

## Quantitative assessment of *Naegleria fowleri* and *Escherichia coli* concentrations within a Texas reservoir

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### ABSTRACT

Previous presence/absence studies have indicated a correlation between the presence of the pathogenic amoeba *Naegleria fowleri* and the presence of bacteria, such as the fecal indicator *Escherichia coli*, in environmental surface waters. The objective of this study was to use quantitative real-time polymerase chain reaction (qPCR) methodologies to measure *N. fowleri* and *E. coli* concentrations within a Texas reservoir in late summer, and to determine if concentrations of *N. fowleri* and *E. coli* were statistically correlated. *N. fowleri* was detected in water samples from 67% of the reservoir sites tested, with concentrations ranging up to an estimated 26 CE (cell equivalents)/100 mL. *E. coli* was detected in water samples from 60% of the reservoir sites tested, with concentrations ranging up to 427 CE/100 mL. In this study, *E. coli* concentrations were not indicative of *N. fowleri* concentrations.

**Key words** | Lake Granbury, microbial water quality, primary amoebic meningoencephalitis, qPCR, quantitative real-time PCR, recreational water quality

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### INTRODUCTION

The free-living amoeba *Naegleria fowleri* is the causative agent of the rare brain disease primary amoebic meningoencephalitis (PAM) (Marciano-Cabral 1988). The disease is particularly troublesome because it mostly affects previously healthy children and young adults (Marciano-Cabral 1988) and is virtually always fatal; of the 138 documented cases in the United States (1937–2012), only one patient survived the infection (Yoder *et al.* 2010; J. S. Yoder, Centers for Disease Control and Prevention, USA, personal communication, 2012, 2013).

*N. fowleri* is both pathogenic, demonstrated by the amoeba's ability to lyse mammalian nerve cells on contact (Marciano-Cabral & Cabral 2007), and thermophilic, defined as having the ability to grow at 40 °C or higher (De Jonckheere 2002). The majority of *N. fowleri* infections are acquired intranasally as a result of swimming in warm, fresh or brackish surface water containing the amoeba (Heggie 2010). Under circumstances that are not completely understood, one or more amoebae may flow into a

swimmer's nasal cavity, attach to the nasal mucosa and migrate along the olfactory nerves into the brain (John 1982; Marciano-Cabral & Cabral 2007). The disease progresses rapidly, causing death within an average of 10 days from exposure (Yoder *et al.* 2010).

*N. fowleri* has been detected on every continent except Antarctica (De Jonckheere 2011) and has been isolated from several natural lakes and reservoirs in the USA (Wellings *et al.* 1979; Kyle & Noblet 1985; Dettlerline & Wilhelm 1991; Jamerson *et al.* 2009). The widespread distribution of *N. fowleri* is likely due to its ability to tolerate a range of physical conditions (temperature, pH and dissolved oxygen) and to encyst when conditions are unfavorable for growth (Brown *et al.* 1983; Marciano-Cabral 1988; Dettlerline & Wilhelm 1991).

Laboratory experiments have shown that a *Naegleria* spp. population will increase proportional to the availability of bacterial food sources when physical and chemical conditions are favorable (Chang 1958; Danso & Alexander 1975). The bacterium *Escherichia coli*, spread over agar

plates, is commonly used as a growth medium for *N. fowleri* (John 1982; De Jonckheere 2002). In laboratory experiments by Marciano-Cabral & Cline (1987), *N. fowleri* showed a preference for *E. coli* over several other bacterial species and a preference for *E. coli* over nerve cells.

Only a handful of studies have considered a possible link between populations of *N. fowleri* and *E. coli* in fresh surface waters. In a study of New Zealand hot springs, Brown *et al.* (1983) reported that most of the pathogenic free-living amoebae were isolated from those thermal pools with a 'high coliform count'. In a study of the vertical distribution of free-living amoebae in a South Carolina lake, Kyle & Noblet (1985) found a correlation between the presence of thermotolerant amoebae and the presence of bacteria ( $p < 0.005$ ), and they observed that 'significant numbers of [free-living amoebae] were isolated from layers of filamentous blue-green algae'. More recently, in a survey of mud-spring recreation areas in Taiwan, Hsu *et al.* (2009) found that water samples positive for *Naegleria* spp. were also higher in heterotrophic bacteria, including *E. coli* ( $p < 0.05$ ).

All of the aforementioned studies were reliant on culture methods, requiring enrichment of water samples that hamper quantification of the amoeba. Only one study (Jamerson *et al.* 2009) appears to have previously quantified both *N. fowleri* and coliforms (including *E. coli*) in surface water samples. Using a combination of techniques including nested polymerase chain reaction (PCR), culture methods and microscopy, Jamerson *et al.* (2009) found no statistically significant correlation between populations of the target microorganisms in Lake Anna, Virginia. *E. coli* concentrations averaged a relatively low 25 colony-forming units (CFU)/100 mL over the study sample period (Jamerson *et al.* 2009).

