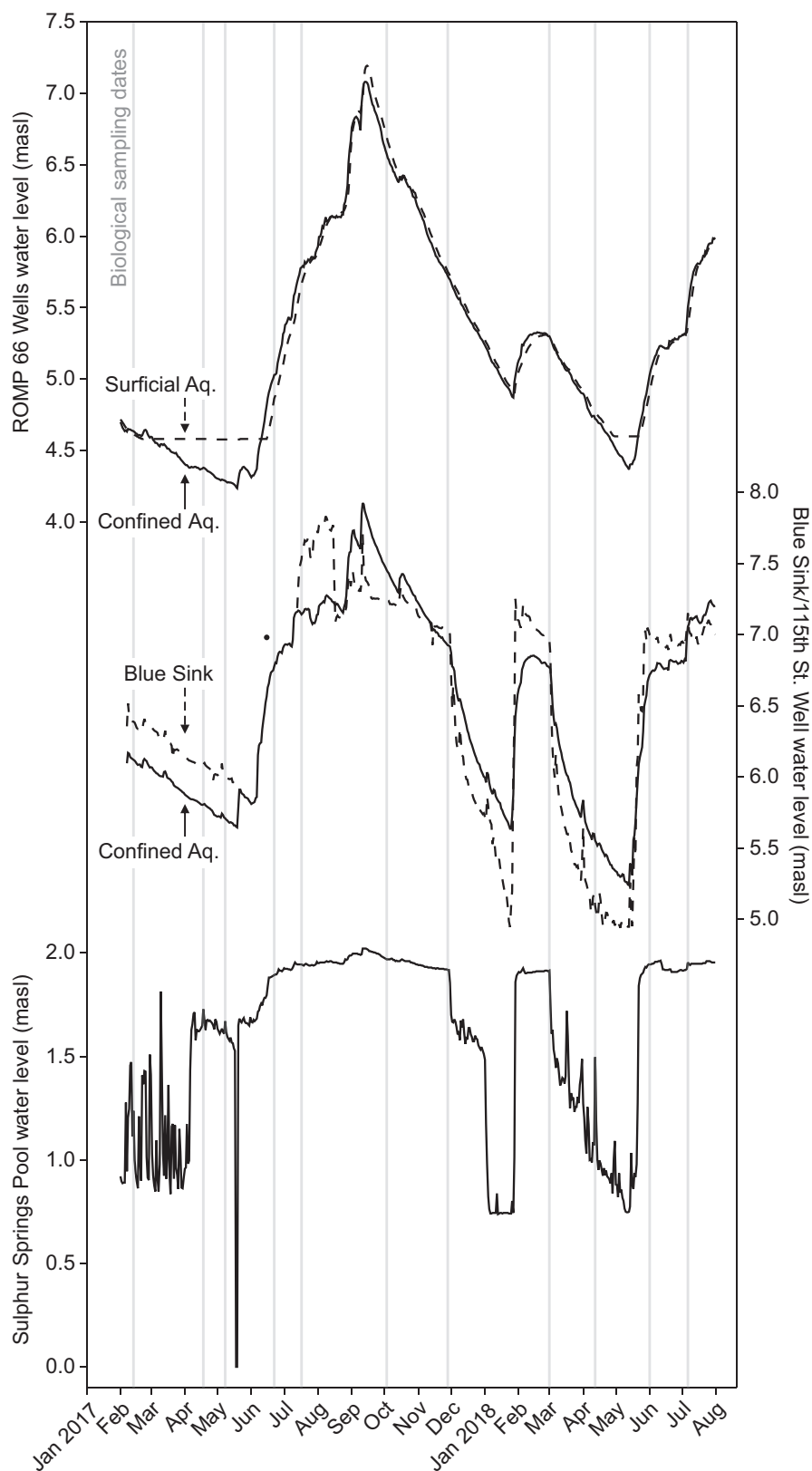


FIGURE 8 Sites used to develop hydrological predictor variables for linear regression models. Panel a: Lithologies of the ROMP 66 Wells, Blue Sink and the 115th Street Well. Diagonal hash marks in the well illustrations represent screened intervals of the wells. Nonhashed portions of the well illustrations represent nonpermeable casing material. Water levels shown (inverted triangles) are mean levels from February 2017–July 2018 for each site. Panel b: Part of Blue Sink, looking southwest. The water surface partly covered by floating aquatic vegetation. The 115th Street Well is located on the bank directly behind where the photographer was standing

