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Short Communication

Attempted Detection of *Toxoplasma gondii* Oocysts in Environmental Waters Using a Simple Approach to Evaluate the Potential for Waterborne Transmission in the Galápagos Islands, Ecuador

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Abstract: Toxoplasmosis is a health concern for wildlife and humans, particularly in island ecosystems. In the Galápagos Islands, exposure to *Toxoplasma gondii* has been found in marine avifauna on islands with and without domestic cats. To evaluate potential waterborne transmission of *T. gondii*, we attempted to use filtration and epifluorescent microscopy to detect autofluorescent *T. gondii* oocysts in fresh and estuarine surface water samples. *T. gondii* oocyst-like structures were microscopically visualized but were not confirmed by polymerase chain reaction and sequence analyses. Further research is needed to refine environmental pathogen screening techniques and to evaluate disease risk of waterborne zoonoses such as *T. gondii* for wildlife and humans, particularly in the Galápagos and other naive island ecosystems.

Keywords: *Toxoplasma gondii*, waterborne zoonosis, Galápagos, epifluorescent microscopy

The Galápagos islands attracts nearly 200,000 visitors yearly and human development has contributed to a growing list of challenges for conservation related to population growth, resource consumption, and invasive species (Watkins and Cruz 2007; González et al. 2008). Introduced species, such as domestic cats, represent the greatest conservation threat to endemic flora and fauna (Kaiser 2001; Snell et al. 2002). Direct links between domestic cat predation and island species extinctions are well described (Nogales et al. 2004), but the impacts of cat-associated diseases have received less attention.

As the only definitive host for *Toxoplasma gondii*, felid species play a pivotal role in the introduction and maintenance of the pathogen (Wallace et al. 1972). In the Galápagos Islands, feral domestic cats (*Felis catus*), which are now ubiquitous on inhabited islands and some areas of

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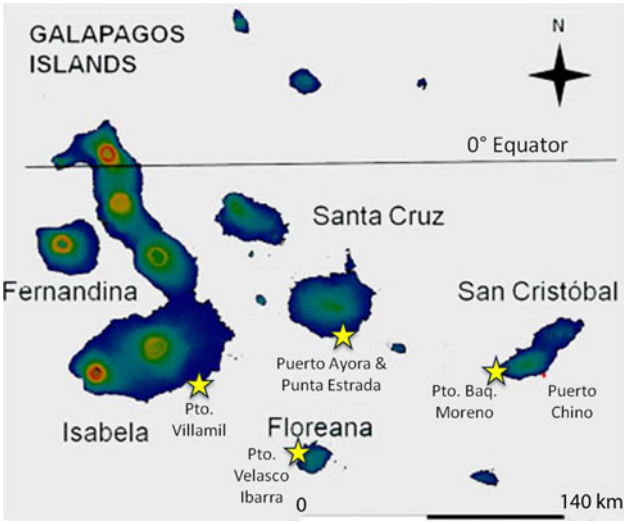

Figure 1. Map of water sampling locations on four islands in the Galápagos. Water sampling sites were primarily located near towns (*stars*) on inhabited islands. Floreana and Isabela have resident penguin colonies in and near town harbors, respectively. Flash flooding and surface runoff were observed on San Cristóbal & Santa Cruz in 2010 in proximity to sampling sites. *Color gradient* indicates elevation from low (*blue*) to high (*red*) (Color figure online).

the Galápagos National Park, have been shown to have a high seroprevalence of *T. gondii* (Levy et al. 2008). Exposure to *T. gondii* has been documented in domestic chickens (Gottdenker et al. 2005), Galápagos penguins (*Spheniscus mendiculus*), and flightless cormorants (*Phalacrocorax harrisi*) in the archipelago (Deem et al. 2010). Clinical toxoplasmosis or mortality has not been observed in the Galápagos, but has been documented in avian species elsewhere (Dubey 2002) including in a Little Penguin (*Eudyptula minor*) (Mason et al. 1991), and naive island species can be highly susceptible (Work et al. 2000, 2002).

