THE OCCURRENCE OF FREE LIVING AMOEBAE IN WATER

by

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2012
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SIGNED: Laura Yvette Sifuentes
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DEDICATION

I dedicate this work first and foremost to God, without whom nothing is possible. I give glory and honor to him in all of the things I accomplish.

This dissertation is also dedicated to my loving husband, Steven Hernandez, who has been incredibly loving and supportive. I love you dearly and I can’t imagine my life without you!

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The amoebae *Naegleria fowleri*, *Acanthamoeba spp.*, and *Balamuthia mandrillaris* are free-living amoebae found in both water and soil. They are opportunistic pathogens in humans. *Acanthamoeba* is the most common cause of illness, usually infecting the eyes and sometimes causing a sight-threatening keratitis. *Acanthamoeba* spp. and *B. mandrillaris* can cause granulomatous amoebic encephalitis, in addition to infections of the lungs and skin. *N. fowleri* causes primary amoebic meningoencephalitis. There is little known regarding the ecology and occurrence of these organisms.

A total of 36 high-use recreational surface waters in Arizona were surveyed over a period of two years to assess the occurrence of *N. fowleri* and seasonal and environmental factors. Overall, 9.3% of the warm weather samples collected were positive for *N. fowleri*, whereas 16.3% of the samples were positive during cold weather. Although the presence of *N. fowleri* could not be significantly correlated with physical and chemical parameters such as temperature, pH, turbidity, conductivity, and the presence of heterotrophic bacteria, total coliforms, and *Escherichia coli*, a weak correlation (0.52) with live amoebic activity was observed. Five lakes to the north and northeast of Phoenix tested positive for the *N. fowleri* on more than one occasion over multiple seasons.
Finished drinking water samples (n= 785) from a municipal potable distribution system were evaluated for the presence of *N. fowleri*, *B. mandrillaris* and *Acanthamoeba* spp. from 18 different regions during three different sampling periods. Physical and chemical parameters were also evaluated but provided no significant correlations with the occurrence of amoebae or indicator organisms. A total of 138 samples (17.9%) were positive for viable amoebae in distribution water with more than an adequate chlorine residual (average of 0.86 mg/L). Microorganisms that are typically used to monitor microbial water quality such as coliforms and *E. coli* would likely not be found under these circumstances. Clusters with three or more samples testing positive for viable amoebae per region were observed during all three periods. Viable amoebae may not only provide a better assessment of the microbial quality of water, but such clustering could reveal areas with potential water quality issues within the distribution system.
INTRODUCTION

Problem Definition:

Waterborne disease remains an important issue in the United States. Because water is a human necessity, every human being is exposed to potential risk of disease caused by waterborne and water-based pathogens. The occurrence of these pathogens needs to be monitored in order to assess the human risk from a variety of illnesses ranging from gastroenteritis, keratitis, and infections of the lungs and skin to more serious illnesses such as aseptic meningitis, primary amoebic meningoencephalitis (PAM), and granulomatous amoebic encephalitis (GAE) (Visvesvara et al. 2007). Waterborne diseases may arise from the contamination of water through human or animal feces infected with pathogenic viruses, bacteria, or protozoa. In addition, water-based pathogens occur naturally in the water and include bacteria such as *Legionella* spp. and the protozoan, *Naegleria fowleri*. This dissertation is focused on water-based pathogens that present a serious risk of disease when present in recreational waters and finished drinking water.

There were 398 recreational water outbreaks documented in the U. S. between 1999 and 2008 (Lee et al. 2002; Yoder et al. 2004; Dziuban et al. 2006; Yoder et al. 2008; Hlavsa et al. 2011). *Naegleria fowleri* has been well documented to occur in recreational waters including swimming pools, ponds (Cerva 1971), and natural hot
springs, but also in hot tubs (Rivera et al. 1993), domestic water supplies (Marciano-Cabral et al. 2003), and artificially heated industrial water sources (De Jonckheere & Voorde 1977). Although rare, the disease caused by N. fowleri (PAM) has a 99% case fatality rate. Since 2000, the number of deaths caused by water-based amoebae has doubled and recreational water outbreaks have tripled (Lee et al. 2002; Hlavsa et al. 2011). Between 1998 and 2011, N. fowleri killed 46 people in the United States, all in warmer regions. Despite a subsequent increase in public awareness, there is little known regarding the ecology and occurrence of this organism.

Despite effective treatment of drinking water, waterborne disease outbreaks are still attributed to exposure to pathogens in treated drinking water (Reynolds et al. 2008; Reynolds et al. 2008; Craun et al. 2010a; Craun et al. 2010b). More than 880 such outbreaks were documented in the US between 1971 to 2008; many more occurred that were either not reported or not recognized (Craun et al. 2010b; Brunkard et al. 2011). Specifically, problems within water distribution systems such as biofilm formation and water main breaks have been linked to a number of outbreaks (Blackburn et al. 2004). Water-based free living amoeba may be found in groundwater, surface water, and even finished drinking water worldwide, though at this time, the public health significance is unknown (Cerva 1971; De Jonckheere & Voorde 1977; Rivera et al. 1993; John & Howard 1995; Marciano-Cabral et al. 2003; Maclean et al. 2004; Blair et al. 2008).
Free-living amoeba

The free living amoebae are ubiquitous in aquatic environments and are aerobic, mitochondriate, eukaryotic protists (Visvesvara et al. 2007). They are often referred to as amphizoic amoebae due to their ability to live freely without a host in addition to having the capability to invade a host and live as parasites (Page 1976; Visvesvara et al. 2007). Humans are continually exposed to these amoebae due to their ubiquitous occurrence in the environment. These organisms can also be found in drinking water (Hoffmann & Michel 2001). Free living amoebae have been shown to colonize surfaces by adhering and secreting metabolic substances, thereby creating a biofilm (Hoffmann & Michel 2001). A biofilm is composed of bacteria held in a polymeric matrix and can form on the pipe wall of water distribution system (Hallam et al. 2001). These biofilms have been shown exert a chlorine demand, pipe corrosion, promote coliform growth and affect water taste and odor problems in the water (Hallam et al. 2001).

*Naegleria fowleri, Acanthamoeba spp., and Balamuthia mandrillaris* are pathogenic free-living amoebae found in the environment in both water and soil. They are opportunistic pathogens in humans, causing infections of the central nervous system
(CNS) (Visvesvara et al. 2007) in healthy young children and adults (Visvesvara et al. 2007).

(Visvesvara et al. 2007). Of the three, Acanthamoeba is the most common cause of illness, usually infecting the eyes and sometimes causing a sight-threatening keratitis. 

*N. fowleri* infects its host via the nasal mucosa following the forceful inhalation of contaminated water and causes primary amoebic meningoencephalitis (PAM). The organism destroys the host brain tissue, resulting in a 99% case fatality rate principally in healthy children and young adults (Visvesvara et al. 2007; Centers for Disease Control and Prevention (CDC) 2008; Yoder et al. 2010a). Acanthamoeba spp. and *B. mandrillaris* can also cause a highly fatal CNS infection known as granulomatous amoebic encephalitis (GAE), in addition to infections of the lungs and skin (Martinez & Visvesvara 1997; Schuster & Visvesvara 2004a; Visvesvara et al. 2007).

*Naegleria fowleri*

*Naegleria* belongs to the family *Vahlkampfiidae* (Sawyer & Griffin 1975) (Ma et al. 1990). There have been over 40 species of *Naegleria* described to date, but only *N. fowleri* is pathogenic to humans; *N. australiensis* has been shown to be pathogenic in mice (De Jonckheere 2004). *Naegleria fowleri* was first identified as a human pathogen in 1965 in Australia (Fowler & Carter 1965). The first case in the United States was reported in 1966 and was described as primary meningoencephalitis (Martinez 1985).
Prior to this documented case, the free living amoebae were not considered to be pathogenic.

*N. fowleri* is considered to be a frank pathogen as it is able to infect healthy children and young adults (Barnett et al. 1996; Rose et al. 2001; Cervantes-Sandoval et al. 2007). It primarily infects individuals in younger age groups (children and teenagers) that are more likely to engage in recreational water activities that involve submerging the head under water. This amoeba is thermophilic, able to proliferate in temperatures of up to 45°C, but does best in temperatures ranging from 35°C to 45°C (Visvesvara et al. 2007).

**Occurrence**

The occurrence of *N. fowleri* is worldwide (Cabanes et al. 2001). The majority of identified cases are in countries with tropical and subtropical climates (Parija & Jayakeerthee 1999). *N. fowleri* is found in a variety of warm fresh and brackish waters including swimming pools (Cerva 1971), hot tubs (Rivera et al. 1993), domestic water supplies (Marciano-Cabral et al. 2003), well water (Blair et al. 2008), ponds, lakes, streams, rivers, natural hot springs, sewage (Wellings et al. 1977)(John & Howard 1995)(De Jonckheere & Voorde 1977; Ettinger et al. 2003; Sheehan et al. 2003), and thermally polluted run-off from industrial zones (De Jonckheere & Voorde 1977). Water sources do not have to be contaminated in order to contain *N. fowleri* (Heggie 2010) as it is a water-based organism. In the United States, the occurrence of *N. fowleri* has been
documented in surface waters in Virginia, Oklahoma, Florida, New Mexico, and Arizona (Wellings et al. 1977; John & Howard 1995; Lee et al. 2002; Marciano-Cabral et al. 2003). More recently, the United States Environmental Protection Agency (USEPA) added *N. fowleri* to their Contaminant Candidate List 3 (CCL3). The CCL is established by a process of identifying the waterborne infectious agents of concern in source waters by evaluating significant health effects and occurrence of unregulated contaminants (United States Environmental Protection Agency (USEPA) 2008). The CCL 3 includes 12 microbiological pathogens of concern in water (Table 1) (United States Environmental Protection Agency 2009).
Table 1. Contaminant Candidate List 3 for Microbial Contaminants.

<table>
<thead>
<tr>
<th>Microbial Contaminant Name</th>
<th>Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenoviruses</td>
<td>Viruses that cause respiratory illness and occasionally gastrointestinal illness</td>
</tr>
<tr>
<td>Caliciviruses</td>
<td>Viruses (includes norovirus) causing mild self-limiting gastrointestinal illness</td>
</tr>
<tr>
<td>Campylobacter jejuni</td>
<td>Bacterium causing mild self-limiting gastrointestinal illness</td>
</tr>
<tr>
<td>Enteroviruses</td>
<td>Group of viruses including polioviruses, Coxsackieviruses, and echoviruses that can cause mild respiratory illnesses</td>
</tr>
<tr>
<td>Escherichia coli (O157)</td>
<td>Toxin-producing bacterium causing gastrointestinal illness and kidney failure</td>
</tr>
<tr>
<td>Helicobacter pylori</td>
<td>Bacterium sometimes found in the environment that is capable of colonizing the human gut and that can cause ulcers and cancer</td>
</tr>
<tr>
<td>Hepatitis A virus</td>
<td>Virus that causes liver disease and jaundice</td>
</tr>
<tr>
<td>Legionella pneumophila</td>
<td>Bacterium found in the environment including hot water systems causing lung diseases when inhaled</td>
</tr>
<tr>
<td>Mycobacterium avium</td>
<td>Bacterium causing lung infections in those with underlying lung disease, and disseminated infection in the severely immunocompromised</td>
</tr>
<tr>
<td>Naegleria fowleri</td>
<td>Protozoan found in shallow, warm surface and ground water causing primary amebic meningoencephalitis</td>
</tr>
<tr>
<td>Salmonella enterica</td>
<td>Bacterium causing mild self-limiting gastrointestinal illness</td>
</tr>
<tr>
<td>Shigella sonnei</td>
<td>Bacterium causing mild self-limiting gastrointestinal illness and bloody diarrhea</td>
</tr>
</tbody>
</table>

(United States Environmental Protection Agency 2009).
**Life Cycle**

*Naegleria fowleri* is an amoeboflagellate with three life stages: the trophozoite, cyst and flagellate stages (Figure 1). N. fowleri can transform back and forth between the trophozoite and the flagellate stage. The flagellate form is a transitional stage, only lasting an hour or less, in which the organism can neither feed nor divide (Visvesvara et al. 2007). Flagellates are pear-shaped and range in size from 10 to 16 µm, usually with two flagella (Figure 1). The trophozoite is the feeding stage of the organism and also its infectious form, causing primary meningoencephalitis (PAM) in humans (Carter 1970; Parija & Jayakeerthee 1999). Trophozoites usually measure from 10 to 25 µm in size and reproduce by binary fusion. Locomotion is achieved by the use of lobopodia, hemispherical bulges, allowing for rapid movements. The trophozoite can transition into a cyst form when the environmental conditions change or become unfavorable, such as when the food supply diminishes (Rose et al. 2001; Visvesvara et al. 2007).

*N. fowleri* mostly exists as a cyst in the environment, whether in soil or water. The cyst form is resistant to different forms of environmental stress such as desiccation and changes in temperature. The cyst is spherical and is composed of a double cell wall containing a thick endocyst and a thin ectocyst, measuring 9 µm in diameter (Barnett et al. 1996; Rose et al. 2001; Visvesvara et al. 2007). The cysts can also revert (excyst) to a trophozoite when the environmental conditions become suitable (Dorsch et al. 1983;
The flagellate and the cyst forms of \textit{N. fowleri} are known to contain a nucleus and a well defined nucleolus (Schuster & Visvesvara 2004a).

\textbf{Figure 1.} Life Cycle of \textit{Naegleria fowleri}.

\textbf{Primary Amoebic Meningoencephalitis (PAM)}

\textit{Exposure and Infection}

\textit{Naegleria fowleri} causes primary amoebic meningoencephalitis (PAM) which is fulminating and hemorrhagic encephalitis. Most often, humans and other mammals come in contact with \textit{N. fowleri} during water-related activities such as swimming, bathing, or in the case of cattle and domesticated animals, drinking from or swimming in water sources where \textit{N. fowleri} is present. Infections associated with this organism are usually associated with children and young adults swimming in natural springs or warm
water lakes. Another possible route of exposure to *N. fowleri* is when the cyst form is inhaled from dust in arid regions (Ma *et al.* 1990). As seen in Figure 2, the trophozoite is the infective form and is the form found in the cerebral spinal fluid (CSF) and in tissues (Centers for Disease Control and Prevention (CDC) 2012c). Death occurs due to an increase in brain pressure and subsequent herniation which leads to cardiopulmonary arrest and pulmonary edema (Martinez 1985; Visvesvara & Schuster 2008).

The organism is inhaled through the nose through the olfactory neuroepithelium as can be visualized in Figure 2. The amoebae are phagocytosed in the nasopharyngeal mucosa and then migrate via the olfactory nerves where it eventually invades the brain through the cribriform plate (Bottone 1993; Visvesvara *et al.* 2007). *N. fowleri* destroys the tissue by causing a cytopathogenic effect (CPE) thought to occur through the production of amebostomes that eat away at the tissue (Visvesvara *et al.* 2007).
*Naegleria fowleri* has three stages, cysts 1, trophozoites 2, and flagellated forms 3, in its life cycle. The trophozoites replicate by promitosis (nuclear membrane remains intact) 4. *N. fowleri* is found in fresh water, soil, thermal discharges of power plants, heated swimming pools, hydrotherapy and medicinal pools, aquariums, and sewage. Trophozoites can turn into temporary non-feeding flagellated forms which usually revert back to the trophozoite stage. Trophozoites infect humans or animals by penetrating the nasal mucosa 5 and migrating to the brain 6 via the olfactory nerves causing PAM) *N. fowleri* trophozoites are found in CSF and tissue, while flagellated forms are occasionally found in CSF. Cysts are not seen in brain tissue (Centers for Disease Control and Prevention (CDC) 2012c)
*Naegleria fowleri* has the ability to evade the host immune system where it then has the advantage with its ability to attach to the nasal mucosa, move quickly through locomotion and release cytolytic molecules (Marciano-Cabral & Cabral 2007). Resistance to an *N. fowleri* infection may be attributed to the host innate immunity rather than acquired immunity seen by the activity of complement neutrophils and macrophages (Marciano-Cabral & Cabral 2007). However, most PAM patients die before the immune response can be detected (Visvesvara *et al.* 2007).

