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Article

Naegleria fowleri Detected in Grand Teton National Park Hot Springs

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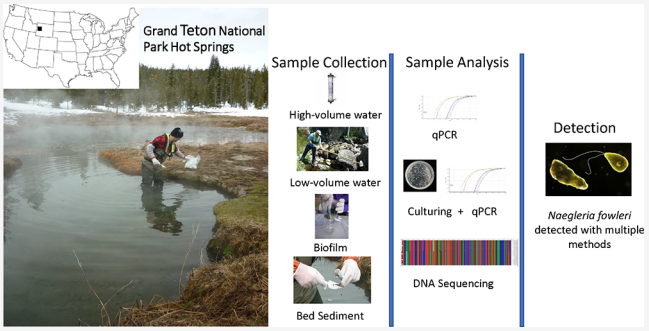
ABSTRACT: The free-living thermophilic amoeba *Naegleria fowleri* (*N. fowleri*) causes the highly fatal disease primary amoebic meningoencephalitis. The environmental conditions that are favorable to the growth and proliferation of *N. fowleri* are not well-defined, especially in northern regions of the United States. In this study, we used culture-based methods and multiple molecular approaches to detect and analyze*N. fowleri* and other *Naegleria* spp. in water, sediment, and biofilm samples from five hot spring sites in Grand Teton National Park, Wyoming, U.S.A. These results provide the first detections of *N. fowleri* in Grand Teton National Park and provide new insights into the distribution of pathogenic *N. fowleri* and other nonpathogenic *Naegleria* spp. in natural thermal water systems in northern latitudes.



KEYWORDS: *Naegleria fowleri, Grand Teton National Park, hot springs, water, amoebae, amoeba*

INTRODUCTION

Naegleria fowleri (*N. fowleri*) is a thermophilic free-living amoeba and the causative agent of primary amoebic meningoencephalitis (PAM), a rare brain infection that usually results in death within 3−7 days after the onset of symptoms.[1] PAM is primarily contracted through recreational activity with untreated water where *N. fowleri* enters the nose then migrates along the olfactory nerves and through the cribriform plate where it invades the central nervous system.[1,2] Between 1937 and 2018, there were 381 confirmed PAM cases worldwide and only 7 confirmed survivors,[3] although it is possible that some cases may go undiagnosed.[4]

N. fowleri has been detected in naturally or artificially heated spas, hot springs, water distribution systems, freshwater lakes and ponds, rivers, sediments, and effluents from power plants.[5−9] The laboratory-determined upper thermal limit for growth is 46 °C,[10] and the amoeba can persist for short durations (24−96 h) at 48 °C.[11] The amoeba also can persist at lower temperatures with viable cells isolated from water distribution biofilm samples collected at 10 °C.[12] Laboratory studies suggest that the optimum growth temperature for *N. fowleri* varies with other environmental factors (e.g., nutrient availability).[13]

In the United States, most PAM cases have historically occurred in warm, southern states such as Texas, Arizona, Florida, South Carolina, and California.[14] More recently, cases have also occurred in Iowa, Nebraska, Indiana, Maryland, and most notably, Minnesota.[14] Surface water collected from a Minnesota lake where the exposure likely occurred was positive for *N. fowleri* based on real-time PCR results, and the lake had a temperature range of 22.1−24.5 °C at the time of sampling several weeks following case exposure.[15] The August 2010 average air temperature near the exposure site was 25 °C, and this August was the third warmest in the Minneapolis area from 1891 to present (2023).[15,16] In the context of changing climate trends and increasing surface water temperatures, the Minnesota case underscores the northern geographical expansion of PAM cases and the need to better understand conditions in northern latitudes under which *N. fowleri* persists and can threaten human health.[17]

There are approximately 100 protected and managed hot springs within the combined boundaries of Grand Teton National Park (GRTE) and the John D. Rockefeller Jr. Memorial Parkway (JODR; also administered by GRTE). Previous work by O'Dell et al.[18] and Ramaley et al.[19] isolated *Naegleria* spp. from Huckleberry and Polecat Creek Hot Springs in GRTE (Figure 1), which have outflow temperatures of 40−60 °C. In these hot springs, *Naegleria* spp. isolates accounted for almost half of the amoebae observed.[19] *Naegleria australiensis*