FIGURE 9 Water levels measured at different sites used to develop hydrological predictor variables for linear regression models. Measurements are mean daily water elevations (NAVD 88). Biological sampling dates are shown with grey vertical bars



These measurements, along with Sulphur Springs Pool levels, were included in linear regression models to explain the relative abundances of unattached *Enterobacteriales* and *Betaproteobacteriales*. Potential leakage between the surficial and confined aquifers was measured as the difference between water levels in the two ROMP

66 wells (Figures 8, 9, 10). There were two dates in the biological time series that corresponded to days when the surficial aquifer water table was apparently below the ROMP 66 surficial aquifer well (18 April 2017 and 08 May 2017; Figure 9), so these dates were not included in the models. The potential for Blue Sink to inject

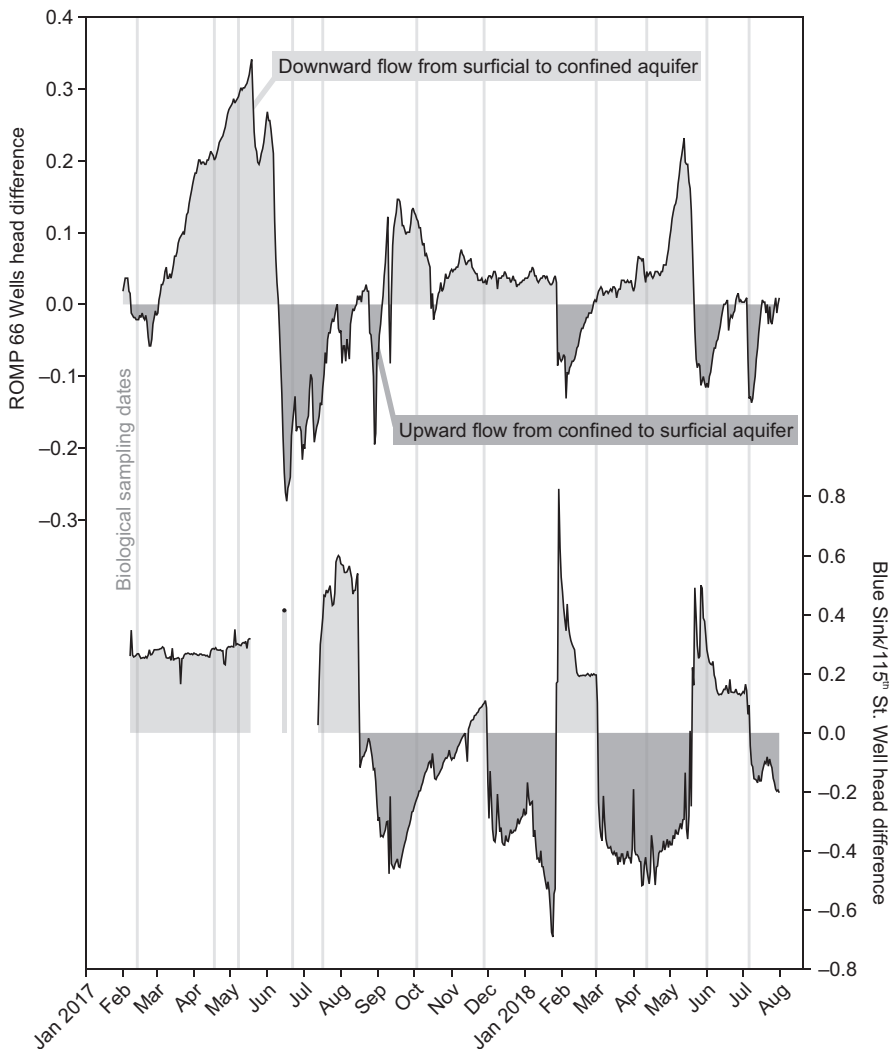


FIGURE 10 Water level differences measured at the ROMP 66 Well station (surficial aquifer levels minus confined aquifer levels) and at Blue Sink (Blue Sink levels minus 115th Street Well levels). Biological sampling dates are shown with grey vertical bars. These values were used as hydrological predictor variables for linear regression models

