HHS Public Access

Author manuscript

J Environ Qual. Author manuscript; available in PMC 2019 November 11.

Published in final edited form as:

J Environ Qual. 2010 ; 39(1): 147–153. doi:10.2134/jeq2009.0062.

Identification of *Naegleria fowleri* in Warm Ground Water Aquifers

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Abstract

The free-living amoeba *Naegleria fowleri* was identified as the etiological agent of primary amoebic meningoencephalitis that caused the deaths of two children in Peoria, Arizona, in autumn of 2002. It was suspected that the source of *N. fowleri* was the domestic water supply, which originates from ground water sources. In this study, ground water from the greater Phoenix Metropolitan area was tested for the presence of *N. fowleri* using a nested polymerase chain reaction approach. Phylogenetic analyses of 16S rRNA sequences of bacterial populations in the ground water were performed to examine the potential link between the presence of *N. fowleri* and bacterial groups inhabiting water wells. The results showed the presence of *N. fowleri* in five out of six wells sampled and in 26.6% of all ground water samples tested. Phylogenetic analyses showed that β- and γ-proteobacteria were the dominant bacterial populations present in the ground water. Bacterial community analyses revealed a very diverse community structure in ground water samples testing positive for *N. fowleri.*

The waterborne pathogen *Naegleria fowleri* has long been associated with the fatal disease of primary amoebic meningoencephalitis. Healthy children and young adults with a history of swimming in freshwater lakes or ponds are usually among those who acquire the infection, which is fatal if not identified early (Carter, 1968; Carter, 1970; Carter, 1972;

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Marciano-Cabral, 1988). *Naegleria fowleri* trophozoites infect humans by entering the nasal cavity, attach to the nasal mucosa, and enter the central nervous system. In the USA, 121 people died by infection from *N. fowleri* between the years of 1937 to 2007, including six deaths in 2007 (Centers for Disease Control and Prevention, 2007).

Previous studies have shown the presence of *N. fowleri* in soil, surface water, treated water, thermal effluents, environmental waters with elevated temperatures (including hot springs), and water distribution systems (MacLean et al., 2004; Marciano-Cabral, 1988). Recently, *N. fowleri* detected in the residual water from household pipes and sinks was identified as the causative agent in the primary amoebic meningoencephalitis infections that resulted in the death of two children in Arizona (Marciano-Cabral et al., 2003). In the latter case, the suspected vector of infection was the potable water supply from a warm water aquifer in the area. Moreover, it is assumed that *N. fowleri* (ranging in size from 7–20 μm) could not be present in well water due to the low organic content of the water and the barriers provided by well casing, which typically screens materials larger than 5 μm.

Although detection of *N. fowleri* has traditionally required cultivation methods, most species of *Naegleria* are morphologically indistinguishable, requiring molecular methods to further identify species of interest. Specifically, the *Mp2Cl5* gene has recently been used to discriminate between *N. fowleri* and closely related species (Reveiller et al., 2002; Blair et al., 2008). In this study, the *Mp2Cl5*–polymerase chain reaction (PCR) assay was used to detect the presence of *N. fowleri* in well water samples from the greater Phoenix Metropolitan area. A recent study (Blair et al., 2008) detected heterotrophic bacteria and *N. fowleri* in ground water samples, suggesting that these bacteria may serve as a potential food source for the amoebae. Thus, phylogenetic analyses of 16S rRNA gene ground water clones were also performed to determine the potential correlation between bacterial community composition and the presence of *N. fowleri*. The overall objectives of this study were to identify the presence of *N. fowleri* in warm ground water aquifer samples and to associate its presence with specific microbial communities in the water samples. The information from this study will lead to a better understanding of the ecology of *N. fowleri* in warm ground water environments.

Materials and Methods

Environmental Wells Sampled

Well samples were collected and processed from six public water supply wells (labeled as TON1, VAL1, SAF3, PX244, SC85, and SC86) from the greater Phoenix Metropolitan area from winter 2004 through autumn 2005. Representative sites were selected based on temperature and pump type. Sites were chosen based on variable temperatures (29–48.1°C) to investigate if *N. fowleri* presence was a potentially temperature-dependent phenomenon. Pump type (submersible or oil-lubed turbine) was also considered because the oil lubricant was thought to serve a potential food-source for bacteria, which in turn could serve as a food source for *N. fowleri*. In addition, well water samples from the Mason Drinking Water Treatment Plant in Cincinnati, Ohio, were collected and used for detection limit experiments and as environmental negative controls, as described below.

