Journal of the Arkansas Academy of Science

Volume 72 Article 9

2018

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Cover Page Footnote
We wish to thank Hot Spring National Park staff for their assistance in collecting the samples and accessing decommissioned bathhouse facilities. The Hendrix College Odyssey Program provided generously provided funds for this research.

Microbial Diversity in the Thermal Springs within Hot Springs National Park

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The authors of this manuscript declare no conflicts of interest.
Running Title: Microbial Diversity in Arkansas Hot Springs

Abstract

The thermal water systems of Hot Springs National Park (HSNP) in Hot Springs, Arkansas exist in relative isolation from other North American thermal systems. The HSNP waters could therefore serve as a unique center of thermophilic microbial biodiversity. However, these springs remain largely unexplored using culture-independent next generation sequencing techniques to classify species of thermophilic organisms. Additionally, HSNP has been the focus of anthropogenic development, capping and diverting the springs for use in recreational bathhouse facilities. Human modification of these springs may have impacted the structure of these bacterial communities compared to springs left in a relative natural state. The goal of this study was to compare the community structure in two capped springs and two uncapped springs in HSNP, as well as broadly survey the microbial diversity of the springs. We used Illumina 16S rRNA sequencing of water samples from each spring, the QIIME workflow for sequence analysis, and generated measures of genera and phyla richness, diversity, and evenness. In total, over 700 genera were detected and most individual samples had more than 100 genera. There were also several uncharacterized sequences that could not be placed in known taxa, indicating the sampled springs contain undescribed bacteria. There was great variation both between sites and within samples, so no significant differences were detected in community structure between sites. Our results suggest that these springs, regardless of their human modification, contain a considerable amount of biodiversity, some of it potentially unique to the study site.

Key Words: Hot Springs National Park, Microbial Diversity, Next Generation Sequencing, Thermophiles

Introduction

Constraints governing the biochemical processes at work in thermophilic bacteria have yielded discoveries with far-reaching implications, ranging from biogeochemistry to biotechnology (Leis *et al*. 2015; Shrestha *et al*. 2018; Undsworth and Koutsopoulos 2007). Evolving in relative isolation from other extremophiles provides opportunities for unique communities to develop, resulting in community structures that vary tremendously, even among similar study sites (Amin *et al*. 2017). Investigation of individual microbes in extreme environments has yielded the discovery of thermostable enzymes critical to biotechnological advances such as PCR (Brock 1969), treatment of industrial waste (Shrestha *et al*. 2018), and industrial chemistry (Leis *et al*.2015).

The in-depth characterization these communities warrant cannot be accomplished with culture-dependent methods alone, as it has been hypothesized that less than 1% of environmental bacteria are likely to be cultured in laboratory settings (Staley and Konopka 1985). Given the stringent growth requirements for extremophiles, and the observation that some thermophiles appear to be co-culture dependent (Stewart 2012), the proportion of culturable thermophiles is likely smaller. The limitations of culture-dependent characterization render these methods insufficient for improving understanding of these microbial communities. However, with the increasing accessibility of modern high-throughput sequencing techniques, researchers have improved understanding of high-temperature microbial community structure, allowing the development of metagenomic libraries specific to thermophiles (Mirete *et al*. 2016). In spite of these advances, many isolated regions of thermophilic activity remain unexplored, limiting knowledge of prospective biodiversity.

Hot Springs National Park is an isolated thermophilic environment located in the Ouachita Mountains of Arkansas, and is the only hot water thermal spring complex with average water temperatures above 50°C in the central U.S. (NOAA NCEI 2018). Given its geographic isolation, opportunities for biological exchange with other North American thermophile communities are likely very

limited. While the hot water springs have been used for human recreation for thousands of years, intense development of the area began in the 1830s (Hanor 1980). Modern development has included heavy landscaping and the capping and diversion of the springs to recreational facilities, such as bath houses. Today, only ten spring complexes are uncapped and open to the environment (Yeatts 2006). In the open springs, a presumably more complex ecological community exists with thermophilic algae (Smith 2010) and crustaceans (Meg O'Connor, personal correspondence). By contrast, the capped springs, which lack a light source, are likely to contain a simpler community consisting mostly of chemotrophs (probably chemolithotrophs). Our goal in this study was to characterize the microbial communities in both capped and uncapped hot springs within Hot Springs National Park. We hypothesized that the microbial diversity would be lower and the community structure distinctive in capped springs compared to the uncapped springs. While some novel thermophiles have been identified in this system via traditional laboratory culture and 16S rRNA sequencing at specific sites within the park (Marks *et al*. 2012), the community structures of these different springs have not been broadly compared with culture-independent methods. Therefore, our study may provide insight into the biodiversity in this isolated system, detect human impacts on the communities, and assess the conservation value of the capped and uncapped springs.