Galápagos penguins and flightless cormorants primarily live on Isabela and Fernandina (Vargas and Wiedenfeld 2004; Jiménez-Uzcátegui and Vargas 2008), islands separated by a narrow ocean channel less than 5-km wide. Interestingly, *T. gondii* antibodies have been found in these species on both islands, despite a lack of cats on Fernandina (Deem et al. 2010). Galápagos penguins do move between islands within their range (Nims et al. 2008), but flightless cormorants remain within 1 km of shore and are distinct populations between islands (Duffie et al. 2009). Thus, exposure to *T. gondii* may be occurring by waterborne routes either directly through oocyst transport, or through prey such as fish that can act as mechanical vectors and migrate between islands (Miller et al. 2008; Massie et al. 2010). Similar to what has been described in other coastal ecosystems (Miller et al. 2002; Kreuder et al. 2003; Conrad et al. 2005), we propose that marine avifauna are being exposed to *T. gondii* oocysts via contaminated freshwater runoff. To evaluate this hypothesis, we collected fresh and estuarine water samples from islands with known cat populations and used membrane filtration and epifluorescent microscopy to analyze them for autofluorescent *T. gondii* oocysts (Lindquist et al. 2003). The utility of this simple and inexpensive approach for oocyst detection has been demonstrated under laboratory conditions (Shapiro et al. 2010), but has not been previously described in environmental samples. Our objectives were to test the field application of this new technique for detection of *T. gondii* oocysts on coastal surface waters of the Galápagos Islands and use this simple method to evaluate the potential for waterborne transmission of *T. gondii* to marine species within near-shore environments.

Water samples were collected from four islands (Santa Cruz, San Cristóbal, Isabela, and Floreana) during February 2010 at sites selected based on previous hydrological studies (Fig. 1) (d'Ozouville 2007, 2008; Pryet et al. 2012). At each site, a pump was used to collect 10 l of water from the mid-water column where fresh and saltwater mix. Water samples were qualitatively ranked using an ordinal scale of turbidity with 1 (clear), 2 (slightly turbid), and 3 (turbid/opaque). Salinity, pH, and temperature measurements were collected directly from the sampling location using a Multi 350i handheld water meter (WTW GmbH, Weilheim, Germany). In the laboratory, each 10-l sample was siphoned into one liter aliquots and up to one liter per aliquot was filtered through a modified MicroFil® filtration funnel housing a 5-µm pore size 25-mm mixed cellulose membrane as described by Shapiro et al.(2010). For the most turbid samples (score 3), accumulation of debris on the membrane restricted filtration volumes. To determine the recovery rates of *T. gondii* oocysts, 200 autofluorescent microspheres, which have been validated as *T. gondii* surrogates (Shapiro et al. 2009), were spiked into one liter aliquots of water per turbidity score and similarly filtered. Membranes were then placed on a glass slide in duplicate, covered with glycerol mounting media and a cover slip, and stored at 4°C.

The membranes were examined for *T. gondii* oocysts using a Nikon E800 Hyperspectral microscope with a UV emission filter set (DAPI: excitation 350/50×, emission 460/50 nm band pass filter; Chroma #11000 v3). Structures similar to *T. gondii* oocysts were identified based on

morphology and blue autofluoresence of the sporocyst and oocyst walls. Membranes that contained *T. gondii* oocyst-like structures were further analyzed using nucleic acid extraction and polymerase chain reaction (PCR) analysis for *T. gondii*. Prior to DNA extraction, each membrane was suspended in 5 ml de-ionized water and sonicated. Individual membrane suspensions were divided into three 1.5 ml aliquots. Following centrifugation at 14,000 rpm for 10 min, the supernatant was removed and the 25-µl pellet remaining for each aliquot was subjected to one freeze–thaw cycle. DNA was extracted from each aliquot following a modified QIAamp DNeasy Blood and Tissue kit protocol (Qiagen) for tissue samples with 50 µl of 95°C RNase and DNase-free sterile water was used in the final elution. Two nested PCR reactions were performed on each extraction sample (three aliquots per membrane) using *T. gondii*-specific primers (Homan primers) targeting a 200–300 copy repetitive element (Homan et al. 2000; Shapiro et al. 2010), and the 35 copy B1 gene (Grigg and Boothroyd 2001; Rejmanek et al. 2009). Positive controls [DNA extracted from Type II *T. gondii* tachyzoites in tissue cell pellets and oocysts (Shapiro et al. 2010)] and negative controls (PCR reagents and sterile water) were included in each reaction. Specific primer sequences, PCR reaction components, and thermocycler conditions are provided in supplemental Table 1. Amplification products from internal PCR reactions were separated by electrophoresis in a 2% agarose gel and visualized by UV transillumination. Following purification with ExoSAP-IT®, PCR products were submitted for sequence analysis at the University of California, Davis Division of Biological Sciences DNA Sequencing Facility. Complementary strand sequences were manually edited and assembled with CodonCode Aligner and compared to sequences in GenBank.