Greater Phoenix Metropolitan Area Ground Water Sampling and Filtration

The following parameters were measured for selected water samples in situ: water temperature, pH, dissolved oxygen, and conductivity measurements. Conductivity, pH, and temperature were measured using an Oakton Portable pH/Con 10 Series Meter (Oakton Instruments, Vernon Hills, IL). Dissolved oxygen was measured using an Orion Model 830 Dissolved Oxygen Meter (Thermo Fisher Scientific, Inc., Waltham, MA).

Heterotrophic bacteria were measured for selected water samples collected from the greater Phoenix Metropolitan area and the Mason Drinking Water Treatment Plant in Cincinnati, Ohio. Water samples were collected and transported back to the lab and analyzed for heterotrophic bacteria according to standard method 9215B (Clesceri et al., 1998).

Wells from the greater Phoenix Metropolitan area were sampled during winter (December), late summer (August), and early autumn (September) to determine if seasonality had an impact on the detection of *N. fowleri* in the environment. In the field, each well water sample was passed through two serially arranged filters (pore size, 0.5 and 1.0 μm, respectively) (Micro-Wynd II D-PPPY cartridge filter; Cuno Inc., Meriden, CT). The volume of water filtered for each well ranged from 236.6 to 4402.1 L. Filters were transported to the laboratory in iced coolers and stored at –80°C until further processing.

Mason Ground Water Sampling, Spiking, and Filtration

Four 100-L control well water samples were collected from the Mason Drinking Water Treatment Plant in Cincinnati, Ohio, and transported to the laboratory immediately for processing. These water samples were considered negative environmental controls because *N. fowleri* levels were below the limit of detection for the assay used in this study. One of the four 100-L samples was directly filtered using two serially arranged filters (pore size, 0.5 μm) (Micro-Wynd II D-PPPZ cartridge filter), while three remaining 100-L control samples were first spiked with 10^3, 10^4, and 10^5 *N. fowleri* cells and then filtered as mentioned above. Pure culture *N. fowleri* (ATCC 30894) was grown in Cline medium (Cline et al., 1983) at 37°C. *Naegleria fowleri* cell counts were performed using a hemacytometer injected with 10 μL of well-mixed *N. fowleri* culture at stationary growth phase. Cell counts were performed by counting all 25 sections of the hemacytometer via a light microscope.

DNA Extraction

A portion of each filter (approximately 1.61 cm^2) was removed aseptically using a sterile razor blade and placed in a 2-mL tube containing bead beating solution from a MoBio Soil DNA Extraction Kit (Mo Bio Laboratories, Inc., Carlsbad, CA). Genomic DNA extractions were performed as suggested by the manufacturer's instructions. The DNA extracts from each filter were used as a template in PCR assays to determine the presence or absence of *N. fowleri* in each sample. DNA was also extracted from 1 mL of *N. fowleri* (ATCC 30894) pure culture cell suspension using the same DNA extraction method.

Molecular Methods

Naegleria fowleri DNA extracts were serially diluted in sterile water (1:1, 1:10, 1:100, and 1:1000). Aliquots were then used to determine the detection limits of PCR assays targeting

N. fowleri (Table 1). Primers and genes targeted in this study are listed in Table 1. Detection of *N. fowleri* was performed using a nested-PCR assay as described previously (Reveiller et al., 2002). DNA extracts from *N. fowleri* cultures and each filter were used as a template in the first PCR assays, which targeted the *Mp2Cl5* gene unique to the *Naegleria* genus. Polymerase chain reaction products from the first PCR reaction were used as a DNA template for the second PCR assay, which targeted a conserved region within the amplified *Mp2Cl5* gene unique to *N. fowleri*. All PCR assays were performed in triplicate. Polymerase chain reaction products were visualized using a 2% ethidium bromide–stained agarose gel.

