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Ashleigh Streby Date

Comparison of real-time PCR methods to detect *Naegleria fowleri* in environmental samples

By

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Comparison of real-time PCR methods to detect *Naegleria fowleri* in environmental samples

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Abstract

Comparison of real-time PCR methods to detect *Naegleria fowleri*

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By Ashleigh Streby

Naegleria fowleri is a thermophilic free-living amoeba found in freshwater environments worldwide. It is the cause of a rare, but potentially fatal disease in humans known as primary amoebic meningoencephalitis (PAM). The purpose of this study was to compare four different real-time PCR methods (Jothikumar et al., Qvarnstrom et al., Puzon et al., Robinson et al.) for the detection of *N. fowleri* from surface water and sediment. It was hypothesized that either the Qvarnstrom et al. or Jothikumar et al. assay would perform the best based on the use of a specifically designed fluorescent reporter probe in these assays. The assays were compared in terms of thermodynamic stability, analytical sensitivity and specificity, detection limits, humic acid inhibition effects, performance with seeded environmental matrices and performance with samples previously tested as positive by the CDC Water Sanitation and Hygiene (WASH) Laboratory. Twenty-one amoeba isolates were included in the DNA panel used for analytical sensitivity and specificity analysis. *N. fowleri* genotypes I and III were used to determine detection limits whereas *N. fowleri* genotype I was used in humic acid inhibition and performance with seeded environmental matrix analyses. Two of the assays were removed from further investigation due to lower sensitivity (71%, Robinson et al.) and higher detection limit for *N. fowleri* genotype III (0.8 *N. fowleri* amoeba per real-time PCR reaction, Puzon et al.). Based on relatively equivalent outcomes at each stage of analysis, it was determined that both the Jothikumar et al. and Qvarnstrom et al. assays should be effective for use in future analyses with environmental matrices. The use of these assays should be useful contributors to studies investigating the potential risk factors for human exposure to *N. fowleri.*

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Introduction

Naegleria fowleri is a thermophilic species of free-living amoeba found in soil and freshwater worldwide that can tolerate temperatures of up to 45°C [9, 43]. It has three morphological forms: trophozoite, flagellate and cyst. The trophozoite form is the vegetative, feeding stage and also is the form responsible for infection, whereas the flagellate form is the non-dividing, non-feeding stage. The cyst form is a protective stage that can be formed in response to certain environmental pressures such as nutrient deprivation or desiccation [29].

Of the more than forty currently recognized *Naegleria* species, *N. fowleri* is the only one known to cause disease in humans [11, 43]. There are eight known *N. fowleri* genotypes [11]. Of these eight genotypes, three have been identified in the United States (Genotypes I, II and III), one of which is unique to this region (Genotype I) [11]. Virulence of the eight different genotypes is thought to be comparable. However, these genotypes vary in genetic characteristics; primarily through differences in internal transcribed spacer (ITS) region lengths and nucleotides within the 5.8S rDNA region [11]. It is important to consider the geographical needs of a study when considering which assay to use.

N. fowleri causes primary amoebic meningoencephalitis (PAM) which almost always results in death within 3-7 days after the onset of symptoms [45]. For infection to occur, *N. fowleri* must enter the nasal cavity. Then, it is hypothesized that *N. fowleri* trophozoites enter the central nervous system (CNS) via phagocytosis of cells lining the olfactory neuroepithelium [43]. Subsequently, the amoebas migrate through the cribiform plate, penetrate into the sub-arachnoid space and continue on to the brain parenchyma where they provoke inflammation and tissue destruction [21, 43]. The clinical presentation of PAM may include headache, fever, nausea, vomiting, and neck stiffness with later progression to loss of balance, seizures, coma, hallucinations and death [7]. The similarity

of the clinical presentation of *N. fowleri* infection to other infections affecting the CNS often results in misdiagnosis and delayed or lack of treatment for PAM cases. As a consequence, the majority of patients die before they are able to receive appropriate clinical intervention. However, use of amphotericin B alone or with other antibiotics remains the gold standard of *N. fowleri* treatment and has reportedly been an effective cure for one individual [42].

Although *N. fowleri* is ubiquitous in bodies of warm freshwater worldwide, a systematic review of PAM case reports from 1962-2008 indicated only 111 cases in the United States [45]. Worldwide, approximately 235 cases of PAM have been reported [11]. The comparatively high number of cases in the U.S. is likely due to increased surveillance activities, not a higher disease burden. The majority of the reported U.S. infections occurred in previously healthy, young males(aged ≤13 years) and only one case survived [44]. Most infections have been attributed to swimming in freshwater bodies including lakes, ponds, rivers, canals and streams during warmer months. Other water sources that have been associated with exposure in the U.S. are geothermally heated water, improperly chlorinated swimming pools and untreated drinking water used for recreational purposes [45]. Most recently, two cases of PAM in the southern U.S. have been associated with the use of commercial nasal irrigation systems in which non-sterilized tap water was used for nasal rinsing (Hill, personal communication). Studies have demonstrated the presence of humoral antibodies against different species of pathogenic free-living amoebae, including *N. fowleri,* in human sera both in the U.S. and New Zealand [8, 27]. This suggests that other *Naegleria* spp. can infect humans, resulting in an immune response but no disease. Similarly, the fact that antibodies specific for *N. fowleri* have been detected in human sera indicates a widespread exposure to this pathogen despite the low number of identified PAM cases.

Beyond water temperature, the environmental risk factors for exposure to *N. fowleri* are not well understood. Studies attempting to elucidate the relationship between the presence of *N. fowleri* in a body of freshwater and various environmental parameters such as water temperature, air temperature or bacterial counts often produce results that conflict with one another [4, 5, 6, 12, 13, 14, 19, 23, 24, 25, 26, 40, 41]. Although the majority of these studies found a correlation between water temperature and the presence of *Naegleria* spp., some did not [5, 6, 14, 19, 25]. This discrepancy illustrates the need for more rigorous studies to characterize the natural environment and ecology of *N. fowleri* so that an evidence base can be developed to support public health risk communications and potential interventions.

To develop a better understanding of the environmental factors affecting the presence of *N. fowleri*, it is important to develop and validate improved detection and enumeration methods that are both sensitive and specific for the characterization of this pathogen in environmental samples such as sediment and water. Established detection methods rely on conventional culture techniques and biochemical and morphological examination, followed by nucleic acid sequencing and subsequent phylogenetic analysis. Although traditional methods are effective, these methods can be time-consuming and often require a combination of techniques in order to be highly specific for *N. fowleri*. For example, *N. fowleri* can be grown either monoxenically on non-nutrient agar (NNA) plates with *Escherichia coli* or axenically in a liquid medium [9]. However, when plating environmental samples, competition from other species of amoeba may inhibit the growth of *N. fowleri,* thereby resulting in a significant decrease in population number. In addition, traditional quantification using culture requires a most probable number estimation approach that is laborious and time consuming. Similarly, morphological analyses are done via microscopy but require an experienced eye in order to correctly differentiate pathogenic

Naegleria from non-pathogenic *Naegleria* and other closely related amoebae. Molecular methods offer an opportunity for simpler and faster detection (and possible quantification) of *N. fowleri* in environmental samples.

Molecularly, many species of *Naegleria* are recognized based on their small subunit ribosomal deoxyribonucleic acid (SSU rDNA), large subunit ribosomal DNA (LSU rDNA) and the ITS regions, including the 5.8S rDNA [18]. However, the major advance in typing *N. fowleri* isolates, and identifying different *Naegleria spp.* came with determining the ITS1, 5.8S rDNA and ITS2 sequences [11]. Current molecular assays target these gene sequences and exploit the differences in nucleic acid sequence between these regions among different *Naegleria spp.* Currently, molecular analytical methods are the most feasible approach for confirming the presence of *N. fowleri* in a sample. Multiple methods have recently been reported for the molecular detection and/or quantification of *Naegleria spp.* [1, 2, 3, 24, 28, 33, 34, 35, 37]. Although most of these assays report successful detection of *Naegleria spp.*, there is still a need to determine which method has better performance characteristics, taking into consideration elements of sensitivity, specificity, complexity, and robustness for a wide range of environmental samples. One drawback of molecular methods, such as PCR, for environmental applications is reaction inhibition. Humic compounds are the most commonly reported group of inhibitors in environmental samples [44]. Although the actual mechanism behind humic acid inhibition of PCR reactions is somewhat unclear, it is believed that the phenolic groups of humic compounds denature biological molecules [46]. However, the addition of polyvinylpolypyrrolidone (PVPP) or polyvinylpyrrolidone (PVP) to an agarose gel electrophoresis experiment has been shown to overcome this particular type of inhibition [46].

For this research project, four real-time PCR assays for the detection of *N. fowleri* were compared using the following parameters: assay specificity and sensitivity, limit of

detection, inhibition effects with humic acid, performance with seeded environmental matrices and performance with a panel of DNA from environmental samples previously identified by the Centers for Disease Control and Prevention (CDC) Environmental Microbiology Laboratory as being positive for *N. fowleri*. Based on the performance of these assays at each stage of analysis, those that performed less effectively were removed from further study to facilitate identification of the most effective assay for environmental testing [Figure 1].

The first real-time PCR assay is currently unpublished and was developed by the Environmental Microbiology Laboratory at CDC (Atlanta, GA). This method (Jothikumar et al.) was intended for use in environmental applications and includes the use of a fluorescent probe specifically designed to bind to a specific *N. fowleri* DNA sequence (5.8S rRNA gene and ITS region). The second assay was developed by Qvarnstrom et al. and was published in 2006 as part of a multiplex PCR assay [34]. This assay was developed for diagnostic applications to detect and differentiate between three pathogenic species of amoeba simultaneously (*Balamuthia mandrillaris, Naegleria fowleri and Acanthamoeba spp.*). The Qvarnstrom et al. assay also uses a fluorescent probe to bind to a specific *N. fowleri* DNA sequence (18S rRNA gene). The third assay was developed by Robinson et al. and uses primers that are specific for thermophilic *Naegleria* spp. and the closely related amoeba *Willaertia magna* by targeting the 5.8S rRNA gene [37]. Unlike the Jothikumar et al. and Qvarnstrom et al. assays, this method utilizes a DNA intercalating dye (SYTO9) for melt-curve analyses which is performed after PCR to differentiate *N. fowleri* from other *Naegleria* spp. based on the melting temperature of the PCR product. The last assay investigated in this research project was developed by Puzon et al. and also utilizes melt-curve analysis with SYTO9 for confirmation of amplified DNA [33]. In contrast to the Robinson et al. assay, the forward and reverse primers in the Puzon et al. assay were specific to *N. fowleri* by

targeting the ITS gene region and were specifically designed to detect this pathogen in biofilm and bulk water samples from a water distribution network [33].

