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Cryptosporidium Species in Coastal California Ecosystems

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Cryptosporidium Species in Coastal California Ecosystems

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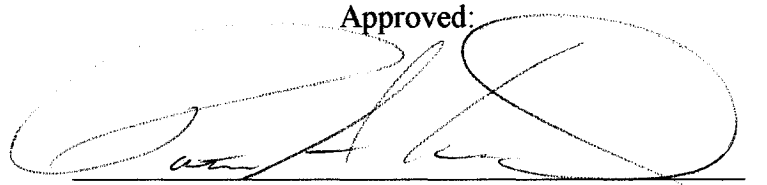

Committee in Charge

2004

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Cryptosporidium Species in Coastal California Ecosystems

Abstract

Cryptosporidium spp. are protozoan parasites that cause gastrointestinal disease in humans and animals, and are spread directly by fecal-oral transmission or indirectly by fecal contamination of water and food supplies. In California, there is evidence of significant fecal pathogen pollution flowing from terrestrial to aquatic ecosystems along the coast. To develop ecosystem monitoring and management strategies, we tested the hypothesis that *Cryptosporidium* spp. are detectable in nearshore filter feeding bivalves and in terrestrial storm runoff collected from fecal-impacted coastal ecosystems in California. The goals of this dissertation research were 1) to evaluate innovative detection methods for *Cryptosporidium* spp. in bivalve and water samples, 2) to evaluate bivalves such as mussels and clams as bioindicators of fecal contamination with *Cryptosporidium* spp. in marine, estuarine, and freshwater ecosystems, and 3) to evaluate farm management solutions to reduce the load of *Cryptosporidium* discharged into downstream waterways from coastal dairies. First, innovative molecular and immunologic methods were evaluated for detection of *Cryptosporidium* spp. in marine mussels (*Mytilus californianus*) and freshwater clams (*Corbicula fluminea*) using tissue spiking and tank exposure experiments. The most analytically sensitive detection method was immunomagnetic separation in combination with immunofluorescent detection of oocysts in mussel digestive tissues. Second, field studies tested 4800 mussels and 600 clams from marine, estuarine, and riverine ecosystems along the California coast for

Cryptosporidium spp. Genotypes detected in bivalves included the anthropozoonotic *C. parvum* genotype 2, as well as the host specific *C. andersoni* and *C. felis* that are shed by cattle and cats, respectively. In addition, 2 novel *Cryptosporidium* genotypes were detected in mussels. Third, a 3-year study evaluated the distribution of *Cryptosporidium* on coastal dairies and the efficacy of various farm management practices to reduce the load of *Cryptosporidium* oocysts in storm runoff. Calves were found to be important loading sources of *Cryptosporidium* on the farms, and vegetative buffers were found to effectively reduce the load of *Cryptosporidium* oocysts in storm runoff. In conclusion, *Cryptosporidium* spp. were detected in terrestrial runoff and nearshore bivalves, suggesting that *Cryptosporidium* spp. will be useful bioindicators of fecal contamination in ecosystem monitoring and management programs.

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The PhD is an adventure, you get as much as you give,
And if you're part of a team, you can still have your life to live.

So many questions to ask, which ones to pursue?
Choose your projects carefully, what means the most to you?

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Last but not least, my husband Cam, my friends, my family,
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Thank you, for everything. Wout

To all who read this:

Carpe Diem

Cryptosporidium in Coastal California Ecosystems

Woutrina Miller

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CHAPTER 1

INTRODUCTION

INTRODUCTION

Chapter Overview

This chapter provides an introduction to *Cryptosporidium* spp., apicomplexan protozoan parasites that cause gastrointestinal disease in humans and animals. Previous publications have provided broad overviews (Fayer, 1997; Thompson et al., 2003) or focused on the zoonotic potential of *Cryptosporidium* spp. (Graczyk, 2003a; Monis and Thompson, 2003). The goal of this review, in addition to providing background for the dissertation projects, is to demonstrate how innovative ecosystem monitoring for *Cryptosporidium* spp. can be utilized to evaluate fecal pathogen pollution and assess the impact of management programs.

Cryptosporidium Biology

Tyzzer (1907) first described the life cycle of *Cryptosporidium* as it occurs in the murine intestinal tract. In the latter half of the 20th century, *Cryptosporidium* spp. were recognized as significant disease-causing agents in livestock, domestic animals, wildlife, and humans (Nime et al., 1976; Pohlenz et al., 1978; Tarwid et al., 1985; Hoerr et al., 1986; Monticello et al., 1987). These studies demonstrated that clinical disease in mammals usually consisted of gastrointestinal symptoms, while cryptosporidiosis in birds also included respiratory pathology.