To identify predominant bacterial populations associated with well water samples, DNA extracts from selected samples were used to develop 16S rRNA gene clone libraries. Clone libraries were developed using primers 8F and 926R and the protocol described by Liu et al. (1997). Polymerase chain reaction products were cloned into chemically competent *Escherichia coli* cells using the TOPO TA Cloning Kit (TOPO10 Electrocomp Cells; Invitrogen Corp., Carlsbad, CA) following the manufacturer's instructions. M13 primers were used in PCR assays to screen for transformants containing correct size product inserts. Polymerase chain reaction products ($n = 209$) were submitted to Cincinnati Children's Hospital Medical Center DNA Sequencing Facility for partial sequencing using primer 338F (Stackebrandt and Goodfellow, 1991). Nucleic acid sequences were edited using BioEdit v7.0 (Hall, 1999) and aligned using ARB software before constructing phylogenetic trees (Ludwig et al., 2004). Aligned sequences of the environmental samples and their close relatives were used to construct phylogenetic trees using inferred from 705 sequence positions using the neighbor-joining algorithm with the Kimura correction. Additionally, 16S rRNA gene sequences were assigned to phylogenetically consistent high-order bacterial taxonomy using the Ribosome Database Project classifier tool to describe the bacterial community structure of four different well samples (Wang et al., 2007).

Data Analysis

To determine potential parameters driving *N. fowleri* presence, physical and chemical parameters for each different well tested were correlated to *N. fowleri* PCR-positive frequency using linear regression. A Student's *t* test was performed to determine if there were significant differences between physical and chemical parameters for PCR-positive and PCR-negative samples.

Results and Discussion

Naegleria fowleri Detection and Environmental Parameters

The detection limit of the nested *N. fowleri* PCR assay was determined using genomic DNA and spiked ground water samples. Experiments with spiked ground water samples showed that the assay could detect 10 cells per mL (Fig. 1). No PCR signals were detected in the negative control Mason ground water samples. When the *N. fowleri* nested PCR assay was applied to ground water samples collected in Phoenix, approximately 27% of all samples tested positive for *N. fowleri* (Table 2; Fig. 2). Five out of the six wells sampled tested positive during this study, although all samples that tested positive for *N. fowleri* were taken from wells during the late summer and early autumn. Although seasonal patterns have been

previously observed for *N. fowleri* (Dorsch et al., 1983; Griffen, 1972; Griffen, 1983; Marciano-Cabral et al., 2003), our results indicate that there was no correlation between PCR-positive detection of *N. fowleri* and water temperature (Table 3). *Naegleria fowleri* was detected in water samples with temperatures ranging from 29 to 47°C, suggesting the thermo-tolerance of this microorganism. None of the physical or chemical parameters tested for in this study showed a correlation to PCR detection of *N. fowleri* (Table 4).

A potentially important factor in the ecology of *N. fowleri* was that the occurrence of *N. fowleri* in the sampled locations could relate to the use of oil-lubed turbine pumps because lubricant released from the pumps could influence the growth of bacterial populations in the ground water, which consequently could serve as a food source for *N. fowleri* (Singh and Dutta, 1984; Marciano-Cabral et al., 2003). However, in this study, *N. fowleri* was not restricted to a specific pump type (Table 5), and therefore no connection could be made between the presence of *N. fowleri* and potential impact of oil on the ground water bacterial populations. No pattern was evident between the presence of *N. fowleri* and pH and conductivity. This is not surprising because pH does not appear to be important factor controlling the ecology of *Naegleria* species (Kyle and Noblet, 1985). Similarly, heterotrophic counts, which ranged from <100 to 800 cfu per 100 mL, did not appear to correlate with the presence of *N. fowleri*.