Methods using quantitative real-time PCR (qPCR) are advantageous because they allow quantification and are very sensitive, enabling detection of a single amoeba in a water sample (Guy *et al.* 2003; Qvarnstrom *et al.* 2006). Additionally, qPCR methods are rapid, often requiring only 3 hours from the time the sample is delivered to the lab until the results are available (Kephart & Bushon 2009). Several studies have demonstrated the potential for qPCR methodologies for recreational water quality monitoring, particularly with regard to monitoring of fecal indicator bacteria (FIB) such as *E. coli* and *Enterococcus* spp.

(Haugland *et al.* 2005; Kephart & Bushon 2009; Lavender & Kinzelman 2009; Marion *et al.* 2010; Noble *et al.* 2010; Wade *et al.* 2010; Clark *et al.* 2011; Schoen *et al.* 2011; Converse *et al.* 2012; Kelty *et al.* 2012). Recreational water quality monitoring of *E. coli* (and other FIB) by state and local agencies is currently easier and less expensive than monitoring for the numerous pathogens that are associated with fecal contamination (Meays *et al.* 2004). For *E. coli* in fresh water, the 2012 US Environmental Protection Agency (EPA) recreational water quality criteria recommendations include a geometric mean of 126 CFU/100 mL (US EPA 2012).

The goal of this study was to determine if *E. coli* could potentially serve as an indicator for the pathogenic amoeba *N. fowleri* in a Texas reservoir. The specific objectives were (1) to use qPCR methodologies to quantify *N. fowleri* and *E. coli* in environmental water samples, and (2) to statistically test the results for significant correlations between (a) *N. fowleri* and *E. coli* concentrations quantified by qPCR and (b) *E. coli* concentrations quantified by qPCR and *E. coli* concentrations quantified by the Colilert method.

## METHODS

### Site description

Lake Granbury, located about 30 miles southwest of Fort Worth, Texas, is a popular reservoir for recreation (Brazos River Authority 2006) and is used as a municipal, industrial and agricultural water source. Land uses within 1 mile of the lakeshore are predominantly single-family residential (42%), followed by rangeland (24%) and cropland and pasture (22%) (Lake Granbury Watershed Protection Plan Stakeholders Committee 2010).

Due to concerns about elevated *E. coli* levels in coves around the lake, the Brazos River Authority (BRA) has sampled several sites on a monthly basis since 2002, and is currently working with federal, state and local entities to implement a Watershed Protection Plan focused on reducing bacterial contamination (Brazos River Authority 2011). Through the planning process, the BRA has identified several potential sources of *E. coli* contamination, including human waste from improperly functioning waste water

treatment systems, as well as animal waste from pets, livestock, birds, and other wildlife (Lake Granbury Watershed Protection Plan Stakeholders Committee 2010).

### Sample collection

Between 11 August and 8 September 2010, a total of 102 environmental water samples were collected from 42 different sites around Lake Granbury. All 42 sites were sampled when the weather was steadily hot and dry ('baseflow conditions'). Daily temperatures were relatively constant and there was very little rainfall between sampling events. Additionally, eight of the sites were resampled on 8 September following the first heavy autumn rainfall ('stormflow conditions').

For qPCR analysis, water samples were collected at two depths. Duplicate 40 mL water samples were collected at the lake surface (0 m depth) and subsurface, at a depth of approximately 0.3 m. Samples were generally collected within 3 m of the BRA sample and data collection point(s). Labeled sample bottles were immediately put on ice and were frozen within 6 hours of collection. Sample bottles were stored at  $-80^{\circ}\text{C}$ .

At each site, BRA personnel collected water samples (at 0.3 m of depth) for *E. coli* quantification using the Colilert method (IDEXX Laboratories, Inc., Westbrook, ME). After 24 hours of incubation, this commercially available kit provides *E. coli* results in terms of most-probable number (MPN). Additionally, BRA personnel collected samples for chemical analysis and measured several physical parameters at each site. For the majority of the sampled sites, these parameters included water temperature, pH, dissolved oxygen, conductivity, salinity, Secchi depth, macrophytes and inorganics (N as  $\text{NO}_3^-$ , P as  $\text{OPO}_4^{3-}$ ,  $\text{Cl}^-$  and  $\text{SO}_4^{2-}$ ) (Painter 2011).

### Sample filtration and DNA extraction

Water samples were filtered using a technique similar to that described by Kephart & Bushon (2009). After thawing in a cold-water bath, 40 mL samples were agitated and filtered under vacuum using a magnetic filter funnel and 47 mm diameter, 0.4  $\mu\text{m}$  pore size polycarbonate filter (HTTP; Millipore Corp., Billerica, MA). Once dry, the filter was aseptically folded and placed in a 2 mL microcentrifuge tube with

cap. The funnel was rinsed between samples using sterile phosphate-buffered saline (PBS). Additionally, a blank was filtered using PBS approximately every five samples.

