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*Toxoplasma gondii* Oocysts in Water

By

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DISSERTATION

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of the

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Approved:



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Committee in Charge

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2008

*Toxoplasma gondii* Oocysts in Water

Abstract

*Toxoplasma gondii*, a ubiquitous parasitic protozoan, is emerging as an aquatic biological pollutant. Infections can result from ingesting environmentally-resistant oocysts in drinking water. However, recommendations regarding water treatment for oocyst inactivation have not been established. As part of this doctoral thesis, the efficacies of two commonly used chemicals, sodium hypochlorite and ozone, as well as physical methods of ultraviolet (UV) radiation and radiofrequency (RF) power were evaluated for their ability to inactivate *T. gondii* oocysts in water. With chemical treatments, oocysts were exposed to either 100 mg/L of chlorine for 30 min, 2 hr, 4 hr, 8 hr, 16 hr, and 24 hr or 6 mg/L of ozone for 1, 2, 4, 8, and 12 min. Oocysts were exposed to pulsed and continuous UV exposure doses ranging from 20 to 1000 mJ/cm<sup>2</sup> and to various RF energy levels to induce 50, 55, 60, 70 and 80°C temperatures. Post-treatment, oocyst viability was determined by mouse bioassay with serology, immunohistochemistry, and *in vitro* parasite isolation as the test methods to confirm *T. gondii* infection in mice. Initially, mouse bioassay experiments were conducted to compare the analytical sensitivity of these 3 detection methods prior to completing the chemical inactivation experiments. *Toxoplasma gondii*

infection was confirmed by at least 1 of the 3 detection methods in mice inoculated with all doses ( $10^5$  -  $10^0$ ) of oocysts. Results of the chemical exposure experiments indicated that neither sodium hypochlorite nor ozone effectively inactivated *T. gondii* oocysts, even when used at high concentrations. Both pulsed and continuous UV irradiation results indicated that some *T. gondii* oocysts were inactivated when exposed to UV radiation, but not with 100% efficiency over the entire range of UV doses evaluated. Oocysts were inactivated with RF-induced thermal levels of  $\geq 60^\circ\text{C}$  in an initial experiment, but not in subsequent experiments conducted under similar conditions. These results indicated that UV treatment may be useful to sanitize drinking water and sewage effluent but the impact of factors such as water heating time, cooling time, and the volume of water treated must be considered when evaluating any method for oocyst inactivation.

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Reportedly, in a letter to fellow scientist, Robert Hooke, Sir Isaac Newton once said "If I have seen further, it is by standing on the shoulders of giants." In the spirit of Newton, I would like to acknowledge my "giants":

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"And when she wraps her hand  
Around my finger,  
Oh it puts a smile in my heart.  
Everything becomes a little clearer;  
I realize what life is all about.  
It's hangin' on when your heart  
Has had enough;  
It's giving more when you feel like giving up;  
I've see the light  
It's in my daughter's eyes"



## DEDICATION

"Our deepest fear is not that we are inadequate. Our deepest fear is that we are powerful beyond measure. It is our light, not our darkness that most frightens us. We ask ourselves, Who am I to be brilliant, gorgeous, talented, fabulous? Actually, who are we not to be? ... Your playing small does not serve the world. There is nothing enlightened about shrinking so that other people won't feel insecure around you. We are all meant to shine, as children do....It's not just in some of us; it's in everyone. And as we let our own light shine, we unconsciously give the other people permission to do the same. As we are liberated from our own fear, our presence automatically liberates others."

~Marianne Williamson

*Toxoplasma gondii* Oocysts in Water

Katlyn Wainwright

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## CHAPTER 1

### *Toxoplasma gondii* in Water

#### Introduction

Discovered in a North African rodent (*Ctenodactylus gondii*) in 1908, *Toxoplasma gondii* is an obligate, intracellular parasite that can infect most warm-blooded animals, including humans (Nicolle and Manceaux, 1908; Schwartzman and Pfefferkorn, 1982; Dubey and Beattie, 1988). With a worldwide distribution, the success of *T. gondii* transmission is remarkable with global estimates of over 1 billion people infected with this parasite (Dubey and Beattie, 1988; Joynson, 2001). Historically described as a pathogen affecting terrestrial animals, there is growing awareness of *T. gondii* as an aquatic, biological pollutant (Hay and Hutchison, 1983; Slifko, 2000; Dubey, 2004). This is supported by the increasing detection of *T. gondii* infections in marine mammals, where infections appear to be having an adverse effect on marine mammal health including an increased risk of mortality (Thomas and Cole, 1996; Dubey et al., 2003; Kreuder et al., 2003; Fayer et al., 2004). Marine mammals affected include cetaceans such as dolphins and toothed-whales (Cruickshank et al., 1990; Inskeep II et al., 1990; Migaki et al., 1990; Mikaelian et al., 2000; Jardine and Dubey, 2002; Resendes, et al., 2002a and b; Bowater et al., 2003; Cabezon et al., 2004; Murata et al., 2004); pinnipeds such as seals and sea lions (Van Pelt and Dietrich, 1973; Migaki et al., 1977; Holshuh et al., 1985; Lamborn et al., 2001; Lindsay et al., 2001; Miller et al., 2001; Dubey et al., 2004; Gajadhar et al., 2004; Measures et al., 2004), and sirenians including

manatees and sea otters (Buergelt and Bonde, 1983; Cole et al., 2000; Kreuder et al., 2003). In these animals, infections with *T. gondii* have resulted in encephalitis, pneumonia, lymphadenopathy, heart, spleen and liver necrosis, brain lesions with necrosis, and death (Van Pelt and Dietrich, 1973; Migaki et al., 1977; Buergelt and Bonde, 1983; Holshuh et al., 1985; Cruickshank et al., 1990; Inskeep II et al., 1990; Migaki et al., 1990; Cole et al., 2000; Mikaelian et al., 2000; Lambourn et al., 2001; Lindsay et al., 2001; Miller et al., 2001; Jardine and Dubey, 2002; Resendes et al., 2002; Dubey et al., 2003; Kreuder et al., 2003; Dubey et al., 2004). Consequently, as top consumers in aquatic food chains, their demise will disrupt the balance of the marine ecosystem. As “apex” species, the health of these marine mammals will also both affect and reflect the general condition of the aquatic environment.

An epidemiological study conducted by Miller et al. (2002), and reviewed by Conrad et al. (2005), suggests that contaminated freshwater sources, such as streams, rivers, sewage effluent, and run-off, emptying into coastal waters serve as a significant risk factor for *T. gondii* infections for threatened southern sea otters in California. These freshwater sources are also used by humans for drinking water, recreation, agriculture and livestock production thus exposing humans and food animals to infection with *T. gondii* (Aramini et al, 1998 and 1999; Miller et al., 2002; Fayer et al., 2004). In addition to marine mammals, outbreaks of toxoplasmosis in human populations have increased recognition of the risks of waterborne transmission of *T. gondii* to humans (Benenson et al.,

1982; Bowie et al., 1997; Aramini et al., 1999; Hall et al. 1999; Bahia-Oliveira et al., 2003; de Moura et al., 2006; Stroka et al., 2006). Water-related outbreaks of human disease have also made apparent that methods to remove or inactivate the environmentally persistent oocyst stage of *T. gondii* from treated drinking and wastewater are inadequate (American Water Works Association, 1999; Slifko et al, 2000; Dubey, 2004).

### **LIFE CYCLE**

Belonging to the phylum Apicomplexa, *T. gondii* is a coccidium with a three-stage life cycle (Levine, 1985). Members of the family *Felidae* serve as definitive hosts, with the *T. gondii* parasites replicating both sexually and asexually in domestic and wild felines (Ogino and Yoneda, 1966; Dubey et al., 1970a; Frenkel et al., 1970; Jewell et al., 1972; Miller et al., 1972; Wallace, 1973b). In contrast, *T. gondii* replicates only asexually in intermediate hosts such as birds and warm-blood mammals, including humans (Ogino and Yoneda, 1966; Dubey et al., 1970a; Miller et al., 1972). Tachyzoites, bradyzoites, and sporozoites (found in sporulated oocysts) are the 3 stages that can infect intermediate or definitive hosts (Frenkel, 1973; Dubey and Beattie, 1988; reviewed by Dubey et al., 1998). Tachyzoites are crescent to oval in shape and are 2-4  $\mu\text{m}$  wide and 4-8  $\mu\text{m}$  in length (Sheffield and Melton, 1968; Mehlhorn and Frenkel, 1980; Speer et al., 1998). Bradyzoites, which are contained within tissue cysts, are morphologically similar to tachyzoites but express many functionally different stage-specific

molecules. Sporozoites are crescent in shape and  $2 \times 6$  to  $8 \mu\text{m}$  in size (Mehlhorn and Frenkel, 1980; Speer et al., 1998). The most common transmission routes include ingestion or inhalation of sporulated oocysts in water and soil or on fruits and vegetables; ingestion of latent bradyzoite tissue cysts in raw or undercooked meat of intermediate hosts; or transplacental transmission of motile tachyzoites from a mother to her fetus (Dubey and Beattie, 1988; Tenter et al., 2000).

The tachyzoite can gain entry to virtually any nucleated cell via active penetration and will form parasitophorous vesicles around themselves within the host cell (Sheffield and Melton, 1968; Werk, 1985; reviewed by Bonhomme, 1992). In the intermediate host, the tachyzoite multiplies rapidly by endodyogeny in any host cell, filling this cell with parasites (Ogino and Yoneda, 1966; Sheffield and Melton, 1968; Ferguson et al., 1974). When the host cell can no longer contain the parasites, the cell bursts and releases them to invade new cells. The organism may disseminate throughout the host's body via macrophages and dendritic cells (Da Gama et al., 2004; Lambert et al., 2006). This dissemination of tachyzoites can result in severe tissue damage if not controlled by the host's immune system (Frenkel, 1988; reviewed by Denkers and Gazzinelli, 1998). After several rounds of asexual replication, approximately 10 days following initial infection, tachyzoites transform into slowly dividing bradyzoites for the formation of tissue cysts (Brown and McLeod, 1990). The signals that initiate this transformation are unknown, but may result from

immunological pressure of the host's innate and adaptive immune system, including host physiological responses such as high or low pH, heat shock (i.e. fever), and production of nitric oxide (Lyons et al., 2002). Host cytokines and chemokines such as interferon (IFN)- $\gamma$  and tumor necrosis factor (TNF) may help to control tachyzoite growth or trigger encystment (Suzuki et al., 1989; Johnson, 1992; Gazzinelli et al., 1993; Scharon-Kersten et al., 1996). Tissue cysts (containing slowly dividing bradyzoites) typically form in neural, muscular, and cardiac tissue (Dubey et al., 1988; Petersen and Dubey, 2001; Tenter et al., 2000) and tissue cysts may persist for the life of the host (Frenkel et al., 1970; Dubey and Beattie, 1988). However, if the host becomes immunocompromised, such as humans with clinical AIDS or undergoing immune suppressive therapy, the cysts may break down, releasing bradyzoites which can transform into tachyzoites. With the reactivation of a latent infection, the tachyzoites can reinvade host cells and produce new tissue cysts and severe, even fatal encephalitis (Luft and Remington, 1992; Tenter et al., 2000).

The definitive host may acquire infection via ingestion of any of the 3 infectious stages. As mentioned, the parasite has two cycles in felines: sexual and asexual. For both cycles, the parasite initially replicates asexually in the epithelial cells of the small intestine (Hutchison et al., 1971; Dubey and Frenkel, 1972; Ferguson et al., 1974). Each sporulated oocyst contains 2 sporocysts (without Stieda bodies), each with 4 sporozoites (Dubey et al, 1970a; Ferguson et al., 1979b, 1979c, & 1979d). Following oocyst ingestion, sporozoites excyst

through openings created as plates of the sporocyst wall fold inward along suture lines and then escapes from the oocyst as the oocyst wall itself ruptures following rupture of at least one of the sporocysts (Christie et al., 1978; Ferguson et al., 1979a; Ferguson et al., 1982). The sporozoites or bradyzoites (from tissue cysts) then penetrate the lamina propria of the intestinal epithelium and further multiply as tachyzoites. Tachyzoites then disseminate through out the host's body and eventually form tissue cysts (Dubey and Frenkel, 1972; Dubey and Frenkel, 1976).

In cats, some of the parasites multiplying asexually in the epithelial cells of the intestine develop into gametes. After fertilization of macrogametes (female gametes) by microgametes (male gametes), oocysts are produced as a wall forms around the new zygotes (Dubey and Frenkel, 1972; Ferguson et al., 1975; Dubey and Frenkel, 1976; Speer and Dubey, 2005). The oocyst ruptures from the cell and is expelled into the intestinal lumen. From here, the unsporulated oocyst is passed out into the environment in feces (Hutchinson, 1965; Frenkel et al., 1970; Hutchinson et al., 1970; Sheffield et al., 1970). Unsporulated oocysts are spherical, 10 to 12  $\mu\text{m}$  in diameter and non-infectious (Dubey et al., 1970a; Frenkel et al., 1970). Within 1 to 5 days, in a moist environment, warm temperatures, and aeration, the oocysts will sporulate and become infectious (Dubey et al., 1970a; Frenkel et al., 1970). Sporulated oocysts are spherical to ellipsoidal (11 x 13  $\mu\text{m}$ ), with a specific gravity of 1.05 -1.14 (Dubey, 1970; Dumetre and Darde, 2004). Older and/or degenerated oocysts may have a



greater density than fresh oocysts attributable to modifications of their content or an increase of interactions between the oocyst surface and the surrounding environmental debris (Dumetre and Darde, 2004).

The number of oocysts necessary to infect intermediate and definitive hosts varies with the host species. A single *T. gondii* oocyst can be infective to susceptible pigs, rats, or mice (Dubey, 1996b; Dubey et al., 1996a; Dubey et al., 1997). Ingestion of 100 oocysts can result in infection and oocyst shedding in cats, and result in further contamination of the environment with oocysts (Dubey, 1996a). High environmental burdens of oocysts may increase the likelihood that feline prey species (rodents and birds) will become infected. Predation of infected intermediate hosts by cats can further propagate the cycle of oocyst production, shedding, and environmental contamination (Hay and Hutchison, 1983; Tenter et al., 2000).

Cats are pivotal in oocyst transmission since toxoplasmosis is not found in areas without cats (Wallace, 1973b). Ruiz and Frenkel (1980) found that where cats defecate is a major factor influencing whether humans become infected with oocysts. Risk for transmission of oocysts increased when cats defecate in or around households. This risk is further increased given the persistence of oocysts in the environment (Afonso et al., 2006). However, despite the potential risk for transmission of oocysts from cats, cat ownership has not been a risk factor for contracting toxoplasmosis in individuals infected with HIV (Wallace et al., 1993).

The occurrence and prepatent period of oocyst shedding may also vary with the stage that infected the feline host. While most cats will shed oocysts after ingesting tissue cysts, less than half will shed after the ingestion of tachyzoites or oocysts (Dubey and Frenkel, 1976). The prepatent period is from 3 to 10 days (with tissue cyst ingestion), 19 + days (with tachyzoite ingestion), and 20 + days after ingestion of oocysts (Dubey et al, 1970a; Freyre et al., 1989). Cats can shed oocysts for 7 to 16 days and a single cat can produce  $10^5$  to  $10^8$  oocysts (Frenkel and Dubey, 1972; Dubey, 1996a; Dubey, 2001). Additionally, feline transplacental infections can occur and once born, infected kittens can further excrete oocysts into the environment (Dubey and Carpenter, 1993; Sato et al., 1993).

Once a cat has been infected and shed oocysts, further shedding from reinfection is considered rare (Dubey and Frenkel, 1974). However, recent studies have suggested that lifetime immunity does not persist. In experiments, cats inoculated with *T. gondii* tissue cysts 6 years after an experimentally-induced primary infection with *T. gondii* (in which the cats shed oocysts), were able to shed oocysts a second time (Dubey, 1995). The ability to re-shed may also occur when the cat has a superinfection with other coccidians, such as *Isospora felis* (Chessum, 1972; Dubey, 1976).

### **The Oocyst**

The sporulated oocyst is highly resistant to many environmental conditions, including extreme temperatures, desiccation, and irradiation (reviewed by

Dumetre and Darde, 2003 and Dubey, 2004). First isolated and identified in cat feces in 1970 (Sheffield and Melton, 1970; Frenkel et al., 1970), the oocyst wall is comprised of three distinct layers and the external wall of the oocyst will auto-fluoresce blue when exposed to 330 - 385 nm ultraviolet (UV) light (Speer et al., 1998; Lindquist et al., 2003). The oocyst is reportedly surrounded by a fine reticulate "veil" that is electron-lucent, loosely adheres to oocyst wall, and has an unknown function (Speer, et al, 1998). The sporulated oocyst consists of 3 layers: an electron-dense outer layer (20 - 50 nm thick) which may be removed following exposure to 1.3% sodium hypochlorite; an electron-lucent middle layer (8 - 10 nm thick) which consists of remnants of two membranes that were laid down between the inner and outer layers during wall formation; and a 50 - 90 nm thick lucent to moderately electron-dense inner layer (Speer, et al, 1998).

Oocyst wall formation is initiated by fertilization and the formation of the zygote when a 5-layer wall forms around the pellicle of the gametes (Ferguson et al., 1975; Dubey et al, 1998a). Two different types of wall-forming bodies (WFB) have been observed during the wall formation. The type I WFB is 0.35  $\mu\text{m}$  in diameter, electron dense, and is no longer observed following the formation of layer 4 of the wall. The type II WFB is 1.2  $\mu\text{m}$  in diameter, moderately electron dense, is less abundant than type I WFB, and disappears with the formation of 5<sup>th</sup> layer of the oocyst wall (Ferguson et al., 1975; Ferguson et al., 2000).

The oocyst has a single, small, randomly located micropyle (a 540 - 610 nm-diameter indentation) in the wall. The micropyle layers are continuous with the

3 layers of the oocyst wall except that the outer layer is thin (10 - 20 nm thick) and moderately electron-dense and the inner layer is electron dense, disk-shaped and slightly thicker than the inner layer of the oocyst wall itself (80 - 160 nm Speer, et al, 1998). Upon oocyst ingestion, CO<sub>2</sub> in the host's intestines may compromise the oocyst wall at the site of the micropyle, which leads to oocyst wall rupture. Rupturing allows digestive enzymes such as trypsin and bile salts to enter the oocyst and stimulate the sporozoites to excyst from the sporocyst, transform into tachyzoites and begin invading host tissues (Christie et al., 1978; Ferguson et al., 1979a; Ferguson et al., 1982; Dubey et al., 1998a; Speer et al., 1998).

### **Human toxoplasmosis**

In most animals and humans, infections with *T. gondii* result in a chronic, asymptomatic infection (Dubey and Beattie, 1988; Frenkel, 1990). However, infections can be problematic and severe in high-risk groups that include non-immune pregnant women and immunocompromised individuals. Infections in pregnant women can result in congenital transmission and fetal toxoplasmosis. This can cause abortion, neonatal death, or fetal and newborn abnormalities such as encephalomyelitis, retinochoroiditis, intracranial calcifications, and hydrocephalous (reviewed in Jones et al., 2001 and Petersen, 2007). Surviving infants may suffer from progressive mental retardation, deafness, visual impairments, and other neurological deficiencies (Jones et al., 2001). Reactivation of latent *T. gondii* infections in immunocompromised individuals may result in

encephalitis and disseminated toxoplasmosis (Frenkel, 1990; Luft and Remington, 1992; Montoya and Liesenfeld, 2004).

### **Oocyst transmission to humans**

To date, there have been multiple documented cases of direct or suspected terrestrial and waterborne-transmission of *T. gondii* oocysts to humans. Several reports describe oocyst transmission through ingestion or inhalation of contaminated soil or sand. A 1971 investigation of toxoplasmosis in a mother and son showed that soil and sand samples collected from the family's garden were infectious for mice (Fleck et al, 1972). This case provides evidence of how outdoor activities such as gardening or playing in residential areas could result in accidental oocyst transmission.

A 1977 outbreak of toxoplasmosis due to oocyst inhalation occurred in a riding stable in Atlanta, Georgia, where 37 people became ill with acute toxoplasmosis or were serologically positive for *T. gondii* exposure. Adult cats on the premises were serologically positive for *T. gondii* and both kittens and mice trapped on the stable's property had detectable *T. gondii* cysts in their tissues (Teutsch et al., 1979; Dubey et al, 1981). This outbreak demonstrated that inhalation of oocysts is one possible route for *T. gondii* transmission to humans.