The immune response leads to swelling of the brain and PAM (Marshall *et al.* 1997). Death usually follows within 7 to 10 days after infection (Carter 1970; Martinez 1985; Barnett *et al.* 1996; Martinez & Visvesvara 1997; Marciano-Cabral & Cabral 2007). Because death occurs so quickly, the exact immune response is not easy to detect. There is some evidence, seen in a survivor of the disease, to show that the antibody response generated is IgM. Over 80% of the patients with PAM who were hospitalized had some IgG, but mostly IgM antibodies to *N. fowleri* (Visvesvara *et al.* 2007).
Incidence of illness

There have been approximately 235 cases of PAM documented worldwide (De Jonckheere 2011), with more than 100 in the United States (Visvesvara et al. 2007; Yoder et al. 2008; Visvesvara 2010; Yoder et al. 2010a; De Jonckheere 2011) and at least six in Arizona (Willaert 1974; John & Nussbaum 1983)(Marciano-Cabral et al. 2003; The Arizona Republic 2006). The most recent cases of PAM are shown in Table 3. A few of these cases may have resulted from nasal exposure of non-disinfected tap waters via bathing in bathtubs, pools, or hot tubs. The only cases associated with drinking water have occurred in Australia, Arizona, and Louisiana (Marciano-Cabral et al. 2003; Okuda et al. 2004). The two cases which occurred in 2002 in Arizona were small children that had been exposed to the same water supply (The Arizona Republic 2006). Recently, another two cases in Louisiana were attributed to drinking water contaminated with \textit{N. fowleri}. In these cases, the victims did not use distilled water as directed and instead used finished drinking water that was contaminated with \textit{N. fowleri} to make their own saline solutions to use in nasal rinses, directly exposing themselves to become infected (Wolchover 2011).

Other recent cases have occurred in Minnesota and Kansas (Katranjidan 2011; Louisiana Office of Public Health 2011; Kemble \textit{et al.} 2012). These cases are significant because these states were not previously considered to be areas at risk for \textit{N. fowleri} infections like the states in routinely warmer areas such as the Southern United States.
As changes in the global climate result in warmer surface water temperatures, there could potentially be new environmental niches established in which *N. fowleri* is able to proliferate (Heggie 2010). Such changes in climate have induced increased rainfall and water runoff from storms which can result in contamination of surface waters and lead to waterborne disease outbreaks (Rose *et al.* 2001). Temperature increases at a rate of 0.2°C to 0.5°C per decade are expected to occur (Houghton *et al.* 1996). Potential climate changes resulting in warmer water temperatures worldwide could increase the risks associated with specific microbial pathogens that prefer these warmer temperatures.

According to a recent review of *N. fowleri* cases from 1962 to 2008, over 50% of occurred in Texas and Florida (30 and 29, respectively) (Yoder *et al.* 2010a). The majority of the *N. fowleri* infections during this period was due to exposure to lakes, ponds or reservoirs and occurred mostly in young adult males and children (Yoder *et al.* 2010a). It is thought that young males are more likely to engage in recreational activities such as water sports, or other activities that involve submerging the head under water.
Table 2. Incidence of PAM in the United States from 2007-2012.

<table>
<thead>
<tr>
<th>Year</th>
<th>Month</th>
<th>Victim Sex</th>
<th>Victim Age (years)</th>
<th>Venue</th>
<th>Location (State)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>2007</td>
<td>June</td>
<td>Male</td>
<td>14</td>
<td>Lake</td>
<td>Florida</td>
<td>(Centers for Disease Control and Prevention (CDC) 2008)</td>
</tr>
<tr>
<td>2007</td>
<td>August</td>
<td>Male</td>
<td>11</td>
<td>Lake</td>
<td>Florida</td>
<td>(Centers for Disease Control and Prevention (CDC) 2008)</td>
</tr>
<tr>
<td>2007</td>
<td>September</td>
<td>Male</td>
<td>10</td>
<td>Lake</td>
<td>Florida</td>
<td>(Centers for Disease Control and Prevention (CDC) 2008)</td>
</tr>
<tr>
<td>2007</td>
<td>August</td>
<td>Male</td>
<td>12</td>
<td>Lake</td>
<td>Texas</td>
<td>(Centers for Disease Control and Prevention (CDC) 2008)</td>
</tr>
<tr>
<td>2007</td>
<td>August</td>
<td>Male</td>
<td>22</td>
<td>Lake</td>
<td>Texas</td>
<td>(Centers for Disease Control and Prevention (CDC) 2008)</td>
</tr>
<tr>
<td>2007</td>
<td>September</td>
<td>Male</td>
<td>14</td>
<td>Lake</td>
<td>Arizona</td>
<td>(Centers for Disease Control and Prevention (CDC) 2008)</td>
</tr>
<tr>
<td>2008</td>
<td>July</td>
<td>Male</td>
<td>9</td>
<td>Lake</td>
<td>California</td>
<td>(Centers for Disease Control and Prevention (CDC) 2008)</td>
</tr>
<tr>
<td>2008</td>
<td>August</td>
<td>Female</td>
<td>14</td>
<td>Lake</td>
<td>Oklahoma</td>
<td>(Yoder et al. 2010b)</td>
</tr>
<tr>
<td>2009</td>
<td>September</td>
<td>Male</td>
<td>22</td>
<td>Watersports Complex</td>
<td>Florida</td>
<td>(Yoder et al. 2010b)</td>
</tr>
<tr>
<td>2009</td>
<td>August</td>
<td>Male</td>
<td>10</td>
<td>Lake</td>
<td>Florida</td>
<td>(Pacheco &amp; Prieto 2009; Postal 2009)</td>
</tr>
<tr>
<td>2010</td>
<td>July</td>
<td>Female</td>
<td>10</td>
<td>Lake</td>
<td>South Carolina</td>
<td>(Rudie 2009)</td>
</tr>
<tr>
<td>2010</td>
<td>August</td>
<td>Female</td>
<td>7</td>
<td>Lake</td>
<td>Minnesota*</td>
<td>(Gosling 2010)</td>
</tr>
<tr>
<td>2010</td>
<td>August</td>
<td>Male</td>
<td>7</td>
<td>Lake</td>
<td>Texas</td>
<td>(Kemble et al. 2012)</td>
</tr>
<tr>
<td>2010</td>
<td>August</td>
<td>Male</td>
<td>7</td>
<td>Lake</td>
<td>Arkansas</td>
<td>(Radosevich &amp; Seltzer 2011)</td>
</tr>
<tr>
<td>2011</td>
<td>August</td>
<td>Female</td>
<td>16</td>
<td>Lake</td>
<td>Florida</td>
<td>(KARK 4 News 2010)</td>
</tr>
<tr>
<td>2011</td>
<td>August</td>
<td>Male</td>
<td>9</td>
<td>Lake</td>
<td>Virginia</td>
<td>(Begley 2011)</td>
</tr>
<tr>
<td>2011</td>
<td>June</td>
<td>Male</td>
<td>20</td>
<td>Nasal Rinse</td>
<td>Louisiana</td>
<td>(Begley 2011)</td>
</tr>
<tr>
<td>2011</td>
<td>December</td>
<td>Female</td>
<td>51</td>
<td>Nasal Rinse</td>
<td>Louisiana</td>
<td>(Louisiana Office of Public Health 2011)</td>
</tr>
<tr>
<td>2011</td>
<td>August</td>
<td>Female</td>
<td>16</td>
<td>River</td>
<td>Florida</td>
<td>(Louisiana Office of Public Health 2011)</td>
</tr>
<tr>
<td>2011</td>
<td>September</td>
<td>NA**</td>
<td>NA**</td>
<td>Lake</td>
<td>Kansas*</td>
<td>(Hutchinson 2011)</td>
</tr>
</tbody>
</table>

*First case reported in this state
**NA – Not Available
Symptoms

The time from exposure to a freshwater source and the first onset of illness is 5 to 7 days, but could be as short as 24 hours (Katranjidan 2011). The most common symptoms of the disease at presentation are headache, stiff neck, seizures, and coma (Visvesvara et al. 2007). A list of the most frequently reported initial and latent symptoms adapted from a review of the literature are presented in Table 2 (Visvesvara et al. 2007).

Table 3. List of Symptoms Associated With PAM.

<table>
<thead>
<tr>
<th>Initial Symptoms</th>
<th>Late Infection Symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Headaches (bifrontal or bilateral)</td>
<td>Irritability and restlessness</td>
</tr>
<tr>
<td>High fever</td>
<td>Photo phobia</td>
</tr>
<tr>
<td>Alteration in taste or smell</td>
<td>Swelling in the brain</td>
</tr>
<tr>
<td>Nuchal rigidity (positive Kernig and Brudzinski signs)</td>
<td>Nose bleeds</td>
</tr>
<tr>
<td>Nausea</td>
<td>Swollen lymph nodes</td>
</tr>
<tr>
<td>Vomiting</td>
<td>Rapid and shallow breathing</td>
</tr>
<tr>
<td></td>
<td>Confusion</td>
</tr>
<tr>
<td></td>
<td>Cranial nerve palsies</td>
</tr>
<tr>
<td></td>
<td>Cardiac rhythm abnormalities</td>
</tr>
<tr>
<td></td>
<td>Seizures</td>
</tr>
<tr>
<td></td>
<td>Coma</td>
</tr>
</tbody>
</table>
**Diagnosis and Treatment**

Laboratory diagnosis can be accomplished by evaluating the cerebral spinal fluid (CSF) for color and red blood cells. The CSF of a patient with PAM appears grayish to yellow and contains from 250 to 24600 mm$^3$ red blood cells, depending on the progression of the disease. The CSF pressure is typically elevated and actively moving trophozoites can be visualized under the microscope (Carter 1970; Martinez 1985; Barnett *et al.* 1996; Martinez & Visvesvara 1997; Marciano-Cabral & Cabral 2007; Visvesvara *et al.* 2007; Heggie 2010). The trophozoites can be differentiated from host cells by the nucleus and the nucleolus.

Early diagnosis and treatment is key to treating PAM; however, most cases are diagnosed post-mortem due to the rapid progression of the disease. The similarity of the early symptoms to the common cold makes the disease difficult to diagnose and treat in a rapid manner. Another issue is that it is often mistaken for bacterial meningitis and thus not treated properly. Although there is no standard treatment, Effective treatments for PAM can be administered if the patient is known to have engaged in a recreational activity and exhibits the symptoms of a *N. fowleri* infection. Unfortunately, this is not usually the case. As such, there have only been eight known survivors (Visvesvara *et al.* 2007). One of these survivors was treated with a combination of intravenous and intrathecal amphotericin B and micanozole, and oral rifampin (Gibbs & Johnson 2006). This patient remained completely healthy after a
four-year follow-up (Seidel 1985). Other patients that have been successfully treated have shown signs of physical or cognitive impairment in spite of surviving the illness (Visvesvara et al. 2007). The combination of amphotericin B and miconazole are thought to have a synergistic effect against the amoeba as demonstrated by laboratory studies (Gibbs & Johnson 2006). The drug of choice for treatment of PAM is amphotericin B (Schuster & Visvesvara 2004a). A comparison of amphotericin B, miltefosine, and chlorpromazine determined that chlorpromazine was the most effective against *N. fowleri*. This could be a possible effective treatment in humans since it resulted in a 75% survival rate in mice infected with PAM (Visvesvara 2010).

**Morbidity and Mortality Risk**

Researchers have attempted to estimate the risk of acquiring PAM from swimming in bodies of water where *N. fowleri* has been detected. This risk assessment used an animal model to assume the dose of infection in humans and determined the mortality risk to be 1 in 1 million when there are 100 *N. fowleri* amoebae per liter (Kim et al. 2008; Visvesvara 2010). The French health authorities have used this risk estimate to establish guidelines in areas of high risk. For instance, French watercourses are not to exceed 100 *N. fowleri* per liter in areas where human exposure exists (Cabanès et al. 2001). A risk assessment for drinking water exposure to *N. fowleri* is needed,
particularly in light of the recent deaths attributed to drinking water being used for nasal rinses.

**Prevention**

The risk of infection is considered to be low due in part to the specificity of the route of exposure. There are some preventative measures that could be taken to reduce or minimize the risk of infection. A recent paper outlined a list of measures that can be taken in order to reduce the risk of acquiring PAM (Cabanes et al. 2001):

1) Avoiding water-related activities in warm freshwaters, hot springs, and thermally polluted water.

2) Avoiding water-related activities in warm freshwater during high temperatures and low water volumes.

3) Using nose plugs/clips or holding the nose shut when submerging the head under water in warm freshwater lakes, rivers, etc.

4) Avoiding digging or stirring up sediment in shallow, warm freshwater bodies of water.

**Detection Methods**

There are a variety of detection methods that have been developed to detect *N. fowleri* both in the environment as well as from cerebral spinal fluid and brain tissue.
These methods have been developed to specifically identify *N. fowleri* and include tests such as combining agglutination to examine isoenzyme electrophoresis to determine specificity (Yoder *et al.* 2010a). Isoenzyme analyses have been developed for environmental samples including soil and water (Kilvington *et al.* 1984; Pernin *et al.* 1985). Cerebral spinal fluid specimens can be identified with a monoclonal antibody that was developed for *N. fowleri* (Visvesvara & Healy 1980; De Jonckheere 1982; Moss *et al.* 1988). Polyclonal antibodies were also developed but exhibited cross-reactivity with other *Naegleria* species (Visvesvara *et al.* 1987). A powerful tool that was thought to be reliable but time consuming was an enzyme-linked immunoSorbent assay (ELISA) which was developed for the specific identification of *N. fowleri* from environmental water samples (Wellings *et al.* 1977).

More recently, the advent of PCR has provided an effective alternative for the specific identification of *N. fowleri* that is both rapid and sensitive for environmental samples and cerebral spinal fluid or tissue specimens. The recent molecular methods that have been developed are reviewed in greater detail in Appendix A of this dissertation.
*Acanthamoeba* species

*Acanthamoeba* belongs to the family *Acanthamoebaebidae* (Reveiller et al. 2003). Acanthamoeba was first isolated in 1913 and named *Acanthamoeba polyphagus* (Marciano-Cabral & Cabral 2003). To date, there have been more than 24 species of *Acanthamoeba* described. These are categorized into three groups according to cyst size and morphology (Pushkarew 1913). Group I is composed of larger amoebae with cysts that range in size from 16 to 30 µm. Group II consists of the most number of species, with amoebae whose cysts measure 18 µm or less, with a wrinkled ectocyst and a stellate, polygonal, triangular, or oval endocyst (Page 1967). Group III also consists of amoebae with cysts measuring 18 µm or less, but have differences in morphology (Marciano-Cabral & Cabral 2003), exhibiting a thin, smooth ectocyst and a round endocyst (Marciano-Cabral & Cabral 2003).