Bacterial Population Ecology of Selected Water Samples

To determine whether there was a link between the presence of *N. fowleri* and predominant bacterial populations in the well water, 16S rRNA gene clone libraries were developed for selected well samples that tested positive (n = 126 sequences) or negative (n = 83 sequences) for *N. fowleri* (Table 5). Three of the samples were from one well, two of which were negative for the presence of *N. fowleri* (TON1A and TON1B). The other two samples (TON1C and SC85A) tested positive for *N. fowleri*. A total of 209 clones were analyzed and used in the development of a phylogenetic tree (Fig. 3). Most clones were closely related to β-proteobacteria, specifically to *Caldimonas* (n = 75), *Aquabacterium* (n = 17), and *Leptothrix* (n = 7). Members of these genera are commonly found in aquatic habitats. For example, *C. taiwanensis* was recently isolated from a hot spring with recorded growth temperatures between 35 and 60°C (Chen et al., 2005), and *Aquabacterium* species were originally detected and isolated from drinking water in Germany (Kalmbach et al., 1999). Sequences closely related to γ-proteobacteria were also abundant, with a total of 56 clones, most of which were closely related to uncultured *Pseudomonas* species. Proteobacteria of the α- and δ-subclasses were not as abundant. Sequences related to *Nitrospira* (n = 7) and *Nocardia* (n = 2) were also retrieved from the water samples.

Phylogenetic classification indicated that sequences retrieved from well samples TON1A and TON1B, which were negative for *N. fowleri*, consisted only of β-proteobacteria. Clones derived from two samples testing positive for *N. fowleri* (SC85A and TON1C) comprised a much more diverse community structure, including members from at least four different bacterial phyla (Fig. 4). Although further sequencing efforts are needed to saturate bacterial diversity within these samples, these data suggest a potential association between high bacterial diversity and the presence of *N. fowleri* in well water. Several studies have

examined the feeding habits and growth rates of free-living amoeba, including *Naegleria* species (Brown et al., 1983; Danso and Alexander, 1975; Danso et al., 1975; Singh and Dutta, 1984). Most of these studies have been performed in laboratory settings with specific bacteria, such as *E. coli*, *Klebsiella* species, *Enterobacter cloacae*, and *Rhizobium meliloti*. In contrast, little is known about the feeding preference of free-living amoeba in environmental settings. Free-living amoebae, including *N. fowleri*, have been isolated from waters containing high levels of filamentous cyanobacteria (Kyle and Noblet, 1985). In such cases, cyanobacteria were assumed to serve as a food source. *Naegleria* species have also been isolated from waters with high coliform levels, but no clear patterns have been established because typically the levels of enteric bacteria in surface waters do not support amoebal survival (Brown et al., 1983). A small number of bacterial sequences from samples SC85A and TON1C (wells that tested positive for *N. fowleri*) were members of the Bacteroidetes and Firmicutes phyla; previous research has shown that these enteric bacterial groups positively correlated with the occurrence of *N. fowleri* (Brown et al., 1983).

Summary

In this study, β- and γ-proteobacteria were present in samples that were both positive and negative to *N. fowleri*. Samples that were negative for *N. fowleri* were primarily associated with clones within the *Caldimonas* and *Leptothrix* clades. These genera are known to oxidize iron and manganese (Takeda et al., 2002; van Veen et al., 1978). These metals have been reported to positively influence the levels of free-living amoeba in environmental waters (Duma, 1981; Kyle and Noblet, 1985), although the mechanism of action is unknown. According to drinking water utilities who participated in this study, iron and manganese concentrations in the region where the samples were collected are generally low, and therefore the presence of metal oxidizers might influence the availability of metals for amoeba. Although additional evidence is needed to understand the importance of metal-oxidizing bacteria in the survival of amoeba, it should be noted that amoeba prefer gram-negative, nonpigmented bacteria (Singh and Dutta, 1984), and pigments are known to possess iron chelating activity.

In conclusion, this study has confirmed the presence of *N. fowleri* in warm ground water aquifers using a nested PCR assay. Although physical and chemical parameters did not correlate with PCR detection of *N. fowleri*, phylogenetic and Bayesian community structure analyses revealed a more rich community structure associated with wells positive for *N. fowleri*. Future studies should address the ecology and in situ feeding habits of *N. fowleri*, including the role metal availability has in the survival of this pathogenic amoeba.

Acknowledgments

The authors thank Greater Cincinnati Water Works and Dr. Jorge Santodomingo. Funding was provided by the US EPA (PR-OH-03-00572) to DBO.