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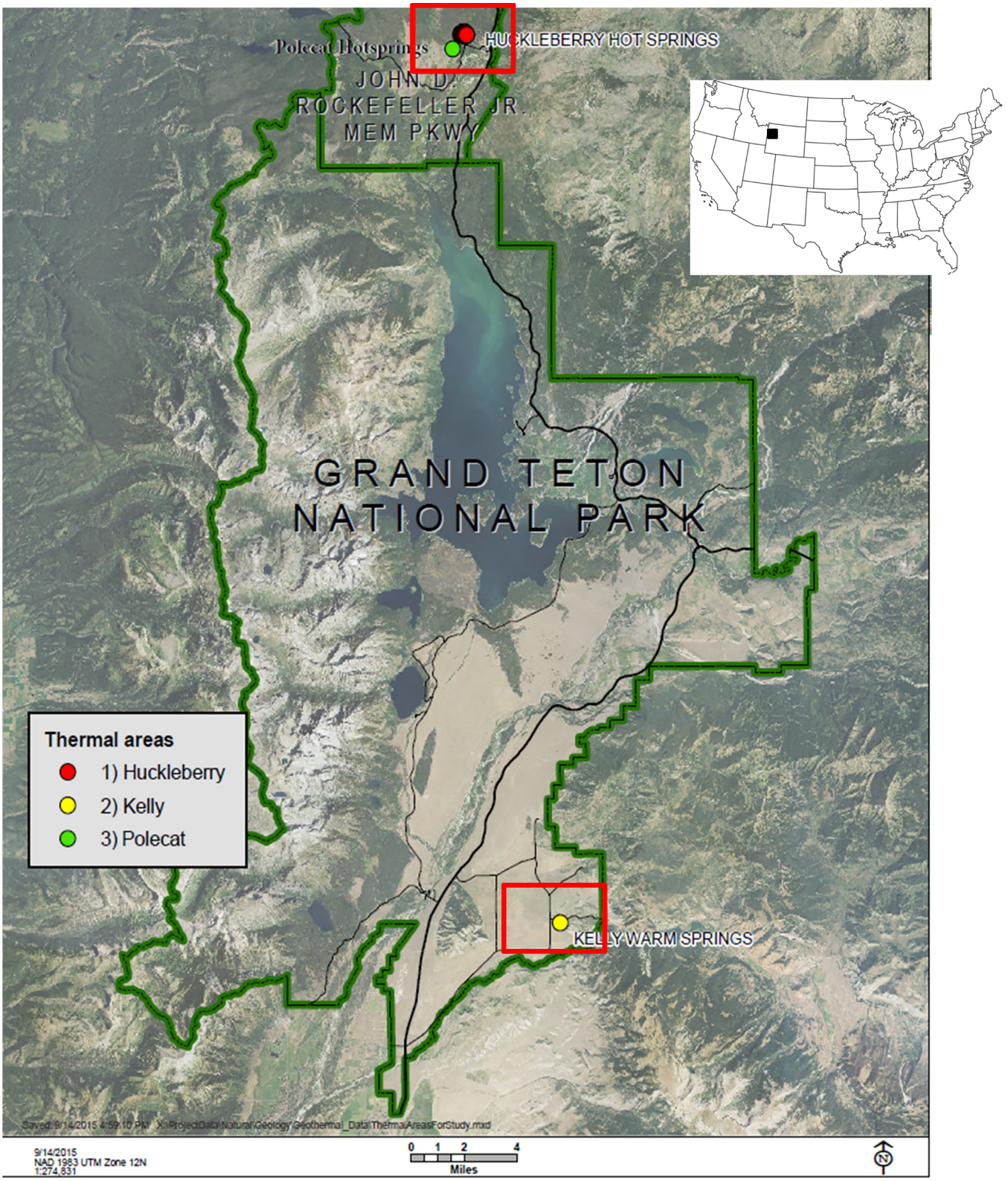

Figure 1. Map of the study area located in northern Wyoming, United States.

(*N. australiensis*), which has been found to be virulent in mice when inoculated intranasally but has never been identified in human infections, was isolated, but *N. fowleri* was not detected.[19,20] Sheehan et al.[5] used PCR amplification, cloning, and sequencing to detect the internal transcribed spacer (ITS) region of *Naegleria* spp. from 23 geothermal sites in Yellowstone National Park (YNP) and GRTE. They detected 3 genotypes representing previously undescribed *Naegleria* spp. and a fourth genotype identified as *N. fowleri* in YNP samples but not in samples from GRTE. Since these studies, no additional research has occurred to assess the presence or absence of *N. fowleri* in GRTE hot springs. Here, we used amoeba culturing and

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multiple molecular methods to provide the first positive detections of *N. fowleri* from water, biofilm, and sediment samples collected from GRTE hot springs during different seasons over the course of a year.[21]

METHODS

Sampling Sites. Surface water, sediment, and biofilm samples were collected from 5 sites in GRTE during three seasons: July 2016 (midsummer), November 2016 (late autumn), and March 2017 (early spring). Water, biofilm, and sediment samples were also collected in March 2016 (early spring) for DNA amplicon sequencing only. Five popular sites for recreational soaking were selected within three thermal areas of GRTE and JODR: Huckleberry Hot Springs (sites H1, H2, and H3), Kelly Warm Springs (K1), and Polecat Hot Springs (P1) (Figure 1 and Figure S1). Site H1, the largest Huckleberry Hot Spring pool, is a geothermal water source with little-to-no surface water influence. Site H2, a spring pool at the confluence of the H1 outlet and Huckleberry Creek, is a mixture of geothermal and surface waters. This site showed evidence of visitor damming to increase the swimming area at the time of sampling. H3, in Huckleberry Creek 234 m downstream of H2, is also a mixture of geothermal and surface waters. P1, a pool along Polecat Creek, of geothermal origin has manmade dams, again indicating frequent recreational use. Site K1 in Kelly Warm Spring has been a popular swimming area for several decades, visited by local daycare centers, summer camps, and other children's groups. Soaking in H1 and P1 is prohibited in GRTE due to hazards associated with geothermal water being the sole source of recharge, but visitor use was observed during most sampling events.