Stagno et al. (1980) reported on 8 cases of acute toxoplasmosis, along with 16 additional individuals seropositive for *T. gondii* exposure in Dothan, Alabama. A family cat and her litter of kittens were suspected as the source of exposure.

Many of those infected were preschool-aged children (8 with a history of geophagia), highlighting the susceptibility of children to orally inoculate themselves with oocysts.

In a report of an outbreak of toxoplasmosis on a dairy farm in Brazil, mice inoculated with soil samples collected from a vegetable garden tested positive for *T. gondii* infection (Coutino et al., 1982). This finding supports the hypothesis that humans can become accidentally infected from handling and/or ingesting oocyst-contaminated soil and that there is a potential risk for oocyst transmission when handling cultivated foods. Individuals handling soil should use gloves and thoroughly wash their hands when done working.

In a boarding school in Turkey, 171 students were seropositive for *T. gondii* following a suspected outbreak of toxoplasmosis in September - October, 2002 (Doganci et al., 2006). No common source of infection was identified. However, there was a large stray cat population near the dining hall and transmission may have been due to contact with oocyst-contaminated cat litter. These data support the need for awareness of potential risks of oocyst transmission and that precautions (like wearing gloves and thorough hand washing) should be used when handling cat feces and cat litter to prevent accidental ingestion or inhalation of oocysts.

The largest documented outbreak of human toxoplasmosis occurred in 1995 in Victoria, British Columbia and resulted from oocyst transmission in municipal

drinking water (Bowie et al., 1997). The drinking water had been treated with chloramines, but was not filtered. Although 100 acute cases of toxoplasmosis were identified, the actual number of asymptomatic, infected individuals was estimated between 2,894 and 7,718 persons. This outbreak illustrates that the true prevalence of *T. gondii* infections in humans is likely underestimated, because most infected individuals remain asymptomatic. Oocyst exposure was believed to have originated from fecal contamination of reservoir water by domestic and feral cats (*Felis catus*), as well as cougars (*Felis concolor*) known to inhabit the watershed.

To evaluate the hypothesis that feces from domestic cats or cougars contaminated the Victoria watershed, Aramini et al. (1999), assessed domestic cats and cougar activity in the area, serologically evaluated these felines for *T. gondii* antibodies and examined fecal samples for the presence of oocysts. Trapped deer mice (*Peromyscus maniculatus*) were also serologically evaluated. With the tested domestic cats, 21.9% were serologically positive for *T. gondii*, but no oocysts were recovered from fecal samples. Only one cougar was captured from the Victoria watershed and it was serologically positive. Oocysts were also recovered from one cougar fecal sample collected from the Victoria watershed. In addition, 10% of the mice had antibodies against *T. gondii* and therefore could serve as a *T. gondii*-infected prey source for cats. The results of this study suggest that an endemic *T. gondii* cycle among wild and domestic animals could have contaminated the Victoria watershed with oocyst-containing feces. Such feline -

*T. gondii* transmission cycles could exist in many watersheds and result in rapid, widespread dispersal of oocysts throughout the environment via surface run-off.

Several other documented cases of toxoplasmosis further illustrate the importance of the waterborne transmission route of *T. gondii* to humans. In 1979, 35 American soldiers became ill with acute toxoplasmosis following a training course in a Panamanian jungle (Benenson, et al., 1982). Evidence suggested that the soldiers became infected by drinking oocyst-contaminated creekwater and indicated that precautions are necessary when drinking any sources of untreated water. Similarly, Hall, et al. (1999), reported that *T. gondii* infections prevalent among Jains, a religious group in India, may be due to ingestion of oocysts in contaminated water. These individuals are strict vegetarians who do not keep cats, do not farm, and meticulously wash the fruits and vegetables they consume. Therefore, direct ingestion of oocysts in water or consumption of produce washed in contaminated water are the most likely modes of transmission. Limited research has been done on oocyst-contaminated food (Kniel et al., 2002), but the Jains example indicates that this potential route needs further investigation.

In a prevalence survey from 1997-1999 for *T. gondii* infections in Brazilians, higher rates of infection were found in lower socioeconomic groups (84%) as compared to middle (63%) and upper socioeconomic (23%) groups. In the lower socioeconomic groups, the majority of households used untreated, unfiltered water and use of unfiltered drinking water was found to be a significant risk



factor for becoming infected with *T. gondii* in that study (Bahia-Oliveira et al., 2003).

In a suspected waterborne outbreak of toxoplasmosis in Brazil over 2001-2002, *T. gondii* was isolated from sources of water suspected of being contaminated. Membrane-filtered water samples were bioassayed in mice, chickens, pigs and cats or analyzed with PCR. The inoculated animals tested positive for *T. gondii* and cats fed water-exposed pig tissue shed oocysts. With PCR, the *T. gondii* B1 gene was amplified from DNA extracted from the membrane filter. The researchers proposed that a domesticated cat and her kittens were living near a reservoir of human drinking water which became contaminated with oocysts (de Moura et al., 2006). This outbreak of toxoplasmosis further reinforces how environmental contamination of oocysts from feline feces can pollute drinking water.

In a study investigating the occurrence of *T. gondii* in drinking water on farms in eastern Poland, 114 water samples were evaluated microscopically and with PCR for the presence of *T. gondii* (Stroka et al., 2006). Oocysts were observed in 15 water samples and *T. gondii* DNA was detected in 31 samples analyzed by PCR, indicating that farm water could be a source of infection to both humans and livestock. The research of both de Moura et al. (2006) and Stroka et al. (2006) demonstrated how molecular tools can be useful in detecting oocysts in water and that further efforts to develop detection methods would be

useful for evaluating environmental samples for the presence of *T. gondii* oocysts. Such information could be used to increase understanding about the natural dissemination of oocysts through ecosystems.

Collectively, these reports suggest that precaution is warranted when ingesting untreated or unfiltered water and that water treatment methods to inactivate or remove *T. gondii* oocysts are needed to ensure the safety of municipal water supplies. In addition, these documented cases of *T. gondii* oocyst transmission to humans highlight the importance of understanding the natural history and transmission of *T. gondii* oocysts through both terrestrial and aquatic environments. The infectious oocyst dose for humans is unknown, but could be as low as a single oocyst, as indicated by animal infectivity studies (Dubey, 1996; Dubey et al., 1996; Dubey et al., 1997). While filtering water is ideal for physical removal of many biological contaminants or pathogens, including *T. gondii* oocysts, it is not always practical or possible. For example, filtration is not always available in rural areas, water treatment facilities serving small communities or with sources of water that do not contain sufficient particulate matter for conventional coagulation and sedimentation processes (Bowie et al., 1997; Betancourt and Rose, 2004; Goh et al., 2005). Therefore, reliable methods to detect or inactivate *T. gondii* oocysts in water are needed.

## ENVIRONMENTAL DISTRIBUTION OF OOCYSTS

Oocysts can be widely dispersed in soil or water, can persist for extended periods, and can be spread by invertebrates, wind, rain, and surface water through terrestrial and aquatic environments (Dubey and Beattie, 1988; Tenter et al, 2000).

### *Toxoplasma gondii* oocysts in soil

In several studies, oocysts have been recovered from soil samples. In 1973, Ruiz et al., highlighted the presence of infective oocysts in yards and gardens in Costa Rica. In this study, soil samples were collected from a suburb that was inhabited by many owned and stray domestic cats. Soil samples collected from shaded or moist sites (20-27°C), where found to be infectious to inoculated mice. In contrast, dry soil samples, with measured soil temperatures of 40-43°C were not infectious to inoculated mice. These findings indicate that cool and moist environments can preserve oocyst viability and suggest that oocysts can persist under these conditions.

Frenkel et al. (1975) first reported the natural persistence of oocysts in soil. Cat feces containing *T. gondii* oocysts were labeled with strontium 85 and then superficially buried (3 - 9 cm deep) to mimic the natural disposal actions of cats in Costa Rica and in Kansas. Oocysts recovered after 51 weeks from the soil of 3 shaded sites (2 areas were moist and 1 area relatively dry with temperature ranges of 15 to 30°C) in Costa Rica remained infectious to mice inoculated with the soil samples. In Kansas, oocysts remained infective to mice following 18

months (including 2 winter periods with temperature ranges of -20 to 35°C) in the environment. These findings reinforce the natural resistance *T. gondii* oocysts to environmental exposure. Oocysts can withstand exposure to extreme temperature variations and remain infective for months to years in soil. The researchers also noted that when humans dig in soil, 7-13 mg of oocyst-contaminated soil can be deposited underneath fingernails and then individuals could potentially orally inoculate themselves with 10-100 oocysts.

Ito et al. (1975) identified that swine can be directly infected by consuming soil naturally contaminated with *T. gondii* oocysts on a hog farm. Ingestion of a single, microscopic *Toxoplasma gondii* oocyst can infect susceptible swine and leads to the formation of parasite cysts in their tissues (Dubey et al., 1996a).

Humans can become infected by eating inadequately cooked pork from infected pigs and the importance of *T. gondii* as a zoonotic, food-borne pathogen is well documented (Jacobs et al., 1960; Choi et al., 1997; Mead et al., 1999). This risk is enhanced in facilities that do not raise their pigs in total confinement and allow cats access to the pigs, feed storage buildings, and water sources (Dubey et al., 1995; Weigel et al., 1995; NAHMS, 2000; Lehmann et al., 2003). To date, since there are no prophylactic methods to prevent wild or domestic cats from shedding oocysts, control efforts must focus on destruction of oocysts in the environment (Dubey, 1996c).

### **Mechanical transmission of *Toxoplasma gondii* oocysts**

Mechanical transmission of *T. gondii* oocysts by animals and invertebrates are also potential modes of oocyst distribution through the terrestrial environment. In transmission experiments, Lindsay et al. (1997) evaluated dogs as potential mechanical oocyst vectors. Viable oocysts were recovered in from feces of dogs fed sporulated *T. gondii* oocysts. The dogs developed positive titers to *T. gondii*, but did not exhibit clinical disease. When nonsporulated *T. gondii* oocysts were placed on dog hair and skin, mice inoculated with dog fur and skin scrapings did not become infected. This suggested that oocysts did not sporulate on the dog's fur, although the researchers concluded that dogs may be involved in mechanical transmission of *T. gondii* to humans.

In a 5-year prospective study examining seroconversion to *T. gondii* in children in Panama, Frenkel et al. (1995) reported that strong relative risk (RR) factors for seroconversion included contact with nursing dogs (RR = 5.8), weaned dogs (RR = 4.7), and 12-month-old dogs (RR = 3.4). The researchers postulated that children could become infected by ingesting oocysts transferred to their hands from petting oocyst-contaminated dogs. Dogs could be contaminated externally by rolling in cat feces. It was also suggested that dogs can disseminate oocysts in their feces following ingestion of oocyst-containing feline feces (coprophagy). Increased risk for seroconversion to *T. gondii* was also found for children in contact with flies (RR = 3.6), weaned cats (RR = 3.0), 6 to 12-month-old cats (RR = 2.7), nursing cats (RR = 2.5), garbage (RR = 2.4), and roaches (RR =

2.2). Since people are not likely to give up their pets, hand washing after contact with cats or dogs may decrease risks of oocyst exposure. Sanitary practices such as cleaning up cat feces and garbage may also decrease the risk of transmission. Garbage is likely to attract pests, such as roaches and flies that may act as oocyst vectors (as described below). Subsequent studies support the findings of Frenkel et al. (1995) by suggesting that *T. gondii* oocysts may be spread through the environment by flies, cockroaches, earthworms, dung beetles, and other coprophagous invertebrates. These invertebrates are commonly found in areas inhabited by people and may serve as a link in the transmission route of oocysts to humans.

In a series of experiments, Wallace tested the ability of filth flies and cockroaches to act as transport hosts for *T. gondii* oocysts. In 1971, Wallace found that filth flies (common house flies, *Musca domestica*, and oriental blow flies, *Chrysoma megacephala*) can contaminate human food with viable oocysts for 1 to 2 days after contact or feeding on cat feces containing oocysts. In addition, *T. gondii* infections were detected in mice fed fly larvae and pupae reared in infectious cat feces, but not in mice fed the newly emerged adult flies. The location of the oocysts in or on the flies was not determined. In 1972, Wallace withheld food from Madeira cockroaches (*Leucophaea maderae*) and American cockroaches (*Periplanta americana*) for 48-72 hours and then gave them access to infectious cat feces. He then fed the cockroach feces and digestive tracts to mice and found that roach feces collected 9-10 days after feeding on contaminated cat

feces were infectious to mice and remained infectious when stored at room temperature for up to 17 days. Subsequent experiments demonstrated that *T. gondii* infections could be detected in mice fed digestive tracts from roaches (*L. maderae*) killed on days 7, 13, and 20, but not from roaches killed on  $\geq 27$  days following exposure to infectious cat feces. Smith and Frenkel (1978) conducted similar experiments with German and American cockroaches (*Blatella germanica* and *P. Americana*, respectively) and that both types of roaches could serve as transport vectors for *T. gondii* oocysts. Overall, these research reports suggested that roaches could potentially spread oocysts via their feces for almost 3 weeks.

Chinchilla and Ruiz (1976), examined the preference of roaches (*L. maderae*, *P. americana*, and *Periplaneta australasiae*) to consume fresh or dried cat feces when also presented with other sources of food, including dough, sugar, bread, and cheese. Both *L. maderae* and *P. australasiae* were found to consume fresh or dried cat feces even when other household foods were available. The results from this experiment also suggest that roaches could directly transfer oocysts derived from feces to human food when scavenging for food.

Ruiz and Frenkel (1980) investigated potential intermediate and transport hosts in San Jose, Costa Rica. Trapped houseflies (*Musca domestica*), German cockroaches (*Blatella gemanica*), Australian cockroaches (*P. australasiae*), Madeira cockroaches (*L. maderae*), and wood roaches (*Eurycotes biolleyi*) from garbage and household areas, and unidentified earthworms collected from residential patios and yards were evaluated for *T. gondii* by mouse bioassay. *Toxoplasma gondii* was

detected in mice inoculated with earthworms, showing that they could also serve as transport hosts.

A potential earthworm-bird-cat cycle was evaluated by Markus (1974) in studies using *Eimeria tenella*. Passage of oocysts through the worm's gut was found not to affect their viability. Following 1 week exposure to soil containing *E. tenella* oocysts, guts and castings from exposed earthworms (*Lumbricus terrestris*) remained infectious to 3-day-old Apollo cockerel chicks.

Research by Bettiol et al. (2000) provided further evidence that worms can be transport host for *T. gondii* oocysts. Eastern barred bandicoots (*Perameles gunnii*) fed earthworms (*Lumbricus rubellus* and *Perioyn excavatus*) exposed to oocyst-contaminated soil died from toxoplasmosis within 14 days post-inoculation. Oocysts in the alimentary tract of the worm appeared to be responsible for the bandicoots' infections based on the observation that bandicoots remained uninfected when fed worms that were first passed through oocyst-containing soil and then subsequently passed through sterile soil. This suggested that these worms become non-infective by excreting oocysts in their castings before being fed to bandicoots.

The role of dung beetles (*Onthophagus* spp.) as transport hosts for *T. gondii* oocysts was examined by Saitoh and Itagaki (1990). They found that oocysts could adhere to the bodies of Dung beetles, were not easily detached, and could be transported by beetles 25 days post-exposure to oocyst-containing feces. Oocyst passage through beetles did not inhibit oocyst sporulation and oocysts



could be detected in the beetles' feces for at least 3 days post-exposure to the contaminated feces. These findings are significant because dung beetles often defecate when immersed in water, thereby contaminating water with oocysts.

### **Transmission on vegetation**

Ingestion of agricultural products irrigated with oocyst-contaminated water may result in oocyst transmission to susceptible hosts (Ortega et al., 1997). Kniel et al. (2002) individually inoculated raspberries and blueberries with  $10^1$  to  $2 \times 10^4$  sporulated *T. gondii* oocysts. Blueberries stored at 4° C for up to 8 weeks remained infectious to mice and mice fed raspberries inoculated with  $\geq 10^1$  oocysts or blueberries inoculated with  $\geq 10^3$  oocysts became infected with *T. gondii*. These experiments demonstrated how oocysts can adhere to food products and therefore, this should be considered a potential route of oocyst transmission. Further studies should be conducted to evaluate patterns of transmission and relative risks of different food products.

### **ENVIRONMENTAL RESISTANCE OF OOCYSTS**

A major contributing factor to the environmental dispersal of *T. gondii* oocysts is the ability of oocysts to remain viable following exposure to many physical and chemical elements, including extreme temperatures, dessication, disinfectants, and irradiation.

#### **Temperature exposure**

Several studies have demonstrated the resistance of *T. gondii* oocysts to inactivation following exposure to extreme temperatures, including the ability of

oocysts to survive temperatures ranging from  $-20^{\circ}\text{C}$  to  $55^{\circ}\text{C}$  under experimental condition. Hutchison (1967) first reported that oocysts maintained in tap water at room temperature ( $20\text{-}22^{\circ}\text{C}$ ) remained infective to inoculated mice. Dubey et al. (1970b) used the mouse bioassay to show that oocysts in water were inactivated upon exposure to  $55^{\circ}\text{C}$  for 30 minutes, but not with 30 minute exposures to 37, 40, 45, and  $50^{\circ}\text{C}$ .

Yilmaz and Hopkins (1972) evaluated oocyst resistance by placing paired oocyst preparations in covered or uncovered Petri dishes at different temperatures. Oocysts were then exposed to  $4^{\circ}\text{C}$ ,  $22.5^{\circ}\text{C}$ , or  $37^{\circ}\text{C}$  under indoor settings, as well as to sunny (temperature range of  $6\text{-}36^{\circ}\text{C}$ ) or shady (temperature range of  $5.5\text{-}35.5^{\circ}$ ) outdoor settings. At  $4^{\circ}\text{C}$ , oocysts kept either uncovered or covered for up to 410 days remained infective to inoculated mice. When left at room temperature ( $22.5^{\circ}\text{C}$ ), uncovered oocysts maintained infectivity for up to 306 days while covered oocysts held for 410 days were infective to mice. Under  $37^{\circ}\text{C}$ , uncovered oocysts remained infective following 91 days of heat exposure, while covered oocysts remained infective for up to 306 days. In outdoor sunny conditions, uncovered oocysts survived up to 122 days of exposure while covered oocysts remained viable following 306 days of exposure. With outdoor shady conditions, uncovered oocysts lasted up to 153 days, while covered oocysts remained viable following 410 days of exposure. These laboratory experiments support the findings that oocysts have increased survival in cool, shaded, and moist conditions in natural environments as previously described

(Ruiz et al., 1973; Frenkel et al., 1975). In addition, researchers found that the oocysts in water (particularly for samples left uncovered) retained greater resistance to environmental exposure when compared to oocysts mixed with fecal matter (oocysts in water maintained viability 50% to 67% longer). The higher resistance of oocysts in water is an important finding, because it indicates oocysts in aquatic environments may remain infectious longer than oocysts in terrestrial environments and that increased energy or longer exposure times may be necessary to inactivate oocysts in water.

Frenkel and Dubey (1972) evaluated the effect of boiling water on oocyst viability. Sporulated oocysts mixed with feline feces were treated with either 75°C or 85°C boiling water and allowed to cool for 1 hour before being inoculated into mice. Mice fed feces exposed to either 75°C or 85°C did not become infected with *T. gondii* suggesting that heat treatment of at least 75°C can inactivate *T. gondii* oocysts.

In another set of experiments, Frenkel and Dubey (1973) found that both sporulated and unsporulated oocysts were more resistant to low, fluctuating temperatures than those exposed to constant low temperatures. In addition, unsporulated oocysts were more readily inactivated following exposure to low temperatures than were sporulated oocysts. Either 5,000 sporulated or unsporulated oocysts were mixed into feline feces, exposed to -6 or -20°C, and then inoculated into mice. Prior to mouse inoculation, unsporulated oocysts were allowed to sporulate at room temperature for 1 week. Sporulated oocysts

could withstand exposure to  $-20^{\circ}\text{C}$  for 28 days, while unsporulated oocysts remained infective following exposure to  $-6^{\circ}\text{C}$  for 7 days. Collectively, these results suggest that unsporulated oocysts are less resistant to environmental extremes and that the process of sporulation facilitates resistance. Further studies are needed to compare the wall structure of unsporulated to sporulated oocysts to try to determine the biochemical factor that results in these differences in resistance.