As stated above, two of the real-time PCR assays employ the use of double-stranded DNA-binding dyes as the reporter molecule [33, 37] whereas the remaining two assays employ the use of fluorescent reporter probes that were designed based on a specific *N. fowleri* DNA sequence [34, Jothikumar et al]. Generally, assays that utilize a unique fluorescent reporter probe tend to be more specific than assays using a double-stranded DNA binding dye such as SYBR Green or SYTO9 because probe-based assays require hybridization of primers and a probe to generate fluorescence signals, whereas intercalating dye-based assays generate fluorescence signals based only on hybridization of primers. In addition, DNA intercalating dyes will bind non-specifically to any double-stranded DNA being created in the real-time PCR reaction, including DNA product formed by primer dimers [22]. Therefore, it was hypothesized that the Qvarnstrom et al. or Jothikumar et al. assay would perform the best based on the use of a specifically designed fluorescent reporter probe.

Figure 1. Experimental Plan for Comparison of *N. fowleri* Real-Time PCR Assays

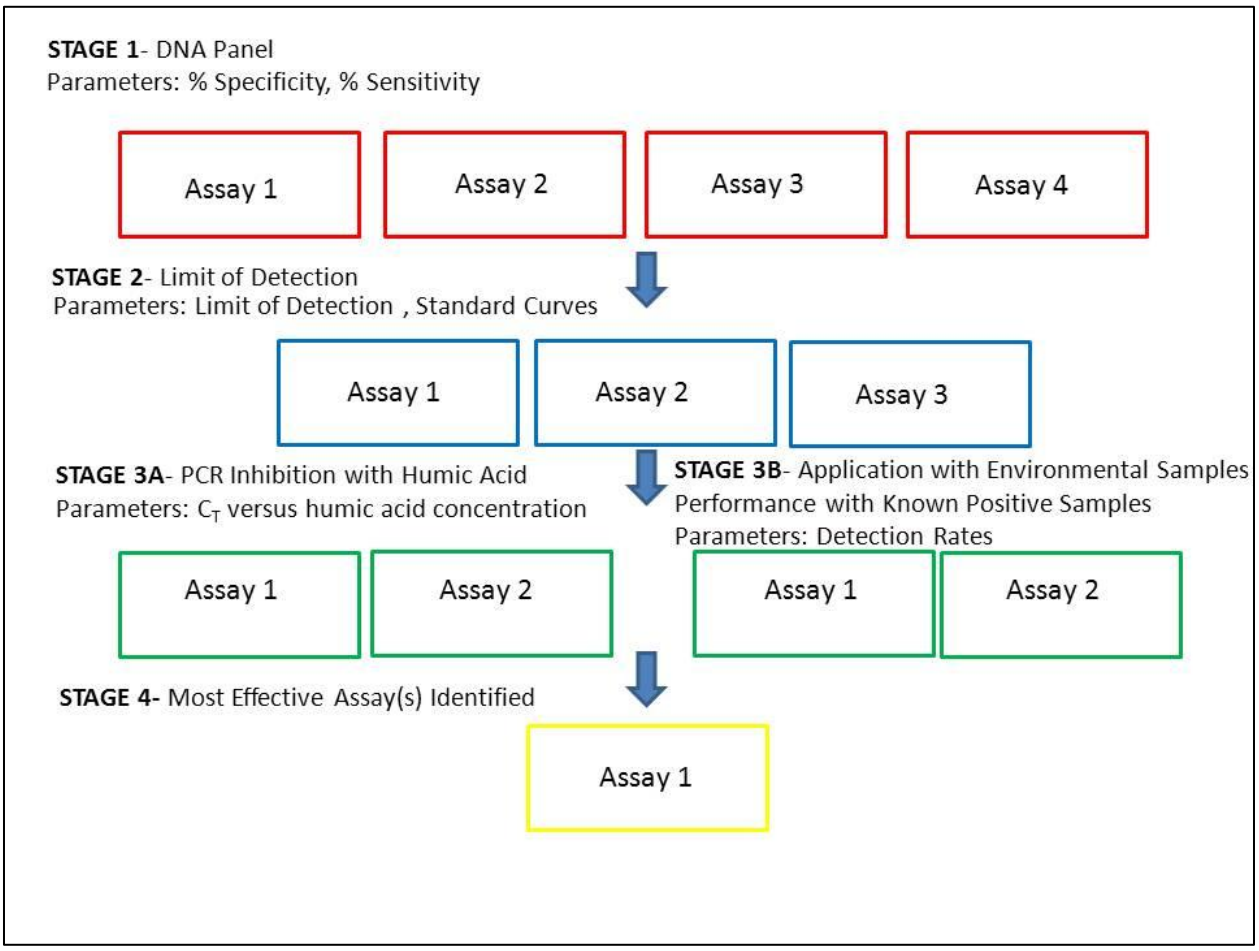


Figure 1: This graphic shows each stage of analysis and the parameters that were used to eliminate assays in a stepwise fashion. Assay numbers do not refer to any particular assay but instead refer to the number of assays remaining after each stage of analysis.

Methods

Amoebae Isolates: DNA from all amoeba isolates was extracted and provided by Dr. Govinda Visvesvara and Bonnie Mull, from the Water, Sanitation and Hygiene (WASH) Laboratory Team at the CDC. After culture, amoeba concentrations were determined by counts on a Thoma hemacytometer, using 400X total magnification on a standard light microscope. Isolates included in this study [Table 1] comprise several genotypes of *N. fowleri*, non-pathogenic *Naegleria* strains and other types of amoebae typically found in freshwater environments.

Table 1. Amoebae Isolates Used in Study

Sample	Genus and/or Species	Origin (Country/State)
30898	*Acanthamoeba castellanii*	Well (OH)
50171	*Echinamoeba exundans*	Hot water tank (CA)
50237	*Hartmannella vermiformis*	Hospital Cooling Tower Drain (SD)
30958	*N. australiensis*	Flood Drainage Water (Australia)
30544	*N. clarki*	Sewage effluent (OH)
PRA-166	*N. dunnebackei*	Water
CDC:V020	*N. fowleri* (Genotype I)	CSF (TX)
CAMP	*N. fowleri* (Genotype II)	CSF (CA)
30462	*N. fowleri* (Genotype IV)	Australia
CDC:V212	*N. fowleri* (Genotype I)	CSF (Mexico)
CDC: V515	*N. fowleri* (Genotype III)	CSF (AZ)
CDC: V511	*N. fowleri* (Genotype I)	CSF (GA)
30877	*N. gruberi*	Freshwater (AL)
PRA-153	*N. italica*	Freshwater (Peru)
30900	*N. jadini*	Swimming Pool (Belgium)
30811	*N. lovaniensis*	Canal (Belgium)
30467	*N. lovaniensis*	Water Supply (Australia)
30703	*Tetramitus jugosus*	Stream
30965	*Vahlkampfia inornata*	Freshwater (WI)
30298	*Vahlkampfia lobospinosa*	Cattle feces (TN)
50036	*Willaertia magna*	Thermal polluted water (Belgium)

The majority of the isolates are from the American Type Culture Collection (ATCC). *N. fowleri* genotypes I, II, III and IV isolates are from patient cerebrospinal fluid (CSF) samples submitted to the CDC for diagnostic purposes. *N. fowleri* Genotype I (CDC:V212) was used

as the positive control for all real-time PCR reactions. Nuclease-free water was used as the negative control for all reactions.

Thermodynamic Stability of Assays: The optimal annealing temperature for each set of primers was determined experimentally using the temperature gradient option on a BioRad iQ5 real-time PCR thermal cycler (BioRad, Hercules, CA). The highest annealing temperature for which there was no appreciable impact on cycle threshold (C_T) value was chosen as the annealing temperature for all subsequent real-time PCR reactions performed with the given assay. This was determined by plotting annealing temperature versus C_T value in MS Excel.

Real-Time PCR Assays: Four real-time PCR assays were chosen for a comparative analysis; one of which was an unpublished method developed by the CDC Environmental Microbiology Laboratory (Jothikumar et al). The table below [Table 2] shows method source, target gene and organism and real-time PCR cycling conditions used in this study.

Table 2. Real-Time PCR Assays Included in Study

Method	**Target Gene (Organism)**	**Cycling Conditions**		
		Temp	*Time*	*# Cycles*
Jothikumar et al.	5.8S rRNA gene and ITS region (*N. fowleri)*	95°C 95°C 63°C	10 min* 15 s 33 s	45
Qvarnstrom et al. 2006	18S rRNA gene (*N. fowleri)*	95°C 95°C 63°C	10 min* 15 s 60 s	45*
Robinson et al. 2006	5.8S rRNA gene (all *Naegleria* spp.)	95°C 94°C 50°C 72°C 80°C 75-95°C, Δ0.5°C	10 min* 20 s 20 s 20 s 6 s* 20 s	45*
Puzon et al. 2009	ITS region (*N. fowleri*)	95°C 95°C 52°C 72°C 80°C 75-95°C, Δ0.2°C	10 min* 30 s 30 s 45 s 6 s 10 s	45*

Within the table, real-time PCR cycling conditions marked with an asterisk (*) represent deviations from original assay conditions. The initial denaturation conditions were standardized to 95°C for 10 minutes for all assays due to the denaturation conditions specified for the TaqMan® Environmental Master Mix 2.0 (Applied Biosystems, Foster City, CA) that was used for all reactions. The number of real-time PCR cycles was set at 45 for each assay because the cutoff for classifying a reaction as positive was established for this study as C_T=42. For the Robinson et al. assay, the time for acquisition of fluorescence data was changed from 1 second to 6 seconds due to a minimum time limit of 6 seconds for any PCR step required by the Bio-Rad iQ5 thermal cycler used in this study.

