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Epidemiology of Fecal Protozoa at the Human, Animal, and Environmental Interface

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Epidemiology of Fecal Protozoa at the Human, Animal, and Environmental Interface

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ABSTRACT

Cryptosporidium and *Giardia* are important disease agents for both humans and animals worldwide. Unlike many pathogens, their capacity for surviving in soil and water for extended periods of time enables environmental transmission. Both parasites replicate within warm-blooded vertebrate hosts and are excreted as an environmentally resistant oocyst or cyst, respectively. New hosts become infected through ingestion of oocysts or cysts in contaminated water, food, or soil. The objective of this thesis work was to utilize an epidemiologic approach to identify environmental factors associated with the prevalence of *Cryptosporidium* and *Giardia* spp. in California coastal wetlands, as well as to ascertain the prevalence and identify potential transmission pathways within the animal community in the Virunga Massif, Rwanda.

Because *Cryptosporidium* and *Giardia* are waterborne parasites, key factors in wetland ecology were examined for their impact on parasite levels in water. First, concentrations of these parasites within three coastal California wetlands were determined. Proximity to livestock operations and recent rainfall events were associated with increased pathogen levels. Second, experimental models mimicking wetland characteristics were used to isolate the effects of several parameters. Vertical settling columns identified increased salinity as leading to slower settling of *Giardia* and horizontal flow mesocosm tanks identified increased salinity as leading to slower removal of *Cryptosporidium*. Furthermore, presence of vegetation led to enhanced

removal of *Cryptosporidium* oocysts, *Giardia* cysts, and *Toxoplasma gondii* surrogates from surface water in the mesocosm tanks.

The impact of *Cryptosporidium* and *Giardia* on animal health is important not only because many wildlife species are suspected to be reservoirs of these parasites for humans, but also because the effect of infection with these parasites on health is of concern in vulnerable wildlife species. To assess the impact of these parasites on mountain gorillas (*Gorilla beringei beringei*), a critically endangered primate restricted to two forests located in Rwanda, Democratic Republic of Congo and Uganda, an investigation was conducted comparing protozoal prevalence and strain identity in mountain gorillas, domestic cattle, and forest buffalo (*Syncerus caffer nanus*). *Giardia* was found in all three species, and genotyping results identified genotypic assemblage B in the gorilla samples and assemblage E in the cattle samples. While this study did not confirm a transmission pathway between cattle and gorillas using the forest buffalo as an intermediary, it suggests that future work should also evaluate humans as potentially contributing to fecal pathogen contamination within the forest.

Environmentally transmitted protozoa are of global health concern due to their extended survival outside of a host, coupled with the relative difficulty and expense of detection in environmental samples. The drivers for protozoal disease transmission, as detailed in Chapter 1, emphasize the key role that environmental change has on *Giardia* and *Cryptosporidium*. Some of these changes are evaluated in Chapters 2 and 3, which highlight research conducted on protozoal transport through coastal wetlands. Health impacts of these protozoa on wildlife and livestock cannot be discounted and Chapter 4

surveys the presence of *Cryptosporidium* and *Giardia* in a complex wildlife community in Rwanda.

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Introduction: Drivers for the Transmission of Environmentally Transported Protozoal Diseases

Introduction

Environmental transmission plays a key role in the epidemiology of fecal-oral pathogens. The conceptual epidemiologic triad model includes the environment in addition to host and pathogen as key contributors to disease, yet the relationship between the environment and the pathogen is not always addressed. For parasites that are transmitted via environmental exposure (e.g., in water or soil), factors related to the changing environment are critically important (Figure 1.1). Environmentally-transmitted pathogens can include bacteria (e.g., *Escherichia coli, Salmonella*), viruses (e.g., Adenovirus, Norovirus, Rotavirus), and helminthic parasites (e.g., *Fasciola, Ascaris*), as well as protozoa such as *Cryptosporidium, Giardia,* and *Toxoplasma*. These protozoans can persist in the environment in oocyst or cyst forms for extended periods of time, and therefore alterations to the environment may directly impact the transmission and the spread of disease between humans and animals. This dissertation evaluates how environmental changes affect the transmission and transport of protozoa in wetland and terrestrial systems.

Parasite Overview

Cryptosporidium

Cryptosporidium is a member of the Apicomplexa phylum, and is related to other parasites of human health importance such as the vector-borne parasites *Plasmodium* and

Babesia, and the environmentally transmitted parasites *Cyclospora* and *Toxoplasma*. *Cryptosporidium* exists in the environment as an oocyst, and once it infects the host through contaminated food or water, it becomes an obligate intracellular parasite [1]. There are nineteen described species of *Cryptosporidium*, of which six have been identified as zoonotic: *C. parvum* is responsible for the majority of zoonotic human infections [2]. *Cryptosporidium* is of particular public health concern because the ID_{50}, or the number of parasites required to establish infection in a human, is low: for human volunteers who received three different isolates of *C. parvum* oocysts, the ID_{50} was found to be as few as 9 oocysts [3]. Pre-weaned ruminants (e.g., cows, sheep, and goats) are particularly susceptible to infection [1], and are capable of shedding large numbers of oocysts in their feces, thereby contaminating soil and water [4].

Giardia

Giardia is a member of the Diplomonada phylum. Like *Cryptosporidium, Giardia* is fecal-orally transmitted between vertebrate hosts. Once in the host, *Giardia* does not invade cells, but rather excysts in the stomach with each cyst releasing two trophozoites, which either multiply by binary fission on the luminal surface of the intestine, or encyst in order to be excreted and be infective for a new host. There are five species of *Giardia*, of which only *Giardia duodenalis* (syn. *G. intestinalis, G. lamblia*) has been identified as infectious to humans; and its zoonotic potential being first recognized by the World Health Organization (WHO) in 1979. There are seven assemblages or genotypes of *G. duodenalis*, currently identified as A-G. Assemblages A and B are considered to be zoonotic, as they have been identified in both human and animal hosts. The remaining five genotypes are characterized by their host specificity: assemblages C and D are found

in canids, assemblage E is found in artiodactylids (e.g., cattle and sheep), assemblage F is found in felids, and assemblage G is found in rodents [2] and [5]. As is characteristic of *Cryptosporidium*, *Giardia* has a low ID_{50} in humans, of only 10 cysts [6].

Toxoplasma

Toxoplasma gondii is related to *Cryptosporidium* as they are both members of the Apicomplexa phylum, but has a very different life cycle as compared to either *Cryptosporidium* or *Giardia*. Two phases of asexual development occur in intermediate hosts, which include warm blooded animals and humans. One of these phases is the formation of tissue cysts, which can occur in any tissue in the host, but have a predilection for neural tissue. Ingestion of these tissue cysts by the definitive host, members of the Felidae family (cats), leads to sexual multiplication and oocyst formation in the small intestine of the host. These oocysts are then excreted and survive in the environment to be ingested by a new intermediate host. A single cat may shed over 100 million oocysts in the environment [7], which is of concern because the ID_{50} of *Toxoplasma* in mice is only 24 oocysts [8], with some studies reporting ID_{50}s as low as 1 in rodents [9] and [10].

Environmental Survival of Oocysts and Cysts

Cryptosporidium, *Giardia*, and *Toxoplasma* persist in the environment as oocysts or cysts, which are resistant to a variety of environmental conditions (Table 1.1): *Cryptosporidium* oocysts can survive for over 7 months in cool water at low salinity [1] and [11], *Giardia* cysts can survive for 11 weeks under similar conditions [11], and *Toxoplasma gondii* oocysts can survive for 6-54 months [12] and [13]. Most critical for the survival of these parasites is the presence of moisture, as desiccation is a primary

means for inactivation. There are a number of other factors that affect (oo)cyst survival, such as predation by other organisms, including heterotrophic bacteria and rotifers [1] and [14].

The difficulty in removing and inactivating parasites from drinking water sources is a constant concern for public health protection. Effective water treatment for human drinking water requires the application of multiple methods for protozoal removal, including physical filtration and disinfection through ozone or ultraviolet (UV) exposure. The level of disinfection required varies among protozoa. Ultraviolet inactivation of *Cryptosporidium* and *Giardia* requires contact time concentrations of 1-50 mJ/cm^2 [15]; however, UV inactivation of *Toxoplasma* requires at least 100 mJ/cm^2 [16]. Typical UV exposure in water treatment plants range from 40-186 mJ/cm^2 [17]. *Cryptosporidium* is also inactivated by exposure to 3-4 mg/L x 1 min of ozone, but *Toxoplasma* remains infectious even after exposure to 6 mg/L x 12 min of ozone [18]. A review of water treatment plants in the Netherlands found that the average contact time for ozone treatment was 2.2 mg/L for surface water and 2.3 mg/L for groundwater – drastically insufficient for protozoal inactivation, prompting requirements that upgrades and new facilities would begin implementing ozone doses of over 6 mg/L, thus targeting microbial contamination of water more effectively [19].

Detection of Protozoal Pathogens

Detection of protozoan parasites in both water and feces is challenging. While many bacteria can be selectively cultured, enabling straightforward detection, (oo)cysts do not readily replicate, therefore necessitating the application of methods that directly detect a typically small number of parasites in any given environmental sample. To

circumvent this limitation, studies have been conducted to determine if fecal indicator bacteria can be used as proxies for *Cryptosporidium* and *Giardia*: these studies were not able to demonstrate a strong linear correlation between protozoal and bacterial counts [20] and [21]. However, Chapter 2 confirmed that fecal indicator bacterial (*Escherichia coli*) levels (high versus low), as determined according to Environmental Protection Agency (EPA) guidelines, can be a good indicator for the presence of protozoa, but cannot be used for linear correlations between absolute numbers of *E. coli* and protozoa [22].

Consistent with this limitation, the EPA guidelines for detection and quantification of *Cryptosporidium* and *Giardia* in water [23] require passing environmental water though an expensive filtration device and performing immunomagnetic separation (IMS) to concentrate parasites, followed by staining the resulting concentrate with direct fluorescent antibody (DFA) and visualizing (oo)cysts under epifluorescent microscopy. While this is the standard methodology, several researchers have since suggested modifications to increase yield of (oo)cysts and thereby decrease false negative results and underreporting, including using heat dissociation of the IMS process rather than standard acid dissociation [24] and [25], and using ultrafiltration devices that are more cost effective and can concentrate a wider range of microbes [26] and [27]. Additionally, *Toxoplasma* is not detectable using available IMS assays, although these alternate ultrafiltration methods have been shown to be effective for concentrating *Toxoplasma* oocysts from water for quantification [28].

Microscopy and DNA amplification by PCR methods are both used for detection of parasites in feces. However, microscopy may underestimate prevalence, and PCR may

overestimate it, depending on the specificity of the locus: one study found that *Giardia* prevalence as determined by microscopy (visualization of intact parasites) ranged from 3-5%, but that prevalence as determined by PCR amplification (detection of *Giardia* antigen) of the same samples was dramatically higher, at 20-80% [5]. An advantage of PCR methods for the detection of protozoa is that it allows for the identification of genotypic assemblages or subtypes, offering additional insight on the pathogen. That said, the preferred locus for genotypic analysis of *Giardia* assemblages remains a topic of debate, and may change as the molecular characterization field continues to evolve [29], [30] and [31].

Zoonotic Transmission

A large number of recent studies using PCR for genotypic level characterization of *Cryptosporidium* and *Giardia* has greatly increased our understanding of these protozoa , but has fueled debates surrounding whether or not these parasites are in fact zoonotic. It is generally agreed that some genotypes of *Cryptosporidium* are zoonotic [32]. However, many studies evaluating the molecular epidemiology of *Giardia* and assessing its zoonotic potential have been conducted in developed countries, in dense study populations like daycare centers and nursery schools, where the risk of transmission among humans is high, but the risk of transmission between humans and animals is low. Such studies have concluded that *Giardia* should not yet be defined as a zoonotic pathogen [5] and [33]. Inappropriate comparisons of populations have been made to support this hypothesis: a review of genotyping studies conducted worldwide [32] showed that *Giardia* subtypes have been found in both humans and domestic animals, but claimed that differences in subtype distribution (e.g., animals were more

likely to have subtype AI, whereas humans were more likely to have subtype AII) did not support zoonotic transmission of *Giardia*. However, this review did show that of the thirteen countries compared for human *Giardia* infection, four were among the nine countries compared for animal *Giardia* infection, and in these four countries, the *Giardia* subtypes found in animals were also found in humans. Furthermore, the review did not include any studies evaluating *Giardia* cysts of animals in developing countries, where human-animal contact may be more frequent.

Many studies evaluating the zoonotic potential of *Giardia* also note that their results do not consider the effect of waterborne transmission through creeks, wetlands, and other water sources contaminated by wildlife or livestock feces [5] and [33]. A recent study posited that the occurrence of *Giardia* in aquatic wildlife suggested the possibility of zoonotic transmission, but acknowledged that based on their isolates from human and animal fecal samples, it was difficult to determine which came first: wildlife fecal contamination or human sewage contamination of surface water [34].

Although the debate over *Giardia*'s zoonotic potential may be ongoing, future studies should continue to consider the environmental aspects of the cyst's transmission, such as the work presented herein, and utilize appropriate population comparisons in determining zoonotic transmission.

Impacts of Protozoa

Human Health Impact

Zoonotic pathogens are a significant source of disease in human populations. Globally, 56 zoonoses are responsible for 2.5 billion cases of human illness and 2.7

million human deaths every year [35]. Of these, zoonotic gastrointestinal diseases, including protozoa as well as bacteria and other pathogens, contribute to 1.5 million human deaths [35]. Immunosuppression and the occurrence of HIV have amplified the effects of environmentally transmitted protozoa in humans; infection of an immunosuppressed host by *Cryptosporidium* can be more virulent and more fatal than in immune-competent hosts [36].

The impact of protozoal pathogens on human health varies greatly across levels of development of countries. In developed countries, the proportion of gastrointestinal disease burden associated with food-borne zoonoses, including the environmentally transmitted protozoa, varies from 30-50% [35]: in the United States, the highest incidence of reported giardiasis was 30 cases per 100,000 people, while in Europe the reported incident rate was 58.1 cases per 100,000 people [37]. Comparatively, in developing countries the prevalence of protozoal infection is very high: in a study of 1,398 schoolchildren in Agboville, Côte d'Ivoire, 13.9% were positive for *Giardia* by microscopy [38]; in 583 children younger than five years old in Butare, Rwanda, 19.8% were positive for *Giardia* by microscopy [39]; and in 1,636 children in Nigeria, the prevalence of *Cryptosporidium* ranged from 15.6-19.6% [40]. While children are more likely to be susceptible to *Giardia* and *Cryptosporidium* infections, the higher prevalence observed in developing countries may also be attributed to differences in the children's environments, such as lower hygiene status, less access to clean water, and higher contact with animals; however, none of these risk factors were measured in the studies evaluating protozoal prevalence in the developing world.

Toxoplasma gondii infection has serious effects on human health by its transmission through environmental, foodborne, and congenital means. Worldwide, 2 million people are thought to be infected with *Toxoplasma*, which causes approximately 10,000 human deaths per year [35]. *Toxoplasma* infections in immuno-competent humans are typically asymptomatic, although recent evidence has linked acquired (non-congenital) *Toxoplasma* infections with retinochoroiditis and other ocular diseases [7] and [41]. However, primary infection of pregnant women with *Toxoplasma* has been shown to cause spontaneous abortion, stillbirth, and congenital toxoplasmosis is associated with severe ocular and neurological effects [42]. More recently, evidence suggests an association between *Toxoplasma* infection and increased rates of suicide and schizophrenia, perhaps related to tissue cyst formation in the brain [43], [44], [45] and [46].

Animal Health Impact

Animals, both domestic and wild, play key roles in the environmental transmission of protozoan parasites. Both host-specific and zoonotic genotypes and assemblages of *Cryptosporidium* and *Giardia* have been documented in a wide range of animal species. Multiple mammalian hosts are known to be reservoirs for human infection with *Cryptosporidium* [47]. As well, the presence of livestock near water sources is a major factor for oocyst contamination of the environment: livestock can shed 2.8×10^4 to 1.4×10^5 *Cryptosporidium* oocysts per animal per day [48], which can then be directly or indirectly transmitted to other hosts, and contribute to the environmental contamination of parasites in soil and water.

Environmental contamination provides a route of transmission between humans, domestic animals, and wildlife. Evaluation of environmentally transmitted protozoa in wild mountain gorillas and domestic cattle in both the Virunga Massif and the Bwindi Impenetrable National Forest revealed that both species have a high prevalence of *Cryptosporidium* and *Giardia*, suggesting a possible transmission route [29], [49], [50] and [51]. To further investigate the epidemiology of these parasites in this tropical ecosystem, in Chapter 4 I examined the prevalence of *Giardia* in mountain gorilla, forest buffalo, and domestic cattle in the Volcanoes National Park, Rwanda. While *Giardia* is often asymptomatic in wildlife [52], given the critically endangered nature of the mountain gorilla, it is important to consider the long-term effects these parasites may have on the health, survival and reproduction of this endangered species [53]. Furthermore, the large numbers of people who enter the gorillas' protected forest habitat are also at risk for contracting zoonotic pathogens from the mountain gorillas through fecal contamination of the environment.

While the definitive hosts for *Toxoplasma* are felids, a wide range of warm-blooded vertebrates can become infected by *Toxoplasma* oocyst consumption and develop clinical disease. That said, foodborne transmission of *Toxoplasma* may be less impactful than environmental transmission in some regions: cattle are considered poor hosts for *Toxoplasma*; and the prevalence of *Toxoplasma* in pigs has significantly decreased in US facilities as better management practices have been implemented; few studies have looked at *Toxoplasma* in commercial poultry, although one study did not find any viable oocysts from chicken breast meat samples [41].

Conversely, environmental transmission has been implicated in *Toxoplasma* infections in numerous marine mammal species [41], which do not have direct contact with felids, nor are terrestrial mammals prey for marine mammals. *Toxoplasma* was identified as a primary cause of death for 16% (17/105) of California sea otters (*Enhydra lutris nereis*) attributed to severe encephalitis associated with inflammatory brain infections with confirmed *T. gondii* tachyzoites [54]. It has been suggested that *Toxoplasma* is transported from inland water sources to the near-shore marine ecosystem, where it is able to infect marine mammals, perhaps through infected invertebrate prey [55].

Economic Impact

In addition to the health effects of protozoan parasites on humans and animals, fecal-oral protozoal infections result in significant economic costs to society for prevention, treatment, and remediation. For example, the 1993 *Cryptosporidium* outbreak in Milwaukee, WI, which affected more than 400,000 people [56], is estimated to have cost $96.2 million for medical treatment costs and productivity losses [57]. Individual infections with protozoa like *Cryptosporidium* and *Giardia* are costly as well: New Zealand spends approximately NZ$1.5 million per year to treat approximately 2,400 people [58].

To minimize the risk of infection, developed countries expend considerable resources on drinking water treatment. Treatment of surface water for microbial pathogens in the United States costs an estimated $307 million annually, with $118.6 million of that attributed to treating water for livestock production [59]. The worldwide use of ozone as a water disinfection method for microbial pathogens is estimated to cost

$361 million as of 2010, and expected to exceed $597 million by 2016. Approximately 83.5% of this ozone usage is centered in three highly developed regions: the East Asian Pacific, North America, and Western Europe [19]. Developing countries, where treatment costs are prohibitive and therefore rarely undertaken, therefore suffer from much higher levels of protozoal contamination of drinking water.

Drivers of Protozoal Transmission

Landscape Change

Landscape change has led to disease transmission; most studies have focused on the emergence of vector-borne pathogens [60], [61], [62] and [63]. Landscape change, defined as habitat alteration through deforestation, fragmentation and climate change, certainly affects vector dynamics, but it also alters the environmental transmission and transport of protozoa, while altering direct transmission dynamics through changes in the nature and frequency of human - animal contact.

Environmentally transmitted protozoa contaminate the environment regardless of any external drivers leading to outbreaks and transmission. Given the public health concerns about *Cryptosporidium* and *Giardia*, most developed countries routinely monitor drinking water sources and surface waters for protozoa to establish background levels of contamination, as well as to identify increases in concentrations that may lead to an outbreak [20] and [64]. In order to identify risk factors related to increases in protozoal concentrations, Chapter 2 describes efforts to monitor the prevalence of *Cryptosporidium*, *Giardia*, and fecal indicator bacteria over a 2-year period in California coastal wetlands that transport water between agricultural and livestock operations and downstream human- utilized recreational waters [22].

Failure to detect and respond to elevated protozoal parasite levels in drinking and surface waters has resulted in numerous human disease outbreaks. In the United States, 143 waterborne outbreaks between 1971 to 2006 were confirmed to be caused by protozoal contamination of drinking water [65], including the 1994 *Cryptosporidium* outbreak in Milwaukee, WI that affected more than 400,000 people [56]. Abroad, a *Cryptosporidium* outbreak in Northwest Wales in 2005 led to 231 laboratory-confirmed cases [66], while numerous small outbreaks have been documented throughout Australia [67]. In addition to water contamination, soil can serve as a route of transmission for these protozoan parasites, as well as *T. gondii* oocysts: two recent studies surveyed soil collected from public parks and identified *Toxoplasma* DNA in 17.8% of samples from locations throughout Poland, including 15.7% in children's sand boxes [68], and 16% of samples taken from locations in Wuhan, Hubei, China [69].

Climate change-related reductions in freshwater sources for drinking water supply, greater precipitation, and increased adverse climate events such as storms, flooding, and wetter conditions, may affect the prevalence of environmentally transmitted protozoa like *Cryptosporidium* and *Giardia*. Rainfall and flooding have been associated with increased protozoal contamination of surface water [20], [22], [61] and [71] in part due to pathogens in animal manure and animal environments washing into human water supplies. In a 1997 study, scientists who evaluated drinking water security in the United Kingdom hypothesized that climate change may alter the availability of subterranean freshwater, leading to the use of more readily contaminated surface sources of drinking water [70]. Furthermore, alterations to water conditions, such as turbidity and salinity, may directly improve the survival of *Cryptosporidium* and *Giardia* [72].

Habitat changes such as deforestation and fragmentation, changes in host distribution, or general conversion of natural landscapes also lead to gastrointestinal protozoal disease transmission [52] and [74]. A study of parasites in red-tail guenons in Kibale National Park, Uganda found a higher prevalence of seven parasites, including *Giardia* and *Entamoeba histolytica*, in heavily logged sections of the forest as compared to undisturbed sections [73]. A study of land conversion to dairy farming identified an increased public health risk for *Giardia* infection [75], and a study of *Toxoplasma* transport in wetlands showed that increased degradation (as defined by absence of vegetation and higher water velocities) led to greater concentrations of protozoa recovered downstream [76]. In order to more specifically identify wetland characteristics that reduce protozoa in surface waters, Chapter 3 describes utilizing controlled settling column and wetland mesocosm tank methodologies that found the presence of vegetation and salinity were effective at protozoal removal from surface water. As well, in Chapter 4, the prevalence of *Giardia* was determined in mountain gorilla, forest buffalo, and cattle in the Volcanoes National Park, Rwanda, an area which is under extreme pressure from human settlements adjacent to the park as well as daily visits from human researchers and tourists.