A total of 135 l of water from 20 sites was filtered and analyzed by epifluorescent microscopy. Debris in the water samples variably affected the ability to visualize small fluorescent structures, such as autofluorescent oocysts (Fig. 2). Membranes from 50% of sites contained considerable debris (score 3), 35% had moderate debris (score 2), and 15% were relatively clear (score 1). Of the 100 membranes examined, two contained structures similar to *T. gondii* oocysts (Fig. 3). One suspect structure was seen on a membrane from San Cristóbal (Puerto Chino) and two suspect structures were seen on a membrane from Santa Cruz (Punta Estrada) (Table 1). These structures measured 5.3, 7.5, and 9.8 µm in diameter, respectively. Recovery of surrogate microspheres in all samples was low, ranging from 3 to 4% for clear (score 1) to 1% for turbid water (score 3).

DNA was amplified from the two membranes containing suspect structures using Homan primers targeting the 200–300 copy repetitive element, but PCR amplification products were not consistent in size with *T. gondii* positive controls (Fig. 4). DNA was not amplified in aliquots from

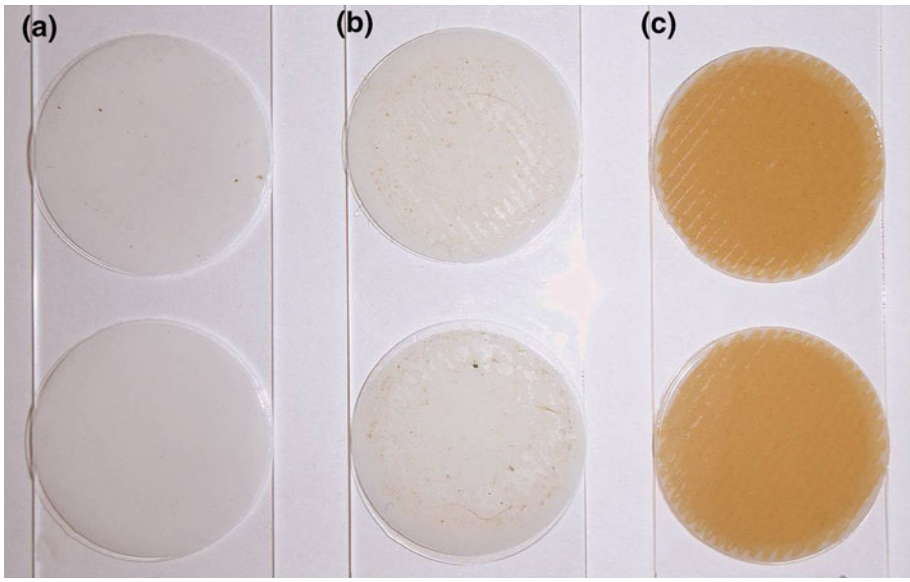

Figure 2. Comparison of membranes acquired from filtration of water samples of varying turbidity scores. Filtration of water samples through cellulose membranes produced variable amounts of debris on membranes. Representative duplicate membranes from water samples with subjective qualitatively ranked turbidity scores of 1 (**a**), 2 (**b**), and 3 (**c**) are shown.

either sample using the B1 primers. Sequence analyses of PCR products produced a clean reverse primer sequence for the San Cristóbal sample, which was highly similar to unclassified marine metagenome samples recovered from surface waters near the Sargasso Sea, Panama Canal, and the Galápagos (GenBank: AACY024030902.1) and Ecuador (GenBank: EJ768524.1) (J Craig Venter Institute 2008). A clear sequence was not attainable from the Santa Cruz sample.

