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Article

Detection of *Naegleria fowleri* in Thermally Impacted Recreational Waters of Western United States National Parks

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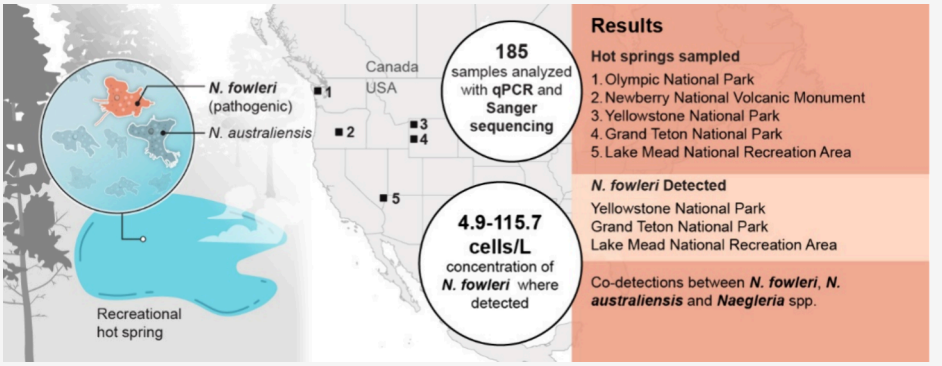

ABSTRACT: *Naegleria fowleri* is a thermophilic free-living amoeba (FLA) and the causative agent of primary amoebic meningoencephalitis, posing public health risks in warm freshwater environments. This multiyear, multiagency study surveyed 40 thermally impacted recreational waters across five western United States national parks and recreation areas—Yellowstone National Park, Grand Teton National Park, Olympic National Park, Newberry National Volcanic Monument, and Lake Mead National Recreation Area—to assess *N. fowleri* presence, concentration, and associated environmental conditions. A total of 185 water samples were analyzed by qPCR and Sanger sequencing, revealing widespread detection of *N. fowleri* in 34% of samples with positive detections from Lake Mead, Yellowstone, and Grand Teton hot springs and thermally impacted waters, with concentrations ranging from 4.9 to 115.7 cells/L. Multiple codetections of *N. fowleri* with nonpathogenic species including *Naegleria australiensis* were identified, suggesting they may inhabit similar ecological niches in the natural systems in contrast to engineered systems. These findings indicate that *N. fowleri* is present in thermally impacted areas across the western United States and underscore the use of enhanced monitoring, public awareness, and risk management strategies in thermally influenced recreational waters.

KEYWORDS: *Naegleria, hot springs, water, pathogens, amoebae, amoeba*

1. INTRODUCTION

Naegleria fowleri is a thermophilic free-living amoeba (FLA) that lives in freshwater and soil worldwide and is the causative agent of primary amoebic meningoencephalitis (PAM).[1] PAM is caused by *N. fowleri* in water entering the nasal cavity, and then migrating to the central nervous system.[2] PAM typically presents with early symptoms including headache, fever, and nausea, then progresses to confusion, coma, and seizures with death occurring 7 to 10 days post infection at a fatality rate of 98%.[3] Fatal *N. fowleri* cases have occurred in recreational waters with reported water temperatures starting at 22 °C and rising to temperatures >30 °C.[4,5] Swimming/diving is the most common recreational activity linked to *N. fowleri* infections; however, multiple aquatic recreational activities (e.g., water skiing, splashing, water festivals) have been linked to other confirmed *N. fowleri* fatalities.[6] Deaths from *N. fowleri* have also been connected to other lower impact recreational activities such as bathing in geothermal waters.[6,7] The use of nasal irrigation products (such as Neti pots) for flushing nasal cavities is also a notable source of PAM associated brain infections.[8] As of 2024, there have been approximately 500 recorded PAM cases diagnosed worldwide; however, PAM is likely underreported due it is rarity, lack of public awareness, and similar symptoms to other more common infections.[6,9,10] Currently there are 47 described *Naegleria* species, but *N.*

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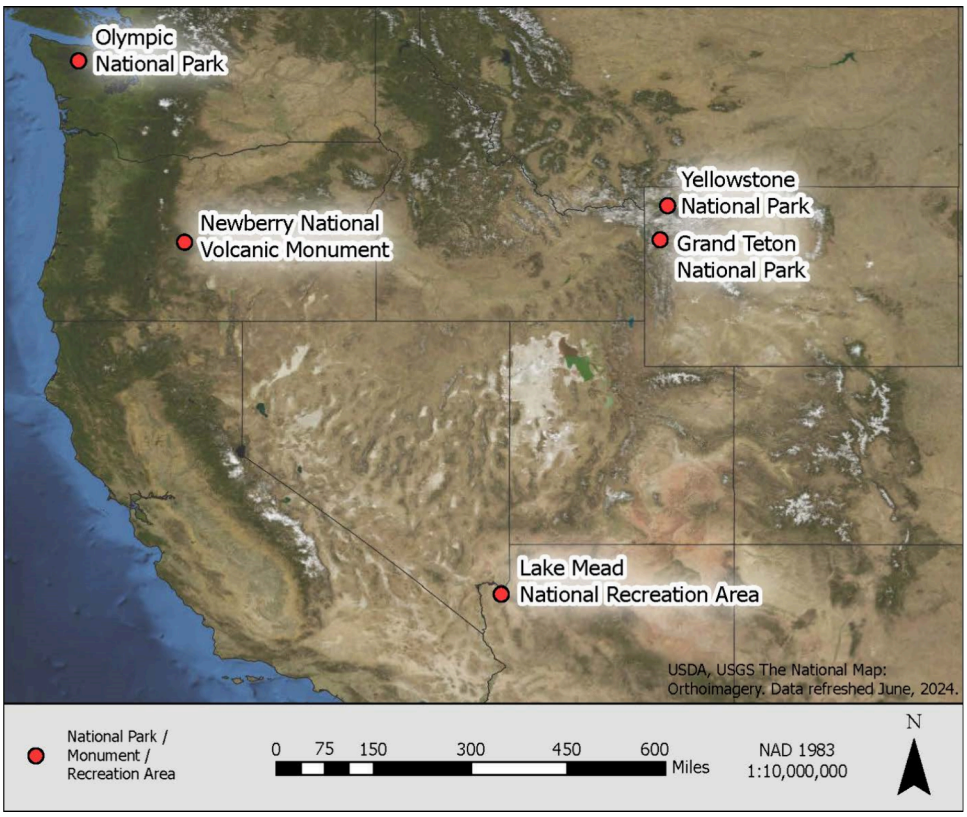

Figure 1. Map of the western United States showing all five sample regions, including Grand Teton National Park, Lake Mead National Recreation Area, Newberry National Volcanic Monument, Olympic National Park, and Yellowstone National Park.

fowleri is the only known human pathogen in the genus.[11] *Naegleria australiensis* and *Naegleria italica* have demonstrated pathogenicity in mice; however, these species have never been isolated from a human PAM infection.[12,13]

Naegleria fowleri has three life stages: cyst, feeding trophozoite, and motile biflagellated stage. *N. fowleri* is capable of quickly alternating between life stages in the environment to survive unfavorable conditions, highlighting the environmental stability of this pathogen. The pathways by which *N. fowleri* disperses to new geographic regions remain undetermined; however, current evidence suggests the organism likely originates in soils and moves to water sources during events such as flooding.[14] In the biflagellated form, flagella enable motility and the search for a preferential food source.[3] *N. fowleri* can encyst when exposed to stress, such as extreme temperatures, high salinity, high or low pH, or nutrient stress.[15] In addition, the encystment of FLA is thought to allow the organisms to become dormant for extended periods of time and propagate once conditions become favorable. *N. fowleri* is thermophilic and can grow in the laboratory at temperatures from 20 °C up to 46 °C while cysts can survive at temperatures below 10 °C and up to 65 °C.[15−18] This wide temperature tolerance allows *N. fowleri* to proliferate in natural and engineered water systems. Accordingly, *N. fowleri* has been isolated from natural hot springs, lakes, ponds and rivers, artificially heated power plants, private wells, recreational water parks, water tanks from recreational vehicles, and drinking water distribution systems (DWDS).[19−28]

Cases of PAM in the United States (U.S.) have seen a northerly geographical expansion since 1962, and the trend is predicted to continue as global temperatures increase.[4,10] In addition to PAM cases, detections of *N. fowleri* have occurred in areas such as hot springs in Wyoming, private wells in Arizona, sediment from Lake Pontchartrain, and DWDS in Louisiana.[22,23,25,29−31] The high fatality rate and expanding geographic range of *N. fowleri* infections indicate that improved pathogen surveillance can progress research in the field. In Australia, regular monitoring of *N. fowleri,* specific protocols in response to detections, and increased public awareness programs have helped limit the number of fatalities to 19 since 1965.[32,33] Broadening surveillance for *N. fowleri* in the U.S. could help inform the public of the expansive geographic range of this pathogen.