surficial aquifer and/or surface water into the underlying confined aquifer was measured as the difference between water levels in Blue Sink and the 115th Street Well (Figures 8, 9, 10). No measurement of Blue Sink water level was recorded on biological sampling date 22 June 2017, so the Blue Sink/115th Street Well head difference measured on the nearest date was used instead (15 June 2017; Figures 9, 10).

3.3 | Linear regression model results

The relative abundances of unattached Enterobacteriales and Betaproteobacteriales were best explained by Sulphur Springs Pool level and measurements of potential confining unit leakage (Table 5). Enterobacteriales relative abundance was inversely related to Sulphur Springs Pool level and directly related to leakage potential measured at both sites (Table 5a), producing a strong model (adjusted $R^2 = .89$, $p = .002$, $n = 9$; Table 5b). All predictors in this model had individual significant relationships with the regressor, and "ROMP 66 Wells head difference" was the strongest predictor (Table 5a). The relationship between Betaproteobacteriales

relative abundance and the hydrological predictors was weaker, but still significant (adjusted $R^2 = .63$, $p = .049$, $n = 9$; Table 5b). Betaproteobacteriales relative abundance responded directly to Sulphur Springs Pool level and inversely to leakage potential measured at both sites (Table 5a). The strongest predictor in this model was "Blue Sink/115th Street Well head difference," and this was also the only predictor that exhibited an individual significant relationship with Betaproteobacteriales relative abundance (Table 5a). Overall, the relative abundances of Enterobacteriales and Betaproteobacteriales responded in opposite ways to the same set of hydraulic conditions (Table 5a).

Unattached Campylobacteriales relative abundance was best explained by Sulphur Springs Cave-specific conductance, ammonium-N concentration and total-P concentration (adjusted $R^2 = .70$, $p = .017$, $n = 10$; Table 5b). Ammonium-N concentrations and specific conductance levels were high in the sampled Sulphur Springs Cave saltwater vent relative to the main conduit (Table 2), and these predictors varied directly with Campylobacteriales relative abundance (Table 5a). The coefficient for specific conductance was much smaller than that of ammonium-N concentration, but both had individual significant relationships with the regressor (Table 5a). Total-P

TABLE 5 Component (A) and whole-model (B) results of linear regression models developed to explain the relative abundances of unattached Enterobacteriales, Betaproteobacteriales and Campylobacteriales in Sulphur Springs Cave

A. Model components						
Regressor (proportion relative abundance)	Predictor	Coefficient sign	Coefficient	SE	t Value	p Value
Enterobacteriales	Sulphur Springs Pool elevation	-	-0.48	0.08	-6.31	.001
Enterobacteriales	Blue Sink/115th St. Well head diff.	+	0.57	0.10	5.77	.002
Enterobacteriales	ROMP 66 Wells head difference	+	0.88	0.30	2.97	.031
Betaproteobacteriales	Sulphur Springs Pool elevation	+	0.18	0.08	2.39	.062
Betaproteobacteriales	Blue Sink/115th St. Well head diff.	-	-0.29	0.10	-2.93	.033
Betaproteobacteriales	ROMP 66 Wells head difference	-	-0.20	0.30	-0.67	.535
Campylobacteriales	Phosphorus concentration	-	-0.06	0.01	-4.87	.003
Campylobacteriales	Ammonium-N concentration	+	0.04	0.01	3.69	.010
Campylobacteriales	Specific conductance	+	1.35E-04	5.04E-05	2.68	.037
B. Whole Models						
Regressor (proportion relative abundance)	Adjusted R ²	p Value	F	df	n	
Enterobacteriales	.89	0.002	22.81	3, 5	9	
Betaproteobacteriales	.63	0.049	5.48	3, 5	9	
Campylobacteriales	.70	0.017	7.91	3, 6	10	

concentrations were not significantly different between the salt-water vent and the main conduit ($t(2) = 4.2$, $p = .90$; Table 2), and this predictor was inversely related to Campylobacteriales relative abundance (Table 5a).