Sample Collection and Processing. Immediately prior to sampling, *in situ* measurements of water temperature, specific conductance, pH, and dissolved oxygen concentration were recorded using a YSI EXO 2 water quality monitor (Yellow Springs, Ohio). Surface water samples were collected from the water column for *Naegleria* spp. genomic testing without culture from the streambank at a single point in the water column using a Nasco swing sampler (Whirl-Pak, Madison, WI) with a sterile 1 L polypropylene bottle from near the midpoint of the hot spring. At site K1, entering the pool could not be avoided because of its large size. Here, a 3 L composite sample was made from 3 locations (1 L each) to better represent the pool's extent. At all sites, a replicate sample was collected with a Nasco swing sampler (Whirl-Pak, Madison, WI) with a sterile 1 L bottle for water quality as described in the USGS National Field Manual[22] and was analyzed by the U.S. Geological Survey (USGS) National Water Quality Laboratory (NWQL). Total nitrogen and total phosphorus concentrations were determined using colorimetry[23] and alkaline persulfate digestion.[24] Dissolved organic carbon (DOC) was analyzed using high-temperature combustion, standard method 5310b.[25] Total particulate carbon (TPC) was analyzed using standard method 440.0.[26] *Escherichia coli* (*E. coli*) were cultured, identified, and enumerated using the modified mTEC method.[27]

Large-volume water samples were collected with dead-end ultrafiltration for cultivation studies and real-time PCR. Using a peristaltic pump, 40 L of water was passed through a hollow-fiber ultrafilter capturing microbes in the cartridge.[28,29] These samples were shipped overnight at ambient temperature in an insulated cooler to the Centers for Disease Control and Prevention (CDC) for *N. fowleri* testing by culture and real-time PCR. Ultrafilters were backflushed using WB saline with 0.1% Tween 80.[28,30]

Small-volume grab water samples collected for real-time PCR and DNA sequencing without culture were filtered in the field using a hand pump connected to a bottle top 0.22 μm poly(ether sulfone) membrane filter (Autofil, Foxx Life Sciences, Londonderry, NH) until the filter clogged. Volumes collected for each sample site and time are published.[21] Sediment from the bottom of the hot spring and floating biofilm samples sampled for *N. fowleri* real-time PCR and DNA sequencing were collected from each hot spring using a sterile scoop to fill a 50 mL Falcon tube with approximately 20 mL of the sample and stored on dry ice until they were stored at −80 °C. Samples for real-time PCR and DNA sequencing (small-volume filters, sediment, and biofilm) were stored on dry ice in the field and then at −80 °C in the laboratory. Biofilm and sediment samples for culturing were maintained at 42 °C in a portable incubator and processed in a nearby laboratory at Montana State University within 24 h of collection.

Amoeba Cultivation and Detection of *N. fowleri*. The large-volume concentrated ultrafilter backflush samples (750 μL to 1.5 mL depending on the concentrate volume) were plated onto 1.5% non-nutrient agar (NNA) plates containing a lawn of *E. coli* (ATCC 25922) following standard methods.[31] *N. fowleri* was cultured at 44 °C, and plates were inspected by microscopy daily for up to 7 days for the presence of active amoebic growth: trophozoites or cysts. If amoebic cysts or trophozoites were observed, the growth was scraped and resuspended with WB saline. A portion of the growth suspension was subjected to nucleic acid extraction as previously described.[32]

To test for viable amoebae, including *Naegleria* spp., in the biofilm and sediment, samples were first briefly shaken (~5 s) to dislodge amoebae from the sediment or biofilm, filtered through a 100 μM membrane filter to remove large particles, and centrifuged at 1200*g* for 20 min. After removing the supernatant, the pellet was resuspended in 500 μL of Page's saline (PS). A 50 μL aliquot was placed in a circle in the middle of NNA plates containing a lawn of *E. coli* (ATCC 25922) and incubated at room temperature for 10−20 min for the sample to absorb. The inoculated NNA plates were sealed with a parafilm and incubated at 42 °C to select for a thermophilic amoeba. Plates were inspected daily by phase contrast microscopy for up to 14 days for the presence of amoeba growth fronts, trophozoites, or cysts (Figure 2). Once amoeba growth fronts, trophozoites, or cysts appeared, plates were scraped into 2 mL of PS and used for the detection of *N. fowleri* with real-time PCR. Additionally, some NNA plates that had areas of amoeba growth indicative of *N. fowleri* were collected by removing an agar block containing viable trophozoites. The agar was transferred to fresh NNA plates, cultured at 42 °C, and passaged on NNA plates. The entire plate was then scraped into PS and analyzed with real-time PCR. DNA was extracted from cultured amoeba as previously described[33] from sediment and biofilm samples using an MP FAST DNA extraction kit (MP Biomedical, Solon, OH).

Real-Time PCR of *N. fowleri* in Surface Water, Biofilm, and Sediment Samples. Small-volume water and both cultured and noncultured biofilm and sediment samples were analyzed using a previously described real-time PCR assay.[34] The assay was modified to accommodate use of a proof-reading high-fidelity DNA polymerase, the Kapa HiFi HotStart ReadyMix (Roche Sequencing and Life Science), according to the manufacturer's instructions.[35] The primers amplify a 123 bp segment of the ITS region. Reaction conditions were 95 °C for 1