Here we present a collaborative, multiyear (2016−2024), geographically expansive study of amoebae in thermally influenced waters used for recreational soaking encompassing 40 different sites across the western United States, including sites in the greater Grand Teton National Park (GRTE) and Bridger -Teton National Forest region, Lake Mead National Recreation Area (LAKE), Newberry National Volcanic Monument (NNVM), Olympic National Park (OLYM) and Yellowstone National Park (YELL). A total of 185 samples were collected and analyzed for amoeba presence, physical and chemical conditions, and *N. fowleri* concentrations. This study characterized the ecological conditions supporting the presence of pathogenic *N. fowleri* and other nonpathogenic *Naegleria* spp. and amoebae. This work has identified locations which may benefit from enhanced public awareness and provides insight into conditions supporting *N. fowleri* and other amoebae in natural systems.

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2. METHODS

2.1. Sites and Field Conditions (Temperature and pH Measurements Only)

This study encompasses results from multiple sampling efforts by the U.S. Geological Survey (USGS), Commonwealth Scientific and Industrial Research Organization (CSIRO), and Montana State University (MSU) between 2016 and 2024. Samples were collected in the 5 main park regions (YELL, GRTE, OLYM, LAKE and NNVM) between 2018 and 2024 (Figure 1). There were 24 sample areas, with 40 specific sample sites resulting in 185 total samples collected in this study. Between 2016 and 2018, samples were collected by the USGS, in 2019 by the USGS and CSIRO, and 2022−2024 by CSIRO and MSU. No sampling occurred between 2020 and 2021 because of travel restrictions due to the COVID-19 pandemic. Sample sites included warm water pools, or areas of hot spring effluent mixing with freshwater, and sites known to be used for recreational soaking. Surface water was sampled at sites within each national park, national recreation area or national monument. Areas within YELL were in the Bechler region (Mr. Bubbles Hot Springs and Dunanda Falls), Lower Geyser Basin (Mushroom Hot Spring, Octopus Hot Springs and White Creek), Heart Lake Geyser Basin (Witch Creek), the southern shore of Lewis Lake, the Yellowstone River, the Firehole River and the Boiling River (Figure S1). Five areas were sampled in the greater GRTE region: Astoria, Granite, Huckleberry, Polecat and upper Polecat Hot Springs (Figure S2). The sampled hot springs in OLYM were Sol Duc (commercial) and Olympic Hot Springs (natural) (Figure S3). Because Sol Duc Hot Spring is a commercial pool, samples were collected from the runoff where the thermal water drains to the Sol Duc River. Hot springs within LAKE were Nevada, Boy Scout, Blue Point, Rogers and Arizona Hot Springs (Figure S4). NNVM hot springs were Paulina Lake and East Lake Hot Springs which are both natural and used for recreation (Figure S5).

2.1.1. USGS Sampling. Water samples were collected from 16 sites during 2018−2019; the USGS sampled 11 sites in the Greater Yellowstone Area, and the National Park Service sampled 5 sites in the LAKE. Prior to sampling, water temperature and pH were measured using a YSI EXO 2 Water Quality Monitor (Yellow Springs, Ohio). Water samples were processed following the methods of Barnhart et al. 2024.[25] To minimize contamination risk, water samples were collected from the center of each hot spring using a sterile, 1-L polypropylene bottle attached to an extendable sampling rod. Water samples were collected for both chemical and molecular analysis. Water chemistry samples were processed onsite and shipped to the USGS National Water Quality Laboratory in Lakewood, Colorado for analysis. Equipment decontamination and sample processing procedures for chemistry samples are described in the USGS National Field Manual (USGS, 2009). Results for water chemistry samples are available at the Water Quality Portal (https://www.waterqualitydata.us).[34] Water samples for molecular analysis were collected into two, 10 L sterile cubitainers and were filtered onsite using a peristaltic pump with 0.2 and 1.0 μm pore size filters (Autofil, Foxx Life Sciences, Londonderry, NH). Water was pushed through each filter until either the 10 L had been completely filtered, or the filter became clogged. Three 1.0 μm filters and two 0.2 μm filters were collected for each sample. After each sample was filtered, the filter was removed from the filter holder using sterile forceps, folded several times, and inserted into a sterile 2 mL microcentrifuge tube and labeled. RNAlater (ThermoFisher Scientific, Waltham, Massachusetts, USA) stabilization solution was added to each tube until it completely covered the filter. Tubes were put into labeled Whirl-Pak bags and stored on ice until they were taken to the laboratory for processing and analysis.

2.1.2. MSU/CSIRO Sampling. Samples collected by MSU used a hand-held thermometer and pH-indicator strips (Merck Millipore, Ireland) for temperature and pH readings. For a secondary analysis, 50 mL of water was filtered through a sterile 0.22 μm syringe filter into a sterile 50 mL Falcon tube to measure pH in the laboratory. Samples (3× per site) were collected using sterile techniques in sterile 1 L polyethylene terephthalate bottles (Corning, USA) by dipping bottles into the water in the center of the hot spring. After collection, samples were filtered onto 0.22 μm filters using EZ-FIT filtration units (Millipore, USA), then filters were transferred to sterile 50 mL tubes (Corning, USA) and preserved with 1 mL of DNA/RNA Shield (Zymo Research, USA). Samples were stored in a closed container for transport back to the laboratory at ambient temperatures (20−22 °C) for DNA extraction.

2.2. DNA Extraction

USGS extracted DNA from samples using two different methods as follows. DNA was extracted at the Upper Midwest Environmental Sciences Center (UMESC) from filters collected in 2018 using the IBI gMax Mini Kit (IBI Scientific; Dubuque, IA) according to the manufacturer's directions. DNA extracts were stored at −80 °C. DNA was extracted at the Northern Rocky Mountain Science Center (NOROCK) from filters collected in 2019 using the DNeasy PowerWater Kit (Qiagen; Germantown, MD) according to the manufacturer's directions.

MSU and CSIRO extracted DNA from concentrated bulk water samples using the DNeasy PowerWater kit (Qiagen; Germantown, MD). The DNA isolation kit was used according to the manufacturer's protocol. Apart from UMESC, DNA extracts were stored at −20 °C prior to qPCR analysis and Sanger sequencing confirmation.

2.3. Detection and Enumeration: Quantitative Polymerase Chain Reaction (qPCR)

For USGS samples, primers and thermal cycling conditions were used for qPCR analyses run at UMESC on CFX96 Touch thermal cyclers (Bio-Rad; Hercules, CA) following Mull et al. (2013) for *N. fowleri* as described in Barnhart et al. (2024) (Table S1).[25,35] This qPCR method provided only detection and not enumeration. Final qPCR volumes were 25 μL with 500 nM of primers, 250 nM of probe, 12.5 μL of ToughMix Mastermix (Quantbio, Beverly, MA, USA), and 2 μL of template DNA. Samples were run in quadruplicate on 96-well plates with one no-template control (NTC) per sample, and three sample replicates spiked with synthetic DNA (gBlocks; Integrated DNA Technologies; Coralville, Iowa) to test for PCR inhibition. Four positive controls for *N. fowleri* using gBlocks and an additional four NTCs were also run on each plate. Standard curves were run on each plate with concentrations from 20,000 to 20 copies of DNA. Samples that were PCR-inhibited were cleaned using the OneStep PCR inhibitor Removal Kit (Zymo Research; Irvine, CA) and qPCR were repeated. Plates were considered contaminated and data

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Table 1. Total Number of Detections for Each Free-Living Amoeba Species Detected Throughout the Entire Study with Their Corresponding Temperature and pH Ranges and Averages

Free-living amoeba detected	Number of detections	Temperature range (°C)	Average temperature (°C)	pH range	Average pH
Naegleria fowleri	63	17.4−54.9	40.3	5.5−8.4	6.9
Naegleria australiensis	74	16.3−66.9	38.6	5.5−8.9	6.7
Naegleria lovaniensis	5	40.2−47.8	43.1	5.5−7	6.3
Vermamoeba vermiformis	7	38−45.9	41.4	5.5−7	6.2
Other *Naegleria* spp.	16	18.5−54.4	41.9	6−9	6.8
No detection	46	13.4−77.6	38.1	6−9.1	7.1

discarded if detection occurred in any NTC on a plate. Samples were rerun if there was enough DNA extract available.