Samples were extracted using a silica extraction technique developed for extracting DNA from plant and insect samples (Brady *et al.* 2011) after favorable comparison to three commercial DNA extraction kits (Painter 2011). Slight modifications to the published extraction technique (Brady *et al.* 2011) were made, generally to accommodate the larger filter volume. A reusable steel-dowel pin along with 750  $\mu\text{L}$  extraction buffer (with 0.1% bovine-serum albumin or BSA) and 225  $\mu\text{L}$  potassium acetate were added to each 2 mL microtube. Following filter disruption in a bead beater, microtubes were centrifuged at 4,000  $\text{g}$  for 1 min and 700  $\mu\text{L}$  of the supernatant was transferred to a fresh 2 mL tube. Next, 1,050  $\mu\text{L}$  of DNA binding buffer and 10  $\mu\text{L}$  of acid-washed silica were added and vortex-mixed, followed by room-temperature incubation for 5 min. Tubes were centrifuged at 4,000  $\text{g}$  for 1 min to pellet the silica/DNA and the pellet was washed twice with 500  $\mu\text{L}$  of wash buffer. After removing any visible liquid, tubes were dried in a  $65^{\circ}\text{C}$  oven for 30 min. The silica was vortex-mixed with 120  $\mu\text{L}$  of elution buffer (with BSA added to 0.1% final concentration), followed by incubation at  $65^{\circ}\text{C}$  for 10 min. Lastly, tubes were centrifuged at 8,000  $\text{g}$  for 1 min to pellet the silica and 100  $\mu\text{L}$  of the supernatant (extracted DNA) was transferred to a fresh microtube.

Although an internal control qPCR assay was used to quantify inhibition (allowing for correction of the data), it was necessary to reduce the level of inhibition in the eluted DNA as much as possible (see Opel *et al.* (2010) for an in-depth discussion of PCR inhibitors). To accomplish this, a post-extraction clean-up technique using the ion-chelating agent Chelex<sup>®</sup>100 (Bio-Rad Laboratories, Hercules, CA) and the solubilization/binding buffer QX1 (Qiagen Inc., Valencia, CA) was developed and applied to all 102 samples. Briefly, the technique was performed as follows. In a 96-well plate, a 14  $\mu\text{L}$  20% Chelex solution was added to 25  $\mu\text{L}$  of eluted DNA, followed by incubation for 5 min on an orbital shaker. The plate was centrifuged, and any supernatant was pipetted into a new plate. Then 90  $\mu\text{L}$  QX1 solution and 5  $\mu\text{L}$  acid-washed silica (as above) were added to each well, and the plate was again incubated on an orbital shaker. The samples were centrifuged for 5 min

at 1,000 g, and the pellets were washed twice in 200  $\mu$ L QX1 and then twice in 200  $\mu$ L wash buffer (as above). The DNA was eluted as described previously.

### qPCR analysis

The qPCR analysis for *N. fowleri* targeted a 153 base-pair (bp) fragment of the multi-copy 18S rRNA gene as described by Qvarnstrom *et al.* (2006) (Table 1). Alignment of the Qvarnstrom primers and probe identified an apparent typographic error relating to the direction of the forward primer (corrected in this paper). A Basic Local Alignment Search Tool (BLAST) search (Altschul *et al.* 1990) verified that the target DNA sequence is unique to *N. fowleri* and that the probe will not bind to other *Naegleria* spp. included in the databases.

For *E. coli*, the qPCR analysis targeted an 83 bp fragment of the single-copy *uidA* gene as described by Frahm & Obst (2003) and Noble *et al.* (2010). The *uidA* gene codes for the enzyme  $\beta$ -D-glucuronidase and has been the target of multiple qPCR studies relating to water quality, including those by Cook & Bolster (2007), Noble *et al.* (2010), Maheux *et al.* (2011) and Srinivasan *et al.* (2011).

Both assays utilized TaqMan probes and primers synthesized by Eurofins MWG Operon (Huntsville, AL). Amplification reaction mixtures (5  $\mu$ L) were the same for both assays: 1  $\mu$ L DNA extract, 1  $\mu$ L sterile H<sub>2</sub>O, 0.25  $\mu$ L 10  $\mu$ M forward and reverse primer mix, 0.25  $\mu$ L 10  $\mu$ M

probe stock and 2.5  $\mu$ L of 2X probe master mix. The latter comprises 80 mM Tris-Cl, pH 9.0, 50 mM KCl, 10 mM MgCl<sub>2</sub>, 1.2 mM dUTP (Roche Applied Science, Indianapolis, IN), 0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dGTP (Promega, Madison, WI), 0.5 U Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA), 0.5 U LightCycler uracil-DNA glycosylase (Roche Applied Science, Indianapolis, IN) and 1 mM Na<sub>2</sub>EDTA, pH 8.0.

Quantification for both the *N. fowleri* and *E. coli* assays relied on external standard curves based on a dilution series (10<sup>-1</sup> to 10<sup>-12</sup>) (Painter 2011). Genomic DNA, provided by F. Marciano-Cabral (Virginia Commonwealth University), was utilized for the *N. fowleri* standard curve and as a standard across multiple qPCR assays (ATCC 30894, trophozoite stage). For the *E. coli* assay, the target DNA sequence was amplified from a laboratory strain (TOP10, Life Technologies, Grand Island, NY).