Increased Trade and Travel

In addition to landscape changes, the increased global movement of humans and animals for both travel and trade affects disease transmission [62], [77] and [78]. "Traveler's diarrhea" is often caused by environmentally-transmitted protozoa obtained through consuming contaminated food and water. A review of the FoodNet database found that of 64,039 gastrointestinal infections, 13% were in people who had recently

travelled [79]. Specifically, *Cryptosporidium* was identified at a rate of 2.8 cases per 100,000 travelers [79]. Contaminated food is also easily transported across the globe: foodborne disease outbreaks such as a 1996 United States outbreak of *Cyclospora* was associated with imported raspberries from Guatemala [80] and two outbreaks in Missouri, in 1999 and in British Columbia, Canada in 2001 were associated with imported basil contaminated with *Cyclospora* [81] and [82]. Importation of illegally traded wildlife and wildlife products is also a concern with regards to movement of parasitic diseases: *Giardia* was found in illegally imported birds, including the MacQueen's Bustard [83], and *Toxoplasma* has been found in exotic bushmeat traded and imported for consumption [84] and [85].

Global and Local Management Failure

Global poverty and water scarcity are highly related to disease burden. Of the 20 most impoverished countries, measured by malnutrition due to inadequate energy or protein intake, 13 were identified as having the highest burden of zoonotic disease, and 11 of these countries were identified as having the highest levels of endemic (versus outbreak or emerging) zoonotic disease prevalence; most of the countries with outbreak scenarios rather than endemic zoonotic disease were from developed countries [35]. The difference in endemic versus outbreak levels of disease is a symptom of the variation in protozoal transmission and risk factors between developed and developing countries. Gastrointestinal infections differ between developed and developing countries in numerous ways including prevalence of co-infections with other parasites, co-morbidity with metabolic diseases such as malnutrition, and exposure to other environmental risk factors, such as poor hygiene [86] and [87].

Water scarcity is an important factor contributing to protozoal disease transmission because it forces people to use unconventional and potentially contaminated water resources [88]: in Yemen, the World Health Organization calculated that only 25% of the population has access to clean water, and consequently, the prevalence of protozoal infection was 31%, including *Giardia* and *Entamoeba histolytica/dispar* prevalences of 17.7% and 17.1%, respectively in Yemen [89].

Civil upheaval can lead to disease transmission when breakdowns in civil structures, functions and measures lead to public health risks. For example, the Vaal River Barrage in South Africa was originally built as a storage facility for potable water, but the fall of apartheid in 1994 and subsequent governmental changes and social upheaval led to periodic breakdowns of government capacity, and precluded local authorities from addressing contamination of the stored Vaal River Barrage water with fecal pathogens including protozoa such as *Cryptosporidium* and *Giardia* [90].

Conclusions

Cryptosporidium, Giardia, and *Toxoplasma* and other environmentally transported pathogens are important causes of human and animal disease. Drivers of disease transmission, such as water security and availability, affect environmental pathogen transport throughout the world. However, drivers of disease transmission differ among countries with varying economic statuses. Intensive molecular epidemiologic studies occurring predominantly in developed countries have biased us away from studying environmentally-transmitted diseases in developing countries, which underestimates the impact of environmentally-transmitted pathogen burden in developing countries. Future studies should strive for a global focus on these pathogens. Importantly,

Cryptosporidium, *Giardia*, *Toxoplasma*, and other environmentally transmitted protozoa should be evaluated from a One Health perspective, simultaneously considering the interrelatedness of the effects on humans, animals, and the environment.

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Table 1.1: Comparison of *Cryptosporidium*, *Giardia*, and *Toxoplasma* survival under various environmental conditions

			Cryptosporidium		***Giardia***		***Toxoplasma***	
Material	**Temperature***	**Salinity****	**Survival**	**Reference**	**Survival**	**Reference**	**Survival**	**Reference**
Water	Frozen		>3 months	[11]	<1 week	[11]	1 month	[13], [91]
	Cold	Low	3- 7 months	[1], [11], [92], [93], [94]	3 months	[11]	6-54 months	[12], [13]
		High	1-3 months	[1], [92], [95]				
	Warm	Low	2-3 months	[1], [11], [92], [93]	2 weeks	[11]	1-3 months	[12], [13]
		High	1-2 months	[92]				
Soil	Frozen		2.5 months	[11]	<1 week	[11]		
	Low		2 months	[11]	2 months	[11]	3-7 months	[8]
	High		1 month	[11]	1 week	[11]		
Feces	Frozen		>3 months	[11]	<1 week	[11]		
	Low		2 months	[11]	1 week	[11]	2-11 months	[13], [96], [97]
	High		1 month	[11]	1 week	[11]	12-18 months	[13], [96], [97]

*Temperature Frozen = <= -4 °C, Low = 4-20 °C, High= 25-40 °C

**Salinity Low = 0-15 ppt, Salinity High = greater than 15 ppt

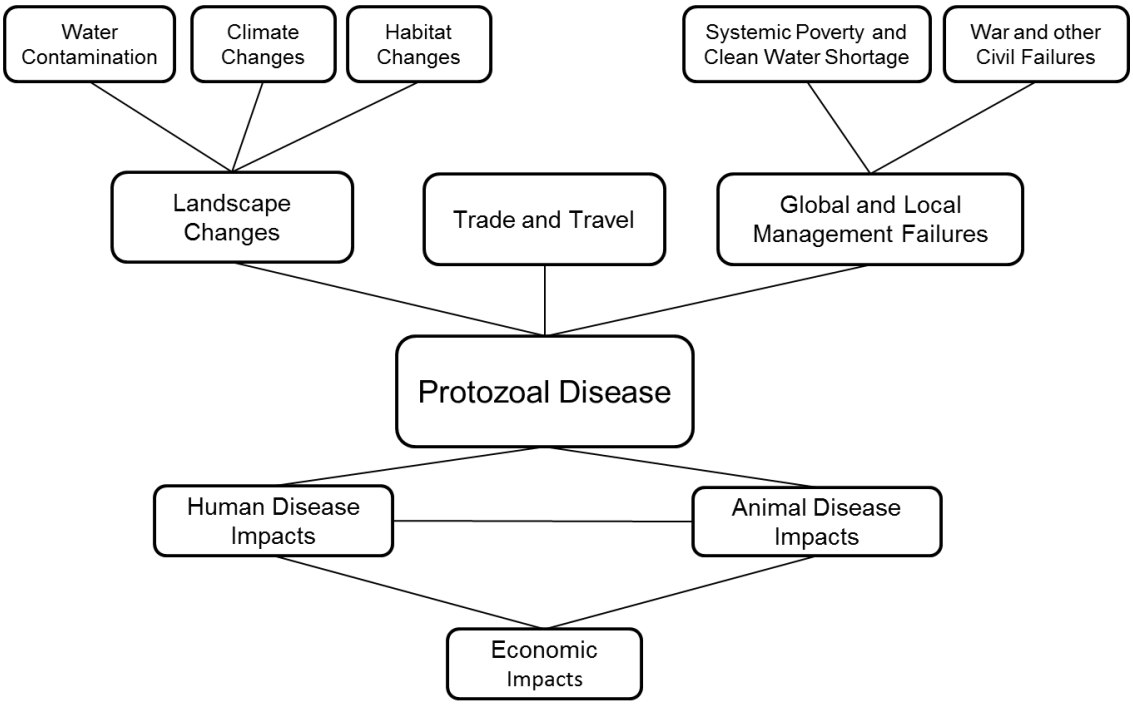

Figure 1.1: Schematic illustration of the drivers for protozoal disease transmission.

CHAPTER 2

Longitudinal Poisson regression to evaluate *Cryptosporidium*, *Giardia*, and fecal indicator bacteria epidemiology in coastal California wetlands

Longitudinal Poisson regression to evaluate *Cryptosporidium*, *Giardia*, and fecal indicator bacteria epidemiology in coastal California wetlands

Running Title: Fecal pathogen epidemiology in coastal wetlands

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ABSTRACT

Fecal pathogen contamination of watersheds worldwide is increasingly recognized, and natural wetlands may have an important role in mitigating fecal pathogen pollution flowing downstream. Given that waterborne protozoa such as *Cryptosporidium* and *Giardia* are transported within surface waters, this study evaluated associations between fecal protozoa and various wetland-specific and environmental risk factors. This study focused on three distinct coastal California wetlands: 1) a tidally-influenced slough bordered by urban and agricultural areas, 2) a seasonal wetland adjacent to a dairy and 3) a constructed wetland that receives agricultural runoff. Wetland type, seasonality, rainfall, and various water quality parameters were evaluated using longitudinal Poisson regression to model effects on protozoa and indicator bacteria (*Escherichia coli* and total coliform) concentrations. Among wetland types, the dairy wetland exhibited the highest protozoal and bacterial concentrations, and despite significant reductions in microbe concentrations, the wetland could still be seen to influence water quality in the downstream tidal wetland. Additionally, recent rainfall events were associated with higher protozoal and bacterial counts in wetland water samples across all wetland types. Notably, detection of *E. coli* concentrations greater than 400 MPN (most probable number) per 100 mL were associated with higher *Cryptosporidium* oocyst and *Giardia* cyst concentrations. These findings show that natural wetlands draining agricultural and livestock operation runoff into human-utilized waterways should be considered potential sources of pathogens; and that wetlands can be instrumental in reducing pathogen loads to downstream waters.

INTRODUCTION

Fecal pathogen contamination of waterways is an important public health consideration in multi-use ecosystems. Sources of pathogens may include agricultural runoff containing livestock feces, sewage outfalls containing human and pet feces, and storm runoff containing feces of domestic and wild animals (12, 36). Zoonotic waterborne protozoa, specifically *Cryptosporidium* and *Giardia* spp., commonly cause diarrheal disease that can be severe or even fatal in immuno-compromised individuals (1, 8, 11, 26). As surface waters move down a watershed, pulses of pathogen contamination from upstream sources contribute to downstream loads that affect recreational uses. Additionally, environmental persistence of *Cryptosporidium* oocysts and *Giardia* cysts, and their low infectious dose (ID_{50}), contribute to the health risks posed to humans and animals that are in contact with surface waters (9, 13, 28).

The importance of wetlands for reducing select agricultural and chemical pollutants and pathogens in impaired surface water has been previously studied (21), and supports wetland use for improving water quality downstream from agricultural lands, livestock operations, and urban communities. However, most studies to date have focused on wetlands receiving sewage effluent (7, 18, 27), and little is known about how natural wetlands function with respect to transport and persistence of *Cryptosporidium* oocysts and *Giardia* cysts from upstream sources. In California and elsewhere, identifying ecologically sustainable management practices to improve water quality is of particular interest to resource managers and agencies.

To evaluate protozoa and fecal indicator bacteria distribution and transport in natural wetlands, microbial concentrations among three wetland types along the central

California coast were compared: 1) a medium sized, tidally-influenced slough that connects urban and agricultural runoff to Monterey Bay, 2) a small seasonal wetland adjacent to a dairy that receives runoff from cattle, and 3) a small unidirectional constructed wetland that filters water from an adjacent slough dominated by agricultural runoff. Associations between fecal protozoa and fecal indicator bacteria (FIB) were evaluated to determine the effectiveness of FIB as a proxy for protozoa in this setting. Multiple sites within each wetland were monitored during a two-year period to evaluate how environmental factors, such as rainfall and water quality measures, are associated with pathogen detection and transport from land to sea.

MATERIALS AND METHODS

Study Sites. The three central California coast wetland study sites (Figure 2.1) were sampled repeatedly between April 2008 and June 2010. The tidal wetland was originally an estuary that drained into a multi-use harbor and recreational area. Degradation of the wetland due to landscape conversion to agriculture, including livestock operations, has altered the region such that area waters remain brackish to hyper-saline most years. For water collection, seven sites spaced approximately 500 m apart, ranging from 4000 m upstream to the harbor tide gates, were sampled seasonally (Figure 2.1A). The tidal wetland received runoff from the dairy wetland described below. A total of 38 samples were collected for fecal protozoa analysis, and 25 samples were also tested for fecal indicator bacteria (FIB).

The dairy wetland was a seasonally occurring wetland that receives runoff from cattle pastures, and eventually drains into the tidal wetland described above. Surface

water was present in the dairy wetland predominantly during the wet season (November to April), and was sampled at five sites, spaced approximately 150 m apart along a 1000 m track from the dairy down to the outflow into the tidal slough described above (Figure 2.1A). A total of 26 samples were collected for protozoal analysis, and 21 samples were also tested for fecal indicator bacteria.

The constructed wetland (Figure 2.1B) was built as a field research site with water pumped in from an adjacent slough that drains a large expanse of agricultural fields. It has an upper snake-like channel section that is characterized by unidirectional flow and was sampled at locations spaced approximately 100 m apart. Additional samples were collected from the lower section of the constructed wetland, where a large floodplain permits multi-directional flow depending on tidal and weather conditions, as well as from the source water in the adjacent slough. California bulrush (*Schoenoplectus californicus*) lined the banks of the upper snake-shaped channel, and the same plants formed 10 2-meter wide vegetative buffers, or berms, that were perpendicular to the main water channel throughout this upper section. The lower floodplain section consists of a variety of plants including California bulrush and slough sedge (*Carex obnupta*). Water samples were collected from the constructed wetland once weekly over four consecutive weeks seasonally, resulting in a total of 87 samples for protozoal analysis, and 76 samples tested for fecal indicator bacteria.

At each sampling location in all three wetlands, 10 L of surface water were collected in a sterile plastic container for protozoal analysis, along with 100 mL of water for bacterial analysis, as described below. Water quality parameters were also recorded at

each wetland on each sampling day, including water temperature (˚C), turbidity (NTU), salinity (ppt), total dissolved solids, dissolved oxygen (mg/L), and pH.

Matrix spikes were prepared periodically during the study to determine percent recovery for protozoal detection methods. Easy Seed ™ (BTF Bio, Pittsburgh, PA, USA) aliquots containing 100 inactivated *Cryptosporidium* oocysts and 100 inactivated *Giardia* cysts were added to 10 L of surface water from the wetland. These matrix samples were processed in the same way as the un-spiked field wetland water samples.

Microbial Detection. Samples for protozoal analysis were processed according to the EPA Method 1623 (33) for detection of *Cryptosporidium parvum* and *Giardia lamblia* in water through filtration using Envirochek® cartridges, followed by immunomagnetic separation (IMS) for parasite purification using Dynabeads GC-Combo (Invitrogen Life Sciences, Carlsbad, CA, USA) and direct fluorescent antibody tests (DFA) for oocyst or cyst identification using EasyStain (BTF Bio, Pittsburgh, PA, USA) and quantification using microscopy under both fluorescein isothiocyanate (FITC) and 4',6-diamidino-2-phenylindole (DAPI) epifluoresence. Particles with apple-green fluorescence in an oval or spherical shape (3-7 μm in diameter) with bright, highlighted edges under FITC and light blue internal staining with a green rim or up to four distinct, sky-blue nuclei under DAPI were classified as a *Cryptosporidium* oocyst. Particles with apple-green fluorescence in a round to oval shape (6-15 μm in diameter) with bright, highlighted edges under FITC and light blue internal staining with a green rim or up to four distinct, sky-blue nuclei under DAPI were classified as a *Giardia* cyst. Results were expressed as oocyst or cyst count/10 L of water sampled (33).

To compare protozoal concentrations with the more commonly measured indicator bacteria, samples for FIB analysis were examined using Colilert-18 (IDEXX Laboratories, Inc.), a standard EPA-approved method for detection of total coliforms and *Escherichia coli* in surface water samples. Briefly, water samples were mixed with Colilert-18 reagent packs and incubated at 35˚C in a Quanti-Tray/2000 (IDEXX Laboratories, Inc.) for 18 hours, with total coliform concentrations estimated by the number of yellow wells, and *E. coli* counts estimated by number of fluorescent wells. The results were expressed as most probable number (MPN) per 100 mL.

Statistical Analyses. A variety of wetland characteristics and environmental factors were evaluated as risk factors for associations with protozoal and indicator bacterial concentrations. Risk factors included wetland type (tidal, dairy, constructed), sample location within each wetland, and season (wet versus dry). Along the central California coast, the rainy season extends November through April, and the dry season extends May through October. Rainfall events were described as rain occurring within the 12 hours prior to sampling or within the preceding four days, as determined by the nearest weather station. Turbidity, total dissolved solids, and pH were evaluated as continuous variables. Water temperature and dissolved oxygen values were defined as "increased" or "decreased" with respect to the mean of collected values for each wetland. Salinity values less than 1 ppt were defined as "fresh water", while samples with salinity greater than or equal to 1 ppt were defined as "brackish". Categories for high FIB contamination of surface water were defined as *E. coli* concentrations greater than 400 Most Probable Number (MPN), and total coliform concentrations greater than 10,000

MPN. *Escherichia coli* and total coliform counts falling below these established cutoffs were deemed to have low to moderate FIB contamination.

The four primary outcomes of interest were the surface water concentrations of *Cryptosporidium, Giardia, E. coli,* and total coliforms. Descriptive statistics were calculated to compare these pathogen groups, with geometric mean concentration calculated for each protozoa species and Regression on Order Statistics (ROS) calculated as a measure of central tendency for indicator bacteria. The ROS method was used to take into account censored data for samples with concentrations below the method detection limits (1 organism / 100 mL). Initial analyses revealed that the protozoal means were equal to the variance, supporting the use of Poisson regression models for subsequent statistical procedures. Log-linear longitudinal Poisson regression (14) was used to determine the effect of each independent variable on each study outcome, in order to identify risk factors that were significantly associated with higher protozoal or bacterial counts. Poisson regression is a form of statistical regression analysis used to model count data, which is assumed to have a Poisson distribution. One assumption of this model is that there is no over-dispersion, meaning that the variance does not exceed the mean. This assumption was not violated with the study data. Longitudinal regression was used to account for repeated sampling at the same sites over time; specifically, sampling sites were treated as random effects. Non-parametric Spearman's rho and Chi-square tests were calculated to determine potential co-linearity between the parameters of interest. Factors significant in bivariate analyses were then used to build a multiple Poisson model to test several predictor variables simultaneously. Additionally, Spearman's rho correlation coefficient was calculated for protozoal and bacterial counts. Statistical

analyses were completed using SAS software, Version 9.2 (SAS Institute Inc., Cary, NC, USA), and *P*-values<0.05 were considered statistically significant.

RESULTS

Microbial Detection. A total of 151 samples were tested for fecal protozoa and 122 samples for fecal indicator bacteria (FIB), with all target organisms detected at all wetland types and sampling locations. The prevalence, mean concentration, and range of *Cryptosporidium, Giardia*, total coliforms, and *E. coli* in water samples collected from the three wetlands are shown in Table 2.1. Within the tidal wetland, 39% of water samples were positive for *Cryptosporidium* oocysts, while 21% were positive for *Giardia* cysts. The dairy wetland exhibited the greatest prevalence of *Cryptosporidium* oocysts and *Giardia* cysts, with 69% and 50% of water samples testing positive, respectively. The constructed wetland had a 44% *Cryptosporidium* oocyst prevalence and a 13% *Giardia* cyst prevalence. Furthermore, surface water collected from the dairy wetland exhibited the highest geometric mean concentrations of both protozoa (168 oocysts and 85 cysts per 10 L), followed by the tidal wetland (31 oocysts and 36 cysts per 10 L), and the constructed wetland (5.5oocysts and 3.2 cysts per 10 L). Matrix spikes from eight surface waters showed *Cryptosporidium* recovery ranged from 2 – 26%, with an average of 13% (SE 3.4%) and *Giardia* recovery ranged from 0-30%, with an average of 15% (SE 4.6%).

As expected, 100% of samples from all three wetlands tested positive for *E. coli* and total coliforms across all sample points and time periods. Surface water collected from the constructed wetland exhibited the highest mean concentrations by Regression on Ordered Statistics (ROS) of *E. coli* (2913 MPN/ 100 mL), followed by the dairy wetland

(870 MPN/ 100 mL) and then the tidal wetland (316 MPN/ 100 mL). For total coliforms, the dairy wetland exhibited the highest concentrations (173590 MPN/ 100 mL), followed by the constructed wetland (155973 MPN/ 100 mL) and the tidal wetland (8571 MPN/ 100 mL).

The concentrations of *Cryptosporidium*, *Giardia*, *E. coli* and total coliform by sample location for each wetland are shown in Figure 2.2, with the "distance downstream" representing the distance from the most upstream sampling location to each sampling location in the wetland. Microbe levels in the tidal wetland were strongly influenced by sample location, particularly with regard to where the outflow from the dairy wetland joined the tidal wetland. For both protozoa and bacteria, microbe concentrations at the tidal wetland were highest at sites immediately upstream and downstream of the dairy wetland outflow, and then decreased thereafter. Results from the dairy wetland showed that *Cryptosporidium, Giardia*, and FIB concentrations were significantly higher (P-value<0.0001) in the site closest to the dairy, when compared to sample locations further downstream. Although very low concentrations of protozoa were detected in the constructed wetland, FIB enumeration demonstrated a similar trend, with highest concentrations at the sample location in the source water from the adjacent slough (sample distance 0 m), and lower counts in water sampled from the subsequent downstream sites within the channel portion of the constructed wetland.

Risk Factors. Analysis of associations for *Cryptosporidium* and *Giardia* concentrations with environmental factors was examined using Poisson regression, as the protozoal means were not over-dispersed. Without adjusting for wetland effects, rainfall in the 12 and 96 hours prior to sample collection was associated with detection of

significantly higher concentrations (*P*-value<0.0001) of both *Cryptosporidium* oocysts and *Giardia* cysts. The odds ratio for rainfall in the prior 12 hours with *Cryptosporidium* counts as the outcome was 30, meaning that in water samples collected from sites that received rainfall within the 12 hours prior, the *Cryptosporidium* oocyst counts were likely to be 30 times greater than for water samples collected when there was not rainfall in the preceding 12 hours. For *Giardia*, the association between rainfall and cyst enumeration was even more pronounced, at 217. Thus, for every *Giardia* cyst in a water sample when there was no rainfall in the prior 12 hours to collection, there was likely to be 217 times as many *Giardia* cysts in water samples collected when rainfall had occurred just previously. The wet season was also associated with significantly higher protozoal counts compared to dry season sampling, with odds ratios of 45 for *Cryptosporidium*, and 1510 for *Giardia* detection in wet season surface waters (*P*-value<0.0001).