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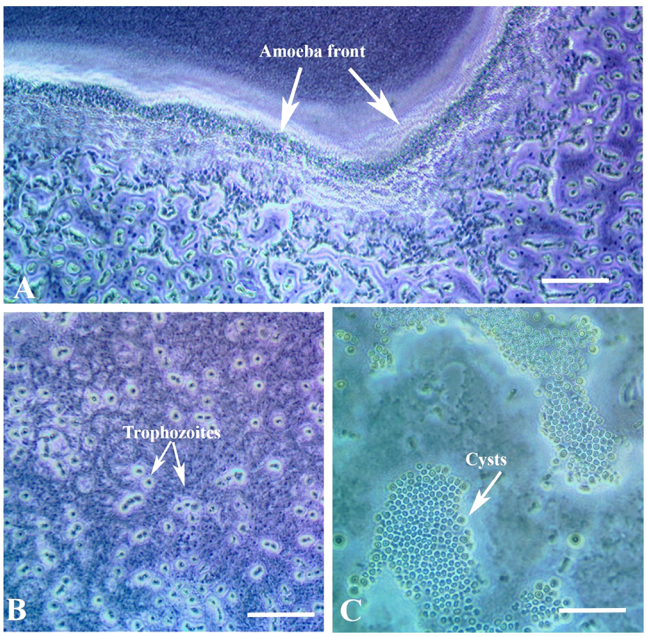

Figure 2. Growth of amoeba on NNA plates from GRTE hot spring samples showing (A) amoeba growth fronts at 10× (the scale bar represents 100 μm), (B) trophozoites, and (C) cysts at 20× (the scale bar represents 50 μm). Trophozoites are characterized by an ameboid shape with clear vacuoles evident, while cysts have a spherical shape with a smooth outline, indicative of *N. fowleri* cysts.

min followed by 45 cycles of 95 °C for 3 s and 63 °C for 33 s. Fluorescence was measured at the end of each PCR cycle. All samples were analyzed in triplicate with a StepOnePlus real-time PCR system (Qiagen). Synthetic DNA g-blocks (Integrated DNA Technologies) that included the 123 bp segment of the ITS region were used as positive controls, and DNA-free water was used for no-template negative controls. Cultured and noncultured samples were considered to have detectable *N. fowleri* genomic material if duplicate reactions were positive, and the C_T (cycle threshold; cutoff for classifying a reaction as "positive") cutoff for positive detection was 42 since this was the cutoff used previously for uncultured field samples.[36]

Cultures sampled from large-volume water samples were analyzed by the CDC with the same assay described above targeting *N. fowleri* and using conditions modified from Mull et al.[34] Assays were performed in duplicate on an ABI 7500 thermocycler with 5 μL of the template in 50 μL reactions consisting of the following: ABI Environmental Master Mix 2.0, 0.5 mg/mL bovine serum albumin, and 25 μg/mL of T4 gene 32 protein. Reaction conditions were as follows: 95 °C for 10 min followed by 45 cycles of 95 °C for 15 s followed by annealing and fluorescence acquisition at 63 °C for 1 min. A sample was considered to have detectable *N. fowleri* genomic material if duplicate PCRs reached C_T values in less than 42 cycles. The negative control for these reactions was nuclease-free water, and the positive control was DNA extracted from cultured *N. fowleri* V212 from the CDC *N. fowleri* collection.

Amplicon Sequencing. DNA results from noncultured biofilm, sediment, and small-volume water samples were further analyzed by amplicon sequencing of a PCR product that includes regions of the ITS1, 5.8S rRNA gene, and ITS2 regions using *Naegleria* ITS primers: for (5′-GAACCTGCGTAGGGATCATTT-3′) and ITS Rev (5′-TTTCTTTTCCTCCCCTTATTA-3′).[37] Samples were diluted with nuclease-free water at a 1:10 ratio for PCR, and reaction conditions were 95 °C for 5 min followed by 30 cycles of 95 °C for 15 s, 55 °C for 90 s, 72 °C for 90 s, and an extension at 72 °C for 10 min. An appropriately sized (300−450 base pairs) amplification product was confirmed for each reaction by electrophoresis of 5 μL of the reaction product through a 1.2% I.D.NA agarose gel (Cambrex Corporation, East Rutherford, NJ) at 100 V for 45 min. PCR products were cleaned with a QIAquick PCR purification kit (Qiagen CT no. 28104, Valencia, CA). Libraries were made following the 16S Metagenomic Sequencing Library Preparation Protocol[38] and quantified using the Qubit dsDNA HS askay Kit (Thermo Fisher Scientific, Grand Island, NY). DNA size spectra for these samples were determined using an Agilent DNA 1000 kit (Agilent Technologies, Savage, MD). Samples were diluted in 10 mM Tris buffer (pH 8.5) to a final concentration of 5 ng/μL. A final 6pM preparation was created with a 15% PhiX control spike, following the Illumina protocol (Illumina, San Diego, CA). DNA libraries were loaded onto an Illumina MiSeq with a 600v3 cartridge. Machine-processed sequencing output was deposited in the NCBI Sequence Read Archive under BioProject PRJNA486492.

DNA Sequence Processing and Taxonomic Analysis. *Quality Control.* Paired-end reads (300 bp) were trimmed of primer, exogenous adapters, and poor-quality sequences using CLC Genomics Workbench v. 9.5.3 (Qiagen). Bases were trimmed at an error probability of 0.05. Reads with more than two ambiguous bases or less than 100 bases in length after trimming were discarded. Trimmed read pairs (355.4 bp average with a range of 108−559 bp for the 46,866 reads) were merged using the scoring parameters +3 for a match, −4 for a mismatch, and a total score of 30 or greater. Unmerged reads were discarded.

Analysis of the Naegleria-Targeting Locus. For the *Naegleria* locus, preliminary searches with the Basic Local Alignment Search Tool (BLAST)[39] with subsamples of reads indicated levels of off-target amplification, particularly with matches to bacteria. Therefore, additional read filtering was performed by aligning reads to a *Naegleria* locus-specific reference database (described below) with the Basic Local Alignment Search Tool Nucleotide (BLASTN)[39] using default discontinuous megablast settings and then realigning matched reads against NCBI's nucleotide (nt) database[40] to ensure the first reported hit was a *Naegleria* sequence. The 46,866 merged reads that mapped to *Naegleria* references were further reduced to 40,582 prior to clustering by eliminating those with ambiguity characters.