This study provides a preliminary investigation of waterborne transmission of *T. gondii* in the Galápagos Islands using a newly applied approach for *T. gondii* oocyst detection in environmental waters. Structures morphologically similar to *T. gondii* oocysts were observed by epifluorescent microscopy, but PCR and sequence analyses were negative for the parasite. Although these structures were smaller than the reference range for *T. gondii* oocysts (10–12 μm), apparent oocyst size and morphology can become altered under vacuum filtration, as is demonstrated by images of positive control *T. gondii* oocysts on filter membranes in Figure 3b, c which are deformed and smaller than 10 μm. The small number of structures per membrane (1–2) may also have been insufficient to adequately amplify target DNA based on previous detection limits (Shapiro et al. 2010). Alternatively, these structures may have been similar autofluorescent apicomplexan parasites (Davies and Stewart 2000; Lindquist et al. 2003), non-pathogenic organisms, or debris of similar size and fluorescence.

Waterborne transmission of *T. gondii* and subsequent health risks have received considerable attention due to toxoplasmosis and mortality in marine wildlife (Kreuder et al. 2003; Conrad et al. 2005), as well as water-associated outbreaks in humans (Bowie et al. 1997; Jones and Dubey 2010). In addition, numerous other waterborne coccidian species, notably *Cryptosporidium* sp., *Cyclospora cayetanensis*, and other recently identified species, pose potential health risks for humans and marine wildlife (Reid et al. 2010; Baldursson and Karanis 2011; Carlson-Bremer et al.