For MSU and CSIRO samples, DNA extracts were aliquoted, then independently analyzed by qPCR in triplicate by both CSIRO and MSU using a Bio-Rad CFX96 instrument (BioRad, USA) and an Applied Biosystems QuantStudio 7 Pro (Applied Biosystems, USA), respectively. Final qPCR volumes were 25 μL with 12.5 μL of HotStarTaq Master Mix (Qiagen, USA), 500 nM of primers, 2 μM of SYTO9 dye (Molecular Probes, USA), and 2 μL of template DNA. Methods for *Naegleria* spp. and *N. fowleri* detection were used as previously described by Puzon et al. (2009) with either a *N. fowleri* specific primer set (FWS and PITSR)[36] or a consensus primer set for *Naegleria* spp. (PITSF and PITSR)[37] (Table S1). Amoeba detection and speciation were further confirmed by melt-curve analysis conducted from 75 to 95 °C raising the temperature 0.2 °C every 10 s. The product was considered confirmed as *Naegleria* spp. or *Vermamoeba vermiformis* if the melting point was between 78 and 85 °C and matched previously reported melt curve signatures.[36] Samples were run with DNA extraction controls (Instagene Matrix and DNeasy PowerWater elution buffer), negative controls (Milli-Q water), and genomic DNA positive controls (*N. fowleri*, *Vermamoeba* spp., or *N. australiensis*).

For *N. fowleri* quantification, standard curves and linear regression eqs (Figure S6) were produced following the method of Puzon et al., 2009 by CSIRO and MSU.[36] Known *N. fowleri* concentrations were enumerated by 10-fold serial dilutions and spiked into 1 L of reverse osmosis (RO) water at 10 to 10^5 cells/L. Samples were filtered through disposable Millipore EZ-Fit 0.22 μm filtration units (Millipore, USA) to isolate whole *N. fowleri* cells. DNA was extracted using the DNeasy PowerWater Kit (Qiagen, USA). Methods for *N. fowleri* specific detection by qPCR melt curve analysis were conducted using a Bio-Rad CFX96 instrument (BioRad, USA) as previously described above.

To determine the relative concentrations of *N. fowleri* and *N. australiensis*, the tested samples were initially amplified by qPCR in triplicate using general primers to ascertain total *Naegleria* spp. concentration (i.e., *N. fowleri* and *N. australiensis*). Specific primers were then used on the same samples to determine *N. fowleri* concentration alone. Both qPCR analyses were performed in conjunction with a *N. fowleri* and *N. australiensis* standard curve (starting quantities of 10 cells/L up to 100,000 cells/L) and cell concentrations were determined using linear regression. Concentrations of each species were calculated using the relative amplifications from each primer set. Standard deviations were also calculated for each sample.

2.4. Sanger Sequencing for MSU and CSIRO *N. fowleri* Positives

Confirmation of a subset of *Naegleria* spp. and every *N. fowleri* qPCR product was done by isolation of amplified DNA bands on 1% agarose gels in 1X Tris buffer and ethidium bromide. Gels were visualized with a Dual LED Blue Light Transilluminator (Invitrogen, USA) at a wavelength of 470 nm. Bands were isolated using the GeneJET Gel Extraction Kit (Thermo Scientific, USA), purified using the QIAquick PCR & Gel Cleanup Kit (Qiagen, USA), and DNA concentrations were measured using the Qubit dsDNA Quantification Assay (Invitrogen; Waltham, MA). MSU samples were sent to Idaho State University Molecular Research Core Facility (RRID:SCR_012598) for sequencing on a Seq Studio Flex 8-capillary genetic analyzer and CSIRO samples were sequenced at Macrogen, Korea. Sequences were uploaded to National Center for Biotechnology Information (NCBI) GenBank and the accession numbers are in Table S3.

2.5. DNA Sequence Processing and Genotyping

Sequences positive for *N. fowleri* were aligned to *N. fowleri* types 1 (AY376149), 2 (X96564), 3 (X96562) (North America) and 5 (X96565) (Oceania) using Geneious Prime.[38−40] Sequences of the internal transcribed spacer subunit 1 (ITS1) containing the 5.8S rRNA gene were downloaded from the NCBI GenBank. Sequences were aligned to the M1 sequence of the ITS1 subunit, a 15 base pair sequence found in all genotypes of *N. fowleri*.[40] The primer set used for *N. fowleri* does not include the entire ITS1 subunit, so genotyping was based off the 31st base pair of the 5.8S subunit (Table S2) described by De Jonckheere et al. (2011). Alignments to the type 5 *N. fowleri* were made to test for contamination from positive controls from Australia (CSIRO).

2.6. Statistical Analysis

Temperature, pH, and other amoebae species were analyzed to detect potential associations between these variables and the presence or absence of *N. fowleri*. Firth's bias-reduced penalized-likelihood logistic regression was used for analysis to reduce small-sample bias in parameter estimates and improve robustness of inference.[41,42] Hypothesis testing and interval estimation were performed using penalized likelihood ratio tests and profile penalized likelihood confidence intervals, as implemented in the R package logistf.[43] Given the sparsity of other species, *Naegleria* spp., *N. italica*, *N. lovaniensis*, *N. dobsoni*, *Vahlkampfia* and unclassified Eukaryotes were combined into a new variable called 'other.sp' that was given a value of 1 if any of those were present and 0 if absent. Analyses were performed in R version 4.3.3 (2024-02-29). An $\alpha < 0.05$ was considered statistically significant. No corrections for multiplicity were performed.

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Table 2. Concentrations of *Naegleria fowleri* from Quantitative Polymerase Chain Reaction Enumeration Methods Following Methods of Puzon et al. (2009)

National park	Site	Date	Temperature (°C)	pH	Concentration (cells/L)	Standard deviation (cells/L)
Yellowstone National Park	Boiling River	9/7/2023	45	7	6.2	1.5
Grand Teton National Park	Huckleberry (H-1)	9/14/2024	42.4	6	6.4	4.7
	Huckleberry Metal Ring (H-MR)	9/14/2024	41.9	6	8.4	3.9
	Huckleberry Metal Ring (H-MR)	9/8/2023	43.9	6	11.6	4.3
	Lewis Lake JS-1	9/9/2023	37.3	8	8.2	3.7
	Polecat (P-1)	9/14/2024	43.8	6	78.7	12.8
	Polecat (P-1)	9/17/2024	38.1	7	16.2	4.3
	Polecat (P-1)	9/8/2023	42.4	7	115.7	29.5
	Upper Polecat P-2	9/15/2024	43.6	5.5	16	5.3
	Upper Polecat P-1	9/15/2024	42.2	6	4.9	4.1

3. RESULTS

3.1. FLA Detections and Environmental Conditions

FLA detections, temperature, pH, sample date, and sample site coordinates for each of the 185 samples, 40 sample sites and 24 samples areas collected are shown in Table S4. The number of detections per FLA and associated temperature and pH ranges are shown in Table 1. *N. fowleri* was the second most abundant FLA species across the 5 sampling regions, where it was detected in 34% (63/185) of samples. The most abundant species was *N. australiensis*, which was detected in 40% (74/185) of samples. Other FLA species were rare, with no other species being detected in more than 5% of the samples. No FLA were detected in 24% (46/185) of samples. The samples without FLA consisted of hot springs with a cold background used to test for negative controls (e.g., Boiling River cold stream) or the direct source of the hot spring (e.g., Heart Lake). There were no detections of *N. fowleri* or *Naegleria* spp. in 2016 and 2017 sampling.

At YELL, temperatures of samples ranged from 10.8 to 77.6 °C and pH range of 5.0 to 9.1 (Table S4). *N. fowleri* was detected in well-mixed rivers (Firehole River), and in effluent streams from hot springs (Boiling River and Lewis Lake hot springs) (Table S4). The regions of YELL without *N. fowleri* detections were the Heart Lake Network, Bechler River region, the Yellowstone River at Nez Perce Picnic Area and thermal features in the Lower Geyser Basin. *N. fowleri* was detected at the Boiling River in July, September and November of 2018, July 2019 and September 2023. The Firehole Canyon Swimming Area was positive for *N. fowleri* in 2018 and 2019, and the Firehole River near Goose Lake was positive in 2023. *N. fowleri* was detected in multiple hot springs at Lewis Lake in 2023; however, it was not detected in 2024. Nonpathogenic *Naegleria* spp. were detected in every region we sampled within YELL (Table S4). *N. australiensis* was detected in every region sampled between 2023 and 2024. In addition, *N. lovaniensis* was detected at the Boiling River and the Heart Lake Network in Witch Creek. *N. dobsoni* was detected at Firehole (FP-1) in September 2019 but not in any other sampling.