The retained *Naegleria* reads were further denoised using Swarm v2[41] with the "fastidious" option invoked but otherwise default settings. This option initially identifies all 1-step sequence variants but uses abundance information to graft low-abundance operational taxonomic units (OTUs) that are more likely artifacts onto higher-abundance OTUs. The initial number of Swarm OTUs was 6,631, which were then screened for chimeras against the matched *Naegleria* reference sequences (Table S1) using default UCHIME[42] parameters as implemented in vsearch v. 2.14.1.[43] The number of retained OTUs was 6,358 after this stage.

Taxonomic classification of amplicon-derived sequences was based on an explicitly phylogenetic approach rather than an alignment-based global-similarity approach. To create a comprehensive reference database, *Naegleria* sequences present in the NCBI nucleotide were downloaded via the taxonomy

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Table 1. *N. fowleri* Detections as Presence (+) or Absence (−) Based on Real-Time PCR of Cultured and Uncultured Biofilm, Sediment, and Filtered Water Samples[21]

	water			sediment			biofilm		
site	July2016	Nov.2016	March2017	July2016	Nov.2016	March2017	July2016	Nov.2016	March2017
H1	+[c]	+[c]	+[c]	−	+[a]	−	−	−	+[b]
H2	−	+[c]	+[a]	−	+[b]	−	−	+[a]	+[b]
H3	+[a]	+[c]	+[c]	−	−	−	−	+[a]	−
P1	+[b]	+[c]	+[c]	−	+[b]	+[b]	+[a]	+[b]	−
K1	−	−	−	+[b]	−	+[b]	−	−	−

[a]Detection in noncultured samples only. [b]Detection in cultured samples only. [c]Detection in noncultured and cultured samples.

browser and aligned with the manually selected seed sequences representing the primer-trimmed amplicon. Matches among the downloaded *Naegleria* sequences were parsed on HSP coordinates, generating a set of 370 partial or complete *Naegleria* reference sequences for the expected amplicon. We extracted a core region of 251 aligned positions, which begins at position two of the 5.8S rRNA and continues through approximately the first 67 bases of the ITS2 region using the annotated *Naegleria lovaniensis* (*N. lovaniensis*) reference NCBI sequence X96568.1[44] as a guide. We masked a 5 bp stretch within this region that contained recurring retained ancestral variation (Figure S2); this masked region includes a C/T nucleotide polymorphism used in some strain-typing studies.[45]

After the 6,358 Swarm OTUs and the 370 NCBI *Naegleria* accessions matched in the initial binning step, they were trimmed and then realigned with Multiple Alignment using Fast Fourier Transform MAFFT v. 7.271,[46] and any partial sequence (i.e., with more than 10 gap positions at either end of the alignment) was removed. Sequences were then dereplicated with the Cluster Database at High Identity with Tolerance CD-HIT v. 4.6[47] by setting the identity parameter to 100% and the overlap parameter to 0.9. Dereplication was necessary because many Swarm clusters differed only in regions that had subsequently been trimmed.

A neighbor-joining phylogeny of aligned amplicons and references was created with Molecular Evolutionary Genetics Analysis MEGA7[48] using the Kimura two-parameter model[49] and assuming uniform-rate variation. Individual clades were manually chosen as OTUs based on bootstrap support or consistency with current taxonomic nomenclature. The tree topology was confirmed with maximum likelihood statistics using default settings in MEGA11[50] (Figure S3). Sequence names within each clade were exported using the subtree options in MEGA7[48] and given the corresponding OTU assignment, whereas the remaining sequences not falling within one of the designated clades were left unclassified. Note that the rRNA locus is extrachromosomal in *Naegleria* and is carried on a high-copy number plasmid.[51]

This process of delineating phylogenetic bins was applied to all merged reads of the pooled data, resulting in a final dereplicated reference database of both reference and environmental sequences. To estimate the relative abundance of these phylogenetic bins in each sample, sample merged reads were mapped to the reference database with Bowtie2[52] using the "very-sensitive-local" setting. We only counted reads that aligned to the top reference match reported by Bowtie2 v. 2.2.8 for more than 200 bases with a combined edit distance of three or less (mismatch or gap position), as determined by parsing mismatch and indel positions from the corresponding "MD:Z" and "CIGAR" tags of the alignment file (see the SAM file specification[53] for details). One singleton of the OTU was discarded after this counting procedure. Raw read counts were transformed using a log-ratio normalization, which is required for linear analysis of compositional data.[54] Specifically, nonzero counts were recast as proportions, divided by the mean of all nonzero proportions in each sample, and then, the $\log_{10}$ of those ratios was calculated. A scalar was then added to make all nonzero cell values positive.