Abbreviations

PCR	polymerase chain reaction

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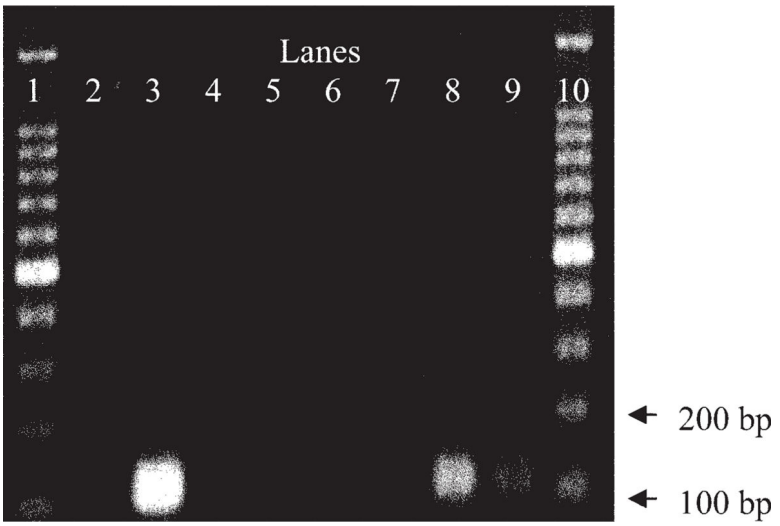

Fig. 1.

Gel image (2% agarose gel stained with ethidium bromide showing the sensitivity of *Naegleria fowleri* nested PCR assay in Mason ground water spiked with increasing concentrations of *N. fowleri.* Lane 1: 100-bp DNA ladder; lane 2: negative control with sterile water; lane 3: positive control with *N. fowleri* genomic DNA; lane 4: Mason ground water filter sample with 0.1 *N. fowleri* cells per mL; lane 5: Mason ground water duplicate filter sample with 0.1 *N. fowleri* cells per mL; lane 6: Mason ground water filter sample with 1.0 *N. fowleri* cells per mL; lane 7: Mason ground water duplicate filter sample with 1.0 *N. fowleri* cells per mL; lane 8: Mason ground water filter sample with 10.0 *N. fowleri* cells per mL; lane 9: Mason ground water duplicate filter sample with 10.0 *N. fowleri* cells per mL; lane 10: DNA ladder.

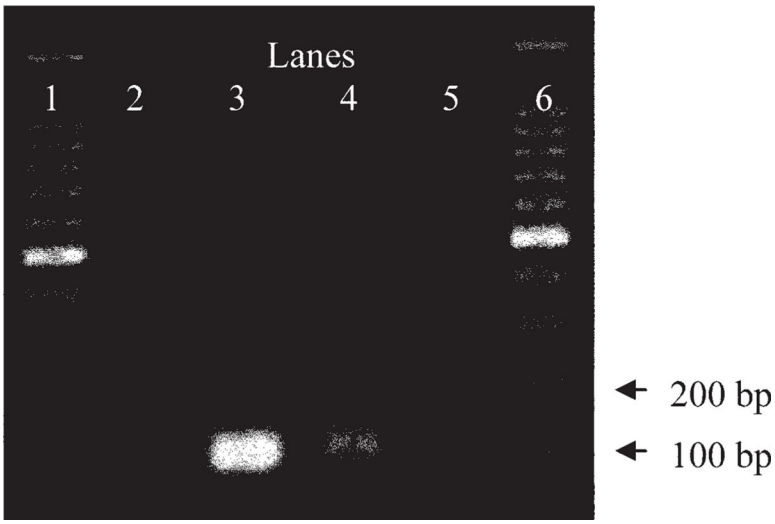

Fig. 2.

Gel image (2% agarose gel stained with ethidium bromide) showing the intensity of *Naegleria fowleri* polymerase chain reaction assay amplification products on environmental samples. Lane 1: 100-bp DNA ladder; lane 2: negative control with sterile molecular grade water; lane 3: positive control with *N. fowleri* genomic DNA; lane 4: TON1 well site sampled during early fall from the greater Phoenix Metropolitan area; lane 5: TON1 well site sampled during early fall from the greater Phoenix Metropolitan area; lane 6: DNA ladder.