Temperatures of hot springs in the greater GRTE region ranged from 32.9 to 47.6 °C and pH 5.5 to 8.3 (Table S4). FLA were detected in every sample collected in the greater GRTE region (Table S4). *N. fowleri* was detected at Granite Hot Spring in 2019 in the pool above the recreational area, Polecat and Huckleberry Hot Springs in all but one time-point between 2019 and 2024, and upper Polecat Hot Spring in 2024. Huckleberry Hot Springs originates in a geothermal pool (H-1) and flows into a creek with several pools for soaking, including a pool with a metal ring in the center to sit on (H-MR). *N. australiensis* was detected consistently in Huckleberry and Polecat Hot Springs between September 2022 and September 2023. Co-detections of *N. fowleri* and *N. australiensis* were observed in samples between 2022 and 2024 from Polecat and Huckleberry Hot Springs. *N. australiensis* was detected in both samples taken at Astoria Hot Springs in September and November of 2019. Polecat Hot Springs was sampled twice in September of 2024, with the first sampling trip by MSU and the second with MSU and USGS. The second sample day (9/17/2024) had substantial rainfall and the temperature in Polecat Hot Springs dropped significantly (43.8 to 38.1 °C). Upper Polecat Hot Springs also had an upper pool (P-1) and lower pool (P-2) which were both positive for *N. fowleri.*

The temperature of Sol Duc and Olympic Hot Springs in OLYM ranged from 25.0 to 44.2 °C and pH ranged between 5.5 to 8.0 (Table S4). *N. australiensis* was detected at Sol Duc and Olympic Hot Springs at every sample point. NNVM has two hot springs on neighboring lakes, Paulina Lake and East Lake. These springs had a temperature range from 21.1 to 37.0 °C and pH range 5.5 to 7.0 (Table S4). *N. australiensis* was detected at both East Lake and Paulina Lake hot springs, and *N. lovaniensis* was detected at Paulina Lake hot springs.

Hot springs around LAKE ranged in temperature between 27.9 to 41.4 °C with pH ranges between 7.1 to 8.3 (Table S4). *N. fowleri* was detected in Blue Point, Boy Scout, Nevada and Roger's Hot Springs. The site without a *N. fowleri* detection was Arizona Hot Spring, which had a temperature range between 30.2 to 36.1 °C and pH 7.6 to 8.1.

3.2. Concentration of *N. fowleri* in Bulk Water

N. fowleri concentrations were quantified for the 2023 and 2024 samples from YELL and GRTE using qPCR standard curve analysis with a serial dilution of known concentrations of *N. fowleri* as described in Puzon et al. (2009)[36] (Table 2 and Figure S6). Triplicate water samples were analyzed (1 L each) in triplicate qPCR reactions to calculate average concentrations and standard deviations. In 2023, the GRTE samples had a mean *N. fowleri* concentration of 116 cells/L at Polecat Hot Spring, 6.4 cells/L at the Huckleberry Hot Spring pool 1 (H-1), and 11.6 cells/L at Huckleberry Hot Spring metal ring (H-MR). In 2024, replicate 1 L GRTE Polecat (P-1) samples had

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a mean *N. fowleri* concentration of 78.8 cells/L and site H-MR showed 8.4 cells/L. In 2024, the upper Polecat upper pool had a *N. fowleri* concentration of 4.9 cells/L and the connected upper Polecat lower pool had a *N. fowleri* concentration of 16.1 cells/L. In the YELL samples, the 2023 Lewis Lake (Site JS-1) 1 had a mean *N. fowleri* concentration of 8.2 cells/L. The Boiling River had a *N. fowleri* concentration of 6.2 cells/L in 2023 and was not detected in 2024.

3.3. Codetections of FLA in Hot Springs

Co-occurrence of FLA species was specific only to the samples analyzed using the Puzon et al. (2009) qPCR methods for detection of multiple species. Unknown qPCR products without melt-curve identification were sequenced and compared to the NCBI GenBank database via NCBI Basic Local Alignment Search Tool (BLAST) and matched based on the highest maximum score. There were 80 samples positive for FLA out of 104 samples analyzed. Of these 80 samples, there were 105 total FLA detections, indicating some samples had multiple FLA species present (Table S4). A co-occurrence heatmap (Figure 2) shows the total number of individual

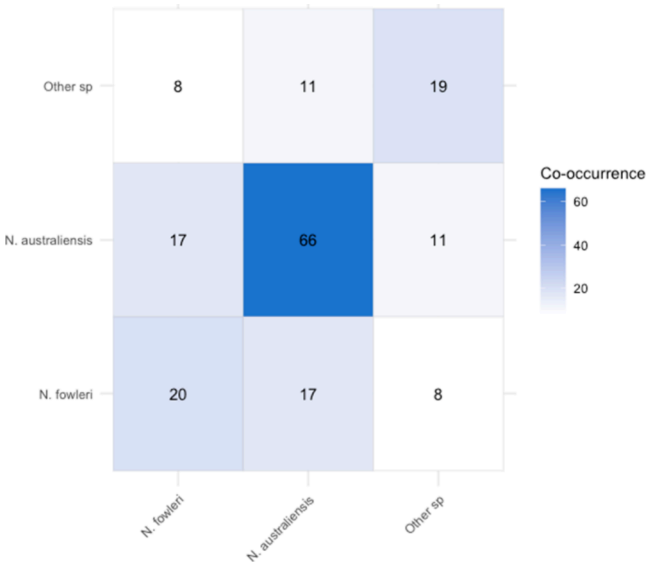

Figure 2. Number of codetections of amoeba in the same sample analyzed following the Puzon et al. (2009) quantitative polymerase chain reaction method. The total number of occurrences of each amoeba species along the *z*-axis, and the number of co-occurrences in corresponding grids among species.

species detections, as well as the number of multispecies detections. *N. australiensis* was the most prevalent species, with 66 total detections (Figure 2). *N. fowleri* was the second most prevalent species, with 20 total detections. In addition, there were detections of *N. italica, N. lovaniensis, Naegleria* spp. NG334, unclassified *Naegleria* spp., *Vahlkampfia,* and *N. dobsoni.* These detections were grouped into "other sp." as detections were rare and did not affect statistical analyses. The unclassified *Naegleria* spp. represented 6 total detections. Interestingly, of the 20 positive samples for *N. fowleri,* 17 had codetections with *N. australiensis* (Figure 2).

In hot springs with codetections of *N. fowleri* and *N. australiensis, N. australiensis* was observed to be at much higher concentrations than *N. fowleri* (Figure 3). In these hot springs, concentrations of *N. fowleri* ranged between 4.9 and 115.7 cells/L while *N. australiensis* ranged from 590 to 8557 cells/L.

3.4. Genotyping of *N. fowleri* Detections

All three genotypes of *N. fowleri* known in North America were detected in this study (Table S2). As stated in the methods, it was not possible to differentiate the genotype 2 or 3 of *N. fowleri* because of the FWS primer set used. In GRTE and YELL, genotyping showed a distinct separation of *N. fowleri* type split by geographical areas. In the GRTE region, the main genotype was the type 2/3, with only one detection of genotype 1 in Granite Hot Spring. In YELL, Lewis Lake hot springs showed genotype 1, while the Firehole River and Boiling River showed type 2/3. These results indicate potentially different origins of *N. fowleri* in the area. However, in LAKE, Nevada Hot Spring showed both the type 1 and type 2/3 *N. fowleri* at differing sample points.

3.5. Statistical Analysis

Statistical analyses were performed separately on samples analyzed by the two qPCR methods. The aim of the analysis was to assess associations between the presence or absence of *N. fowleri* and the temperature and pH, adjusting for park, month, year, and other species detected. For the USGS samples (following Mull et al. [2013]), there was a significant difference in the presence of *N. fowleri* with year ($p = 0.005$), but not with month ($p = 0.228$) or park ($p = 0.56$) (Figure 4, OR and 95% CI in Supporting Information Table S5). The presence of *N. fowleri* in these samples was dominated by sampling in the years of 2018 and 2019. A similar trend was observed in the samples analyzed following Puzon et al. (2009) method. A significant difference between sample years was observed while comparing 2023 samples to 2018/2022 samples (OR (95%CI): 21.44 (2.14, 2097.70), $p = 0.015$) (Figure 5, OR and 95% CI in Supporting Information Table S6). However, there was a significant difference associated with location (i.e., Park sampled), with GRTE ($p = 1.1e-07$) showing an increased likelihood of *N. fowleri* presence, and YELL (OR [95% CI]: 0.0026 [0.000014, 0.037]), OLYM YELL (OR [95% CI]: 0.0018 [0.0000032, 0.052]), and NNVM YELL (OR [95% CI]: 0.0096 [0.0000002, 0.031]) showing a relative absence of *N. fowleri.* No significant relationship was found between pH and the presence/absence of *N. fowleri* and temperature and the presence/absence of *N. fowleri* at the sample sites included in this study (Figures 4 and 5).