RESULTS

***N. fowleri* Detection and Environmental Conditions in Water.** *N. fowleri* was detected with real-time PCR from low-volume noncultured and large-volume cultured water samples at every time point (midsummer (July 2016), late autumn (November 2016), and early spring (March 2017)) at site H1 (Table 1). Sites P1 and H3 had positive noncultured real-time PCR and large-volume culture *N. fowleri* detections in November 2016 and March 2017. There were also detections in July 2016 with H3 only positive for noncultured and P1 only positive following culturing of the large-volume samples. Kelly Warm Spring (site K1) had no detections from water samples and had the lowest average water temperatures (mean of 27.5 °C; Table S1). The average water temperatures of H1 and P1 were the highest compared to the other hot springs sampled (means of 41.8 and 42.3 °C, respectively; Table S1). Sites H2 and H3 showed the widest range in water temperatures (18.6−46.1 and 15.8−44.8 °C, respectively) with maximum water temperatures in July and minimum temperatures in March, indicating seasonal fluctuations because of ambient surface water entering the hot springs at these sites. The pH at all sites remained neutral to slightly alkaline, ranging from 6.97 at site H2 (March 2017) to 8.01 at site H3 (July 2016) (Table S1). Organic carbon was mostly in the dissolved form, and the concentrations were highest at H2 (max DOC of 4.56 mg/L, November 2016) and H3 (max DOC of 6.18 mg/L, March 2017). Site K1 showed the lowest DOC concentrations, which were below the detection limit during 2 of 3 sampling events (Table S1). *E. coli* was present in most samples (>1 CFU/100 mL in 80% of samples collected) at low densities (Table S1).

***N. fowleri* Detections in the Sediment and Biofilm.** The real-time PCR detection rates for *N. fowleri* were lower in noncultured and cultured biofilm (20 and 20%, respectively) and sediment (7 and 33%, respectively) samples than in surface water samples (60 and 67%, respectively) when considering all the sites and time points (n = 15). (Table 1). In sediment and biofilm samples, more detections occurred in November (6 total detections) and March (4 total detections) than in July (2 total detections) (Table 1). *N. fowleri* was not detected from site K1 biofilm samples and only from K1 sediment samples during July 2016 and March 2017 following culturing (Table 1).

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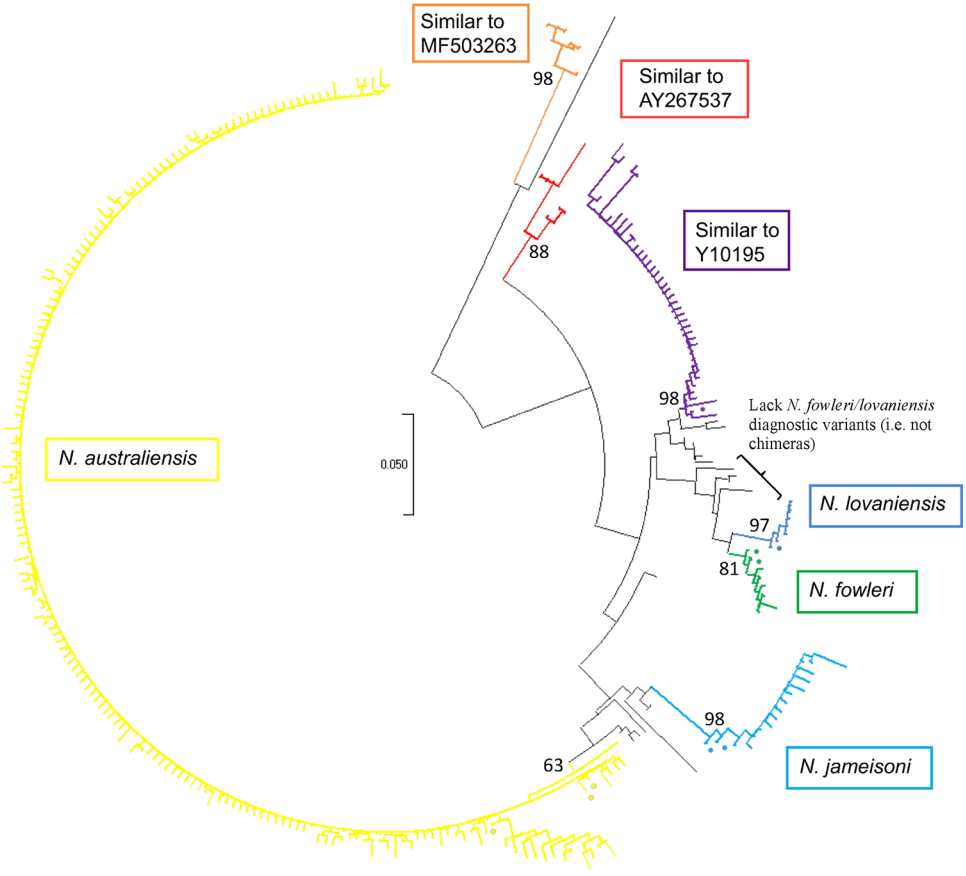

Figure 3. Phylogenetic binning of all 6,358 retained *Naegleria* OTUs as well as reference sequences with counts in the data set. The tree was constructed across the 5 sampling sites with the neighbor-joining algorithm and the Kimura 2-parameter model.[63] GenBank[64]-derived accessions are indicated by dots at branch tips.