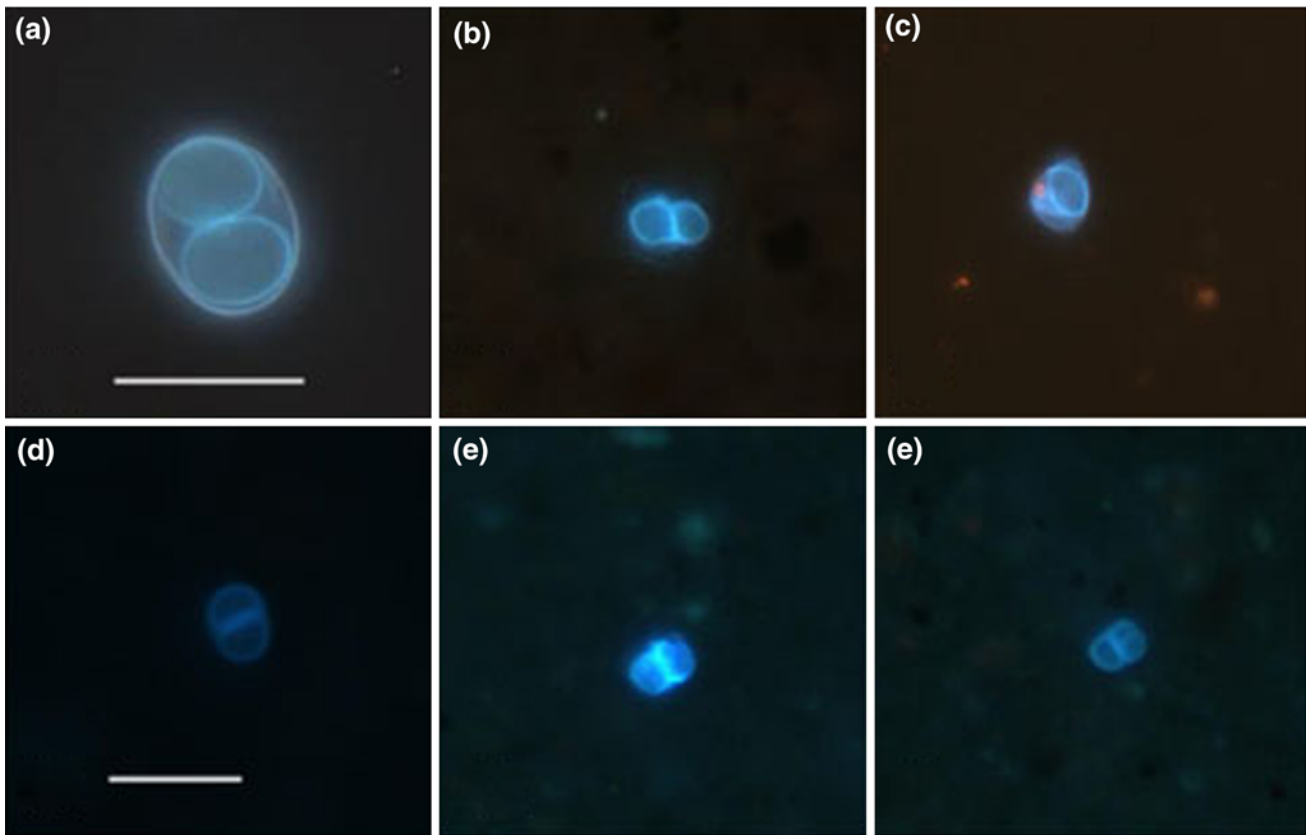

Figure 3. Appearance of *T. gondii* oocysts and oocyst-like structures visualized on filter membranes under epifluorescent microscopy. *Toxoplasma gondii* (Type II) oocysts (Shapiro et al. 2010) shown as positive controls at: (**a**) ×1000 on a wet mount demonstrating characteristic morphology; and (**b–c**) at ×400 on membranes depicting the distortion of typical morphology and size that can result from vacuum filtration. Oocyst-like structures observed on filter membranes from samples collected at (**d**) Puerto Chino and (**e–f**) Punta Estrada at ×400. Structure sizes measured 5.3, 7.5, and 9.8 μm in diameter, respectively. *Scale bars* represent 10 μm.

Table 1. Site characteristics and test results for water sample locations.

Island sample location	Turbidity score (1–3)	Salinity (ppt)	Volume of water filtered (L) Total per site/per membrane	Number of oocyst-like structures seen
Floreana				
Cana Parada	2	34	10/1	0
Finca El Trebol	3	0	5/0.5	0
Laguna de la Montura	2	32	10/1	0
Punta Cormorant	2	34	2.5/0.25	0
Isabela				
Concha la Perla	2	25	10/1	0
El Estero	3	26	5/0.5	0
El Estero outflow	3	8	5/0.5	0
Laguna Cuidad	3	35	2.5/0.25	0
Manzanillo	1	0	10/1	0
San Cristóbal				
Cero Gato	3	0	5/0.5	0
La Policia	3	0	5/0.5	0
Puerto Chino	2	17	10/1	1[a]
La Toma	3	0	5/0.5	0
Tongo Reef	3	32	5/0.5	0
Santa Cruz				
El Baranco	3	1	10/1	0
El Baranco 2	2	0	10/1	0
La Camiseta	1	1	10/1	0
Las Grietas	2	24	10/1	0
La Mision	1	2	10/1	0
Punta Estrada	3	30	5/.05	2[a]

Structures morphologically similar to *T. gondii* oocysts were visualized using epifluorescent microscopy in samples from sites on Santa Cruz and San Cristóbal.

[a]*T. gondii* not confirmed by PCR and sequence analyses.

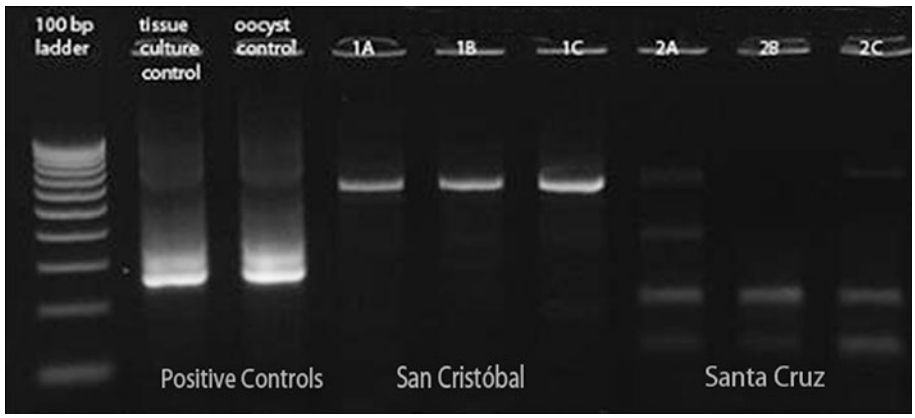

Figure 4. Gel electrophoresis of PCR amplified DNA products from membranes that contained structures resembling *T. gondii* oocysts. PCR analysis using Homan nested primers targeting a 200–300 copy repetitive element of *T. gondii* amplified DNA in the suspect membranes. However, amplicon sizes were not consistent with *T. gondii* positive controls (Type II isolate tachyzoites in tissue culture cells and oocysts).