4. DISCUSSION

This multiyear study documented the presence of FLA in recreational hot springs across the western United States. *N. fowleri* was found to be more widespread than previously identified within three national parks where it has been detected (YELL, GRTE, and LAKE), and this study serves as a baseline for two previously untested recreational areas (OLYM and NNVM) where nonpathogenic *Naegleria* spp. were detected and *N. fowleri* was not detected in the samples. This study also confirms the presence of *N. fowleri* in multiple hot springs within YELL and GRTE where it has previously been reported[20,25,44,45] and also LAKE where there have been warnings regarding potential presence of *N. fowleri.*[46] Concentrations of *N. fowleri* in hot springs ranged from 0 to 115.7 *N. fowleri* cells/L. The only published *N. fowleri* concentration limit for recreational water is from the Public Health Ministry of France which has established a maximum of 100 *N. fowleri* cells/L at sites with human recreation.[47] However, Moussa et al. (2013) showed that a fatality occurred

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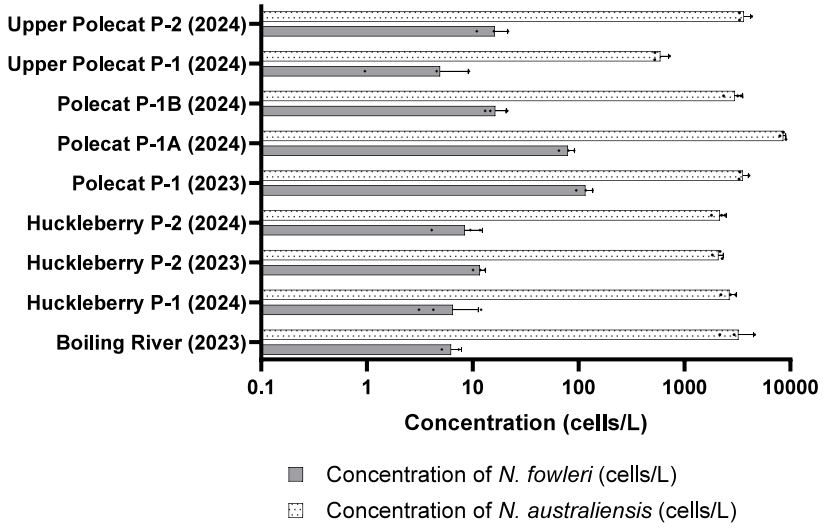

Figure 3. Concentrations of *Naegleria fowleri* and *Naegleria australiensis* at sites with codetection of each species. Each bar represents the average concentration (cells/L), with individual values represented as dots and error bars plotted.

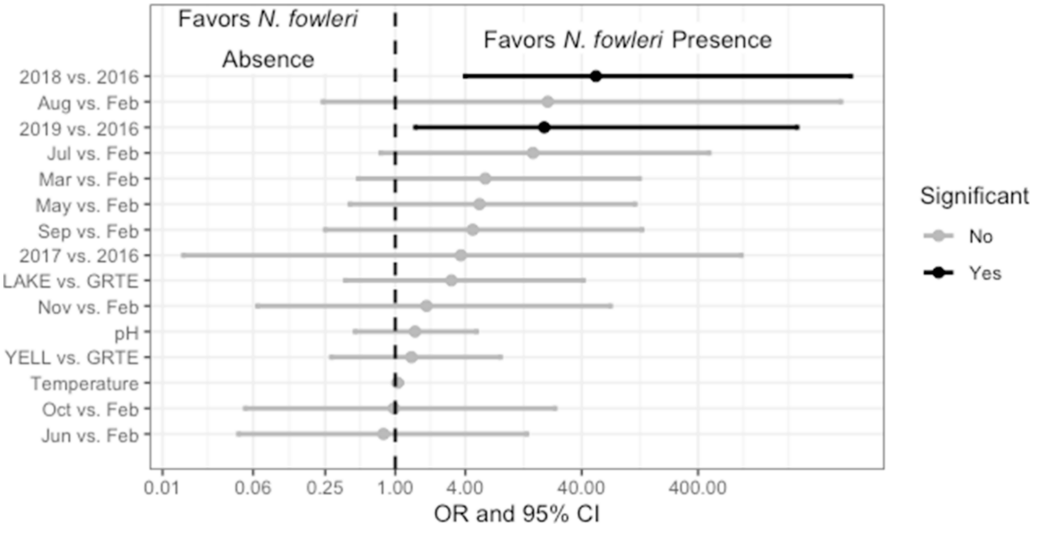

Figure 4. Statistical analysis from samples analyzed following Mull et al. (2013) quantitative polymerase chain reaction methods showing odds ratio (OR) and confidence interval (CI). The *y*-axis represents variables under comparison (year or month sampled, area sampled, and geochemistry), with *x*-axis representing the odds ratio. Corresponding lines are the 95% confidence interval of each comparison, with the significant *p*-value of $p < 0.05$.

in hot springs in the French West Indies with *N. fowleri* concentrations up to 22 *N. fowleri* cells/L via culturing.[5] These cell density numbers were most likely underestimated as culturing methods have proven to be difficult for enumerating low concentrations of *N. fowleri*.[48] Polecat Hot Springs in GRTE, a popular area with recreational hot spring soakers, had concentrations of *N. fowleri* of 78, 16, and 115.7 cells/L during a two-year period. It should be noted that the Australian drinking water quality guideline recommends a much lower limit of 2 *N. fowleri* cells/L,[33] which all the *N. fowleri* positive sites in this study were above. While a fatal PAM infection has not been reported at any of the sites sampled, the concentrations of *N. fowleri* observed here are within the limits of known PAM infections, which highlights potential benefits of informational signs, as well as public warnings, at sites to help prevent a future fatality.

To our knowledge, this is the first study to report *N. fowleri* detections in Lewis Lake Hot Springs, upper Polecat Hot Spring, Nevada Hot Spring, Boy Scout Hot Spring, Blue Point Hot Spring and Rogers Hot Spring. In addition, four of the other sample sites, Huckleberry Hot Spring, Polecat Hot Spring, the Boiling River, and the Firehole River confirmed past studies showing *N. fowleri* detections.[20,25,45] All *N. fowleri* detections in GRTE and YELL are associated with direct thermal influence except for the Firehole Canyon Swimming Area. Although the Firehole Canyon Swimming Area does not have the *direct* thermal influence, the Firehole River itself receives significant thermal input from the Upper, Midway and Lower Geyser Basins located between 6 and 13 miles upstream.[49] The detections of *N. fowleri* near Goose Lake are upstream of the Firehole Canyon Swimming Area and the site is near thermal springs with temperatures around 37 °C (Table S4). At LAKE, four of the five sample sites tested positive for *N. fowleri*. The Blue Point and Rogers Hot Springs feed directly into Lake Mead, potentially serving as a source for *N. fowleri* presence and persistence in Lake Mead. Nevada has

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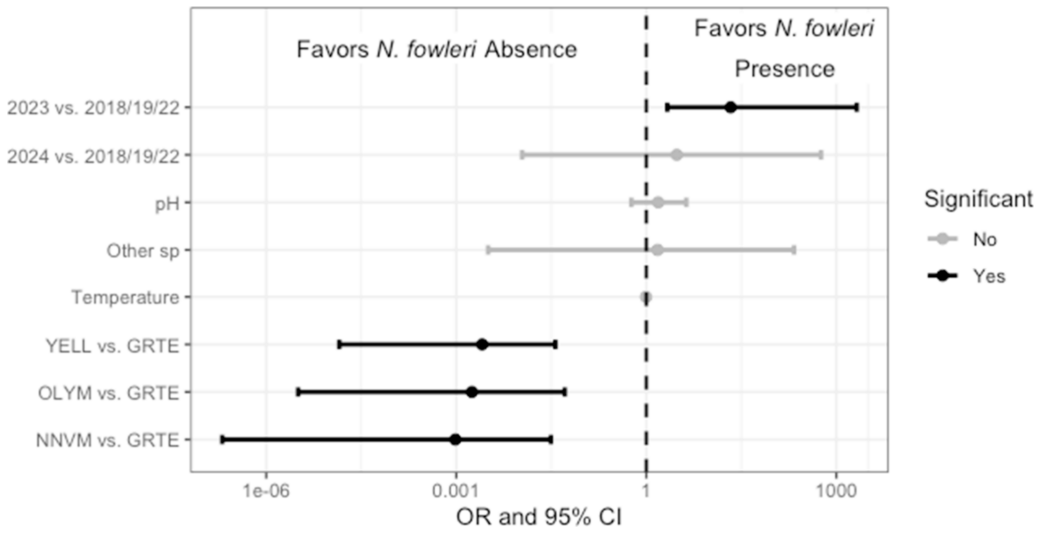

Figure 5. Statistical analysis from samples analyzed following Puzon et al. (2009) quantitative polymerase chain reaction methods showing odds ratio (OR) and confidence interval (95% CI). The *y*-axis represents variables under comparison (year or month sampled, area sampled, and geochemistry), with *x*-axis representing the odds ratio. Corresponding lines are the 95% confidence interval of each comparison, with the significant *p*-value of $p < 0.05$.

recorded multiple reported fatalities from *N. fowleri*, one from Ash Hot Springs located 100 miles north of Las Vegas, and one in Lake Mead in 2022.[50−52] The detections around the LAKE follow the trend of an increase in PAM cases observed in Nevada.