Due to the primary focus of this project being molecular in nature, only the real-time PCR portion of the reported methods was followed. In order to effectively compare the candidate assays, several parameters were standardized. The reaction volumes were made uniform so that each assay contained the same DNA per reaction volume ratio (5 µL of extracted DNA in 50 µL of total reaction volume). Additional steps not listed in the table pertain to the melt-curve analysis of the Puzon et al. and Robinson et al. real-time PCR assays. The PCR products were subjected to melt curve analysis by the iQ5 for species-specific *Naegleria* detection. Samples were denatured by ramping the temperature from 75 to 95°C in 0.2 °C increments (0.5°C increments for the Robinson et al. assay). Fluorescent dye emission was detected for 10 s at each 0.2 °C increment (Puzon et al.) and for 20 s after each step (Robinson et al.). Melt curve profiles were automatically created by the iQ5 system software.

Assay Sensitivity and Specificity: The analytical sensitivity and specificity of the four assays was determined using a DNA panel that included six *N. fowleri* genotypes, six *Naegleria spp.*, two *Naegleria lovaniensis* genotypes, two *Vahlkampfia spp.*, *Acanthamoeba*

castellanii, Echinamoeba exundans, Hartmannella vermiformis, Tetramitus jugosus, and *Willaertia magna* for a total of twenty-one isolates. The concentration of the extracted DNA from isolates was equivalent to 200 amoebae/µL (1000 amoebae per real-time PCR reaction). Each reaction was performed in duplicate. The assay that demonstrated the lowest sensitivity or specificity was removed from further analyses. Analytical sensitivity (%) and specificity (%) were calculated as follows:

% Sensitivity = # True Positives / (# True Positives + # False Negatives) * 100

% Specificity = # True Negatives / (# True Negatives + # False Positives) * 100

Standard Curves and Limits of Detection: The limit of detection for the three remaining assays was determined with DNA extracted from stocks of *N. fowleri* genotypes I and III containing a known number of amoebae and serially diluted in TE buffer. These specific genotypes were chosen because they represent two of the three genotypes that cause PAM cases within the U.S. (De Jonckheere, 2011). The best fit of C_T value was calculated as a function of the log of the amoeba titer per reaction by linear regression to produce a standard curve. PCR Efficiency of each assay for both genotypes was determined using the following equation:

$$\text{PCR Efficiency (\%)} = -1 + 10^{(-1/\text{Slope})}$$

Each real-time PCR reaction was performed in triplicate. In conjunction with each standard curve, 1:2 dilutions were made from the last DNA concentration where all three reactions were positive and these dilutions were analyzed in 5 replicate reactions to more accurately estimate the limit of detection as the DNA template amount corresponding to a detection rate of ≥80%. The assay that exhibited the lowest limit of detection was removed from further analyses.

Humic Acid Inhibition Effects: To evaluate potential inhibitory effects of humic acid on the two remaining assays, varying concentrations (0, 5, 10, 20, 30 ng/µL) of Suwanee River Humic Acid Standard II (Cat # 2S101H, International Humic Substances Society, St. Paul, MN) were added to the real-time PCR reaction mixtures, which contained an equal amount of DNA. The relationship between humic acid concentration and real-time PCR C_T value for each assay was evaluated by plotting C_T values as a function of humic acid concentration. Each real-time PCR reaction was performed in triplicate with *N. fowleri* Genotype I (CDC:V212) as the source of DNA.

Application of Real-time PCR to Sediment and Water Samples: The performance of the two most effective real-time PCR assays was analyzed using seeded surface water and sediment samples. Sediment was included in this analysis because *N. fowleri* is commonly found in soil. In addition, sediment is a matrix commonly analyzed for this amoeba by the CDC Environmental Microbiology laboratory. Six 1-L surface water and six 1-L sediment samples were collected from Murphey Candler Lake located in DeKalb County, Georgia. Surface water and sediment samples were each seeded with approximately 83 *N. fowleri* amoebae (CDC:V212 , Genotype I). After seeding, surface water samples were subsequently centrifuged for 15 minutes at 15000 x *g* to pellet the *N. fowleri* trophozoites and cysts. Sediment samples were first washed with 1 L of WB saline (pH 6.5, prepared by CDC media/solutions production group) and the supernatant was processed using the same procedures performed for surface water samples.

Three surface water and three sediment samples were processed using immunomagnetic separation (IMS) prior to PCR analysis, whereas the remaining samples were analyzed directly without IMS. An IMS method developed by the CDC for the recovery of *N. fowleri* from water samples was used. CDC staff pre-coated Dynabeads with Biotin-

labeled anti-*Naegleria fowleri* monoclonal Antibody Nf-5D12 (Indicia Biotechnology Oullins, France) using Dynabeads Biotin Binder (Invitrogen 110.47) at a concentration of 2 µg of biotinylated antibodies per 50 µL of Dynabeads. These IMS beads were then used in experiments within 2 weeks. IMS beads were mixed with sample concentrates, then the mixture was allowed to incubate for 60 minutes with gentle rotation, then placed on a magnet and washed with buffer (0.01M PBS with 0.1% BSA and 2mM EDTA, pH 7.4) to remove any unbound antibodies. Pellets were resuspended in WB saline and 50 µL of freshly vortexed bead-antibody complex was added to each sample. Samples were then incubated at room temperature for 30 minutes on a rotator. The incubated Leighton tubes were then placed on the magnet for 3 minutes and the supernatant was subsequently discarded. The bead pellet was resuspended with 1 mL of buffer and was transferred to a 1.5 mL centrifuge tube and placed on the magnet for 2 minutes. The supernatant was discarded and the bead pellet was again resuspended in 100 µL of buffer and vortexed for 30 seconds.

Nucleic acid from all of the surface water and sediment samples (IMS and non-IMS) was extracted using the same protocol. Briefly, samples were added to approximately 200 mg each of 0.2 mm and 0.5 mm yttrium (III) oxide-stabilized zirconium oxide (ZrOx) beads and 750 µL of lysis buffer containing 4.5M guanidinium isothiocyanate. Samples were bead-beated at maximum speed for 1 minute in a Mini-Bead-Beater-8 (Biospec, Bartlesville, OK). The supernatant of each sample was added to a DNA-binding silica column (Omega Biotek, Norcross, GA) and centrifuged at 10,000 x *g* for 1 minute. Samples were subsequently washed with 100% ethanol and 70% ethanol, and centrifuged at 10,000 g for 1 minute after each wash. DNA was eluted with 80 µL of TE buffer and was then run through a Zymo-Spin™ IV-HRC column (Zymo Research Corporation, Irvine, CA) in order to remove potential PCR inhibitors. Each real-time PCR reaction was performed in triplicate for each sample

with DNA template volumes of both 5 µL and 2 µL to increase chances of detection in the presence of any remaining inhibitors.

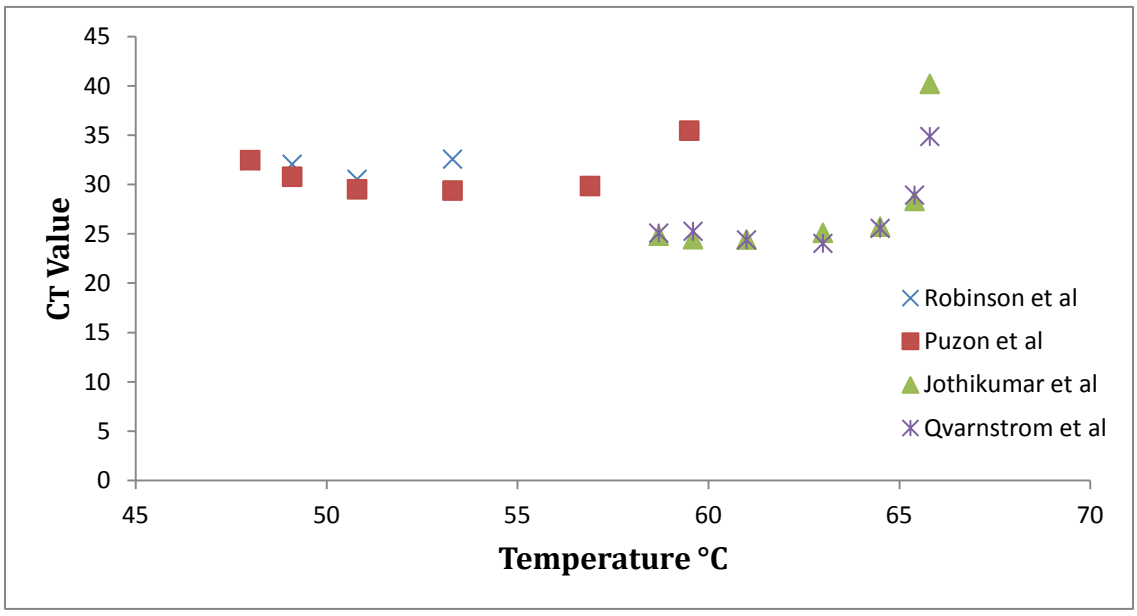
Real-time PCR Analysis of Known Positive Samples: The performance of the two most effective assays was further analyzed using surface water and sediment samples from Minnesota and Florida that CDC determined to be positive for *N. fowleri* in previous testing. DNA from these samples had been previously extracted and stored at -20°C. Each real-time PCR reaction was performed at least in duplicate for the present study.

Statistical Analysis: For the last two analyses (Stage 3A and 3B), Fisher's exact test was used to determine whether or not the assays performed differently based on the results for seeded environmental samples at a significance level of 0.05. Analyses were performed in OpenEpi (Version 2.3.1).

Results (Raw data presented in Appendix B)

Thermodynamic Stability of Assays: Using the temperature gradient option on the BioRad iQ5 real-time PCR thermal cycler, the optimal annealing temperature was determined experimentally for each set of assay primers. C_T values were plotted as a function of temperature using MS Excel. The protocol specified annealing temperature was found to be optimal for each assay. The Robinson et al. assay was found to yield consistent C_T values up to an annealing temperature of 53°C, after which it resulted in false-negative reactions (Figure 2). The Puzon et al. assay was found to have somewhat greater thermodynamic stability, with consistent C_T values up to 57 °C, after which substantially higher C_T values and false-negative results were obtained (Figure 2). The Jothikumar et al. and Qvarnstrom et al. assays were found to be more thermodynamically stable than the Robinson et al. and Puzon et al. assays, with consistent C_T values obtained up to ~65 °C (Figure 2).