Acanthamoeba are considered to be opportunistic pathogens, able to infect patients with AIDS and other immunocompromised individuals (Culberston 1961; Jones *et al.* 1975; Martinez & Janitschke 1985; Kong & Pollard 2002; Marciano-Cabral & Cabral 2003; Visvesvara *et al.* 2007). *Acanthamoeba* are the causative agents of granulomatous amebic encephalitis, a life threatening disease of the central nervous system and amebic keratitis, a painful disease of the eyes (Visvesvara *et al.* 2007). In the case of amebic keratitis, it is a non-opportunistic pathogen which primarily infects individuals who use contact lenses (Gullett *et al.* 1979; Martinez & Janitschke 1985; Fiedland *et al.* 1992;

**Occurrence**

*Acanthamoeba* is ubiquitous in the environment worldwide (Stehr-Green *et al.* 1989; Illingworth *et al.* 1995; Marciano-Cabral & Cabral 2003). These amoebae have been isolated from a variety of environments including soil, dust, air (Page 1967; Rivera *et al.* 1989; Mergeryan 1991; Rivera *et al.* 1991; Rodriguez-Zaragoza 1994), sediments, fresh water, brackish water (Kingston & Warhurst 1969; Rivera *et al.* 1987), sea water, swimming pools, domestic tap water, drinking water treatment plants, bottled water, dental treatment units (Visvesvara *et al.* 2007), eyewash stations (Barbeau & Buhler 2001), hospital and dialysis units (Paszko-Kolva *et al.* 1991), sewage, contact lenses and lens cases, ventilating and air conditioning units, cooling towers of electric and nuclear power plants, heating pools, Jacuzzi tubs, and hydrotherapy pools in hospitals (Casemore 1977). *Acanthamoeba* have also been found to be contaminants in bacterial (Visvesvara & Stehr-Green 1990; De Jonckheere 1991; Mergeryan 1991; Michel *et al.* 2001; Marciano-Cabral & Cabral 2003; Visvesvara *et al.* 2007; Visvesvara *et al.* 2007), yeast (Shin & Hadley 1936) and mammalian cell cultures (Castellani 1930). *Acanthamoeba* spp. are able to survive in a range of osmolarities, allowing them to survive in distilled water, body fluids, tissue culture media, and sea water (Jahnes *et al.* 1957).
Life Cycle

The life cycle of *Acanthamoeba* consists of two stages: trophozoite and cyst. The trophozoite actively feeds on bacteria, algae and yeast present in the environment and reproduces by binary fission (Martinez 1985; Martinez & Visvesvara 1997; Schuster & Visvesvara 2004a; Visvesvara et al. 2007). The trophozoite ranges in size from 15 to 50 µm with a unique characteristic of fine, thorn-like acanthopodia on the surface of the body (Marciano-Cabral & Cabral 2003; Visvesvara et al. 2007). The cyst on the other hand is a dormant form which is also more resistant to environmental stress (Marciano-Cabral & Cabral 2003; Visvesvara et al. 2007). Cysts range in size from 10 to 25 µm and contain a double wall (Marciano-Cabral & Cabral 2003; Visvesvara et al. 2007). The cyst stage is formed when adverse environmental conditions such as food deprivation, desiccation, and changes in temperature and pH (Visvesvara et al. 2007).

Granulomatous Amebic Encephalitis

Exposure and Infection

Granulomatous Amebic Encephalitis (GAE) is an opportunistic disease of the central nervous system that may involve the lungs (Bowers & Korn 1969; Chagla & Griffiths 1974; Marciano-Cabral & Cabral 2003). There are several species that have been associated with GAE including *A. castellani*, *A. polyphaga*, *A. culberstoni*, *A. healyi*, and *A. divionensis* (Duma et al. 1978; Martinez & Janitschke 1985; Marciano-Cabral &
Acanthamoeba spp. have been shown to primarily cause disease in HIV/AIDS patients and other immunocompromised individuals (Marciano-Cabral & Cabral 2003). Acanthamoeba are thought to enter the body by inhalation through the nasal passage and lungs or through skin lesions (Figure 3) (Marciano-Cabral & Cabral 2003). Infection can take weeks to months before clinical signs are apparent (Marciano-Cabral & Cabral 2003).

**Cutaneous Acanthamebiasis**

Acanthamoeba have also been shown to cause cutaneous infections primarily in immunocompromised populations, with or without GAE (Visvesvara et al. 2007). The route of exposure is through the skin, sinuses, or respiratory tract (Figure 3) (Marciano-Cabral & Cabral 2003).

**Amebic Keratitis**

There are several species of Acanthamoeba that have been shown to cause amebic keratitis. These include: A. castellani, A. polyphaga, A. hatchetti, A. culbertsoni, A. rhysodes, A. griffini, A. quina and A. lugdunensis (Helton et al. 1993; Murakawa et al. 1995; Duloul et al. 1996; Casper et al. 1999). Amebic keratitis can be sight threatening and is characterized by painful corneal disease (Marciano-Cabral & Cabral 2003). Unlike
granulotomous amebic encephalitis and cutaneous acanthamebiasis caused by
*Acanthamoeba*, amebic keratitis is a disease that generally affects immunocompetent
persons (Moore *et al.* 1985; Moore *et al.* 1987; Bacon *et al.* 1993; Schaumberg *et al.*
1998; Marciano-Cabral & Cabral 2003). The major risk factor associated with amebic
keratitis is wearing contact lenses and using poor lens hygiene (Marciano-Cabral &
Cabral 2003). The route of exposure is primarily the eyes through corneal abrasions
(Figure 3) (Salter *et al.* 1994; Sison *et al.* 1995).

**Figure 3.** The Life Cycle and Route of Infection of *Acanthamoeba* spp.

*Acanthamoeba* spp. have been found in soil; fresh, brackish, and sea water; sewage; swimming
pools; contact lens equipment; medicinal pools; dental treatment units; dialysis machines;
heating, ventilating, and air conditioning systems; mammalian cell cultures; vegetables; human nostrils and throats; and human and animal brain, skin, and lung tissues. Unlike *N. fowleri*, *Acanthamoeba* has only two stages, cysts and trophozoites, in its life cycle. No flagellated stage exists as part of the life cycle. The trophozoites replicate by mitosis (nuclear membrane does not remain intact). The trophozoites are the infective forms, although both cysts and trophozoites gain entry into the body through various means. Entry can occur through the eye, the nasal passages to the lower respiratory tract, or ulcerated or broken skin. When *Acanthamoeba* spp. enters the eye it can cause severe keratitis in otherwise healthy individuals, particularly contact lens users. When it enters the respiratory system or through the skin, it can invade the central nervous system by hematogenous dissemination causing granulomatous amebic encephalitis (GAE) or disseminated disease, or skin lesions in individuals with compromised immune systems. *Acanthamoeba* spp. cysts and trophozoites are found in tissue (Marciano-Cabral & Cabral 2003).

**Symptoms**

Clinical signs of granulomatous amebic encephalitis and cutaneous acanthamebiasis can take weeks to months to become apparent (Centers for Disease Control and Prevention 2012a). The symptoms of amebic keratitis can occur days after exposure (Marciano-Cabral & Cabral 2003). A list of symptoms compiled for granulomatous amebic encephalitis, amebic keratitis, and cutaneous acanthamebiasis is shown in Table 4 (Marciano-Cabral & Cabral 2003).
Table 4. List of Symptoms Associated with *Acanthamoeba* infections.

<table>
<thead>
<tr>
<th>Granulomatous Amebic Encephalitis</th>
<th>Amebic Keratitis</th>
<th>Cutaneous Acanthamebiasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Headache</td>
<td>Eyelid Ptosis</td>
<td>Skin Lesions</td>
</tr>
<tr>
<td>Fever</td>
<td>Conjunctival hyperemia</td>
<td>Inflammation</td>
</tr>
<tr>
<td>Mental Status Abnormalities</td>
<td>Photophobia</td>
<td>Nodules</td>
</tr>
<tr>
<td>Seizures</td>
<td>Eye Tearing</td>
<td>Sinus lesions</td>
</tr>
<tr>
<td>Stiff Neck</td>
<td>Blurred Vision</td>
<td>Sinusitis</td>
</tr>
<tr>
<td>Muscular Weakness</td>
<td>Ocular Pain</td>
<td></td>
</tr>
<tr>
<td>Irritability</td>
<td>Corneal Ring</td>
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</tr>
<tr>
<td>Nausea and Vomiting</td>
<td>Perineural infiltrates</td>
<td></td>
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<tr>
<td>Hemiparesis</td>
<td>Opacities</td>
<td></td>
</tr>
<tr>
<td>Cranial Nerve Palsies</td>
<td>Loose Corneal Epithelium</td>
<td></td>
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<tr>
<td>Hallucinations</td>
<td>Eye Irritation</td>
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<tr>
<td>Gait Ataxis</td>
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<tr>
<td>Diplopia</td>
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<td>Photophobia</td>
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<tr>
<td>Sleep Disturbances</td>
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<tr>
<td>Anorexia</td>
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<tr>
<td>Babinski’s Sign</td>
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<tr>
<td>Kernig’s Sign</td>
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</tbody>
</table>

*Incidence of illness*

The first reported cases of illness for granulomatous amebic encephalitis, amebic keratitis, and cutaneous amebiasis caused by *Acanthamoeba* spp. were in the 1970s (Marciano-Cabral & Cabral 2003). There were 208 cases of diagnosed amebic keratitis reported for the Centers for Disease Control and Prevention between 1976 and 1986 (Kenney 1971; Jager & Stamm 1972; Robert & Rorke 1973; Naginton *et al.* 1974; Jones...
et al. 1975; Ringsted et al. 1976; Willaert & Stevens 1976; Gullett et al. 1979; Martinez & Janitschke 1985, Visvesvarya et al., 2007). Since then cases have only been reported in individuals with AIDS, but the number of cases are rare (approximately 39 cases) (Visvesvarya et al. 2007).

**Diagnosis and Treatment**

Granulomatous amebic encephalitis is diagnosed though a brain biopsy or a cerebrospinal fluid microscopic examination and confirmed through a polymerase chain reaction assay (Walochnik et al. 2000; Marciano-Cabral & Cabral 2003). Treatment should be administered early and includes petamidine, azole compounds, flucocytosine and sulfadiazine (Maugda 1989). Unfortunately, misdiagnosis occurs frequently and often accurate diagnosis only occurs postmortem. Very few patients survive the disease although a mortality rate has not been determined (Visvesvarya et al. 2007).

Amebic keratitis is diagnosed through a corneal scrape or biopsy and microscopy (Visvesvarya et al. 2007; Visvesvarya 2010); however, it can be difficult to diagnose and treat since it can often be mistaken for a fungal or viral keratitis (Marciano-Cabral & Cabral 2003). Early diagnosis is key as formation of resistant amebic cysts can occur in the tissue (Maugda 1989; Tay-Kearney et al. 1993; Schaumberg et al. 1998). Treatment for this type of infection is rather successful and includes chlorhexidine, polyhexamethylene biguanide, propamidine isethionate, edibromopropamide
isethionate, neomycin, paromomycin, polymixin B, clotimazole, ketoconazole, miconazole, and itraconazole (Marciano-Cabral & Cabral 2003)

Cutaneous acanthamebiasis is diagnosed through a skin lesion biopsy, culture or indirect immunofluorescence of tissue (Schuster & Visvesvara 2004b). Treatment is often unsuccessful, but when diagnosed without association with the central nervous system, improvement has been shown (Marciano-Cabral & Cabral 2003). The mortality rate for cutaneous acanthamebiasis for individuals without any disease of the central nervous system in approximately 73%, compared to 100% when it exists in combination (Visvesvara & Stehr-Green 1990). A combination of treatments that has been demonstrated to be successful includes itraconazole, pentamidine, 5-fluocytosine, and topical chlorhexidine gluconate and ketoconazole cream (Hunt et al. 1995; Sison et al. 1995; Dunand et al. 1997).

Prevention

There is no prevention or control for GAE or cutaneous acanthamebiasis since it occurs in immunocompromised individuals and Acanthamoeba is prevalent in many environments. Nonetheless, early diagnosis is essential (Helton et al. 1993; Salter et al. 1994). However, for amebic keratitis, educating contact lens wearers about proper lens hygiene would aid in prevention. This would include instructing contact lens wearers to avoid wearing contact lenses during swimming or any recreational water activity in freshwater, sea water, or pool water (Visvesvara et al. 2007).
Balamuthia mandrillaris

*Balamuthia mandrillaris* is an opportunistic, free-living amoeba related to *Acanthamoeba*. The diseases it causes are similar to those of *Acanthamoeba* spp., including granulomatous amebic encephalitis and cutaneous lesions (Visvesvara et al. 2007). *B. mandrillaris* is the only *Balamuthia* species that causes disease in humans and animals (Visvesvara et al. 2007). *B. mandrillaris* was first recognized as a separate genus and species from other amoebae that produce cysts in the brain in 1993 (Marciano-Cabral & Cabral 2003).

**Life Cycle**

*B. mandrillaris* has two life stages: trophozoite and cyst. Trophozoites are uninucleate, but have sometimes been shown to be binucleate and can measure from 12 to 60 µm (Visvesvara & Stehr-Green 1990; Visvesvara et al. 1993). The cyst is the environmentally resistant form of the amoeba and measure 12 to 30 µm. Cysts appear to have a double wall microscopically, but ultrastructurally, the cyst has three walls: an outer thin layer endocyst, a thick endocyst, and a middle amorphous mesocyst (Visvesvara et al. 2007). The life cycle and routes of entry for *B. mandrillaris* are shown in Figure 4 (Visvesvara et al. 1993; Visvesvara et al. 2007).
Figure 4. The Life Cycle and Route of Infection of *Balamuthia mandrillaris*.

*Balamuthia mandrillaris* has only recently been isolated from the environment and has also been isolated from autopsy specimens of infected humans and animals. *B. mandrillaris* has only two stages, cysts and trophozoites, in its life cycle. No flagellated stage exists as part of the life cycle. The trophozoites replicate by mitosis (nuclear membrane does not remain intact). The trophozoites are the infective forms, although both cysts and trophozoites gain entry into the body through various means. Entry can occur through the nasal passages to the lower respiratory tract, or ulcerated or broken skin. When *B. mandrillaris* enters the respiratory system or through the skin, it can invade the central nervous system by hematogenous dissemination causing granulomatous amebic encephalitis (GAE) or disseminated disease, or skin lesions in individuals who are immune competent as well as those with compromised immune systems. *B. mandrillaris* cysts and trophozoites are found in tissue (Centers for Disease Control and Prevention (CDC) 2012b).
**Exposure and Incidence of illness**

*B. mandrillaris* is found in soil and can enter the human body through the olfactory epithelium, the skin, or the respiratory tract (Centers for Disease Control and Prevention 2012c). These amoebae have been found to cause disease in areas of the Southern United States and have also been reported to cause disease in Hispanic Americans (Marciano-Cabral & Cabral 2003). The disease progression may take weeks to months before clinical signs are present (Marciano-Cabral & Cabral 2003).

*B. mandrillaris* has also been reported to cause disseminated infections including infections of the lungs, prostate, and uterus (Visvesvara et al. 2007; Diaz 2011). *B. mandrillaris* has been reported to cause infections in both healthy and immunocompromised individuals such as AIDS patients (Schuster & Visvesvara 2008; Visvesvara 2010). Young children and the elderly are primarily affected (Marciano-Cabral & Cabral 2003; Schuster & Visvesvara 2008; Schuster et al. 2009). There have been more than 150 cases of Balamuthiasis reported worldwide since 1990, of which only seven patients have survived (Visvesvara et al. 2007; Centers for Disease Control and Prevention 2008). Most recently, *B. mandrillaris* was reported for the first time to be transmitted through organ transplantation (Galarza et al. 2002; Bakardjiev et al. 2003; Deetz et al. 2003; Jung et al. 2004; Schuster & Visvesvara 2004a; Visvesvara et al. 2007; Schuster & Visvesvara 2008; Schuster et al. 2009; Cary et al. 2010; Bravo et al. 2011).
Symptoms

*B. mandrillaris* causes balamuthiasis, a disease similar to the granulomatous amoebic encephalitis caused by *Acanthamoeba* spp. (Centers for Disease Control and Prevention 2010). A list of symptoms for balamuthiasis has been summarized in Table 5 (Visvesvara *et al.* 2007).