In this study, the temperature range 17.4 to 46 °C of sites with *N. fowleri* fall within ranges previously reported in the literature (16−47 °C).[53] Both qPCR methods are able to detect cysts;[35,36] however, our methodology of collecting bulk water samples most likely missed the cysts embedded in the river bed sediment. The lowest temperature with a positive *N. fowleri* detection was 17.4 °C in the Firehole Canyon Swimming Area in September of 2018. *N. fowleri* is reported to stop replication around 20 °C; however, cysts can survive for extended periods below 10 °C.[15,17,18] The lowest temperature for a confirmed *N. fowleri* fatality was in Minnesota with the surface water temperature of 22 °C.[4] The coldest sample in this study was collected in the Firehole Canyon Swimming Area at 10.8 °C in November 2018 and was negative for *N. fowleri*. At low temperatures such as those during winter months, *N. fowleri* can encyst and remain dormant while embedded in sediment to proliferate once conditions become favorable.[54] The highest temperature for *N. fowleri* detection in this study was 54.9 °C at the Boiling River in November 2018. In fact, *N. fowleri* was detected in the Boiling River at four sample points at temperatures greater than 49 °C between July 2018-November 2018 and once in July 2019. The maximum temperature for *N. fowleri* growth is reported to be 46 °C,[16] which correlates with the upper temperature threshold of our samples.

The pH range of *N. fowleri* detections were from pH 5 to 8.4. In the laboratory, *N. fowleri* is reported to persist between pH 2 to 11, with attrition at the upper and lower limits as determined by 72 h survival.[53,55,56] Witch Creek, located within the Heart Lake Geyser Basin, did not have any detections for *N. fowleri*; however, *N. australiensis* was detected. Witch Creek is an alkaline creek with a pH around 9 nearest to the thermal features, and down to pH 7 once it reaches Heart Lake. Although this is within the pH range reported in the literature, higher pH ranges in the environment may tend to favor *N. australiensis* over *N. fowleri* colonization.

In this study, *N. australiensis* was detected at all sample sites in OLYM and NNVM, and at multiple sites within YELL, GRTE and LAKE. *Naegleria australiensis* is found in freshwater and drinking water systems worldwide.[57−59] Previous studies have shown that *N. australiensis* is prevalent in hot springs in YELL and GRTE.[25,45] Our study confirms this and further demonstrates the broad distribution of the species across hot springs in the western United States. *N. australiensis* has been shown to be pathogenic in mice studies, however it has not been associated with PAM in humans.[12] The more virulent strains of *N. australiensis* (virulence in mice) found in the Greater Yellowstone Ecosystem were from Huckleberry Hot Spring and a commercial spring North of YELL.[45] It remains unknown if *N. australiensis* is pathogenic in humans. Further, in this study *N. dobsoni* and *N. lovaniensis* were detected in YELL; however, these are reported as nonpathogenic *Naegleria* species.[60]

Co-detections of *N. fowleri* and *N. australiensis* were observed at the Boiling River, Polecat, Huckleberry, and Lewis Lake Hot Springs (Figure 3). The Boiling River in 2024 showed detections of both *N. australiensis* and *N. lovaniensis*. Barnhart et al. (2024) showed an overall negative association of *N. fowleri* and *N. australiensis* in hot spring samples collected in GRTE.[25] However, in this more expansive study, although *N. fowleri* and *N. australiensis* are positively associated in hot springs (Figures 2 and 3), the codetections were not statistically significant. The concentration of *N. australiensis* was shown to be much greater (10^2−10^3 fold concentration) than *N. fowleri* in all samples with codetection (Figure 3). The concentration of *N. australiensis* ranged between 590 and 8557 cells/L, compared to 4.9−115.7 *N. fowleri* cells/L (Figure 3). Since samples in our study were taken from the same months in different years, it is unclear whether these *Naegleria* spp. are coinhabitants of the hot springs, if a seasonal shift is occurring, or if multiple food sources supporting *N. fowleri* and *N. australiensis* are present.[26] Further study with more sampling

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times could help enhance understanding of the complex relationship between these *Naegleria* species.

The genotyping of *N. fowleri* is important during detection studies because certain genotypes are continent-specific and give insights into the migration of *N. fowleri*. In North America, the genotype 1 of *N. fowleri* has only been found in California from 2 patients.[40] Interestingly, in our study genotype 1 had the most detections in LAKE (3/6 detections) compared to YELL (2/4 detections) and GRTE (1/15 detections). This could provide more evidence of the genotype 1 being predominately in western states, and the transition to genotype 2/3 moving to eastern states. Interestingly, our results show a geographical split in genotypes of GRTE and YELL, while LAKE showed a codetection in the same hot spring. The codetection of different *N. fowleri* genotypes in the same body of water has been shown in Europe;[40] however, we are unaware of any studies showing long-term population dynamics between different genotypes of *N. fowleri*. These results show the potential of genotyping to allow for mapping of *N. fowleri* genotypes by water body.

Interestingly, *N. fowleri* detections were significantly different between year and not by month (Figures 4 and 5). This indicates that smaller, seasonal variations may not alter the presence or absence of *N. fowleri*. Instead, there may be larger, more impactful events occurring between years which alters the microbial community; however, we do acknowledge that thermal waters and thermally impacted waters are less likely to see large seasonal variations in temperature, which result in minimal variation between sample months, but the data indicate that the health risk of *N. fowleri* infection persists in thermal and thermal impacted waters even when surrounding air temperatures area low.

In addition, MSU/CSIRO samples showed statistically significant association between *N. fowleri* present in GRTE compared to YELL, OLYM and NNVM. Stahl et al. (2025) predicted potential *N. fowleri* ecological niches by watershed and showed sites at risk are most likely correlated with dense human populations in proximity to natural waters; however, they did state that national parks pose an interesting case as research into *N. fowleri* in remote areas is limited to few studies.[61] All four sites within GRTE sampled by MSU/CSIRO (Polecat, upper Polecat, and Huckleberry Hot Springs network) are relatively close to one another. It is unclear if these hot springs are connected via groundwater; however, they are in the same watershed which could have caused *N. fowleri* to spread through the area. Further study of a larger number of GRTE sites would likely provide additional insight on the prominence of *N. fowleri* detections in this area and how entire watersheds may be affected by *N. fowleri* colonization.

Although PAM is a rare disease, cases are reported globally and are predicted to become more frequent.[62] Recently, in Kerala, India there have been five cases of amoebic meningoencephalitis between May and July of 2024.[63] In Pakistan, an outbreak of *N. fowleri* in 2023 resulted in 7 fatalities.[64] In the United States, while there has not been an increase in PAM cases per year, incidence of PAM has seen northern movement into new states since recording started in 1978.[10] These states include Minnesota, Iowa, Nebraska, Nevada and Arizona, showing a geographical trend of cases moving north and west.[4] In addition, recent studies have shown *N. fowleri* colonizing the brackish water in Lake Pontchartrain and numerous drinking water distribution systems in Louisiana.[22,24] The PAM cases from Ash Hot Springs in Nevada, Lake of Three Fires in Iowa, a splash pad in Arkansas, and nasal rinse users in the southeastern United States show *N. fowleri* colonizes many types of water systems in the United States.[8,27,28,51,65] Further study into the hot springs that were positive for *N. fowleri* may provide insight into the spread, colonization, and conditions supporting these amoebic pathogens as global water temperatures increase. In this study, we present results demonstrating that long-term monitoring of *N. fowleri* is feasible across a broad geographic area of the United States over multiple years. Building on this, long-term monitoring could support the development of statistical models to better predict the occurrence and distribution of *N. fowleri* across diverse environmental and geographic conditions.

5. CONCLUSIONS

This study provides the first broad survey of FLA in recreational thermal waters across the western United States. Our findings indicate that *N. fowleri* is geographically widespread, with detections in YELL, GRTE and LAKE. *N. fowleri* was detected in well-known and previously untested hot springs, including sites with high recreational use. The detection of *N. fowleri* across a broad range of environmental conditions, spanning temperatures between 17.7 to 54.9 °C and pH 5.0 to 8.4, shows the robustness of *N. fowleri* in natural water systems. Measured concentrations of *N. fowleri* ranged from 4.9 to 115.7 cells/L, showing that sites within YELL and GRTE are at concentrations higher than those known to have caused *N. fowleri* (PAM) infections previously. Co-detections with other FLA species, specifically *N. australiensis*, in these natural systems suggests there may be a complex interspecies dynamic in natural water systems which appears to differ with engineered water systems. These results contribute to a growing body of evidence characterizing the environmental distribution of *N. fowleri* and highlight the utility of DNA-based monitoring approaches across broad spatial and temporal scales. These long-term results provide the foundation for future risk models that can be used to understand the factors that influence *N. fowleri* occurrence and distribution.

■ ASSOCIATED CONTENT

Data Availability Statement

USGS metadata used for this study: qPCR Results for An Assessment of Pathogens in National Park Hot Springs | U.S. Geological Survey (https://www.usgs.gov/data/qpcr-results-assessment-pathogens-national-park-hot-springs).[34]

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsestwater.5c01243.