Microbial detection and concentrations were significantly associated with most of the measured water quality parameters, including dissolved oxygen, water temperature, total dissolved solids and salinity (*P*-value <0.05), while turbidity and pH were not. Increased dissolved oxygen was negatively associated with protozoal and bacterial counts. Increases in total dissolved solids were also negatively associated with protozoal counts (*P*-value <0.0001). Warmer water temperature was inversely associated with protozoal counts (*P*-value <0.0001), but positively associated with total coliform values (*P*-value <0.005). When stratified by wetland type, water samples with higher salinity were negatively associated with both *Cryptosporidium* (*P*-value<0.05) and *Giardia* (*P*-value <0.0001) counts, meaning that water samples from sites with lower marine influence were more likely to have high protozoal concentrations. Mean salinity levels

for the tidal wetland tended to be brackish, at 11.1 ppt, whereas the dairy and constructed wetlands exhibited lower salinity, at 1.3 ppt and 2.6 ppt, respectively.

Protozoal relationship with fecal indicator bacteria. When the Spearman's rho correlation coefficient was calculated to compare protozoal counts to fecal indicator bacterial counts, no FIB-protozoal combinations were found to be significant. However, when a Poisson model approach was taken to look at the association between protozoal concentrations with "high" or "low" FIB levels, significant associations were observed. Using this approach, *E. coli* counts greater than 400 MPN/ 100 mL were associated with higher protozoal counts; for example the odds ratio of having high levels of *E. coli* with increased *Giardia* cyst counts in water samples was 210 (*P*-value <0.0001). Thus, if a sample was found to have *E. coli* counts greater than 400 MPN, the sample would be predicted to have a count of 210 times more *Giardia* than if there was an *E. coli* concentration of below 400 MPN. Conversely, concentrations of total coliforms exceeding 10,000 MPN were associated with lower protozoal oocyst or cyst concentrations.

Regression Models. Longitudinal Poisson regression was used to further assess relationships between protozoal counts in surface water and various environmental and water quality parameters and FIB measures. In these models, *Cryptosporidium* and *Giardia* concentrations in surface water samples were best predicted by simultaneously considering wetland type, FIB levels and rainfall variables. After adjusting for the difference in protozoal count by wetland type, rainfall was assessed to account for both the overall effect of rain as well as the difference in rainfall between the three wetlands

through the use of interaction terms in the model. These results are tabulated in Table 2.2 and the associated predictive models are summarized in Figure 2.3.

For *Cryptosporidium*, recent rainfall exposure was the best predictor of protozoal concentrations when evaluated as the volume of rainfall (in mm) in the 12 hours prior to sampling. Rainfall occurring within 12 hours prior to water sampling at the dairy and constructed wetland sites was associated with a 1.7- and 1.4-fold increase in *Cryptosporidium* oocyst concentrations, respectively (*P*-value <0.05 for both). Inclusion of FIB level into this model also had an impact on *Cryptosporidium* oocyst detection: "High" *E. coli* counts of greater than 400 MPN were associated with 4.8-fold increased concentrations of *Cryptosporidium* oocysts. Conversely, total coliform counts of greater than 10000 MPN in surface water were associated with lower concentrations of *Cryptosporidium* oocysts, similar to what was noted in univariate analyses. The predictive model in Figure 2.3 Panel A shows that as rainfall volume increases, the *Cryptosporidium* concentration is predicted to increase in the dairy wetland, but decrease in the tidal wetland.

For *Giardia*, the longitudinal Poisson regression model showed similar trends to the *Cryptosporidium* model, with one difference being that increased *Giardia* counts were associated with the occurrence of rainfall in the 12 hours prior to sampling rather than the total volume of rainfall. The odds ratio shows that *Giardia* counts were 104 times greater in water samples collected when there was preceding rainfall, regardless of wetland type, when compared to no rainfall within the prior 12 hours. Additionally, the effect of fecal contamination (classified as water samples with *E. coli* concentrations above 400 MPN) was much greater for the *Giardia* model than for *Cryptosporidium*, with

an odds ratio of 265. The *Giardia* model utilizing rainfall as a continuous variable did not converge, so no predictive model is shown.

The longitudinal Poisson regression models for the bacterial indicators included the risk factors wetland type, FIB level, and total rainfall within the prior 96 hours (Table 2.2 and Figure 2.3). For the *E. coli* model, salinity was also an important factor, with an odds ratio of 0.3 (*P*-value <0.0001) for brackish water compared to fresh. Thus, fresh water streams had greater concentrations of *E. coli* than brackish water estuaries, as expected if fecal pollution is coming from freshwater sources up the watershed. For total coliforms, both the dairy and constructed wetland had odds ratios greater than four when compared to the tidal wetland (*P*-value >0.0001), meaning that significant increases in total coliform counts were noted between these two freshwater wetlands compared to the more marine-influenced tidal wetland. When stratified by wetland type, total rainfall (mm) was negatively associated with levels of total coliforms for the dairy wetland, but a slight positive association was noted for the constructed wetland when compared to the tidal wetland.

DISCUSSION

While the majority of studies on protozoal transport have focused on constructed wetlands as a mitigating factor for sewage treatment (15, 18, 32, 35), livestock operations, domestic pets, and wildlife can also contribute substantially to environmental loading (2, 4, 5, 22). All three central California coastal wetlands examined in this study contained the protozoal pathogens *Cryptosporidium* and *Giardia*, as well as fecal indicator bacteria (FIB) in surface waters, and all three wetlands showed reductions of

both protozoal and bacterial concentrations within the wetlands. For the tidal and dairy wetlands in particular, the pathogen concentrations were high enough to be a public health concern for humans utilizing these water sources for recreation, especially considering that these wetlands drain into a harbor and sanctuary that is heavily used for recreation (31). The public health importance of the parasites depends on the particular species of *Cryptosporidium* or *Giardia*, as there are host specific species and genotypes which may are not commonly linked to disease in humans, such as the cattle-specific *C. andersoni* and *G. lamblia* assemblage E. However, the transport dynamics are expected to be similar across genotypes, and this study highlights the importance of wetlands in reducing pathogen loads.

The dairy wetland, which forms naturally during the wet season and drains into the adjacent tidal wetland, exhibited the greatest concentration of *Cryptosporidium*, *Giardia*, and FIB. Increased concentrations of both protozoa and bacteria were also noted at the tidal wetland when the dairy wetland was discharging water to sample sites closest to the junction of the two wetlands. Increased livestock density, increased water flow rates, or further degradation of this wetland habitat could lead to increased pathogen transported to adjacent recreational waters. It is acknowledged that results may be affected by unaccounted-for variation between study sites, such as differing water volumes, watershed areas, and surrounding land uses between each wetland. Furthermore, the actual concentrations of *Cryptosporidium* and *Giardia* in the wetlands are underestimated, as shown by the matrix spikes where protozoal recovery ranged from 0-30% of spiked parasites in wetland water samples. The use of acid dissociation may underestimate the number of parasites compared to heat dissociation (29, 37), but acid

was used to be consistent with the standard EPA 1623 protocol for *Cryptosporidium* and *Giardia* identification in water (33). Ultimately, this study illustrates the ability of natural and reconstructed wetlands to reduce pathogen loads from a variety of sources.

Protozoal contamination from livestock feces is of particular concern because cattle are potential reservoirs for *Cryptosporidium parvum* and *Giardia lamblia* assemblage A (11, 17, 39), both of which are known to infect humans. Previous studies have examined the role of dairies and other intensive livestock operations in pathogen transmission and identified potential risk factors for nearby surface water contamination, including application of manure on agricultural fields (30) and surface runoff from areas with high animal densities, such as dairies and beef cattle operations (23). Consistent with these results, the data from this study demonstrate that the wetland in closest proximity to a livestock operation exhibited the greatest protozoal and bacterial loading. We also observed that a tidal wetland receiving water from this same dairy wetland had the highest microbial concentrations at sites nearest to the outflow from the dairy wetland. Tidal wetland sites immediately upstream and downstream of the junction with the dairy wetland exhibited greater parasite concentrations because this tidally-influenced wetland experiences bi-directional flow (tide was not controlled for in the wetland sampling scheme).

While intensive livestock operations have significant potential to contribute to downstream pathogen loads, several Best Management Practices (BMPs) have been proposed to reduce pathogen contamination in runoff flowing from pastures to adjacent receiving waters. First, reduction of cattle density may reduce the spread of infection within the herd and reduce the amount of contaminated manure released into the

environment (23, 24). Second, reducing or more carefully controlling field applications of manure and slurry can greatly decrease the number of pathogens released into the environment (16). Finally, incorporation of vegetative filter strips or "buffer strips" can reduce the number of oocysts and cysts transported out of intensive livestock operations (3, 16, 23, 24). Implementation of these BMPs at dairies and other livestock operations could considerably reduce their contribution to pathogen loading of adjacent wetlands.

In addition to the close proximity of fecal pathogen sources, rainfall and specific water quality parameters were also associated with *Cryptosporidium*, *Giardia*, and FIB counts in surface water. Rainfall events just prior to water sampling were associated with increases in oocyst or cyst concentrations across wetland types. Rainfall occurring within the previous 12 hours was associated with increased *Cryptosporidium* and *Giardia* counts in the dairy wetland water, likely due to enhanced runoff from fecally-contaminated soils. In livestock operations that utilized vegetative buffer strips to reduce watershed contamination from storm runoff, previous studies have documented reduced pathogen loading of adjacent waterways (6, 23, 24). Reductions in water quality, characterized by increased water temperature and total dissolved solids, and decreased dissolved oxygen, was also associated with higher *Cryptosporidium* and *Giardia* concentrations. Runoff from intensive livestock and agricultural operations can adversely affect these water quality measurements (19) and may also directly and indirectly contribute to pathogen transport.

Wetlands can be used to reduce microbial pollutant loads through several processes, including adsorption, sedimentation, and vegetative uptake (20). As with prior studies, our data indicate a general reduction in *Cryptosporidium*, *Giardia*, and FIB

counts as contaminated water travels downstream through the wetlands. The data from the dairy wetland clearly showed that when protozoa and FIB were detected in upstream samples, samples collected further downstream exhibited lower pathogen concentrations. In a similar pattern, the tidal wetland had increased microbial concentrations nearest the dairy inlet, but downstream samples had returned to the low concentrations seen upstream of the inlet. There was an increase in *E. coli* concentrations in the last tidal wetland site, but this could be attributed to other potential fecal contamination sources, including live-aboard boats in the adjacent harbor and a substantial wildlife population residing near the downstream site. While the background levels of *Cryptosporidium* and *Giardia* were too low to document an effect in the constructed wetland, the total coliform and *E. coli* counts decreased sequentially throughout the water channel portion of the wetland. In contrast, the lower floodplain section was observed to be periodically inundated with undiluted water from the adjacent slough, perhaps explaining the increase in pathogen concentrations at the last sample site.

Surface water quality monitoring programs generally rely on FIB levels to represent risk of pathogen exposure and associated health risks to humans. This approach is taken in part because FIB testing is less expensive and easier than direct pathogen testing. When FIB were analyzed as predictors of protozoal pathogen concentrations in coastal wetland systems, the detection of *E. coli* counts exceeding EPA guidelines (greater than 400 MPN) were positively associated with increased concentrations of protozoal oocysts or cysts in water samples. However, detection of total coliform levels exceeding EPA guidelines (greater than 10,000 MPN) was negatively associated with protozoal counts. In general, *E. coli* are considered a more reliable indicator of fecal

contamination than total coliforms (10), the latter of which can vary with environmental conditions and bacterial populations (34). In contrast to FIB that can multiply in the environment, *Cryptosporidium* oocysts and *Giardia* cysts are shed in the feces and do not multiply in the environment. Thus, the association of protozoal counts with a more reliable marker for fecal contamination (*E. coli* counts), compared to the less specific total coliform counts is not surprising. However, it can be noted that although increased *E. coli* counts correlated with greater protozoal concentrations, no significant correlations were identified between the absolute numbers of *E. coli* and protozoa detected in the same water samples. This contrasts with results from prior studies that evaluated *Cryptosporidium* and *Giardia* contamination of drinking water sources and reported linear correlations between protozoal concentrations and FIB concentrations (25, 38).

In conclusion, given that fecal pathogens can enter waterways through a multitude of routes and sources, natural wetlands may serve as a sustainable BMP to improve water quality in downstream waters. The study results show that protozoal and bacterial concentrations can be reduced as water travels through the coastal wetland, and also that rainfall events are important to consider when identifying highest risk times for fecal pollution entering waterways. Recognizing the potential of natural and reconstructed coastal wetlands for reducing transport of pathogens downstream holds promise for improving ecosystem health while also conserving natural habitat that is important for wildlife and a variety of recreational uses.

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Table 2.1: Prevalence and concentrations using geometric means of *Cryptosporidium* oocysts and *Giardia* cysts, and prevalence and concentrations using Regression on Ordered Statistics of *Escherichia coli*, and total coliforms in coastal California wetlands.

	Cryptosporidium	***Giardia***	**Total Coliforms**	***E. coli***
Tidal Wetland				
Prevalence	39% (15/38)	21% (8/38)	100% (25/25)	100% (25/25)
Mean (per 10 L)	31	36	8571	316
Range (per 10 L)	0-389	0-15000	100-41060	46-1890
Dairy Wetland				
Prevalence	69% (18/26)	50% (13/26)	100% (21/21)	100% (21/21)
Mean (per 10 L)	168	85	173590	870
Range (per 10 L)	0-54044	0-36417	2000-2419600	200-4840
Constructed Wetland				
Prevalence	46% (40/87)	13% (11/87)	100% (78/78)	100% (78/78)
Mean (per 10 L)	5.5	3.2	155973	2913
Range (per 10 L)	0-148	0-14	1320-2420000	4-46400

Table 2.2: Odds Ratios (OR) from multiple Poisson regression models for significant variables predicting protozoa and fecal indicator bacteria concentrations in surface waters from coastal wetlands.

		Cryptosporidium **Concentration per 10 L**		***Giardia*** **Concentration per 10 L**		***Escherichia coli*** **MPN per 100 mL**		**Total Coliform MPN per 100 mL**	
Predictor Variables	**N**	**OR**	***P*-value**	**OR**	***P*-value**	**OR**	***P*-value**	**OR**	***P*-value**
Wetland Type									
Tidal	38	1		1		1		1	
Dairy	26	12.13*	0.0020	3.93	0.1565	0.86	0.7146	32.97*	<0.0001
Constructed	104	0.07*	0.007	0.08*	0.0105	2.85*	0.0189	4.37*	<0.0001
Indicator Bacteria									
Total coliforms-Below 10,000	86	1		1		1			
Total coliforms-Above 10,000	82	0.01*	<0.0001	0.12	0.0035	2.51*	<0.0001		
E. coli-Below 400	102	1		1				1	
E. coli-Above 400	66	4.75*	0.0096	265.39*	<0.0001			1.94*	0.0340
Salinity									
Fresh water	76					1			
Brackish water	92					0.34*	<0.0001		
Rainfall (mm)									
Total Rainfall on Sample Date (mm)	155	0.73*	0.0254						
Rainfall (mm) × Tidal	38	1							
Rainfall (mm) × Dairy	21	1.75*	0.0252						
Rainfall (mm) × Constructed	96	1.42*	0.0226						
Rainfall Occurrence									
No Rainfall on Sample Date	127			1					
Rainfall on Sample Date	41			103.86*	<0.0001				
Rainfall × Tidal	7			1					
Rainfall × Dairy	5			0.68	0.7473				
Rainfall × Constructed	29			0.01*	0.0082				
Rainfall (mm)									
Total Rainfall within prior 96 hours (mm)	172					0.99	0.5339	0.99	0.5904
Rainfall (mm) × Tidal	38					1		1	
Rainfall (mm) × Dairy	26					1.11*	0.0172	0.73*	0.0122
Rainfall (mm) × Constructed	108					1.04*	0.0255	1.06*	0.0353

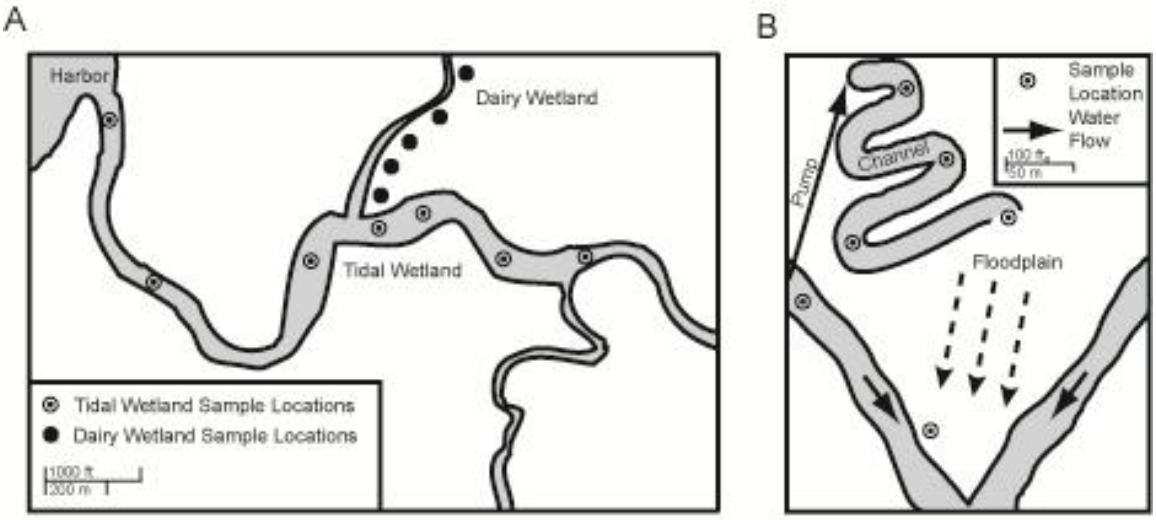

Figure 2.1: Maps of field sampling locations. A-Tidal and Dairy Wetlands; B-Constructed Wetland

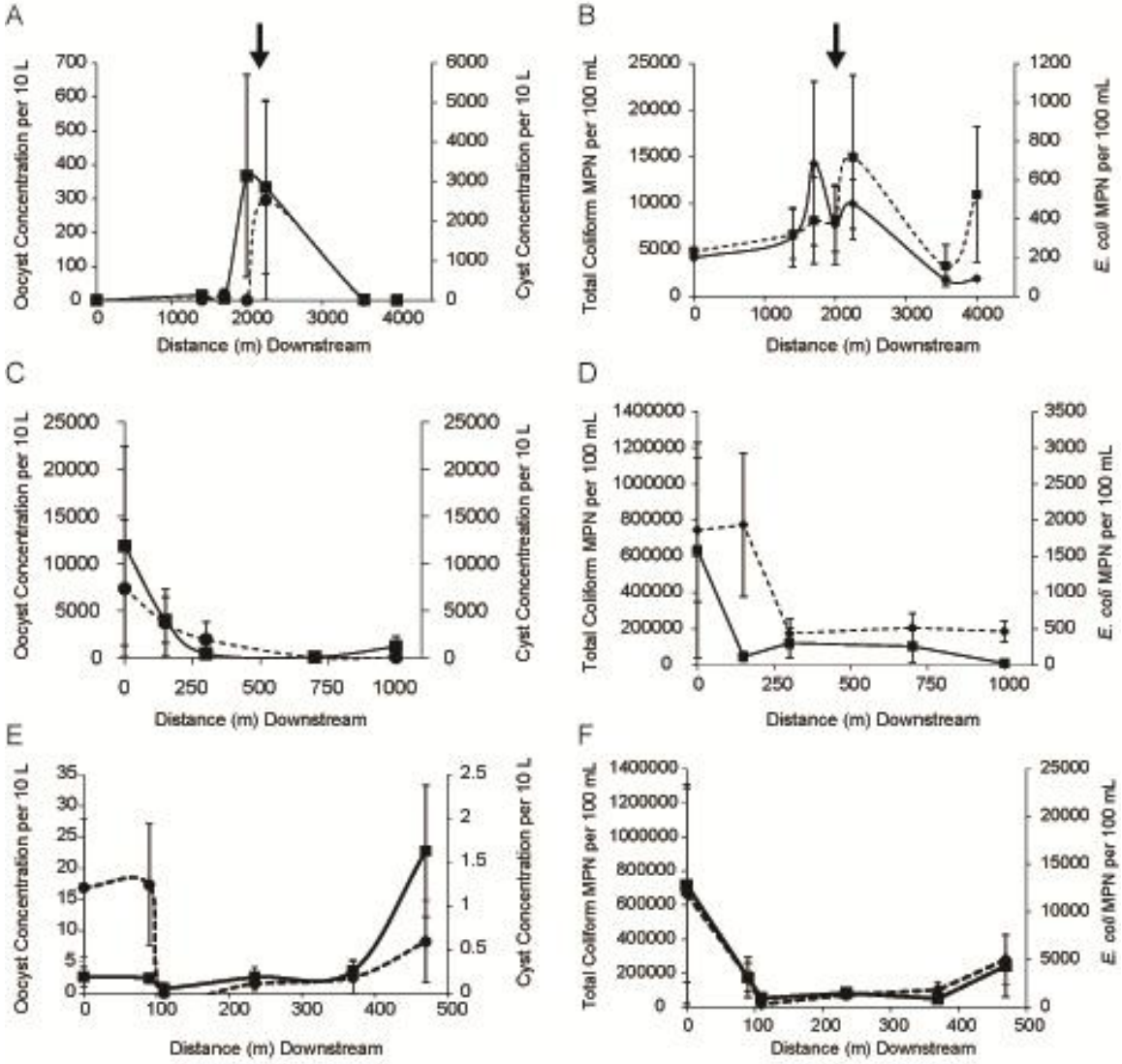

Figure 2.2: Mean concentrations of *Cryptosporidium* oocysts, *Giardia* cysts, total coliforms, and *Escherichia coli* by site in the tidal wetland (A and B), dairy wetland (C and D) and constructed wetland (E and F). Arrow indicates where dairy wetland joins tidal wetland. For A, C, and E, solid line represents *Cryptosporidium* oocyst concentration and dashed line represents *Giardia* cyst concentration. For B, D, and F, solid line represents total coliform concentration and dashed line represents *E. coli* concentration.