4 | DISCUSSION

This study of the Sulphur Springs Cave microbial communities had two goals. The first was to address knowledge gaps in the relationship between autochthonous aquifer biofilms and presumably allochthonous microbial indicator taxa collected from groundwater (Epting et al., 2018; Page et al., 2017). The second goal was to trace sources of urban contamination and groundwater in an anthropogenically impacted karst aquifer ecosystem by analysing the relative abundances of dominant unattached microbial taxa collected from the site. Though 16S rRNA gene analysis offers relatively coarse taxonomic resolution and does not provide information on whether sequenced microbes are active or dead/dormant, the results of this study were corroborated by community structure analyses conducted with several techniques at two taxonomic levels (Tables 1, 3–5; Figures 5–7).

4.1 | Validating the microbial indicator concept for use in groundwater

The hypothesis guiding the first goal of this study was that autochthonous aquifer biofilms would influence the community structure dynamics of groundwater-borne bacterial communities. If this were true, current interpretations of the presence of presumably allochthonous indicator microbes in groundwater wells and springs would be called into question. The results of the present investigation did not support this hypothesis. At the taxonomic resolutions of order and genus, the Sulphur Springs Cave brown biofilm and the adjacent unattached bacterial community were significantly distinct from each other (Figure 6, Table 3), and patterns of change in the structures of these two communities were not related (Figure 7, Table 4). These results suggest that presumed microbial indicators of anthropogenic contamination collected from groundwater are most likely truly allochthonous, with relevant autochthonous aquifer biofilms having little effect on the presence of these indicators.

The emergent properties exhibited by microbial biofilms may explain why the Sulphur Springs Cave unattached bacterial community was not simply comprised of microbes sloughed from the abundant