All qPCR assays were performed using a LightCycler 480 Real-Time PCR System with software version 1.5.0 (Roche Diagnostics Corp., Indianapolis, IN). Assays for *N. fowleri* and *E. coli*, as well as an internal positive control (IPC) assay to quantify inhibition, were each run separately. For the *N. fowleri* and *E. coli* assays, all Lake Granbury water samples (and the associated blanks) were run in replicates of five along with 30 negative controls and five positive controls on 384-well plates. The IPC assay was based on Schaad *et al.* (2002) and it targeted the 16S–23S internal

**Table 1** | Summary of qPCR assay conditions used in this study

Target organism	Target gene	Primer or probe name	Sequence (5' → 3')	Program	Reference
<i>Naegleria fowleri</i>	18S rRNA	NaegIF192	GTG CTG AAA CCT AGC TAT TGT AAC TCA GT	Denature: 95 °C for 3 min	Qvarnstrom <i>et al.</i> (2006)
		NaegIR344	CAC TAG AAA AAG CAA ACC TGA AAG G	Amplify: (95 °C for 10 s, 63 °C for 10 s, 72 °C for 10 s) × 45	
		NfowlP	HEX <sup>a</sup> -ATA GCA-ATA TAT TCA GGG GAG CTG GGC-BHQ1 <sup>b</sup>		
<i>Escherichia coli</i>	<i>uidA</i>	784F	GTG TGA TAT CTA CCC GCT TCG C	Denature: 95 °C for 3 min	Frahm & Obst (2003)
		866R	AGA ACG GTT TGT GGT TAA TCA GGA	Amplify: (95 °C for 10 s, 60 °C for 10 s, 72 °C for 10 s) × 45	
		Ec807	FAM <sup>c</sup> -TCG GCA TCC GGT CAG TGG CAG T-TAMRA <sup>d</sup>		

<sup>a</sup>HEX, 6-carboxyhexachlorofluorescein, fluorescence reporter dye, Em = 556 nm.

<sup>b</sup>BHQ1, Black Hole Quencher –1, non-fluorescent quencher.

<sup>c</sup>FAM, 6-carboxyfluorescein, fluorescence reporter dye, Em = 525 nm.

<sup>d</sup>TAMRA, carboxytetramethylrhodamine, quencher.

transcribed spacer (ITS) region of the plant bacterium *Xylella fastidiosa*, a xylem-limited plant pathogen that adheres to polysaccharide-coated surfaces (Killiny & Almeida 2009) and is unlikely to be found in aquatic environments. In order to assess the relative level of inhibition, extracted DNA for each environmental water sample was mixed with a standard amount of IPC target DNA and the qPCR results were compared to an IPC target DNA mixed with sterile water.

Quantification of ribosomal DNA copy number in *N. fowleri* (ATCC 30894, trophozoite stage) was made by amplifying the 18S ribosomal locus as previously described (Qvarnstrom *et al.* 2006) and comparing Cp (crossing point) values to qPCR amplifications of the Mp2C15 gene as previously described (Behets *et al.* 2007). Relative quantification using multiple points in a dilution series of the same *N. fowleri* template DNA was conducted using the  $2^{-\Delta\Delta C_p}$  method (Pfaffl 2001), where  $-\Delta\Delta C_p = -(\Delta C_p \text{ 18S ribosomal gene} - \Delta C_p \text{ Mp2C15 single copy gene})$  as well as the Roche LightCycler software Relative Quantification module.

### Data post-processing

Results were corrected for inhibition based on differences between expected and actual Cp values in the IPC assay. For example, in the surface sample from Site 9, the quantity calculated by the LightCycler software averaged 3.03 copies/well. The IPC assay indicated that the sample was inhibited as the amplification efficiency was 0.794 (1.0 indicates no inhibition). Dividing the original copy number by the amplification efficiency returns a normalized quantity of 3.82 copies/well.

### Statistical analysis

Data were statistically evaluated to test for significant correlations between (a) *N. fowleri* and *E. coli* concentrations quantified by qPCR and (b) *E. coli* concentrations quantified by qPCR and *E. coli* concentrations quantified by the Coli-ert method. Additionally, *N. fowleri* and *E. coli* concentrations were evaluated by depth of sample.

For the purposes of the statistical analysis, the method detection limit (MDL) was defined as the lowest detected copy number by run and any zeros (non-detects) were

assigned a value that was half of the MDL. For *N. fowleri* and *E. coli* data, all runs were arithmetically averaged. Data by sample depth (surface or subsurface) were separately evaluated.

The distribution of each parameter was visually assessed and then evaluated using a Shapiro-Wilks test for normality. Data were transformed using a natural log function, as is commonly done with water quality data (Helsel & Hirsch 2002), and the distribution of the data for each parameter was reevaluated. The natural-log transformation reduced skewness and improved normality of the data for each parameter, although in most cases a normal distribution was still not obtained. Spearman rank correlations were therefore calculated to evaluate relationships between parameters. Spearman correlations are non-parametric and unaffected by the statistical distribution of the data. In addition, the Wilcoxon Signed Rank test was used to evaluate significant differences between samples by depth (surface and subsurface). Statistical analyses were performed using the SAS software package (version 8.2, SAS Institute Inc., Cary, NC).