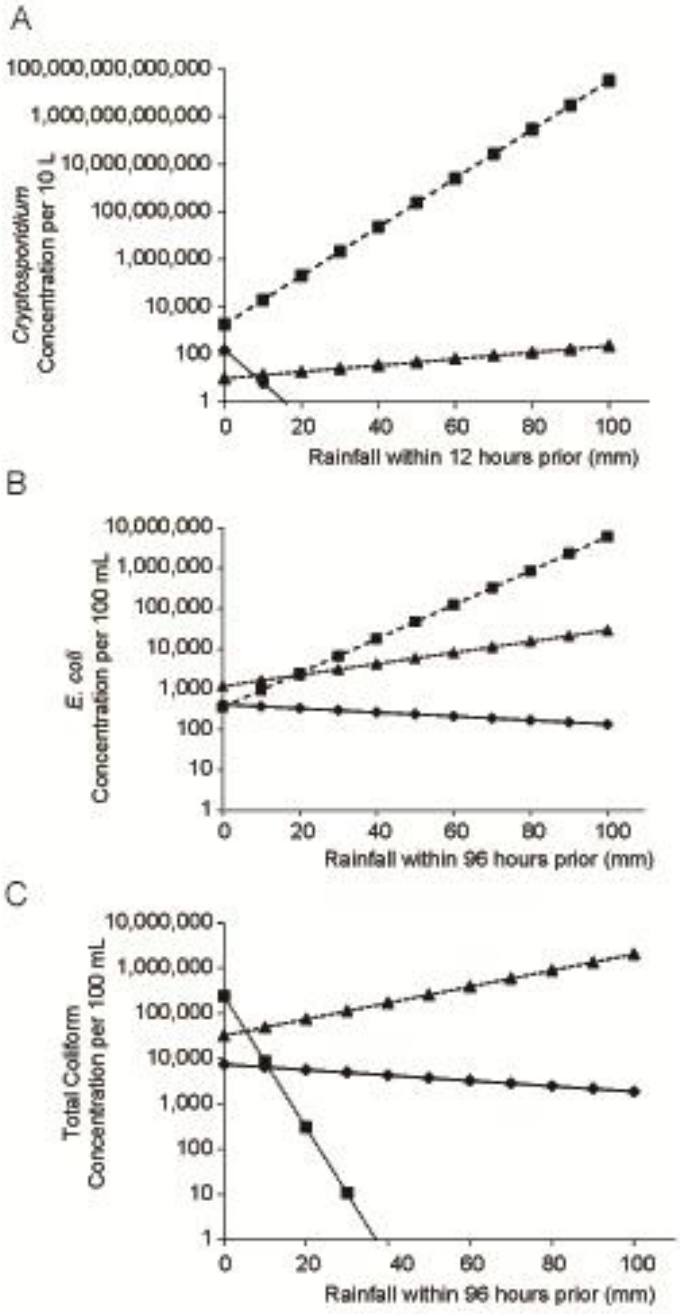

Figure 2.3: Predictive models for *Cryptosporidium* oocysts (A), total coliforms (B), and *E. coli* (C) utilizing Poisson regression models where variables other than rainfall were held stable.

CHAPTER 3

Hydrologic and vegetative removal of *Cryptosporidium parvum*, *Giardia lamblia*, and *Toxoplasma gondii* surrogate microspheres in coastal wetlands

Hydrologic and vegetative removal of *Cryptosporidium parvum*, *Giardia lamblia*, and *Toxoplasma gondii* surrogate microspheres in coastal wetlands

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Abstract

Constructed wetland systems are used to reduce pollutants and pathogens in wastewater effluent, but comparatively little is known about pathogen transport through natural wetland habitats. Fecal protozoans including *Cryptosporidium parvum, Giardia lamblia,* and *Toxoplasma gondii* are waterborne pathogens of humans and animals, which are carried by surface waters from land-based sources into coastal waters. This study evaluated key factors of coastal wetlands for the reduction of protozoal parasites in surface waters using settling column and re-circulating mesocosm tank experiments. Settling column experiments evaluated the effects of salinity, temperature, and water type (pure versus environmental) on the vertical settling velocities of *C. parvum, G. lamblia,* and *T. gondii* surrogates, with salinity and water type found to significantly affect settling of the parasites. The mesocosm tank experiments evaluated the effects of salinity, flow rate, and vegetation parameters on parasite and surrogate counts, with increased salinity and presence of vegetation found to be significant factors for removal of parasites in a unidirectional transport wetland system. Overall, this study highlights the importance of water type, salinity, and vegetation parameters on pathogen transport within wetland systems, with implications for wetland management, restoration efforts, and coastal water quality.

Keywords

Cryptosporidium, *Giardia*, salinity, *Toxoplasma*, transport, vegetation, wetlands

1. Introduction

Natural and constructed wetlands have the capacity for waterborne pollutant and pathogen reduction and removal. Construction of wetlands near dairies, farms, and wastewater treatment facilities may help reduce pollutant loads entering adjacent surface waters (Kao et al., 2001; Karim et al., 2004; Quinonez-Diaz et al., 2001). Few published studies have focused on the efficacy of naturally occurring coastal wetlands to filter polluted water (Quinonez-Diaz et al., 2001; Shapiro et al., 2010a). As a conduit for microbial transport from land to sea, natural wetlands may have an ameliorating effect on concentrations of potential pathogens including *Cryptosporidium parvum, Giardia lamblia* (syn. *G. intestinalis* and *G. duodenalis*), and *Toxoplasma gondii*, reducing pathogen contamination in downstream waters that may be used for recreation, drinking water, food harvest, and wildlife habitat (Millenium Ecosystem Assessment, 2005).

Waterborne protozoa including *C. parvum*, *G. lamblia*, and *T. gondii* are important human and animal pathogens in coastal ecosystems (Fayer et al., 2004; Miller et al., 2002). The environmentally resistant oocyst and cyst stages of *C. parvum* and *G. lamblia*, respectively, facilitate parasite survival, rendering them potentially infectious to susceptible hosts as they are transported through waterways (Slifko et al., 2000).The presence of these protozoal parasites in surface waters that are used for human recreation, drinking, or food harvest is of significant public health interest, because infectious doses are relatively low. Both *C. parvum* and *G. lamblia* are transmitted through fecal-oral routes and can cause severe gastrointestinal illness (Furness et al., 2000; Juranek, 1995; Rose et al., 1991). *Toxoplasma gondii* oocysts are shed in the feces of cats, the only known definitive host of the parasite; humans and other animals are intermediate hosts

(Dubey and Beattie, 1988). *Toxoplasma gondii* can adversely affect the fetus if a pregnant woman acquires the parasite, and immunocompromised individuals may suffer severe, even fatal, infections (Montoya and Liesenfeld, 2004). *Toxoplasma gondii* can also infect and cause disease in marine mammals, including the threatened California sea otter (*Enhydra lutris nereis*) that ranges along the California coast. Otters may become infected following exposure to oocysts that are transported from land-to-sea through contaminated freshwater runoff (Kreuder et al., 2003; Miller et al., 2002).

Previous studies have examined parasite transport through surface waters to identify hydrologic factors that result in protozoal reduction. For example, *C. parvum* counts were reduced more quickly in water flumes containing sediment when compared to those without (Searcy et al., 2006), which is consistent with results from studies examining the vertical settling velocity of *C. parvum* and *G. lamblia* (Dai and Boll, 2006; Searcy et al., 2005). Wetland vegetation has also been demonstrated to reduce water pollution and prevent pathogen run-off near dairies through application of various vegetation types and configurations in constructed wetlands (Atwill et al., 2006; Davies et al., 2004; Karim et al., 2008). For example, in a study of natural estuarine wetlands, vegetation-lined waterways significantly reduced transport of *T. gondii* surrogates, when compared with non-vegetated mud flats (Shapiro et al., 2010a).

Our aim was to establish a more robust model of estuarine wetland ecology and its role in removing waterborne pathogens of public health significance. Our objectives were to: 1) compare the effects of physical factors (salinity, temperature, and water type) on the vertical settling velocities of *C. parvum*, *G. lamblia*, and *T. gondii* surrogates; and 2) compare the effects of hydrologic factors (salinity and flow rate) and vegetation

(presence, species, and configuration) on removal of these parasites from environmental surface water using wetland mesocosm tanks.

2. Materials and methods

2.1. Source of protozoa and surrogates

Heat inactivated *C. parvum* oocysts and *G. lamblia* cysts were obtained from the Wisconsin State Laboratory of Hygiene (Madison, WI) and Waterborne Inc. (New Orleans, LA), respectively. Oocysts and cysts were obtained no more than one week prior to use for both settling column and mesocosm tank studies. Dragon Green (DG) microspheres (Product No. FC07F/5493, Bangs Laboratories, Inc., Fishers, IN) and Glacial Blue (GB) microspheres (Product No. PC06N/8319, Bangs Laboratories, Inc., Fishers IN) have been previously evaluated as surrogate particles for *T. gondii* oocysts based on their surface properties (Shapiro et al., 2009).

2.2. Settling column experiments

To investigate the effects of hydrologic factors on settling velocities, experiments were conducted in vertical settling columns (Figure 3.1). Each settling column held 1 L of water with sampling ports at 100 mm and 300 mm from the top of the water column. Protozoan and microsphere counts at the two ports over time were used to assess differences in settling velocities for the various water conditions that were evaluated. Eight treatment conditions in a complete blocked design were evaluated in triplicate to measure the effects of water type, salinity level, and water temperature on settling velocity of parasites and microspheres. Two water types were evaluated: reverse osmosis purified distilled water from a Milli-Q® water system (“pure”) and environmental water

collected from Tembladero Slough near Castroville, CA ("environmental"). Two salinity levels were evaluated to parameterize the variability in salinity levels in coastal wetlands from freshwater to marine influence: "low salinity" at baseline saline levels (0 ppt in Milli-Q® water and 0.1 ppt in environmental water) and "high salinity" at 30 ppt, which was achieved through the addition of salt (Coralife Scientific Grade Marine Salt®, Franklin, WI).Two water temperatures, 4°C and 27°C, were also evaluated for each water type and salinity level to assess the effect of water temperature on protozoan and microsphere settling properties.

Immediately prior to each experiment, $1x10^6$ each of *C. parvum* oocysts, *G. lamblia* cysts, and *T. gondii* oocyst surrogates (DG and GB) were mixed together into a 1 L container of water and homogenized by shaking. Once mixed, water was poured into the settling column and subsamples were taken at 0, 5, 15, 30, 60, and 90 min, and 2, 4, 8, 24, and 48 hrs. At each sample point, 1 mL of water was removed from the column using an 18 gauge needle and 100 µL was expressed onto a microscope slide and air-dried. Slides were stained with direct fluorescent antibody (DFA) for oocyst and cyst identification using Aqua-Glo™ G/C Direct (Waterborne, Inc., New Orleans, LA, USA) and parasites were enumerated under epifluorescent illumination using fluorescein isothiocyanate (FITC) and 4',6-diamidino-2-phenylindole (DAPI) emission filter sets. Particles with apple-green fluorescence in an oval or spherical shape (3-7 µm in diameter) with bright, highlighted edges under FITC and light blue internal staining with a green rim or up to four distinct, sky-blue nuclei under DAPI were classified as *C. parvum* oocysts. Particles with apple-green fluorescence in a round to oval shape (6-15 µm in diameter) with bright, highlighted edges under FITC and light blue internal staining with

a green rim or up to four distinct, sky-blue nuclei under DAPI were classified as *G. lamblia* cysts. Surrogates were enumerated using epifluorescent microscopy as previously described (Shapiro et al., 2010b).

Settling velocities were calculated by linear regression using the equation derived by Dai and Boll (2006):

$$\ln\left(\frac{N_0}{N_t}\right) = \frac{v_s}{H}t$$

where N_0 is the number of parasites or microspheres enumerated from a single port at time 0, N_t is the number of parasites or microspheres enumerated at time t, H is the height (mm) from the top of the water column to the sampling port, and v_s is the settling velocity expressed as mm/hr. These settling velocities were converted to µm/sec. Any settling velocities estimated using regression to be below zero were truncated to 0 µm/sec, and were classified as NS, "no settling". Regression on Ordered Statistics (ROS) analysis was used to determine mean settling velocity and the 95% Confidence Interval for each experimental condition by adjusting for the truncated values (Shumway et al., 2002). Due to the non-normal distribution of the settling velocity results, the non-parametric Kruskall-Wallis test was used to evaluate if there were differences between any of the treatment groups or combinations.

2.3. Wetland mesocosm release experiments

To investigate the effect of wetland factors (salinity, flow rate, and vegetation) on reduction of particle numbers, experiments were completed in re-circulating wetland mesocosm tanks (Figure 3.2A). Three tanks were used in this study, and each was a closed system measuring 3 m by 0.5 m. A depth of one cm of commercially available Monterey Beach silica sand was used on the bottom of the tank as substrate. Water for

the tanks was obtained from Tembladero Slough in Castroville, CA. Each tank was filled with 450 L of water to achieve a depth of 30 cm. Ten treatment conditions were compared: non-vegetated tanks at high and low salinity, California bulrush (*Schoenoplectus californicus*) at low salinity in two configurations, and slough sedge (*Carex obnupta*) at low salinity. Each of these conditions was tested at fast and slow water flow rates. The inflow velocity of water was manipulated to compare flow rates of 0.1 cm/sec (slow) and 1 cm/sec (fast), which approximate water flow rates observed in regional natural and reconstructed coastal wetlands (Shapiro et al., 2010a).

In non-vegetated tanks, salinity was increased from baseline (0.1 ppt; low) to 30 ppt (high) through addition of Coralife Scientific Grade Marine Salt® to the tank. For experimental conditions requiring vegetation, California bulrush and slough sedge were transplanted from Molera Constructed Wetland near Castroville, CA and placed in tanks at 150 cm from the water inflow and between the two sampling locations in either of two configurations: buffer or channel. The buffer configuration consisted of stands of bulrush being placed across the width of the tanks (Figure 3.2B). The root and substrate mass reached a height of 15 cm, and plant stalks continued past the top of the water. The channel configuration was similar to the buffer configuration, but an opening 25 cm wide was left between two stands of bulrush (Figure 3.2C). For slough sedge, only the buffer configuration was used. Three tank replicates were evaluated for each combination of salinity, flow rate, and vegetation.

At the beginning of each experiment, aliquots of 4.5×10^6 each of *C. parvum*, *G. lamblia*, and DG *T. gondii* surrogates were homogenized into a 5 mL volume of phosphate buffered saline (PBS). Prior to particle injection, baseline water samples were

taken to determine prevalence of naturally occurring *C. parvum* or *G. lamblia*. Particles were then injected as a bolus at a depth of 10 cm and a distance of 10 cm from the inflow. Fifty mL samples were collected downstream at a 15 cm depth at two locations: 76 cm and 226 cm from the inflow, at post-injection times of 1, 5, 10, 15, 30, 60, 90 min, and 2, 4, 6, 24, 48, and 72 hr. Water quality data, including pH, temperature, salinity, conductivity, turbidity, and dissolved oxygen, were also collected for each tank once at the start of the experiment.

After collection, samples were centrifuged at 1000 g for 15 min and the upper 45 mL of supernatant was discarded. The remaining pellet was stored at 4º C and processed within 48 hr. *Cryptosporidium parvum* and *G. lamblia* were concentrated using immunomagnetic separation (IMS) with a half dose of magnetic beads (Miller et al., 2005) (Dynabeads® Invitrogen Life Sciences, Carlsbad, CA, USA) and visualized using DFA (Easy Stain™, BTF Bio, Pittsburgh, PA, USA) as described in section 2.2. Results were expressed as the (oo)cyst count per 50 mL of water. Supernatant from the IMS process was directly filtered through a 5µm pore size membrane filter and *T. gondii* surrogates trapped on the filter surface were enumerated under epifluorescence microscopy (Shapiro et al., 2010b).

To estimate the time for water to fully circulate within the tank and allow complete dispersion, concurrent tracer experiments were conducted for each treatment condition. Using a single bolus injection of sodium bromide (NaBr), the salt reached equilibrium in each tank after 15 min, so samples taken at 15 min or earlier were excluded from subsequent analyses in order to focus on post-dispersion parasite counts.

Mesocosm tank experimental data were analyzed using longitudinal negative binomial (NB) regression models to determine associations between cyst counts and key factors including sample location, flow rate, salinity, vegetation presence, vegetation species (bulrush versus sedge), configuration of bulrush (buffer versus channel), and time post-particle injection. The models were fitted using generalized estimating equations (GEE) because this approach inherently allows for temporal autocorrelation, which we expected to be present in our data by virtue of repeated sampling of each tank; a first-order autoregressive correlation structure was specified in the GEE formulation after an initial examination of the covariance structures (Liang and Zeger, 1986). Negative binomial variance was specified in the models because the descriptive statistics revealed that the mean counts for all three parasites were over-dispersed, with the variances far exceeding the means. A logarithmic link function was employed in order to assure that modeled mean counts were non-negative, as is typical for negative binomial regressions (Ver Hoef and Boveng, 2007). Predictors were included for each variable assessed in the study, including time post-particle injection. The importance of each predictor was inferred from its odds ratio, which in turn was calculated by exponentiation of the estimated parameter value corresponding to that predictor (Ozminkowski et al., 2002).The effect of individual covariates was modeled first, and those determined to be significant were then used to build models with multiple covariates. Quasi-likelihood under the Independence model Criterion (QIC) values were used to determine model fit. Statistical analyses were conducted using SAS software, Version 9.2 (SAS Institute Inc., Cary, NC, USA, PROC GENMOD, with options REPEATED, CORR=AR(1), and DIST=NEGBIN), and $p<0.05$ was considered statistically significant.

3. Results

3.1. Settling column experiments

A total of eight conditions were examined in settling column experiments, each run in triplicate, and 48 individual settling velocities were determined, which included one for each condition per replicate and sampling port. The mean settling velocities (± standard error) were 0.44 (±0.13) µm/sec for *C. parvum*, 0.84 (±0.16) µm/sec for *G. lamblia*, and 1.05 (±0.19) µm/sec and 1.14 (±0.19) µm/sec for DG and GB *T. gondii* surrogates, respectively. No statistical difference in settling velocities was observed between the top and the bottom ports within a single column, so data from both ports was pooled for subsequent analysis.

Settling velocity means and standard errors for *C. parvum*, *G. lamblia*, and both *T. gondii* surrogates are shown in Table 3.1. When the settling velocities were compared across individual predictor variables of water type, salinity, and temperature, the DG surrogates' settling velocities were significantly different between the pure and environmental water matrices (Kruskall-Wallis $p < 0.05$). When these predictor variables were examined in combination, significant differences were found for the settling velocities of both *C. parvum* and *G. lamblia:* in pure water, increased salinity led to faster settling; however, in environmental water, increased salinity led to slower settling (Kruskall-Wallis $p < 0.05$, for both parasites). No significant differences were found between settling velocities at high and low temperatures.

3.2. Wetland mesocosm release experiments

Based on the significant effect of salinity on the settling velocities of protozoa in the settling column experiments, salinity was also examined in the subsequent mesocosm

release experiments using environmental waters. With the NB regression model evaluating the effects of individual predictors (Table 3.2), the *C. parvum* odds ratio for the effect of salinity was 1.33 (p <0.05), indicating that as salinity increased, 1.33 oocysts would be found per 50 mL in more saline water for every 1 oocyst found per 50 mL in low salinity water. No significant difference was observed between slow and fast flow rates for either parasite or *T. gondii* surrogate counts with the NB model (Table 3.2). There was no significant difference between sampling locations or flow rates in the non-vegetated system in the NB analyses.

Both California bulrush and slough sedge significantly enhanced removal of *G. lamblia* and *T. gondii* surrogates from the surface water in the wetland mesocosm tanks, compared to experimental replicates where no vegetation was used. Figure 3.3 shows the mean parasite count changes over time for *C. parvum, G. lamblia,* and *T. gondii* surrogates in surface water from the vegetated and non-vegetated tanks. To illustrate the effect that individual variables had on parasite and surrogate counts, Table 3.2 shows the odds ratios for individual vegetation parameters. For the *T. gondii* surrogate counts, the presence of any vegetation (regardless of species or configuration) was significant (OR = 0.51; p <0.05), indicating that fewer surrogates were recovered from the water column of mesocosm tanks with vegetation, when compared to mesocosm tanks without vegetation.

Individual vegetated conditions also showed significant trends in parasite or surrogate recovery, as shown in Table 3.2. Recovery of *T. gondii* surrogates from all three vegetated conditions was significantly lower than recovery counts from the non-vegetated replicates, with odds ratios of less than 1 (p <0.01). The effect of vegetation species and California bulrush configuration comparisons varied between the parasites

and surrogates (Table 3.2). When comparing buffer and channel configurations of California bulrush, a significant effect on recovery counts occurred for *T. gondii* surrogates only, with an odds ratio of 0.75 (p <0.001), indicating that the buffer strip configuration removed more surrogates than the channel configuration. Conversely, there was no difference in effect of channel or buffer configurations of California bulrush on counts of either *C. parvum* or *G. lamblia.* When comparing vegetation species in a buffer configuration, significant differences were noted for both *G. lamblia* and *T. gondii* surrogates (p <0.001) with odds ratios of 0.45 and 0.7, respectively, which suggests that wetland mesocosm tanks containing bulrush removed more parasites than those containing sedge.

Evaluation of the combined effect of the hydrologic and vegetation parameters on the rate of removal in the mesocosm tanks further emphasizes the role of vegetation in removing *G. lamblia* and *T. gondii* surrogates from surface water. Using longitudinal NB multiple regression analysis (Table 3.3), parameters significant to the final model included the effects of either vegetation type when compared to absent vegetation, plus the effect of increasing salinity for *C. parvum* and *G. lamblia*. For *C. parvum*, both bulrush in the channel configuration and slough sedge had significant effects on parasite counts, with odds ratios of 1.5 and 1.6, respectively (p <0.01), as compared to absent vegetation. The inclusion of salinity was also significant with an odds ratio of 1.8 (p <0.0001), indicating that at increased salinity more *C. parvum* oocysts were retained in surface water than at lower salinity. For *G. lamblia*, bulrush in the buffer configuration was significant with an odds ratio of 0.56 (p <0.05), indicating that when California bulrush is present, fewer cysts were recovered. For *T. gondii* surrogates, all three

vegetation conditions had odds ratios of less than 1 ($p<0.05$), indicating that when vegetation is present, fewer surrogates are recovered. In summary, addition of vegetation significantly enhanced removal of *G. lamblia* cysts and *T. gondii* surrogates, while decreased water salinity enhanced removal of *C. parvum* oocysts in wetland mesocosm tanks.

4. Discussion

Wetlands reduce the concentration of numerous water pollutants and pathogens in effluent waters, but the mechanisms responsible for pathogen removal vary by wetland and pathogen (Hogan et al., 2012; Quinonez-Diaz et al., 2001). This study aimed to better characterize the physical and hydrological conditions that enhance removal of *Cryptosporidium*, *Giardia*, and *Toxoplasma* in fecal pathogen-polluted surface water that enters coastal wetlands. We determined that salinity, water type, and vegetation type and configuration are key factors for reduction of suspended protozoal parasites in water. Although field investigations in the Tembladero Slough wetland system have also been conducted to evaluate its role in pathogen transport (Hogan et al., 2012), experimental laboratory approaches in simulated natural wetlands conditions utilized during this study enabled examination of key factors not easily controlled in the field setting.