Cryptosporidium spp. were originally classified as coccidial parasites, but recent studies have shown with cell culture techniques and phylogenetic analyses that *Cryptosporidium* may in fact be more closely related to the gregarine apicomplexan family (Carreno et al., 1999; Hijjawi et al., 2002, 2004; Leander et al., 2003a, 2003b). Differences between the coccidia and *Cryptosporidium* spp., such as the ability of

Cryptosporidium spp. to replicate extracellularly (Hijjawi et al., 2004), may help explain why anti-coccidial chemotherapeutics have not been very effective in treating cryptosporidiosis (Armson et al., 2003). Within the genus *Cryptosporidium*, molecular and biologic studies of *Cryptosporidium* spp. from a variety of hosts have led to the designation of new *Cryptosporidium* spp. (Sargent et al., 1998; Lindsay et al., 2000; Morgan-Ryan et al., 2002). Some *Cryptosporidium* spp. are thought to be host-specific, such as *C. felis, C. andersoni,* and *C. hominis* shed in the feces of cats, cattle, and humans, respectively, while *C. parvum* genotype 2 can infect many human and animal hosts (Fayer, 1997; Peng et al., 1997).

Transmission of *Cryptosporidium* spp. occurs when environmentally resistant oocysts are shed in the feces and are eventually ingested by a new host. The infectious dose of *Cryptosporidium* oocysts in human volunteer studies has been as low as 10-100 oocysts (Okhuysen et al., 1999). Infected hosts begin shedding oocysts a few days after initial infection, they often exhibit diarrheal symptoms at the same time, and they can shed millions of oocysts during the first 1-2 weeks of infection (Fayer et al., 1997, 1998). Most immunocompetent hosts recover uneventfully, though in immunocompromised hosts cryptosporidiosis can become chronic and eventually fatal because there is no fully effective treatment regimen (Hunter and Nichols, 2002).

Cryptosporidium oocysts have caused outbreaks of diarrheal disease after fecal contamination of surface, pool, and drinking waters (Gallaher et al., 1988; Mackenzie et al., 1994; McAnulty et al., 1994). Human and livestock fecal sources have been implicated in these outbreaks, though identification of the exact source can be difficult due to the time lag between oocyst exposure and clinical symptoms. One of the largest

outbreaks occurred in Milwaukee in 1993, when fecal contamination of the public water supply led to an estimated 400,000 human cases and 70 deaths (MacKenzie et al., 1994). The spread of *Cryptosporidium* oocysts from humans and terrestrial animals into freshwater environments, and eventually into downstream estuarine and marine ecosystems, may pose a health risk to the humans and animals that utilize these ecosystems (Fayer et al., 2004).

Cryptosporidium Detection

A variety of *Cryptosporidium* detection techniques are available for use on fecal and environmental samples. *Cryptosporidium* oocysts are shed in high numbers in the feces during initial infection, but then are dispersed and diluted in the environment. Because low numbers of oocysts may still be significant in environmental samples, concentrating *Cryptosporidium* oocysts is critical for sensitive detection. Water samples can be initially concentrated by centrifugation or filtration (Xiao et al., 2000; Sturbaum et al., 2002). Further concentration of water or fecal pellets can be done with oocyst floatation on gradients such as sucrose, sodium, zinc sulfate, or cesium chloride, but significant oocyst loss has been reported (Fayer, 1997; Massanet-Nicolau, 2003).

A more sensitive and accurate method for oocyst concentration is immunomagnetic separation (IMS), a technique that utilizes magnetic beads attached to anti-*Cryptosporidium* antibodies to trap oocysts against a magnet while the debris and sample supernatant are discarded. Sample concentration by IMS has increased the analytic sensitivity limit by 1-2 $\log_{10}$ units in water and fecal samples (Johnson et al., 1995; Pereira et al., 1999), and is part of Method 1623 that is recommended by the Environmental Protection Agency for detection of *Cryptosporidium* and *Giardia* spp. in

water samples (USEPA, 2001). In Chapters 2 and 4, IMS is evaluated as a concentration method for use on marine mussels and freshwater clams, and in Chapter 5, IMS is used to concentrate storm water samples from coastal farms.

Oocysts can be visualized and quantified using light microscopy or immunofluorescent detection (Fayer, 1997). Alternatively, polymerase chain reaction (PCR) can be used to amplify *Cryptosporidium* DNA, and is often followed by DNA sequence analysis or restriction fragment length polymorphism for genotype identification (Morgan et al., 1997; Xiao et al., 1999). A fairly new technology, real-time TaqMan PCR, has potential advantages over conventional PCR techniques because TaqMan PCR provides quantitative results and can be incorporated into a robotic system that increases sample throughput (Batt, 1997). The potential for false-positive results has been demonstrated for both immunofluorescent and PCR detection techniques (Bull et al., 1998; Sturbaum et al., 2002). These studies showed that the immunofluorescent anti-*Cryptosporidium* antibodies cross-reacted with spores of the gregarine *Monocystis* spp., and that PCR amplification of water samples could amplify dinoflagellate DNA in addition to *Cryptosporidium* DNA. In Chapter 2, a TaqMan real-time PCR technique is compared with conventional PCR and direct immunofluorescent antibody (DFA) techniques for *Cryptosporidium* detection in mussel tissues. In Chapters 3-5, DFA and conventional PCR methods are used to detect *Cryptosporidium* spp. in mussel, clam, and storm water samples from coastal California ecosystems.