Figure 2: Thermodynamic Stability of Assays



Assay Sensitivity and Specificity: The ability of each real-time PCR assay to successfully amplify the given target organism DNA was evaluated with a panel of extracted DNA from a total of twenty-one *N. fowleri, Naegleria spp.,* and other freshwater free-living amoebae.

For the Puzon et al. assay, DNA from all six *N. fowleri* strains successfully amplified whereas DNA from other *Naegleria* spp. and freshwater free-living amoebae did not, resulting in 100% sensitivity and 100% specificity [(6 / (6+0)) * 100 = 100% ; (15 / (15+0)) * 100 = 100%]. In addition, melt curves from the Puzon et al. assay had two peaks in the interval 78°C – 81.6°C; peak 1 at 78.3°C ± 0.14°C and peak 2 at 81.0°C ± 0.55°C when resolved at 0.5°C. Melt curve positions reported from the Puzon et al. study were different with peak 1 at 81.3°C ± 0.3°C and peak 2 at 84.2°C ± 0.4°C [33]. However the temperature difference between peak 2 and peak 1 was similar for both studies (2.9°C in Puzon et al. and 2.7°C in the current study). Slight variations in melt curve profiles could be the result of differences between the real-time PCR assay reaction components including the master mix. Both the Jothikumar et al. and Qvarnstrom et al. assays yielded 100% sensitivity and 94% specificity [(6 / (6+0)) * 100 = 100% ; (14 / (14+1)) * 100 = 93%]. The Qvarnstrom et al. assay amplified *Willertia magna* at a concentration of 1000 amoebae/reaction. However, the strength of the cross-reaction was weak due to the high C_T value at which the positive reaction was observed (average C_T=40.44) versus C_T values of ~30 when stocks of *N. fowleri* at 1000/reaction were amplified. The Jothikumar et al. assay amplified *Hartmannella vermiformis* at a concentration of 1000 amoebae/reaction. The strength of this cross-reaction was also weak (average C_T= 40.25) versus C_T values of ~30 when stocks of *N. fowleri* at 1000/reaction were amplified.

For the Robinson et al. assay, DNA from 10 of the targeted *Naegleria* spp. strains amplified whereas DNA from 4 of the remaining targeted *Naegleria* spp. strains did not, resulting in 71% sensitivity, but 100% specificity (10 / (10+4) * 100 = 71% ; 7 / (7+0) * 100

= 100%). In addition, melt curves from this assay had 1-2 peaks in the interval 78°C-82°C when resolved at 0.5°C. Melt curve results from the Robinson et al. assay are displayed in Table 3. Melt curve profiles reported from the Robinson et al. study are italicized below the results obtained from the current study, including the third peak for *N. fowleri* that Robinson et al. reported but which was not observed in the present study. Differences in melt curve profiles are most likely the result of a different thermal cycler being used in the current study than was used by Robinson et al. As stated in Robinson's study, the method of analysis of the DNA melting-curve data is critical for the resolution of multiple melting domains [37]. Due to the relatively low percent sensitivity of the Robinson et al. assay and the fact that it failed to amplify one of the strains of *N. fowleri* used in subsequent limit of detection analyses, it was removed from further investigation. Results are displayed in Table 4.

Table 3. Positions of Melt Curve Peaks for Robinson et al. Assay

Species	**Strain**	**Peak 1 (°C)**	**Peak 2 (°C)**
		Reported	Reported
N. fowleri	CDC:V212, CDC:V511, CAMP, 30462	78.2 ± 0.26	81.2 ± 0.26
		80.46 ± 0.11	82.25 ± 0, 3rd peak at 84.75 ± 0
N. lovaniensis	30811	77.8 ± 0.35	81.5 ± 0
		81.55 ± 0.05	84.75 ± 0
N. dunnebackei	PRA-166	78.8 ± 0.35	81.0 ± 0
		Not used in Robinson study	Not used in Robinson study
N. australiensis	30958	81.5 ± 0	None
		83.75 ± 0	85.15 ± 0
N. gruberi	30877	79.5 ± 0	82 ± 0
		82.4 ± 0	84.85 ± 0.025
N. clarki	30544	78 ± 0	81.3 ± 0.35
		Not used in Robinson study	Not used in Robinson study
N. italica	PRA-153	78.5 ± 0	81.5 ± 0
		82.25 ± 0	84.75 ± 0

Table 4. Sensitivity and Specificity of Real-Time PCR Assays

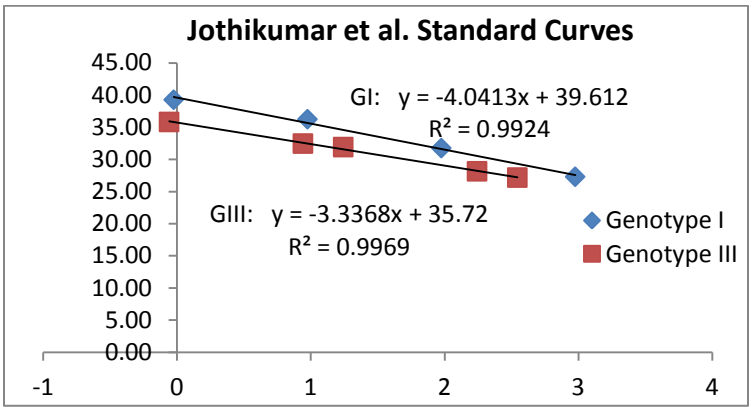
Method (# Target Organisms)	**Sensitivity**	**Specificity**
Jothikumar 2011 (CDC) (6 *N. fowleri*)	100%	93%
Qvarnstrom et al. 2006 (6 *N. fowleri*)	100%	93%
Robinson et al. 2006 (14 *Naegleria*)	71%	100%
Puzon et al. 2009 (6 *N. fowleri*)	100%	100%

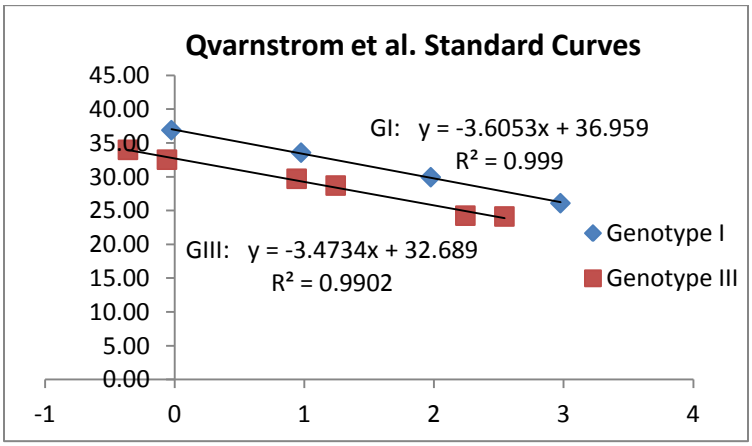
Standard Curves and Limits of Detection: The R^2 of the best fit line for each standard curve was ≥0.9902. The Puzon et al. assay had lower overall PCR efficiency for both genotype I (82.9%) and III (71.5%). The Jothikumar et al. assay had the lowest PCR efficiency for genotype I (76.8%), but the highest for genotype III (99.4%). The Qvarnstrom et al. assay had more consistent, and overall higher, PCR efficiency for both genotypes I (89.4%) and genotype III (94.0%).

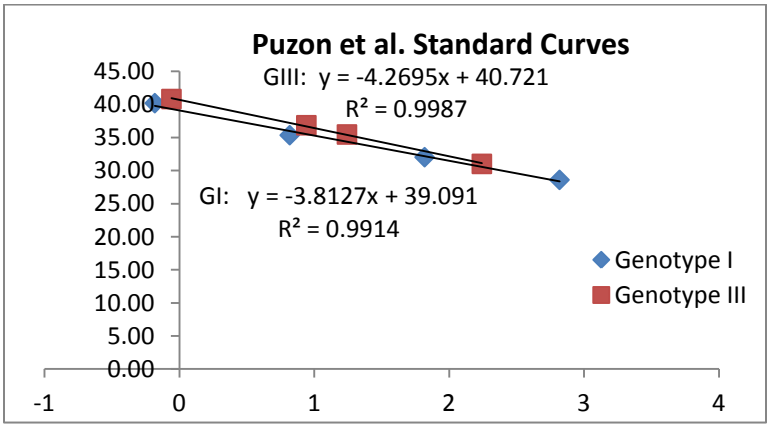
The limit of detection for the Jothikumar, Qvarnstrom et al. and Puzon et al. assays was determined experimentally using DNA extracted from *N. fowleri* genotypes I and III and serially diluted in TE buffer. Standard curves were plotted in MS Excel as C_T versus the log of amoeba titer per reaction and are shown in Figure 3A-3C. For the Jothikumar et al. assay, a product was amplified from extracted DNA equivalent to 0.2 amoebas per reaction for genotype I and 0.05 amoebas for genotype II. For the Qvarnstrom et al. assay, a product was amplified from extracted DNA equivalent to 0.1 amoebas per reaction for genotype I and 0.2 amoebas per reaction for genotype III. For the Puzon et al. assay, a product was amplified from extracted DNA equivalent to 0.2 amoebas per reaction for genotype I and 0.8 amoebas per reaction for genotype III. In environmental applications, sensitivity is an important parameter to consider when designing a PCR assay. Therefore, the Puzon et al.

assay was eliminated from further analysis based on lower overall PCR efficiency for the assay and a higher limit of detection for *N. fowleri* genotype III.