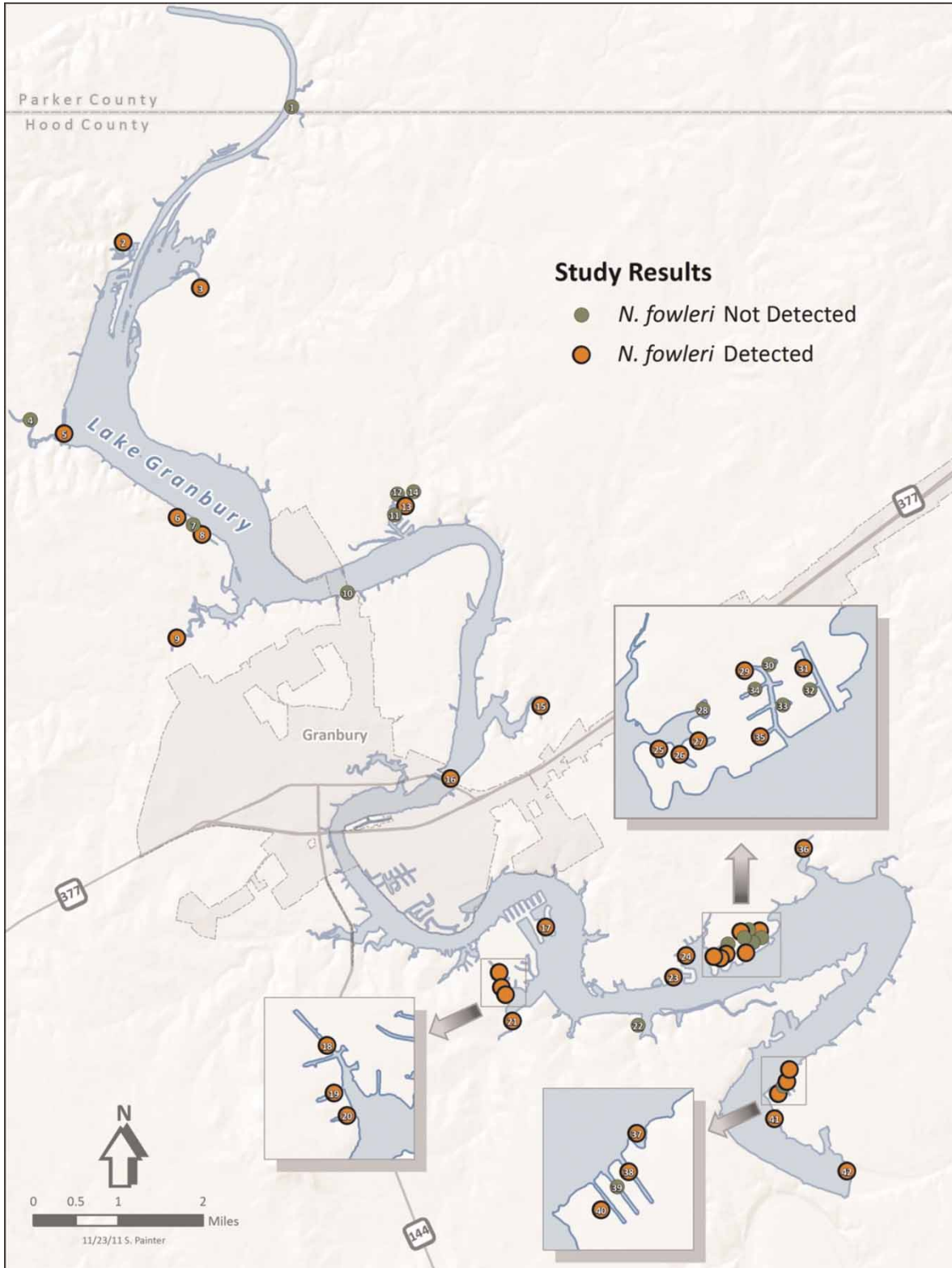
## RESULTS

### *N. fowleri* concentration results

The *N. fowleri* target DNA sequence was detected at 66.6% ( $n = 28$ ) of the 42 sites around Lake Granbury and in 50% (42 of 84) of the water samples that were collected during baseflow conditions (Figure 1, Table 2). These results indicate that *N. fowleri* was widespread in Lake Granbury during the study period.

The units for all intermediate qPCR results are given as target DNA copies per 100 mL of environmental water (copies/100 mL), written simply as 'copies'. Multiple copies of the target DNA sequence are equivalent to one *N. fowleri* cell, as described in the 'Copy number quantification' section below.

For water samples in which *N. fowleri* was detected, quantities of target DNA ranged from a low of seven copies to a high of 256 copies (Site 2); the mean quantity was 42 copies. The *N. fowleri* target DNA sequence was only detected during baseflow conditions; none were detected in any of the 16 stormflow samples (Table 3). In the baseflow samples, the average number of *N. fowleri*



**Figure 1** | Map showing locations where *N. fowleri* was detected using the described qPCR assay. Samples were collected during baseflow conditions.