Materials and Methods

Sampling Sites

All sampling sites were located within Hot Springs National Park (HSNP) (34°30'53'' N, 93°03'12'' W). While the park contains 43 thermal springs located in a 5.6 Ha section of the park (Yeatts, 2006), all sampling sites were located on Bath House Row, where over the last 150 years, 33 of the thermal springs have been highly modified by human activity. In this system, rain water seeps slowly into cracks in Hot Springs rock formations, where it is heated before being forced back to the surface. Temperatures of the springs average 61.4°C. The rock in the area is largely shale, chert, novaculite, and sandstone, of which only shale impedes the ground water movement in the area (Yeatts 2006).

Sites within HSNP were sampled between 10:30-12:00am CST on 26 Sept 2017. Of the 43 individual springs where heated water rises to the surface, four individual springs were selected for sampling. Of these four springs, two sites were capped springs, where bathhouse buildings were constructed on top of the individual flows and previously used for recreation and tourism. The two capped sites, the Fordyce Bath House Spring (F) and the Hale Bath House Spring (H), have been decommissioned for public use. The remaining sites, the Lamar Display Spring (L) and the Tunnel Display Spring (T), are open-air springs on display to the public.

Sample Collection and Preparation

Duplicate water samples from each site were collected aseptically at the surface level using sterile 50 mL conical tubes and stored immediately on ice in transit to the laboratory for further processing. Samples were processed within four hours of collection for DNA extraction. All water samples were filtered via a Millipore® filtration apparatus containing a filter with a pore size of 0.45 μm. DNA extraction was performed using the Zymo Research® ZR Fecal DNA Miniprep Kit (Catalog No: D6010) according to manufacturer specifications. Purified product was quantified by spectrophotometry and stored at -80°C until preparation for Illumina Sequencing.

Sequencing and Analysis

PCR-amplification targeted variable regions V3 & V4 of the 16S ribosomal subunit and amplified from 515bp to 806 bp within the gene using the Illumina 515FB/806RB primer pair (Caporaso *et al*. 2011; Parada *et al*. 2016) for a total of 291bp. This PCR-amplification, as well as barcoding and high-throughput sequencing on an Illumina platform, was performed by Wright Labs (Huntingdon, PA 16652) using primers utilized in previous studies. All sequence data was analyzed with access to computing cluster located at Juniata College using Quantitative Insights Into Microbial Ecology (QIIME version 1.9.1). Before using this program, sequences were trimmed to 253bp and discarded if sequence overlap was less than 200 bp. The USEARCH version 7 algorithm filtered sequences with an expected error rate of <1%, and with a minimum of 5,000 reads required for retention. This resulted in a total of 646, 651 quality reads which were then analyzed using QIIME program (v1.9.1). Operational taxonomic units (OTU's) were selected using the open reference OTU UCLUST algorithm (Edgar 2010), and were defined by 97% sequence similarity. Taxonomy was assigned using the RDP Classifier and Greengenes 16S rRNA gene database (DeSantis *et al*. 2006, 13-8 release).

For alpha diversity measurements, we counted the number of genera present in each sample within each site. To determine the general similarity between sites,

we calculated genera richness (S), the Shannon diversity index (H'), and evenness (J'). To examine general community structure, we performed a nonmetric multidimensional scaling (NMDS, Kruskal 1964) analysis and plotted the results on a two-dimensional graph. We utilized the Bray-Curtis method to measure beta diversity, or dissimilarity between samples.