At low salinity, faster settling of all four particles (*C. parvum* oocysts, *G. lamblia* cysts, DG and GB *T. gondii* surrogates) was observed in environmental water as compared to pure water, a finding that is consistent with previous studies (Medema et al., 1998; Searcy et al., 2005). Higher salinity correlated with faster settling of *Cryptosporidium* oocysts and *Giardia* cysts in pure water, but higher salinity correlated

with slower settling of *Giardia* cysts in environmental water. This latter observation was surprising in light of recent findings that increased numbers of *T. gondii* oocysts and surrogate microspheres were recovered from settled aggregates in environmental waters with increased concentrations of salt (Shapiro et al., 2012). The effect of salinity on aggregation and subsequent particle settling may be countered by increased buoyancy of particles when suspended in higher density aquatic solutions. In addition, the experimental setup described here was conducted on spiked parasites and surrogates added directly to settling columns, without incubation or rolling conditions that enhance formation of aggregates (Shapiro et al., 2012). Thus, the apparent impact of salinity on pathogen settling may be confounded by experimental methodology, altered surface properties of the organism, and/or interactions between the protozoa and suspended sediments (Dumètre et al., 2012).

Previous studies aiming to quantify the settling velocities of *C. parvum* and *G. lamblia* in pure and environmental waters at low salinity have demonstrated a wide range of results (Dai and Boll, 2006; Medema et al., 1998; Searcy et al., 2005). Our study showed that salinity has a significant effect on the vertical settling of *Cryptosporidium*, *Giardia* and *Toxoplasma* surrogates and that water type also contributes significantly to variation in settling properties, thus accounting for the wide range of results in prior studies. The impact of environmental water salinity on parasite transport was further evaluated in the mesocosm tank studies. For *Giardia* and *T. gondii* surrogates in the settling columns, increased salinity in environmental water correlated with slower settling velocities, and in the mesocosm tanks increased salinity correlated with slower removal

of *Cryptosporidium*. The impact of salinity is relevant for coastal wetlands, which are greatly affected by tidal influence.

Beyond the interaction of water type and salinity affecting the settling of protozoa, the presence or absence of vegetation was also shown to be a critical factor in protozoal removal. Wetlands can reduce microbial pollutant loads through several processes, including adsorption, sedimentation, and vegetative uptake (Kao et al., 2001; Karim et al., 2008; Knox et al., 2008). When vegetation was included in the experimental wetland mesocosm tanks, *Giardia* and *T. gondii* surrogate counts were significantly lower in the surface water as compared with mesocosm tanks without vegetation, suggesting that the presence of vegetation can reduce the concentrations of parasites that remain suspended in surface waters.

The utility of vegetation for microbial removal has been demonstrated in prior studies, which have shown that vegetated buffer strips can effectively remove microbial pathogens from contaminated run-off (Atwill et al., 2006; Miller et al., 2007; Miller et al., 2008; Winkworth et al., 2008). However, different aquatic plants may vary in their ability to remove parasites due to distinct surface properties, unique biofilms, and differential effects on water flow and drag (Lightbody and Nepf, 2006; Nepf, 1999). Therefore, plant selection is an important consideration for wetlands managers in understanding how to best restore degraded wetlands or conserve existing ones (Millenium Ecosystem Assessment, 2005; Shapiro et al., 2010a; Zedler and Kercher, 2005). This study compared California bulrush, a dense, reed-like plant, to slough sedge, a more grass-like plant. Both are found in California coastal wetlands and are commonly used in landscape restoration. The presence of bulrush was more likely to reduce parasite

counts for both *Giardia* and *T. gondii* surrogates; however no difference was seen between the two vegetation treatment groups for *Cryptosporidium*. A further consideration for wetlands managers is how to best integrate vegetation into the landscape, as this can also affect the flow and drag of water (Nepf, 1999). Interestingly, the channel and buffer configurations of California bulrush in the mesocosm tanks showed no significant difference in removal of *Cryptosporidium* or *Giardia*, despite the presence of a 15 cm gap between stands of bulrush in the channel configuration. Although wider gaps still need to be studied, our results support the use of either configuration in restoration efforts, particularly if the waterway is narrow.

In coastal habitats where land-to-sea transport of parasites such as *Cryptosporidium*, *Giardia*, and *Toxoplasma* is of concern for the commercial and sport harvest of invertebrates for human consumption, recreational water use, and marine wildlife health, the comparatively low-cost water purification services provided by wetlands should not be discounted. The ability of wetland habitats to reduce outflow of parasites and other fecal pathogens to receiving coastal waters could play a key role in preserving or restoring adequate water quality that will promote human and wildlife health. Salinity and the presence of vegetation play an important role in moderating wetlands' capacity to remove parasites through adsorption, sedimentation, and uptake (Knox et al., 2008). Because much of the coastal wetlands in the United States have been degraded, there are numerous efforts currently underway to restore these habitats; the inclusion of vegetation within wetland restoration projects will not only promote healthier ecosystems, but will also enhance parasite removal from surface waters.

5. Conclusions

- In settling column experiments, increased salinity in environmental water led to slower settling of *Giardia lamblia* and *Toxoplasma gondii* surrogates, and in mesocosm tank experiments, increased salinity correlated with slower removal of *Cryptosporidium parvum.*
- In experimental wetland mesocosm tanks, addition of vegetation led to enhanced removal of *Giardia lamblia* and *Toxoplasma gondii* surrogates from surface water.
- Experimental wetland mesocosm tanks containing California bulrush (*Schoenoplectus californicus)* removed greater numbers of *Giardia lamblia* cysts and *Toxoplasma gondii* surrogates when compared to slough sedge (*Carex obnupta*).
- Assessment of two configurations of California bulrush (*Schoenoplectus californicus*) in wetland mesocosm tanks revealed that both buffer and channel configurations work equally well at removal of C*ryptosporidium parvum*, *Giardia lamblia*, and *Toxoplasma gondii* surrogates from surface water.

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Table 3.1: Settling velocities (µm/sec) as measured in settling column experiments utilizing *Cryptosporidium parvum* oocysts, *Giardia lamblia* cysts, and *Toxoplasma gondii* surrogate microspheres (DG-Dragon Green; GB-Glacial Blue), stratified by water type and relative salinity

			Cryptosporidium		***Giardia***		**DG surrogates for *T. gondii***		**GB surrogates for *T. gondii***	
Water Type	**Salinity (ppt)**	**n**	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**
Pure	Low (0)	6	0.08	0.04	0.14	0.04	0.42	0.13	0.84	0.26
Pure	High (30)	6	0.93	0.44	1.04	0.14	0.84	0.50	0.81	0.51
Environmental	Low (0)	6	0.86	0.28	1.48	0.49	1.82	0.48	1.95	0.48
Environmental	High (30)	6	NS		0.79	0.29	1.11	0.30	1.15	0.23

NS=No Settling

Table 3.2: Effect of individual hydrologic or vegetation parameters on counts of *Cryptosporidium parvum* oocysts, *Giardia lamblia* cysts, and Dragon Green (DG) *Toxoplasma gondii* surrogate microspheres in wetland mesocosm tanks using longitudinal negative binomial regression.

		Cryptosporidium		***Giardia***		***T. gondii* surrogates**	
Predictor	**n**	**OR**	***p***	**OR**	***p***	**OR**	***p***
Salinity-Low	480	1		1		1	
Salinity-High	120	1.33	0.0167*	0.79	0.3377	1.30	0.3422
Flow-Slow	300	1		1		1	
Flow-Fast	300	1.02	0.8796	1.12	0.5475	1.19	0.4052
Tank Location 1	300	1		1		1	
Tank Location 2	300	0.99	0.8384	1.00	0.988	1.04	0.2221
Time since injection (continuous)	600	0.97	<0.0001*	0.97	<0.0001*	0.97	<0.0001*
Vegetation absent	240	1		1		1	
Vegetation present	360	1.04	0.7114	0.97	0.9147	0.51	<0.0001*
Vegetation							
Absent (reference)	240	1		1		1	
Bulrush Buffer	120	0.92	0.5737	0.66	0.0729	0.41	<0.0001*
Bulrush Channel	120	1.05	0.7271	0.82	0.3809	0.54	0.0006*
Sedge Buffer	120	1.14	0.3366	1.46	0.1003	0.59	0.0026*
Bulrush Configuration							
Channel (reference)	120	1		1		1	
Buffer	120	0.88	0.4504	0.81	0.3775	0.75	0.0006*
Vegetation Species							
Sedge (reference)	120	1		1		1	
Bulrush	120	0.81	0.1225	0.45	<0.0001*	0.70	<0.0001*

OR = odds ratio = e^{β}, n = number of water samples included in analysis for factor, *Effect of factor is significant with $p<0.05$

Table 3.3: Effect of multiple hydrologic and vegetation parameters on counts of *Cryptosporidium parvum* oocysts, *Giardia lamblia* cysts, and Dragon Green (DG) *Toxoplasma gondii* surrogate microspheres in wetland mesocosm tanks using longitudinal negative binomial regression.

		Cryptosporidium		***Giardia***		***T. gondii* surrogates**	
Predictor	**n**	**OR**	***p***	**OR**	***p***	**OR**	***p***
Salinity-Low (reference)	480	1		1		1	
Salinity-High	120	1.8	<0.0001*	0.69	0.2922	0.77	0.3427
Vegetation							
Absent (reference)	240	1		1		1	
Bulrush Buffer	120	1.3	0.1381	0.56	0.0167*	0.36	<0.0001*
Bulrush Channel	120	1.5	0.0075*	0.69	0.1445	0.48	0.0033*
Sedge Buffer	120	1.6	<0.0001*	1.23	0.3045	0.52	0.0079*

OR = odds ratio = e^{β}
n = number of water samples included in analysis for factor
*Effect of factor is significant with $p<0.05$

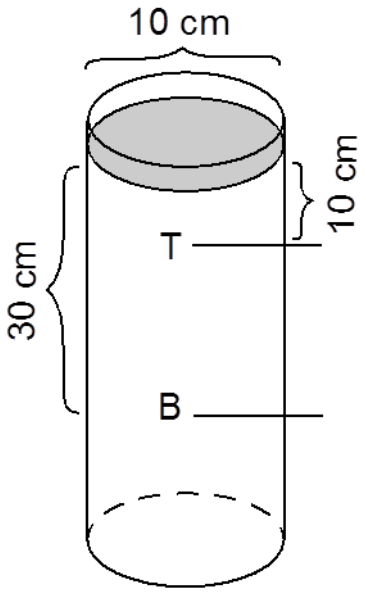

Figure 3.1: Schematic diagram of an experimental settling column where T is top sampling port and B is bottom sampling port.

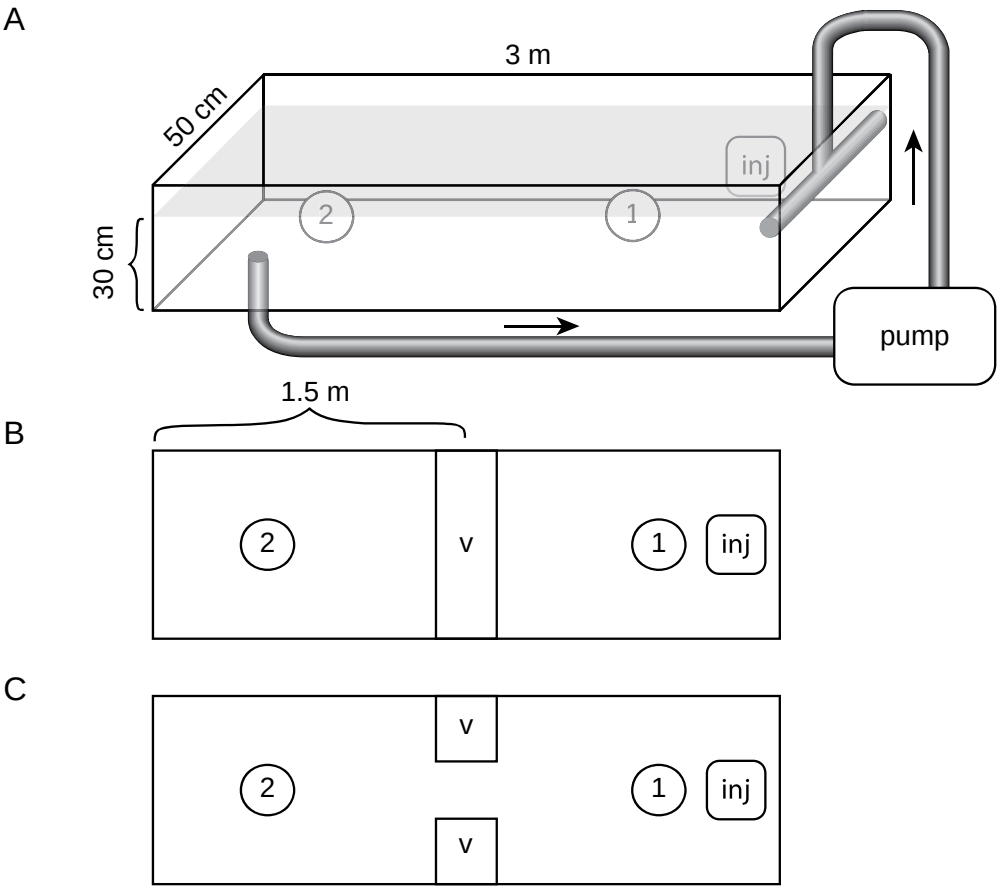

Figure 3.2: Schematic diagrams of an experimental wetland mesocosm tank where 1 and 2 indicate sampling locations, and "inj" indicates the injection point for *Cryptosporidium parvum*, *Giardia lamblia* and DG *Toxoplasma gondii* surrogates. (A) Entire mesocosm tank with water flow directionality; (B) Mesocosm tank view from above with vegetation (v) in buffer strip configuration; (C) Mesocosm tank view from above with vegetation (v) in channel configuration.

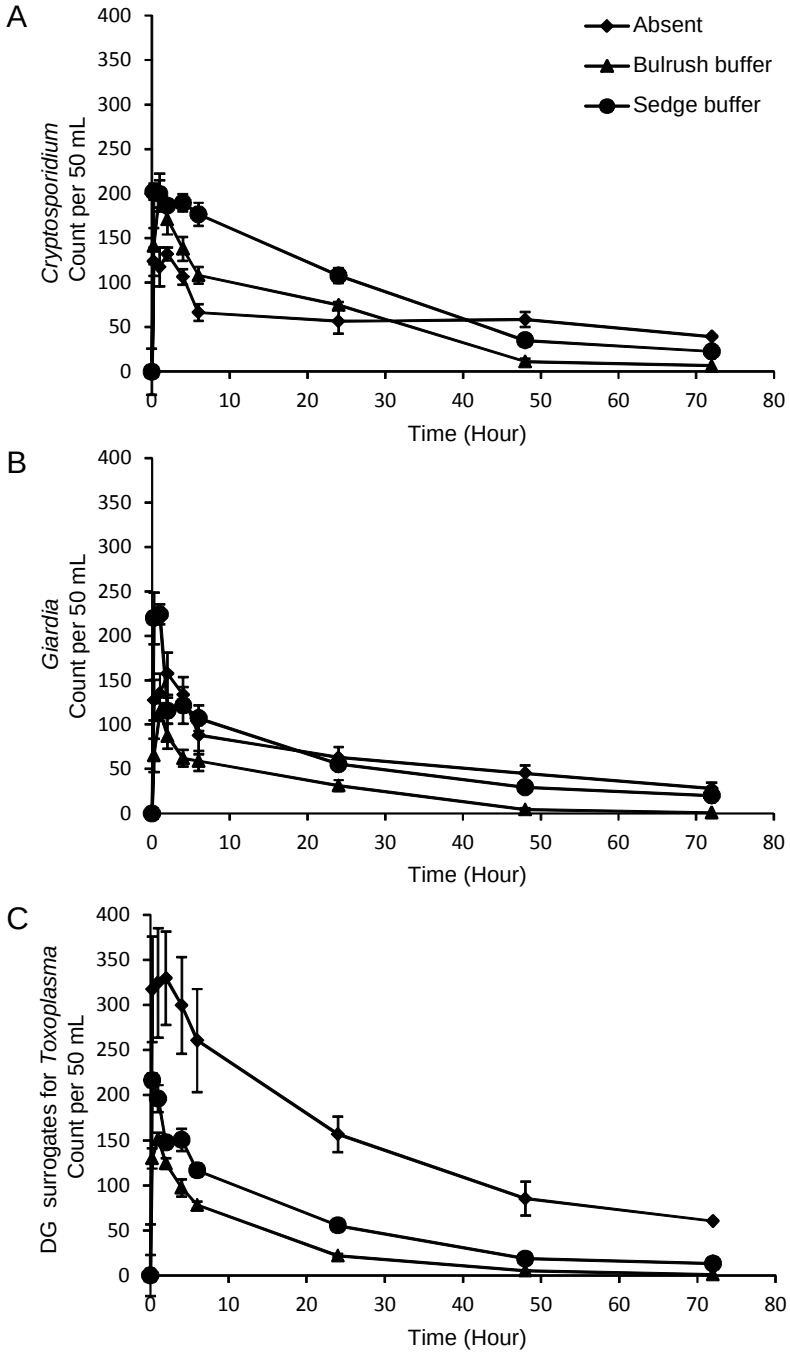

Figure 3.3: Concentrations of *Cryptosporidium parvum*, *Giardia lamblia*, and Dragon Green *Toxoplasma gondii* surrogates per 50 mL volume of surface water in mesocosm tanks plotted over time. Three replicates of each wetland condition were tested to evaluate the effect of vegetation presence and species on removal of parasites from water column. (A) *Cryptosporidium*, (B) *Giardia*, and (C) *Toxoplasma* surrogates.

CHAPTER 4

Giardia in mountain gorillas (*Gorilla beringei beringei*), forest buffalo (*Syncerus caffer*), and domestic cattle in Volcanoes National Park, Rwanda

Giardia in mountain gorillas (*Gorilla beringei beringei*), forest buffalo (*Syncerus caffer*), and domestic cattle in Volcanoes National Park, Rwanda

Running Title: Hogan et al.—*Giardia* in Rwandan wildlife and cattle

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ABSTRACT

Mountain gorillas (*Gorilla beringei beringei*) are critically endangered primates surviving in two isolated populations in protected areas within the Virunga Massif of Rwanda, Uganda, and the Democratic Republic of Congo, and in Bwindi Impenetrable National Park in Uganda. Mountain gorillas face intense ecologic pressures due to their close proximity to humans. Both the human communities living outside the national parks as well as the numerous human activities occurring within the national parks, including research, tourism, illegal hunting, and anti-poaching patrols, lead to a relatively high degree of contact between mountain gorillas, wildlife, domestic animals, and humans. To investigate key interfaces and assess the pathogen transmission potential between wildlife and livestock, feces of mountain gorillas, forest buffalo (*Syncerus caffer nanus*) and domestic cattle (*Bos taurus*) in Rwanda were examined for the parasites *Giardia* and *Cryptosporidium*. *Giardia* was found in 9% of mountain gorillas, 6% of cattle, and 2% of forest buffalo. Our study represents the first report of *Giardia* prevalence in forest buffalo. *Cryptosporidium*-like particles were also observed. Molecular characterization of *Giardia* isolates identified zoonotic genotype assemblage B in the gorilla samples and assemblage E in the cattle samples. A significant spatial clustering of *Giardia*-positive samples was observed in one sector of the park. While this study did not find evidence for transmission of protozoa from forest buffalo to mountain gorillas, the genotypes of *Giardia* samples isolated from gorillas suggest that the importance of humans in this ecosystem should be more closely evaluated.

KEY WORDS (4-8): *Cryptosporidium*, disease transmission, ecosystem health, *Giardia*, mountain gorilla, one health, Rwanda

INTRODUCTION

Mountain gorillas are a critically endangered, charismatic species living in a restricted range in Africa in close proximity to people. Of the world's remaining 786 mountain gorillas, approximately 480 live in the Virunga Massif, which spans the borders of Rwanda, Uganda, and the Democratic Republic of Congo, and includes Volcanoes National Park in Rwanda. The park is bordered by dense human communities averaging 300 people/ km^2 (Gray and Kalpers, 2005), which practice subsistence crop and animal agriculture. Additionally, there is considerable human activity inside the park for research, tourism, illegal hunting and harvest, and anti-poaching patrols. Livestock are grazed on lands directly abutting the park boundary, while wildlife, including forest buffalo (*Syncerus caffer nanus*), which live inside the park and share habitat with gorillas, routinely enter and exit the park. The buffalo utilize domestic livestock habitat and come into direct contact with both cattle and mountain gorillas.

The protozoal parasites *Giardia lamblia* (syn. *G. intestinalis*, *G. duodenalis*) and *Cryptosporidium parvum* (Xiao and Fayer, 2008) are significant fecal-oral pathogens for both people and animals, infect a wide range of hosts, and cause disease in their hosts (Fayer et al., 2004, Gillespie and Chapman, 2006). Both *Giardia* and *Cryptosporidium* have been identified in humans, domestic cattle, and mountain gorillas (Nizeyi et al., 1999, Sleeman et al., 2000, Graczyk et al., 2002). Whether or not these parasites infect other wildlife in the Virunga Massif is not known, nor is it known whether other wildlife could serve as a potential reservoir for transmission of these parasites to or from mountain gorillas.

The environmentally resistant cyst or oocyst stage allows these parasites to survive in soil, feces, and water for up to a year before infecting a new host (Olson et al.,

1999, Slifko et al., 2000). In an ecosystem such as the Virunga Massif, all mammals, humans included, may be sharing parasites through contaminated soils and vegetation (Olson et al., 1999, Dumètre et al., 2012) as a result of overlapping habitat and shared foraging grounds. For *Giardia* in particular, the genotypic assemblages A and B have zoonotic potential (Cacciò and Ryan, 2008), creating the possibility for cross-species transmission in this ecosystem.

Understanding the transmission cycle of parasites within this ecosystem is critical for identifying beneficial interventions to reduce pathogen spread between species and to improve human and animal health in this region. This study focused on the cattle-forest buffalo-mountain gorilla transmission cycle for *Cryptosporidium* and *Giardia*. Using fecal samples from mountain gorillas within the park, domestic cattle living at the boundary of the park, and forest buffalo that are known to cross the boundary frequently, the prevalence, spatial distribution and genotypic diversity of *Giardia* and *Cryptosporidium* were determined in order to elucidate whether forest buffalo serve as an intermediary for parasite transmission between mountain gorillas and domestic cattle.

MATERIALS AND METHODS

Study site and sample collection. This study was conducted in September 2010 in the Volcanoes National Park, which comprises Rwanda's protected portion of the Virunga Massif, and covers approximately 130 km^2. Samples were collected in the Shingiro, Nyange, and Kinigi sectors that border the park, in which buffalo and other wildlife were anecdotally known to frequently exit the park to forage in farmlands.