Images identifying the different sampling areas within each national park and methods, results and amoebae sequences, data for genotyping, metadata of all sample sites (PDF)

Results, amoebae sequences, data for genotyping, and metadata of all sample sites (PDF)

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Kofman, A.; Guarner, J. Infections Caused by Free-Living Amoebae. *J. Clin Microbiol* **2022**, *60* (1), No. e0022821.

(2) Grace, E.; Asbill, S.; Virga, K. *Naegleria fowleri*: pathogenesis, diagnosis, and treatment options. *Antimicrob. Agents Chemother.* **2015**, *59* (11), 6677−6681.

(3) Güémez, A.; García, E. Primary Amoebic Meningoencephalitis by *Naegleria fowleri*: Pathogenesis and Treatments. *Biomolecules* **2021**, *11* (9), 1320.

(4) Kemble, S. K.; Lynfield, R.; DeVries, A. S.; Drehner, D. M.; Pomputius, W. F., 3rd; Beach, M. J.; Visvesvara, G. S.; da Silva, A. J.; Hill, V. R.; Yoder, J. S.; et al. Fatal *Naegleria fowleri* infection acquired in Minnesota: possible expanded range of a deadly thermophilic organism. *Clin Infect Dis* **2012**, *54* (6), 805−809.

(5) Moussa, M.; De Jonckheere, J. F.; Guerlotté, J.; Richard, V.; Bastaraud, A.; Romana, M.; Talarmin, A. Survey of *Naegleria fowleri* in geothermal recreational waters of Guadeloupe (French West Indies). *PLoS One* **2013**, *8* (1), No. e54414.

(6) Gharpure, R.; Bliton, J.; Goodman, A.; Ali, I. K. M.; Yoder, J.; Cope, J. R. Epidemiology and Clinical Characteristics of Primary Amebic Meningoencephalitis Caused by *Naegleria fowleri*: A Global Review. *Clin Infect Dis* **2021**, *73* (1), e19−e27.

(7) Abrahams-Sandí, E.; Retana-Moreira, L.; Castro-Castillo, A.; Reyes-Batlle, M.; Lorenzo-Morales, J. Fatal meningoencephalitis in child and isolation of *Naegleria fowleri* from hot springs in Costa Rica. *Emerg Infect Dis* **2015**, *21* (2), 382−384.

(8) Yoder, J. S.; Straif-Bourgeois, S.; Roy, S. L.; Moore, T. A.; Visvesvara, G. S.; Ratard, R. C.; Hill, V. R.; Wilson, J. D.; Linscott, A. J.; Crager, R.; et al. Primary amebic meningoencephalitis deaths associated with sinus irrigation using contaminated tap water. *Clin Infect Dis* **2012**, *55* (9), e79−85.

(9) Matanock, A.; Mehal, J. M.; Liu, L.; Blau, D. M.; Cope, J. R. Estimation of Undiagnosed *Naegleria fowleri* Primary Amebic Meningoencephalitis, United States. *Emerg Infect Dis* **2018**, *24* (1), 162−164.

(10) Gharpure, R.; Gleason, M.; Salah, Z.; Blackstock, A. J.; Hess-Homeier, D.; Yoder, J. S.; Ali, I. K. M.; Collier, S. A.; Cope, J. R. Geographic Range of Recreational Water-Associated Primary Amebic Meningoencephalitis, United States, 1978−2018. *Emerg Infect Dis* **2021**, *27* (1), 271−274.

(11) Dereeper, A.; Allouch, N.; Guerlais, V.; Garnier, M.; Ma, L.; De Jonckheere, J. F.; Joseph, S. J.; Ali, I. K. M.; Talarmin, A.; Marcelino, I. *Naegleria* genus pangenome reveals new structural and functional insights into the versatility of these free-living amoebae. *Front Microbiol* **2023**, *13*, 1056418.

(12) De Jonckheere, J. F.; Pernin, P.; Scaglia, M.; Michel, R. A comparative study of 14 strains of *Naegleria australiensis* demonstrates the existence of a highly virulent subspecies: *N. australiensis italica* n. spp. *J. Protozool* **1984**, *31* (2), 324−331.

(13) Robinson, B. S.; Monis, P. T.; Henderson, M.; Gelonese, S.; Ferrante, A. Detection and significance of the potentially pathogenic amoeboflagellate *Naegleria italica* in Australia. *Parasitol Int.* **2004**, *53* (1), 23−27.

(14) Moussa, M.; Tissot, O.; Guerlotté, J.; De Jonckheere, J. F.; Talarmin, A. Soil is the origin for the presence of *Naegleria fowleri* in the thermal recreational waters. *Parasitol Res.* **2015**, *114* (1), 311−315.

(15) Chang, S. L. Resistance of pathogenic *Naegleria* to some common physical and chemical agents. *Appl. Environ. Microbiol.* **1978**, *35* (2), 368−375.

(16) Griffin, J. L. Temperature tolerance of pathogenic and nonpathogenic free-living amoebas. *Science* **1972**, *178* (4063), 869−870.

(17) Cerva, L. Some further characteristics of the growth of *Naegleria fowleri* and *Naegleria gruberi* in axenic culture. *Folia Parasitol.* **1978**, *25* (1), 1−8.

(18) Puzon, G. J.; Miller, H. C.; Malinowski, N.; Walsh, T.; Morgan, M. J. *Naegleria fowleri* in drinking water distribution systems. *Current Opinion in Environmental Science & Health* **2020**, *16*, 22−27.

(19) Sykora, J. L.; Keleti, G.; Martinez, A. J. Occurrence and pathogenicity of *Naegleria fowleri* in artificially heated waters. *Appl. Environ. Microbiol.* **1983**, *45* (3), 974−979.

(20) Sheehan, K. B.; Fagg, J. A.; Ferris, M. J.; Henson, J. M. PCR detection and analysis of the free-living amoeba *Naegleria* in hot springs in Yellowstone and Grand Teton National Parks. *Appl. Environ. Microbiol.* **2003**, *69* (10), 5914−5918.

(21) Blair, B.; Sarkar, P.; Bright, K. R.; Marciano-Cabral, F.; Gerba, C. P. *Naegleria fowleri* in well water. *Emerg Infect Dis* **2008**, *14* (9), 1499−1501.

(22) A Shahin, S.; H Alarcon, J.; Brosky, H. N.; Lamori, J. G.; Dada, A. C.; Xue, J.; Gyawali, P.; P Sherchan, S. Occurrence of *Naegleria fowleri* and faecal indicators in sediments from Lake Pontchartrain, Louisiana. *J. Water Health* **2022**, *20* (4), 657−669.

(23) Mapili, K.; Rhoads, W. J.; Coughter, M.; Pieper, K. J.; Edwards, M. A.; Pruden, A. Occurrence of opportunistic pathogens in private wells after major flooding events: A four state molecular survey. *Science of The Total Environment* **2022**, *826*, No. 153901.

(24) Ward, L.; Sherchan, S. P. Surveillance of *Naegleria fowleri* in Louisiana's public water systems. *J. Water Health* **2023**, *21* (11), 1627−1631.

(25) Barnhart, E. P.; Kinsey, S. M.; Wright, P. R.; Caldwell, S. L.; Hill, V.; Kahler, A.; Mattioli, M.; Cornman, R. S.; Iwanowicz, D.; Eddy, Z.; et al. *Naegleria fowleri* Detected in Grand Teton National Park Hot Springs. *ACS ES&T Water* **2024**, *4*, 628.

(26) Malinowski, N.; Morgan, M. J.; Wylie, J.; Walsh, T.; Domingos, S.; Metcalfe, S.; Kaksonen, A. H.; Barnhart, E. P.; Mueller, R.; Peyton, B. M.; et al. Prokaryotic microbial ecology as an ecosurveillance tool for eukaryotic pathogen colonisation: *Meiothermus* and *Naegleria fowleri*. *Water Res.* **2024**, *254*, No. 121426.

(27) Dulski, T. M.; Montgomery, F.; Ramos, J. M.; Rosenbaum, E. R.; Boyanton, B. L., Jr.; Cox, C. M.; Dahl, S.; Kitchens, C.; Paul, T.; Kahler, A.; et al. Fatal Case of Splash Pad-Associated *Naegleria fowleri* Meningoencephalitis - Pulaski County, Arkansas, September 2023. *MMWR Morb Mortal Wkly Rep* **2025**, *74* (10), 167−172.

(28) Smith, O.; Tillman, W.; Lewis, J. B.; et al. Notes from the Field: Primary Amebic Meningoencephalitis Associated with Nasal Irrigation Using Water from a Recreational Vehicle - Texas, 2024. *MMWR Morb Mortal Wkly Rep* **2025**, *74*, 334−335.

(29) Kelly, R. B.; Francine, M.-C.; Charles, P. G. Occurrence of *Naegleria fowleri* in Arizona drinking water supply wells. *Journal AWWA* **2009**, *101* (11), 43−50.