Ito et al. (1975) examined the effect of heat on the sporulation rate of unsporulated oocysts and the viability of sporulated oocysts. Following heat treatment, unsporulated oocysts were allowed to sporulate for 72 hours at  $25^{\circ}\text{C}$ . Oocysts were then examined for sporulation and a sporulation rate was determined. Sporulation was inhibited following heat exposure of  $70$  or  $60^{\circ}\text{C}$  for 10 seconds,  $55^{\circ}\text{C}$  for 30 seconds,  $50^{\circ}\text{C}$  for 2.5 minutes,  $45^{\circ}\text{C}$  for 1 hour, and  $37^{\circ}\text{C}$  for 48 hours. With sporulated oocysts, oocysts remained infectious to inoculated mice following heat treatment of  $50^{\circ}\text{C}$  for 25 minutes,  $55$  or  $60^{\circ}\text{C}$  for 10 minutes, and  $70^{\circ}\text{C}$  for 1 minute. However, oocysts were inactivated by  $50^{\circ}\text{C}$  for 30 minutes,  $55$  or  $60^{\circ}\text{C}$  for 15 minutes,  $70^{\circ}\text{C}$  for 2 minutes,  $80^{\circ}\text{C}$  for 1 minute, and  $90^{\circ}\text{C}$  for 30 seconds. These results suggest that boiling water ( $100^{\circ}\text{C}$ ) should rapidly ( $< 30$  seconds) inactivate *T. gondii* oocysts. However, these experiments were likely conducted using small amounts of water (exact volume not indicated). If heat treatment is to be practically applied to inactivate *T. gondii* oocysts in water, treatment of larger volumes should also be evaluated.

Kuticic and Wikerhauser (1994) exposed sporulated oocysts in water to either 58°C for 15 and 30 minutes or to -20°C for 2 or 3 weeks. Oocysts were inactivated following heating to 58°C for 15 or 30 minutes, which is in agreement with the results of Ito et al., 1975. Kuticic and Wikerhauser also found that oocysts remained infective after 2 weeks of exposure to -20°C, but were inactivated following 3 weeks of exposure. However, Frenkel and Dubey (1973), reported oocyst inactivation following 28 days of exposure to -20°C; this discrepancy may be attributable to differences in the analytical sensitivity of the methods used to detect *T. gondii* infections in mice. Frenkel and Dubey (1973) used Giemsa-stained impressions of lymph nodes, lungs, and brain samples and serology, while Kuticic and Wikerhauser (1994) used microscopic examination of mouse brains for the presence of tissue cysts. This finding underscores an important concept: difficulties often arise when comparing results and efficacy of inactivation treatments based on different published reports, because there is no gold standard for confirmation of *T. gondii* infections using mouse bioassay. The sensitivity and specificity of different detection methods must be considered to more effectively evaluate published findings.

In a series of experiments, Dubey (1998) evaluated effects of temperatures ranging from -10°C to 70°C on oocyst survivability. Oocysts did not lose infectivity following exposure to -10°C or -5°C for 106 days, 0°C for 13 months, 4°C for 1620 days, 10°C and 15°C for 200+ days, 30°C for 107 days, 35°C for 32 days, 40°C for 9 days, 45°C for 1 day, 50°C for 60 minutes, 52°C for 5 minutes, and

55°C for 1 minute. In contrast, inactivation occurred following heat treatment of 35°C for 62 days, 40°C for 28 days, 45°C for 2 days, 50°C for 120 minutes, 55°C for 2 minutes, and 60°C for 1 minute. These are the most successful findings of oocyst inactivation by heat treatment to date and suggest that *T. gondii* oocysts may be more sensitive to heat inactivation than previously reported. Heating water at 55°C for 2 minutes or 60°C for 1 minute may have an application in drinking water treatment. Studies on heat treatment of larger volumes of water, such as might be used for practical disinfection of drinking water are indicated.

Lindsay et al. (2002) examined the ability of unsporulated oocysts to survive at 4°C. Cat feces containing unsporulated oocysts were held for up to 11 weeks at 4°C. Sub-samples of the feces were collected weekly at weeks 6-11 and 200 oocysts were microscopically evaluated for initial sporulation. The samples were then held at room temperature for 1 week to allow for any further sporulation and then re-examined. They found that oocysts did not sporulate while under the 4°C condition, but sporulated at room temperature after being held up to 11 weeks at 4°C and remained infectious to mice. In spite of prior work suggesting decreased resistance of unsporulated oocysts to environmental exposures (Frenkel and Dubey, 1973), these data suggest that unsporulated oocysts shed by felines near water could remain viable for almost 3 months in aquatic environments while being dispersed via waterways through the ecosystem. Survivability may even be enhanced in water, given Yilmaz and Hopkin's (1972)

previous findings that oocysts had a higher resistance to environmental exposures when in water versus mixed into cat feces.

### **Drying**

The effect of relative humidity and desiccation on the viability of oocysts has been previously assessed. Dubey et al. (1970b) reported that sporulated oocysts held at room temperature remained viable for 30 days at 100% relative humidity, but appeared to be nonviable after being held at both 80.5 and 58.3% relative humidity for 30 days. At 18.8% and 37.1% relative humidity, oocysts maintained viability for 3 and 7 days, respectively. In another experiment, oocysts in 0.10 ml of water were placed in a beaker and the water was allowed to evaporate over 24 hours at room temperature. These oocysts were non-infectious to mice. Frenkel and Dubey (1972) found that oocysts at 22°C to 26°C maintained infectivity (as determined by mouse bioassay) for 11 days following exposure to 37% or 58% relative humidity and also remained infective for 18 days at 80% relative humidity. In 1994, Kuticic and Wikerhauser placed sporulated oocysts mixed in water onto filter paper in Petri dishes and left the samples uncovered at 21°C (with relative humidity of 60-68%) and 23°C (relative humidity of 30-42%). Between weeks 3 - 7, oocysts were collected weekly from the paper strips for mouse inoculation. All mice were found to be infected with *T. gondii* 6 weeks post inoculation with treated oocysts indicating that oocysts could remain infective for at least 7 weeks of exposure to relative humidity of 30-42%. These reports support that *T. gondii* oocyst viability is preserved longer in moist

environments, such as buried in moist soil or in water. Despite high resistance to many environmental factors, oocysts are prone to desiccation. While desiccation may be a treatment option in dry environments, this is not a viable method for inactivation of *T. gondii* oocysts in water. However, these data suggest that the oocyst wall is semi-permeable because water molecules are moving across the oocyst wall. Therefore, potential water disinfection methods might include use of compounds that could permeate the oocyst wall and inactivate sporozoites.

### **Disinfectants**

*Toxoplasma gondii* oocysts have been shown to have a remarkably high resistance to chemical inactivation. In a series of chemical exposure experiments, Dubey et al. (1970b) treated oocysts mixed with feline feces with various chemicals and assessed inactivation by mouse bioassay. As listed in Table I, oocysts were inactivated with exposure to sulfuric acid (63%) + dichromate (7%) for 24 hours, ethanol (95%) + acetic acid (5%) for 24 hours, and 5% ammonium hydroxide for 30 minutes. Frenkel and Dubey (1972) reported that oocysts were no longer viable following treatment with ammonia for 3 hours, 2% iodine for 3 hours, and 7% iodine for 10 minutes (Table 1).

In 1975, Ito et al., tested the efficacy of several chemicals to inhibit sporulation of unsporulated oocysts and to inactivate sporulated oocysts (Table I). Sporulation was completely inhibited by treatment with 1% Lomasept (coccidiocidal disinfectant developed for the poultry industry) for 15 minutes, 5% Lomasept for 5 minutes, and 1% Neo Kurehasol (another coccidiocidal



disinfectant for the poultry industry) for 120 minutes. Kuticic and Wikerhauser (1993a and 1993b) also tested the ability of various chemicals to inactivate sporulated oocysts (Table I).

In a recent study, Dumetre et al. (2008) evaluated ozone as a potential disinfectant to treat drinking water and found that *T. gondii* oocysts could not be inactivated following an exposure of up to 9.4 mg X min/L. In comparison, *C. parvum* oocysts could be inactivated following exposure to 3 or 4 mg X 1 min/L of ozone (Finch et al., 1993). However, *C. parvum* oocysts are chlorine-resistant. These data suggest that *T. gondii* oocysts are more difficult to inactivate than *C. parvum* oocysts and are likely to also be resistant to chlorination.

Of the 42 different chemicals and concentrations described above (Table I), *T. gondii* oocysts were sensitive to 16 chemicals, including 10% formalin, 99% ethanol, methanol, and ammonia. However, contact times required for inactivation were as long as 96 hours (10% formalin). Such extended contact times are not practical for chemical disinfection of drinking water. The shortest required contact time was 10 minutes for 7% iodine, but this concentration is for treatment of potable water. Commercial iodine tablets (8mg/L iodine) are ineffective at inactivating *Cryptosporidium* oocysts and *Giardia* cysts (Gerba et al., 1997; Butkas et al., 2005), and would probably be equally ineffective in inactivating *T. gondii* oocysts. The *C. parvum* oocyst wall is comprised of 2 to 3 layers, ranging from 40 to 49.7 nm in total width (Reduker et al., 1985; Harris and Petry, 1999) while the *T. gondii* oocyst is comprised of 3 layers that are

approximately 100 nm wide (Speer et al., 1998). In addition to having a thicker oocyst wall, *T. gondii* also has an internal sporocyst wall surrounding the sporozoites that is not present in *C. parvum* (Dubey et al., 1998a; Harris and Petry, 1999). Both of these characteristics may confer additional protection to *T. gondii* sporozoites. Water disinfection using chemicals that have been shown to inactivate *T. gondii* oocysts in the laboratory (Table 1) are unlikely to be practical or safe to apply to treat large volumes of drinking water, in part because the removal or inactivation of these chemicals post-treatment would be cost prohibitive and technically difficult.

### **Salinity**

Lindsay et al. (2003) examined the effects of salinity on oocyst sporulation and survival. Unsporulated oocysts were held up to 3 days in either 2% sulfuric acid or 15 ppt (parts per thousand) or 32 ppt artificial seawater at 24°C and examined daily for sporulation. Sub-samples were also inoculated into mice and all inoculated animals died from acute toxoplasmosis. In a second experiment, sporulated oocysts were stored in 15 ppt seawater at 4°C or room temperature for 1, 2, 3, or 6 months and then inoculated into mice; all inoculated mice became infected with *T. gondii*. The ability of *T. gondii* oocysts to survive in seawater for prolonged periods greatly facilitates dissemination through different waterway environments. For example, oocysts shed by felines into freshwater rivers could remain viable when dispersed into estuarine or seawater locations. This

increased resistance likely enhances the probability of transmission to marine species and broadens the range of available hosts.

### **High Pressure**

High pressure processing (HPP) was evaluated for efficacy to inactivate oocysts (Lindsay et al., 2005). Oocysts were treated with 0, 100, 140, 200, 270, 340, 400, 480, and 550 MPa for 1 minute and then orally-inoculated into mice. Results indicated that pressures of at least 340 MPa can inactivate oocysts. While not a practical method to disinfect water, HPP has been shown to inactivate *C. parvum* oocysts in apple and orange juice (Slifko et al., 2000) and may have application in treating oocyst-contaminated produce (Kniel et al., 2002) or treat shellfish, which are known to bioaccumulate *T. gondii* oocysts (Lindsay et al., 2001; Arkush et al., 2003; Miller et al., in press).

### **Irradiation resistance**

*Toxoplasma gondii* oocysts have been shown to resist gamma irradiation exposure (Dubey et al., 1996b and 1996c). Sporulated oocysts were exposed to a range of emitted doses (0.10, 0.15, 0.20, 0.25, 0.30, 0.40, or 0.50 kGy) at room temperature or the same dose range with the absorbed dose measured at 5°C, using a  $^{137}\text{Cs}$   $\gamma$ -irradiation source. Following oocyst treatment, viability was assessed by mouse and cat bioassays. Tissue cysts could not be observed microscopically in mice inoculated with oocysts irradiated with  $\geq 0.25$  kGy or detected in sub-inoculated mice. However, *T. gondii* specific antibodies were detected in at least 25% of inoculated mice, indicative of activation of the

humoral response by tachyzoites. This suggests that irradiated but viable sporozoites were able to excyst and replicate and therefore, were not completely inactivated by irradiation. The lack of visible tissue cysts in these animals may be influenced by the level of parasitemia and host immune response: fewer parasites survive to encyst in animals with an effective and rapid immune response (Brown and McLeod, 1990). In the cat bioassay, oocysts were not detected in cat feces, cats did not develop any *T. gondii* antibodies, and *T. gondii* was not isolated from any cat tissues. This supports the conclusion that tissue cysts did not form in mice inoculated with oocysts irradiated with  $\geq 0.25$  kGy; as ingestion of a single bradyzoite can result in oocyst shedding in cats (Dubey, 2001). Mice were also pre-inoculated with irradiated oocysts, then challenged with lethal doses of non-treated oocysts; 71% of mice inoculated with oocysts irradiated at 0.20 or 0.40 kGy, then given challenge doses of  $10^4$  or  $10^5$  oocysts died from toxoplasmosis. No mice challenged with  $10^3$  oocysts died. However, all mice orally inoculated with 100 non-irradiated oocysts (and treated with sulfadiazine), then challenged 6 months later with  $10^3$ - $10^5$  non-irradiated oocysts survived, but were positive for *T. gondii*. The results suggest that: 1) vaccinating with irradiated oocysts provides only partial protection against subsequent *T. gondii* exposures; 2) a more vigorous immune response occurred following inoculation with non-irradiated oocysts when compared to irradiated oocysts. Mice that were pre-exposed to untreated oocysts (with concurrent sulfadiazine administration) all survived, while most mice vaccinated with irradiated oocysts

died from toxoplasmosis following oocyst challenge. Lund et al. (1961) reported that *T. gondii* tachyzoites irradiated at 0.30 kGy did not multiply following penetration into host cells. This lack of replicating parasites may have resulted in a sub-optimal host immune response.

Dubey et al. (1998b), orally vaccinated 2-3 month old pigs with  $10^5$  sporulated oocysts treated with 0.3 or 0.4  $^{137}\text{C}$  gamma irradiation at  $5^\circ\text{C}$ . Twelve pigs were vaccinated once with treated  $10^5$  oocysts and an additional 11 pigs were vaccinated twice. After 11-13 weeks, these pigs and non-exposed controls were challenged with either high ( $10^5$  or  $10^6$ ) or low ( $10^2$  or  $10^3$ ) doses of non-irradiated sporulated oocysts. All control pigs inoculated with high doses of oocysts were clinically ill by 5 days post-inoculation. Vaccinated pigs challenged with high or low oocyst doses and control pigs exposed to low oocyst doses, remained clinically normal. Mouse bioassay performed using tissues from these pigs revealed that vaccination of pigs either once or twice did not prevent tissue cyst formation. In addition, both cats fed vaccinated pig tissue and those fed tissues from control pigs shed oocysts. However, fewer tissue cysts were observed in mice inoculated with challenged, vaccinated pig tissues, as compared to mice inoculated with challenged, control pig tissue. These data suggest that vaccination may decrease the density of tissue cysts in pig tissues, but does not adequately prime the immune response to provide complete immunity against challenge with competent *T. gondii* oocysts.

To summarize, these vaccination studies (Dubey et al., 1996b, 1996c, 1998b) suggest that while irradiation may have a negative effect on sporozoite infectivity, it is not 100% effective at inactivating oocysts. Future experiments should evaluate the effects of UV irradiation on oocyst viability, since UV treatment could be useful for water disinfection.

Dubey et al. (1998c) examined the effects of gamma irradiation on unsporulated and sporulated oocysts and its applicability for inactivation of oocysts on fruits and vegetables. In a 1<sup>st</sup> series of experiments, sporulated oocysts were irradiated with either 0.4 kGy or 0.5 kGy of <sup>137</sup>Cs gamma irradiation and then fed to mice. Using transmission electron microscopy (TEM), morphologically-normal *T. gondii* sporozoites were observed in the mesenteric lymph nodes, spleen, and intestinal tissues of mice up to 5 days post-inoculation (p.i.) of oocysts exposed to 0.5 kGy, but parasite numbers declined by 24 hours p.i. No parasites were observed at day 7 p.i. on TEM or histopathology for mice fed oocysts irradiated at 0.5 kGy. Thus, while the sporozoites were able to excyst and penetrate cells, they could not multiply sufficiently to sustain *T. gondii* infection. These data support prior reports that the tissue cyst burden is decreased or non-detectable in mice fed irradiated oocysts (Dubey et al, 1996b; 1996c; 1998b). In a 2<sup>nd</sup> series of experiments, the effects of irradiation on unsporulated and sporulated oocysts were compared. Unsporulated oocysts were treated with 0, 0.2, 0.4, 0.6, and 0.8 kGy irradiation, allowed to sporulate for 30 days at room temperature, and then evaluated microscopically for sporulation

and by mouse bioassay for infectivity. Oocysts sporulated following irradiation treatment of 0.8 kGy, but the percentages of sporulated oocysts were inversely proportional to the dosage of irradiation. Morphologically abnormal oocysts were observed at the higher irradiation doses and infective sporozoites did not develop in oocysts irradiated with doses of 0.35, 0.4, 0.6, and 0.8 kGy, as demonstrated by negative mouse bioassay. These findings demonstrate that unsporulated oocysts are more susceptible to inactivation than are sporulated oocysts, and that these inactivation treatments would be most effective for unsporulated oocysts. However, unsporulated oocysts are not ideal targets, because oocysts readily sporulate in the environment. In a final set of experiments, the efficacy of 0.40 kGy of irradiation to inactivate sporulated oocysts on raspberries or in water was assessed. Sporulated oocysts were either sprayed on or inoculated into the berries, irradiated, then processed for mouse inoculation. *Toxoplasma gondii* infections were not observed in mice inoculated with berry-oocyst mixtures or sporulated oocysts in water irradiated at 0.4 kGy. However, 1 of 5 mice inoculated with sporulated oocysts in water irradiated at 0.35 kGy was infected, suggesting that irradiation treatment is not 100% effective. In addition, *T. gondii* antibodies were detected in 15 of 20 mice fed the berry-oocyst mixture irradiated at 0.40 kGy, and in all mice inoculated with oocysts in water irradiated at 0.35 or 0.40 kGy, suggesting that irradiated sporozoites can elicit an immune response, but do not progress to a persistent *T. gondii* infection.

Since using a sensitive method to detect oocyst viability that does not require use of live animals is idea, study of other possible detection methods, such as excystation ratios is warranted. Dumetre et al. (2008) reported a 4-log reduction in sporozoite excystation when *T. gondii* oocysts were exposed to 20 or 40 mJ/cm<sup>2</sup> of irradiation. However, evaluating excystation ratios to assess oocyst viability may not be as sensitive as mouse bioassay. In this report, *T. gondii* oocysts appeared to be inactivated with exposure to 40 mJ/cm<sup>2</sup> of irradiation. However, irradiation was not 100% efficient, even at 40 mJ/cm<sup>2</sup>, because 1 mouse inoculated with 50,000 irradiated oocysts was positive for toxoplasmosis. These data demonstrate a log-reduction of infectivity, but not complete oocyst inactivation. Given the potential for UV irradiation to inactivate *T. gondii* oocysts in water, further evaluation of this potential disinfectant method is of interest.

#### **DETECTION OF OOCYSTS IN THE ENVIRONMENT**

Unlike the related enteric protozoan *C. parvum*, there are no assays for rapid detection of *T. gondii* oocysts in water (Rose et al., 1989; Anusz et al., 1990; McCuin et al, 2001; Sturbaum et al., 2002). Proposed methods to improve detection include oocyst concentration by filtration, centrifugation, and flocculation (Issac-Renton et al., 1998; Kourenti et al., 2003). However, if oocyst numbers are low, these approaches are inefficient, costly, and relatively insensitive. Were et al. (1999), proposed using guinea pigs as preferred bioassay models since they are infected with low doses of *T. gondii* oocysts and can tolerate larger doses of test substrate when compared to mice. Oocysts have



rarely been recovered from environmental samples due to a lack of analytically sensitive methods for detection. To date, oocysts have only once been recovered from naturally contaminated water (Sroka et al., 2006).