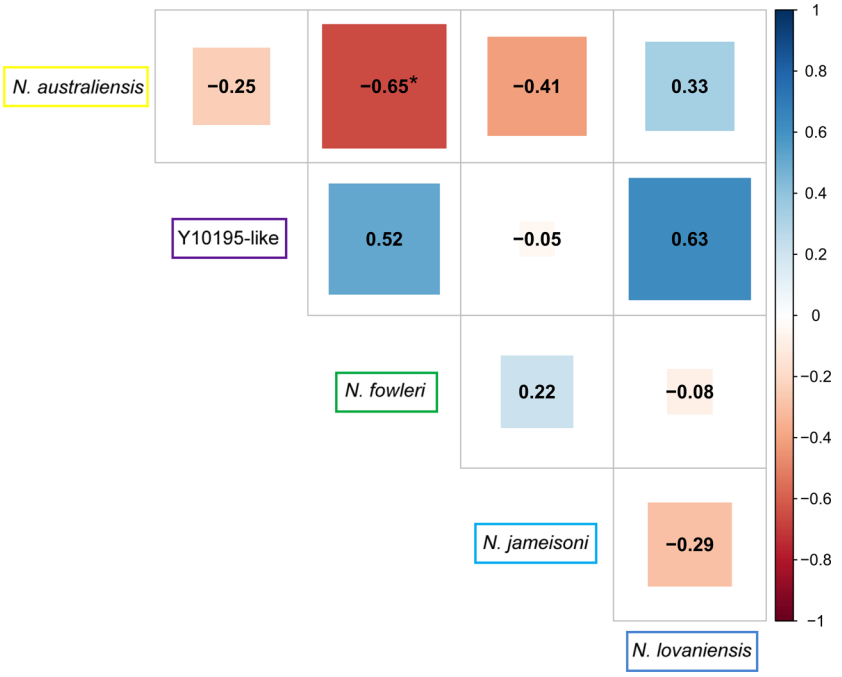

Figure 4. Pairwise Pearson correlations between "major" *Naegleria* clades as defined previously. Values were center log-ratio transformed. The value 1 indicates a strong positive correlation, and −1 indicates a strong negative correlation. The asterisk indicates a significant two-sided test ($P = 0.010$) between *N. australiensis* and *N. fowleri*.

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***Naegleria* Diversity in Hot Springs.** Biofilm, small-volume water, and sediment samples from each hot spring were analyzed with amplicon sequencing using primers specific to the ITS regions for the *Naegleria* genus. A total of 1,115 sequences with >98.5% identity to *N. fowleri* were identified after quality filtering (Table S2). A sediment sample from H2 and a water sample from P1 collected in March 2017 and a water sample from H3 collected in November 2016 contained the highest relative percentage of *N. fowleri* sequences (11.5, 11.5, and 7.4%, respectively) and were the only samples with over 100 *N. fowleri* sequences detected (Table S2 and Figure S4). All these samples were also positive when cultured, indicating that there were viable *N. fowleri* present. Most of the non-*N. fowleri* OTUs from the hot spring samples were most closely related to *N. australiensis*, while several clades were closely related to sequences previously observed by Sheehan et al.[5] (AY247814, AY274813, AY267537, and Y10195) (Figure 3). There was minimal codetection of *N. fowleri* with *N. lovaniensis* (Table S1) and overall a negative association of *N. fowleri* and *N. australiensis* sequences in hot spring samples collected in this study (Figure 4).

DISCUSSION

Using different field-sampling techniques, both culture and molecular-based real-time PCR tests, and phylogenetic analyses based on sequencing of the highly variable ITS region, we were able to (1) provide the first positive detections of *N. fowleri* during different seasons in multiple GRTE hot springs in different media (sediment, water, and biofilm) and (2) provide new insight into the diversity of *Naegleria* spp. in GRTE hot springs.

This study resulted in positive detections of *N. fowleri* in GRTE hot springs when previous studies sampling the same hot springs did not detect *N. fowleri*.[5,18,19] One difference with our study was a higher volume used in the filtered water samples for culturing (26.4−40 L) and most grab water samples that were filtered until the filters clogged (750 mL−14.5 L)[21] In the previous studies, Ramaley et al.[19] filtered 750 mL and O'Dell et al.[18] filtered 45 mL from each hot spring in their culturing, which could have decreased the sensitivity of detecting lower-concentration *N. fowleri*. Sheehan et al.[5] did not filter water and, instead, analyzed 5 mL of biofilm or sediment samples from each GRTE hot spring without culturing. Our results indicated increased chances of detection, especially from sediment (Table 1). Sheehan et al.[5] also used a different PCR-based approach which did not result in amplification of *N. fowleri* from the same GRTE sites analyzed in the presented study. There were fewer *N. fowleri* detections in biofilm (6 total detections) and sediment (6 total detections) samples than in surface water samples (19 total detection; Table 1). The different detection rates among sample types could be due to varying sensitivities and volumes collected by sample type or the life stages (e.g., cyst versus trophozoite) of *N. fowleri* in the different samples.

A previous study indicated that *N. fowleri* was detected less frequently in sediment than associated water samples but that the concentrations of *N. fowleri* could be higher in the sediments since only 100 mg of sediments was used for each sample versus 500 mL for water samples.[7,8,55] The present study also analyzed a much smaller volume of sediment and biofilm (~0.5 mL) compared to much higher volumes of water (750 mL−40,000 mL) which could account for the higher detection rate in the water samples versus biofilm and sediment (Table 1). With positive detections in water, sediment, and biofilm, a next step could be to examine the differences in *N. fowleri* density based on sample type and location within the hot springs to help understand where *N. fowleri* is concentrated in these environments.