**Table 5.** List of Symptoms Associated with *Balamuthia mandrillaris* infection.

<table>
<thead>
<tr>
<th>Balamuthiasis</th>
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<tbody>
<tr>
<td>Slurred Speech</td>
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<tr>
<td>Muscle Weakness</td>
</tr>
<tr>
<td>Headache</td>
</tr>
<tr>
<td>Nausea</td>
</tr>
<tr>
<td>Seizures</td>
</tr>
<tr>
<td>Stiff neck</td>
</tr>
<tr>
<td>Hydrocephalus</td>
</tr>
<tr>
<td>Sinus infection</td>
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<tr>
<td>Nodule formation</td>
</tr>
</tbody>
</table>
Diagnosis and Treatment

Diagnosis for *B. mandrillaris*, like other pathogenic amoebae, is difficult and often diagnosed postmortem (Marciano-Cabral & Cabral 2003; Visvesvara *et al.* 2007; Visvesvara 2010). The treatment for *B. mandrillaris* infections consists of a combination of pentamidine, azithromycin, fluconazole, and fluocytosine (Schuster & Visvesvara 2004a; Schuster & Visvesvara 2004a; Visvesvara *et al.* 2007; Schuster & Visvesvara 2008). Unfortunately, there are no preventive methods that could be taken to avoid infection, though treatment appears to be successful if diagnosed and treated early (Visvesvara *et al.* 2007; Visvesvara 2010).
Dissertation Format:

This dissertation is composed of four appendices. Each of the first three appendices will be submitted to peer-reviewed scientific journals for publication. The candidate completed two different projects in order to fully evaluate the ecology of *Naegleria fowleri* in water.

In Appendix A, the occurrence of *N. fowleri* was evaluated in surface waters. A multi-seasonal surveillance study of this organism was conducted including recreational surface waters with varying physical, chemical and biological characteristics from across Arizona to determine the organism’s occurrence and distribution and if its presence can be correlated with seasonal or other environmental factors.

Appendix B is focused on the detection of free living amoeba in finished drinking water. In this study, we determined the occurrence of specific free living amoeba, *N. fowleri, Acanthamoeba*, and *B. mandrillaris*, in finished chlorinated drinking water from across a municipal potable distribution system using polymerase chain reaction (PCR) assay. We also determined the temporal and/or seasonal effects and physio and/or chemical and routine microbial findings associated with the occurrence of these amoebae.

The current methods for the detection of *Naegleria fowleri* in water samples are reviewed in Appendix C. In order to use an appropriate detection method for different source waters, a literature review revealed the absence of a standard method for the
detection of *N. fowleri* in water. With documented deaths attributed to *N. fowleri* it is essential that a standard method be proposed and evaluated to monitor various source waters which could pose a public health risk.

Appendix D is an informational brochure that was created to make the public aware of the risks of engaging in water recreation activities in Arizona. This brochure will be distributed throughout the lakes of Arizona. The state of Arizona is an area of the United States that is thought to contain *N. fowleri* due to its warm lake, pond and hot spring temperatures. There have been some deaths associated with *N. fowleri* infections from exposure to both lake and drinking water (Marciano-Cabral 2003; Arizona Republic, 2006). The potential for infections of *N. fowleri* in Arizona exists and it is important to educate the public to prevent more deaths.
The amoebae *Naegleria fowleri*, *Acanthamoeba* spp., and *Balamuthia mandrillaris* are free-living organisms found in the environment in both water and soil. They are opportunistic pathogens in humans. *Acanthamoeba* is the most common cause of illness, usually infecting the eyes and sometimes causing a sight-threatening keratitis. *Acanthamoeba* spp. and *B. mandrillaris* can cause granulomatous amoebic encephalitis (GAE), in addition to infections of the lungs and skin. *N. fowleri* infects its host via the nasal mucosa following the forceful inhalation of contaminated water and causes primary amoebic meningoencephalitis (PAM). Although rare, the disease has a 99% fatality rate. Between 1998 and 2012, *N. fowleri* killed 45 people in the United States. There is little known regarding the ecology and occurrence of this organism.

The goal of the manuscript titled “The occurrence of *Naegleria fowleri* in recreational waters in Arizona” shown in Appendix A was to determine the occurrence of *N. fowleri* in surface waters throughout Arizona to assess if its presence could be correlated with seasonal or other environmental factors. In order to assess the organism’s seasonality, samples were collected from 36 different recreational waters (lakes, rivers, and hot springs) across Arizona over two years. One liter grab samples were concentrated and analyzed using polymerase chain reaction (PCR) specific for *N. fowleri* and a live amoebic assay. Overall, 9.3% of the warm weather samples collected were positive for *N. fowleri*, whereas 16.3% of the samples were positive during the cold
weather sampling periods. Although the presence of *N. fowleri* could not be significantly correlated with physical and chemical parameters such as temperature, pH, turbidity, conductivity, and the presence of heterotrophic bacteria, weak correlations (> 0.40) with the presence of *Escherichia coli* and live amoebic activity were observed.

Appendix B is a second manuscript titled “Detection of free living amoebae in a drinking water distribution system.” In this study we collected a total of 785 samples of finished chlorinated drinking water from across a municipal potable distribution system. Physio/chemical parameters (chlorine residual, temperature, pH, turbidity, and conductivity) were measured for all samples. In addition, all samples were assayed for the presence of viable amoebae. Of these 785 samples, 138 (17%) were positive for the presence of live amoebae, 2 (1.5%) positive for *N. fowleri* and 2 (1.5%) were positive for *Acanthamoeba* spp. This distribution water had more than an adequate chlorine residual (~0.8 ± 0.2 mg/L). Indicator microorganisms that are typically used to monitor microbial water quality such as coliforms and *Escherichia coli* were not found under these circumstances. The locations determined to have live amoebae were scattered throughout the distribution system as single point positives. More interestingly, some of these locations were found in clusters within the potable distribution system, suggesting that multiple water sources, hydraulic characteristics, and poor maintenance of the distribution system environment itself may be contributing to the presence of these pathogenic water-based organisms. Therefore, a viable amoebae assay would not only
provide a better indication of the water-based microbial quality of water, but such clustering could potentially reveal other problem areas within the distribution system.

All of the free living amoebae may be found in ground water, surface water, and even finished drinking water worldwide although the public health significance is unknown at this time. There is a need for a standard method for monitoring these waters. A review of the significance of *N. fowleri* and the current detection methods are described in the manuscript titled “Current Methods for the Detection of *Naegleria fowleri in Water*” in Appendix C.

It was important to communicate our findings to and to educate the Arizona community in order to prevent future cases of PAM. An informational brochure attached as Appendix D was created to educate the public of the risk of exposure when engaging in recreational water activities such as swimming, jet skiing, diving, and wake boarding. This was accomplished by creating an easy to read yet informative short leaflet explaining the importance of *N. fowleri* and the potential risk to human health from engaging in these recreational activities. This was of particular importance since some of the temperatures in lakes in Arizona maintain a relatively warm temperature ideal for the survival of *N. fowleri*. 
REFERENCES


30. Craun G. F., Brunkard J. M., Yoder J. S., Roberts V. A., Carpenter J., Wade T.,
outbreaks associated with drinking water in the United States from 1971 to


Disease.* **13**(Suppl. 5), S385-S387.


pathogenic amoeboflagellate Naegleria fowleri. *Infect Genet Evol.* **11**(7), 1520-
1528.


36. De Jonckheere J. F. (1982). Isoenzyme patterns of pathogenic and non-
pathogenic Naegleria spp. using agarose isoelectric focusing. *Ann Microbiol
(Paris).* **133**(2), 319-342.


(accessed March 29 2012).


159. Wiser M. F. Life Cycle of *Naegleria fowleri*.


161. Yoder J., Roberts V., Craun G. F., Hill V., Hicks L. A., Alexander N. T., Radke V.,
Calderon R. L., Hlavsa M. C., Beach M. J., Roy S. L. and Centers for Disease
Control and Prevention (CDC). (2008). Surveillance for waterborne disease and
outbreaks associated with drinking water and water not intended for drinking--

162. Yoder J. S., Blackburn B. G., Craun G. F., Hill V., Levy D. A., Chen N., Lee S. H.,
Surveill Summ.* **53**(8), 1-22.

epidemiology of primary amoebic meningoencephalitis in the USA, 1962-2008.
*Epidemiol Infect.* **138**(7), 968-975.

164. Yoder J. S., Hlavsa M. C., Craun G. F., Hill V., Roberts V., Yu P. A., Hicks L. A.,
Alexander N. T., Calderon R. L., Roy S. L., Beach M. J. and Centers for Disease
Control and Prevention (CDC). (2008). Surveillance for waterborne disease and
outbreaks associated with recreational water use and other aquatic facility-
**57**(9), 1-29.
APPENDIX A

THE OCCURRENCE OF *NAEGLERIA FOWLERI* IN RECREATIONAL WATERS IN ARIZONA

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ABSTRACT

Naegleria fowleri is a free-living amoebae found primarily in soil and water in warmer regions which can cause primary amebic meningoencephalitis (PAM) in children, young adults, and older or immunocompromised individuals. Although rare, the disease has a 99% case fatality rate. Despite a recent increase in public awareness, little is known regarding the ecology and occurrence of this organism. The goal of the current project was to determine the occurrence of N. fowleri in recreational surface waters from across Arizona to assess if its presence could be correlated with seasonal or other environmental factors. One-liter grab samples were collected from 33 different recreational waters in Arizona over a two-year period. The samples were concentrated and analyzed using a polymerase chain reaction (PCR) assay specific for N. fowleri and an amoebae viability assay. Overall, 16.3% (8 of 49) of the samples collected from waters with temperatures ≤ 19°C were positive for N. fowleri, whereas only 9.3% (5 of 54) of the samples were positive at temperatures above this. Nevertheless, the spring and fall seasons, though having the same average water temperature (17.9°C), had vastly different N. fowleri occurrence data (18.2% versus 5.9%, respectively). The presence of N. fowleri could not be significantly correlated with not only water temperature, but also pH, turbidity, conductivity, and the presence of heterotrophic bacteria and Escherichia coli. A weak correlation (0.52) with live amoebic activity was observed. Five lakes to the north and northeast of Phoenix tested positive for the
pathogen on more than one occasion over multiple seasons. This could represent a potential threat to public health as these are some of the most frequented recreational waters in the state. Future monitoring for N. fowleri should therefore focus on these lakes to further elucidate why these particular waters appear to be a reservoir for the organism.
INTRODUCTION

Human-induced global climate changes caused by greenhouse gases are predicted to cause increases in temperatures and increases in precipitation and to produce storms of greater intensity (Karl et al. 1995). Increased rainfall and water runoff from storms can result in contamination of surface waters and lead to waterborne disease outbreaks (Rose et al. 2000). The global mean surface temperature is expected to increase at a rate of 0.2°C to 0.5°C per decade (Houghton et al. 1996). With these potential climate changes resulting in warmer water temperatures worldwide, there exists the possibility that there will be a concomitant rise in specific microbial waterborne pathogens that prefer these warmer temperatures.

The amoebae *Naegleria fowleri* have been found primarily in waters in warmer regions in the United States such as in Arizona and the Southwest. There have only been over 400 cases worldwide, *N. fowleri* is known to be a warm water amoebae, preferring 30°C and above, making surface waters an ideal habitat (Yoder et al., 2004). It is also able to survive in a pH range between 4.0 and 9.1 (Fliermans et al., 1979).

*N. fowleri* is a free-living protozoan found primarily in soil and water which can cause primary amoebic meningoencephalitis (PAM) in children, the elderly and immunocompromised individuals. With 20% of the U.S. population considered immunocompromised (Geiss 2011), understanding exposure to and infection by *N. fowleri* is increasingly important. Specifically, *N. fowleri* infects its host via the nasal
mucosa and causes primary amoebic meningoencephalitis (PAM). Although rare, the
disease has a 99% fatality rate. PAM destroys the host’s brain tissue, typically causing
death in four to six days (Visvesvara 2007). Since 2000, the number of deaths caused by
water-based amoebae has doubled and recreational water outbreaks have tripled (CDC, 2002). This increase has been most notable in underdeveloped nations (Visvesvara et al. 2007; Visvesvara 2010). Nevertheless, between 1998 and 2011, N. fowleri killed 46 people in the United States, all in warmer regions. Despite a recent increase in public awareness, there is little known regarding the ecology and occurrence of this organism.

Environmental sources of these pathogens are freshwater lakes, streams, thermal
springs, and soil (CDC, 2008). N. fowleri has been found not only in a variety of
recreational waters including swimming pools, ponds (Cerva, 1971), natural hot springs,
but also in hot tubs (Rivera et al., 1993), domestic water supplies (Marciano-Cabral et
al., 2003), and artificially heated industrial water sources (De Jonckheere et al., 1977).

In the current study, we conducted a multi-seasonal surveillance study of this
organism in recreational surface waters with varying physical, chemical and biological
characteristics from around Arizona to determine its occurrence and distribution and if
its presence can be correlated with seasonal or other environmental factors. It is
unclear how often this water-based pathogen may be present in recreational waters in
Arizona and at what level it becomes a public health problem. Because of the warm
surface waters found in Arizona, this organism may pose a particular threat to human
health in this state.
MATERIALS AND METHODS

Sampling Sites

Surface water samples were collected from a total of 33 rivers, lakes, creeks and hot springs from 2008 to 2010 (Figure 1). These locations were selected because of a high use by bathers and for other recreational activities. Many of the lakes are artificial and used for recreation, water storage, and power generation. Three of these lakes have also been associated with documented cases of PAM. The recreational waters varied in size (e.g., creeks, rivers, and lakes) and water temperatures (e.g., collected from cold and warm regions), and were located in different regions of the state. In addition, samples were collected from each site in different seasons over the period of two years.

Some of the most commonly frequented lakes in Arizona are those created by dams on the Salt River to the northeast of the city of Phoenix. These include Roosevelt Lake, Apache Lake, Saguaro Lake, and Canyon Lake. These lakes vary in size and depth based on their proximity from the dam. The largest is Roosevelt Lake which covers over 21,000 acres with a length of 22 miles and 128 miles of shoreline. It has a maximum depth of 300 feet. Roosevelt Lake is one of the most popular sites for recreational water activities in Arizona including boating, swimming, jet-skiing, wake boarding, and fishing and is visited by 1 to 1.5 million people each year (Arizona Leisure Vacation Guide,
2012). Apache Lake is over 17 miles long and Saguaro Lake is approximately 22 miles long and 118 feet deep when full. Canyon Lake is the smallest of these lakes with 28 miles of shoreline and the nearest to Phoenix (Arizona Leisure Vacation Guide, 2012).

Other popular recreational lakes in Arizona include Lake Pleasant to the north of Phoenix, Lake Powell on the border with Utah, and Lake Havasu on the border with California. At over 180 miles long with over 2,000 miles of shoreline, Lake Powell is the second largest man-made lake in the U.S., and is more than 4,000 feet deep. It welcomes over 3,000,000 visitors a year (Arizona Leisure Vacation Guide, 2012). Lake Pleasant is over 50 miles long and over 10,000 acres. It is the largest lake in the Greater Phoenix area (Arizona Leisure Vacation Guide, 2012) and is thus a popular recreational spot for residents. Lake Havasu, fed by the Colorado River, is over 45 miles long with a capacity of 648,000 acre feet and is visited by over 2.5 million people every year (Arizona Leisure Vacation Guide, 2012).

**Sample Collection**

Each water sample consisted of two 1-liter grab samples collected by submerging sterile 1-liter polypropylene bottles beneath the surface of the water until completely filled. The samples were transported at 4°C to the laboratory for further analysis. Global positioning system (GPS) coordinates were used to ensure the same location was
sampled over the course of the multi-seasonal study. A map of sample sites was created using GPS coordinates (Figure 1). The samples sites included the boat launch and beach areas for most of the locations, which showed the greatest amount of recreational activity. In some cases, the recreational water locations were frozen over (e.g., during the winter months) or the bed was dry (due to a lack of rainfall) and thus could not be sampled. This occurred mostly with lakes in the Northern region of the state.