Figures 3A-3C. Standard Curves for *N. fowleri* Genotypes I and III



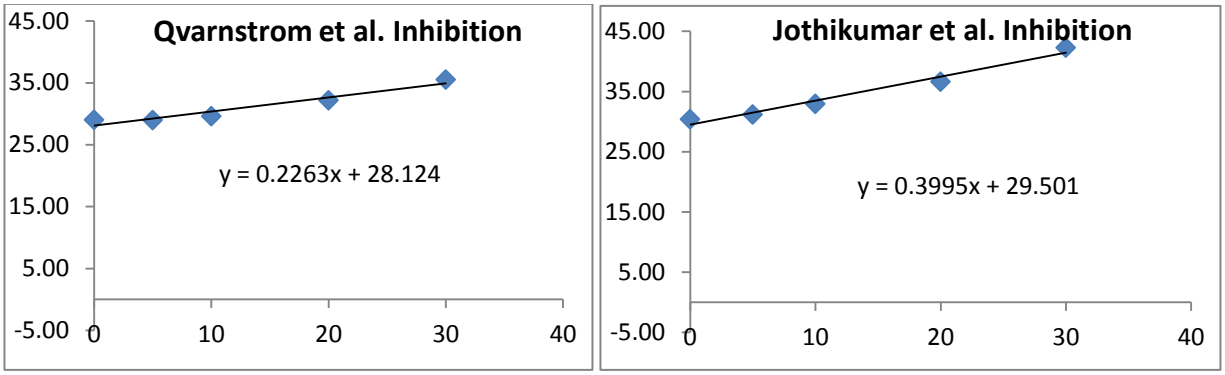




Humic Acid Inhibition Effects: Using a range of 0-30 ng/µL of humic acid, the Qvarnstrom et al. assay demonstrated less susceptibility to inhibition [Figure 4], with the ΔC_T value at 30 ng/µL

(versus 0 ng/µL) = 7.0. When this concentration of humic acid was present in Jothikumar et al. assays, the ΔC_T value (versus 0 ng/µL) was 11.8. The slope of the C_T value versus humic acid concentration relationship was 0.2263 for the Qvarnstrom et al. assay and 0.3995 for the Jothikumar et al. assay.

Figure 4. C_T Value as a Function of Humic Acid Concentration



Application of Real-time PCR to Sediment and Water Samples: Results from the Qvarnstrom et al. and Jothikumar et al. real-time PCR assays performance with seeded sediment and surface water matrices are displayed in Table 5.

Table 5. Application to Environmental Sediment and Water Samples Results

Assay	Matrix	Direct Analysis (no IMS)		With IMS	
		5 µL DNA	*2 µL* DNA	*5 µL* DNA	*2 µL* DNA
Jothikumar	Sediment	0/3	0/3	0/3	0/3
	Water	0/3	1/3	1/3	1/3
Qvarnstrom	Sediment	0/3	0/3	0/3	0/3
	Water	2/3	0/3	1/3	1/3

Overall, three of the seeded samples that were analyzed with the Jothikumar et al. assay were positive whereas four of the seeded samples that were analyzed with the Qvarnstrom et al. assay were positive. This difference in detection between the two assays was not statistically significant (p=0.500). For both assays, the IMS procedure was not found to be associated with significantly higher real-time PCR detection of *N. fowleri* (p = 0.5 for Jothikumar et al assay, p =

0.705 for Qvarnstrom et al. assay). Assaying a smaller volume of sample (specifically,2 µL) can result in improved nucleic acid amplification in the presence of inhibitors, but this technique did not appear to improve detection rates for either assay, with the possible exception of *N. fowleri* detection in water using the Jothikumar et al assay.

Real-time PCR Analysis of Known Positive Samples: The Jothikumar et al. and Qvarnstrom et al. assays were used to analyze sediment and water samples collected in Minnesota and Florida. These samples were previously determined by CDC to be positive for *N. fowleri*. Results are displayed in Table 6. Out of the 14 samples analyzed, both assays detected *N. fowleri* in 7 of the samples. In most cases, the Jothikumar et al. and Qvarnstrom et al. assays yielded the same results. However, the sediment sample #2 from Minnesota was identified as positive by the Jothikumar et al. assay but not by the Qvarnstrom et al. assay. Similarly, the water sample #6 from Minnesota was identified as positive by the Qvarnstrom et al. assay but not by the Jothikumar et al. assay.

Table 6. Assay Performance with *N. fowleri* Positive Samples

State	Sample ID (Matrix)	Outcome	
		Jothikumar	Qvarnstrom
Minnesota	1 (Sediment)	Positive	Positive
	2 (Sediment)	Positive	Negative
	3 (Water)	Negative	Negative
	4 (Sediment)	Negative	Negative
	5 (Sediment)	Negative	Negative
	6 (Water)	Negative	Positive
	7 (Sediment)	Positive	Positive
	8 (Sediment)	Negative	Negative
Florida	1 (Water)	Positive	Positive
	2 (Water)	Negative	Negative
	3 (Sediment)	Negative	Negative
	4 (Sediment)	Positive	Positive
	5 (Sediment)	Positive	Positive
	6 (Sediment)	Positive	Positive

Assay Choice: As demonstrated by the results of the three different stages of analysis, the performance of the Robinson et al. and Puzon et al. assays was determined to be lower than the

Jothikumar et al. and Qvarnstrom et al. assays, and thus were eliminated from extensive investigation in this study. On the contrary, the Qvarnstrom et al. and Jothikumar assays performed well at each stage of analysis. Comparatively speaking, both the Jothikumar et al. and Qvarnstrom et al. assays had similar performance at each stage of analysis. The assays had identical percent sensitivity and specificity, thermodynamic stability, and performance with seeded environmental matrices and *N. fowleri* positive samples. The Jothikumar et al. assay had a lower detection limit for *N. fowleri* genotype III whereas the Qvarnstrom et al. assay had a somewhat lower detection limit for *N. fowleri* genotype I. In terms of humic acid inhibition, the Qvarnstrom et al. assay was more robust. However, the Jothikumar et al. assay appeared to perform better when the seeded environmental samples were processed via IMS versus when samples were processed directly, suggesting that humic acid inhibition could be overcome with a few modifications to the laboratory processing protocol. Although the Qvarnstrom et al. assay performed slightly better with seeded environmental matrices (4 positive samples / 24 seeded samples versus 3 positive samples / 24 seeded samples with the Jothikumar et al. assay), this difference in assay performance was not statistically significant. Therefore, both assays were chosen as suitable for future use in environmental applications. The framework for arriving at this conclusion is presented in Table 7.

Table 7. Performance of Real-Time PCR Assays

	Performance			
Parameter	**Jothikumar**	**Qvarnstrom**	**Puzon**	**Robinson**
% Sensitivity	100%	100%	100%	67%
% Specificity	93%	93%	100%	100%
Thermodynamic Stability	≤ 65°C	≤ 65°C	≤ 57°C	≤ 53°C
Limit of Detection GI/GIII	0.2/0.05	0.1/0.2	0.2/0.8	N/A
Humic Acid Inhibition (slope)	0.3395	0.2263	N/A	N/A
Performance with Seeded Environmental Matrices*	3/24	4/24	N/A	N/A
Performance with *N. fowleri* Positive Samples**	7/14	7/14	N/A	N/A

* Represents number of samples that were positive out of 24 total seeded samples (combined IMS and Direct)

** Represents number of samples that were positive out of the 14 known positive samples

Discussion

Robinson et al. and Puzon et al. Assays: The four real-time PCR assays were characterized in the following areas: analytical sensitivity and specificity, limit of detection, inhibition with humic acid and performance with seeded environmental samples and samples previously determined to be positive by the CDC. It was determined that there were no major performance differences between the Jothikumar et al. and Qvarnstrom et al. assays, therefore both were recommended for future use in environmental applications. The Puzon et al. and Robinson et al. assays were removed from further investigation based on relatively lower analytical sensitivity (Robinson et al.) with the amoeba isolates used in this study and a relatively higher detection limit for *N. fowleri* genotype III (Puzon et al.).

The relatively lower performance of the Robinson et al. and Puzon et al. assays observed in this study should not be considered as suggesting that these assays cannot be effective in general or for specific applications. The Robinson et al. assay was not designed to be specific for *N. fowleri*. However, the researchers determined that the *Naegleria* species could be differentiated based on unique melt curve profiles. As displayed in Table 3, the current study was unable to replicate the melt-curve results from Robinson et al., thereby making it difficult to differentiate *N. fowleri* from other *Naegleria* species. In addition, the analytical sensitivity of the Robinson et al. assay (67%) was lower than the other assays. One possible reason for this is that the amoeba isolates used in the current study included several *Naegleria* species that were not tested in the Robinson et al. study, namely *N. jadini* and *N. fowleri* genotype III, both of which did not amplify. Another reason is the use of a different master mix in the current study versus that used in the Robinson et al. study. Lastly, the detection limit of an environmental assay should be relatively low in order to have confidence in detecting target organisms whose concentration is largely unknown. Because the Puzon et al. assay had a higher detection limit for *N. fowleri* genotype III (0.8 amoebas/reaction), it was removed from further analyses. However, the Puzon et al. assay was developed by an

Australian research group and genotype III has not caused any known PAM cases in Australia [11]. This might also explain the lower detection limit of 0.01 cells measured in the Puzon et al. assay due to the use of different *N. fowleri* strains although the exact genotype was not specified [33].

In general, melt curve analysis lengthens the real-time PCR procedure time and interpretation of melt curves can be difficult, especially if performed on different instruments using different analytical software. As stated by Robinson et al., the method of analysis of the DNA melting-curve data is critical for the resolution of multiple melting domains [37]. However, a melt curve assay has the potential to serve well for characterization of an environmental sample if this is the goal of a study, rather than rapid detection of a specific organism. In addition, if PCR primers for non-probe assays are not specific to the target organism, then the melt temperatures must be characteristic of the target organism or else the risk of false positives is increased.

Geographical Context of Assay: *N. fowleri* genotypes I, II and III have been identified in the United States [11]. Both the Jothikumar et al. assay and Qvarnstrom et al. assay amplified all three genotypes, in addition to genotype IV which has been identified in Europe. Therefore, both of these assays are robust for environmental samples from different geographical areas where at least one of these genotypes have been identified (United States, Mexico, Asia or Europe) [11].