Detection of *N. fowleri* in GRTE in this study but not in previous studies could be due to updated analytical methods or due to potential increased *N. fowleri* concentrations since previous studies were performed on the same hot springs more than 20 years ago. Previous studies have suggested that warming climate trends and higher surface water temperatures have facilitated a northern geographical expansion of PAM cases in the United States.[17] Even though our study focused on hot springs with geothermal water features which have deep subsurface thermal influences unrelated to climate change, atmospheric temperatures and snowmelt runoff also impacted temperatures of the hot springs as evidenced by the large fluctuation in temperature in H2 and H3 during spring runoff (Table S2).[21] While the existing data could support the hypothesis that range expansion into GRTE hot springs may have occurred since the previous studies, the current data are insufficient to make this conclusion. Additional sampling with the improved techniques described here could provide sufficient data to determine the areas where *N. fowleri* expansion may occur and help educate people who live in or live near hot springs.

Another goal of our study was to compare *N. fowleri* detections between different seasons and geochemical conditions, although with hot springs, this can be complicated because geothermal water can buffer season influences. We observed that the highest real-time PCR detection prevalence was at sites H1 and P1 (8 and 9 total detections, respectively) where the average temperature (mean of 41.5 °C and mean of 41.6 °C, respectively) was the highest compared to the other hot springs (Table 1 and Table S2). The fewest detections occurred in K1 (2 detections total), which had the lowest average temperature mean of 27.6 °C, and detections only occurred following culturing (Table 1 and Table S2). Interestingly, when considering all positive samples, there were more positive real-time PCR detections in November and March (14 and 11, respectively) when the average temperatures for all the hot springs were lower (31.8 and 28.4 °C, respectively) versus in July with 6 positive detections with an average temperature of 41.5 °C (Table 1). A previous lake study in Arizona detected *N. fowleri* more frequently in winter and spring (20%) than in summer and fall (7.9%),[56] while a lake study in Louisiana detected higher concentrations of *N. fowleri* during summer.[57] In an engineered water system, Puzon et al.[12] showed a multiyear seasonal correlation, but with differences for *N. fowleri*'s presence in water versus biofilm. Future investigations with additional sampling over several seasons could help define specific abiotic factors that impact *N. fowleri* prevalence in hot springs.

Naegleria-specific sequencing and phylogenetic analysis successfully differentiated *Naegleria* spp. in this study and provided new insight into the types of *Naegleria* within GRTE hot springs. A phylogenetic approach was necessary because environmental variants often aligned most closely to accessions of unnamed *Naegleria*. Furthermore, few informative sites were present in aligned amplicons and reference sequences, such that sequencing error could be a significant source of classification error for alignment-based approaches. Additionally, the ITS1 and ITS2 sequence flanking the 5.8S region contained variable numbers of small insertions (Figure S5), consistent with those

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reported by De Jonckheere.[44] The extent to which plasmid sequences accurately represent the evolutionary history of each species has not been fully determined.[58] However, previous phylogenies have used the ribosomal locus, and our unrooted tree (Figure 3) is consistent with those reports.[59]

In-depth phylogenetic analysis indicated that most of the non-*N. fowleri* OTUs from the hot spring samples were most closely related to *N. australiensis*, which aligns with previous studies that isolated *N. australiensis* from Huckleberry Hot Spring.[19] *N. australiensis* has been found to cause PAM in laboratory mice and has been associated with the brains of fish but has not been reported to cause PAM in humans.[60,61] Our phylogenetic analysis revealed several related clades of sequences previously identified by Sheehan et al.[5] with clone library analysis (AY247814, AY274813, AY267537, and Y10195) (Figure 3). *N. fowleri* and *N. lovaniensis* appeared to be embedded within a larger clade of weakly resolved or unclassified sequences in the hot spring samples. There was no indication that reference branches that were sister clades to *N. fowleri* or *N. lovaniensis* were chimeric. Clearly, identification based on the ITS region did not provide information on the traits associated with this novel clade. A log-ratio normalized Pearson correlation indicated that there was a significant negative association between *N. fowleri* with *N. australiensis* (a significant two-sided test (P = 0.010)) in the hot spring samples (Figure 4). Competition and negative associations between *N. fowleri* with *N. lovaniensis* were previously identified in drinking water distribution systems in Australia,[62] and our study also identified a slight negative association between *N. fowleri* with *N. lovaniensis* (Figure 4). These results provide new insight into the *Naegleria* spp. and its associations in GRTE hot springs.

CONCLUSIONS

With larger sample sizes, multiple sample types, and updated analytical methods, the present study provides the first detection of the presence and viability of *N. fowleri* in surface water, sediment, and biofilm samples from GRTE hot springs. These results provide new insights into the distribution of both pathogenic *N. fowleri* and nonpathogenic *Naegleria* in natural thermal water systems in northern latitudes. The *N. fowleri* detection rates were higher in both noncultured and cultured water samples than in biofilm and sediment samples. *N. fowleri* was detected more from sites with higher average temperatures, although overall, there were fewer real-time PCR detections in July when average temperatures were higher compared to November and March. Further analysis of temperature and the presence of *N. fowleri* over space and time, for example, could help to understand the seasonality of *N. fowleri* occurrence. Sequencing analysis also identified a negative association between *N. fowleri* and *N. australiensis*. These findings have implications for understanding the distribution of deadly pathogens in GRTE, and the methods used in this study could be applied to better understand the distribution of *N. fowleri* in other thermal springs. Consideration of GRTE thermal springs and other thermal springs as potential water exposures for suspected PAM cases may improve healthcare, so that appropriate treatment can be started as early as possible.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsestwater.3c00650.

Additional information including images of sampling sites, counts of *Naegleria* species, geochemical parameters of hot springs, sequence alignments, phylogenetic tree, and relative abundance of *Naegleria* species (PDF)

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Notes

The authors declare no competing financial interest.

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