A maximum of 50 fecal samples were collected from each study species (mountain gorillas, forest buffalo, and cattle) in each of the three sectors; sample size calculation was based on prevalence of protozoal parasites reported in previous studies (Nizeyi et al., 1999, Sleeman et al., 2000, Graczyk et al., 2002). If a sector had less than 50 individual animals of each study species living in that sector, then all samples were used in the study. Collection was conducted on a single day per gorilla family group, cattle sector, or buffalo sector, to avoid oversampling any given individual. All fecal samples were collected and stored in individually numbered bags on ice in the field, or in a refrigerator in the laboratory, until initial processing within 24 hours of collection. At each sampling site, a GPS location was recorded for subsequent spatial analysis.

Mountain gorillas live in family groups, the members and home range of which have been well characterized. Fifteen family groups were identified as having a portion of their home range adjacent to one of the three sectors of this study, and were therefore the groups targeted for sampling. Park rangers collected all feces deposited in the night nests of each individual mountain gorilla in the targeted family group. Multistage cluster sampling by sector and family group was utilized to obtain 50 samples per sector, while collecting a proportionate number of samples per family group. The total number of cattle per farm or household was determined. If a sector had greater than 50 cattle living in the area, simple random sampling was utilized to determine which cows in that sector were to be sampled for the study. For cattle samples, all farms within the three sectors were visited and cattle were either observed defecating and a sample was collected, or feces were collected from night quarters where cattle were individually housed. Body condition score (DEFRA, 2001), sex, breed, age class, and pregnancy status was determined for all

cattle sampled in the study, as these have been considered risk factors for protozoal infection and increased shedding in cattle (Fayer, 2004, Thompson, 2004). The population of the forest buffalo was unknown at the time of this study; to collect samples, trackers followed fresh trails into the forest in each of the three sectors and collected any fresh buffalo feces observed.

Parasite identification. Fresh fecal samples were processed similarly to previously published studies (e.g., Gaydos et al., 2008), with slight modification. Briefly, 3 g of feces were mixed with 12 mL of 0.1% Tween 80, sieved through disposable gauze, centrifuged for 10 min at 1000g at 4°C and the supernatant discarded. The fecal slurry consisting of the top half of the remaining pellet with fine particulate matter was analyzed. For direct fluorescent antibody (DFA) tests to observe parasites and determine prevalence 50 µl of 10% formalin was mixed with 5 µl of fecal slurry and air dried onto hydrophobic DFA slides for microscopy. Slides were stained with EasyStain™ DFA tests (BTF Bio, Pittsburg, PA) and examined under epifluorescent illumination using fluorescein isothiocyanate (FITC) and 4',6-diamidino-2-phenylindole (DAPI) emission filter sets. Particles with apple-green fluorescence in a round to oval shape (6-15 µm in diameter) with bright, highlighted edges under FITC and light blue internal staining with a green rim or up to four distinct, sky-blue nuclei under DAPI were classified as *Giardia* cysts. Particles with apple-green fluorescence in an oval or spherical shape (3-7 µm in diameter) with bright, highlighted edges under FITC and light blue internal staining with a green rim or up to four distinct, sky-blue nuclei under DAPI were classified as *Cryptosporidium* oocysts. (U.S. Environmental Protection Agency, 2005). A subset of samples was selected for preservation. For those samples, 1 mL of fecal slurry was heated

to 72°C for 30 min and re-suspended in 1 mL of RNAlater® (Qiagen, Valencia, CA) and stored at 4°C for future DNA isolation.

Molecular Characterization. Samples positive for *Giardia* by fluorescent microscopy for which we had preserved fecal slurry were processed further to isolate *Giardia* cysts by immunomagnetic separation (IMS) using Dynabeads® G-C Combo (Invitrogen Life Technologies, Grand Island, NY). For *Giardia* positive samples for which no preserved fecal slurry was available, fecal material was scraped from the DFA slide for DNA isolation (Gaydos et al., 2008). Parasite DNA was isolated using DNeasy® Blood & Tissue kits (Qiagen, Valencia, CA), with three consecutive freeze/thaw cycles in liquid nitrogen and boiling water to more effectively break apart the cyst walls.

The molecular characterization of *Giardia* cysts was accomplished through nested PCR amplification and sequencing of a 432-bp region of glutamine dehydrogenase (GDH), as described previously (Read et al., 2004) using external forward primer GDHeF: (5′-TCAACGTYAAYCGYGGYTTCCGT-3′), internal forward primer GDHiF: (5′-CAGTACAACTCYGCTCTCGG-3′) and reverse primer GDHiR: (5′-GTTRTCCTTGCACATCTCC-3′). Additionally, a nested PCR amplification and sequencing of a 384 bp region of β-giardin was completed as described previously (Cacciò et al., 2002) using external forward primer G7 (5′-AAGCCCGACGACCTCACCCGCAGTGC-3′), internal forward primer G376 (5′-CATAACGACGCCATCGCGGCTCTCAGGAA-3′), and reverse primer G759 (5′-GAGGCCGCCCTGGATCTTCGAGACGAC-3′). For both GDH and β-giardin, PCR amplification conditions were as described, except that HotStarTaq® DNA polymerase (Qiagen, Valencia, CA) was used, and the reactions included 0.4 µg/ml BSA. PCR

products were treated with ExoSAP-IT® (Affymetrix, Santa Clara, CA) according to the manufacturer's instructions before sequencing. Type and subtype were determined by comparison of determined sequences to previously described reference sequences.

Statistical analysis. Prevalence for each species was calculated as the number of positive fecal samples / total collected number of fecal samples. Additionally, prevalence was calculated per sector for each species and per age class (pre-weaned versus weaned), body condition score, and sex of cattle to characterize demographics and possible risk factors for *Giardia* infection. In order to evaluate the spatial distribution and clustering of *Giardia* cases, spatial analysis using a multinomial Bernoulli modeling approach (Kulldorff and Inc, 2006, Jung et al., 2010) was conducted using SaTScan™ v.9.1.1 (Boston, MA, USA) to determine the most likely circular cluster of cases within the distribution of a single species and also within the distribution of all samples collected from all three species: mountain gorillas, cattle, and buffalo. $P < 0.05$ were considered significant.

RESULTS

Prevalence of *Giardia* and *Cryptosporidium*. Using epifluorescent microscopy, *Giardia* was detected in all three study species: mountain gorilla, domestic cattle, and forest buffalo (Table 4.1). Overall, 9% (11 / 130) of mountain gorilla feces were positive for *Giardia*; the parasite was detected in fecal samples from 33% of the gorilla groups (5 / 15 groups surveyed). Of these five infected groups, three were habituated for research activities, and two were habituated for tourism. All positive groups were identified as typically residing within the Kinigi sector of the park. Similarly, 6% (8 / 135) of cattle

fecal samples were positive for *Giardia*; all infected cows were age one year or older. Of the cattle for which demographic data was available, 7 / 114 females and 1 / 19 males were positive for *Giardia*. No pregnant females (n=14) were positive for *Giardia*. Three of the *Giardia* positive cattle resided in Kinigi, four in Nyange, and one in Shingiro sectors. Of forest buffalo samples, 2% (1 / 55) were positive for *Giardia*; the one positive forest buffalo sample was collected in Kinigi sector. *Cryptosporidium*-like particles were observed in 1% (1 / 130) of mountain gorilla, 3% (4 / 135) of cattle, and 36% (20 / 55) of buffalo samples. Due to low numbers of these *Cryptosporidium*-like particles (<50 oocyst-like particles per sample), there was insufficient genomic material for molecular characterization and genotyping purposes.

All of the *Giardia*-positive gorilla samples were collected in the Kinigi sector of the park (Figure 4.1); SaTScan analysis was conducted to evaluate whether this trend indicated a statistically significant cluster. *Giardia*-positive mountain gorilla fecal samples produced a space-cluster in the Kinigi sector with a radius of 5.24 km (relative risk=31.94, $P < 0.0001$). No significant clustering was observed among *Giardia*-positive cattle or buffalo samples. When evaluating all species using a Bernoulli multinomial approach, the *Giardia*-positive cluster was still significant, and located in the Kinigi sector as the gorilla-only cluster, which also encompassed the infected buffalo sample (relative risk=5.25, $P <0.0001$).

Molecular characterization. Because the genetic diversity of *Giardia* among host species is an important factor for consideration of shared parasitism, we identified the *Giardia* genotype of each positive sample using molecular tools (Table 4.2). Due to insufficient DNA recoverable from low numbers of cysts, particularly from slide

scrapings, PCR products were obtained for just 6 of 11 gorilla samples, 1 of 8 cattle samples, and 0 of 1 buffalo samples. Nevertheless, sequence analysis of the GDH locus of the *Giardia* detected in the mountain gorilla samples revealed that five samples were of the B-IV subtype, covering three family groups, and one sample from a different family group was of the B-III subtype. Sequence analysis of the β-giardin locus confirmed the B-IV subtype in the five samples, and assemblage B in the one sample. Sequence analysis of the cattle sample with both GDH and β-giardin loci revealed that it was *Giardia* assemblage E, which is known to be host specific to cattle.

DISCUSSION

Mountain gorillas surviving in the Virunga Massif face a variety of ecological pressures, including sharing their limited habitat with humans (Woodford et al., 2002, Cranfield, 2008). While gastrointestinal disease is not a primary cause of gorilla mortality (Cranfield, 2008), and in many cases may be asymptomatic (Gillespie et al., 2004), it is important to consider the long term effects that gastrointestinal parasites may have for general health, survival and reproduction (Gillespie and Chapman, 2006). Furthermore, continued interaction and contact with humans and livestock may result in altered transmission rates and virulence of gastrointestinal parasites for mountain gorillas (Gillespie et al., 2004), and may well provide a route of transmission for other pathogens.

Our findings suggest that *Giardia* prevalence in mountain gorilla has increased to 9% (11 / 130), compared to the 3% (2 / 70) prevalence detected in 1997 (Sleeman et al., 2000), although another study in 2003 determined the prevalence to be 10% (16/169)

(Gaffikin et al., 2003). On the other hand, we determined that *Giardia* prevalence in domestic cattle in the region has decreased to 6% (8 / 135) from 16% (25 / 153) in 2003 (Gaffikin et al., 2003). Although pre-weaned cattle are more susceptible to *Giardia* infections and shed higher number of cysts (Thompson, 2000), none of the *Giardia* positive domestic cows evaluated in this study were younger than one year of age. Age distribution was not available for the 2003 study (Gaffikin et al., 2003). Interestingly, *Cryptosporidium* has been found in both cattle and mountain gorillas previously (Nizeyi et al., 1999, Nizeyi et al., 2002, Gaffikin et al., 2003), but no confirmed *Cryptosporidium* infections were detected in this study. Additionally, we detected a low level of *Giardia* in forest buffalo, with a prevalence of only 2% (1 / 55). This represents a first report for *Giardia* in this host.

The cross-sectional design of this study coupled with a limited volume of examined feces may have led to an underestimate of the prevalence of both *Giardia* and *Cryptosporidium* in our study species, especially considering that infected animals can be low or intermittent shedders. Additionally, it is possible that we oversampled buffalo, as individuals were not visualized prior to collection, which could have led us to underestimate parasite prevalence in this species.

The lower *Giardia* prevalence in cattle observed in this study compared to earlier published reports may indicate that livestock management changes implemented after the other studies were conducted are improving the parasite prevalence in the cattle. Zero-grazing is a livestock management strategy that mandates restrictions on cattle range, with cut grass replacing pastured grazing. In 2006, the Rwandan government implemented a “one cow per family” program with the goals of reducing chronic

childhood malnutrition, increasing household food security, and generating alternative income. Farmers receiving cows through this program were required to implement a zero-grazing livestock management system (Kim et al., 2012). In addition to the reduced pressures on land use in low carrying-capacity regions, this control strategy has also been shown to reduce a number of cattle-specific and zoonotic diseases. For example, a tick survey implemented to develop a control strategy for East Coast fever (*Theileria parva*; ECF) showed that the widespread use of zero-grazing in northwestern Rwanda resulted in a delayed age-at-first-contact with ticks, and thus fewer losses to ECF in northwestern Rwanda compared to other regions of the country (Bazarusanga et al., 2007). Additionally, a lower prevalence of brucellosis was observed in zero-grazing areas in sub-Saharan African countries (McDermott and Arimi, 2002). A zero-grazing management system is most effectively implemented with small landholders who own few cattle per household (Lukuyu et al., 2009), which characterizes the farms surrounding the Volcanoes National Park. While this study did not specifically set out to examine the benefits of zero-grazing on the health of cattle in this area, the decrease in *Giardia* prevalence since zero-grazing was implemented may suggest that decreased parasitism is an added benefit of this strategy.

This study detected two separate genotypic assemblages of *Giardia* infecting our study species: the multi-host species genotype assemblage B in mountain gorillas and the cattle-specific assemblage E in cattle. Within assemblage B, two subtypes were found: B-III and B-IV. Our findings differ from a previous study reporting assemblage A in mountain gorillas, humans and cattle in Bwindi Impenetrable National Park, which suggested cross-transmission between the species may have been occurring (Graczyk et

al., 2002). The SSU-rDNA locus used in that study has low phylogenetic resolution and is prone to misclassification bias (Traub et al., 2005, Johnston et al., 2010); in contrast, we employed two loci sensitive enough to distinguish between assemblages. Our discovery of assemblage B is interesting because it has been observed in a wide range of mammalian species, including humans (Appelbee et al., 2005, Xiao and Fayer, 2008). While there is limited knowledge of *Giardia* diversity in mountain gorillas, it has been established that assemblage B infects non-human-primates (NHP): a survey of isolates from 31 captive NHP species, including western and eastern gorillas, documented assemblage B in 48% of the samples (Levecke et al., 2009). Furthermore, assemblage subtype B-IV has been implicated in human-to-wild NHP (colobus and guenon monkey) transmission in Uganda, particularly when coupled with a high degree of ecological overlap (Johnston et al., 2010).

While this study may not have directly identified the transmission cycle of *Giardia* into the mountain gorilla population, the genotypic subtype results suggest that humans may play a role. Of all *Giardia* sequences, 83% were characterized as assemblage subtype B-IV, and 17% were characterized as assemblage subtype BIII; a survey of 1,658 human *Giardia* isolates from ZOOPNET-database comparing the geographic distribution of B-III and B-IV in humans found that in Africa, B-III was detected in 81% of the samples and B-IV was found in 19% of the samples. In North American samples, the opposite trend was found, with 14% of samples characterized as B-III and 86% of samples characterized as B-IV. The distribution in other geographic regions fell somewhere in between (Sprong et al., 2009).

Previous studies have postulated that the local community and park workers may be introducing gastrointestinal parasites into gorilla population, in part due to the suboptimal sanitation in communities and the daily forays of park workers into gorilla habitat (Graczyk et al., 2002). Given the relative prevalence of the subtypes of *Giardia* we observed in this study, it might also be appropriate to examine tourists and researchers for their potential role in the transmission cycle of *Giardia* in this area.

While many studies emphasize that human interaction with mountain gorillas is a significant risk factor for zoonotic disease transmission (Graczyk et al., 2002, Chapman et al., 2005, Goldberg et al., 2007), it is important to consider that the conservation status of the mountain gorilla is highly dependent upon contact with humans. This contact includes implementation of behavioral research programs involving daily human observation, anti-poaching patrols to protect the integrity of the park and its wildlife community, and gorilla ecotourism, which generates significant revenue for both the park and surrounding communities, while educating global citizens about these endangered animals. Disease prevention measures have been implemented to reduce disease transmission from humans to gorillas directly or via environmental transmission (Cranfield, 2008), but these have been mainly enforced for those in daily contact with the animals, such as the researchers or park rangers. Tourism brings individuals from all across the world to an ecosystem with an endangered species sensitive to human pathogens. Only a limited number of studies have been conducted on the role that tourism plays in zoonotic disease transmission in primate-based ecotourism endeavors (Muehlenbein and Ancrenaz, 2009, Muehlenbein et al., 2010).

Giardia was found in all of the species examined in this study - mountain gorilla, cattle, and forest buffalo - in and around the Volcanoes National Park in Rwanda. Compared to studies conducted seven to thirteen years earlier, *Giardia* prevalence has not changed for mountain gorillas, but its prevalence in cattle was lower in this study, perhaps due to livestock management changes implemented in the interim. Additionally, the genetic subtype analysis here suggests that tourism or research may play a larger role in disease transmission of these gastrointestinal parasites, and thus should be included in future studies involving gastrointestinal parasites. These future studies should more deeply examine the role of human-primate contact on disease transmission to endangered populations.

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Table 4.1: *Giardia* prevalence in mountain gorillas (*Gorilla beringei beringei*), forest buffalo (*Syncerus caffer*) and domestic cattle in three sectors of Volcanoes National Park, Rwanda, as determined by direct fluorescent antibody testing.

Sampled Group	Mountain Gorilla			Domestic Cattle			Forest Buffalo		
	n	**Number Positive**	**Prevalence (%)**	***n***	**Number Positive**	**Prevalence (%)**	***n***	**Number Positive**	**Prevalence (%)**
Overall	130	11	9	135	8	6	55	1	2
Sector									
Kinigi	50	11	9	50	3	6	28	1	4
Shingiro	50	0	0	37	1	3	16	0	0
Nyange	30	0	0	48	4	8	11	0	0
Age									
Pre-weaned (<1 year)				24	0	0			
Post-weaned (>1 year)				111	8	7			
Body Condition Score									
1				8	1	13			
2				46	2	4			
3				63	4	6			
4				15	1	7			
Sex									
Male				19	1	5			
Female (pregnant)				14	0	0			
Female (not pregnant)				100	7	7			

Table 4.2: *Giardia* genotypes in mountain gorilla (*Gorilla beringei beringei*), forest buffalo (*Syncerus caffer*), and domestic cattle in Volcanoes National Park, Rwanda using glutamate dehydrogenase (GDH) and β-giardin loci to determine assemblage and sub-type genetic information.

Species	*n*	Assemblage result by GDH	Accession # and Reference	Assemblage result by β-giardin	Accession # and Reference
Mountain Gorilla	5	BIV*	JF773755 (Lebbad et al., 2010)	BIV*	AB618785 (Abe and Teramoto, 2012)
Mountain Gorilla	1	BIII*	DQ923581 (Robertson et al., 2007)	B**	EU014389 (Teodorovic et al., 2007)
Domestic Cattle	1	E*	DQ18265 (Langkjaer et al., 2007)	E*	DQ116621 (Di Giovanni et al., 2006)

*100% match to reference sequence

**90% match to reference sequence

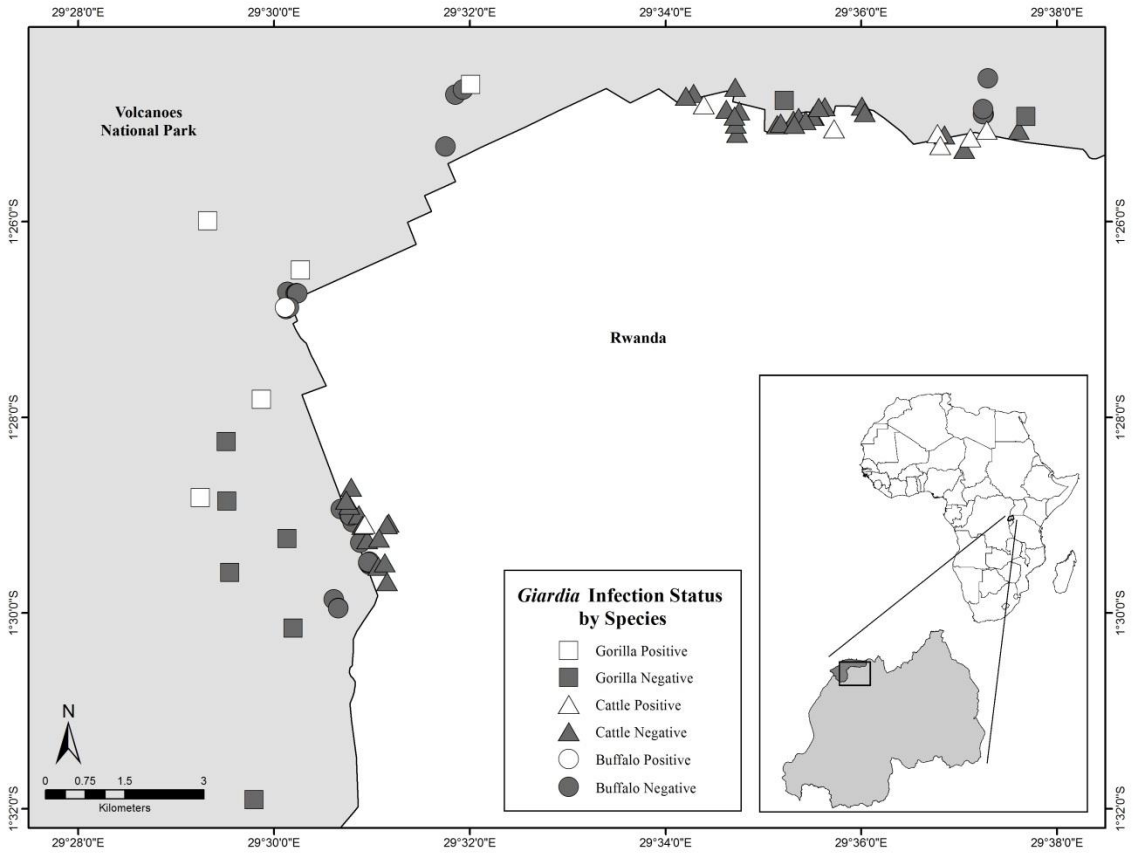

Figure 4.1: Map of project sampling sites in and just outside Volcanoes National Park, Rwanda.

CHAPTER 5

Summary and Conclusions

Summary and Conclusions

The epidemiology of fecal protozoa at the intersection of human, animal, and environmental health encompasses complex ecosystems driven by a number of factors. The baseline prevalence and dynamics of *Cryptosporidium* and *Giardia* in the environment are modulated by the presence of protozoa in humans and animals, as well as environmental factors such as both hydrologic (salinity, flow rate, temperature) and landscape effects such as vegetation. Furthermore, these factors are constantly in flux, as the environment is constantly changing. This dissertation used epidemiologic approaches to assess the effect that environmental changes have on protozoal transport and disease transmission. The knowledge gained can help identify ways to reduce protozoal persistence in hosts and their environments, as well as offering new directions for future studies.