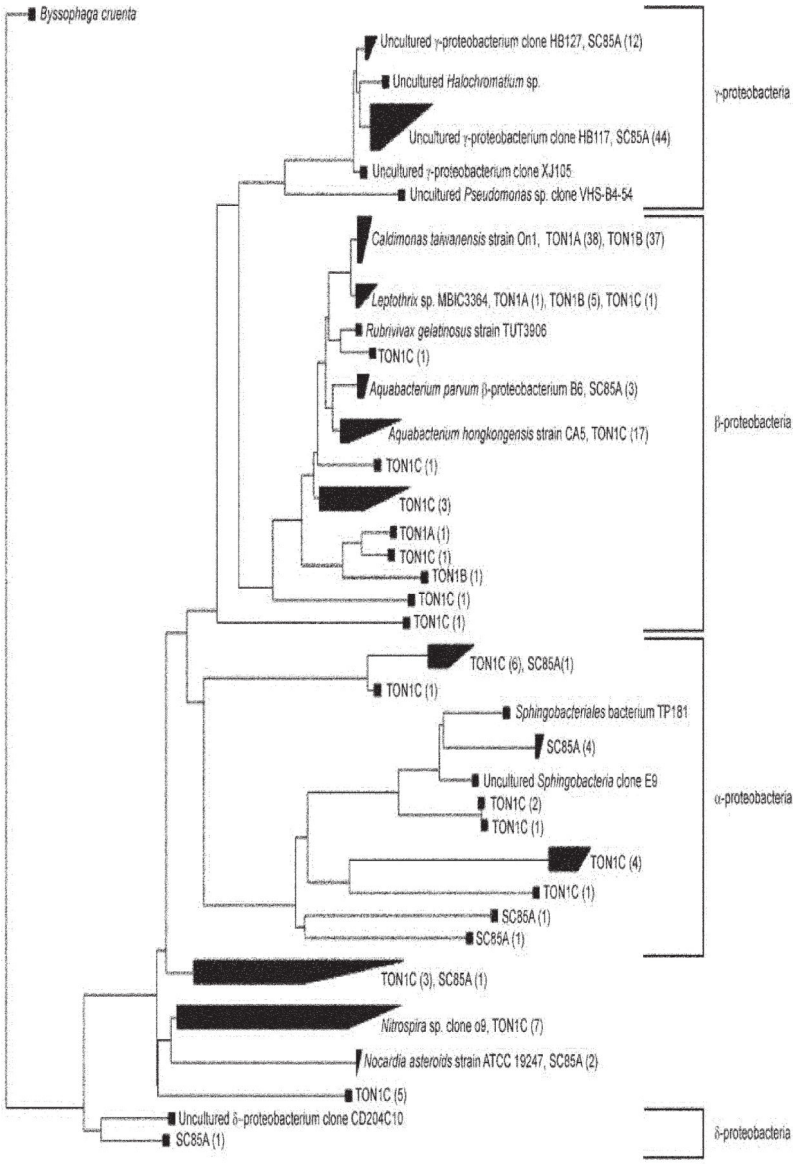

Fig. 3.

Phylogenetic tree of 16S rRNA gene sequences derived from well samples SC85A, TON1A, TON1B, and TON1C and bacterial members from the α, β, γ, and δ proteobacteria, as well as *Nitrospira and Nocardia* genera as points of reference. Samples TON1A (n = 40 sequences) and TON1B (n = 43 sequences) tested negative for *N. fowleri*, whereas SC85A (n = 70 sequences) and TON1C (n = 56 sequences) tested positive for *N. fowleri*. The phylogenetic tree was constructed in ARB using the neighbor joining algorithm with a Kimura correction (Ludwig et al., 2004). Numbers in parentheses indicate the number of sequences derived from a given well sample.