Following an outbreak of human toxoplasmosis in Victoria, British Columbia, Issac-Renton et al. (1998) attempted to recover oocysts from the likely source of this outbreak (Humpback Reservoir). Large volumes of water (1,000 liters) were filtered with 1- $\mu$ m pore size filter cartridges and contents were collected, aerated to allow for oocyst sporulation, and inoculated orally into mice. No mice died and all remained seronegative for *T. gondii* by MAT, highlighting the difficulty of recovering *T. gondii* oocysts from environmental samples.

Kourenti et al. (2003) evaluated 2 concentration methods (flocculation and centrifugation) to recover sporulated and unsporulated oocysts from non-turbid water samples seeded with  $10^4$  or  $10^5$  oocysts. Oocysts were sedimented and concentrated with either of two flocculants (ferric sulfate or aluminum sulfate), or concentrated at different g values using centrifugation. Recovered oocysts were counted using phase-contrast microscopy. Oocysts recovered by centrifugation were assessed for viability via mouse bioassay and were infectious to mice; oocysts recovered by flocculation were not assessed for viability. Higher numbers of sporulated oocysts were recovered using aluminum sulfate (96.5% recovery) compared to ferric sulfate (93.1% recovery) or centrifugation (at 2,073 g; 82.5% recovery). Similar results were obtained for unsporulated oocysts, with

more oocysts recovered using ferric sulfate (100.3%) compared to aluminum sulfate (90.4%) or centrifugation (at 2,565 g; 97.2%). Further experiments are needed to assess recovery rates when there are few (such as 1- 100 oocysts) oocysts in water samples, such as would be expected in a natural setting.

Villena et al., 2004, used a 3 step process to detect *T. gondii* oocysts in water:

1) concentration and filtration, 2) elution and purification using density gradients, and 3) detection by PCR or mouse bioassay. Over a 20 month period, 139 water samples were evaluated, including deionized water (100 L samples seeded with 100 or 1000 sporulated oocysts), public drinking water (100 L samples seeded with  $10^2$ ,  $10^3$ , or  $10^4$  sporulated oocysts), and untreated surface water (40 L samples seeded with  $10^2$ ,  $10^3$ , or  $10^4$  sporulated oocysts). Following the concentration and filtration procedures, particulate matter was eluted from filters with detergents (Tween 80 and Laureht-12) to break hydrophobic bonds between the oocyst and the filter, thus increasing oocyst recovery rates. The eluates were purified using a sucrose gradient and the final product was evaluated with real time-PCR (targeting the *T. gondii* B1 gene) or by oral inoculation of mice. Results indicated that PCR was more sensitive for oocyst detection than was mouse bioassay. This suggests that PCR can serve as a rapid technique to survey water for the presence (but not viability) of *T. gondii* oocysts.

Kourenti et al. (2004) also evaluated PCR for oocyst detection in water.

Deionized and tap water were spiked with  $10^5$  purified sporulated or unsporulated oocysts. Prior to DNA extraction, the spiked water samples were

divided into 5 treatment groups: 1) centrifugation in distilled water; 2) centrifugation in tap water; 3) ferric sulfate flocculation; 4) aluminum sulfate flocculation; 5) no treatment (controls). For PCR, both universal and specific primers for 18s rRNA were used. PCR results were positive for all treatment groups except ferric sulfate flocculation, suggesting that ferric ions might inhibit PCR or induce DNA base damage. A theoretical detection limit of 0.1 oocysts was reported; however, this theoretical limit corresponds to detection of DNA from 0.1 oocysts, amplified from a pool of DNA extracted from an inoculum of thousands of oocysts. Therefore, the assay's analytical sensitivity may be much lower if the water sample contains only a few oocysts. Thus, these experiments should be repeated to determine detection limits when initial water samples contain  $10^0$  - $10^4$  oocysts.

For the recovery of *C. parvum* oocysts from environmental samples, the Environmental Protection Agency (2001) recommends the combined use of filtration, immunomagnetic separation (IMS), and direct fluorescent antibody (DFA) detection. Both IMS and DFA require use of monoclonal antibodies against intact oocysts. *Toxoplasma gondii* oocysts could also be separated from environmental debris by IMS cell sorting, then detected by DFA or PCR (das Gracas et al., 1999; Sturbaum et al., 2002; Dumetre and Darde, 2003; Dumetre and Darde, 2005). However, no monoclonal antibodies have been developed against the external oocyst wall of intact *T. gondii* oocysts. Antigens have only been described for the internal oocyst wall, sporozoites, and tachyzoites (Kasper et al.,

1983, 1984, 1985; Kasper, 1989; Everson et al., 2002; Dumetre and Darde, 2005).

Although the external oocyst wall layer may have low or no antigenicity, further studies on outer wall antigenic composition are needed (Dumetre and Darde, 2003). A monoclonal antibody against antigens on the intact oocyst wall will allow for concentration and rapid detection of oocysts, thereby providing a powerful tool for environmental surveillance.

## CONCLUSION

*Toxoplasma gondii* is gaining recognition as a waterborne pathogen (Slifko, et al., 2000; Dubey, 2004; Fayer et al, 2004), but at present there are no Environmental Protection Agency recommendations for methods to detect or destroy *T. gondii* oocysts in water sources. Research in this area has been limited and oocysts have been demonstrated to be highly resistant to most widely used chemical and physical disinfectants (Dumetre and Darde 2003; Dubey 2004). Given the public health risk for *T. gondii* infections, on-going studies to evaluate the efficacy of various disinfectant methods to treat oocyst-contaminated water are warranted. The purpose of the work described in this doctoral thesis was to evaluate potential chemical and physical methods to inactivate *T. gondii* oocysts in water. In chapter 2, an evaluation of the efficacy of sodium hypochlorite and ozone to inactivate *T. gondii* oocysts is presented. In addition, the analytical sensitivity of 3 detection methods: serology, immunohistochemistry, and *in vitro* parasite isolation for the detection of *T. gondii* infection in oocyst-inoculated mice are compared. Chapter 3 describes the results of experiments to assess the ability

of pulsed and continuous ultraviolet (UV) radiation to inactivate *T. gondii* oocysts. Finally, in Chapter 4, results of studies to determine the efficacy of thermal heating via radiofrequency to inactivate *T. gondii* oocysts are presented.

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Table I. Disinfectant Treatment of *Toxoplasma gondii* Oocysts

Chemical	Exposure time	Inactivation	Evaluation of Mouse Bioassay	Reference
33% Aldesol (5 g benzalchoniumchloride, 6 g glutaraldehyde, and 8 g gloxal in 100 g of solution)	24 hours	No	brain tissue cyst detection	Kuticic & Wikerhauser, 1993a
0.1% ammonia water	60 minutes	No	mortality rate; brain tissue cyst detection	Ito et al., 1975
1.0%	60 minutes	No		
10%	30 minutes	Yes		
28%	30 minutes	Yes		
Ammonia	60 minutes 3 hours	No Yes	determination of mean day of death; serology; detection of brain tissue cysts	Frenkel & Dubey, 1972
5% ammonium hydroxide	10 minutes 30 minutes	No Yes	giemsa staining; acid-Schiff hematoxylin staining; serology; subinoculation in mice	Dubey et al., 1970b
0.5% ammonium sulfide	30 minutes 60 minutes	No Yes	mortality rate; brain tissue cyst detection	Ito et al., 1975
<i>n</i> -Butyl alcohol	48 hours	No	mortality rate; brain tissue cyst detection	Ito et al., 1975
3% carbolic acid	48 hours	No	mortality rate; brain tissue cyst detection	Ito et al., 1975

0.1% cetyl trimethyl ammonium	24 hours	No	giemsa staining; acid-Schiff hematoxylin staining; serology; subinoculation in mice	Dubey et al., 1970b
75% ethanol	1 hour 2 hours 4 hours 24 hours	No No No No	brain tissue cyst detection	Kuticic & Wikerhauser, 1993b
95% ethanol	1 hour 2 hours 4 hours 24 hours	No No No No	brain tissue cyst detection	Kuticic & Wikerhauser, 1993b
99% ethanol	24 hours	Yes	mortality rate; brain tissue cyst detection	Ito et al., 1975
Ethanol (95%) + acetic acid (5%)	60 minutes 24 hours	No Yes	giemsa staining; acid-Schiff hematoxylin staining; serology; subinoculation in mice	Dubey et al., 1970b
1% formalin	48 hours	No	mortality rate; brain tissue cyst detection	Ito et al., 1975
10% formalin	96 hours	Yes	mortality rate; brain tissue cyst detection	Ito et al., 1975
10% formalin	1 hour 2 hours 4 hours 24 hours	No No No No	brain tissue cyst detection	Kuticic & Wikerhauser, 1993b
0.5% hibitane solution	48 hours	No	mortality rate; brain tissue cyst detection	Ito et al., 1975
3% hydrogen peroxide	48 hours	No	mortality rate; brain tissue cyst detection	Ito et al., 1975



2% iodine	10 minutes 3 hours	No Yes	determination of mean day of death; serology; detection of brain tissue cysts	Frenkel & Dubey, 1972
7% iodine	10 minutes	Yes	determination of mean day of death; serology; detection of brain tissue cysts	Frenkel & Dubey, 1972
Isoamyl alcohol	48 hours	No	mortality rate; brain tissue cyst detection	Ito et al., 1975
0.02% Izosan-G	24 hours	No	brain tissue cyst detection	Kuticic & Wikerhauser, 1993a
1% Lomasept	60 minutes 3 hours	No Yes	mortality rate; brain tissue cyst detection	Ito et al., 1975
3% Lomasept	60 minutes 3 hours	No Yes	mortality rate; brain tissue cyst detection	Ito et al., 1975
5% Lomasept	60 minutes 3 hours	No Yes	mortality rate; brain tissue cyst detection	Ito et al., 1975
100% Methanol	6 hours 12 hours	No Yes	mortality rate; brain tissue cyst detection	Ito et al., 1975
100% Methanol	1 hour 2 hours 4 hours 24 hours	No No No No	brain tissue cyst detection	Kuticic & Wikerhauser, 1993b
1% Neo Kurehasol	3 hours	Yes	mortality rate; brain tissue cyst detection	Ito et al., 1975
5% Neo Kurehasol	24 hours	No	mortality rate; brain tissue cyst detection	Ito et al., 1975
Ozone	Contact time = 9.4 mg x min/L	No	sporozoite excystment evaluation	Dumetre et al., 2008

5% Peracetic acid	24 hours 48 hours	No Yes	mortality rate; brain tissue cyst detection	Ito et al., 1975
<i>n</i> -Propyl alcohol	48 hours	No	mortality rate; brain tissue cyst detection	Ito et al., 1975
3% saponate cresol solution	48 hours	No	mortality rate; brain tissue cyst detection	Ito et al., 1975
6% Sodium hypochlorite	24 hours	No	giemsa staining; acid-Schiff hematoxylin staining; serology; subinoculation in mice	Dubey et al., 1970b
1 N sodium hydroxide solution	48 hours	No	mortality rate; brain tissue cyst detection	Ito et al., 1975
0.1% sodium lauryl sulfate	24 hours	No	giemsa staining; acid-Schiff hematoxylin staining; serology; subinoculation in mice	Dubey et al., 1970b
0.1% sublimate solution	48 hours	No	mortality rate; brain tissue cyst detection	Ito et al., 1975
Sulfuric acid (63%) + dichromate (7%)	30 minutes 24 hours	No Yes	giemsa staining; acid-Schiff hematoxylin staining; serology; subinoculation in mice	Dubey et al., 1970b
Tincture of Hibisept (0.5 g chlorhexidine gluconate in 70% ethanol in 100 ml of solution)	24 hours	No	brain tissue cyst detection	Kuticic & Wikerhauser, 1993a

0.1% Tween 80	24 hours	No	giemsa staining; acid-Schiff hematoxylin staining; serology; subinoculation in mice	Dubey et al., 1970b
1% Virkon 2% Virkon	24 hours 24 hours	No No	brain tissue cyst detection	Kuticic & Wikerhauser, 1994

## CHAPTER 2

### CHEMICAL INACTIVATION OF *TOXOPLASMA GONDII* OOCYSTS IN WATER

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**ABSTRACT:** The protozoan parasite *Toxoplasma gondii* is increasingly recognized as a waterborne pathogen. Infection can be acquired by drinking contaminated water and conventional water treatments may not effectively inactivate tough, environmentally resistant oocysts. The present study was performed to assess the efficacy of 2 commonly used chemicals, sodium hypochlorite and ozone, to inactivate *T. gondii* oocysts in water. Oocysts were exposed to 100 mg/L of chlorine for 30 min, or for 2, 4, 8, 16, and 24 hr or to 6 mg/L of ozone for 1, 2, 4, 8, and 12 min. Oocyst viability was determined by mouse bioassay. Serology, immunohistochemistry, and in vitro parasite isolation were used to evaluate mice for infection. Initially, mouse bioassay experiments were conducted to compare the analytical sensitivity of these 3 detection methods prior to completing the chemical inactivation experiments. *Toxoplasma gondii* infection was confirmed by at least 1 of the 3 detection methods in mice inoculated with all doses ( $10^5$  -  $10^0$ ) of oocysts. Results of the chemical exposure experiments indicate that neither sodium hypochlorite nor ozone effectively inactivate *T. gondii* oocysts, even when used at high concentrations.

*Toxoplasma gondii* is a protozoan parasite whose worldwide importance as a zoonotic, food-borne pathogen is well documented (Choi et al., 1997; Mead et al., 1999). In recent years, increasing reports of major outbreaks of human toxoplasmosis associated with public water supplies in several countries have been attributed to waterborne transmission (Benenson et al., 1982; Bowie et al., 1997; American Water Works Association, 1999; Aramini et al., 1999; Hall et al., 1999; Bahia-Oliveira et al., 2003; de Moura et al., 2006). Humans and other warm-blooded mammals and birds all serve as intermediate hosts for *T. gondii*. However, only wild and domestic felids are definitive hosts (Dubey and Beattie, 1988; Frenkel, 1990; Tenter et al., 2000). In felids, the parasite multiplies sexually in the intestine, producing millions of environmentally resistant oocysts that are shed in the feces (Tenter et al., 2000; Dubey, 2004). Once shed, these oocysts may sporulate and become infectious within 24 hr, are widely dispersed, and may survive in terrestrial and aquatic environments for months or years (Dubey and Beattie, 1988; Tenter et al., 2000; Lindsay et al., 2003; Dubey, 2004). Humans and livestock can be infected directly by drinking water contaminated with *T. gondii* oocysts or by ingesting vegetation irrigated with oocyst-contaminated water (Ortega et al., 1997; Slifko et al., 2000; Kniel et al., 2002; Dubey, 2004). Humans can also acquire an infection by consuming inadequately cooked meat products from infected livestock or from contact with infected cat feces (Tenter et al., 2000). In most immunocompetent people, *T. gondii* infections are mild to asymptomatic and parasites ultimately encyst in the host tissues as quiescent bradyzoite cysts.

However, during periods of immunosuppression, encysted bradyzoites can transform into invasive, pathogenic tachyzoites and cause severe encephalitis (Frenkel, 1990; Luft and Remington, 1992; Montoya and Liesenfeld, 2004). The risk is also great for women who are infected during pregnancy; transplacental transmission of *T. gondii* may result in fetal death, abortion, congenital malformations, blindness, or mental retardation (Jones et al., 2001).

At present there are no specific regulations or approved methods to control *T. gondii* oocysts if present in public water supplies, and research in this area has been limited (Dubey et al., 1970; Lindsay et al, 2002; Lindsay et al, 2003). The purpose of this study was to evaluate 2 chemicals that are most commonly used to treat drinking water, chlorine (sodium hypochlorite) and ozone (Betancourt and Rose, 2004), for their ability to inactivate *T. gondii* oocysts in water. Resistance to chlorine has been demonstrated with other coccidian organisms. In prior research, 90% inactivation of *Cryptosporidium parvum* oocysts was achieved following exposure to 80 mg/L of free chlorine for 90 min (Korich et al., 1990). *Cryptosporidium parvum* oocysts treated with 5.25 % aqueous sodium hypochlorite (undiluted household bleach) for a maximum of 120 min remained infectious to neonatal mice (Fayer, 1995). Additionally, oocysts from both *Cryptosporidium* and *Eimeria* species are routinely exposed to 1.75 - 5.25% aqueous sodium hypochlorite for 15 to 30 min to purify oocysts from bacteria and debris (Wagenbach et al, 1966; Bonnin et al, 1991). Given this high resistance of coccidian oocysts to at least chlorine, *T. gondii* oocysts were exposed to

chlorine and ozone at concentrations greater than would typically be used for water disinfection in the present study. Typical chlorine concentrations used to disinfect drinking water range from 0.2 – 2 mg/L; for ozone disinfection, concentrations of ozone range from <0.1 to 1 mg/L (USEPA, 1999a). Thus the chemical concentrations used in this study for *T. gondii* oocyst inactivation are  $\geq$  50 times higher than typical for chlorination and  $\geq$  6 times for ozonation of drinking water. Post-treatment oocyst viability was assessed using the mouse bioassay. Mouse infection was confirmed using 3 methods: serology, immunohistochemistry, and in vitro parasite isolation.

## **MATERIALS AND METHODS**

### **Animals**

All animal experiments were conducted with the approval and oversight of the Institutional Animal Care and Use Committee (IACUC) at the University of California, Davis, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International (AAALAC). Female Swiss-Webster (SW) and C57BL/6 mice (Charles River Laboratories, Wilmington, Massachusetts), 20 to 22 g in weight, and 9- to 12-wk-old specific pathogen free (SPF) kittens (Nutrition and Pet Care Center, Department of Molecular Biosciences, University of California, Davis, CA) were used for oocyst production. An indirect fluorescent antibody test (IFAT) was used to prescreen mouse and kitten sera at 1:10 and 1:40 dilution, respectively, for the presence of antibodies to *T. gondii* (Arkush et al, 2003; Dabritz, 2006).

## Oocyst production

To produce bradyzoite cysts in mouse brains, 64 female mice (32 SW and 32 C57BL/6) were inoculated with culture-derived tachyzoites from a well-characterized type II *T. gondii* strain isolated from a southern sea otter (*Enhydra lutris nereis*) in California (Miller et al., 2004). Half of the mice were inoculated subcutaneously (s.c.) with  $10^6$  tachyzoites (16 SW and 16 C57BL/6) and the remaining half (16 SW and 16 C57BL/6) were inoculated with  $10^8$  tachyzoites. When tissue cysts were detected by immunohistochemical examination of brains selected from randomly sampled mice, the remaining mice were killed. Their brains were removed and fed to 2 kittens that were serologically and fecally negative for *T. gondii*. Each kitten received a total of 16 brains from seropositive mice 48 - 49 days post-infection (PI). From the same group of mice fed to the kittens, 24 half-brain sections were also submitted for immunohistochemistry. At the time of death, blood samples were collected from all mice and screened with the IFAT at a 1:40 dilution. From the day of acquisition of the kittens until 7 days beyond the last day of oocyst detection, feces were collected from the kittens and evaluated for the presence of oocysts by microscopic examination after a zinc sulfate flotation. Once oocysts were detected PI, all feces were collected and processed daily to recover oocysts. Sodium chloride (1.2 specific gravity) was used to concentrate the oocysts by flotation. Briefly, feces were mixed with 0.1% Tween 80 to form a smooth slurry at approximately 10 ml of Tween per g of feces. This mixture was poured through a fine-mesh tea strainer



into 50-ml tubes and centrifuged for 10 min at 1,000 g (Sorvall RT 6000D centrifuge; Rotor H-1000B, Thermo Fisher Scientific, Waltham, Massachusetts). The supernatant was decanted and sodium chloride solution was added to a final volume of 25 ml. The fecal pellet was thoroughly resuspended in the salt solution and centrifuged for 10 min at 1,250 g. The top 15 ml of the flotation fluid containing the oocysts was aspirated and placed into a clean 50-ml tube. The oocysts were then washed 3 times, once with 0.1% Tween 80 and twice with double distilled water (ddH<sub>2</sub>O). Following the final wash, the oocyst pellet was resuspended in 5 ml of 2% sulfuric acid. Oocysts were incubated and aerated for 2 wk at room temperature (~22 C) to allow for sporulation. They were then stored in the 2% sulfuric acid at 4 C and used within 3.5 mo. Prior to use, the oocysts were washed 3 times, the resultant pellet resuspended in ddH<sub>2</sub>O, and the proportion of sporulated oocysts determined using a hemocytometer. All oocysts used for the chemical inactivation experiments were from a single batch of oocysts and oocyst concentrations were determined counting the proportion of sporulated (and presumed viable) oocysts.