The research presented here aimed to utilize various methods to elucidate some of the factors associated with protozoal prevalence in the environment and animal communities. A literature review in Chapter 1 assessed what is known currently regarding the major factors in protozoal disease transmission. In Chapter 2, a two-year longitudinal study quantified the parasite and fecal indicator bacterial prevalence in California coastal wetlands. Next, in Chapter 3 a series of experimental studies using settling columns and mesocosm wetland tanks measured the individual effects that specific wetland factors (water temperature, salinity, flow rate, and vegetation) had on parasite concentrations in surface water. Finally, a cross-sectional study described in

Chapter 4 evaluated the potential transmission pathways of these parasites among mountain gorilla, domestic cattle, and forest buffalo in the Volcanoes National Park, Rwanda.

Water issues were identified as one of the key factors in protozoal disease transmission; this includes both water parameter alterations such as salinity, as well as water security issues, such as drinkable water for both humans and animals. Infective oocysts of *Cryptosporidium* and *Toxoplasma*, as well as cysts of *Giardia*, are readily transported through water and their transmission is affected by variations in water and in the environment. However, to fully assess these issues, studies must be designed to more thoroughly examine the effects of pathogen transport by variations in the water and the environment. Critical information needed to assess these effects includes understanding what is the baseline protozoal contamination in the environment, what changes are predicted to occur in the environment, and how this baseline contamination may be altered over time given those predicted—or unpredicted—changes. This information can then be used to both propose remediation efforts to reduce current disease risk and model potential scenarios to identify ways of lowering future disease risk.

The baseline risk of parasites in California coastal wetlands was assessed by measuring the number of parasites collected in three different wetlands (constructed, dairy, and tidal) over two years and measuring the effects of factors such as rainfall, seasonality, and proximity to livestock on protozoal concentrations. It was determined that rainfall events occurring within 72 hours prior to sample collection was a key risk factor for increased protozoal counts. Additionally, the wetland nearest the dairy had the highest protozoal counts, and the concentrations decreased as the dairy wetland sample

locations were further from livestock. Dairies and other sources of zoonotic pathogens will always be near surface water sources, which may or may not drain to recreational waters. To combat this, a number of studies have evaluated best management practices of the dairies to reduce their parasite load to surface waters (Atwill et al., 2002, Atwill et al., 2006, Miller et al., 2007, Miller et al., 2008).

Climate change is predicted to have an effect on rainfall and precipitation events in many parts of the world by increasing their intensity and frequency (Michener et al., 1997, Burkett and Kusler, 2000, Hunter, 2003). Incorporating the current background risk of protozoa concentrations in surface waters due to increased rainfall with known predictions for climate change, we can begin to assess how future disease transmission rates may be affected. These predictions can help to identify areas for remediation. Other ways of predicting these effects is through experimental means. Here, vertical settling columns and horizontal flow wetland mesocosm tanks were used experimentally to assess the effects of numerous water parameters on protozoal concentrations. Factors including temperature, salinity, and flow rate may be altered due to predicted climate change (Burkett and Kusler, 2000). In the research presented here, increased salinity was determined to be highly significant to protozoal removal through faster settling for *Cryptosporidium* oocysts, *Giardia* cysts, and *Toxoplasma* surrogate microspheres. Furthermore, vegetation presence, species, and configuration were all determined to be important. The addition of vegetation in the mesocosm wetland tanks led to enhanced removal of all three parasites evaluated. California bulrush removed greater numbers of *Giardia* and *Toxoplasma* surrogates, while both bulrush and slough sedge removed

Cryptosporidium equally well; these vegetation factors may be key to wetland remediation efforts.

Beyond just looking forward towards predicted environmental alterations, some inferences can be made in understanding changes that have already occurred. To evaluate the prevalence of *Giardia* in mountain gorillas, cattle, and forest buffalo in the Volcanoes National Park, Rwanda, a cross-sectional study was implemented. Cross-sectional studies tends to give limited information, as there is no longitudinal component and just the prevalence at a single time-point is evaluated; however, it was noted that the prevalence of *Giardia* infection in cattle had decreased by almost half from studies conducted 10-15 years prior. Furthermore, the *Giardia* prevalence in mountain gorillas has remained relatively constant. When considering modifications to both livestock management and gorilla tourism and research in the interceding period, it was noted that the implementation of zero-grazing policies and the "One Cow per Family" program had taken place. These policies were structured to reduce chronic childhood malnutrition, increasing household food security and generating alternative income (Kim et al., 2012), but other studies showed how they were also beneficial to livestock disease reduction including East Coast Fever (*Theileria parva*; ECF) and brucellosis (McDermott and Arimi, 2002, Bazarusanga et al., 2007). It is conceivable that these policies may also have reduced the prevalence of *Giardia* infection. No substantial increase or decrease in *Giardia* prevalence was observed in mountain gorillas, indicating that either the beneficial effects of the program on livestock did not extend into the national park's wildlife, or that cattle were not as significant of a reservoir as previously had been thought (Graczyk et al., 2002, Nizeyi et al., 2002).

Environmentally transmitted protozoa are constantly affected by changes occurring in the ecosystem. By utilizing these various epidemiologic study design methods and approaching analyses with appropriate statistical methods, inferences can be made to help us mediate the effects of future disease transmission in humans, wildlife, and domestic animals. Protozoal disease does not solely affect humans, but is tied to the effects of disease in animals, and both are connected to environmental variations. By applying a One Health perspective to protozoal pathogens, the complexities of disease dynamic can be assessed. The research presented here evaluated ways in which environmental change may lead to protozoal disease transmission. This was accomplished by both focusing on the environmental transport of protozoa in water, and by seeking to understand protozoal transmission between three animal species living in close proximity.

Factors significantly affecting waterborne pathogen transport identified during preliminary assessments in both a field study of coastal California wetlands and a mesocosm tank model should be explored and validated with additional experiments. In looking at the California coastal wetlands study, one concern identified was that parasites shed from inland livestock were capable of being transported downstream to recreational waters. What is the level of risk in those recreational waters, and how much can be attributed to the dairies versus general human usage and contamination of the water? Identifying which *Cryptosporidium* and *Giardia* genotypes are being shed by livestock, or found in the surface water near dairies, and then comparing that to the protozoal diversity found in recreational waters, shellfish, or water users (human or animal) would be a logical next step. In our experimental studies, the presence of vegetation was found

to be a significant factor for reduction of parasite concentrations. Does this hold true as well in field situations? Comparing protozoal concentration and prevalence in recreational waters and with the population of water users in areas that have either been highly degraded, relatively untouched and/or restored would be an interesting validation of this environmental component and would strengthen the call for using vegetation as a remediation tool.

The role of humans in zoonotic pathogen transmission is a question worth addressing based on the *Giardia* prevalence and genotyping results in our Rwanda study. Humans play a large role in the Virunga Massif ecosystem and frequently come into contact with mountain gorillas and other wildlife. Previous studies have looked at protozoal prevalence in local human communities, revealing that there was a slightly higher *Giardia* prevalence amongst park employees as compared to the local community. (Gaffikin et al., 2003). Thus far, no study has looked at the role of tourists or researchers in the transmission cycle of zoonotic protozoal parasites in the area despite the fact that both the Virunga Massif and Bwindi Impenetrable National Park are visited by non-locals on a daily basis for both research and tourism purposes. As the tourists visiting the park change frequently, one way of determining the risk of introducing protozoa to the gorillas could be through implementing a human pathogen surveillance system. This would require considerable political will and innovation to ensure participation.

Cryptosporidium and *Giardia* are important pathogens throughout the world, with profound disease impacts on many human and animal populations. However, the differences in their epidemiology seem to vary between developed and developing countries. This has just been alluded to in reviews of *Cryptosporidium* and *Giardia*

epidemiology and zoonotic potential (Thompson, 2004, Feng and Xiao, 2011); however, no previous study has methodically looked for these distinctions. Although the differences may be due in part to endemic versus epidemic levels of disease, risk factors for human *Giardia* infection in developing countries have not been examined to the same depth as in developed countries, and few studies have used molecular characterization methods to evaluate zoonotic potential on samples from developing countries. Given that many drivers for disease transmission detailed in Chapter 1 are occurring in developing countries, and that recognized risk factors such as human contact with wildlife, livestock, or contaminated water occur more frequently in developing countries, future studies should recognize this bias and respond accordingly. A meta-analysis reviewing epidemiologic studies on these parasites throughout the world should also be considered.

The epidemiology of fecal protozoa is critically important in detailing the complexities of disease transmission at the human, animal, and environmental interface. Through assessing environmental change on protozoal transport in surface waters and evaluating cross-species transmission in a complex ecosystem, new information has been gained to aid in mitigating disease transmission and deepened the foundation for future studies. With greater environmental alterations predicted to occur, any knowledge regarding these environmentally transmitted pathogens will help to reduce the disease impacts of these parasites. The effects of fecal protozoa on humans and animals are intricately coupled to the environment, and by evaluating them with a One Health perspective, a deeper understanding of transmission and disease emergence can be gained.

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APPENDIX

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Longitudinal Poisson Regression To Evaluate the Epidemiology of *Cryptosporidium*, *Giardia*, and Fecal Indicator Bacteria in Coastal California Wetlands

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Fecal pathogen contamination of watersheds worldwide is increasingly recognized, and natural wetlands may have an important role in mitigating fecal pathogen pollution flowing downstream. Given that waterborne protozoa, such as *Cryptosporidium* and *Giardia*, are transported within surface waters, this study evaluated associations between fecal protozoa and various wetland-specific and environmental risk factors. This study focused on three distinct coastal California wetlands: (i) a tidally influenced slough bordered by urban and agricultural areas, (ii) a seasonal wetland adjacent to a dairy, and (iii) a constructed wetland that receives agricultural runoff. Wetland type, seasonality, rainfall, and various water quality parameters were evaluated using longitudinal Poisson regression to model effects on concentrations of protozoa and indicator bacteria (*Escherichia coli* and total coliform). Among wetland types, the dairy wetland exhibited the highest protozoal and bacterial concentrations, and despite significant reductions in microbe concentrations, the wetland could still be seen to influence water quality in the downstream tidal wetland. Additionally, recent rainfall events were associated with higher protozoal and bacterial counts in wetland water samples across all wetland types. Notably, detection of *E. coli* concentrations greater than a 400 most probable number (MPN) per 100 ml was associated with higher *Cryptosporidium* oocyst and *Giardia* cyst concentrations. These findings show that natural wetlands draining agricultural and livestock operation runoff into human-utilized waterways should be considered potential sources of pathogens and that wetlands can be instrumental in reducing pathogen loads to downstream waters.

Fecal pathogen contamination of waterways is an important public health consideration in multiuse ecosystems. Sources of pathogens may include agricultural runoff containing livestock feces, sewage outfalls containing human and pet feces, and storm runoff containing feces of domestic and wild animals (12, 36). Zoonotic waterborne protozoa, specifically *Cryptosporidium* and *Giardia* spp., commonly cause diarrheal disease that can be severe or even fatal in immunocompromised individuals (1, 8, 11, 26). As surface waters move down a watershed, pulses of pathogen contamination from upstream sources contribute to downstream loads that affect recreational uses. Additionally, environmental persistence of *Cryptosporidium* oocysts and *Giardia* cysts, and their low infectious dose, contribute to the health risks posed to humans and animals who are in contact with surface waters (9, 13, 28).

The importance of wetlands for reducing select agricultural and chemical pollutants and pathogens in impaired surface water has been previously studied (21) and supports wetland use for improving water quality downstream from agricultural lands, livestock operations, and urban communities. However, most studies to date have focused on wetlands receiving sewage effluent (7, 18, 27), and little is known about how natural wetlands function with respect to transport and persistence of *Cryptosporidium* oocysts and *Giardia* cysts from upstream sources. In California and elsewhere, identifying ecologically sustainable management practices to improve water quality is of particular interest to resource managers and agencies.

To evaluate the distribution and transport of protozoa and fecal indicator bacteria (FIB) in natural wetlands, microbial concentrations among three wetland types along the central California coast were compared: (i) a medium-sized, tidally influenced slough that connects urban and agricultural runoff to Monterey Bay, (ii) a small seasonal wetland adjacent to a dairy that receives runoff from cattle, and (iii) a small, unidirectionally constructed wetland that filters water from an adjacent slough dominated by agricultural runoff. Associations between fecal protozoa and indicator bacteria were evaluated to determine the effectiveness of FIB as a protozoal proxy in this setting. Multiple sites within each wetland were monitored during a 2-year period to evaluate how environmental factors, such as rainfall and water quality measures, are associated with pathogen detection and transport from land to sea.

MATERIALS AND METHODS

Study sites. The three central California coast wetland study sites (Fig. 1) were sampled repeatedly between April 2008 and June 2010. The tidal wetland was originally an estuary that drained into a multiuse harbor and recreational area. Degradation of the wetland due to landscape conversion to agriculture, including livestock operations, has altered the region such that area waters remain brackish to hypersaline in most years. For water

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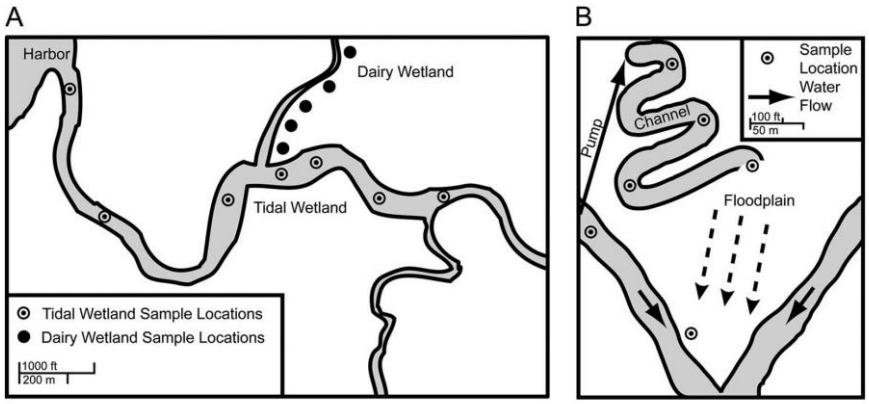

FIG 1 Maps of field sampling locations. (A) Tidal and dairy wetlands. (B) Constructed wetland.

collection, seven sites spaced approximately 500 m apart, ranging from 4,000 m upstream to the harbor tide gates, were sampled seasonally (Fig. 1A). The tidal wetland received runoff from the dairy wetland described below. A total of 38 samples were collected for fecal protozoan analysis, and 25 samples were also tested for fecal indicator bacteria (FIB). The dairy wetland was a seasonally occurring wetland that received runoff from cattle pastures and eventually drained into the tidal wetland described above. Surface water was present in the dairy wetland predominantly during the wet season (November to April) and was sampled at five sites, spaced approximately 150 m apart along a 1,000-m track from the dairy down to the outflow into the tidal slough described above (Fig. 1A). A total of 26 samples were collected for protozoal analysis, and 21 samples were also tested for FIB.

The constructed wetland (Fig. 1B) was built as a field research site with water pumped in from an adjacent slough that drains a large expanse of agricultural fields. It has an upper snake-like channel section that is characterized by unidirectional flow and was sampled at locations spaced approximately 100 m apart. Additional samples were collected from the lower section of the constructed wetland, where a large floodplain permits multidirectional flow depending on tidal and weather conditions, as well as from the source water in the adjacent slough. California bulrush (*Schoenoplectus californicus*) lined the banks of the upper snake-shaped channel, and the same plants formed 10 two-meter-wide vegetative buffers, or berms, that were perpendicular to the main water channel throughout this upper section. The lower floodplain section consists of a variety of plants, including California bulrush and slough sedge (*Carex obnupta*). Water samples were collected from the constructed wetland once weekly over four consecutive weeks seasonally, resulting in a total of 87 samples for protozoal analysis and 76 samples tested for FIB.

At each sampling location in all 3 wetlands, 10 liters of surface water was collected in a sterile plastic container for protozoal analysis along with 100 ml of water for bacterial analysis, as described below. Water quality parameters were also recorded at each wetland on each sampling day, including water temperature (°C), turbidity (nephelometric turbidity units [NTU]), salinity (parts per thousand [ppt]), total dissolved solids (mg/liter), dissolved oxygen (mg/liter), and pH.

Matrix spikes were prepared periodically during the study to determine percent recovery for protozoal detection methods. Easy Seed (BTF Bio, Pittsburgh, PA) aliquots containing 100 inactivated *Cryptosporidium* oocysts and 100 inactivated *Giardia* cysts were added to 10 liters of surface water from the wetland. These matrix samples were processed in the same way as the unspiked-field wetland water samples.

Microbial detection. Samples for protozoal analysis were processed according to EPA Method 1623 (33) for detection of *Cryptosporidium parvum* and *Giardia lamblia* in water through filtration using Envirochek cartridges, followed by immunomagnetic separation (IMS) for parasite purification using Dynabeads GC-Combo (Invitrogen Life Sciences, Carlsbad, CA) and direct fluorescent antibody tests (DFA) for oocyst or cyst identification using EasyStain (BTF Bio, Pittsburgh, PA) and quantification using microscopy under both fluorescein isothiocyanate (FITC) and DAPI (4′,6-diamidino-2-phenylindole) epifluorescence. Particles with apple-green fluorescence in an oval or spherical shape (3 to 7 μm in diameter) with bright, highlighted edges under FITC and light blue internal staining with a green rim or up to four distinct, sky-blue nuclei under DAPI were classified as *Cryptosporidium* oocysts. Particles with apple-green fluorescence in a round to oval shape (6- to 15-μm in diameter) with bright, highlighted edges under FITC and light blue internal staining with a green rim or up to four distinct, sky-blue nuclei under DAPI were classified as *Giardia* cysts. Results were expressed as the oocyst or cyst count/10 liters of water sampled (33).

To compare protozoal concentrations with those of the more commonly measured indicator bacteria, samples for FIB analysis were examined using Colilert-18 (IDEXX Laboratories, Inc.), a standard EPA-approved method for detection of total coliforms and *Escherichia coli* in surface water samples. Briefly, water samples were mixed with Colilert reagent packs and incubated at 35°C in a Quanti-Tray/2000 (IDEXX Laboratories, Inc.) for 18 h, with total coliform concentrations estimated by the number of yellow wells and *E. coli* counts estimated by the number of fluorescent wells. The results were expressed as the most probable number (MPN) per 100 ml.

Statistical analyses. A variety of wetland characteristics and environmental factors were evaluated as risk factors for associations with protozoal and indicator bacterial concentrations. Risk factors included wetland type (tidal, dairy, constructed), sample location within each wetland, and season (wet versus dry). Along the central California coast, the rainy season extends from November through April and the dry season extends from May through October. Rainfall events were described as rain occurring within the 12 h prior to sampling or within the preceding 4 days, as determined by the nearest weather station. Turbidity, total dissolved solids, and pH were evaluated as continuous variables. Water temperature and dissolved oxygen values were defined as "increased" or "decreased" with respect to the mean of the collected values for each wetland. Salinity values of less than 1 ppt were defined as "freshwater," while samples with salinity greater than or equal to 1 ppt were defined as "brackish." Categories for high FIB contamination of surface water were defined as *E. coli* concentrations greater than 400 MPN and total coliform concentrations greater than 10,000 MPN. *Escherichia coli* and total coliform counts falling

TABLE 1 Prevalence and concentrations in coastal California wetlands of *Cryptosporidium* oocysts and *Giardia* cysts using geometric means and of *Escherichia coli* and total coliforms using regression on ordered statistics

Wetland and characteristic[a]	Value for each protozoan or FIB			
	Cryptosporidium	*Giardia*	Total coliforms	*E. coli*
Tidal wetland				
Prevalence (% [no. of positive samples/total no. of samples])	39 (15/38)	21 (8/38)	100 (25/25)	100 (25/25)
Mean concn	31	36	8,571	316
Concn range	0–389	0–15,000	100–41,060	46–1,890
Dairy wetland				
Prevalence (% [no. of positive samples/total no. of samples])	69 (18/26)	50 (13/26)	100 (21/21)	100 (21/21)
Mean concn	168	85	173,590	870
Concn range	0–54,044	0–36,417	2,000–2,419,600	200–4,840
Constructed wetland				
Prevalence (% [no. of positive samples/total no. of samples])	46 (40/87)	13 (11/87)	100 (78/78)	100 (78/78)
Mean concn	5.5	3.2	155,973	2,913
Concn range	0–148	0–14	1,320–2,420,000	4–46,400

[a] Concentrations are oocysts or cysts per 10 liters for *Cryptosporidium* and *Giardia* and MPN per 100 ml for total coliforms and *E. coli*.

below these established cutoffs were deemed to have low to moderate FIB contamination.

The four primary outcomes of interest were the surface water concentrations of *Cryptosporidium*, *Giardia*, *E. coli*, and total coliforms. Descriptive statistics were calculated to compare these pathogen groups, with geometric mean concentrations calculated for each protozoal species and regression on order statistics (ROS) calculated as a measure of central tendency for indicator bacteria. The ROS method was used to take into account censored data for samples with concentrations below the method detection limits (1 organism/100 ml). Initial analyses revealed that the protozoal means were equal to the variance, supporting the use of Poisson regression models for subsequent statistical procedures. Log-linear longitudinal Poisson regression (14) was used to determine the effect of each independent variable on each study outcome in order to identify risk factors that were significantly associated with higher protozoal or bacterial counts. Poisson regression is a form of statistical regression analysis used to model count data, which are assumed to have a Poisson distribution. One assumption of this model is that there is no overdispersion, meaning that the variance does not exceed the mean. This assumption was not violated with the study data. Longitudinal regression was used to account for repeated sampling at the same sites over time; specifically, sampling sites were treated as random effects. Nonparametric Spearman's rho and chi-square tests were calculated to determine potential colinearity between the parameters of interest. Factors significant in bivariate analyses were then used to build a multiple Poisson model to test several predictor variables simultaneously. Additionally, Spearman's rho correlation coefficient was calculated for protozoal and bacterial counts. Statistical analyses were completed using SAS software, version 9.2 (SAS Institute Inc., Cary, NC), and P values of <0.05 were considered statistically significant.

RESULTS

Microbial detection. A total of 151 samples were tested for fecal protozoa and 122 samples were tested for fecal indicator bacteria (FIB), with all target organisms detected in all wetland types and sampling locations. The prevalence, mean concentration, and concentration range of *Cryptosporidium*, *Giardia*, total coliforms, and *E. coli* in water samples collected from the three wetlands are shown in Table 1. Within the tidal wetland, 39% of water samples were positive for *Cryptosporidium* oocysts while 21% were positive for *Giardia* cysts. The dairy wetland exhibited the greatest prevalence of *Cryptosporidium* oocysts and *Giardia* cysts, with 69% and 50% of water samples testing positive for each, respectively. The constructed wetland had a 44% *Cryptosporidium* oocyst prevalence and a 13% *Giardia* cyst prevalence. Furthermore, surface water collected from the dairy wetland exhibited the highest geometric mean concentrations of both protozoa (168 oocysts and 85 cysts per 10 liters), followed by water collected from the tidal wetland (31 oocysts and 36 cysts per 10 liters) and the constructed wetland (5.5 oocysts and 3.2 cysts per 10 liters). Matrix spikes from eight surface waters showed that *Cryptosporidium* recovery ranged from 2 to 26%, with an average of 13% (standard error [SE], 3.4%), and *Giardia* recovery ranged from 0 to 30%, with an average of 15% (SE, 4.6%).