Qvarnstrom et al. and Jothikumar et al. Cross-reactivity: The Qvarnstrom et al. assay was found to have weak cross-reactivity with *Willertia magna* whereas the Jothikumar et al. assay was found to have weak cross-reactivity with *Hartmannella vermiformis.* However, identifying these amplifications as cross-reactions is solely based on the determined C_T cutoff value. A difference of ~10 C_T values was observed for the cross-reactions for both assays, indicating that these non-target amoebas would need to be present in a sample at a concentration ~10^3 higher than *N. fowleri* (assuming a standard curve slope of 3.3x relating C_T values to microbe concentration) in order to

represent a substantial false-positive risk for an environmental sample. *W. magna* and *N. fowleri* are closely related species so cross-reaction with *W. magna* is not surprising. In addition, *W. magna* was not specifically tested for cross-reactivity in the published Qvarnstrom et al. study due to the assay's clinical/diagnostic nature, rather than environmental focus [34]. The fact that the two assays demonstrated cross-reactivity to different species indicates the possibility of using both of them to reduce the possibility of obtaining a false positive for an environmental sample.

Humic Acid Inhibition: In this study, the effects of inhibition on the Jothikumar et al. and Qvarnstrom et al. assays was determined using only humic acid. However, environmental samples have a wide range of inhibitors including phenolic compounds and heavy metals which could potentially have different affects on the two assays [43]. The use of a 2-µL DNA template volume in the seeded environmental samples experiment may have been effective for improving the detection rate for the Jothikumar et al. assay. Therefore, the results from this study suggest that two or more different sample volumes (e.g., 5 µL, 2 µL) should be assayed when applying the Jothikumar et al. assay to environmental samples.

Results of Seeded Environmental Matrices and Known Positive Samples Analysis: Ideally, all of the seeded environmental sediment and water samples and the known positive samples should have been identified as positive by both assays. For the seeded environmental matrices, detection rates for both assays were relatively low (Jothikumar et al. = 3/24, Qvarnstrom et al. = 4/24). However, this indicates the difficulty in assessing environmental samples and suggests the importance of additional processing steps to remove potential inhibitors (such as using a PVPP column during the DNA extraction process or IMS). Similarly, 7/14 of the known positive samples were identified as positive by both assays, although *N. fowleri* was detected in 8/14 samples between the two assays. These results suggest that the archived DNA from these samples might have degraded during

storage, but lend additional support for the recommendation to use both assays when testing environmental samples.

Limitations: Due to the comparative nature of this study, several real-time PCR parameters were standardized across the four assays in order to effectively compare the results. First, the master mix used in the current study differed from the ones specified for the Qvarnstrom et al., Puzon et al., and Robinson et al. studies. It is likely that the differences in the melt curve profiles generated for the current assay versus those of the Robinson et al. assay were a result of performing the assay using a different master mix and real-time PCR instrument. However, deviations between assay conditions published for the Qvarnstrom et al., Puzon et al., and Robinson et al. assays and those of the current study were kept to a minimum and should not have dramatically affected the experimental outcomes.

Another limitation of the current study is the sample size of the seeded environmental matrices. In total, there were only six sediment and six surface water samples that were seeded with *N. fowleri* amoebae. A larger sample size was probably needed in order to measure any significant difference in the two assay's detection rates. Similarly, the laboratory processing of the pellet results in a relatively small amount of sample (750 µL) that subsequently undergoes nucleic acid extraction and real-time PCR analysis. This indicates that the majority of the seeded *N. fowleri* could be missed, especially with sediment which tends to be more difficult to process than surface water.

Strengths: First, the current study was a fairly robust analysis due to the fact that the assays were characterized based on several parameters. Assays were removed from further consideration in a stepwise fashion until the strongest real-time PCR assays remained. Second, a large number of amoeba isolates were used in the analytical sensitivity and specificity portion of this study,

including *N. fowleri* genotypes directly obtained from cerebrospinal fluid (CSF) from PAM case patients. All three genotypes found in the United States were also included in this DNA panel in addition to a variety of other amoeba species commonly found in environmental samples. Analysis of the Florida and Minnesota samples that were previously tested as positive provided a unique opportunity to test the performance of the Jothikumar et al. and Qvarnstrom et al. assays. Finally, the same analyst and same PCR thermal cycler were used throughout the study which eliminated the need to control for these variables when comparing assay outcomes.

Conclusion and Recommendations:

In conclusion, both the Jothikumar et al. and Qvarnstrom et al. assays should be effective for use in future analyses with environmental matrices. It is advantageous to have two effective real-time PCR assays to use, especially if the results of one assay are inconclusive. In this case, the other assay could be used for confirmation purposes. The assays were designed to amplify different target genes of *N. fowleri* [specifically, the 18S rRNA and 5.8S rRNA genes], so when used in conjunction with one another they could potentially have a greater chance of detecting environmental concentrations of *N. fowleri* versus one method used individually.

Based on the uncertain results obtained in the analyses with seeded environmental matrices and known positive samples, it would be beneficial to conduct more of these experiments with both assays. The seed amount used in the current study was 83 *N. fowleri* amoebae/liter of sediment or water. In future analysis, it might be valuable to study at least one higher *N. fowleri* seed level to determine the range of concentrations that both assays can detect in water and sediment samples. In addition, a larger sample size would increase the power to detect any notable differences between the Jothikumar et al. and Qvarnstrom et al. assays.

In terms of laboratory analysis, using IMS prior to the nucleic acid extraction process and is recommended to remove potential inhibitors existing in environmental samples. Similarly, a

smaller DNA template volume than what was used in the current study (5 µL in a 50-µL reaction volume) might further decrease the risk of real-time PCR inhibition. Humic acid was the only inhibitor used in the current study. If time allows, both assays should be characterized with other PCR inhibitors such as fulvic acid, phenolic compounds or heavy metals to evaluate the effects of a range of potential inhibitor compounds.

Geographically, both the Jothikumar et al. and Qvarnstrom et al. assays are useful for detecting genotypes commonly found in the United States. However, to increase the utility of these assays in global applications, additional genotypes could be added to the amoeba isolate DNA panel. Genotypes V-VIII were not used in the current study but all have been identified in Europe.

Worldwide, *N. fowleri* continues to be a tragic source of water-related mortality. In addition, ecological changes related to global climate change could potentially increase the risk for PAM in new geographical areas. Little is known about the ecology of *N. fowleri* in the environment. However, understanding ecological parameters that govern this pathogen's behavior and survival in surface water and sediment is crucial for identifying potential risk factors related to PAM infection. Characterization of real-time PCR methods for detection of *N. fowleri* is important to identify the assay(s) with the highest analytical sensitivity and specificity, lowest PCR inhibition effects and robustness for both sediment and surface water samples. The two molecular methods recommended (Jothikumar et al. and Qvarnstrom et al.) should be useful contributors to studies investigating the potential risk factors for human exposure to *N. fowleri.*

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Appendix A: Assay Protocols

Qvarnstrom, Y., Visvesvara, G. S., Sriram, R., & da Silva, A. J. (2006). Multiplex Real-Time PCR Assay for Simultaneous Detection of *Acanthamoeba spp., Balamuthia mandrillaris,* and *Naegleria fowleri*. *Journal of Clinical Microbiology, 44*(10), 3589-3595.

"For the detection of *Naegleria fowleri*, primers NaegIF192 (3'-GTG CTG AAA CCT AGC TAT TGT AAC TCA GT-5') and NaegIR344 (5'-CAC TAG AAA AAG CAA ACC TGA AAG G-3') were used to amplify a 153-bp long fragment, detected by the hexachlorofluorescein (HEX)-labeled probe NfowlP (5'-HEX-AT AGC AAT ATA TTC AGG GGA GCT GGG C-BHQ1-3'). The triplex reaction mix contained 1x Platinum Quantitative PCR Supermix-UDG with ROX (Invitrogen, Carlsbad, Calif.), 0.2 μM of each primer, 0.1 μM of each probe, and 1 μL of DNA in 20 μL total reaction volume. PCRs were performed in a Mx3000P real-time thermocycler (Stratagene, La Jolla, Calif.), with two initial holds at 50°C for 2 min (incubation for uracil-DNA-glycosylase activity) and 95°C for 2 min (activation of Platinum *Taq* DNA polymerase), respectively, followed by 40 cycles of 95°C for 15 seconds and 63 for 15 seconds and 63°C for 60 seconds. Fluorescence was measured at the end of each 63C incubation. The results were analyzed using the Mx3000P version 2.0 software."

Puzon, G. J., Lancaster, J. A., Wylie, J. T., & Plumb, J. T. (2009). Rapid Detection of Naegleria fowleri in Water Distribution Pipeline Biofilms and Drinking Water Samples. *Environmental Science & Technologh, 43*(17), 6691-6696

"RT-PCR melt curve analysis of the *Naegleria* intragenic spacer region (ITS) was done using a BioRad iQ5 (BioRad, US) following the method of Robinson et al. Each 25 μL reaction contained, 12.5 μL Hot Star Taq Master Mix (Qiagen, US), 500 nM forward primer, 500 nM reverse primer, 2.0 μM SYTO9 (Molecular Probes, US), and 2 μL of DNA template or nuclease free water for controls. Primer pairs amplifying the ITS of all *Naegleria* species as well as other amoebae, e.g., consensus primers (CP), were used for competition experiments. For specific detection of *N. fowleri* the primer pair designed by Pelandakis et al. was initially used, however the forward primer was found to misprime in the opposite direction generating short fragments not suitable for RT-PCR melt curve analysis. A new forward primer (FWS) (5′GTGAAAACCTTTTTTCCATTT3′), specific for the *N. fowleri* ITS, was designed and combined with the consensus reverse primer (5′TTTCTTTTCCTCCCCTTATTA3′) to form a primer pair (SP) specific for *N. fowleri*. RT-PCR cycling conditions were 95 °C 15 min followed by 50 cycles of 95 °C 30 s, 52 °C 30 s, 72 °C 45, with a 6 s pause at 80 °C for fluorescent dye detection. Cycle thresholds (*C*T) were determined automatically by the iQ5 optical system software. After completion of amplification, the PCR products were subjected to melt curve analysis by the iQ5 for species-specific *Naegleria* detection. Samples were progressively denatured by ramping the temperature from 75 to 95 °C in 0.2 °C increments. Fluorescent dye emission was detected for 10 s at each 0.2 °C increment. Melt curve profiles were automatically plotted by the iQ5 system software. Each sample was assayed in triplicate."

Robinson, B. S., Monis, P. T., & Dobson, P. J. (2006). Rapid, Sensitive, and Discriminating Identification of *Naegleria* spp. by Real-Time PCR and Melting-Curve Analysis. *Applied and Environmental Microbiology, 72*(9), 5857-5863.