All samples were transported to the laboratory for immediate processing and assayed within 24 hours of collection. One liter of the water was used of in tests to quantify the other physical and chemical water parameters. All water quality parameters were tested using Standard Methods (APHA, 2005). These included temperature, pH, specific conductance, turbidity, and the presence of total coliform bacteria, *Escherichia coli*, and heterotrophic bacteria.

**Identification of Naegleria fowleri**

The sample was concentrated for PCR by filtering one liter of water through a polyethylene filter (2 μm pore size; Millipore, Bedford, MA) to concentrate amoebic cysts. The filters were then placed in 1 ml of Page’s Amoebae Saline (1.08 g NaCl, 0.036 g MgSO₄, 0.036 g CaCl₂ x 2 H₂O, 1.275g Na₂HPO₄, 1.22 g KH₂PO₄, 9.0 L distilled water), agitated, and stored at -80°C (Page, 1976). To lyse the amoebae, the samples were
frozen and thawed three times. These concentrates were then assayed based on established methods for the detection of *N. fowleri* using a nested polymerase chain reaction (PCR) method (Marciano-Cabral et al., 2003; Reveiller et al., 2002). The forward primer, Mp2Cl5.for (5′-TCTAGAGATCCAACCAATGG-3′) and the reverse primer, Mp2Cl5.rev (5′-ATTCTATTCACCTCCACAATCC-3′), were used to amplify a 166-base pair fragment of the Mp2Cl5 gene that is unique to *N. fowleri*. The reaction was performed in a 50-μl volume consisting of 1× *Taq* DNA polymerase buffer (10 mM Tris-HCl, 50 mM KCl, 2.5 mM MgCl₂), a 0.2 mM concentration of each deoxynucleoside triphosphate, 0.6 μM primer, and 2.5 U of AmpliTaq DNA polymerase. The PCR was conducted under the following conditions: initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 65°C for 1 min, and extension for 2 min at 72°C.

An additional nested PCR assay was conducted in order to increase the sensitivity of the method, which consisted of another set of internal primers, a forward primer Mp2Cl5.for-in (5′-GTACATTGTTTTTATTAATTTCC-3′) and a reverse primer Mp2Cl5.rev-in (5′-GTCTTTGTGAAAACATCACC-3′), which amplified a 110-bp fragment of the Mp2Cl5.P sequence. The second PCR reaction was also performed in a 50μl volume consisting of 1× *Taq* DNA polymerase buffer, 0.2 mM of dNTPs, 0.5 μM of each primer, 2.5 U of AmpliTaq Polymerase, and 2 μl of the first PCR product. The conditions for the nested PCR were as follows: initial denaturation at 95°C for 5 min followed by 35 cycles
of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension for 2 min at 72°C.

This method detects the presence of *N. fowleri* DNA, but is not able to determine the organism’s viability. The detection limit is 5 to 10 organisms. All samples were assayed in triplicate. Positive (*N. fowleri* Lee strain, ATCC 30894) and negative (DNA free PCR-grade water) controls were also included in triplicate. Gel electrophoresis was used to visualize the PCR products.

*Live Amoebae Assay*

One liter of the collected sample was filtered through a polyethylene filter as before to concentrate amoebic cysts. The filters were then placed in 1 ml of Page’s Amoebae Saline and aliquots were grown on non-nutrient agar (Difco, Sparks, Maryland) seeded with *E. coli* at 44°C. The Petri dishes were observed daily using brightfield light microscopy to detect amoebic growth (clearing in the bacterial lawn). The live amoebae were then harvested using a sterile cell scraper (VWR, Denver, Colorado) to remove each individual plaque (taken from the advancing edge) and transferred to 0.5 ml of sterile polymerase chain reaction (PCR) grade water (I.D.T., Coralville, Iowa) and examined for enflagellation. The detection limit for this assay is one organism. Samples were then frozen/thawed three times to lyse the cells and
release the DNA for subsequent nested PCR analysis to confirm the identification as *N. fowleri*.

**RESULTS**

Table 1 summarizes the range, the mean, and the standard deviations for all of the water quality parameters tested by season. Overall, the samples collected during the winter months (December through February) had the lowest mean temperature of 13.9°C, while the highest temperatures (mean = 26.5°C) were seen in the summer (June through August). The average temperature was 17.9°C for both spring (March through May) and fall (September through November) which were also similar with regard to other water quality parameters. Turbidity was the highest in the fall (mean = 17 NTU, range = 0.7 - 81.7 NTU) and the spring (mean = 15.1 NTU, range = 1.7 – 76.0 NTU). Conductivity was the highest in the summer (mean = 139.2 S/m) and the winter (mean = 109.8 S/m). The range of pH was similar through all the seasons and varied mostly by lake or region rather than by season.

Total coliform bacterial numbers were not significantly different between seasons, averaging between 2.65 and 3.13 Log_{10} most probable number (MPN) per 100 ml (Table 2). The numbers of *E. coli* were similar for winter, spring, and summer, but spiked in the fall. Heterotrophic plate count bacteria were at their lowest levels in the winter; however, there was no significant difference from the levels observed in other seasons.
N. fowleri was detected in 6 sites (18.2%), and live amoebae in 8 sites (24.2%) of the 33 recreational waters sampled over a two-year period. N. fowleri was detected (using PCR) in 13 of 103 samples (12.6%). The organism’s occurrence appeared to be seasonal, with 8 of 40 (20.0%) samples positive in the winter and spring and only 5 of 73 (6.9%) positive in the summer and fall (Table 3). In addition, 61.5% of those samples testing positive were collected from waters with temperatures below 20°C (Table 4). A similar trend was observed for live amoebae with 12 of 40 (30.0%) samples containing live amoebae in the winter and spring and 6 of 46 (13.0%) samples positive in the summer (Table 3). Live amoebae were not detected in any of the samples collected in the fall (n = 27). The majority (66.7%) of the positive samples were found in waters with temperatures below 20°C (Table 4). Two of the 18 samples containing live amoebae were determined to have N. fowleri by PCR analysis.

The presence of N. fowleri could not be correlated with any of the water quality parameters measured or the presence of the bacterial indicators. Nevertheless, there was a weak positive correlation (0.52) between the presence of N. fowleri and the presence of live amoebae. In addition, several lakes (Lake Pleasant, Lake Roosevelt, Apache Lake, Canyon Lake, and Saguaro Lake) clustered near the Phoenix, AZ area tested positive for N. fowleri on more than one occasion and over multiple seasons.
DISCUSSION

*N. fowleri* has been documented in surface waters in Virginia, Oklahoma, Florida, New Mexico, and Arizona. In addition, the majority of cases have occurred during the summer months in these states. Therefore, the low observed occurrence of *N. fowleri* during the warmer months of the current study was unexpected. However, far fewer positive samples were collected in the summer months than during the remainder of the year. When compared to other similar survey studies for *N. fowleri*, this study has the greatest number of sample sites and samples over an extended period of time. Despite this, no correlations could be determined between any of the physical/chemical parameters and the occurrence of *N. fowleri* in these waters. Perhaps with a greater sample size, the weak correlations observed in this study could be established more firmly. There were no significant correlations between the occurrences of *N. fowleri* based on seasonality. Previous research has shown that *N. fowleri* grows best at an acidic pH of 5.5 to 6.5 (Weik and John, 1977). In this study, the pH ranged from 6.7 to 9.0. This could explain the low number of positives in different areas of Arizona.

Previous studies have demonstrated little relation of the occurrence of *N. fowleri* and seasonality. One study surveyed waters in Connecticut and Virginia and no correlations with total coliforms, *E. coli*, or temperature were found (MacLean et al., 2004). The bodies of water sampled in Connecticut had a greater percentage of positives found in a small shallow pond. This pond was believed to have lower organic
matter levels (MacLean et al., 2004). In the current study, we also observed a greater number of positive samples from waters low in organic matter inferred by the physical and chemical parameters of the water. Another study aimed to assess the seasonal distribution of *N. fowleri* in Oklahoma waters (John and Howard 1995). A total of 2,016 samples were tested including swab and water samples from three different sampling locations. This study found more amoebae in the spring and fall seasons. These seasons also had the greatest rainfall, therefore having greater ecological overturn of water in the lakes and ponds. The highest number of positives, however, were found in the pond with the lowest turbidity which also had the least amount of visible organic matter (John and Howard 1995), thus supporting the idea that *N. fowleri* is more likely found in waters with lower levels of organic matter. Previous research also has demonstrated that organically rich water does not favor the growth of *N. fowleri* (De Jonckheere et al, 1977).

Despite the lack of correlations between the presence of *N. fowleri* and the various water quality parameters, there did seem to be a group of lakes that consistently tested positive for *N. fowleri*. These lakes, Lake Pleasant, Roosevelt Lake, Apache Lake, Saguaro Lake, and Canyon Lake, were clustered in the Phoenix, AZ area. All but Lake Pleasant are located on the Salt River. They are popular year round, attracting millions of visitors annually (Arizona Leisure Vacation Guide, 2012). There have also been cases of PAM reported from people using several of these lakes for recreational activities. As such, these recreational waters should be included in all future monitoring
for *N. fowleri* in the state of Arizona and examined further to determine why these waters appear to be a reservoir for the organism.
REFERENCES


Figure 1 B. Global positioning system (GPS) map of recreational water sampling locations.
Table 1 B. Quality of Arizona recreational water samples by season.

<table>
<thead>
<tr>
<th>Water Quality Parameter</th>
<th>Winter</th>
<th>Spring</th>
<th>Summer</th>
<th>Fall</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (±SD)</td>
<td>Range</td>
<td>Mean (±SD)</td>
<td>Range</td>
</tr>
<tr>
<td>Temp. (°C)</td>
<td>13.9 (±2.8)</td>
<td>9 – 20</td>
<td>17.9 (±5.3)</td>
<td>12 – 24</td>
</tr>
<tr>
<td>pH</td>
<td>7.9 (±0.3)</td>
<td>7.3 – 8.4</td>
<td>7.7 (±0.4)</td>
<td>6.7 – 8.1</td>
</tr>
<tr>
<td>Turbidity (NTU)</td>
<td>5.4 (±9.4)</td>
<td>0.3 – 48.1</td>
<td>15.1 (±22.8)</td>
<td>1.7 – 76</td>
</tr>
<tr>
<td>Conductivity (S/m)</td>
<td>109.8 (±27.5)</td>
<td>37 – 149</td>
<td>53.5 (±48.7)</td>
<td>6 – 112</td>
</tr>
</tbody>
</table>
Table 2 B. Indicator organisms found in Arizona recreational water samples by season.

<table>
<thead>
<tr>
<th>Indicator Organism</th>
<th>Winter</th>
<th>Spring</th>
<th>Summer</th>
<th>Fall</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coliforms ((\text{Log}_{10} \text{MPN/100 ml}))</td>
<td>Mean (±SD)</td>
<td>Median</td>
<td>Mean (±SD)</td>
<td>Median</td>
</tr>
<tr>
<td>Coliforms ((\text{Log}_{10} \text{MPN/100 ml}))</td>
<td>2.87 ± 0.44</td>
<td>2.90</td>
<td>2.65 ± 0.33</td>
<td>2.79</td>
</tr>
<tr>
<td>Escherichia coli ((\text{Log}_{10} \text{MPN/100 ml}))</td>
<td>0.75 ± 0.48</td>
<td>0.79</td>
<td>0.30 ± 0.00</td>
<td>0.30</td>
</tr>
<tr>
<td>Heterotrophic bacteria ((\text{Log}_{10} \text{CFU/ml}))</td>
<td>3.47 ± 1.32</td>
<td>3.09</td>
<td>4.25 ±0.41</td>
<td>4.31</td>
</tr>
</tbody>
</table>


Table 3 B. Amoebae found in Arizona recreational water samples by season.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Winter</th>
<th>Spring</th>
<th>Summer</th>
<th>Fall</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td># Positive</td>
<td>% Positive</td>
<td># Positive</td>
<td>% Positive</td>
</tr>
<tr>
<td>Naegleria fowleri (by PCR)</td>
<td>6 / 29</td>
<td>20.7</td>
<td>2 / 11</td>
<td>18.2</td>
</tr>
<tr>
<td>Live Amoebic Activity</td>
<td>9 / 29</td>
<td>31.0</td>
<td>3 / 11</td>
<td>27.3</td>
</tr>
</tbody>
</table>
Table 4B. Amoebae found in Arizona recreational water samples by water temperature.

<table>
<thead>
<tr>
<th>Organism</th>
<th>0 to 19 °C</th>
<th>20 °C</th>
<th>All Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td># Positive</td>
<td>% Positive</td>
<td># Positive</td>
</tr>
<tr>
<td>Naegleria fowleri (by PCR)</td>
<td>8 / 49</td>
<td>16.3</td>
<td>5 / 54</td>
</tr>
<tr>
<td>Live Amoebic Activity</td>
<td>12 / 49</td>
<td>24.5</td>
<td>6 / 54</td>
</tr>
</tbody>
</table>
APPENDIX B

MONITORING FOR THE PRESENCE OF FREE-LIVING AMOEBAE AS AN ALTERNATIVE METHOD FOR ASSESSING WATER QUALITY IN DRINKING WATER DISTRIBUTION SYSTEMS

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ABSTRACT

The occurrence of free-living amoebae was investigated in a municipal drinking water distribution system during three sampling periods (~15 sample collection sites per 18 distinct regions) over a two-year period. A total of 138 of 785 samples (17.6%) were positive for the presence of viable amoebae. Two of these positive samples contained *Naegleria fowleri* and two *Acanthamoeba* species as determined by polymerase chain reaction assays; no *Balamuthia mandrillaris* was detected. Live amoebae were commonly found despite an average free chlorine residual of 0.86 mg/L. Total coliforms and *Escherichia coli* that are typically used to monitor water quality would likely not be found under these conditions. Of the 18 regions within the distribution system, viable amoebae were found in four (22.2%) during all three sampling periods. Only one region had no samples testing positive during the study. Clusters with three or more samples testing positive for viable amoeba were observed during all three periods. Five of these clusters occurred in the same region over multiple periods. More positive samples were identified during periods with higher average water temperatures (≥30.1°C) than during the period with a water temperature of 22.9°C, suggesting seasonal variation; however, temperature did not seem to be involved in the observed clustering of positive samples.

We believe that live amoebae could be used as an alternative method to routinely monitor overall water quality that could be used to complement total coliform rule monitoring of drinking water distribution systems. These organisms may not only
provide a better assessment of the microbial quality than traditional indicator organisms, but such regional clustering could reveal areas with potential water quality issues within the distribution system (e.g., contamination of a particular water source, poor maintenance in a specific region, sediment buildup, and daily flow and pressure changes due to fluctuations in consumer demand).
INTRODUCTION

Despite effective treatment of drinking water, waterborne disease may at times still be attributed to exposure to pathogens in treated drinking water (Reynolds et al. 2008; Craun et al. 2010). Specifically, deficiencies or problems within water distribution systems such as biofilm formation and water main breaks have been linked to a number of outbreaks (Blackburn et al. 2004). Microorganisms that are typically used to monitor microbial water quality such as coliforms and *Escherichia coli* would most likely not be found in adequately treated water distribution systems with an appropriate chlorine residual.