**Table 2** | Target DNA sequences (copy/100 mL) quantified using qPCR for samples collected under baseflow conditions; dash (–) indicates that the value was below the detection limit

Site Number	<i>N. fowleri</i>		<i>E. coli</i>	
	Surface	Subsurface	Surface	Subsurface
1	–	–	–	–
2	256	–	64	–
3	–	26	–	–
4	–	–	112	–
5	37	22	–	–
6	95	57	3	–
7	–	–	–	–
8	31	–	178	267
9	123	38	–	39
10	–	–	–	–
11	–	–	159	241
12	–	–	42	14
13	29	27	–	34
14	–	–	–	75
15	20	18	83	–
16	11	–	–	–
17	11	72	–	–
18	22	7	16	105
19	9	–	–	–
20	25	30	–	–
21	7	29	–	–
22	–	–	51	–
23	18	17	186	427
24	35	33	22	–
25	43	–	–	–
26	11	–	87	–
27	–	20	–	3
28	–	–	–	40
29	43	–	100	–
30	–	–	–	–
31	57	–	–	–
32	–	–	5	146
33	–	–	5	–
34	–	–	234	307
35	25	29	–	19
36	25	–	–	94
37	–	21	–	–
38	68	19	249	99
39	–	–	8	5
40	57	20	–	–
41	–	32	–	–
42	–	17	–	–

**Table 3** | Target DNA sequences (copy/100 mL) quantified using qPCR for samples collected under stormflow conditions. A dash (–) indicates that the value was below the detection limit; NR indicates that there was no result due to inhibition

Site number	<i>N. fowleri</i>		<i>E. coli</i>	
	Surface	Subsurface	Surface	Subsurface
1	–	–	2,905	222
2	–	–	347	794
3	NR	NR	NR	NR
4	–	–	–	2,180
8	–	–	477	404
9	NR	–	NR	7,549
12	–	–	1,423	922
36	–	–	13,940	438

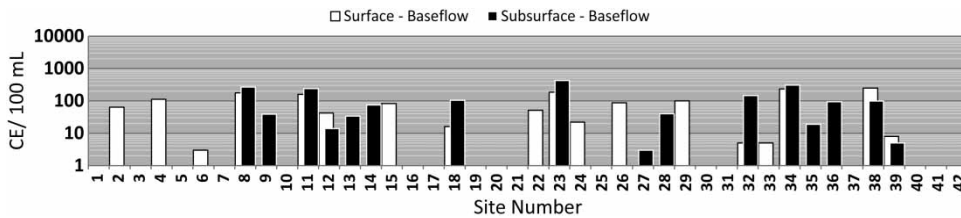
detections was higher in surface (0 m) vs subsurface (0.3 m) samples (23 vs 19 detects, respectively, an average of 46 copies vs 28 copies), although the Wilcoxon Signed Rank test revealed that the difference was not statistically significant ( $p = 0.200$ ).

### *E. coli* concentration results

For the *E. coli* assay, the target DNA sequence was detected at 59.5% ( $n = 25$ ) of the 42 sites around Lake Granbury during baseflow conditions, indicating that *E. coli* was widespread in Lake Granbury during the study period. Since the *uidA* gene is a single-copy gene (Noble *et al.* 2010) one copy is representative of one *E. coli* cell, allowing the expression of results in CE (cell equivalents)/100 mL of environmental water, written simply as 'CE'.

Under baseflow conditions, *E. coli* concentrations ranged from 3 CE to 427 CE (Figure 2, Table 2). For samples where the target DNA was detected under baseflow conditions, the mean quantity was 104 CE. For baseflow conditions, differences between surface and subsurface samples were not pronounced (18 surface detects vs 16 subsurface detects, average target DNA copy number being 123 CE compared to 180 CE). The Wilcoxon Signed Rank test confirmed that the difference in surface and subsurface copy number was not statistically significant ( $p = 0.837$ ).

Compared to baseflow conditions, *E. coli* target DNA concentrations increased considerably during stormflow



**Figure 2** | *E. coli* target DNA sequences (CE/100 mL) quantified using qPCR for samples collected under baseflow conditions. Note logarithmic scale on y-axis.

conditions (Figure 3, Table 3). For the same seven sites, the mean concentration (for sites where *E. coli* was detected) increased from 83 CE to 2,633 CE. Again, there was no statistically significant difference between surface and subsurface copy number ( $p = 0.844$ ).

### *N. fowleri* and *E. coli* correlation results

No statistical correlations were found between the results of the qPCR assays for *N. fowleri* and *E. coli* under baseflow conditions. Using the Spearman correlation coefficient, we found that there was no significant correlation between target microbe abundance in surface ( $p = 0.47$ ,  $r = 0.11$ ) or subsurface ( $p = 0.31$ ,  $r = -0.16$ ) samples.

With qPCR results from baseflow and stormflow conditions combined, a weak correlation was found between *N. fowleri* and *E. coli*. For the surface samples, the relationship between *N. fowleri* and *E. coli* numbers was not statistically significant ( $p = 0.369$ ,  $r = -0.13$ ), but for the subsurface samples there was a significant correlation ( $p = 0.036$ ,  $r = -0.30$ ). In consideration of this result, it is important to recognize that many of the physical and chemical conditions changed dramatically between the baseflow and stormflow samples (Painter 2011). This weak negative correlation was largely driven by the stormflow samples

and is not expected to accurately describe the relationship between the microbe populations when conditions are less variable.