"Real-time PCR and melting-curve analysis were carried out in a RotorGene 3000 (Corbett Research, Sydney, Australia). An advantageous feature of this instrument for our purpose is that it permits the operator to manipulate key conditions of the melting analysis, including the temperature interval and the holding time before fluorescence data are collected at each step. The 20-μl reaction mixture consisted of 200 μM concentrations of deoxynucleoside triphosphates (Promega, Madison, WI), 200 nM forward and reverse primers (NGITSF, 5'-AACCTGCGTAGGGATCATTT, and NGITSR, 5'-TTTCCTCCCCTTATTAATAT) PCR buffer II, 2.5 mM MgCl2 (Applied Biosystems, Branchburg, NJ), 2.0 μM SYTO9 (Molecular Probes, Eugene, OR), 1 U of Ampli*Taq* Gold (Applied Biosystems), and 2.0 μl of sample DNA in Instagene supernatant or nuclease-free water. A range of conditions for realtime PCR were tested. Unless otherwise stated, the conditions for experiments reported here were 10 min at 95°C to activate *Taq* polymerase, followed by 50 cycles of 20 s at 94°C, 20 s at 50°C, and 20 s at 72°C. The ramping between the extension and melting steps included a 1-s pause at 80°C for acquisition of fluorescence data (6-carboxyfluorescein channel, excitation at 470 nm, detection at 510 nm, gain set to maximum of 10). Upon completion of amplification, the program continued directly to a melting-curve analysis in which temperature was ramped from 75 to 95°C in steps of 0.5°C. Fluorescence data were collected after pauses of 60 s on the first step and 20 s on subsequent steps to allow the melting DNA structure to stabilize, again using the 6-carboxyfluorescein channel, but with two gain settings, 3 and 5, to allow for variations in the amount of product amplified. For fine-scale resolution of the melting analysis, data were collected in some experiments at 0.2°C intervals, with pauses of 60 s on the first and 10 s on subsequent steps. The method of analysis of the DNA melting-curve data is critical for the resolution of multiple melting domains. The RotorGene software uses bicubic interpolation to estimate fluorescence between data points when graphing the negative of the first derivative of the raw DNA melting data and provides the option of curve smoothing. For all analyses reported here, no smoothing was used in the generation of melting curves (i.e., the digital filter was set to none)."

Appendix B: Raw Data

Table 1: Thermodynamic Stability of Assays

							Melt Curve Analysis	
Assay	**Temperature °C**	**CT Value**		**Assay**	**Temperature °C**	**CT Value**	**Peak 1 (Temp °C)**	**Peak 2 (T**
Jothikumar et al.	65.8	N/A		Robinson et al.	62	N/A	N/A	N/
	65.4	40.2			61.1	N/A	N/A	N/
	64.5	28.34			59.5	N/A	N/A	N/
	63	25.73			56.9	N/A	N/A	N/
	61	25.11			53.3	32.57	78.5	81
	59.6	24.43			50.8	30.53	78.5	8
	58.7	24.46			49.1	32.06	78.5	8
	58	24.82			48	32.4	78.5	81
Qvarnstrom et al.	65.8	N/A		Puzon et al.	62	N/A	**Peak 1 (Temp °C)**	**Peak 2 (T**
	65.4	34.87			61.1	N/A	N/A	N/
	64.5	28.93			59.5	35.46	N/A	N/
	63	25.56			56.9	29.85	78.4	81
	61	24.04			53.3	29.39	78.4	81
	59.6	24.39			50.8	29.52	78.6	81
	58.7	25.26			49.1	30.79	78.4	81
	58	25.08			48	32.47	78.4	81
							78.6	81

Table 2A & 2B: Analytical Sensitivity and Specificity

TABLE 2A	Jothi			Qvarnstrom			
Isolates	**Samples**	**Ct Value**	**Average Ct**		**Samples**	**Ct Value**	**Average Ct**
	Neg. Control (MM)	N/A			Neg. Control (MM)	N/A	
	Pos. Control CDCV212	23.28			Pos. Control CDCV212	27.68	
N. lovaniensis	30467	N/A			30467	N/A	
N. dunnebackei	PRA-166	N/A			PRA-166	N/A	
N. australiensis	30958	N/A			30958	N/A	
Nf Genotype IV	30462	25.84, 26.28	26.06		30462	26.2, 27.42	26.81
H. vermiformis	50237	40.01, 40.49	40.25		50237	N/A	
N. gruberi	30877	N/A			30877	N/A	
N. clarki	30544	N/A			30544	N/A	
V. inornata	30965	N/A			30965	N/A	
Nf Genotype II	CAMP	23.85, 24.74	24.30		CAMP	29.97, 29.28	29.63
N. lovaniensis	30811	N/A			30811	N/A	
N. italica	PRA-153	N/A			PRA-153	N/A	
T. jugosus	30703	N/A			30703	N/A	
V. lobospinosa	30298	N/A			30298	N/A	
E. exundans	50171	N/A			50171	N/A	
N. jadini	30900	N/A			30900	N/A	
Nf Genotype III	CDC V515	std. curve			CDC V515	std. curve	
Nf Genotype I	CDC V212	std. curve			CDC V212	std. curve	
Nf Genotype I	CDC V020	38.67, 38.47	38.57		CDC V020	35.10, 34.90	35.00
Nf Genotype 1	CDC V511	31.76, 31.69	31.73		CDC V511	26.94, 27.18	27.06
A. castellanii	30898	N/A			30898	N/A	
W. magna	50036	N/A			50036	40.21, 40.67	40.44

TABLE 2B	**Assay**					**Assay**				
	Puzon					Robinson				
				Melt Curve					**Melt Curve**	
Isolates	**Samples**	**Ct Value**	**Average Ct**	**Avg. Peak 1**	**Avg. Peak 2**	**Samples**	**Ct Value**	**Average Ct**	**Avg. Peak 1**	**Avg. Peak 2**
	Neg. Control (MM)	N/A				Neg. Control (MM)				
	Pos. Control (CDCV212)					Pos. Control (CDCV212)	33.41			
N. lovaniensis	30467	N/A				30467	N/A			
N. dunnebackei	PRA-166	N/A				PRA-166	25.68, 25.30	25.49	78.75	81
N. australiensis	30958	N/A				30958	25.33, 25.70	25.52		81.5
Nf Genotype IV	30462	29.77, 29.98	29.88	78.2	81.4	30462	37.68, 38.21	37.95	78	81.5
H. vermiformis	50237	N/A				50237	N/A	N/A		
N. gruberi	30877	N/A				30877	31.85, 30.5	31.18	79.5	82
N. clarki	30544	N/A				30544	31.73, 32.09	31.91	78	81.25
V. inornata	30965	N/A				30965	N/A	N/A		
Nf Genotype II	CAMP	31.25, 30.54	30.90	78.3	81.5	CAMP	32.45, 32.34	32.40	78	81.5
N. lovaniensis	30811	N/A				30811	35.78, 36.06	35.92	77.5	81.5
N. italica	PRA-153	N/A				PRA-153	36.33, 36.35	36.34	78.5	81.5
T. jugosus	30703	N/A				30703	N/A	N/A		
V. lobospinosa	30298	N/A				30298	N/A	N/A		
E. exundans	50171	N/A				50171	N/A	N/A		
N. jadini	30900	N/A				30900	N/A	N/A		

Nf Genotype III	CDC V515	std. curve		78.5	81.2	CDC V515	N/A	N/A		
Nf Genotype I	CDC V212	std. curve		78.3	81.2	CDC V212	35.76, 35.27	35.52	78.25	81.25
Nf Genotype I	CDC V020	39.09, 38.43	38.76	78.5	81.2	CDC V020	N/A	N/A		
Nf Genotype 1	CDC V511	33.52, 31.71	32.62	78.4	81.2	CDC V511	38.69, 38.51	38.6	78	81
A. castellanii	30898	N/A				30898	N/A	N/A		
W. magna	50036	N/A				50036	N/A	N/A		

Table 3A: Qvarnstrom Standard Curves

Assay (Genotype)	Log (pfu)	Dilution	Titer/µL	Titer/rxn		CT Value		Average CT	Std. Dev
Qvarnstrom (GI)		Undilute	1893	9465					
	2.97612062	1.00E-01	189.3	946.5	26.18	26.06	26.07	26.10	0.07
	1.97612062	1.00E-02	18.93	94.65	30.04	29.89	29.92	29.95	0.08
	0.97612062	1.00E-03	1.893	9.465	33.56	33.64	33.55	33.58	0.05
	-0.0238794	1.00E-04	0.1893	0.9465	36.83	36.37	37.53	36.91	0.58
	-1.0238794	1.00E-05	0.01893	0.09465	39.56	N/A	N/A		
	-2.0238794	1.00E-06	0.001893	0.009465	N/A	N/A	N/A		
	Log (pfu)	**Dilution**	**Titer/µL**	**Titer/rxn**		**CT Value**		**Average CT**	**Std. Dev**
Qvarnstrom (GIII)		Undilute	700	3500					
	2.54406804	1.00E-01	70	350	24.06	24.67	23.68	24.14	0.50
	2.24303805	1:2A	35	175	25.26	22.54	24.98	24.26	1.50
	1.24303805	1.00E-02	3.5	17.5	28.73	28.98	28.43	28.71	0.28
	0.94200805	1:2B	1.75	8.75	29.88	29.84	29.43	29.72	0.25
	-0.0579919	1.00E-03	0.175	0.875	31.72	33.13	32.77	32.54	0.73
	-0.3590219	1:2C	0.0875	0.4375	33.74	34.26	33.99	34.00	0.26
		1.00E-04	0.00875	0.04375	35.54	N/A	N/A		

Table 3B: Qvarnstrom Detection Limits

Qvarnstrom (GI)					
Amoeba/rxn			**Ct Values**		
0.2187	37.67	38.1	36.97	37.83	39.46
0.1094	N/A	39.37	36.3	37.55	38.76
0.0547	38.35	N/A	N/A	N/A	N/A
0.0273	39.13	38.42	N/A	N/A	N/A
Qvarnstrom (GIII)					
Amoeba/rxn			**Ct Values**		
0.2187	37.7	37.83	35.94	36.15	35
0.1094	38.2	37.8	36.8	N/A	N/A
0.0547	37.08	N/A	N/A	38.31	41.44
0.0273	38.28	N/A	N/A	N/A	N/A