2011; Colegrove et al. 2011; Rengifo-Herrera et al. 2013). Several direct and molecular methods for oocyst detection in environmental samples have been described with variable success (Davies and Stewart 2000; Lindquist et al. 2003; Dumètre and Dardé 2005; Kourenti and Karanis 2006; Sotiriadou and Karanis 2008; Moulin et al. 2010; Gallas-Lindemann et al. 2013; Karanis et al. 2013). It is clear from this work that detection of *T. gondii* and other coccidian oocysts in environmental water remains challenging due to likely low and highly variable oocyst concentrations, as well as inhibition by other water constituents.

Membrane filtration of water samples provides a relatively fast and inexpensive method for parasite concentration compared with other approaches that use capsule filtration followed by immunomagnetic separation (Dumètre and Dardé 2005) or sucrose gradient separation (Moulin et al. 2010). Under laboratory conditions, Shapiro et al. (2010) demonstrated the utility of this method following initial water filtration steps, and showed that subsequent analysis of the filter membranes using epifluorescent microscopy was more sensitive than conventional PCR for oocyst detection in both fresh and marine water. However, application of this method using membrane filtration as the sole means of parasite concentration for environmental water samples has not previously been described. The simple two-step process described here potentially offers a unique approach for detection of *T. gondii* and other autofluorescent coccidia in relatively isolated and resource limited locations such as, but not limited to, the Galapágos. The low recovery rates of surrogate microspheres and detection of *T. gondii*-like structures in this study suggest that modifications to more selectively preserve oocysts and increase removal of other water constituents may improve the effectiveness of this approach for environmental samples. These observations substantiate previous work that reported lower recovery rates in more complex sample matrices (Shapiro et al. 2010). In addition, the similarity in appearance among autofluorescent apixcomplexans such as *Hemmondia*, *Neospora*, *Sarcocystis*, and *Besnoitia* (Lindquist et al. 2003), can impede definitive identification and necessitates PCR and sequence analysis for confirmation.

Despite the lack of confirmation of *T. gondii* in the samples tested here, contamination of surface waters remains a plausible route for *T. gondii* exposure in marine avifauna of the Galápagos (Conrad et al. 2005; Deem et al. 2010). Feral domestic cats are frequently observed within coastal watersheds, including those with populations of penguins and cormorants (Vargas et al. 2006), and high volumes of surface runoff are known to occur, primarily during El Niño-Southern Oscillation events (Glynn 1988; Trueman and d'Ozouville 2010; Violette et al. 2013). Specifically, the water sample collected at Puerto Chino was from a seasonal freshwater river that had formed following heavy rains 2 days prior and was delivering surface runoff directly into the ocean. Multiple feline scat piles were observed along the river bank during sample collection, and cats are known to be common in the area. Similarly, La Laguna at Punta Estrada is surrounded by residential development, and the area has an abundant cat population. Although the incidence of oocyst shedding among cats is generally low, one cat can shed up to 1 billion oocysts over several days following initial infection (Dubey and Frenkel 1972; Dubey 1995). Oocysts are incredibly resistant to environmental degradation and are known to survive in seawater for at least 2 years (Lindsay and Dubey 2009). Furthermore, unmanaged feral cats are associated with higher *T. gondii* prevalence and odds of oocyst shedding due to primary consumption of intermediate hosts (Konecny 1987; Afonso et al. 2007; VanWormer et al. 2013).

In a broader context, the zoonotic potential of *T. gondii* presents a public health concern for island residents and visitors. The incidence of toxoplasmosis and *T. gondii* seroprevalence in human communities in the Galápagos is unknown but likely high. Globally, highest infection rates of *T. gondii* are associated with rural areas, lower socioeconomic status, and drinking unfiltered water (Bahia-Oliveira et al. 2003). In the Galápagos Islands, fresh water is limited and the geologic structure of porous rock facilitates drainage and rapid mixing of surface and groundwater (Violette et al. 2013). Consequently, the overall lack of waste water treatment, unregulated groundwater pollution, and improper waste management practices are contributing to environmental pathogen pollution and health risks (González et al. 2008; Liu and d'Ozouville 2013). Understanding the epidemiology of toxoplasmosis and other waterborne diseases should be a top priority for protecting the health of wildlife and human communities, particularly in sensitive regions such as the Galápagos. Toward this goal, we suggest that further research is needed with refined environmental detection methods to evaluate pathogen exposure and disease risk.

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