Results

We detected 646,651 quality-reads for the eight samples after removal of low-quality and chimeric sequences (Table 1). These data resulted in a total of 46 bacterial phyla and 3 archaeal phyla across all sampling sites (Table 2). Only one phylum of Archaea (Crenarchaeota) and 12 phyla of Bacteria were found within all of the sites sampled. Although there were some phyla found only in a single sampling site, there was no indication of groups exclusively present in one type of sample (i.e. capped or uncapped). The 12 phyla of bacteria found in all sites, in order of abundance were Proteobacteria, Actinobacteria, Acidobacteria, Firmicutes, Cyanobacteria, Bacteriodetes, Planktomycetes, Chloroflexi, Verrucomicrobia, Nitrospirae, Chlorobi, and Elusimicrobia. The proportion of unassigned taxa in our samples ranged from 0.5 – 19.9% of sequences, indicating a considerable amount of new unknown biodiversity. We detected a total of 706 genera with OTU's >97% similarity to previously described microbes (see supplemental data, Moran 2018).

Table 1. Number of reads returned from next generation sequencing of 16S rRNA variable regions V3 & V4 after quality filtering. L = Lamar Display Spring, F = Fordyce Bathhouse, T = Tunnel Spring, and H = Hale Bathhouse.

NGS Reads within HSNP	
Sample	Number of reads
H1	136748
H2	22666
F1	206444
F2	18165
L1	49789
L2	86089
T1	37401
T2	89349
Total	646651

There was high variation in genus richness between sites and between samples within sites (Table 3). With the exception of one sample (F2), all samples contained over 100 genera, while some had over 400 genera represented. H' and J' values were relatively high in most samples, indicating a general lack of dominance by one particular group. However, the various genera within the Proteobacteria tended to make up over 50% of sequence abundance. The NMDS results at the genus level showed clustering of sites L, H, and T, while both F samples were dramatically different in community structure. (Stress = 0.12, Fig. 1).

When examined at the site level, each location had over 250 genera and each had unique genera not found in other springs (Table 4). However, the proportion of unique genera varied greatly from 4% (Fordyce) to 34% (Hale).

Discussion

Based on our assessment of the four thermal springs in HSNP, the area contains considerable biodiversity. Our sequence data indicates that no single genus of bacteria or archaea are dominant within the springs, but instead a variety of genera thrive in these habitats, ranging from 90 to over 400 across sample sites. The phylum Proteobacteria, however, did typically comprise ~50% of sequences within a given sample. These biodiversity results generally agree with other studies that have examined thermal communities using the 16S rRNA sequence method (Amin *et al.* 2017; Chan *et al.* 2015; De León *et al.* 2013; Vick *et al.* 2010).

Our results contrast with a previous study of the Hale Bathhouse in HSNP, which found high abundance of the phylum Nitrospira (Bacteria) and Thaumarchaeota (Archaea, Marks *et al.* 2012). In our samples, however, Nitrospira was relatively rare (less than 2%) and Thaumarchaeota was absent. However, this study examined biofilms on submerged glass slides, while our study sampled the water column. This difference shows that thermophilic microbial communities may vary greatly within sites, depending on the location and method of collection. It should be noted that the number of reads and community structures in our study vary dramatically, as much as ten-fold, across replicates of the same site, indicating possible sampling error (Table 1). For example, in the low flow spring of Fordyce Bathhouse, one sample (F2) visibly contained more sediment, which may have changed the biodiversity profile for that sample (Dalu *et al.* 2017; Smolders *et al.* 2003) and may explain the large difference in community structure seen in the

Table 2. Phyla diversity and proportional representation based on 16S rRNA sequencing data across four sampled sites in Hot Springs National Park. L = Lamar Display Spring, F = Fordyce Bathhouse, T = Tunnel Spring, and H = Hale Bathhouse.