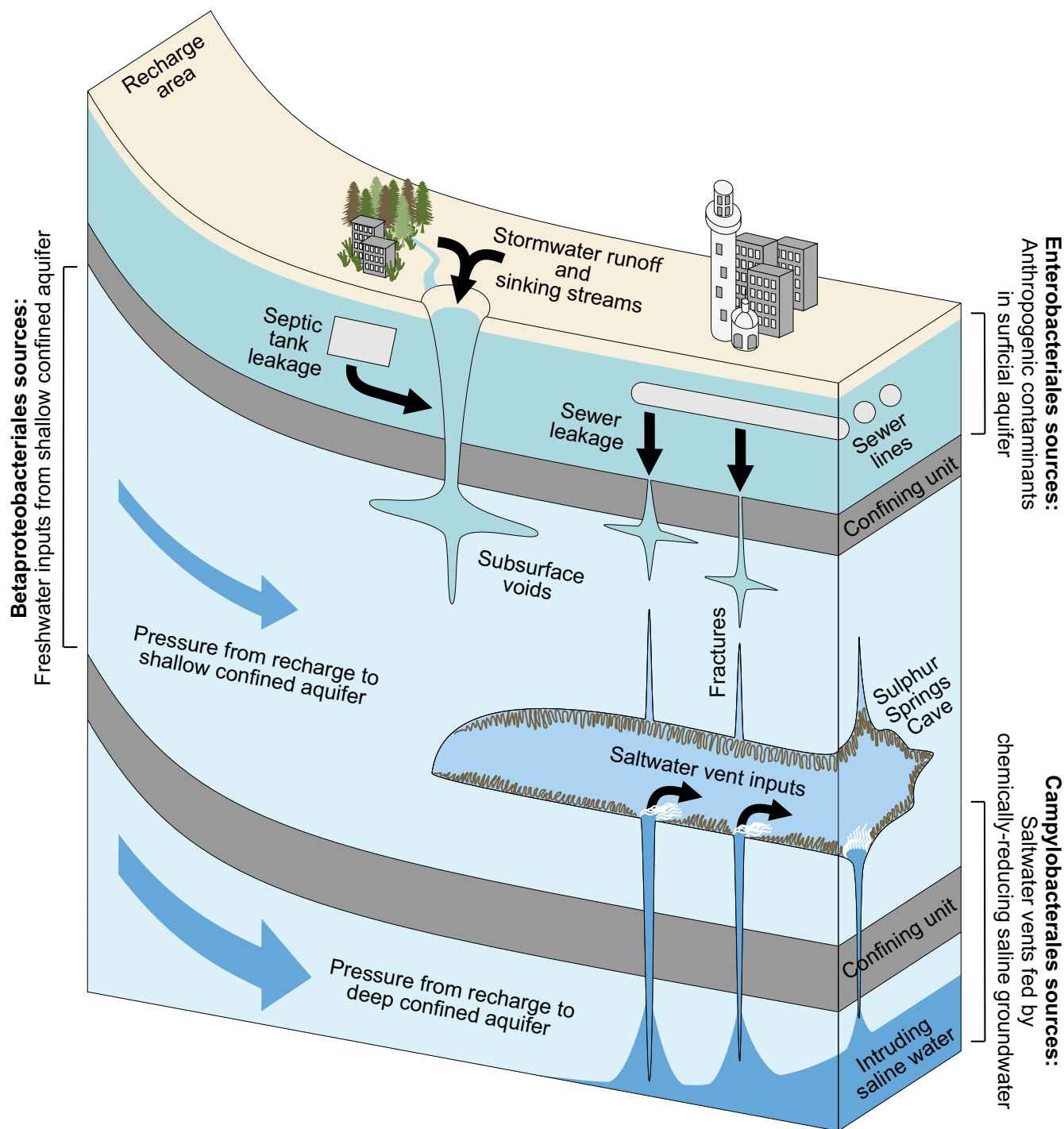


FIGURE 11 Conceptualization of the linear regression models developed in this study

brown biofilm. Biofilms represent a higher level of organization than what is possible for free-living cells (Flemming & Wuertz, 2019) and are specially adapted to endure environmental disturbances (Battin et al., 2016). For example, in aquatic environments with elevated flow velocities, biofilms respond to shear stress by forming anchored streamers that oscillate to dissipate turbulence (Taherzadeh et al., 2012). Additionally, cells living together in biofilms behave synergistically to prevent erosion (Besemer et al., 2007). Even though groundwater moves through Sulphur Springs Cave at relatively high

velocities, the biophysical attributes of biofilms probably allow the brown biofilm living in this cave to maintain a steady-state form.

4.2 | Tracing contaminant and water sources with unattached microbial taxa

The relative abundance of Enterobacteriales, an order that includes *Escherichia coli* and other coliform bacteria of the family

Enterobacteriaceae, in Sulphur Springs Cave was best explained by hydrological variables that indicated increased surficial aquifer inputs into the confined aquifer (Table 5). While several studies have blamed sinkholes as the source of coliform bacteria contamination in Sulphur Springs (MWH, 2009; Stewart & Mills, 1984; Wallace, 1993), these studies also reported much higher coliform counts at the spring than in nearby sinkholes. The model developed in the current study suggests that the main source of coliform contamination in Sulphur Springs is downwards leakage from the surficial aquifer in the general area, rather than from discrete points, such as sinkholes (i.e. "ROMP 66 Wells head difference;" Table 5a). Though the heterogeneous and anisotropic character of karst aquifers imposes limits on conclusions that can be drawn from this model, under certain hydraulic conditions, sewage leaks in the Sulphur Springs area could directly enter the confined aquifer and thus introduce Enterobacteriales into Sulphur Springs Cave (Figure 11).