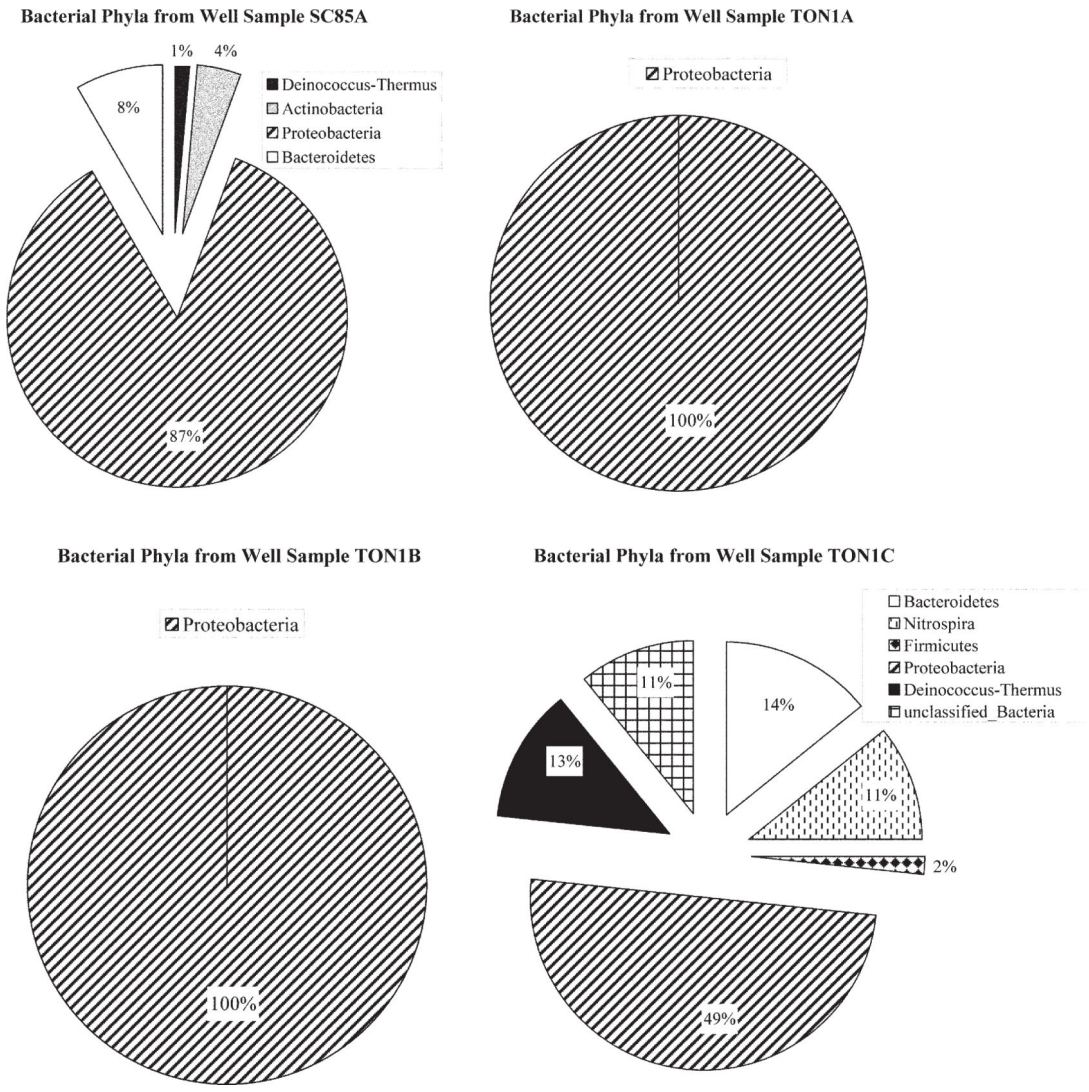

Fig. 4.

Bacterial phyla represented in well samples SC85A, TON1A, TON1B, and TON1C using the Ribosomal Database Project Bayesian classifier (Wang et al., 2007). SC85A and TON1C tested positive for *Naegleria fowleri*, and TON1A and TON1B tested negative for *N. fowleri* by polymerase chain reaction.

Table 1.

Description of polymerase chain reaction assays used in this study.