Phylum	**L1**	**L2**	**T1**	**T2**	**F1**	**F2**	**H1**	**H2**
Unassigned;Other	0.0249	0.0192	0.0053	0.0080	0.1987	0.0147	0.0297	0.0494
Kingdom Archaea								
Crenarchaeota	0.0010	0.0011	0.0121	0.0074	0.3460	0.0087	0.0042	0.0066
Euryarchaeota	< 0.0001	0.0001	0.0022	0.0031	0.0050	0.0003	0.0002	NA
Parvarchaeota	NA	< 0.0001	NA	< 0.0001	NA	NA	0.0004	NA
Kingdom Bacteria								
Other	NA	NA	NA	NA	< 0.0001	NA	NA	NA
k__Bacteria;p__	NA	NA	0.0010	0.0011	0.0002	NA	0.0019	0.0026
AD3	NA	< 0.0001	NA	0.0106	NA	NA	0.0515	0.0014
Acidobacteria	0.0613	0.0399	0.0223	0.2349	0.0543	0.0216	0.1436	0.0498
tinobacteria	0.0147	0.0301	0.1652	0.1624	0.0442	0.0236	0.1053	0.1088
Aquificae	NA	NA	0.0003	NA	NA	NA	NA	NA
Armatimonadetes	0.0019	0.0022	0.0006	0.0071	0.0026	NA	0.0152	0.0114
BHI80-139	NA	NA	NA	< 0.0001	NA	NA	0.0002	NA
BRC1	0.0007	< 0.0001	NA	0.0002	NA	NA	0.0017	NA
Bacteroidetes	0.0115	0.0306	0.0479	0.0160	0.0036	0.1497	0.0168	0.0321
Chlamydiae	0.0035	0.0015	0.0005	< 0.0001	< 0.0001	NA	0.0007	< 0.0001
Chlorobi	0.0031	0.0058	0.0003	0.0007	0.0110	0.0012	0.0024	0.0101
Chloroflexi	0.0058	0.0417	0.0014	0.0865	0.0445	0.0012	0.0695	0.0334
Cyanobacteria	0.0688	0.1764	0.0188	0.0058	0.0012	0.0171	0.0105	0.0230
Deferribacteres	NA	NA	NA	NA	NA	NA	0.0001	0.0003
Elusimicrobia	0.0002	< 0.0001	0.0041	0.0023	0.0017	0.0052	0.0007	0.0008
FBP	NA	0.0002	NA	NA	NA	NA	NA	NA
FCPU426	< 0.0001	< 0.0001	NA	NA	NA	NA	< 0.0001	0.0037
Fibrobacteres	< 0.0001	0.0002	NA	0.0002	NA	NA	< 0.0001	NA
Firmicutes	0.0160	0.0101	0.0309	0.0113	0.0059	0.2833	0.0296	0.0397
GAL15	< 0.0001	< 0.0001	NA	0.0001	0.0211	0.0701	0.0003	NA
GN02	NA	0.0001	NA	< 0.0001	NA	NA	NA	NA
Gemmatimonadetes	0.0044	0.0028	0.0001	0.0090	< 0.0001	NA	0.0038	0.0004
MVP-21	NA	NA	NA	0.0001	NA	NA	0.0002	NA
NC10	NA	NA	NA	NA	0.0152	NA	0.0002	< 0.0001
NKB19	0.0001	NA	NA	< 0.0001	NA	NA	< 0.0001	NA
Nitrospirae	0.0009	0.0004	0.0012	0.0258	0.1083	0.0197	0.0052	0.0153
OD1	< 0.0001	0.0001	NA	NA	NA	NA	< 0.0001	NA
OP1	0.0001	< 0.0001	0.0004	NA	0.0413	0.0076	0.0014	NA
OP11	NA	NA	NA	NA	NA	NA	< 0.0001	NA

Table 2. *continued*.

OP3	0.0011	< 0.0001	NA	< 0.0001	0.0005	NA	0.0005	0.0006
OP8	NA	NA	NA	NA	NA	NA	< 0.0001	NA
Planctomycetes	0.0283	0.0307	0.0328	0.0653	0.0047	< 0.0001	0.1123	0.0285
Proteobacteria	0.7331	0.5875	0.6471	0.2858	0.0815	0.3686	0.2287	0.5041
SBR1093	< 0.0001	0.0002	NA	NA	0.0073	NA	0.0002	NA
Spirochaetes	0.0013	0.0059	NA	NA	NA	NA	0.0052	0.0118
TM6	0.0041	0.0020	NA	< 0.0001	NA	NA	< 0.0001	0.0003
TM7	NA	NA	NA	0.0001	NA	NA	0.0011	NA
Tenericutes	NA	NA	NA	< 0.0001	NA	0.0004	< 0.0001	NA
Thermotogae	NA	NA	NA	NA	NA	NA	< 0.0001	0.0004
Verrucomicrobia	0.0095	0.0081	0.0029	0.0523	0.0001	0.0069	0.1247	0.0146
WPS-2	0.0022	0.0006	0.0018	0.0006	0.0005	NA	0.0167	0.0022
WS2	NA	NA	NA	0.0006	NA	NA	0.0002	NA
WS3	0.0001	NA	NA	0.0022	NA	NA	0.0003	NA
WWE1	NA	NA	NA	NA	0.0003	NA	NA	NA
Thermi	0.0013	0.0022	0.0007	< 0.0001	< 0.0001	NA	0.0147	0.0488