Sewer lines and septic tank systems in Tampa are buried in surficial sands above the clay confining unit, and major sewer line junctions are installed well below the surficial aquifer water table overlying Sulphur Springs Cave (Stewart & Mills, 1984). The top of the confined aquifer lies only 3–4 m below these junctions. The confined aquifer in this area is highly fractured and karstified (Menke et al., 1961; Stewart & Mills, 1984), which probably generates many breaches in the overlying confining unit besides those that have manifested as sinkholes. Sewer line leakage in the United States is very common (Sharp, 2010), and any time surficial aquifer water table levels in the Sulphur Springs area are higher than confined aquifer potentiometric surface levels; leaked sewage could directly infiltrate the confined aquifer. Conditions where sewage would leak into the confined aquifer were exacerbated by artificially lowered Sulphur Springs Pool levels (Table 5a), which induce potentiometric surface level drops up to ~3 km away (MWH, 2009).

While the Enterobacteriales relative abundance model developed in this study was useful in tracing sources of urban contamination to Sulphur Springs, this model and those of Betaproteobacteriales and Campylobacteriales relative abundances were also useful in highlighting the different sources of groundwater that enters Sulphur Springs Cave (Figure 11). The relative abundances of Enterobacteriales and Betaproteobacteriales responded in opposite ways to the same hydrological predictor variables (Table 5). It is clear that Enterobacteriales relative abundance was related to inputs from the local surficial aquifer and nearby sinkholes. Members of the order Betaproteobacteriales are typically the numerically dominant taxa in freshwater environments (Newton et al., 2011), and based on the model developed here, it is likely that the relative abundance of this order in Sulphur Springs Cave corresponded to increased relative inputs from the freshwater confined aquifer that surrounds the cave (Figure 11). Furthermore, the relative abundance of Campylobacteriales in Sulphur Springs Cave was likely related to increased relative inputs from the sulphidic saltwater vents located throughout the cave (Figure 11, Table 5). This order was overwhelmingly represented by *Sulfurimonas*, a

well-characterized genus of sulphur-oxidizing chemolithoautotrophs (Inagaki et al., 2003). Campylobacteriales and other functionally similar taxa, such as *Thiothrix* (Trubitsyn et al., 2014), were also a major component of the white biofilm collected from a saltwater vent in the cave (Figure 5).

5 | CONCLUSIONS

This study addressed knowledge gaps in the concepts behind the use of microbial indicators in groundwater and applied the microbial indicator tool to inform management strategies at a karst spring of conservation concern. A time series analysis of a karst aquifer biofilm community and the adjacent unattached bacterial community suggested that autochthonous biofilms do not influence the presence of microbial taxa that are presumed to be allochthonous indicators of groundwater contamination. That said, some members of the unattached bacterial community present at this site were likely to be autochthonous, though not associated with the dominant aquifer biofilm. Linear regression models developed to explain the relative abundance patterns of dominant unattached microbial taxa at this site led to a new understanding about the sporadic presence of coliform bacteria in Sulphur Springs. These results are applicable to studies and management plans of anthropogenically impacted groundwater-dependent ecosystems worldwide, especially in karst landscapes.

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AUTHOR CONTRIBUTIONS

R.J.S. and J.R.G. designed this study and conducted the fieldwork. R.J.S. performed the laboratory work, analysed the data and wrote the manuscript. J.R.G. was doctoral advisor to R.J.S. and edited the manuscript.

DATA ACCESSIBILITY

DNA sequences: GenBank/DBJ/EMBL accessions KEEI000000000 (unattached bacteria time series), KEEK000000000/KEEH000000000 (brown biofilm time series) and KEEG000000000 (white biofilm). Water level data: Sulphur Springs Pool (waterdata.usgs.gov; station number 02306000), ROMP 66 surficial aquifer well (watermatters.org; site ID 19238), ROMP 66 confined aquifer well (watermatters.org; site ID 18670), 115th Street confined aquifer well (watermatters.org; site ID 670753) and Blue Sink (watermatters.org; site ID 670721).

ORCID

Robert J. Scharping  <https://orcid.org/0000-0003-0366-7794>

James R. Garey  <https://orcid.org/0000-0001-8205-5563>

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