### **Mouse bioassay experiments**

Prior to conducting the oocyst inactivation experiments, the analytical sensitivity of each method of detecting infections in mice (serology, immunohistochemistry, and in vitro parasite isolation) was determined. Five replicates of each log dilution ( $10^5 - 10^0$ ) of sporulated oocysts were suspended in 200  $\mu$ l of ddH<sub>2</sub>O water and inoculated s.c. into SW mice. Negative controls

included 5 mice inoculated with processed fecal material from an uninfected, seronegative cat and 5 mice inoculated with sterile phosphate-buffered saline solution (PBS). This experiment was conducted twice, using 2 batches of oocysts from the same *T. gondii* isolate, but produced at different times. All exposed and control mice were held for 43 - 47 days before being killed and were evaluated for the presence of *T. gondii* infection via serology, immunohistochemistry, and parasite isolation. All evaluations were completed by persons who were blinded to the infection status of each mouse.

### **Chemical inactivation experiments**

*Chlorine:* For the first set of chlorine experiments, oocysts were exposed to approximately 100 mg/L of free chlorine in a sodium hypochlorite solution (made daily from 10% sodium hypochlorite stock) for 30 min, or for 1, 2, 4, 8, 16, and 24 hr. Prior to use, the stock sodium hypochlorite solution (EMD Chemicals, Gibbstown, New Jersey) was stored at 4 C and the percentage of free chlorine was confirmed with iodometric titration (American Public Health Association, 1992). All experiments were performed at room temperature (~22 C) using ddH<sub>2</sub>O (pH 7.25) as diluent that was previously determined to be demand-free of chlorine and organic materials (1.37 ppm TDS). All new glassware and magnetic stir rods were made demand-free by being soaked for 24 hr in Decon\* Contrad\* 70 liquid detergent (Fisher Scientific, Pittsburg, Pennsylvania) and rinsed 3 times with ddH<sub>2</sub>O. For each experiment, the starting concentration of sodium hypochlorite working solution of approximately 100 mg/L ( $\pm$  5 mg/L) free

chlorine was measured 3 times using a LaMotte Chlorine/Bromine Titrator kit (model CL-BR, code 3624, LaMotte, Chestertown, Maryland) as directed by manufacturer's instructions.

For each time point, 50 ml of the working solution of sodium hypochlorite were added to each of 3 125-ml Erlenmeyer flasks containing a Teflon-coated magnetic stir rod. The starting temperature of the solution was recorded and  $10^4$  sporulated oocysts (in 100  $\mu$ l of ddH<sub>2</sub>O) were added to each flask. Each flask was sealed with parafilm and continuously stirred at low speed by placement on a stir plate. Ten min prior to the end of the designated exposure time, the contents of the flasks were transferred to 50-ml tubes and centrifuged for 10 min at 1,250 g to pellet the oocysts and prevent contamination when sampling the supernatant to evaluate chlorine concentration. Five milliliters of the supernatant were collected to determine the end concentration of free chlorine and 3 ml of 0.1 N sodium thiosulfate were added into the remaining supernatant and oocyst pellet to quench any remaining free chlorine. The triplicate samples were pooled, mixed, washed 3 times with ddH<sub>2</sub>O, and evenly divided for s.c. inoculation into 3 mice. Preliminary experiments revealed that replicates of  $10^4$  sporulated oocysts remained viable and infective to mice after exposure to 0.1 N sodium thiosulfate for 2 hr (data not shown).

The chlorine experiments were repeated twice but, based on preliminary results, only the highest (24 hr) exposure time point was retested in mice starting with either  $4 \times 10^4$  or  $4 \times 10^5$  sporulated oocysts. Following 24 hr of sodium

hypochlorite exposure, the oocysts from each flask were washed and processed separately and the entire contents of each flask were inoculated s.c. into 1 of 4 mice. Positive control mice were inoculated with  $10^4$  or  $10^5$  sporulated oocysts suspended in ddH<sub>2</sub>O and stirred for 24 hr. In a final experiment, mice were each inoculated with  $10^4$  oocysts exposed to 50 ml of 5.25 % aqueous sodium hypochlorite (undiluted household bleach) for 24 hr. The bleach was neutralized with 50 ml of 0.1 N sodium thiosulfate and the oocysts were washed 3 times and inoculated s.c. into 4 SW mice.

*Ozone:* The first ozone experiments were conducted to test the ability of 6 mg/L of ozone to inactivate oocysts, when exposed for 1, 2, 4, 8, or 12 min at room temperature. Ozone was generated by passing pure oxygen across tungsten points, as part of a high-voltage discharge and analysis system (Ozone Generator BMT 803 and Ozone Analyzer BMT 964, BMT Messtechnik, Berlin, Germany), and was bubbled continuously at a concentration of 40,000 to 50,000 ppm ozone to oxygen into 48 ml of ddH<sub>2</sub>O in demand-free 125-ml Erlenmeyer flasks. Initial concentrations of ozone in each flask were measured with a test kit (ozone test kit 20644-00, Hach Company, Loveland, Colorado). Following each exposure period, 3 ml of 0.1 N sodium thiosulfate were added to neutralize the ozone. Triplicate samples of  $10^4$  sporulated oocysts were treated and the oocysts from the 3 flasks were pooled, mixed, washed 3 times, and separated into equal doses for s.c. inoculation into 3 mice. For positive controls,  $3 \times 10^4$  sporulated

oocysts were added to 48 ml of ddH<sub>2</sub>O into which pure oxygen was bubbled for 12 min.

Duplicate ozone experiments were conducted with either  $10^4$  or  $10^5$  sporulated oocysts, but only the 8-min ozone exposure was tested in mice. Concentrations of  $4 \times 10^4$  or  $4 \times 10^5$  oocysts were added to each of the 3 flasks of ddH<sub>2</sub>O into which ozone was continually bubbled for the 8-min period. After 3 ml of 0.1 N sodium thiosulfate were added to quench the reaction, the contents of each individual flask were washed and inoculated s.c. into each of 4 mice. Positive controls were performed as previously described, with each of 4 mice being inoculated s.c. with  $10^4$  sporulated oocysts.

#### **Evaluation of oocyst viability**

For all inactivation experiments, the chemically treated oocysts were inoculated s.c. into 20- to 22-g female SW mice (Charles River Laboratories) that had been prescreened for the presence of *T. gondii* antibodies using the IFAT (1:10) before use. Prior to initiation of the chemical inactivation experiments, we evaluated oocyst viability and compared 3 methods (serology, immunohistochemistry, and in vitro cultivation of parasites from brain tissue) of detection of *T. gondii* infection in mice, when killed 46 to 49 days PI. Samples recovered from mice that died prematurely were also evaluated using the same methods. Serum obtained blood samples from each mouse at death was evaluated for the presence and concentration of antibodies to *T. gondii* tachyzoite-specific antigens by 2 independent readers (KW and AP) using the

IFAT at a 1:40 positive cut-off dilution, as previously described (Miller et al., 2001; Arkush et al., 2003). For histological evaluation, the spleen, 1 lung lobe, heart, and half of the brain were fixed in 10% phosphate-buffered formalin for at least 24 hr, but no longer than 72 hr. With the first mouse bioassay experiment, all tissues were cut lengthwise into 2 equal sections for immunohistochemical evaluation. For mouse bioassay experiment #2 and all of the chemical inactivation experiments, the lung lobe and heart were cut lengthwise into 2 equal sections; the spleen was cut in half and then lengthwise to yield 4 equal sections; and the half brain was thinly sliced to yield 6 cross-sections of the cerebrum and cerebellum. These formalin-fixed tissues were submitted to the California Animal Health & Food Safety Laboratory, University of California--Davis, California, for sectioning and preparation for immunohistochemical stains, as previously described (Miller et al., 2001; Arkush et al., 2003). Two experienced pathologists (MM and BB) independently examined 2 slides from each mouse for evidence of positively stained parasites.

The second halves of these same mice brains (removed aseptically) were placed in antibiotic saline at 4 C for 24 hr, homogenized, and incubated in 0.25% trypsin solution at 37 C for 1 hr. The trypsinized homogenate was then layered over monkey kidney cells (MA104, BioWhittaker, Walkersville, Maryland) and incubated at 37 C for 2 hr. After incubation, the homogenate and media were removed and the feeder layer washed once with fresh media (Dulbecco's modified essential medium supplemented with L-glutamine, penicillin G,

streptomycin, HEPES buffer, 2-mercaptoethanol, and fetal bovine serum). Fresh medium was added and replaced every 2 to 3 days and the cultures were microscopically assessed each time for the presence of *T. gondii* tachyzoites. Cultures were considered negative and discarded if no parasites were observed after 30 days PI.

## RESULTS

Mouse bioassay demonstrated that untreated oocysts remained viable and infective after inoculation in the 2 selected strains of mice. In experiment 1 (Table I), *T. gondii* infection was detected by serology, immunohistochemistry, and/or parasite isolation in all 20 mice inoculated with  $10^5 - 10^2$  oocysts and in 4 of 5 mice inoculated with  $10^1$  oocysts. The fifth mouse tested negative for *T. gondii* by all 3 detection methods. Parasite isolation had high analytical sensitivity in detecting infections in all other mice. Serology was also reliable for detecting infections, except in 2 mice, i.e., 1 mouse inoculated with  $10^5$  oocysts (PID = 10) and 1 inoculated with  $10^4$  oocysts (PID = 11). Since both of these mice died within 11 days of exposure, the negative results on IFAT are likely due to insufficient time to achieve a measurable antibody response. However, both of these mice tested positive for *T. gondii* by immunohistochemistry and parasite isolation. For IFAT interpretation, the 2 readers were in agreement within 1 dilution of each other 100% of the time and were 100% consistent in determining the positive or negative status of mice based on IFAT results.

Immunohistochemistry was the least sensitive detection method in experiment 1,

with 100% of infections, as determined by the other 2 tests, detected only in mice inoculated with  $\geq 10^4$  oocysts. The 2 pathologists had 92% agreement on positive samples and only disagreed on the positive status of 3 mice inoculated with low doses of oocysts ( $10^3 - 10^1$ ). In experiment 2 (Table II), infections were detected by all 3 methods in all of the mice except for 1 mouse inoculated with a single oocyst based on serial dilution. The pathologists were in 97% agreement on samples positive by immunohistochemistry, with 1 disagreement on a mouse inoculated with  $10^1$  oocysts. For experiment 2 using the more complete tissue trimming protocol, results of immunohistochemistry were the same or nearly the same as those for serology or parasite isolation, depending on which reader's results were considered. For both bioassay experiments, none of the negative control mice were found to be infected with *T. gondii*.

All mice inoculated with oocysts exposed to approximately 100 mg/L of free chlorine for up to 24 hr became infected with *T. gondii*, demonstrating that under these experimental conditions, sodium hypochlorite did not inactivate *T. gondii* oocysts (Table III). The infections were detectable by all 3 methods, with the exception of a single mouse inoculated with  $10^4$  oocysts exposed to chlorine for 24 hr (Table III, experiment 2) and another mouse inoculated with  $10^5$  oocysts exposed to chlorine for 24 hr (Table III, experiment 2). For the first mouse, no parasites were observed in culture after 30 days but infection was detected with serology and immunohistochemistry. The second mouse died only 11 days PI before a detectable antibody response developed, but parasites were observed in



culture and by immunohistochemistry. For both experiments 1 and 2, the pathologists had 98% agreement on positive samples, disagreeing on only 1 mouse inoculated with  $10^4$  oocysts exposed to sodium hypochlorite for 24 hr (Table III, experiment 2). Oocysts also remained viable and infectious to mice following exposure to undiluted bleach for 24 hr.

Oocysts remained infectious following exposure to 6 mg/L of ozone for up to 12 min (Table IV). Infections were detected by all 3 detection methods in all of the inoculated mice, showing that under these conditions ozone was ineffective in inactivating oocysts. The pathologists had 96% agreement on positive samples, with 1 disagreement in each experiments 1 and 2. *Toxoplasma gondii* infections were also detected in all positive control mice in both the chlorine and ozone experiments.

## DISCUSSION

Both chlorine and ozone are strong oxidizing agents. Chlorine can cause cell death through inhibition of enzymatic activity, alterations in cell permeability, or damage to DNA and RNA (U.S. Environmental Protection Agency, 1999b). In general, chlorination is cost-effective as a water treatment and efficiently inactivates a range of other microbes, has a residual effect after the initial treatment, and is easy to use in sanitizing high volumes of water (Moore and Payne, 2004). In our study, the maximal test dose of 100 mg/L applied to oocysts in water was 4 times the concentration currently recommended to treat raw sewage and  $\geq 50$  times greater than that used to disinfect drinking water

(Palin, 1983; U.S. Environmental Protection Agency, 1999a; Cheremisinoff, 2002).

As previously described, aqueous sodium hypochlorite solutions are used to purify coccidian oocysts. However, oocyst exposure to the chlorine is generally limited to 15 – 30 min. In this study, *T. gondii* oocysts were exposed to 5.25 % aqueous sodium hypochlorite for 24 hr without resulting in oocyst inactivation. To our knowledge, no other coccidian organism has remained viable under comparable experimental conditions. Another research group reported exposing cat feces containing *T. gondii* oocysts to 5.25 % aqueous sodium hypochlorite for 24 hr, also with no success in killing the oocysts (Dubey et al, 1970). The present study differs because the oocysts were purified from the cat feces before the exposure to bleach. This would allow for an increased interaction to occur between the chlorine and the oocyst, instead of chlorine chemically reacting with the organic components of the cat feces itself. Overall, our findings suggest that *T. gondii* oocysts present in either sewage or drinking water that is disinfected with chlorine at standard dosages will likely remain viable.

Ozone is an allotrope of oxygen that forms a highly unstable gas, which can damage cells by modifying cellular components (U.S. Environmental Protection Agency, 1999c). Potential advantages of using ozone to treat drinking water include high efficacy for inactivating a range of microbes, low production of toxic byproducts, and ease of treating large volumes of water (Betancourt and Rose, 2004). For our ozone experiments, a dose of 6 mg/L of ozone was tested because this was the highest concentration of ozone that could

be passively bubbled into the ddH<sub>2</sub>O. This concentration is  $\geq 6$  times that used at many facilities to disinfect drinking water. Previous studies have shown that oocysts of a related chlorine-resistant parasitic protozoan, *Cryptosporidium parvum*, are inactivated ( $\geq 99\%$ ) following exposure to 3 or 4 mg x 1 min/L of ozone (Finch et al., 1993). However, our studies revealed that *T. gondii* oocysts remained viable and infectious for mice at all times when subjected to 6 mg/L of ozone for up to 12 min. Differences in ozone susceptibility between *T. gondii* and *C. parvum* may be attributed to differences in oocyst wall composition. The oocyst wall of *T. gondii* is comprised of 3 distinct layers: a 20- to 50-nm thick, electron-dense outer layer, a 8- to 10-nm thick, electron-lucent middle layer, and a 50- to 90-nm thick moderately electron-dense inner layer (Speer et al, 1998). The *C. parvum* oocyst wall is comprised of 2 or 3 layers, ranging in 40 to 49.7 nm in total width (Reduker et al, 1985; Harris and Petry, 1999). In addition to having a thicker oocyst wall, *T. gondii* also has a sporocyst wall surrounding the sporocysts that is not present in *C. parvum* (Dubey et al, 1998; Harris and Petry, 1999). Both of these characteristics may contribute additional protection to the *T. gondii* sporozoite.

Potential health risks of human exposure to *T. gondii* and reduction in oocyst numbers due to sampling and purification methods following chemical exposure precluded determination of final ozone concentrations after oocyst treatment. Moreover, prior to the addition of oocysts, continuous flow of ozone into the test flasks was shown to maintain a consistent ozone concentration of 6

mg/L. Since there was no evidence of oocyst inactivation at this concentration, no recommendations can be made for the application of this method to treat *T. gondii* oocysts in drinking water.

For both chlorine and ozone experiments, flasks of treated sporulated oocysts were initially pooled by treatment group prior to mouse inoculation to increase the likelihood that each mouse would be inoculated with an equivalent dose of treated oocysts. In subsequent experiments, replicates were obtained by increasing the number of mice and increasing the oocysts per flask, with the contents of each individual flask being inoculated into 4 mice. However, no significant differences were detected between these experimental approaches and the number of mouse infections that resulted.

Different properties of water, including pH, temperature, turbidity, or organic compound concentration, could affect the ability of both sodium hypochlorite and ozone to inactivate oocysts. Cost and time constraints of oocyst production and application of the mouse bioassay precluded testing a broader range of water conditions. Our chlorine and ozone experiments were conducted at room temperature (22 C) with pH neutral (7.2) ddH<sub>2</sub>O. These parameters were selected to limit any additive effects of temperature and pH on oocyst inactivation, as well as effects on the efficacy of the chemicals tested. The disinfection properties of chlorine are affected by both pH and temperature, i.e., lower temperatures and higher pH both reduce disinfection efficacy. At pH levels of < 8, free chlorine is predominately present as hypochlorous acid, the

most effective disinfectant form of chlorine (Cheremisinoff, 2002; American Water Works Association, 2003). The disinfection rate of ozone is less sensitive to changes in pH and temperature levels than that of chlorine. Ozone is less soluble and stable with increasing water temperatures, but this does not affect disinfection efficiency (U.S. Environmental Protection Agency, 1999a).

The mouse bioassay was selected to evaluate oocyst viability after chlorine or ozone treatment due to the high analytical sensitivity of this assay in previous studies (Hitt and Filice, 1992; Garcia et al., 2006). Mice were held on average for 45 days PI to ensure dissemination of infection and development of *T. gondii* tissue cysts in the brains of infected mice. Preliminary studies (data not shown) demonstrated that the type II *T. gondii* strain selected for oocyst production yielded moderate to high tissue cyst numbers in the brains of the mice strains selected for our experiments. In mouse bioassay experiment 1 (Table I), *T. gondii* infections could be detected with IFAT and parasite isolation in mice inoculated with  $\geq 10^1$  oocysts. Analytical sensitivity increased with both of these detection methods and infections were detected in mice inoculated with  $\geq 10^0$  oocysts in the second mouse bioassay experiment (Table II). The reason(s) for this increase in the sensitivity of detection has not been determined. Analytical sensitivity of immunohistochemistry also increased between the 2 mouse bioassay experiments: infections were detected in mice inoculated with  $\geq 10^4$  oocysts in the experiment 1 (Table I), whereas infections were detected in mice inoculated with  $\geq 10^0$  oocysts in experiment 2 (Table II). Increasing the total surface area of tissue examined on

the microscope between the first and second mouse bioassay could have significantly contributed to the increased sensitivity of immunohistochemical evaluation.

Multiple detection methods were employed to increase the likelihood of correctly determining the true infection status of inoculated mice after chemical treatment of the oocysts. Each of the 3 detection methods offers different advantages and disadvantages. The IFAT is rapid and “user-friendly,” with high sensitivity in determining infection. However, this test can result in false negative results if an animal dies prematurely before a measurable antibody response can develop and, because it is a fluorescent test, results may vary by 1 or more dilutions when results are evaluated by > 1 independent readers. Immunohistochemistry is rapid and sensitive and can also provide visual evidence of tissue distribution of parasites and inflammation, as well as pathologic changes resulting from a *T. gondii* infection. The drawbacks of this test include potential false negatives if insufficient tissue surface area is trimmed, stained, and evaluated; false positives if artifacts from antibody staining are present; the requirement for technical expertise to process and examine tissue samples; and an inherent level of subjectivity, as seen with the IFAT. For the mouse bioassay experiments, we were able to substantially increase analytical sensitivity of immunohistochemistry in experiment 2 (Table II) by increasing the number of tissue sections cut and evaluated from each tissue sample. In both mouse bioassay experiments, the high agreement (92% in experiment 1 and 97%

in experiment 2) between the 2 pathologists indicates that individual subjectivity was not problematic in this study. In vitro parasite isolation is also a sensitive detection method and can be advantageous to definitively establish the presence and viability of parasites from sampled tissues. Potential limits include expense and labor in maintaining tissue cultures, technical expertise required to safely handle and maintain parasite and cell lines, increased time required to obtain results, and the potential for bacteria or mold to contaminate and destroy culture samples.