Free-living amoebae such as *Naegleria fowleri*, *Acanthamoeba* species, and *Balamuthia mandrillaris* can cause rare but fatal diseases such as primary amoebic meningoencephalitis (*N. fowleri*) and granulomatous amoebic encephalitis (*B. mandrillaris* and *Acanthamoeba* spp. Martinez & Visvesvara 1997; Schuster & Visvesvara 2004; Visvesvara et al. 2007). *Acanthamoeba* and *B. mandrillaris* can also cause pulmonary and cutaneous lesions and *Acanthamoeba* can cause sight-threatening keratitis (Visvesvara et al. 2007; Centers for Disease Control and Prevention 2008; Yoder et al. 2010). Little is known about their occurrence or ecology in drinking water, particularly with regard to *N. fowleri* and *B. mandrillaris*. Recently, *N. fowleri* was added to the Environmental Protection Agency’s Candidate Contaminant List (CCL3) as an organism under consideration for future regulation in drinking water (USEPA 2008).
These organisms may persist in biofilms within water distribution systems, which serve to further protect them from environmental insults and any disinfectant residual (Thomas et al. 2004; Langmark et al. 2005; Loret et al. 2005). In addition, water-based bacterial pathogens such as *Legionella* and *Mycobacterium* have been shown to survive within the cysts of these amoebae (Marciano-Cabral 2004; Marciano-Cabral et al. 2010). Such cysts possess a thick impenetrable wall that is more resistant to both desiccation and chemical disinfectants (Marciano-Cabral et al. 2010). As a result, the bacteria are also protected from disinfectants, allowing them to re-colonize the system rapidly (Thomas et al. 2004).

In this study, we determined the specific presence of free living amoeba species in finished chlorinated drinking water from across a municipal potable distribution system using a live amoebic assay followed by a polymerase chain reaction (PCR) assay. We also determined the temporal and seasonal effects on the occurrence of these organisms and if there were any correlations with physico/chemical parameters of water quality or the presence of bacterial indicator organisms.
MATERIALS AND METHODS

Water Sample Collection

A municipal drinking water system in southern Arizona serving 775,000 people was monitored over the course of two years over three different sampling periods. The water utility uses a blend of ground water and surface water from the Colorado River, the ratio of which varies based on seasonal differences in customer demand. In a previous study, 10.6% of 113 groundwater supply wells tested positive for the presence of *N. fowleri* DNA by polymerase chain reaction (PCR) prior to disinfection (Bright et al. 2009). Samples of finished chlorinated drinking water (with an annual average residual of 0.88 ppm free chlorine) were collected during three sampling periods from across the potable distribution system during August to September 2009 (sampling period I), February to May 2011 (sampling period II), and September to October 2011 (sampling period III). Prior to sample collection, the water was first flushed for a minimum of five gallons. Each sample consisted of two 1-liter grab samples collected in sterile 1-liter polypropylene bottles. The bottles were then capped and placed on ice for transport to the laboratory.

A total of 251 samples were collected during the first sampling period and 267 samples were collected during both the second and third sampling periods, for a project total of 785 samples.
Sample Processing

All samples were assayed within 24 hours of collection. The first one-liter grab sample was used for a viable amoeba assay, the second was used in pathogenic amoebae presence/absence PCR assays, and used in tests to determine the other physical and chemical water quality characteristics. All water quality parameters were tested using Standard Methods (American Public Health Association 2005). These included the temperature, pH, specific conductance, turbidity, free chlorine level, and the presence of total coliform bacteria, Escherichia coli, and heterotrophic bacteria.

Live Amoebae Assay

Escherichia coli strain 25922 was obtained from the American Type Culture Collection (ATCC; Manassas, VA) and was maintained on Tryptic Soy Agar (TSA; Difco, Sparks, MD) with incubation for 18 to 24 hours at 37°C. Prior to the start of each experiment, an Erlenmeyer flask containing 100 ml of Tryptic Soy Broth (TSB; Difco, Sparks, MD) was inoculated with the organism and incubated on an orbital shaker (Model G33; New Brunswick Scientific, Edison, NJ) at 300 rpm at 37°C overnight. After incubation, the E. coli were pelleted via centrifugation (9,800 × g, 15 min, 25°C). The pelleted cells were washed by re-suspension in 100 ml of sterile phosphate buffered
saline (PBS; pH 7.4; Sigma-Aldrich, St. Louis, MO) followed by centrifugation as described previously. This step was repeated one additional time. The final pellet was resuspended in 5 ml of PBS.

The first one-liter water grab sample was used to test for the presence of viable amoebae. A five-hundred mL volume was seeded with 3 ml of the pre-prepared *E. coli*. The seeded sample (~500mL) was then concentrated by centrifugation (9,800 x g, 20 min, 25°C). Following centrifugation, all but 20 ml of the supernatant was discarded. The remaining 20 ml was collected and then these concentrates were inoculated separately onto non-nutrient agar (Difco, Sparks, Maryland) using the spread plate method and incubated for three days at 44°C. The inoculated plates were observed daily using brightfield light microscopy to detect amoebic growth (a plaque or clearing in the bacterial lawn). The viable amoebae were then harvested using a sterile cell scraper (VWR, Denver, Colorado) to remove a sample from each individual plaque (taken from the advancing edge) and transferred to 0.5 ml of sterile PCR grade water (I.D.T., Coralville, Iowa) and examined for enflagellation. The detection limit for this assay is one organism. The DNA from all of the samples was then extracted using the UltraClean Soil DNA kit (Mo-Bio), with subsequent real-time PCR analysis (as described previously) to identify any *N. fowleri, Acanthamoeba* spp., or *B. mandrillaris* present.
Identification of pathogenic amoebae

For the *N. fowleri* presence/absence PCR test, the entire one-liter sample was filtered through a polyethylene filter (2 μm pore size; Millipore, Bedford, MA) to concentrate amoebic cysts. The filters were then placed in 1 ml of Page’s Amoeba Saline (1.08 g NaCl, 0.036 g MgSO$_4$, 0.036 g CaCl$_2$ x 2 H$_2$O, 1.275 g Na$_2$HPO$_4$, 1.22 g KH$_2$PO$_4$, 9.0 L distilled water), agitated, and stored at minus 80°C (Page 1976). The DNA was extracted from the concentrates using the Ultra Clean Soil DNA isolation kit (Mo-Bio, Carlsbad, CA). Only those samples that tested positive for live amoeba were assayed with PCR.

For the first sampling period, the DNA was assayed based on established methods for the detection of *N. fowleri* using a nested polymerase chain reaction (PCR) method (Marciano-Cabral et al. 2003; Reveiller et al. 2002). The forward primer, Mp2Cl5.for (5′-TCTAGAGATCCAATGG-3′), and the reverse primer, Mp2Cl5.rev (5′-ATTCTATTCCTCCACAATCC-3′), were used to amplify a 166-base pair fragment of the Mp2Cl5 gene that is unique to *N. fowleri*. The reaction was performed in a 50-μl volume consisting of 1 X Taq DNA polymerase buffer (10 mM Tris-HCl, 50 mM KCl, 2.5 mM MgCl$_2$), a 0.2 mM concentration of each deoxynucleoside triphosphate (dNTP), 0.6 μM of each primer, and 2.5 U of AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA). The PCR was conducted using the following conditions: initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 65°C for 1
min, and extension for 2 min at 72°C.

In order to increase the sensitivity of the method which consisted of another set of internal primers, a forward primer, Mp2Cl5.for-in (5'-GTACATTGTTTTTATTAATTTCC-3'), and a reverse primer, Mp2Cl5.rev-in (5'-GTCTTTGTGAAAACATCACC-3'), which amplified a 110-bp fragment of the Mp2Cl5.P sequence. The second PCR reaction was also performed in a 50 µl volume consisting of 1 X Taq DNA polymerase buffer, 0.2 mM of each dNTP, 0.5 μM of each primer, 2.5 U of AmpliTaq Polymerase, and 2 µl of the first PCR product. The conditions for the nested PCR were as follows: initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension for 2 min at 72°C (Marciano-Cabral et al. 2003; Reveiller et al. 2002).

This method detects the presence of *N. fowleri* DNA, but is not able to determine the organism’s viability. The detection limit is 5 to 10 organisms. All samples were assayed in triplicate. Positive (*N. fowleri* Lee strain, ATCC 30894) and negative (DNA free PCR-grade water) controls were also included in triplicate. Gel electrophoresis was used to visualize the PCR products.

DNA extracts from the second and third sampling periods (2011) were assayed for *Acanthamoeba* spp., *B. mandrillaris*, and *N. fowleri* using previously described TaqMan-based real-time PCR methods (Qvarnstrom et al. 2006) with slight modifications. Only those samples that tested positive for the live amoeba screening were assayed with qPCR.
These assays are believed to have a detection limit of one organism. Briefly, the real-time PCR assays targeting each type of amoeba were performed individually (singleplex format) in a 25-μL reaction volume containing 2.5 μL of DNA, 12.5 μL of iQ Supermix (Bio-Rad, Hercules, CA), 200 nM of each primer, and 100 nM of a TaqMan probe (Biosearch Technologies, Inc., Novato, CA). For the detection of *Acanthamoeba* spp., the forward primer AcantF900 (5’-CCCAGATCGTTTACCGTAA-3’), the reverse primer AcantR1100 (5’-TAAATATTAATGCCCTACTATCC-3’), and the fluorogenic probe AcantP1000 [5’-FAM-CTGCCACCGAATACTAGCATGG-BHQ1-3’ (FAM and BHQ1 are the 6-carboxyfluorescein and black hole quencher 1, respectively)] were used. The forward primer BalaF1451 (5’-TAACCTGCTAAATAGTCATGCCAAT-3’), the reverse primer BalaR1621 (5’-CAAACTTCCCTCGCTAATCA-3’), and the fluorogenic probe BalaP1582 (5’-FAM-AGTACTTCTACCAATCCAACCGCCA-BHQ1-3’) were used to detect *B. mandrillaris*. For the detection of *N. fowleri*, the primers NaeglF192 (5’-GTGCTGAAAACCTAGCTATTGTAACTCAGT-3’) and NaeglR344 (5’-CACTAGAAAAGCAACCTGAAAAG-3’) and the probe NfowlP (5’-FAM-ATAGCAATATATTGGGAGCTGGGC-BHQ1-3’) were used. PCR amplification was performed using the iQ5 real-time PCR detection system (Bio-Rad), and amplification data were collected and analyzed using the iQ5 optical system software version 2.1 (Bio-Rad). Two replicate wells in a 96-well PCR plate were used for each sample. A sample was considered positive when a fluorescent signal was obtained from at least one of the replicates. DNA purified from laboratory-grown *Naegleria fowleri*, *Acanthamoeba*
castellani and Balamuthia mandrillaris served as a positive control, while PCR-grade water lacking DNA template served as a negative control. Positive and negative controls were always included to avoid false-negative and false-positive results, respectively.

RESULTS

The results for the water quality physico/chemical parameters (free chlorine, temperature, pH, conductivity, and turbidity) are shown in Table 1. The only parameter that varied significantly between sampling periods was the temperature. The average temperature of the water samples collected during sampling period II was significantly lower than that of the other two sampling periods. This sampling period occurred during some of the months with colder ambient temperatures. Sampling periods I and III were conducted during the same season in different years and as such, had similar recorded water temperatures. The free chlorine residual within the distribution system remained high throughout all three sampling periods with an average ranging from 0.8 to 0.9 mg/L (ppm) chlorine. Only 0.37% (2 / 534) of samples collected during period II and III were positive for total coliforms and E. coli.

The results for the presence of amoebae in distribution system water samples may be found in Table 2. Viable amoebae were identified in 138 of 785 total samples (17.6%). Of the 251 samples collected during sampling period I, 45 (17.9%) were positive for the presence of viable amoebae. Of these 45 viable amoebae, 2 were determined to
be *N. fowleri*; no *Acanthamoeba* spp. or *B. mandrillaris* were detected. Sampling period III which took place during the same season in a different year had 55 of 267 (20.6%) samples with viable amoebae. None of these viable amoebae were identified as pathogenic species. Sampling period II which occurred during a colder period of the year had a lower number of samples with viable amoebae. Of the 38 of 267 (14.2%) positive samples, only two were identified as *Acanthamoeba*.

The distribution system samples were collected from 18 distinct regions with approximately 15 sample collection sites each (Table 3). Only 17 of these regions were included during the first sampling period. Of these 18 distribution system regions, 4 (22.2%) were found to have samples that were positive for viable amoebae for all three sampling periods (regions 8, 9, 13, and 14). Conversely, region 15 had no samples testing positive for viable amoebae during any of the three sampling periods.

Regional clusters (defined as having 3 or more samples testing positive for viable amoeba) were observed during all of the sampling periods; sampling period I had 5 such regional clusters (of the 17 regions tested), sampling period II had 7 regional clusters, and sampling period III had 7 regional clusters. Five of these positive clusters were found in the same region (4, 7, 8, 10, and 14) during different sampling periods (Table 3). Of these, region 8 had clusters of samples that were positive for viable amoebae during all three sampling periods over the two years. In some of the clusters during sampling periods I and III, a majority of the samples tested positive for the presence of viable amoebae; the highest number of positive samples observed was 12 out of 15
samples (80%).

**DISCUSSION**

A recent review paper revealed that free-living amoebae are consistently found in treated drinking water systems; however, the possible related health risks could not be quantified (Thomas & Ashbolt 2011). Although some of these free-living amoebae are pathogenic species, the majority are likely non-pathogenic. Nevertheless, even the rare occurrence of pathogenic species may be cause for some concern. Cases of keratitis caused by *Acanthamoeba* spp. have been frequently linked to the use of tap water to cleanse contact lenses. In immunocompromised individuals, *Acanthamoeba* and *B. mandrillaris* infections can progress to granulomatous amoebic encephalitis (GAE) (Visvesvara, 2007); however, it is unclear at this time whether or not *B. mandrillaris* infections can be acquired from water. There have been several cases of primary amoebic meningoencephalitis (PAM) caused by *N. fowleri* linked to treated drinking water in Australia. These were primarily associated with unusually high summer temperatures and inadequate chlorine residual (Dorsch 1982). In 2002, two children died from *N. fowleri* infections acquired from bathing in unchlorinated tap water (from groundwater) in Arizona (Marciano-Cabral et al. 2003; Okuda et al. 2004). More recently, two deaths in Louisiana were attributed to an infection by *N. fowleri* acquired from the use of treated tap water in nasal flushing devices (neti pots) (Soule 2011). According to the National Health Interview Survey 13% of people with sinusitis age 18
years in the United States (Sondik et al. 2012). Additionally, higher numbers of people with sinusitis are concentrated in the Southern United States (Sondik et al. 2012). These individuals are potentially at risk if they perform such nasal rinses, thus exposing themselves to *N. fowleri* in treated drinking water. In addition, sensitive populations that are more vulnerable to infections (e.g., the elderly and the immunocompromised) are now estimated to comprise 20-25% of the U.S. population (Reynolds et al. 2008).

A study investigating the occurrence of free-living amoebae in tap water found that their presence was influenced by temperature, with *N. fowleri* being detected in September and *Acanthamoeba* in March with the temperatures ranging from 4 – 28°C for the entire duration of the study (Marciano-Cabral et al. 2010). Similarly in the current study, *N. fowleri* was isolated in August (average temperature of 32.4 ± 0.4) and *Acanthamoeba* in February and March (average temperature of 22.8 ± 3.0). It is important to control the occurrence of even non-pathogenic free-living amoebae since the pathogenic bacteria *Legionella pneumophila* and *Mycobacterium avium* have been shown to have a strong association with these amoebae (Loret & Greub 2010). Marciano-Cabral et al. (2010) found *Legionella* and *Mycobacterium* in both March and September in the tap water samples. Furthermore, the rapid formation of biofilms was observed in the water samples with the presence of both free-living amoebae and bacteria, providing evidence that the persistence or colonization in a distribution system by these organisms is associated with biofilm formation (Marciano-Cabral et al. 2010). Also, *N. fowleri* was detected more commonly in biofilm samples than in water samples.
in an Australian distribution system (Puzon et al. 2009). Previous studies have demonstrated that *N. fowleri* is able to survive in distribution system for extended periods (≥ 5 months) (Biyela et al. 2012). A recent study assessing the prevalence of *Acanthamoeba* spp. and other free-living amoebae in household water and biofilm swab samples detected amoebae in biofilm samples more frequently (Stockman et al. 2011). In this study, a total of 2,454 household water and biofilm samples were evaluated; amoebae were found in 79% of all samples, 51% of which were confirmed to be *Acanthamoeba* spp. (Stockman et al. 2011).