(30) Laseke, I.; Korte, J.; Lamendella, R.; Kaneshiro, E. S.; Marciano-Cabral, F.; Oerther, D. B. Identification of *Naegleria fowleri* in warm ground water aquifers. *J. Environ. Qual* **2010**, *39* (1), 147−153.

(31) Xue, J.; Lamar, F. G.; Zhang, B.; Lin, S.; Lamori, J. G.; Sherchan, S. P. Quantitative assessment of *Naegleria fowleri* and fecal indicator bacteria in brackish water of Lake Pontchartrain, Louisiana. *Science of the total environment* **2018**, *622*−*623*, 8−16.

(32) Dorsch, M. M.; Cameron, A. S.; Robinson, B. S. The epidemiology and control of primary amoebic meningoencephalitis with particular reference to South Australia. *Trans R Soc. Trop Med. Hyg* **1983**, *77* (3), 372−377.

(33) NHMRC. *Australian Drinking Water Guidelines Paper 6 National Water Quality Management Strategy*. National Health and Medical Research Council, N. R. M. M. C., Commonwealth of Australia, Canberra, Ed.; National Water Quality Management Strategy., 2011.

(34) Kageyama, S. A.; Schreier, T. M.; Merkes, C. M.; Barnhart, E. P.; Wright, P. R.; Kinsey, S. M.; Day, J.; Thom, T.; Puzon, G.; Brugger, C. L.*qPCR Results for An Assessment of Pathogens in National Park Hot Springs*: U.S. Geological Survey data release **2024**.

(35) Mull, B. J.; Narayanan, J.; Hill, V. R. Improved Method for the Detection and Quantification of *Naegleria fowleri* in Water and Sediment Using Immunomagnetic Separation and Real-Time PCR. *J. Parasitol Res.* **2013**, *2013*, 608367.

(36) Puzon, G. J.; Lancaster, J. A.; Wylie, J. T.; Plumb, J. J. Rapid Detection of *Naegleria fowleri* in Water Distribution Pipeline Biofilms

https://doi.org/10.1021/acsestwater.5c01243
ACS EST Water 2026, 6, 1704−1715

and Drinking Water Samples. *Environ. Sci. Technol.* **2009**, *43* (17), 6691−6696.
(37) Pélandakis, M.; Serre, S.; Pernin, P. Analysis of the 5.8S rRNA Gene and the Internal Transcribed Spacers in *Naegleria* spp. and in *N. fowleri*. *Journal of eukaryotic microbiology* **2000**, *47* (2), 116−121.
(38) De Jonckheere, J. F. Sequence Variation in the Ribosomal Internal Transcribed Spacers, Including the 5.8S rDNA, of *Naegleria* spp. *Protist* **1998**, *149* (3), 221−228.
(39) Zhou, L.; Sriram, R.; Visvesvara, G. S.; Xiao, L. Genetic variations in the internal transcribed spacer and mitochondrial small subunit rRNA gene of *Naegleria* spp. *J. Eukaryot Microbiol* **2003**, *50* (Suppl), 522−526.
(40) De Jonckheere, J. F. Origin and evolution of the worldwide distributed pathogenic amoeboflagellate *Naegleria fowleri*. *Infect Genet Evol* **2011**, *11* (7), 1520−1528.
(41) Firth, D. Bias reduction of maximum likelihood estimates. *Biometrika* **1993**, *80* (1), 27−38.
(42) Heinze, G.; Schemper, M. A solution to the problem of separation in logistic regression. *Stat Med.* **2002**, *21* (16), 2409−2419.
(43) Georg Heinze, M. P.; Dunkler, D.; Southworth, H.; Jiricka, L.; Steiner, G *logistf: Firth's Bias-Reduced Logistic Regression. R package version 1.26.1* **2025**; http://CRAN.R-project.org/package=logistf
(44) Detterline, J. L.; Wilhelm, W. E. Survey of Pathogenic *Naegleria fowleri* and Thermotolerant Amebas in Federal Recreational Waters. *Transactions of the American Microscopical Society* **1991**, *110* (3), 244−261.
(45) Ramaley, R.; Scanlan, P.; O'Dell, W. Presence of Thermophilic *Naegleria* Isolates in the Yellowstone and Grand Teton National Parks. In *Thermophiles: Biodiversity, Ecology, and Evolution*, Reysenbach, A. L; Springer, 2001; pp 41−50.
(46) NPS. *Lake Mead Recreation Area Safety*. Area, L. M. N. R., Ed.; nps.gov, 2023.
(47) Cabanes, P. A.; Wallet, F.; Pringuez, E.; Pernin, P. Assessing the risk of primary amoebic meningoencephalitis from swimming in the presence of environmental *Naegleria fowleri*. *Appl. Environ. Microbiol.* **2001**, *67* (7), 2927−2931.
(48) Pernin, P.; Pélandakis, M.; Rouby, Y.; Faure, A.; Siclet, F. Comparative recoveries of *Naegleria fowleri* amoebae from seeded river water by filtration and centrifugation. *Appl. Environ. Microbiol.* **1998**, *64* (3), 955−959.
(49) Hurwitz, S.; Hunt, A.; Evans, W. Temporal variations of geyser water chemistry in the Upper Geyser Basin, Yellowstone National Park, USA. *Geochemistry, Geophysics, Geosystems* **2012**, *13*, 12005.
(50) ProMED. *Amebic Meningoencephalitis, Primary - USA (Nevada) Pakistan (SINDH)*; International Society for Infectious Diseases**2022**, (8706319).
(51) ProMED. *Amebic Meningoencephalitis, Primary - USA: (Nevada)*; International Society for Infectious Diseases**2023**, (7/24/2023).
(52) SNHD. *Southern Nevada Health District Release October 19, 2022*; southernnevadahealthdistrict.org **2022**.
(53) Stahl, L. M.; Olson, J. B. Environmental abiotic and biotic factors affecting the distribution and abundance of *Naegleria fowleri*. *FEMS Microbiol Ecol* **2020**, *97* (1), fiaa238.
(54) Wellings, F. M.; Amuso, P. T.; Chang, S. L.; Lewis, A. L. Isolation and identification of pathogenic *Naegleria* from Florida lakes. *Appl. Environ. Microbiol.* **1977**, *34* (6), 661−667.
(55) Lam, C.; He, L.; Marciano-Cabral, F. The Effect of Different Environmental Conditions on the Viability of *Naegleria fowleri* Amoebae. *J. Eukaryot Microbiol* **2019**, *66* (5), 752−756.
(56) Stahl, L. M.; Olson, J. B. Investigating the interactive effects of temperature, pH, and salinity on *Naegleria fowleri* persistence. *J. Eukaryot Microbiol* **2023**, *70* (3), No. e12964.
(57) Edagawa, A.; Kimura, A.; Kawabuchi-Kurata, T.; Kusuhara, Y.; Karanis, P. Isolation and genotyping of potentially pathogenic *Acanthamoeba* and *Naegleria* species from tap-water sources in Osaka, Japan. *Parasitol Res.* **2009**, *105* (4), 1109−1117.
(58) Panda, A.; Khalil, S.; Mirdha, B. R.; Singh, Y.; Kaushik, S. Prevalence of *Naegleria fowleri* in Environmental Samples from Northern Part of India. *PLoS One* **2015**, *10* (10), No. e0137736.
(59) Milanez, G.; Masangkay, F.; Somsak, V.; Kotepui, M.; Tangpong, J.; Karanis, P. Occurrence and the first report of *Naegleria australiensis* presence in a major lake in the Philippines. *Journal of Water and Health* **2019**, *17* (4), 647−653.
(60) Jamerson, M.; da Rocha-Azevedo, B.; Cabral, G. A.; Marciano-Cabral, F. Pathogenic *Naegleria fowleri* and non-pathogenic *Naegleria lovaniensis* exhibit differential adhesion to, and invasion of, extracellular matrix proteins. *Microbiology* **2012**, *158* (Pt 3), 791−803.
(61) Stahl, L. M.; Snead, A. A.; Olson, J. B. Predicting *Naegleria fowleri* freshwater habitat suitability in the United States for the present-day and future using ecological niche modeling. *Water Res.* **2025**, *285*, No. 124018.
(62) Maciver, S. K.; Piñero, J. E.; Lorenzo-Morales, J. Is *Naegleria fowleri* an Emerging Parasite? *Trends Parasitol* **2020**, *36* (1), 19−28.
(63) ProMED. *Amebic Meningoencephalitis, Primary - India: (Kerala)*; International Society for Infectious Diseases**2024**, (8717540).
(64) Nadeem, A.; Malik, I. A.; Afridi, E. K.; Shariq, F. *Naegleria fowleri* outbreak in Pakistan: unveiling the crisis and path to recovery. *Front Public Health* **2023**, *11*, 1266400.
(65) Berger, J. R. Amebic infections of the central nervous system. *Journal of NeuroVirology* **2022**, *28* (4), 467−472.

https://doi.org/10.1021/acsestwater.5c01243
ACS EST Water 2026, 6, 1704−1715