Partial oocyst inactivation may have occurred with chlorine or ozone treatments, but would be undetectable with the 3 detection methods employed. The infectious dose of *T. gondii* oocysts for humans is not known, but animal infectivity studies indicate that such doses could be as low as a single oocyst (Dubey, 1996; Dubey et al., 1996, 1997). Therefore, the aim of our study was to evaluate the efficacy of sodium hypochlorite and ozone for complete inactivation of oocysts under our experimental conditions.

Treatment of drinking water for human consumption is a safety issue of global concern. In 1995, in Vancouver, British Columbia, waterborne transmission of *T. gondii* infected an estimated 2,894 to 7,718 individuals, including 100 individuals identified with acute cases of toxoplasmosis. In this largest known outbreak of water-transmitted *T. gondii*, the contaminated drinking water was chlorinated, but not filtered (Bowie et al, 1997). Filtering water is ideal for physical removal of many biological contaminants or

pathogens, including *T. gondii* oocysts which are 10 X 13  $\mu\text{m}$  in size (Dubey et al., 1998). However, filtration is not always available in water treatment facilities serving small communities or with sources of water that do not contain sufficient particulate matter for conventional coagulation and sedimentation processes (Bowie et al., 1997; Betancourt and Rose, 2004; Goh et al., 2005). Therefore, most water supplies are treated with chemicals, most notably chlorine, to inactivate potential waterborne pathogens. Given the findings of this study, current chemical water treatment practices are insufficient to inactivate *T. gondii* oocysts, if present, in public water supplies. The biochemical properties of the oocyst wall that impart such strong chemical resistance for *T. gondii* oocysts remain unknown. Such information would be useful to determine effective means of inactivating oocysts.

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**Table I. Evaluation of the analytical sensitivity of mouse bioassay (Experiment 1) at 43-47 days post-inoculation of *Toxoplasma gondii* oocysts or at time of death.**

Oocyst dose	No. of mice	IFAT*	Immunohistochemistry		Parasite isolation
			Reader 1	Reader 2	
10 <sup>5</sup>	5	4 <sup>†</sup>	5	5	5
10 <sup>4</sup>	5	4 <sup>†</sup>	5	5	5
10 <sup>3</sup>	5	5	1	2	5
10 <sup>2</sup>	5	5	2	3	5
10 <sup>1‡</sup>	5	4	1	2	4
10 <sup>0</sup>	5	0	0	0	0
Negative controls					
Feces	3	0	0	0	0
Saline	3	0	0	0	0

\*IFAT: Indirect fluorescent antibody test with positive cut-off at 1:40.

†One mouse died ≤11 days post-inoculation and a serologic response was not detectable.

‡One mouse was negative by all three detection methods.



**Table II. Evaluation of the analytical sensitivity of mouse bioassay (Experiment 2) at 43-47 days post-inoculation of *Toxoplasma gondii* oocysts.**

Oocyst dose	No. of mice	IFAT*	Immunohistochemistry		Parasite isolation
			Reader 1	Reader 2	
10 <sup>5</sup>	5	5	5	5	5
10 <sup>4</sup>	5	5	5	5	5
10 <sup>3</sup>	5	5	5	5	5
10 <sup>2</sup>	5	5	5	5	5
10 <sup>1</sup>	5	5	5	5	5
10 <sup>0†</sup>	5	4	3	4	4
Negative controls					
Feces	3	0	0	0	0
Saline	3	0	0	0	0

\*IFAT: Indirect fluorescent antibody test with positive cut-off at 1:40.

†One mouse was negative by all three detection methods.

**Table III. Results of mouse bioassay (46-49 days post-inoculation or at time of death) to assess viability of *Toxoplasma gondii* oocysts after exposure to 100 mg/L chlorine.**

Oocyst dose	Chlorine exposure time	No. of mice	IFAT*	Immuno-histochemistry		Parasite isolation	Average initial chlorine (mg/L)	Average final chlorine (mg/L)	Ct values (mg x min/L) <sup>†</sup>
				Reader #1	Reader #2				
<b>Exp.1</b>									
10 <sup>4</sup>	0 min	3	3	3	3	3	NA <sup>‡</sup>	NA	NA
10 <sup>4</sup>	30 min	3	3	3	3	3	103	102	3,075
10 <sup>4</sup>	2 hrs	3	3	3	3	2/2 <sup>§</sup>	101	99	12,000
10 <sup>4</sup>	4 hrs	3	3	2	2	3	100	100	24,000
10 <sup>4</sup>	8 hrs <sup>‡</sup>	2	2	2	2	2	101	94	46,800
10 <sup>4</sup>	16 hrs	3	3	3	3	3	101	81	87,360
10 <sup>4</sup>	24 hrs	3	3	3	3	3	104	82	133,920
<b>Exp.2</b>									
10 <sup>4</sup>	0 min	4	4	4	4	4	NA	NA	NA
10 <sup>5</sup>	0 min	4	4	4	4	4	NA	NA	NA
10 <sup>4</sup>	24 hrs	12	12	11	12	11	107.5	39.6	105,912
10 <sup>5</sup>	24 hrs <sup>  </sup>	11	10 <sup>#</sup>	11	11	11	108.5	15.3	89,165

\*IFAT: Indirect fluorescent antibody test with positive cut off at 1:40.

<sup>†</sup>mg/L used to calculate Ct (disinfectant concentration x contact time) is the average of initial and final chlorine concentration.

<sup>‡</sup>NA: not applicable.

<sup>§</sup>One culture became contaminated with bacteria and was discarded.

<sup>||</sup> Mouse lost due to early death and cannibalism.

<sup>#</sup>One mouse died at PID = 11 and a serologic response was not detectable.

**Table IV. Results of mouse bioassay (46-49 days post-inoculation) to assess viability of *Toxoplasma gondii* oocysts after exposure to 6 mg/L ozone.**

Oocyst dose	Ozone exposure time	No. of mice	IFAT*	Immuno-histochemistry		Parasite isolation	Average initial ozone (mg/L)	Ct values (mg x min/L)†
				Reader #1	Reader #2			
<b>Exp. 1</b>								
10 <sup>4</sup>	0 min	3	3	3	3	3	NA‡	NA
10 <sup>4</sup>	1 min	3	3	3	3	3	6.00	6.00
10 <sup>4</sup>	2 min	3	3	3	3	3	6.00	12.00
10 <sup>4</sup>	4 min	3	3	2	3	3	6.33	25.32
10 <sup>4</sup>	8 min	3	3	3	3	3	6.17	49.34
10 <sup>4</sup>	12 min	3	3	3	3	3	5.83	69.96
<b>Exp. 2</b>								
10 <sup>4</sup>	0 min	4	4	4	4	4	NA	NA
10 <sup>4</sup>	8 min	12	12	11	12	12	6.00	48.00
10 <sup>5</sup>	8 min	12	11§	12	12	12	6.00	48.00

\*IFAT: Indirect fluorescent antibody test with positive cut off at 1:40.

†mg/L used to calculate Ct (disinfectant concentration x contact time) is the average of initial ozone concentrations.

‡NA: not applicable.

§One serum sample was missing.

### CHAPTER 3

#### PHYSICAL INACTIVATION OF *TOXOPLASMA GONDII* OOCYSTS IN WATER

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**Abstract:** Inactivation of *Toxoplasma gondii* oocysts occurred with exposure to pulsed and continuous UV radiation, as evidenced by mouse bioassay. Even at doses of  $\geq 500$  mJ/cm<sup>2</sup>, some oocysts retained their viability.

Human toxoplasmosis acquired through the consumption of infective *Toxoplasma gondii* oocysts in contaminated drinking water is an increasing public health risk worldwide (1, 5, 17-19). Currently, there are no specific recommendations or approved methods to inactivate *T. gondii* in public water supplies, and limited research indicates that oocysts may be difficult to destroy in water using conventional disinfection methods (5, 8, 15, 20, 21). Although *T. gondii* oocysts can be inactivated by exposure to boiling, freezing, and gamma irradiation (4, 6, 10, 11), the effect of UV radiation on these oocysts has not been evaluated.

The purpose of this study was to evaluate the efficacy of pulsed and continuous UV radiation (PUV and CUV, respectively) at various doses for inactivation of *T. gondii* oocysts in water intended for public consumption. Post-treatment oocyst viability was assessed by mouse bioassay, and by using

serology, immunohistochemistry, and *in vitro* parasite isolation to confirm infection.

*Toxoplasma gondii* oocysts of genotype II (16) were obtained as described elsewhere (20) from feces of two pairs of infected kittens. PUV was administered with an Excimer laser (LPX-200; Lambda Physik, Germany) with 248-nm monochromatic, coherent beams (KrF mode). PUV exposures were calculated from the operating PUV energy fluence (mJ/cm<sup>2</sup>/pulse) measured with a 5-cm-diameter (18.1 cm<sup>2</sup> active area) calibrated joulemeter (Jmax 43, Molectron Detector Inc., Portland, Oregon), the pulse repetition rate (pulses/s), and the target area (cm<sup>2</sup>). Direct, horizontal laser beams were used to expose all targets contained in quartz tubes (1-cm diameter, 10-cm length). All physical and optical devices used in these exposures have been previously reported (14).

In experiment 1, sporulated oocysts were exposed to PUV at 0 (positive control), 100, 200, 500, and 1000 mJ/cm<sup>2</sup>. Oocysts (10<sup>5</sup>) were added to 5 ml of double-distilled H<sub>2</sub>O (ddH<sub>2</sub>O) in a quartz test tube containing a Teflon-coated magnetic stir rod and was then sealed with Parafilm. The oocysts were continuously stirred during the entire treatment at all levels of UV exposure. All experiments were conducted in triplicate, with test replicates treated consecutively. Following treatment, replicate samples at each treatment level were pooled, mixed, and then divided into thirds for subcutaneous inoculation into three mice. Positive control mice (n=3) were inoculated with either 10<sup>5</sup> (experiments 1 and 2) or 10<sup>4</sup> (experiments 3 and 4) sporulated, untreated oocysts

suspended in 3 to 5 ml of ddH<sub>2</sub>O in quartz tubes for 1 h at room temperature prior to inoculation. A similar procedure was repeated for experiment 2, except using PUV doses of 0, 100, 200, 500, and 750 mJ/cm<sup>2</sup>. UV exposure in experiments 3 and 4 was determined with a calibrated joulemeter (Model Jmax 43, Molectron Detector Inc., Portland, Oregon). Oocysts in experiments 3 and 4 were irradiated with 0, 45, 106, 196, 408, 498, or 755 mJ/cm<sup>2</sup>. To prepare the samples, oocysts (10<sup>4</sup> suspended in 100 µl ddH<sub>2</sub>O) were added to 2.9 ml ddH<sub>2</sub>O in quartz cuvettes that were thoroughly resuspended by gently shaking immediately prior to treatment.

A conventional, low pressure Hg lamp (250-MF, 1.5-cm diameter, 30-cm length, Ultra Dynamics Corporation, Santa Monica, CA) was used in all CUV experiments. The energy fluence (mJ/cm<sup>2</sup>) and exposure rates (mJ/cm x s) at a designated perpendicular position (12.5 cm) from the lamp surface were calibrated prior to the experiments. Different UV radiation exposure levels were achieved by varying exposure time. At the chosen position and distance, UV source emittance (mJ/cm<sup>2</sup>s) was calibrated using ferrous sulfate solution (2), whose response to UV energy was standardized and measured with a 5-cm calibrated joulemeter. Experiments 1 to 4 were performed in the same manner as for PUV experiments 1 to 4, except for small differences in UV exposure doses within the selected ranges. Exposure doses of 0, 100, 200, 500, and 1000 mJ/cm<sup>2</sup> were tested in experiment 1 while a narrower range of 0, 20, 40, 80 and 100 mJ/cm<sup>2</sup> was evaluated in experiment 2. In experiments 3 and 4, CUV exposure

doses (0, 160, 186, 228, 335, 412, and 731 mJ/cm<sup>2</sup>) were determined as previously described for PUV experiments 3 and 4 with a single set of positive controls because both experiments were conducted on the same day.

For all inactivation experiments, UV-treated oocysts were inoculated subcutaneously into seronegative 20- to 22-g female SW mice (Charles River Laboratories, Wilmington, MA). To determine if mice inoculated with treated oocysts became infected with *T. gondii*, each mouse was evaluated 43 to 48 days postinoculation by serology, immunohistochemistry, and *in vitro* cultivation of brain tissue for parasite isolation as previously described elsewhere (20).

Results of PUV experiments are shown in Table 1. In experiment 1, infections were not detected in any of the mice inoculated with oocysts exposed to 1000 mJ/cm<sup>2</sup> but at 500 mJ/cm<sup>2</sup>, a single mouse became infected. For experiment 2, PUV exposure doses of 750 mJ/cm<sup>2</sup> appeared to inactivate all oocysts; however, *T. gondii* infection was detected in one of three mice inoculated with oocysts treated with 500 mJ/cm<sup>2</sup> of UV radiation. In experiments 3 and 4, all oocysts appeared to be inactivated with PUV doses  $\geq 106$  mJ/cm<sup>2</sup>, with the exception of a single mouse inoculated with oocysts treated with 755 mJ/cm<sup>2</sup> of UV radiation (experiment 3). This mouse was seropositive for *T. gondii* (1:80) but was negative on immunohistochemistry and parasite isolation.

Results of the CUV experiments are shown in Table 2. In experiment 1, all CUV exposure doses tested from 100 to 1000 mJ/cm<sup>2</sup> effectively inactivated oocysts. In experiment 2, oocysts appeared to be inactivated with CUV doses of

40, 80, and 100 mJ/cm<sup>2</sup>, but not at 20 mJ/cm<sup>2</sup>. In experiment 3, infections were not detected in any mice inoculated with oocysts treated with CUV doses of 160 to 731 mJ/cm<sup>2</sup>, with the exception of one mouse inoculated with oocysts exposed to CUV radiation at 731 mJ/cm<sup>2</sup>. This mouse was positive for *T. gondii* by serology and *in vitro* parasite isolation from brain tissue. Similar results were evident in experiment 4; all of the mice inoculated with oocysts treated with CUV doses of 160 to 731 mJ/cm<sup>2</sup> were negative for *T. gondii* infection, with the exception of one mouse inoculated with oocysts exposed to 160-mJ/cm<sup>2</sup> CUV dose and one mouse exposed to the 335-mJ/cm<sup>2</sup> dose. Both mice were positive by all three detection methods. *T. gondii* infections were detected in all positive control mice. One control mouse (experiment 1) died at postinfection day (PID) 12 before an antibody response was detectable with the indirect fluorescent-antibody test (IFAT).

*T. gondii* is gaining recognition as a waterborne pathogen (1, 5, 17, 18), but how to effectively eliminate this parasite from drinking water remains undetermined. The physical methods (PUV and CUV) tested in this study were more effective at oocyst inactivation than chemical treatments previously tested (20).

Oocyst inactivation occurred at PUV doses as low as 40 mJ/cm<sup>2</sup> and CUV doses as low as 45 mJ/cm<sup>2</sup>. However, oocyst inactivation was not 100% reliable at higher doses of UV administered both by pulsed and continuous sources. Unexpectedly, infections were detected in mice inoculated with oocysts treated



with PUV doses as great as 755 mJ/cm<sup>2</sup> and CUV doses of 731 mJ/cm<sup>2</sup>. The infection detected in the mouse inoculated with oocysts treated with a PUV dose of 755 mJ/cm<sup>2</sup> was supported via seroconversion to a 1:80 titer for *T. gondii* in the IFAT. However, tissues from this mouse were negative for infection using immunohistochemistry and parasite isolation. In all other cases, infection was confirmed by identification of *T. gondii* tachyzoites in mouse tissues at 45 days postinfection by immunohistochemistry and/or parasite isolation. Mouse bioassay is commonly used for detecting *T. gondii* oocyst viability (12, 13, 20). By using serology, immunohistochemistry, and in vitro isolation to detect infection, the sensitivity was increased so that inoculation with a single oocyst was potentially detectable (20).

In this study, similar ranges of UV radiation were tested in both PUV and CUV experiments 3 and 4 to facilitate comparisons between the two UV delivery systems and to confirm the reproducibility of results between experiments. Lower levels of CUV radiation consistently inactivated oocysts compared with PUV. These results were unexpected, and the reasons for this difference are unknown. We had hypothesized that greater inactivation would occur with a lower dose pulse-derived UV energy because PUV delivers the same amount of UV energy as CUV but in shorter periods of time (seconds versus minutes). Thus, we expected that this greater concentration of UV energy delivered in a shorter time period should result in more irreversible DNA damage.

The goal of our study was to evaluate the efficacy of UV treatments for complete inactivation of oocysts under defined conditions with the hope of formulating recommendations for the treatment of drinking water. Partial oocyst inactivation appears to have occurred with UV treatment since some oocysts survived the treatment and remained infectious. The infective dose of *T. gondii* oocysts for humans is not known, but animal infectivity studies indicate that an infectious dose could be as low as a single oocyst (3, 7, 9, 20).

Overall, though, our results suggest that a minimum UV exposure dose of 1000 mJ/cm<sup>2</sup> may be required for either PUV or CUV treatment of water to increase the probability of consistent and complete oocyst inactivation.

Further investigations of UV treatment of *T. gondii* oocysts are warranted to evaluate the effectiveness of UV radiation in water with different levels of turbidity. Combining chemical disinfection treatments with UV treatments might also provide additional benefits of residual disinfection.

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**TABLE 1. Results of mouse bioassay at 44 to 48 days postinoculation to assess viability of *T. gondii* oocysts after exposure to PUV**

Oocyst dose	No. of mice	PUV exposure Dose (mJ/cm <sup>2</sup> )	IFAT <sup>c</sup>	Immunohistochemistry		Parasite Isolation
				Reader 1	Reader 2	
<b>Preliminary tests</b>						
Expt 1						
10 <sup>5</sup>	3	0	3	3	3	2 <sup>a</sup>
10 <sup>5</sup>	3	100	3	2	2	2
10 <sup>5</sup>	3	200	2	1	1	2
10 <sup>5</sup>	3	500	0	1	1	0
10 <sup>5</sup>	3	1000	0	0	0	0
Expt 2						
10 <sup>5</sup>	3	0 <sup>b</sup>	2	3	3	2
10 <sup>5</sup>	3	100	1	0	0	0
10 <sup>5</sup>	3	200	0	0	0	0
10 <sup>5</sup>	3	500	1	1	1	1
10 <sup>5</sup>	3	750	0	0	0	0
<b>Confirmation tests</b>						
Expt 3						
10 <sup>4</sup>	3	0	3	3	3	3
10 <sup>4</sup>	3	45	3	1	1	3
10 <sup>4</sup>	3	106	0	0	0	0
10 <sup>4</sup>	3	196	0	0	0	0
10 <sup>4</sup>	3	408	0	0	0	0
10 <sup>4</sup>	3	498	0	0	0	0
10 <sup>4</sup>	3	755	1	0	0	0
Expt 4						
10 <sup>4</sup>	3	45	1	0	0	1
10 <sup>4</sup>	3	106	0	0	0	0
10 <sup>4</sup>	3	196	0	0	0	0
10 <sup>4</sup>	3	408	0	0	0	0
10 <sup>4</sup>	3	498	0	0	0	0
10 <sup>4</sup>	3	755	0	0	0	0

<sup>a</sup>One culture became contaminated with bacteria and was discarded.

<sup>b</sup>One mouse found dead (PID 12) and cannibalized; only the spleen was recoverable for immunohistochemical testing.

<sup>c</sup>IFAT with a positive cut-off value at 1:40.