According to Trolio et al. (2008), a minimum free chlorine residual of 0.2 mg/L should be maintained throughout the distribution system. Despite the fact that the distribution system in the present study maintained an adequate free chlorine residual (mean = 0.86 mg/L) during all three sampling periods, 17.6% of the samples tested positive for the presence of viable amoebae (average chlorine residual was 0.9 ± 0.2). *Acanthamoeba* spp. cysts have been shown to survive chlorine levels of 100 mg/L for ten minutes, indicating that the typical chlorine residuals in water distribution systems are insufficient for the control of *Acanthamoeba* spp. (Storey et al. 2004). *Naegleria* has also been detected in the presence of high chlorine residuals (Trolio et al. 2008); this was confirmed by the results of the current study in which *N. fowleri* and *Acanthamoeba* spp. were detected in distribution samples with a substantial chlorine residual (average of 0.92 ± 0.1).

A total of 2,454 samples from 467 households were examined. Amoebae were
found in water samples of 371 (79%) households. Sites most likely to contain amoeba were shower heads (52%) and kitchen sprayers (50%).

In the current study, all of the samples collected from region 15 of the distribution system were negative for the presence of viable amoebae during all three sampling periods despite substantial differences in water temperature. Interestingly, 3 of the 4 clusters that recurred between two sampling periods were found between sampling periods II and III, which were undertaken during different seasons of the same year (2011), and not between sampling periods I and III, which took place during the same season in different years (2009 and 2011). This suggests that temporal (year to year), rather than seasonal variations might play a greater role in such clustering effects. Nevertheless, more positive samples were found during the warmer sampling periods I and III (average water temperatures of 31.5°C and 30.1°C, respectively) than during the colder sampling period II (average water temperature of 22.9°C). This suggests that there are seasonal variations in the occurrence of live amoebae in the distribution system; however, these do not seem to be involved in the clustering of positive samples.

Characteristics of the distribution system environment itself may contribute to the occurrence of these free-living amoebae. For instance, sediments in the distribution system could provide a layer of protection to the amoebae from the disinfection residual. A study in Germany isolated a variety of free-living amoebae in water and sediment after different stages in the treatment process and in the drinking water distribution system (Hoffmann & Michel 2001). Sediments were not tested in the
current study, but the distribution system did contain sediments at times. A water utility in Western Australia implemented an ultrafiltration treatment in order to address the issue of sediments in the distribution system (Trolio et al. 2008). The combination of ultrafiltration and proper chlorine residual has been demonstrated to be an effective way to eradicate *Naegleria* in a distribution system for up to two years (Trolio et al. 2008).

The fact that positive samples were often found in clusters within the potable distribution system suggests that multiple groundwater sources (with different supply wells for separate regions), varying ratios of groundwater to surface water blends, and characteristics of the distribution system environment itself may contribute to the presence of these free-living amoebae. Therefore, identifying viable amoebae may not only provide a better indication of the water-based microbial quality of water, but such clustering as observed could reveal areas with potential contributing factors within the distribution system (e.g., physical/mechanical problems, contamination of a particular water source, poor maintenance of that region of the distribution system, multiple blended sources, sediment buildup, and daily flow and pressure changes due to fluctuations in consumer demand). We believe that routine monitoring for the presence of live amoebae could be used as an alternative and perhaps more sensitive method for monitoring the overall water quality in drinking water distribution systems. Such monitoring could be used as a complementary step to the total coliform rule and the groundwater rule monitoring requirements.
REFERENCES


Table 1. Water quality measurements by sampling period.

<table>
<thead>
<tr>
<th>Water Quality Parameter</th>
<th>Period I [mean ± SD (range)]</th>
<th>Period II [mean ± SD (range)]</th>
<th>Period III [mean ± SD (range)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free Chlorine (mg/L)</td>
<td>$0.9 \pm 0.2 (0 – 1.7)$</td>
<td>$0.9 \pm 0.2 (0 – 1.6)$</td>
<td>$0.8 \pm 0.2 (0 – 1.7)$</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>$31.5 \pm 1.7 (23.6 – 37.0)$</td>
<td>$22.9 \pm 2.7 (14.5 – 28.3)$</td>
<td>$30.1 \pm 1.5 (25.9 – 37.0)$</td>
</tr>
<tr>
<td>pH</td>
<td>$7.8 \pm 0.2 (7.1 – 8.1)$</td>
<td>$8.0 \pm 0.2 (7.2 – 8.2)$</td>
<td>$7.9 \pm 0.2 (7.3 – 8.1)$</td>
</tr>
<tr>
<td>Conductivity (umhos/cm)</td>
<td>$589 \pm 144 (234 – 966)$</td>
<td>$692 \pm 121 (219 – 1,003)$</td>
<td>$662 \pm 142 (223 – 978)$</td>
</tr>
<tr>
<td>Turbidity (NTU)</td>
<td>$0.3 \pm 0.1 (0.1 – 1.0)$</td>
<td>$0.3 \pm 0.2 (0.1 – 1.4)$</td>
<td>$0.2 \pm 0.1 (0.1 – 0.9)$</td>
</tr>
<tr>
<td>Heterotrophic Plate Count (CFU/100 mL)</td>
<td>ND</td>
<td>$19.6 \pm 66.3 (0-895)$</td>
<td>$5.0 \pm (0-210)$</td>
</tr>
</tbody>
</table>

ND = Not determined  
SD = Standard deviation
Table 2. Amoebae found in potable water distribution system by collection period.*

<table>
<thead>
<tr>
<th>Organism</th>
<th>Period I</th>
<th>Period II</th>
<th>Period III</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viable amoebae</td>
<td>45 / 251 (17.9%)</td>
<td>38 / 267 (14.2%)</td>
<td>55 / 267 (20.6%)</td>
<td>138 / 785 (17.6%)</td>
</tr>
<tr>
<td>Naegleria fowleri</td>
<td>2 / 45 (4.4%)</td>
<td>0 / 38 (0%)</td>
<td>0 / 55 (0%)</td>
<td>2 / 138 (1.5%)</td>
</tr>
<tr>
<td>Acanthamoeba spp.</td>
<td>0 / 45 (0%)</td>
<td>2 / 38 (5.3%)</td>
<td>0 / 55 (0%)</td>
<td>2 / 138 (1.5%)</td>
</tr>
<tr>
<td>Balamuthia mandrillaris</td>
<td>0 / 45 (0%)</td>
<td>0 / 38 (0%)</td>
<td>0 / 55 (0%)</td>
<td>0 / 138 (0%)</td>
</tr>
</tbody>
</table>

ND = Not determined
* Results are presented as the ratio of the number of positive samples to the total number of samples collected with the percentage testing positive in parenthesis. Only the samples testing positive for viable amoebae were tested for the presence of specific pathogenic amoebae by PCR or qPCR.
Table 3. Number of samples positive for viable amoebae by water distribution system region and sampling period. Positive clusters (regions with three or more positive samples) are highlighted in yellow.

<table>
<thead>
<tr>
<th>Distribution System Region</th>
<th>Period I</th>
<th>Period II</th>
<th>Period III</th>
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<td>2</td>
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<td>18</td>
<td>ND</td>
<td>0</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>45</strong></td>
<td><strong>38</strong></td>
<td><strong>55</strong></td>
<td><strong>138</strong></td>
</tr>
</tbody>
</table>

ND = not determined
APPENDIX C

METHODS FOR THE DETECTION OF *NAEGLERIA FOWLERI IN WATER*

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Naegleria fowleri causes primary amoebic meningoencephalitis (PAM) which is fulminating and hemorrhagic encephalitis. Humans become exposed to N. fowleri via nasal exposure from swimming activities or use of tap water for nasal cleansing or nasal exposure to tap water.

The current methods for the detection of Naegleria fowleri for water have not been standardized. Such methods are needed to assess the risks from N. fowleri in water. These methods have been developed to specifically identify N. fowleri include biochemical tests such as combining agglutination to examine isoenzyme patterns in soil and water. Enzyme-Linked ImmunoSorbent Assay (ELISA) for the specific identification of N. fowleri for water samples is reliable but time consuming (Hlavsa et al. 2011).

More recently, PCR assays have demonstrated to be successful for the identification of N. fowleri in water and are both rapid and sensitive. For use in monitoring, the methods must be quantitative so that risks of infection can be assessed. The most sensitive assays for the detection of N. fowleri in water to date are real-time PCR assays. Based on a current risk assessment, methods should be capable of detecting at least 100 N. fowleri per liter of water. The most sensitive assays for the detection of N. fowleri in water to date are real-time PCR assays. Both the real-time PCR with melting curve analysis and the multiplex real-time PCR (Reveiller et al. 2003) have a high discrimination and a low detection limit of one N. fowleri. The combination
of a culture based assay followed by testing with a real-time PCR assay is currently the best approach to assess infectivity. There is a need for a standard method to monitor *N. fowleri* in water to assess the risk of infection.
INTRODUCTION

*Naegleria fowleri* is an amoeboflagellate that infects its host via the nasal mucosa following the forceful inhalation of contaminated water and causes primary amoebic meningoencephalitis (PAM) (Qvarnstrom *et al.* 2006). Death usually occurs 10-14 days after exposure (Martinez, 1997). *N. fowleri* is considered to be an opportunistic pathogen and primarily infects healthy, children and young adults (Visvesvara *et al.* 2007). It most frequently infects individuals in an age group that is more likely to engage in recreational water activities that involve submerging head under water.

There are over 40 species of *Naegleria* described to date, but only *N. fowleri* is pathogenic to humans, *N. australiensis* has been shown to be pathogenic in mice (Barnett *et al.* 1996; Rose *et al.* 2001; Cervantes-Sandoval *et al.* 2007). *Naegleria* is classified under *Vahlkampfiidae* and *Acanthamoebae* (De Jonckheere 2004); (Sawyer & Griffin 1975). Pathogenic *Naegleria fowleri* is not easily differentiated from other *Naegleria* species due to a similarities including common morphology when observed microscopically and indistinguishable behavior in cell culture (Ma *et al.* 1990). *N. fowleri* was first identified as a human pathogen in 1965 in Australia (Stevens *et al.* 1980; Martinez 1985). The first case in the United States was reported in 1966 and was described as primary meningoencephalitis (Fowler & Carter 1965). Prior to this documented case, free-living amoebae were not considered to be pathogenic.
Although rare, primary amoebic meningoencephalitis (PAM) has a 99% fatality rate. In the last decade, the number of deaths caused by water-based amoebae has doubled and recreational water outbreaks have tripled (Martinez 1985). *N. fowleri* has resulted in the deaths of 46 people in the United States, all in warmer regions between 1998 and 2011. Despite a recent increase in public awareness, there is little known regarding the ecology and occurrence of this organism. It has been suggested that the number of cases of *N. fowleri* are increasing worldwide possibly due to global climate change aiding in the establishment of *N. fowleri* in new regions where it has not been previously reported (Lee et al. 2002; Hlavsa et al. 2011).

Recreational outbreaks occur regularly in United States, with 398 recreational water outbreaks documented between 1999 and 2008 in the United States and Puerto Rico (Heggie 2010; Yoder et al. 2010a). The majority of documented cases of *N. fowleri* have occurred in countries with tropical and subtropical climates (Lee et al. 2002; Yoder et al. 2004; Dziuban et al. 2006; Yoder et al. 2008; Hlavsa et al. 2011). However, *N. fowleri* is found in warm, fresh or brackish water including swimming pools (Parija & Jayakeerthee 1999), hot tubs (Cerva 1971), domestic water supplies (Rivera et al. 1993), well water (Marciano-Cabral et al. 2003), ponds, lakes, streams, rivers, natural hot springs and sewage (Blair et al. 2008)(Wellings et al. 1977); (John & Howard 1995) and thermally polluted run-off from industrial zones (De Jonckheere & Voorde 1977; Ettinger et al. 2003; Sheehan et al. 2003). This amoebae is thermophillic, able to proliferate at
temperatures of up to 45°C, but does best at temperatures ranging from 25°C to 45°C (De Jonckheere & Voorde 1977).

The risk for humans of contracting primary amoebic meningoencephalitis while swimming once in water containing *N. fowleri* was calculated to be $2.5 \times 10^{-6}$ for water containing 100 *N. fowleri* per liter or $8.5 \times 10^{-8}$ for 10 *N. fowleri* per liter and $2.6 \times 10^{-4}$ for 1,000 *N. fowleri* per liter (Cabanes *et al.* 2001). The safe recommended level is a maximum of 100 *N. fowleri* per liter of water as adopted by the French authorities. This risk was based on a quantitative microbial risk assessment assuming the risk for a single swimming event with a mean of 10 ml water inhalation per event. The numbers of *N. fowleri* per liter of water were extrapolated based on the number of *N. fowleri* in Florida water (approximately 40 *N. fowleri* amoebae/liter) and the lowest concentration method detection limit (1 *N. fowleri*/liter) (Cabanes *et al.* 2001).

Currently, there is no standard protocol for the detection of *Naegleria fowleri* in water. Such protocols would be useful for the monitoring to assess potential risks from *N. fowleri* in tap water and recreational waters. A review of the literature for methods detecting *N. fowleri* in water revealed the need for the establishment of a standard method for monitoring.
DETECTION METHODS

Currently, cultural methods are used to differentiate *N. fowleri* from other free living amoebae. Diagnosis of primary amoebic meningoencephalitis (PAM) also relies on microscopic methods and cultural methods followed by confirmation with molecular methods (Visvesvara *et al.* 2007). The organism is concentrated from water then added to non-nutrient agar petri dishes grown on a bacterial lawn at 42 to 45°C (Visvesvara 1999). This quantitative amoebae assay is used to detect live ameba in cerebrospinal fluid (CSF) in combination with the flagellation test (FT) followed by a diagnostic confirmation test (De Jonckheere & Voorde 1977; Schuster 2002).

A variety of methods have been developed for the detection of *N. fowleri* both in water, soil, cerebral spinal fluid and brain tissue specimens. Methods have been developed to specifically identify *N. fowleri* and include biochemical tests such as combining agglutination to examine isoenzyme patterns (Schuster 2002). Isoenzyme analyses have been developed for soil and water samples (Visvesvara & Healy 1980; Kilvington *et al.* 1984; Pernin *et al.* 1985; Nerad *et al.* 1995). *N. fowleri* in cerebral spinal fluid specimens can be identified with a monoclonal antibody (*Visvesvara & Healy 1980; De Jonckheere 1982; Moss *et al.* 1988). Polyclonal antibodies have also been developed but exhibit cross reactivity with other *Naegleria* species (*Visvesvara *et al.* 1987)(*Wellings *et al.* 1977).
Another method for the detection of *N. fowleri* is an Enzyme-Linked ImmunoSorbent Assay (ELISA) which was developed for the specific identification of *N. fowleri* in cooling pond and river water samples (Wellings et al. 1977). This ELISA did not exhibit cross reactivity and 97.1% of the water samples were confirmed to be *N. fowleri*, with a discrimination of 2,000 *N. fowleri* /mL (Reveiller et al. 2003). A solid phase cytometry method combined with immunoflorescent assay was developed to detect *N. fowleri* in water (Reveiller et al. 2003). This method had a detection limit of 200 cells per liter of water and provided results within three hours (Pougnard et al. 2002).