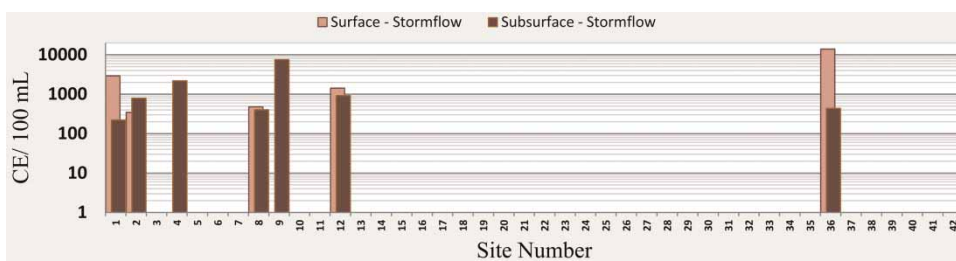
### qPCR *E. coli* and Colilert *E. coli* correlation results

Statistical analysis using the Spearman correlation coefficient revealed that there was considerable agreement between the qPCR *E. coli* assay and the Colilert method (culture) assay (Figure 4). For subsurface samples (stormflow and baseflow), the correlation between the two methods was found to be very highly significant ( $p < 0.0001$ ) and accounted for 48.1% of the sample variability ( $r = 0.694$ ). These results are comparable to results of similar studies (Haugland *et al.* 2005; Kephart & Bushon 2009; Lavender & Kinzelman 2009).

### Copy number quantification

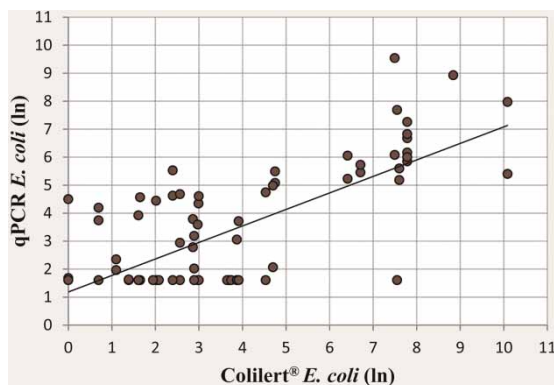
For *E. coli*, absolute quantification is relatively simple because the *uidA* gene is a single-copy gene (Noble *et al.* 2010); one copy is representative of one *E. coli* cell.

For *N. fowleri*, absolute quantification was hampered due to the lack of information available on the target copy number. Optimization trials revealed that the Qvarnstrom



**Figure 3** | *E. coli* target DNA sequences (CE/100 mL) quantified using qPCR for samples collected under stormflow conditions. Note logarithmic scale on y-axis.





**Figure 4** | *E. coli* concentrations for baseflow and stormflow samples, as determined by qPCR (ln CE/100 mL), compared to Colilert method (ln MPN/100 mL).

assay used in this study, which targets the 18S rRNA gene, is more sensitive than assays that have targeted the Mp2Cl5 gene (Behets *et al.* 2007; Madarová *et al.* 2010). As suggested by Qvarnstrom *et al.* (2006), the increased sensitivity of this assay is likely due to the higher copy number (per amoeba) of the putative multi-copy 18S rRNA gene, compared to other targets such as the putative single-copy Mp2Cl5 gene.

A search of the literature revealed only one estimate of copy number for the 18S rRNA gene in any *Naegleria* spp.; that estimate was proposed by Clark & Cross (1987) who investigated the *N. gruberi* genome. Per cell, they estimated between 3,000 and 5,000 copies of the 18S rRNA gene, which is located on a circular plasmid in *N. gruberi*. Puzon *et al.* (2009) used a value of 4,000 copy/amoeba to quantify *N. fowleri* amoebae in pipe wall biofilms in an assay targeting the ITS region of the ribosomal gene. When we conducted a relative quantification of the 18S gene in *N. fowleri* using the putative single-copy Mp2Cl5 gene, we found five 18S genes for every one Mp2Cl5 gene. To illustrate, in the first dilution of a serial dilution, the Cp of Mp2Cl5 was 19.79 and the Cp of the 18S ribosomal gene was 17.435. From the equation,  $\text{ratio} = 2^{-\Delta\Delta C_p}$  (Pfaffl 2001), we have  $\text{ratio} = 2^{-(17.435-19.79)}$ , or  $\text{ratio} = 5.12$ , for this dilution. Other dilutions produced the following ratios of 18S ribosomal genes for every single Mp2Cl5 gene: 5.31, 4.40, 4.81 and 4.92. This method assumes equal efficiencies for both qPCR reactions, which is a false assumption. However, when utilizing the Roche LightCycler relative quantification software suite to quantify the 18S gene (which does not assume equal qPCR efficiencies), similar results were produced that also grouped

around the number 5. We therefore conclude that there are five 18S rRNA genes in *N. fowleri* strain ATCC 30894 for every single copy of the Mp2Cl5 gene.