NMDS results. Testing both the water column and the sediment in each spring may help elucidate the impact of substrate and microhabitats on microbial biodiversity within the springs. With the exception of Fordyce Bathhouse spring however, community structure is relatively similar between sites.

The community structure of these thermophilic environments is complex, perhaps reflecting the diversity of energy sources present in these waters, which includes iron, sulfur compounds, ammonia, and methane (Marks *et al*. 2012). Some of our identified phyla contain species that are known to use chemoautotrophic metabolism, including Crenarchaeota (sulfur, Woese 1984), Nitrospira (ammonia, Marks *et al.* 2012), Crenarchaeota (iron, Kozubal *et al.* 2008), and a variety of methane metabolizing Archaea (Evans *et al.* 2015; Ozuolmez *et al.* 2015; Yang *et al.* 2017). We find it interesting that even the capped springs had high biodiversity, showing that complex bacterial communities are surviving without external energy sources (i.e., light). Therefore, it appears that a large amount of the biodiversity has been maintained in these sites, even with the high degree of human modification.

Table 3. Genera richness (S), Shannon diversity index (H'), and evenness index (J') for each thermophilic sample. L = Lamar Display Spring, F = Fordyce Bathhouse, T = Tunnel Spring, and H = Hale Bathhouse.

Sample	S	H'	J'
L1	345	3.20	0.55
L2	420	3.94	0.65
F1	240	2.59	0.47
F2	90	3.61	0.80
T1	134	2.98	0.61
T2	420	4.18	0.69
H1	466	4.46	0.73
H2	224	4.05	0.75

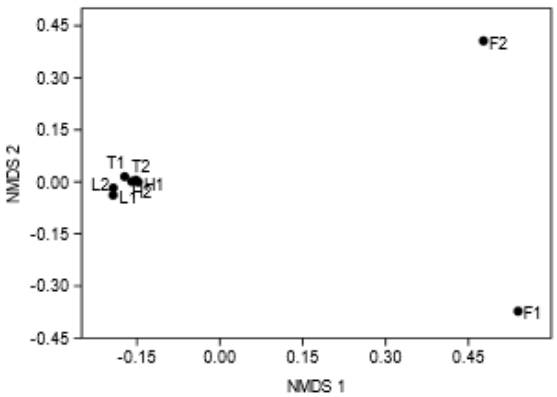

Figure 1. Results of the NMDS analysis showing the similarity between samples. L = Lamar Display Spring, F = Fordyce Bathhouse, T = Tunnel Spring, and H = Hale Bathhouse.

Table 4. Number of unique genera, total genera, and proportional unique at each site. L = Lamar Display Spring, F = Fordyce Bathhouse, T = Tunnel Spring, and H = Hale Bathhouse

	L	H	F	T
Unique Genera	89	171	18	42
Total Genera	468	504	452	272
Proportion Unique	0.19	0.34	0.04	0.15

This study, although limited to a small subset of springs in HSNP, found a high diversity of thermophilic microbes. It is likely that further sampling of the spring system would yield additional genera. We recommend a thorough sampling of microhabitats within each spring (e.g., substrate, water column, and different distances from spring source) to fully document the microbe biodiversity. These springs have been recognized for decades for their unique geological and cultural value, while their biological value has been less well-understood. Our analysis shows that the microbial biodiversity remains rich in HSNP despite continual anthropogenic modification, and should remain a focus of ongoing conservation efforts, as this diversity could be of considerable scientific value.

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