**TABLE 2. Results of mouse bioassay at 45 to 48 days postinoculation to assess viability of *T. gondii* oocysts after exposure to CUV**

Oocyst dose	No. of mice	CUV exposure dose (mJ/cm <sup>2</sup> )	IFAT <sup>c</sup>	Immunohistochemistry		Parasite Isolation
				Reader 1	Reader 2	
<b>Preliminary tests</b>						
Expt 1						
10 <sup>5</sup>	3	0	2 <sup>a</sup>	3	3	2 <sup>b</sup>
10 <sup>5</sup>	3	100	0	0	0	0
10 <sup>5</sup>	3	200	0	0	0	0
10 <sup>5</sup>	3	500	0	0	0	0 <sup>b</sup>
10 <sup>5</sup>	3	1000	0	0	0	0 <sup>b</sup>
Expt 2						
10 <sup>5</sup>	3	0	3	3	3	3
10 <sup>5</sup>	3	20	2	2	2	2
10 <sup>5</sup>	3	40	0	0	0	0
10 <sup>5</sup>	3	80	0	0	0	0
10 <sup>5</sup>	3	100	0	0	0	0
<b>Confirmation tests</b>						
Expt 3						
10 <sup>4</sup>	3	0	3	3	3	3
10 <sup>4</sup>	3	160	0	0	0	0
10 <sup>4</sup>	3	186	0	0	0	0
10 <sup>4</sup>	3	228	0	0	0	0
10 <sup>4</sup>	3	335	0	0	0	0
10 <sup>4</sup>	3	412	0	0	0	0
10 <sup>4</sup>	3	731	1	0	0	1
Expt 4						
10 <sup>4</sup>	3	0	3	3	3	3
10 <sup>4</sup>	3	160	1	0	1	1
10 <sup>4</sup>	3	186	0	0	0	0
10 <sup>4</sup>	3	228	0	0	0	0
10 <sup>4</sup>	3	335	1	0	1	1
10 <sup>4</sup>	3	412	0	0	0	0
10 <sup>4</sup>	3	731	0	0	0	0

<sup>a</sup>One mouse died at PID 12; antibody immune response was not detectable.

<sup>b</sup>One culture became contaminated with bacteria and was discarded.

<sup>c</sup>IFAT with a positive cut-off value at 1:40.

## CHAPTER 4

RADIOFREQUENCY-INDUCED THERMAL INACTIVATION OF  
*TOXOPLASMA GONDII* OOCYSTS IN WATER

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**Impacts**

- *Toxoplasma gondii* is gaining recognition as a waterborne pathogen.
- Water temperatures of 70°C may not reliably kill *T. gondii* oocysts.
- Water heating time and cooling times, as well as water volumes, may affect efficacy of oocyst inactivation by radiofrequency (RF) power.

**Summary:** *Toxoplasma gondii*, a ubiquitous parasitic protozoan is emerging as an aquatic biological pollutant. Infections can result from ingesting environmentally-resistant oocysts in drinking water. However, recommendations regarding water treatment for oocyst inactivation have not been established. In this study, the physical method of radiofrequency (RF) power was evaluated for its ability to inactivate *T. gondii* oocysts in water. Oocysts were exposed to various RF energy levels to induce 50, 55, 60, 70 and 80°C temperatures maintained for 1 min. Post-treatment oocyst viability was determined by mouse bioassay with serology, immunohistochemistry, and *in vitro* parasite isolation to confirm *T. gondii* infections in mice. None of the mice inoculated with oocysts treated with RF-induced temperatures of  $\geq 60^\circ\text{C}$  in an initial experiment became infected; however, there was incomplete oocyst



activation in subsequent experiments conducted under similar conditions. These results indicate that *T. gondii* oocysts may not always be inactivated when exposed to a minimum of 60°C for 1 min. The impact of factors such as water heating time, cooling time, and the volume of water treated must be considered when evaluating RF power for oocyst inactivation.

Key words: *Toxoplasma gondii*; oocyst; radiofrequency; water treatment

## Introduction

*Toxoplasma gondii* is a protozoal parasite that infects a wide variety of warm-blooded animals, including humans (Dubey and Beattie, 1988; Tenter et al., 2000). In recent years, major outbreaks of toxoplasmosis have increased recognition of the risks of waterborne transmission of the environmentally resistant oocyst stage of *T. gondii* (Dubey, 2004; Sroka et al., 2006). Humans and food-producing animals may be directly infected from drinking oocyst-contaminated water or by ingesting agricultural products irrigated with contaminated water (Dubey et al., 1995; Kniel et al., 2002). For example, ingestion of a single, microscopic *T. gondii* oocyst in contaminated water can infect susceptible swine and lead to the formation of tissue cysts (Dubey et al., 1996). Humans can then become infected by eating inadequately cooked pork (Nielsen and Wegener, 1997). The importance of *T. gondii* as a zoonotic, food-borne pathogen is well documented and approximately 10-25% of the U.S. population is positive for antibodies

against *T. gondii*, suggestive of chronic infection (Choi et al., 1997; Jones et al., 2003; Jones et al., 2006).

*Toxoplasma gondii* infections are asymptomatic in most humans with a competent immune system (Dubey and Beattie, 1988). However, encysted parasites can reactivate during periods of immunosuppression, resulting in clinical disease (Frenkel, 1990). In patients with HIV infections or patients undergoing immune suppression therapy prior to organ transplantation, severe and sometimes fatal encephalitis may result from reactivation of encysted bradyzoites that transform to become invasive, pathogenic tachyzoites (Frenkel, 1990; Luft and Remington, 1992). Women who become infected during pregnancy are also at higher risk of disease, as transmission of *T. gondii* to their fetus may result in miscarriage or congenital toxoplasmosis (Baril et al., 1999). Congenitally infected infants and children may develop blindness and mental retardation (Jones et al., 2001).

Although many animals can be infected with *T. gondii*, only domestic and wild felids serve as definitive hosts as reviewed by Dubey (2004), Tenter et al. (2000) and Dabritz and Conrad (2008). Cats become infected by ingesting sporulated oocysts in the environment or by eating raw meat or prey (rodents and birds) containing tissue cysts. Once ingested by a cat, sexual multiplication of the parasite occurs within intestinal cells, resulting in the formation of millions of oocysts which are shed in the feces. As few as 100 oocysts can result in infection and oocyst shedding in cats (Dubey, 1996a). Predation of infected

intermediate hosts by naïve cats further propagates the cycle of oocyst production, shedding, and contamination (Tenter et al., 2000). Once in the environment, these oocysts may become infectious within 24 h of defecation, may persist for long periods and can be widely dispersed in soil or water (Tenter et al., 2000). To date, no practical methods are available to prevent wild or domestic cats from shedding oocysts (Mateus-Pinilla et al., 1999). Therefore, for prevention of zoonotic disease, control efforts must focus on destruction of oocysts already present in the environment (Dubey, 1996b).

Currently, there are no specific recommendations or approved methods to inactivate *T. gondii* oocysts in public water supplies; limited research indicates that oocysts may be difficult to destroy in water using conventional disinfection methods (Dubey, 2004; Dubey et al., 1998). Recent research in our laboratory has demonstrated that high concentrations of 2 common chemical disinfectants commonly used to treat drinking water (sodium hypochlorite and ozone) do not inactivate *T. gondii* oocysts in water under experimental conditions (Wainwright et al., 2007b). Additional experiments evaluating the efficacy of both pulsed and continuous ultraviolet (UV) irradiation revealed that *T. gondii* oocysts were inactivated when exposed to UV radiation, but not with 100% efficacy over the entire range of UV doses evaluated (Wainwright et al., 2007a).

Heating or boiling liquids is another well-established method to inactivate waterborne pathogens (Cheremisinoff, 2002). Prior research has indicated that *T. gondii* oocysts in water can be inactivated following exposure to temperatures  $\geq$

50°C for varying lengths of time (Dubey et al, 1970; Ito et al., 1975; Kuticic and Wikerhauser, 1994; Dubey, 1998). In comparison to thermal heating of water, radiofrequency (RF) power allows for uniform thermal heating of volumes of water without the use of chemicals and has been shown to completely and rapidly inactivate pathogenic bacteria in wastewater treated at 60-65°C for < 1 min (Lagunas-Solar et al., 2005a). Furthermore, RF is more energy-efficient than surface heating (Lagunas-Solar et al., 2005a and 2005b). With RF, a rapidly oscillating electrical field is generated between two electrodes. The continually changing electrical field causes dipole molecules present in the treated material to align and reorient themselves with the changing electrical field. Thermal energy (heat) is produced as a result of friction generated by the constant molecular movement (Lagunas-Solar et al., 2005a). An increase in RF energy-absorbing molecules increases the efficiency of this system. Therefore, RF should be more effective than UV radiation for treating water with high turbidity, such as wastewater.

The purpose of this study was to evaluate the efficacy of RF power as an alternative to chemical disinfectants to inactivate *T. gondii* oocysts in water intended for public consumption. In this study, *T. gondii* oocysts were exposed to varying doses of RF-generated thermal energy levels; post-treatment oocyst viability was assessed by mouse bioassay and serology, immunohistochemistry, and *in vitro* parasite isolation to confirm infection of oocyst-exposed mice.

## Materials and Methods

### Animals

All animal experiments were conducted with the approval and oversight of the Institutional Animal Care and Use Committee (IACUC) at the University of California, Davis, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International (AAALAC). Female Swiss-Webster (SW) and C57BL/6 mice (Charles River Laboratories, Wilmington, Massachusetts) weighing 20 to 22 g, and 9-12 wk old specific pathogen free (SPF) kittens (Nutrition and Pet Care Center, Department of Molecular Biosciences, University of California, Davis, CA) were used for oocyst production. Three mice per treatment group were housed in individual cages and access to a standard diet and water were provided *ad libitum*. Pairs of kittens were housed in a large kennel cage, with dry kitten food and water available *ad libitum*. An indirect fluorescent antibody assay (IFAT) was used to prescreen mouse and kitten sera at dilutions of 1:10 and 1:40, respectively, for the presence of antibodies to *T. gondii* (Arkush et al., 2003; Dabritz, 2006). Feces from pre-infection kittens were processed daily for 10 days with a double-centrifugation flotation technique using zinc sulfate (1.2 specific gravity) and confirmed as negative by microscopic examination for the presence of *T. gondii* oocysts.

### Oocyst production

*Toxoplasma gondii* oocysts were produced as previously described (Wainwright et al., 2007b). Briefly, bradyzoites were produced in the brains of

SW and C57BL/6 mice via s.c. inoculation of culture-derived tachyzoites from a type II *T. gondii* strain isolated from a southern sea otter in California (Miller et al., 2004). When tissue cysts were detected by immunohistochemical examination of brains selected from randomly sampled mice, the remaining mice were euthanized. Their brains were removed and fed to 2 kittens that were serologically and fecally negative for *T. gondii*. Once oocysts were detected in the kittens' feces post-infection, all feces were collected and processed daily to recover oocysts by concentration using sodium chloride solution (1.2 specific gravity) as previously described (Wainwright et al., 2007b). Harvested oocysts were incubated and aerated for 2 wk at room temperature (~22°C) to allow for sporulation, then stored in 2% sulfuric acid at 4°C and used within 3.5 months. Prior to use, the oocysts were washed 3 times, the resultant pellet resuspended in ddH<sub>2</sub>O, and the proportion of sporulated oocysts determined using a hemocytometer. Sporulated oocysts used for the physical inactivation experiments were obtained from oocysts produced by separate pairs of kittens infected with the same *T. gondii* isolate.

### **Radiofrequency Treatments**

All RF-induced thermal effect experiments were conducted with a research-prototype parallel-plate cavity RF system that was developed, constructed and operated at the Crocker Nuclear Laboratory (CNL) at the University of California, Davis. Design, engineering and operating details were as previously described (Lagunas-Solar et al., 2005b). The RF treatment cavity consists of 2

parallel metallic aluminum plates (46 x 60 cm; 2.5-cm thick) connected to a copper-coil resonance inductor and a capacitor-matching network. The RF system employed a synthesized RF signal generator (Model 6060B, 10 kHz to 1050 MHz, Gigatronics Inc., San Ramon, CA, USA) and a 2.5-kW wideband RF amplifier (Model TCCX 2500P, 10 kHz to 250 MHz, Instruments for Industry Inc., Ronkonkoma, NY, USA) to deliver RF energy to the enclosed (RF-shielded) cavity. Several diagnostic devices, such as a RF power meter (Model 4391A, Bird Electronic Corp., Cleveland, OH, USA) and an oscilloscope (Model 54610B, 500 MHz, Hewlett Packard, Palo Alto, CA, USA) were used for electric field measurements within the RF cavity, as sensed with a CNL-built voltage divider. To profile RF of the treated samples during processing, thermocouples were used for off-line measurements and a fiber optic system (Model UMI-4, FISO Technologies, Quebec, Canada) was used for on-line measurements. A digital computer provided data acquisition and storage capabilities.

For RF treatment of water, increased turbidity has been shown to maximize the efficiency of RF heating (Lagunas-Solar et al., 2005a). Therefore, in all of the RF experiments, oocysts were suspended in a sterile feline fecal supernatant to provide additional molecules for turbidity in order to increase heat production efficiency. This slurry was prepared from feces of uninfected, seronegative cats that had been processed by the same methods described previously for oocyst recovery, except that the supernatant was decanted, saved, and centrifuged twice for 10 min at 1000 x g. The final supernatant was collected, autoclaved for 20 min

on the liquid setting, cooled, and stored at 4°C prior to use. For experiment #1,  $3 \times 10^5$  oocysts were added to 100 mL of the fecal supernatant in T75 tissue culture flasks. With one flask per temperature, the oocyst samples were heated with RF power to temperatures of 50, 55, 60 or 70°C, respectively. After treatment, the samples were allowed to cool and the contents of each flask were concentrated, divided equally into 3 aliquots, and inoculated s.c. into each of 3 mice. For controls,  $3 \times 10^5$  oocysts were added to 100 mL of sterile fecal supernatant, left at room temperature for 1 h and then inoculated s.c. into 3 mice. This process was repeated in experiments #2 and #5. Temperatures of 50, 55, and 60°C were used in experiment #2 and temperatures of 50, 55, 60, and 70°C were used in experiment #5. For experiments #3 and #4,  $10^4$  oocysts were mixed with 100 mL of fecal supernatant in a T75 flask and 3 replicates were individually treated at each of the selected temperatures (50, 55, 60 and 70°C). The reduction in oocyst concentration from  $10^5$  to  $10^4$  allowed for individual sample treatment. Post-treatment, the replicate samples were pooled, mixed, and inoculated s.c. into 3 mice.

#### **Evaluation of oocyst viability**

For all inactivation experiments, RF-treated oocysts were inoculated s.c. into seronegative 20 to 22 g female SW mice (Charles River Laboratories). To determine if mice inoculated with treated oocysts became infected with *T. gondii*, each mouse was evaluated 43-48 days post-inoculation by serology, immunohistochemistry, and *in vitro* cultivation of brain for parasite isolation as



previously described (Wainwright et al., 2007b). When possible, samples were collected from mice that died prior to euthanasia and were evaluated for using the same methods. All diagnostic assays were completed in a blinded fashion by examiners with no knowledge of each animal's oocyst exposure or infection status. Blood was collected from all mice at euthanasia or death and serum was tested for the presence and concentration of IgG antibodies to *T. gondii* tachyzoite-specific antigens by two independent readers (KW and AP) using the IFAT at a 1:40 positive cut-off dilution, as previously described (Wainwright et al., 2007b). For histopathological evaluation, the spleen, 1 lung lobe, heart, and half of the brain were fixed in 10% phosphate-buffered formalin for at least 24 h, but no longer than 72 h. To prepare the tissue samples for immunohistochemistry, the lung lobe and heart were cut lengthwise into 2 equal sections; the spleen was cut in half and then lengthwise to yield 4 equal sections; and half of the brain was cut to yield 6 cross-sections of cerebrum, cerebellum, mesencephalon and brainstem. Formalin-fixed tissues were submitted to the California Animal Health & Food Safety Laboratory, University of California, Davis, CA, for sectioning and preparation of immunohistochemical stains, as previously described (Wainwright et al., 2007b). Two experienced pathologists (MM and BB) independently examined all tissues from each mouse for evidence of positively-stained parasites. The second half of each brain was removed aseptically, placed in antibiotic saline at 4°C for 24 h and prepared for *in vitro* parasite isolation as previously described (Wainwright et al., 2007b). Cultures

were microscopically examined for the presence of *T. gondii* tachyzoites and were considered negative and discarded if no parasites were observed after 30 days.

## Results

The results of the RF experiments are shown in Table 1. In experiment #1, oocysts appeared to be inactivated at temperatures of 60°C and above. In experiments #2-5, oocyst inactivation was inadequate, even at temperatures  $\geq$  60°C, and *T. gondii* infections were observed microscopically in brains of some mice. In experiment #2, all mice were positive by all 3 detection methods except for a single mouse in the 55°C test group which was negative on IFAT but positive by immunohistochemistry and parasite isolation. In experiment #3, at 60°C, all 3 mice were positive on IFAT and 2 mice were positive by parasite isolation in culture. However at 70°C, only 1 mouse was positive for *T. gondii* by all 3 detection methods. In experiment #4, 2 mice inoculated with oocysts treated at 60°C were *T. gondii*-positive based on positive serology and parasite isolation. The remaining mouse in this treatment group and all 3 mice in the 70°C treatment group remained uninfected, based on negative histopathologic, culture and serology results. In experiment #5, all 3 mice inoculated with oocysts treated at 70°C were *T. gondii*-positive by at least 1 detection method. Infections were detected in all mice inoculated with oocysts held at room temperature (positive controls).

Table 2 shows the time periods of RF treatment required to reach the target temperatures from the initial temperatures of the fecal slurry. In experiments #3

and #4, the exposure times for 3 samples in each treatment group were averaged since these samples were individually treated and then pooled for inoculation into 3 mice. In experiments #3-5, oocysts were exposed to temperatures  $\geq 60^{\circ}\text{C}$  for periods of 2-5 min.

## Discussion

Since *Toxoplasma gondii* is gaining recognition as a global waterborne pathogen (Tenter et al., 2000; Dubey, 2004) and given the public health risk for *T. gondii* infections, studies to evaluate the efficacy of chemical or physical methods for inactivating oocysts in drinking water are needed. Based on our prior studies, chemicals traditionally used to treat municipal drinking water, such as ozone and sodium hypochlorite, do not inactivate *T. gondii* oocysts (Wainwright et al., 2007b). Physical methods (pulsed UV and continuous UV irradiation) appear to be more effective than chemical treatment to inactivate oocysts in water; however, no method has proven 100% effective and reliable (Wainwright et al., 2007a). Previous research supports the resistant nature of oocysts in water. *Toxoplasma gondii* oocysts are resistant to low environmental temperatures and oocysts can survive exposure to  $-21^{\circ}\text{C}$  for 28 days (Frenkel and Dubey, 1973). Nonsporulated oocysts have been shown to survive refrigeration at  $4^{\circ}\text{C}$  for 11 weeks, then sporulate at room temperature to become infective to inoculated mice (Lindsay et al., 2002). An increase of salinity in an aqueous environment can affect oocyst viability. Lindsay et al. (2003) reported that oocysts can sporulate in seawater (15 parts per thousand, ppt, and 32 ppt artificial seawater)

and remain infectious following storage in seawater at both 4°C and 22-24°C for 6 months. Hutchinson (1967) reported that oocysts remained viable after being held in room temperature (20-22°C) water for 17 months. Dubey and colleagues (1970) found that oocysts remained infective to mice when heat treated at 50°C for 30 min, but were inactivated following exposure to 55°C for 30 min. In 1998, Dubey reported that oocysts suspended in water remained infective to mice following exposure to 4°C for 1,620 days, 10° and 15°C for 200+ days, 30°C for 107 days, 35°C for 32 days, 40°C for 24 h, 52°C for 5 min, and 55°C for 1 min. However, in these same series of experiments, oocysts were inactivated by heat treatment at 55°C for 2 min and 60°C for 1 min. The potential for oocyst inactivation at temperatures  $\geq 60^\circ\text{C}$  provided the rationale for testing the effect of RF treatment.

In the current study, the efficacy of RF to inactivate *T. gondii* oocysts was evaluated because RF is an efficient and effective method to thermally-treat water homogeneously and to inactivate a variety of waterborne pathogens (Lagunas-Solar et al., 2005a and 2005b). Additionally, RF is effective in treating turbid water, unlike other physical methods such as UV irradiation (Lagunas-Solar et al., 2005a). The aim of our study was to evaluate the efficacy of RF treatments for complete inactivation of oocysts under defined conditions.