In order to accurately quantify the number of amoebae based on 18S rRNA copy number, the ploidy should be known. A search of the literature did not reveal any information about *N. fowleri* ploidy; however, the results of recent investigations of the well-studied *N. gruberi* genome may provide some insight. Genomic sequencing by Fritz-Laylin *et al.* (2011) has revealed monoploid (1X), diploid (2X) and tetraploid (4X) strains of *N. gruberi*. For this experiment, *N. fowleri* was assumed to be diploid, resulting in an estimated 10 copies of the 18S rRNA gene per diploid *N. fowleri* cell. At 256 copies/100 mL, the water sample collected at Site 2 had the highest *N. fowleri* target DNA concentration in the study. Based on our estimate, this represents a concentration of about 26 CE (cell equivalents) per 100 mL of water.

## DISCUSSION

The results of this study indicate that *N. fowleri* was distributed throughout Lake Granbury during the sample period (Figure 1). *N. fowleri* was detected in water samples from most of the sites in Lake Granbury (66.6%), with concentrations ranging from an estimated 1–26 CE/100 mL. Based on quantitative estimates by Cabanes *et al.* (2001), the maximum risk of contracting PAM from swimming in the cove with the highest detected *N. fowleri* concentration at the time of sampling is estimated to be 0.0094%, or about 1 in 10,600. We are aware of two confirmed PAM cases, possibly resultant from swimming in Lake Granbury (occurring in 1991 and 2010). Considering the impossibility of knowing exactly when and where the victims were exposed to *N. fowleri*, as well as the amoeba concentrations at exposure, the validity of this estimate cannot be ascertained. Further research that contributes to the refinement of the model proposed by Cabanes *et al.* (2001) should be continued.

Although no measured physical, chemical or biological parameters strongly correlated with *N. fowleri* concentration (results not shown, see Painter (2011)), recorded field observations may provide some insight into conditions

encouraging proliferation of the amoeba. The samples that had the two highest *N. fowleri* concentrations were the surface samples collected under baseflow conditions at Site 2 and Site 9, with 26 and 12 CE/100 mL, respectively. These samples were unique because floating biofilms, or assemblages of microorganisms living at the liquid–air interface (Declerck 2010), were observed during sampling. At Site 2, we noted ‘heavy algae on surface attached to emergent vegetation’, and at Site 9 we noted a ‘scum on the surface of the water’. Only one other observation of a surface film was recorded, at Site 1, where ‘some scum’ was noted, but *N. fowleri* was not detected.

As suggested by Kyle & Noblet (1985), free-living amoebae may exhibit a preference for cyanobacteria because algae may provide a substrate for attachment, a food source or both. Puzon *et al.* (2009) used qPCR techniques to quantify *N. fowleri* in pipe wall biofilms and found high numbers of *N. fowleri* amoebae under these bacteria-rich conditions. Most recently, Goudot *et al.* (2012) found a ‘direct link between the bacteria/amoeba ratio and *N. fowleri* growth’ in biofilms growing spontaneously on glass slides, demonstrating that the availability of a bacterial food source does promote the growth and survival of the amoeba. Taken together, the indications are that biofilms could possibly be a reservoir for *N. fowleri* in surface water environments (Goudot *et al.* 2012).

For the samples that we collected under baseflow conditions in Lake Granbury, the average water temperature (measured at 0.3 m of depth) was 32.1 °C, just below the 35–44 °C range established for optimal *N. fowleri* growth in the laboratory (Wellings *et al.* 1979; Tyndall *et al.* 1989; John & Howard 1996). Most likely, the daytime temperature of the floating biofilm (at the sites where a neustonic layer was noted) was greater than the measured water temperature, potentially enhancing conditions for the amoeba. Goudot *et al.* (2012) confirmed that the effect of temperature on *N. fowleri* growth within a biofilm was substantial; fairly low densities (1–10 *N. fowleri*/cm<sup>2</sup>) occurred at 32 °C while the amoeba proliferated at 42 °C, reaching densities of up to 900 *N. fowleri*/cm<sup>2</sup>.

In this study, *E. coli* concentrations were not indicative of *N. fowleri* concentrations. Further research could investigate the degree to which bacteria besides *E. coli* serve as a food source for the amoeba. Laseke *et al.* (2008) found

that Arizona groundwater samples testing positive for *N. fowleri* tended to have a diverse bacterial structure. Microbial community analysis of surface water samples may reveal relationships that are helpful in assessing the degree to which bacteria-rich waters present an increased risk to swimmers.

## CONCLUSIONS

We used two qPCR assays to rapidly and sensitively quantify the pathogenic amoeba *N. fowleri* and the fecal indicator bacterium *E. coli* in environmental water samples. qPCR inhibitor problems were mitigated by the use of an IPC, along with the addition of a DNA clean-up step. *N. fowleri* was detected in water samples from 67% of the reservoir sites tested, with concentrations ranging up to an estimated 26 CE/100 mL. Under baseflow conditions, *E. coli* was detected in water samples from 60% of the reservoir sites tested, with concentrations ranging up to 427 CE/100 mL. A strong correlation was found between *E. coli* concentrations detected via qPCR vs the Colilert method ( $p < 0.0001$ ;  $r = 0.694$ ). In this study, *E. coli* concentrations were not indicative of *N. fowleri* concentrations.

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