PCR† assay name	Target microorganism	Target gene	Forward primer name	Forward primer sequence (5′–3′)	Reverse primer name	Reverse primer sequence (5′–3′)	Reference
Naegleria assay	general *Naegleria*	Mp2Cl5	Mp2Cl5.for	TCTAGAGATCCAACCAATGG	Mp2Cl5.rev	ATTCTATTCACTCCACAATCC	Reveiller et al., 2002
N. fowleri assay	*N. fowleri*	Mp2Cl5	Mp2Cl5.for-in	GTACATTGTTTTTATTAATTTCC	Mp2Cl5.rev-in	GTCTTTGTGAAAACATCACC	Reveiller et al., 2002
Universal 16S assay	universal DNA	16S rDNA	S-D-Bact-0008-a-S-20	AGAGTTTGATCCTGGCTCAG	S-D-Bact-0926-a-S-20	CCGTCAATTCCTTTRAGTTT	Liu et al., 1997
Sequencing assay	n/a	16S rDNA gene	S-D-Bact-0338-a-S-18	GCTGCC TCCCGT AGGAGT	n/a	n/a	Stackebrandt et al., 1991

†PCR, polymerase chain reaction.

Table 2.

Polymerase chain reaction results for environmental samples from the greater Phoenix Metropolitan area.

	Amplification results for samples collected in summer, autumn, and winter for *Naegleria fowleri* (number positive/samples tested)		
Well name	**Late summer**	**Autumn**	**Winter**
TON1	–	3/6	0/2
VAL1	–	4/13	–
SAF3	–	2/5	0/2
PX244	0/2	0/3	0/2
SC85	2/2	0/3	–
SC86	1/2	0/3	–

Table 3.

Student's *t* test of physical and chemical parameters for samples testing positive and negative for *Naegleria fowleri*.

Temperature		Conductivity		pH		HPC[†]	
Positive	**Negative**	**Positive**	**Negative**	**Positive**	**Negative**	**Positive**	**Negative**
—°C—		**μS**	**S μm^{-1}**			**—cfu per 100 mL—**	
47	43.9	968	840	8.4	8.6	1	2
48.1	44.2	921	896	8.3	8.6	2	3
35.9	47.9	352	910	8.6	8.6	8	11
35.6	46.8	343	912	8.6	8.6	1	1
31.2	35.4	365	2310	8.4	8.1	7	3
31.4	35.1	470	2310	8.2	8	15	2
29.2	34.7	624	2280	7.7	8.3	21	3400
	34.5		2230		8.3		3200
	30.5		326		8.5		50
	30.5		321		8.5		23
	34.7		340		8.8		1
	34.5		340		8.8		1
	34.1		444		8.1		2
	34.3		437		8.1		13
	34.5		381		8.3		1
	34.8		378		8.3		1
	37.9		447		8		1
	37.7		457		8.2		1
	31.2		478		8.2		83
	31.1		487		8.4		32
	29.2		611		7.8		10
	29.1		616		7.6		52
	29		617		7.7		19
$p = 0.652$		$p = 0.149$		$p = 0.794$		$p = 0.152$	

[†]cfu, colony-forming units; HPC, heterotrophic plate count.

Table 4.

Correlation of physical and chemical parameters to polymerase chain reaction–positive detection of *Naegleria fowleri*.

Parameter	Average	Correlation coefficient
pH	8.2	0.1698
Conductivity, µS	547.2	0.119
Temperature, °C	35.1	0.0464
HPC,† cfu per 100 mL	288.6	0.0265
DO, mg L^{-1}	5.4	0.0512

†cfu, colony-forming units; DO, dissolved oxygen; HPC, heterotrophic plate count.

Table 5.

Summary of environmental samples analyzed for bacterial populations.

Sample name	HPC[†]	Water temperature	Dissolved oxygen	pH	Conductivity	Filter size	Pump type	PCR results for *Naegleria fowleri*
	CFU per 100 mL	°C	mg L^{-1}		S μm^{-1}	µm		
TON1A[‡]	200	43.9	4.1	8.6	840	1.0	submersible	negative
TON1B	300	44.2	4.2	8.6	896	0.5	submersible	negative
TON1C	<100	47.0	not measured	8.4	968	1.0	submersible	positive
SC85A	700	31.2	not measured	8.4	365	1.0	oil lube turbine	positive

[†]HPC, heterotrophic plate count; PCR, polymerase chain reaction.

[‡]TON1, VAL1, SAF3, PX244, SC85, and SC86 are well names. Letters A, B, and C listed after well names designate specific samples from the wells.