The RF treatment experiments were conducted using the same methodology and equipment as previously used to inactivate *Salmonella sp.*, *Escherichia coli* O157:H7, and *Mycobacterium avium* subsp. *paratuberculosis*

(Lagunas-Solar et al., 2005a and 2005b). Multiple detection methods (serology, immunohistochemistry, and *in vitro* parasite isolation) were used to increase the sensitivity of detection of *T. gondii* infection in mice used to test the viability and infectivity of oocysts after RF treatment. We have previously shown that these methods have high analytical sensitivity and can detect infections in mice inoculated with a single oocyst (Wainwright et al., 2007b). Fiber-optic technology was employed to monitor the temperature of the fecal slurry and ensure that target test temperatures were reached and maintained throughout the timed treatments. Samples were treated with RF for 7-17 min to reach target test temperatures of  $\geq 60^{\circ}\text{C}$ .

Our experiments indicate that heating water via RF treatment to a minimum of 1 min at 60- 70°C is not sufficient to reliably inactivate *T. gondii* oocysts in water. Our initial results (experiment #1) concur with those of a previous study which reported that *T. gondii* oocysts in water are completely inactivated following exposure to 60°C for 1 min (Dubey, 1998). Despite this initial success, RF treatment failed to reliably inactivate *T. gondii* oocysts at temperatures  $\geq 60^{\circ}\text{C}$  in subsequent experiments using the same range of temperatures and exposure times. Other reports suggest that heat inactivation of *T. gondii* oocysts at 50-70°C may require exposure times longer than 1 min (Ito et al, 1975; Kuticic and Wikerhauser, 1994). Ito et al. (1975) found that sporulated oocysts remained infectious to mice (with ~2,500 oocysts orally inoculated per mouse) following heat treatments of 50°C for 25 min, 55 or 60°C for 10 min, or

70°C for 1 min. These same researchers found that sporulated oocysts were inactivated following exposure to 50°C for 30 min, 55 or 60°C for 15 min, or 70°C for 2 min. Kuticic and Wikerhauser (1994) found that at 58°C *T. gondii* oocyst inactivation required treatment periods of 15 to 30 min.

The effects of gradual heating of water, gradual cooling post-treatment and an increase in the volume of water treated by any single method may be important considerations when using heat for inactivation of *T. gondii* oocysts present in water. Subjecting oocysts to progressive increases in temperature over several minutes (as with our RF experiments) may have allowed the parasites within the oocysts to acclimate to the increasing heat stress and remain viable, compared to instantaneous exposure to high temperatures in prior experiments (Dubey, 1998; Ito et al., 1975). Heat shock proteins produced constitutively by some cells, including protozoa, may facilitate response to rising cellular temperatures through repair and protection of vital cellular proteins, permitting survival during short-term hyperthermia (Alberts, et al., 1994; Polla, 1991). Gradual cooling of oocysts during the RF treatments may also have contributed to oocyst survival, when compared to sudden submersion into cold water following heat treatment as performed by Dubey (1998) and Ito et al. (1975). In addition, during the RF experiments, oocysts were heated within larger volumes of fluid (100 mL of fecal slurry) than the 1 mL samples used by Dubey (1998). These treatment parameters (rate of water heating and cooling, duration of heating and volumes heated) will be critical considerations for future application

of RF or other modalities for water treatment for inactivation of *T. gondii* and related zoonotic protozoa.

The results of this study raise questions not only about the efficacy of RF treatment, but also the reliability of heating water as means of *T. gondii* oocyst inactivation. In developing nations, where heat treatment (preferably boiling) is recommended to kill microorganisms, water in volumes of > 100 mL would be gradually heated and cooled. The reliability of this method for inactivating oocysts may require further evaluation. If thermal heating is to be used in practical applications such as treating drinking or wastewater for *T. gondii* oocyst inactivation, factors of heating time, cooling time, and volume of treated water should be systematically evaluated. Experiments should be conducted to assess whether rapid cooling following heat exposure of  $\geq 60^{\circ}\text{C}$  affects oocyst viability, to evaluate if oocyst viability is affected by immediate exposure to temperatures of  $\geq 60^{\circ}\text{C}$ , when compared to oocysts that are heated more gradually and to evaluate the efficacy of oocyst inactivation following heat exposure of  $\geq 60^{\circ}\text{C}$  in different volumes of liquid. Future RF treatment studies in which the oocyst exposure temperatures and times are increased beyond what is required to kill other microbial pathogens may prove more effective for *T. gondii* oocyst inactivation, while still permitting practical application of RF for water treatment. The personal and societal costs of human toxoplasmosis are sufficiently high to justify efforts to reduce the prevalence of *T. gondii* oocysts in

both aquatic and terrestrial environments (Jones et al., 2007; Roberts and Frenkel, 1990) and to promote further research on methods for water disinfection.

### **Conclusions**

We conclude: (i) *T. gondii* oocysts may not always be inactivated when exposed to a minimum of 60°C for 1 min; (ii) water heating time, cooling time, and the volume of water treated must be considered when evaluating RF power or thermal heating for oocyst inactivation.

### **Competing Interests**

The authors declare that they have no competing interests.

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**Table 1. Positive results of mouse bioassay (43-49 days post-inoculation) to assess viability of *Toxoplasma gondii* oocysts after exposure to radiofrequency (RF)**

Oocyst dose	Number of mice inoculated	Treatment temperature	Number of mice positive by IFAT	Number of mice positive by immunohistochemistry		Number of mice positive by culture
				Reader #1	Reader #2	
Exp #1						
10 <sup>5</sup>	3	RT <sup>a</sup>	3	3	3	3
10 <sup>5</sup>	3	50° C	3	3	3	3
10 <sup>5</sup>	3	60° C	0	0	0	0
10 <sup>5</sup>	3	70° C	0	0	0	0
10 <sup>5</sup>	3	80° C	0	0	0	0
Exp #2						
10 <sup>5</sup>	3	RT	3	3	3	3
10 <sup>5</sup>	3	50° C	3	3	3	3
10 <sup>5</sup>	3	55° C	2	2	3	3
10 <sup>5</sup>	3	60° C	3	3	3	3
Exp #3						
10 <sup>4</sup>	3	RT	3	2	3	3
10 <sup>4</sup>	3	50° C	2	3	3	3
10 <sup>4</sup>	3	55° C	3	2	3	3
10 <sup>4</sup>	3	60° C	3	0	0	2
10 <sup>4</sup>	3	70° C	1	1	1	1
Exp #4						
10 <sup>4</sup>	3	RT	3	2	3	3
10 <sup>4</sup>	3	50° C	3	3	3	3
10 <sup>4</sup>	3	55° C	3	1	1	3
10 <sup>4</sup>	3	60° C	2	0	0	2
10 <sup>4</sup>	3	70° C	0	0	0	0
Exp #5						
10 <sup>5</sup>	3	RT	3	3	3	3
10 <sup>5</sup>	3	50° C	3	3	3	3
10 <sup>5</sup>	3	55° C	3	1	0	3
10 <sup>5</sup>	3	60° C	1	0	0	3
10 <sup>5</sup>	3	70° C	3	2	2	2/2 <sup>b</sup>

IFAT: Indirect fluorescent antibody test with a positive cut off value at 1:40.

<sup>a</sup>RT: oocysts held at room temperature before mouse inoculation.

<sup>b</sup>One culture became contaminated with bacteria and was discarded.

**Table 2. Initial temperatures and heating times of *Toxoplasma gondii* oocysts in solution during radiofrequency (RF) experiments**

	Initial Temperature (Celsius)	Heating Time to Reach Test Temperature (Minutes)	Effectiveness of Inactivation <sup>a</sup>
Experiment #1			
50°C	17.8	7'40"	-
60°C	21.4	8'15"	+
70°C	25.4	9'48"	+
80°C	27.2	12'44"	+
Experiment #2			
50°C	23.3	7'05"	-
55°C	23.0	7'32"	-
60°C	22.8	8'21"	-
Experiment #3 <sup>b</sup>			
50°C	18.4	6'12"	-
55°C	19.5	8'29"	-
60°C	21.6	13'00"	-
70°C	24.4	17'10"	-
Experiment #4 <sup>b</sup>			
50°C	23.1	6'11"	-
55°C	23.1	5'51"	-
60°C	23.5	9'10"	-
70°C	24.3	12'00"	+
Experiment #5			
50°C	21.4	5'06"	-
55°C	21.1	6'01"	-
60°C	21.6	7'07"	-
70°C	21.8	10'02"	-

<sup>a</sup> "+" indicates complete oocyst inactivation at the test temperature (all mice were negative for *T. gondii* infection); "-" indicates incomplete oocyst inactivation at the test temperature (at least 1 of the 3 mice were positive for *T. gondii* infection based on serology, immunohistochemistry and/or parasite isolation in culture).

<sup>b</sup> The initial temperatures and heating times to reach test temperatures are the mean of 3 samples with 10<sup>4</sup> oocysts per flask individually treated with RF at each test temperature and then pooled for inoculation into 3 mice.

## CHAPTER 5

*Toxoplasma gondii* oocysts in water: concluding perspective

*You still don't know what you're dealing with, do you? Perfect organisms. Its structural perfection is matched only by its hostility....I admire its purity; unclouded by conscience, remorse, or delusions of morality. ~Ash to Ripley in Alien (1979)*

The perfect organism: if such a species was to be defined, *Toxoplasma gondii* would have to be a top contender. Living in undeserved obscurity, *T. gondii* just may be one of the most successful human predators. While able to infect, multiply, and eventually encyst in virtually any nucleated cell, the host usually remains unaware of this obligate intracellular organism's activities. An infected immunocompetent human host may suspect nothing more than infection with the flu. *Toxoplasma gondii* increases its own survival and dissemination by not killing its host outright. Instead, an infection with *T. gondii* promotes a response in the host's immune system which results in this parasite encysting in host tissues, preserving itself for transmission to new susceptible host (reviewed by Denkers and Gazzinelli, 1998). Its tactics for dissemination are equally impressive. *Toxoplasma gondii* infections in prey animals (such as rodents) can result in less fearful animals, increasing the likelihood of being caught and eaten by *T. gondii*'s definitive host, a cat (Hutchison et al, 1980). *Toxoplasma gondii* infection in cats can then result in the production of highly environmentally-resistant oocysts. Persisting for extended periods of time in both terrestrial and aquatic environments, the oocyst allows for even further dispersal of *T. gondii*.



For ingestion of a single, microscopic *T. gondii* oocyst can infect a susceptible host, thereby maintaining existence of this extremely successful parasite (Dubey, 1996; Dubey et al., 1996; Dubey et al., 1997).

Although much research has been published on the cellular stages of *T. gondii* (tachyzoites and bradyzoites), much less is known about the environmental stage, the oocyst. Research is probably limited in this area because of the difficulty of producing *T. gondii* oocysts. To date, *T. gondii* oocysts cannot be cultivated *in vitro* and can only be made by infecting cats and harvesting oocysts from their feces. Unfortunately, there is no guarantee that a cat infected with *T. gondii* will even shed oocysts. Despite these potential limitations, the paucity of information on *T. gondii* oocysts prompted the work of this doctoral thesis.

The unique biochemical structure of the oocyst wall of *T. gondii* still remains undetermined, but the oocyst has been shown in this doctoral research to provide protection for the sporozoite against both physical and chemical disinfectant treatments. In the environment, this natural resistance can increase successful transmission between host species. Based on the studies in this thesis, chemicals (sodium hypochlorite and ozone) commonly used to treat municipal drinking water do not inactivate *T. gondii* oocysts (Chapter 2). Sodium hypochlorite at doses 4 times the concentration currently recommended to treat raw sewage and  $\geq 50$  times the concentration used to disinfect drinking water did not inactivate *T. gondii* oocysts. Furthermore, oocysts remained viable following

24 hours of exposure to 5.25% aqueous sodium hypochlorite (undiluted household bleach). Ozone, at the test dose of 6 mg/L, was equally ineffective in inactivating *T. gondii* oocysts.

The physical methods pulsed UV radiation (PUV) and continuous UV radiation (CUV) evaluated in Chapter 3 were more effective than chemical treatment for the inactivation oocysts. Oocyst inactivation was observed at doses as low as 40 mJ/cm<sup>2</sup> of PUV and 45 mJ/cm<sup>2</sup> of CUV. However, inactivations were not 100% effective and infections were detected in mice inoculated with oocysts treated with PUV doses as great as 755 mJ/cm<sup>2</sup> and CUV doses of 731 mJ/cm<sup>2</sup>. To increase the probability of consistent and complete oocyst inactivation, our results suggest that a minimum of 1000 mJ/cm<sup>2</sup> of UV exposure dose may be required for either PUV or CUV treatment of water.

Study results described in Chapter 4 showed that oocysts were inactivated with radiofrequency (RF)-induced thermal levels of  $\geq 60^{\circ}\text{C}$  in an initial experiment, but not in subsequent experiments conducted under similar conditions. These results indicate that *T. gondii* oocysts may not always be inactivated when exposed to a minimum of 60°C for one minute and that the impact of factors such as water heating time, cooling time, and the volume of water treated must be considered when evaluating this method for oocyst inactivation.

Overall, no method of chemical or physical inactivation evaluated in the course of this doctoral research was 100% effective and reliable at inactivating *T.*

*gondii* oocysts. Therefore, the best method to effectively reduce or eliminate this parasite in drinking water remains undetermined. Ultraviolet radiation is an established practical method of water disinfection that has been shown to effectively inactivate bacteria, viruses, and some protozoa, most notably *Cryptosporidium* and *Giardia* spp. (Betancourt and Rose, 2004; Cheremisinoff, 1995; Hijnen et al., 2006). At higher doses, UV irradiation holds some promise for the inactivation of *T. gondii* oocysts in water. Exposure to UV radiation causes damage to DNA and RNA, preventing cell replication. When sufficient energy is delivered, UV radiation often results in cell death because the cell cannot repair the extensive damage to its DNA (American Water Works Association, 2003; Sincero and Sincero, 2003).

Advantages of using UV radiation for water disinfection, when compared with chemical treatments such as chlorine and ozone, include shorter contact times and decreased formation of toxic chemical by-products. In addition, UV radiation can degrade organic debris, its disinfection properties are not affected by water temperature or pH, and treatment overdose is not a concern. The primary disadvantages of UV radiation are a lack of residual disinfection properties after treatment and reduced efficacy for disinfection of turbid water (Betancourt and Rose, 2004; NDWC, 2000). Low-pressure mercury (Hg) lamps are the most commonly used sources of UV radiation for water treatment as they emit CUV radiation at a wavelength of 253.7 nm, which has germicidal effects when absorbed by cellular material (American Water Works Association, 2003;

Sincero and Sincero, 2003). Typical UV doses used to treat drinking water range from 16 to 40 mJ/cm<sup>2</sup> (Zimmer et al., 2003). PUV radiation is an emergent technology that can generate UV radiation with laser technology or by using a non-mercury flashlamp filled with an inert gas such as xenon and helium that provides broadband, non-coherent radiation comprised of a range of the UV spectrum (10 to 380 nm wavelength), but with an ancillary component of visible and sometimes infra-red (IR) radiation (AWWA, 2003; Dunn and Clark, 1995; Kogelschatz, 1992; Smith et al., 2002; EPA, 1999). PUV technology has been shown to successfully inactivate food-borne microorganisms and pasteurize cow's milk against bacterial growth (MacGregor, 1998; Rowan, 1999; Smith et al., 2002).

How oocysts are able to survive treatment at these high doses of radiation is unknown, but might be attributable to several factors. First, although the oocysts were purified from cat feces, minute amounts of fecal debris were still present in treated oocyst samples and may have allowed oocysts to clump, either shielding some oocysts or resulting in low transmission of UV radiation energy through the sample. Wright et al., (2002) found a decrease in the effectiveness of *E. coli* inactivation in unpasteurized apple cider with UV radiation when concentrations of background material, such as yeast and mold, were present in the sample. When developing a thin film photoreactor for sterilizing liquids with UV light, Shama et al., (1996) described how UV transmission decreases in liquids as the concentration of particulate matter or dissolved organic material increases.

Second, a subpopulation of oocysts may exist within the samples with genetic or structural characteristics that allow for increased resistance to the effects of UV exposure. Craik, et al., (2001) found variations in the levels of sensitivity to UV radiation between multiple batches of *Cryptosporidium parvum* oocysts that appeared to occur independent of oocyst age. Other researchers have also found differences in UV susceptibility between different strains of *E. coli* (MacGregor et al., 1998; Sommer, 2000). Third, it is not known if *T. gondii* oocysts are capable of photoreactivation or nucleotide excision repair following UV-induced damage. A novel gene has been reported that encodes for a DNA repair enzyme (TgDRE) in *T. gondii* that when expressed, increased resistance to UV radiation (Dendouga, 2002). A significant increase in resistance to UV-induced DNA damage was observed when an experimental mutant *E. coli* lacking components of DNA repair mechanisms were transformed with TgDRE cDNA (Dendouga et al., 2002). Although not yet proven, this UV repair enzyme in *T. gondii* may increase effectiveness of UV-induced damage repair, resulting in higher resistance to UV radiation.

Pigmentation is known to increase UV resistance in other organisms (Imshenetsky et al., 1979; Moeller et al., 2005; Ruan et al., 2004), but the presence of oocyst wall-pigments has not been demonstrated for *T. gondii*. The oocyst wall of *T. gondii* is known to be comprised of 3 distinct layers: a 20-50 nm thick, electron-dense outer layer, a 8-10 nm thick, electron-lucent middle layer, and a 50-90 nm thick, moderately electron-dense inner layer (Speer et al., 1998). The

spore coat of environmentally-resistant *Bacillus subtilis* consists of 3 distinct layers: an electron-dense outer layer, a thinner lamellar inner layer, and an electron-dispersed undercoat layer (Moeller et al., 2005; Riesenman and Nicholson, 2000). Research on *B. subtilis* spores suggests that the inner coat layer is important for environmental UV resistance (Moeller et al., 2005; Riesenman and Nicholson, 2000). Further studies are needed to determine whether electrons and their density distribution in the different wall layers of the *T. gondii* oocyst may absorb and reduce penetration of UV radiation.

While complete sterilization of water in which all potential pathogens are inactivated is ideal, this may not be practical or achievable, given current technology. Such a process may be too costly to maintain on a large scale because it would require a significant increase in the concentration and exposure times of chemical or physical treatments necessary for 100% inactivation of microorganisms (Cheremisinoff, 1995). Additionally, increased concentration of applied chemical disinfectants would create undesirable levels of potentially toxic disinfection by-products. The primary objective of water disinfection is to render potential human pathogens harmless and to reduce the number of these pathogens present in water designated for human consumption but without increasing the level of toxic byproducts. This decrease in risk of exposure is legislated under government acts such as the Surface Water Treatment Rule and the Interim Enhanced Surface Water Treatment Rule (EPA 2001 and 2002). Currently, water treatment systems are required to inactivate or reduce the levels

of *Cryptosporidium* by 2-log or 99%, *Giardia* by 3-log or 99.9%, and viruses by 4-log or 99.99%.

There are currently no regulations or approved methods to inactivate *T. gondii* oocysts in water sources. However, as demonstrated in this doctoral project, UV radiation at higher doses holds some promise. Further investigations of UV treatment of *T. gondii* oocysts are warranted to evaluate the practical application of this disinfectant method to treat large volumes of water and to assess the capabilities of UV to inactivate oocysts in water samples with varying turbidity. Additional studies should evaluate UV radiation in combination with chemical disinfection treatments that could provide the additional benefits of residual disinfection.

The research road that resulted in the published findings of this doctoral thesis has been long and winding. The production of *T. gondii* oocysts was a far more daunting task than was originally anticipated. Working with millions of infective sporulated oocysts without infecting myself and co-workers was equally challenging at times. Knowing what I now know and have experienced, would I do it all again? Of course I would! I am proud of my doctoral research and the possible benefits it may provide to public health and the treatment of drinking water. Worldwide, 1.4 children die yearly from lack of access to safe drinking water and adequate sanitation (Unicef, 2005). This should not be acceptable. My ultimate goal in life and through my work is to contribute back to the world in which my daughter Makenna belongs: a world she will inherit

and a world that is brighter because she is part of it. How to successfully control *T. gondii* oocysts in the environment has yet to be determined, but hopefully the work of this doctoral thesis will inspire others to continue exploring all possibilities.

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