As expected, 100% of samples from all three wetlands tested positive for *E. coli* and total coliforms across all sample points and time periods. Surface water collected from the constructed wetland exhibited the highest mean concentrations by ROS of *E. coli* (2,913 MPN/100 ml), followed by water from the dairy wetland (870 MPN/100 ml) and then water from the tidal wetland (316 MPN/100 ml). For total coliforms, the dairy wetland exhibited the highest concentrations (173,590 MPN/100 ml), followed by the constructed wetland (155,973 MPN/100 ml) and the tidal wetland (8,571 MPN/100 ml).

The concentrations of *Cryptosporidium*, *Giardia*, *E. coli*, and total coliform by sample location for each wetland are shown in Fig. 2, with the "distance downstream" representing the distance from the most-upstream sampling location to each sampling location in the wetland. Microbe levels in the tidal wetland were strongly influenced by sample location, particularly with regard to where the outflow from the dairy wetland joined the tidal wetland. For both protozoa and bacteria, microbe concentrations at the tidal wetland were highest at sites immediately upstream and downstream of the dairy wetland outflow and then decreased thereafter. Results from the dairy wetland showed that *Cryptosporidium*, *Giardia*, and FIB concentrations were significantly higher (P value < 0.0001) in the site closest to the dairy than in sample locations further downstream. Although very low concentrations of protozoa were detected in the constructed wetland, FIB enumeration demonstrated a similar trend, with the highest concentrations at the sample location in the source water from the adjacent slough (sample distance of 0 m) and lower counts in water

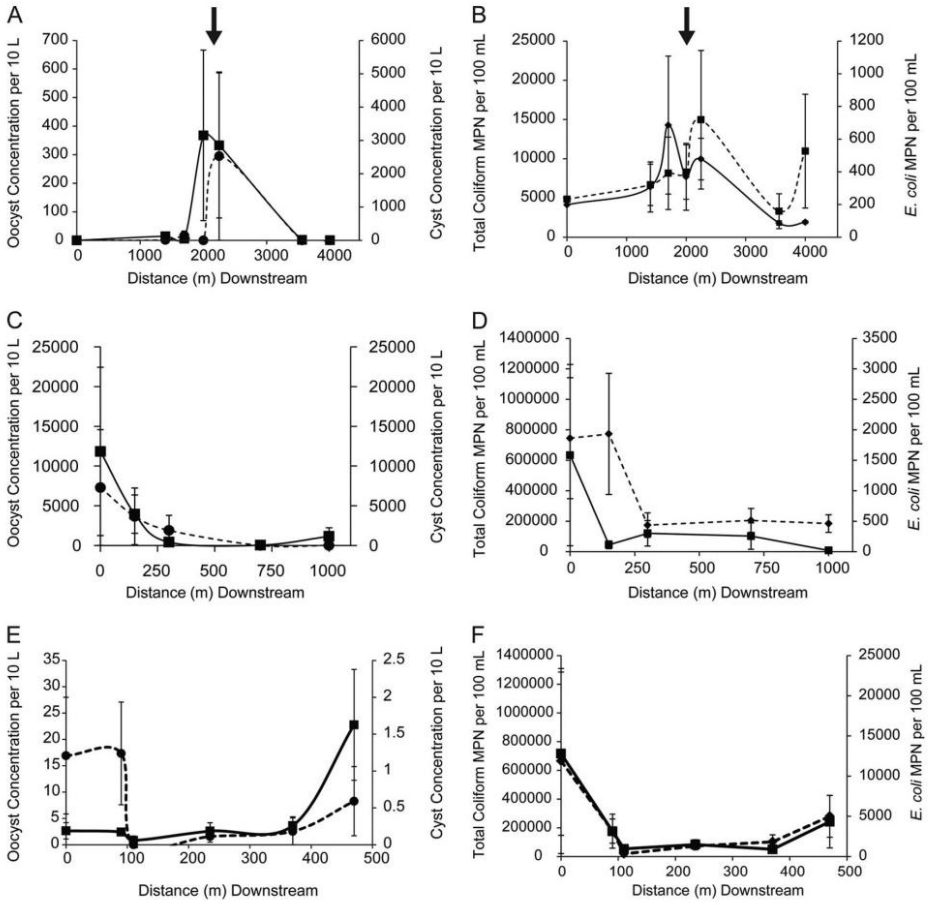

FIG 2 Mean concentrations of *Cryptosporidium* oocysts, *Giardia* cysts, total coliforms, and *Escherichia coli* by site in the tidal wetland (A and B), dairy wetland (C and D), and constructed wetland (E and F). Arrows indicate where the dairy wetland joins the tidal wetland. For A, C, and E, the solid line represents the *Cryptosporidium* oocyst concentration and the dashed line represents the *Giardia* cyst concentration. For B, D, and F, the solid line represents the total coliform concentration and the dashed line represents the *E. coli* concentration.

sampled from the subsequent downstream sites within the channel portion of the constructed wetland.

Risk factors. Analysis of associations for *Cryptosporidium* and *Giardia* concentrations with environmental factors was examined using Poisson regression, as the protozoal means were not overdispersed. Without adjusting for wetland effects, rainfall in the 12 and 96 h prior to sample collection was associated with detection of significantly higher concentrations (P value < 0.0001) of both *Cryptosporidium* oocysts and *Giardia* cysts. The odds ratio (OR) for rainfall in the prior 12 h with *Cryptosporidium* counts as the outcome was 30, meaning that in water samples collected from sites that received rainfall within the 12 h prior, the *Cryptosporidium* oocyst counts were likely to be 30 times greater than those for water samples collected when there was not rainfall in the preceding 12 h. For *Giardia*, the association between rainfall and cyst enumeration was even more pronounced, with an odds ratio of 217. Thus, for every *Giardia* cyst in a water sample when there was no rainfall in the 12 h prior to collection, there were likely to be 217 times as many *Giardia* cysts in water samples collected when rainfall had occurred just previously. The wet season was also associated with significantly higher protozoal counts compared to those of dry season sampling, with odds ratios of 45 for *Cryptosporidium* and 1,510 for *Giardia* detection in wet-season surface waters (P value < 0.0001).

Microbial detection and concentrations were significantly associated with most of the measured water quality parameters, including dissolved oxygen, water temperature, total dissolved solids, and salinity (P value < 0.05), but not turbidity and pH. Increased dissolved oxygen was negatively associated with protozoal and bacterial counts. Increases in total dissolved solids were also negatively associated with protozoal counts (P value < 0.0001). Warmer water temperature was inversely associated with protozoal counts (P value < 0.0001) but positively associated with total coliform values (P value < 0.005). When stratified by wet-

TABLE 2 Odds ratios from multiple Poisson regression models for significant variables predicting concentrations of protozoa and fecal indicator bacteria in surface waters from coastal wetlands[a]

Predictor variable	*n*	OR and *P* value of concn (per 10 liters) of:				OR and *P* value of MPN (per 100 ml) of:			
		Cryptosporidium		*Giardia*		*Escherichia coli*		Total coliform	
		OR	*P* value	OR	*P* value	OR	*P* value	OR	*P* value
Wetland type									
Tidal	38	1		1		1		1	
Dairy	26	12.13*	0.0020	3.93	0.1565	0.86	0.7146	32.97*	<0.0001
Constructed	104	0.07*	0.007	0.08*	0.0105	2.85*	0.0189	4.37*	<0.0001
Indicator bacteria									
Total no. of coliforms (below 10,000 MPN)	86	1		1		1			
Total no. of coliforms (above 10,000 MPN)	82	0.01*	<0.0001	0.12	0.0035	2.51*	<0.0001		
E. coli (below 400 MPN)	102	1		1				1	
E. coli (above 400 MPN)	66	4.75*	0.0096	265.39*	<0.0001			1.94*	0.0340
Salinity									
Freshwater	76					1			
Brackish water	92					0.34*	<0.0001		
Rainfall (mm)									
Total rainfall on sample date	155	0.73*	0.0254						
Rainfall × tidal	38	1							
Rainfall × dairy	21	1.75*	0.0252						
Rainfall × constructed	96	1.42*	0.0226						
Rainfall occurrence									
No rainfall on sample date	127			1					
Rainfall on sample date	41			103.86*	<0.0001				
Rainfall × tidal	7			1					
Rainfall × dairy	5			0.68	0.7473				
Rainfall × constructed	29			0.01*	0.0082				
Rainfall (mm)									
Total rainfall within prior 96 h	172					0.99	0.5339	0.99	0.5904
Rainfall × tidal	38					1		1	
Rainfall × dairy	26					1.11*	0.0172	0.73*	0.0122
Rainfall × constructed	108					1.04*	0.0255	1.06*	0.0353

[a] Asterisks indicate a *P* value of <0.05. OR, odds ratio.

land type, water samples with higher salinity were negatively associated with both *Cryptosporidium* (*P* value < 0.05) and *Giardia* (*P* value < 0.0001) counts, meaning that water samples from sites with lower marine influence were more likely to have high protozoal concentrations. Mean salinity levels for the tidal wetland tended to be brackish, at 11.1 ppt, whereas the dairy and constructed wetlands exhibited lower salinity, at 1.3 ppt and 2.6 ppt, respectively.

Protozoal relationship with fecal indicator bacteria. When Spearman's rho correlation coefficient was calculated to compare protozoal counts to indicator bacterial counts, no FIB-protozoa combinations were found to be significant. However, when a Poisson model approach was taken to look at the association between protozoal concentrations with "high" or "low" FIB levels, significant associations were observed. Using this approach, *E. coli* counts greater than 400 MPN/100 ml were associated with higher protozoal counts; for example, the odds ratio of having high levels of *E. coli* with increased *Giardia* cyst counts in water samples was 210 (*P* value < 0.0001). Thus, if a sample was found to have *E. coli* counts greater than 400 MPN, the sample would be predicted to have a count of 210 times more *Giardia* cysts than if there was an *E. coli* concentration of below 400 MPN. Conversely, concentrations of total coliforms exceeding 10,000 MPN were associated with lower protozoal oocyst or cyst concentrations.

Regression models. Longitudinal Poisson regression was used to further assess relationships between protozoal counts in surface water and various environmental and water quality parameters and FIB measures. In these models, *Cryptosporidium* and *Giardia* concentrations in surface water samples were best predicted by simultaneously considering wetland type, FIB levels, and rainfall variables. After adjusting for the difference in protozoal count by wetland type, rainfall was assessed to account for both the overall effect of rain and the difference in rainfall between the three wetlands through the use of interaction terms in the model. These results are tabulated in Table 2, and the associated predictive models are summarized in Fig. 3.

For *Cryptosporidium*, recent rainfall exposure was the best predictor of protozoal concentrations when evaluated as the volume of rainfall (in mm) in the 12 h prior to sampling. Rainfall occurring within 12 h prior to water sampling at the dairy and constructed wetland sites was associated with 1.7- and 1.4-fold increases in *Cryptosporidium* oocyst concentrations, respectively (*P*

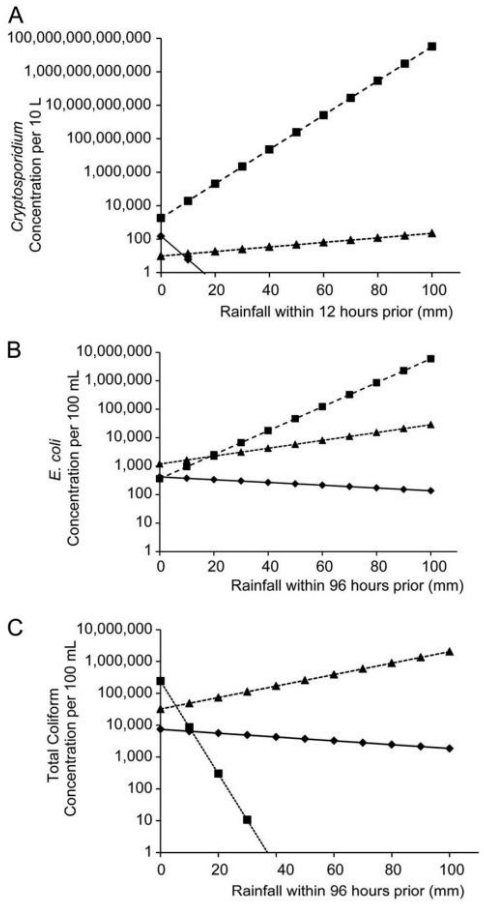

FIG 3 Predictive models for *Cryptosporidium* oocysts (A), total coliforms (B), and *E. coli* (C) utilizing Poisson regression models in which variables other than rainfall were held stable. Diamonds, tidal wetland; squares, dairy wetland; triangles, constructed wetland.

value of <0.05 for both). Inclusion of FIB levels into this model also had an impact on *Cryptosporidium* oocyst detection: "high" *E. coli* counts of greater than 400 MPN were associated with 4.8-fold increased concentrations of *Cryptosporidium* oocysts. Conversely, total coliform counts of greater than 10,000 MPN in surface water were associated with lower concentrations of *Cryptosporidium* oocysts, similar to what was noted in univariate analyses. The predictive model in Fig. 3A shows that as rainfall volume increases, the *Cryptosporidium* concentration is predicted to increase in the dairy wetland but decrease in the tidal wetland.

For *Giardia*, the longitudinal Poisson regression model showed trends similar to those of the *Cryptosporidium* model, with one difference being that increased *Giardia* counts were associated with the occurrence of rainfall in the 12 h prior to sampling rather than the total volume of rainfall. The odds ratio shows that *Giardia* counts were 104 times greater in water samples collected when there was preceding rainfall, regardless of wetland type, than when there was no rainfall within the prior 12 h. Additionally, the effect of fecal contamination (classified as water samples with *E. coli* concentrations above 400 MPN) was much greater for the *Giardia* model than for *Cryptosporidium*, with an odds ratio of 265. The *Giardia* model utilizing rainfall as a continuous variable did not converge, so no predictive model is shown.

The longitudinal Poisson regression models for the bacterial indicators included the risk factors of wetland type, FIB level, and total rainfall within the prior 96 h (Table 2 and Fig. 3). For the *E. coli* model, salinity was also an important factor, with an odds ratio of 0.3 (P value < 0.0001) for brackish water compared to freshwater. Thus, freshwater streams had greater concentrations of *E. coli* than brackish water estuaries, as expected if fecal pollution is coming from freshwater sources up the watershed. For total coliforms, both the dairy and constructed wetlands had odds ratios greater than 4 when compared to the tidal wetland (P value > 0.0001), meaning that significant increases in total coliform counts were noted between these two freshwater wetlands and the more marine-influenced tidal wetland. When stratified by wetland type with tidal wetland as the reference category, total rainfall (mm) was negatively associated with levels of total coliforms for the dairy wetland, but a slight positive association was noted for the constructed wetland compared to the tidal wetland.

DISCUSSION

While the majority of studies on protozoal transport have focused on constructed wetlands as a mitigating factor for sewage treatment (15, 18, 32, 35), livestock operations, domestic pets, and wildlife can also contribute substantially to environmental loading (2, 4, 5, 22). All three central California coastal wetlands examined in this study contained the protozoal pathogens *Cryptosporidium* and *Giardia* as well as FIB in surface waters, and all three wetlands showed reductions of both protozoal and bacterial concentrations within the wetlands. For the tidal and dairy wetlands in particular, the pathogen concentrations were high enough to be a public health concern for humans utilizing these water sources for recreation, especially considering that these wetlands drain into a harbor and sanctuary that is heavily used for recreation (31). The public health importance of the parasites depends on the particular species of *Cryptosporidium* or *Giardia*, as there are host-specific species and genotypes, such as the cattle-specific *Cryptosporidium andersoni* and *G. lamblia* assemblage E, which are not commonly linked to disease in humans. However, the transport dynamics are expected to be similar across genotypes, and this study highlights the importance of wetlands in reducing pathogen loads.

The dairy wetland, which forms naturally during the wet season and drains into the adjacent tidal wetland, exhibited the greatest concentrations of *Cryptosporidium*, *Giardia*, and FIB. Increased concentrations of both protozoa and bacteria were also noted at the tidal wetland when the dairy wetland was discharging water to sample sites closest to the junction of the two wetlands. Increased livestock density, increased water flow rates, or further degradation of this wetland habitat may lead to increased amounts of pathogens being transported to adjacent recreational waters. It is acknowledged that results may be affected by unaccounted-for variation between study sites, such as differing water volumes, watershed areas, and surrounding land uses between each wetland. Furthermore, the actual concentrations of *Cryptosporidium* and *Giardia* in the wetlands are underestimated, as shown by the

matrix spikes in which protozoal recovery ranged from 0 to 30% of spiked parasites in wetland water samples. The use of acid dissociation may underestimate the number of parasites compared to that found by heat dissociation (29, 37), but acid was used to be consistent with the standard EPA 1623 protocol for *Cryptosporidium* and *Giardia* identification in water (33). Ultimately, this study illustrates the ability of natural and reconstructed wetlands to reduce pathogen loads from a variety of sources.

Protozoal contamination from livestock feces is of particular concern because cattle are potential reservoirs for *Cryptosporidium parvum* and *Giardia lamblia* assemblage A (11, 17, 39), both of which are known to infect humans. Previous studies have examined the role of dairies and other intensive livestock operations in pathogen transmission and identified potential risk factors for nearby surface water contamination, including application of manure on agricultural fields (30) and surface runoff from areas with high animal densities, such as dairies and beef cattle operations (23). Consistent with these results, the data from this study demonstrate that the wetland in closest proximity to a livestock operation exhibited the greatest protozoal and bacterial loading. We also observed that a tidal wetland receiving water from this same dairy wetland had the highest microbial concentrations at sites nearest to the outflow from the dairy wetland. Tidal wetland sites immediately upstream and downstream of the junction with the dairy wetland exhibited greater parasite concentrations because this tidally influenced wetland experiences bidirectional flow (tide was not controlled for in the wetland sampling scheme).

While intensive livestock operations have significant potential to contribute to downstream pathogen loads, several best management practices (BMPs) have been proposed to reduce pathogen contamination in runoff flowing from pastures to adjacent receiving waters. First, reduction of cattle density may reduce the spread of infection within the herd and reduce the amount of contaminated manure released into the environment (23, 24). Second, reducing or more carefully controlling field applications of manure and slurry can greatly decrease the number of pathogens released into the environment (16). Finally, incorporation of vegetative filter strips, or "buffer strips," can reduce the number of oocysts and cysts transported out of intensive livestock operations (3, 16, 23, 24). Implementation of these BMPs at dairies and other livestock operations may considerably reduce their contribution to pathogen loading of adjacent wetlands.

In addition to the close proximity of fecal pathogen sources, rainfall and specific water quality parameters were also associated with *Cryptosporidium*, *Giardia*, and FIB counts in surface water. Rainfall events just prior to water sampling were associated with increases in oocyst or cyst concentrations across wetland types. Rainfall occurring within the previous 12 h was associated with increased *Cryptosporidium* and *Giardia* counts in the dairy wetland water, a result which is likely due to enhanced runoff from fecally contaminated soils. In livestock operations that utilized vegetative buffer strips to reduce watershed contamination from storm runoff, previous studies have documented reduced pathogen loading of adjacent waterways (6, 23, 24). Reductions in water quality, characterized by increased water temperature, total dissolved solids, and decreased dissolved oxygen, were also associated with higher *Cryptosporidium* and *Giardia* concentrations. Runoff from intensive livestock and agricultural operations can adversely affect these water quality measurements (19) and may also directly and indirectly contribute to pathogen transport.

Wetlands can be used to reduce microbial pollutant loads through several processes, including adsorption, sedimentation, and vegetative uptake (20). As with prior studies, our data indicate a general reduction in *Cryptosporidium*, *Giardia*, and FIB counts as contaminated water travels downstream through the wetlands. The data from the dairy wetland clearly showed that when protozoa and FIB were detected in upstream samples, samples collected further downstream exhibited lower pathogen concentrations. In a similar pattern, the tidal wetland had increased microbial concentrations nearest to the dairy inlet but downstream samples had returned to the low concentrations seen upstream of the inlet. There was an increase in *E. coli* concentrations in the last tidal wetland site, but this may be attributed to other potential fecal contamination sources, including live-aboard boats in the adjacent harbor and a substantial wildlife population residing near the downstream site. While the background levels of *Cryptosporidium* and *Giardia* were too low to document an effect in the constructed wetland, the total coliform and *E. coli* counts decreased sequentially throughout the water channel portion of the wetland. In contrast, the lower floodplain section was observed to be periodically inundated with undiluted water from the adjacent slough, perhaps explaining the increase in pathogen concentrations at the last sample site.

Surface water quality-monitoring programs generally rely on FIB levels to represent the risk of pathogen exposure and the associated health risks to humans. This approach is taken in part because FIB testing is less expensive and easier than direct pathogen testing. When FIB were analyzed as predictors of protozoal pathogen concentrations in coastal wetland systems, the detection of *E. coli* counts exceeding EPA guidelines (greater than 400 MPN) was positively associated with increased concentrations of protozoal oocysts or cysts in water samples. However, detection of total coliform levels exceeding EPA guidelines (greater than 10,000 MPN) was negatively associated with protozoal counts. In general, *E. coli* is considered a more reliable indicator of fecal contamination than total coliforms (10), the latter of which can vary with environmental conditions and bacterial populations (34). In contrast to FIB that can multiply in the environment, *Cryptosporidium* oocysts and *Giardia* cysts are shed in the feces and do not multiply in the environment. Thus, the association of protozoal counts with a more reliable marker for fecal contamination (*E. coli* counts) rather than the less-specific total coliform counts is not surprising. However, it can be noted that although increased *E. coli* counts correlated with greater protozoal concentrations, no significant correlations were identified between the absolute numbers of *E. coli* cells and protozoa detected in the same water samples. This contrasts with results from prior studies that evaluated *Cryptosporidium* and *Giardia* contamination of drinking water sources and reported linear correlations between protozoal concentrations and FIB concentrations (25, 38).

In conclusion, given that fecal pathogens can enter waterways through a multitude of routes and sources, natural wetlands may serve as a sustainable BMP to improve water quality in downstream waters. The study results show that protozoal and bacterial concentrations can be reduced as water travels through the coastal wetland and also that rainfall events are important to consider when identifying the times of highest risk for fecal pollution entering waterways. Recognizing the potential of natural and recon-

structed coastal wetlands for reducing transport of pathogens downstream holds promise for improving ecosystem health while also conserving natural habitats that are important for wildlife and a variety of recreational uses.

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