More recently, the advent of polymerase chain reaction (PCR) has provided an alternative for the specific identification of *N. fowleri* that is both rapid and sensitive for the identification both in water and soil samples and cerebral spinal fluid and brain tissue specimens. These molecular methods are reviewed to better understand which method would be appropriate as a standard method for monitoring recreational or other types of water.

Some of the PCR assays developed are able to detect one *N. fowleri* cell (Pougnard et al. 2002). The only drawback to some of these methods is that they require the amoebae to be cultured prior to performing the assays. These cultures have to be purified through the use of a commercial DNA extraction kit. Some of the first molecular methods for the detection of *N. fowleri* utilized conventional PCR providing a more rapid method for identification when compared to antibosy assays (Sparagano 1993b; Kilvington & Beeching 1995; Qvarnstrom et al. 2006; Madarova et al. 2010).
Sensitivity of detection was increased by using nested PCR to detect *Naegleria fowleri* and was able to differentiate from other non-pathogenic amoebae with the use of a probe referred to as Mp2C15 to differentiate from other *Naegleria* species. This nested PCR assay was able to detect as few as five *N. fowleri* (McLaughlin *et al.* 1991; Sparagano 1993a; Kilvington & Beeching 1995).

This nested PCR assay was further developed and used for the identification of *Naegleria fowleri* from river water and soil in Virginia and Connecticut (Reveiller *et al.* 2002). The assay was also used to identify *N. fowleri* in domestic water sources in Arizona after two deaths attributed to primary amoebic meningoencephalitis occurred (Maclean *et al.* 2004). For both of these surveys, the samples were cultured on non-nutrient agar first and then tested using a nested PCR assay specifically for *N. fowleri*.

More recently drinking water and biofilm samples from a water distribution pipeline were tested using total DNA extraction followed by real-time PCR melting curve analysis (Marciano-Cabral *et al.* 2003). This method was able to detect one to five organisms and was more rapid due to the use of total DNA extraction, rather than culture. Another group used real-time PCR using hybridization fluorescent labeled probes targeting the Mp2C15 sequence with a detection limit of one copy of the sequence when used with clinical specimens (Puzon *et al.* 2009). Ahmad *et al.* 2011 recently reported on the development of a rapid DNA extraction method and a one-step nested PCR for the detection of *N. fowleri* river water (Madarova *et al.* 2010). The method was used to study river water associated with a nuclear power plant in France.
and the United Kingdom (Ahmad et al. 2011). No *N. fowleri* or *Naegleria* spp. were identified in the water, but this assay could provide a more rapid alternative to the two step nested PCR assay.

Real-time PCR methods have also been developed to identify *N. fowleri* in the environmental and clinical samples. A real-time PCR assay combined with melting-curve analysis was rapid, sensitive, discriminating, and simple since there is no need for electrophoresis to verify the PCR product (Ahmad et al. 2011). In this assay, a single primer set based on the internal transcribed spacer (ITS) region for the *Naegleria* species with SYTO9 as the intercalating dye was used. It was able to distinguish between seven different *Naegleria* species by using the melting-curve analysis (Robinson et al. 2006). Another real-time PCR assay developed to test for three different genera of free-living amoebae (*Naegleria fowleri*, *Balamuthia mandrillaris* and *Acanthamoeba* spp.) simultaneously was developed using TaqMan technology (Robinson et al. 2006). This method demonstrated high specificity and sensitivity with a detection limit of one *N. fowleri* organism. This test only required five hours to complete (Qvarnstrom et al. 2006). A duplex real-time PCR assay for the quantitative detection of *N. fowleri* in water samples and was able to detect as few as 320 *N. fowleri* cells per liter after concentration and DNA extraction (Qvarnstrom et al. 2006). A summary of the molecular methods developed in the last decade are presented in Table 1.
METHODS FOR THE CONCENTRATION OF NAEGLERIA FROM WATER

Most investigators have concentrated water samples for the detection of *N. fowleri*. Water sample sizes have ranged from 15 mL (Reveiller *et al.* 2002) to 1 L (Lares-Villa *et al.* 2010) for recreational water and from 10 (Puzon *et al.* 2009) to 1,000 L for drinking water. Concentrates then added to non-nutrient agar containing heat-killed *E. coli*, to culture the amoebae. Direct plating of water samples onto non-nutrient agar without any prior concentration is also commonly done (Maclean *et al.* 2004).

Concentration of large volumes is possible using Envirocheck HV sampling Capsule, which has been developed for the detection of *Cryptosporidium* and *Giardia* (Pall Corp. East Hills, New York). Water samples are concentrated by filtration (Maclean *et al.* 2004; Lares-Villa *et al.* 2010) or centrifugation (5,000× *g*, 10 min). The pelleted cells were re-suspended in 1 ml of Page’s amoeba saline (Reveiller *et al.* 2002; Marciano-Cabral *et al.* 2003). Water samples have also been concentrated by centrifugation and tested directly by nested PCR (Reveiller *et al.* 2002). Another option is to extract the total DNA from a 250 mL sample directly and analyze by using a real-time PCR melt curve assay (Puzon *et al.* 2009). Unfortunately no studies have been conducted on the efficiency of these methods. A comparison of different concentration methods are needed to determine the efficiency of detecting *N. fowleri* in water.
ADVANTAGES AND DISADVANTAGES OF MOLECULAR METHODS

Major advantages of molecular methods are that they are rapid, sensitive and specific. Although for some of these assays it is necessary to first culture the sample before the molecular assay. These are, however, an attractive alternative to microscopy and culture alone since there needs to be some discrimination between pathogenic and non-pathogenic species of *Naegleria* spp. The use of better, more efficient, extraction methods in combination with PCR methods have proven to be successful in identifying *N. fowleri* from different environmental samples (Behets *et al.* 2007). Water samples could contain very low levels of *N. fowleri*, thought to be anywhere from 1 to 1000 organisms *N. fowleri* per liter of water (Cabanes *et al.* 2001). This can make it difficult to identify, but with molecular methods this problem is overcome with the sensitivity of the assays allowing for detection of one amoebae per liter. Some of the disadvantages of molecular methods are that they do not determine infectivity when not done in tandem with culture methods. A variety of these molecular assays need processing for DNA extraction for the purification of the samples before PCR assays, causing an increase in the time needed to identify *N. fowleri* from environmental samples.
CONCLUSIONS

Several different approaches have been used to detect *Naegleria* spp. in water. Biochemical methods were effective and specific enough for the detection of *N. fowleri* when using monoclonal antibodies for the identification in clinical specimens. However, polyclonal antiserum for *N. fowleri* cannot distinguish from *N. lovaniensis* from other *N.* spp. Isoenzyme analyses were a good starting point as they were specific enough to detect *N. fowleri* from water, soil and clinical specimens (Pelandakis & Pernin 2002; Sheehan *et al.* 2003; Maclean *et al.* 2004; Puzon *et al.* 2009).

In comparison, molecular techniques such as PCR assays have been more successful for the identification of *N. fowleri*. Real-time PCR assays are more rapid than the other PCR assays and can yield results with high specificity and sensitivity. While melting curve analysis has a high discrimination against seven different *Naegleria* spp., the multiplex real-time PCR assay can test for three organisms of public health concern simultaneously.

For use in monitoring, the methods must be quantitative so that risks of infection can be assessed. Based on a current risk assessment, methods should be capable of detecting at least 100 *N. fowleri* per liter of water for a risk $2.5 \times 10^{-6}$. Both the real-time PCR with the melting curve analysis (Visvesvara & Healy 1980; Kilvington *et al.* 1984; Pernin *et al.* 1985; Nerad *et al.* 1995) and the multiplex real-time PCR (Robinson *et al.* 2006) have a high discrimination and a low detection limit of one *N.*
fowleri. In addition, the Taqman based duplex real-time PCR assay is able to detect 3 N. fowleri with 100% specificity in seeded cooling circuit by the seeded evaluation (Behets et al. 2007).

The nature of which method should be used depends on the sample. There is a need for a standard method to monitor N. fowleri in the environment to assess the risk of infection from N. fowleri. The combination of total DNA extraction followed by differentiation with a real-time PCR assay is currently the most rapid approach to monitor N. fowleri in water. Studies are needed to determine the efficiency of N. fowleri by the various methods for their concentration from water.
REFERENCES


Table 1A. Summary of Detection Methods for *Naegleria fowleri*.

<table>
<thead>
<tr>
<th>Study Location</th>
<th>Source Water</th>
<th>Detection Method</th>
<th>Detection Limit</th>
<th>Year</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>France</td>
<td>Cooling ponds and rivers</td>
<td>Multiplex PCR</td>
<td>DNA from as few as five trophozoites or cysts</td>
<td>2002</td>
<td>(Qvarnstrom et al. 2006)</td>
</tr>
<tr>
<td>USA-Virginia and Florida</td>
<td>Mock environmental samples</td>
<td>PCR</td>
<td>5 pg of <em>N. fowleri</em> DNA or 5 intact <em>N. fowleri</em> amoebae</td>
<td>2002</td>
<td>(Pelandakis &amp; Pernin 2002)</td>
</tr>
<tr>
<td>USA - Arizona</td>
<td>Groundwater</td>
<td>PCR*</td>
<td>5 intact <em>N. fowleri</em> in 50 mL of water</td>
<td>2003</td>
<td>(Reveiller et al. 2002)</td>
</tr>
<tr>
<td>Belgium</td>
<td>Cooling water</td>
<td>FT***, ELISA, PCR</td>
<td>2 <em>N. fowleri</em> per liter</td>
<td>2003</td>
<td>(Marciano-Cabral et al. 2003)</td>
</tr>
<tr>
<td>USA - Yellowstone National Park</td>
<td>Hot springs</td>
<td>PCR</td>
<td>No limit of detection mentioned</td>
<td>2003</td>
<td>(Behets et al. 2003)</td>
</tr>
<tr>
<td>USA</td>
<td>CSF Samples</td>
<td>Multiplex Real-Time PCR</td>
<td>1 amoeba</td>
<td>2006</td>
<td>(Sheehan et al. 2003)</td>
</tr>
<tr>
<td>South Australia</td>
<td>Pure cultures</td>
<td>Real-Time PCR and Melting - curve analysis</td>
<td>Detection limit of 0.1 to 0.2 cells. Reaction efficiency calculated from the standard curve was 0.92 for <em>N. fowleri</em>.</td>
<td>2006</td>
<td>(Qvarnstrom et al. 2006)</td>
</tr>
<tr>
<td>France and USA</td>
<td>River Water</td>
<td>Flow Cytometry</td>
<td>0.06 amoeba per ml using a flow rate of 15 ml min⁻¹</td>
<td>2007</td>
<td>(Robinson et al. 2006)</td>
</tr>
<tr>
<td>Belgium</td>
<td>Cooling water</td>
<td>Duplex real-time PCR</td>
<td>3 <em>N. fowleri</em> per liter. Quantification limit was 320 <em>N. fowleri</em> cells per liter</td>
<td>2007</td>
<td>(Johnson et al. 2007)</td>
</tr>
<tr>
<td>Slovakia</td>
<td>Swimming Pool</td>
<td>Real-time PCR</td>
<td>1 copy of Mp2Cl5 DNA sequence</td>
<td>2010</td>
<td>(Behets et al. 2007)</td>
</tr>
<tr>
<td>France and the UK</td>
<td>River Water</td>
<td>One-step nested PCR</td>
<td>&gt;5 - 50 cells of <em>N. fowleri</em></td>
<td>2011</td>
<td>(Madarova et al. 2010)</td>
</tr>
</tbody>
</table>

*PCR = Polymerase Chain Reaction
**ELISA = Enzyme-Linked ImmunoSorbent Assay
***FT = Flagellation Test
APPENDIX D

PLAYING SAFE IN NATURAL WATERS:

HOW TO PROTECT YOURSELF FROM *NAEGLERIA FOWLERI*

WHEN YOU GO SWIMMING

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This leaflet can be found at http://cals.arizona.edu/pubs/water/az1545.pdf

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Playing Safe in Natural Waters: How to Protect Yourself from *Naegleria fowleri* When You Go Swimming

Laura Sifuentes, Charles Gerba, and Charrnah Rock

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Life-cycle of *Naegleria fowleri*. *Naegleria fowleri* has three stages: cysts, trophozoites, and flagellated forms, in its life-cycle. The trophozoites replicate by binary fission (cell division). Trophozoites can turn into temporary non-feeding flagellated forms which usually revert back to the trophozoite stage. Trophozoites infect humans or animals by penetrating the nasal mucosa and migrating to the brain via the olfactory nerves causing Primary Amoebic Meningoencephalitis (PAM). (Ref: [http://www.dpd.cdc.gov/dpdx])
What is Naegleria fowleri?

Naegleria fowleri, pronounced 'nä-ɡlə-ri-a fough-ler-e', is an amoeba (single-celled living organism) that can act as a parasite in animals and humans. Naegleria is commonly found in warm freshwater (for example, lakes, rivers, and hot springs) and soil. It has three life stages: trophozoite, flagellate, and cyst and can measure from 10 μm to 25 μm.

Where is Naegleria fowleri found?

Naegleria fowleri is found around the world. In the United States, the majority of infections have been caused by Naegleria fowleri from freshwater located in southern-tier states. The amoeba is most commonly found in warm bodies of water such as ponds, lakes, rivers, hot springs, and coastal waters and can survive temperatures of up to 45°C.

How common is Naegleria fowleri infection?

Infection with Naegleria fowleri occurrence is rare, with only 400 cases documented worldwide. From 2000 to 2009, 39 infections were reported in the U.S.; at least six of these infections occurred in Arizona. The risk of infection is estimated to be 1 in 2.6 million.

Can I get Naegleria fowleri infection from a swimming pool?

No. You cannot get a Naegleria fowleri infection from a properly chlorinated, cleaned, and regularly maintained swimming pool.

How does infection with Naegleria fowleri occur?

Naegleria fowleri infects people by entering the body through the nose. This typically occurs during recreational activities such as swimming, diving, jet skiing, water skiing, and wake boarding in natural waters. It is not transferred from person to person and cannot be infected by drinking contaminated water.

What are the symptoms of Naegleria fowleri infection?

Naegleria fowleri infection causes Primary Amoebic Meningoencephalitis (PAM), a brain infection that leads to the destruction of brain tissue. Initial symptoms include headache, fever, nausea, vomiting and stiff neck. These symptoms can begin 1 to 14 days following infection. Later symptoms include confusion, inability to focus, seizures, and hallucinations. After the start of symptoms, the disease progresses rapidly and usually causes death within 1 to 12 days. If you experience any of these symptoms, seek immediate medical attention.
Is there medical treatment for *Naegleria fowleri* infections?

Because of the rarity of the infection, there isn’t substantial medical evidence at this time. Several drugs are effective against *Naegleria fowleri* in the laboratory, and in some cases antibiotic treatment in conjunction with other experimental treatments have shown to be effective if *N. fowleri* is detected and diagnosed early in the course of infection.

How can I reduce my risk of *Naegleria fowleri* infection?

It is likely that the low risk of *Naegleria fowleri* infection will always exist with occasional use of warm freshwater lakes, rivers, and hot springs. The low number of infections makes it difficult to know why few people have been infected compared to the millions of other people using the same or similar waters across the U.S. The only certain way to prevent infection is to refrain from water-related activities in warm, untreated, or poorly-treated water.

If you do plan to take part in water-related activities some measures that might reduce risk include:

- Hold nose shut or wear nose plugs when jumping or diving into natural waters such as lakes, rivers, or hot springs.
- Avoid swimming or diving in warm natural water during periods of high temperatures and low water volume.
- Avoid digging in or stirring up sediment in shallow natural waters.

For further questions contact The Arizona Department of Health Services, Office of Infectious Disease Services at 602-542-3975.

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**References**

Centers for Disease Control and Prevention. *Naegleria fowleri*.
https://www.cdc.gov/parasites/naegleria/faq.html