Table 3C: Jothikumar Standard Curves

Assay (Genotype)	Log (pfu)	Dilution	Titer/µL	Titer/rxn		CT Value		Average CT	Std. Dev
Jothi (GI)		Undilute	1893	9465					
	2.97612062	1.00E-01	189.3	946.5	27.32	27.26	27.33	27.30	0.04
	1.97612062	1.00E-02	18.93	94.65	31.79	31.83	31.65	31.76	0.09
	0.97612062	1.00E-03	1.893	9.465	35.84	36.03	36.88	36.25	0.55
	-0.0238794	1.00E-04	0.1893	0.9465	38.86	39.36	39.61	39.28	0.38
	-1.0238794	1.00E-05	0.01893	0.09465	40.34	41.96	40.82		
		1.00E-06	0.001893	0.009465					
Assay (Genotype)	Log (pfu)	Dilution	Titer/µL	Titer/rxn		CT Value		Average CT	Std. Dev
Jothi (GIII)	3.54406804	Undilute	700	3500					
	2.54406804	1.00E-01	70	350	27.08	27.24	27.21	27.18	0.09
	2.24303805	1:2A	35	175	27.74	28.85	27.86	28.15	0.61
	1.24303805	1.00E-02	3.5	17.5	31.91	31.94	31.9	31.92	0.02
	0.94200805	1:2B	1.75	8.75	32.16	32.45	32.78	32.46	0.31
	-0.0579919	1.00E-03	0.175	0.875	35.82	35.9	35.74	35.82	0.08
	-0.3590219	1:2C	0.0875	0.4375	37.18	37.43	38.02	37.54	0.43
	-1.3590219	1.00E-04	0.00875	0.04375	40.4	41.4			

Table 3D: Jothikumar Detection Limits

Jothi (GI)					
Amoeba/rxn			**Ct Values**		
0.4733	35.03	34.75	36.9	35.69	35.04
0.2366	36.32	36.4	35.78	37.62	37.15
0.1183	N/A	36.61	N/A	37.3	36.07
0.0592	N/A	38.03	41.75	N/A	N/A
Jothi (GIII)					
Amoeba/rxn			**Ct Values**		
0.2187	37.64	39.29	38.44	40.27	40.19
0.1094	40.04	40.36	39.67	40.5	41.99
0.0547	41.38	40.84	41.18	40.78	40.86
0.0273	39.16	39.69	42.74	42.02	43.14

Table 3E: Puzon Standard Curves

Assay (Genotype)	Log (pfu)	Dilution	Titer/µL	Titer/rxn		CT Value		Average CT	Std. Dev
Puzon (GI)		Undilute	1317.5	6587.5					
	2.81872063	1.00E-01	131.75	658.75	29.34	28.45	28.06	28.62	0.66
	1.81872063	1.00E-02	13.175	65.875	32.1	32.06	31.98	32.05	0.06
	0.81872063	1.00E-03	1.3175	6.5875	35.42	35.12	35.58	35.37	0.23
	-0.1812794	1.00E-04	0.13175	0.65875	41.26	40.11	39.28	40.22	0.99
	-1.1812794	1.00E-05	0.013175	0.065875	N/A	42.68	41.79		
Assay (Genotype)	**Log (pfu)**	**Dilution**	**Titer/µL**	**Titer/rxn**		**CT Value**		**Average CT**	**Std. Dev**
Puzon (GIII)	3.54406804	Undilute	700	3500					
	2.54406804	1.00E-01	70	350					
	2.24303805	1:2A	35	175	31.34	31.1	30.65	31.03	0.35
	1.24303805	1.00E-02	3.5	17.5	35.47	35.68	35.29	35.48	0.20
	0.94200805	1:2B	1.75	8.75	36.87	36.88		36.88	0.01
	-0.0579919	1.00E-03	0.175	0.875	40.88	40.79	40.85	40.84	0.05
	-0.3590219	1:2C	0.0875	0.4375					
	-1.3590219	1.00E-04	0.00875	0.04375					

Table 3F: Puzon Detection Limit

Puzon (GI)			Ct Values		
Amoeba/rxn					
3.294	36.44	36.14	36.44	36.45	37.14
1.6469	37.46	36.39	37.62	36.92	36.91
0.8234	39.01	38.83	38.36	37.39	37.52
0.4117	39.75	39.46	39.28	38.84	39.47
0.2059	40.68	41.07	39.71	39.33	42.42
0.1029	41.53	N/A	43.19	42.98	N/A

Puzon (GIII): Detection Limit

***Determined directly from standard curve since Ct values for last dilution were 40.88, 40.79, 40.85.**

Table 4: PCR Inhibition

Assay- Jothi						Assay- Qvarnstrom					
Humic Acid Conc (ng/uL)		Ct		Average Ct	Std. Dev	Humic Acid Conc (ng/uL)		Ct		Average Ct	Std. Dev
0	30.46	30.43	30.33	30.41	0.07	0	29.44	29.38	28.26	29.03	0.66
5	31.46	30.99	31.11	31.19	0.24	5	29.09	29.26	28.59	28.98	0.35
10	33.06	33	32.81	32.96	0.13	10	29.74	29.98	29.12	29.61	0.44
20	36.74	37.35	35.82	36.64	0.77	20	32.41	32.42	31.72	32.18	0.40
30	42.29	N/A	N/A	42.29		30	35.15	36.44	35	35.53	0.79

Table 5: Seeded Environmental Matrices

Assay	Method	Matrix		CT		Average	Std. Dev
Jothi - 5 µL	IMS	Soil-1	Neg.	Neg.	Neg.		
		Soil-2	Neg.	Neg.	Neg.		
		Soil-3	41.55	Neg.	Neg.	41.55	
		Water-1	41.76	39.84	39.47	40.36	1.23
		Water-2	Neg.	Neg.	Neg.		
		Water-3	Neg.	Neg.	Neg.		
	Direct	Soil-1	Neg.	Neg.	Neg.		
		Soil-2	Neg.	Neg.	Neg.		
		Soil-3	Neg.	Neg.	Neg.		
		Water-1	Neg.	Neg.	Neg.		
		Water-2	43.31	41.52	42.65	42.49	0.91
		Water-3	Neg.	Neg.	Neg.		
Assay	**Method**	**Matrix**		**CT**		**Average**	**Std. Dev**
Qvarnstrom- 5 µL	IMS	Soil-1	Neg.	Neg.	Neg.		
		Soil-2	Neg.	Neg.	Neg.		
		Soil-3	Neg.	Neg.	Neg.		
		Water-1	38.4	39.41	37.66	38.49	0.88
		Water-2	Neg.	Neg.	Neg.		
		Water-3	39.76	Neg.	Neg.	39.76	
	Direct	Soil-1	Neg.	Neg.	Neg.		
		Soil-2	Neg.	Neg.	Neg.		
		Soil-3	Neg.	Neg.	Neg.		
		Water-1	Neg.	Neg.	Neg.		
		Water-2	39.45	39.41	Neg.	39.43	0.03
		Water-3	40.41	39.72	40.34	40.16	0.38

Table 5 (Continued): Seeded Environmental Matrices

Assay	Method	Matrix		CT		Average	Std. Dev
Jothi - 2 µL	IMS	Soil-1	Neg.	Neg.	42.7	42.70	
		Soil-2	Neg.	41.67	Neg.	41.67	
		Soil-3	Neg.	Neg.	Neg.		
		Water-1	40.93	39.84	41.67	40.81	0.92
		Water-2	Neg.	Neg.	Neg.		
		Water-3	Neg.	Neg.	40.88	40.88	
	Direct	Soil-1	Neg.	Neg.	41.91	41.91	
		Soil-2	Neg.	Neg.	Neg.		
		Soil-3	Neg.	Neg.	Neg.		
		Water-1	Neg.	Neg.	Neg.		
		Water-2	41.97	Neg.	41.74	41.86	0.16
		Water-3	Neg.	Neg.	42.04	42.04	
Assay	**Method**	**Matrix**		**CT**		**Average**	**Std. Dev**
Qvarnstrom- 2 µL	IMS	Soil-1	Neg.	Neg.	Neg.		
		Soil-2	Neg.	Neg.	Neg.		
		Soil-3	Neg.	Neg.	Neg.		
		Water-1	Neg.	37.84	36.91	37.38	0.66
		Water-2	Neg.	Neg.	Neg.		
		Water-3	Neg.	Neg.	Neg.		
	Direct	Soil-1	Neg.	Neg.	Neg.		
		Soil-2	40.4	Neg.	Neg.	40.4	
		Soil-3	Neg.	Neg.	Neg.		
		Water-1	Neg.	Neg.	Neg.		
		Water-2	39.64	Neg.	Neg.	39.64	
		Water-3	Neg.	Neg.	Neg.		

Table 6: Known Positives from MN & FL

Assay	Sample	CT		Average	Std. Dev	Assay	Sample	CT		Average	Std. Dev
Jothi	**FL**					Qvarnstrom	**FL**				
	BW D	40.3	40.48	40.39	0.13		BW D	37.88	37.23	37.56	0.46
	CW D		43.03	43.03			CW D				
	CS IMS						CS IMS				
	BS D	36.28	35.94	36.11	0.24		BS D	33.9	34.61	34.26	0.50
	CS D	40.18	40.7	40.44	0.37		CS D	39.09	39.47	39.28	0.27
	BS IMS	22.16	22.71	22.44	0.39		BS IMS	20.37	20.66	20.52	0.21
	MN						**MN**				
	LIS D	39.15	40.47	39.81	0.93		LIS D	36.83	38.68	37.76	1.31
	BCS IMS		41.9	41.90			BCS IMS		38.56	38.56	
	BCW D						BCW D	41.44		41.44	
	BMW D						BMW D				
	DMS IMS		41.1	41.10			DMS IMS	40.1		40.10	
	ELW D	42.72	43.27	43.00	0.39		ELW D	38.78		38.78	
	LCS IMS	41.83	40.08	40.96	1.24		LCS IMS	37.69	38.54	38.12	0.60
	LIS IMS						LIS IMS				