Ecosystem Monitoring

Innovative ecosystem monitoring programs assess new ways to measure environmental health, which may include testing for pathogens that pose a public health

risk. Monitoring for *Cryptosporidium* spp. in environmental studies has several advantages if used in tandem with coliform counts, the most commonly used bacterial indicator of fecal contamination. First, a goal of environmental monitoring is to understand the risk of exposure to pathogenic microbes, but coliform counts do not always correlate well with pathogenic protozoa such as *Cryptosporidium* and *Giardia* spp. (Goldstein et al., 1996; Bonadonna et al., 2002; Kistemann et al., 2002). The lack of correlation between coliform counts and *Cryptosporidium* load may be because the *Cryptosporidium* oocysts are more environmentally resistant than most bacteria, and thus the oocysts may survive under conditions that are not tolerable for bacteria (Nasser et al., 2003). Another difference is that unlike *Cryptosporidium* spp., bacteria have been shown to reproduce in the environment, suggesting that the bacterial load and genotypes detected in environmental samples may not reliably correlate with the initial fecal loads (Barnes and Gordon, 2004). Finally, *Cryptosporidium* spp. have host-specific genotypes and are endemic in many animal and human populations (Fayer, 1997), thus detecting host-specific genotypes of *Cryptosporidium* in environmental samples may indicate whether animal and/or human fecal loading sources are present in the ecosystem. Most bacteria don't have well recognized host-specific genotypes, so drawing inferences about contributing host requires comparisons of environmental bacterial fingerprints with reference strains (Haack et al., 2003; Liebana et al., 2003). An important reason to use *Cryptosporidium* in tandem with coliform counts as indicators of fecal contamination is that while coliform bacteria are shed in all fecal samples, *Cryptosporidium* oocysts are not. Therefore, an absence of *Cryptosporidium* spp. in environmental samples does not necessarily indicate an absence of fecal contamination.

Cryptosporidium spp. have been detected in a variety of environmental samples, most commonly water. Early studies identified oocysts mainly by DFA detection (Madore et al., 1987; Ongerth and Stibbs, 1987), but as it became clear that not all types of *Cryptosporidium* have the same public health risk, studies began to utilize PCR methods to obtain genotype information as well (Xiao et al., 2000; Ward et al., 2002). *Cryptosporidium* spp. have been identified in many types of water, including marine, estuarine, riverine, storm runoff, pool water, drinking water, and wastewater treatment effluents (McAnulty et al., 1994; Johnson et al., 1995; Goldstein et al., 1996; Xiao et al., 2000; Ward et al., 2002). Zoonotic, human host-specific, animal host-specific, and novel *Cryptosporidium* genotypes have been detected in these studies. Some studies have identified risk factors such as season, turbidity, and livestock density associated with the detection of *Cryptosporidium* in water samples (Robertson and Gjerde, 2001; Horman et al., 2004). Limitations of studies testing water are that 1) often large volumes must be concentrated for analysis, requiring extensive processing and expense, and 2) environmental substances such as humic acid may be present that are inhibitory to PCR techniques, making molecular detection and characterization difficult (Tsai and Olson, 1992). Chapter 5 evaluates the distribution and risk factors associated with detecting *Cryptosporidium* and *Giardia* spp. in storm runoff from coastal California farms.

An alternative to concentrating large volumes of water in the laboratory is to instead harvest bivalve shellfish that act as natural concentrating mechanisms through their filter feeding activities (McMahon and Bogan, 2001). Bivalve molluscs such as mussels, clams, and oysters have been used for decades as bioindicators of organochlorine and metal contamination at the terrestrial-aquatic interface (Camoni et al., 1980; O'Conner,

2002; El-Shenawy, 2004). In recent years, bivalves have also been shown to concentrate bacteria, viruses, and protozoa from fecal contaminated waters, with depuration rates ranging from hours to weeks (Graczyk et al., 2003b; Kingsley and Richards, 2003; El-Shenawy, 2004). *Cryptosporidium* spp. were first reported in bivalves in 1997, when oocysts were detected in marine mussels and river water in Ireland (Chalmers et al., 1997). Since then, *Cryptosporidium* spp., and other protozoa such as *Giardia* and *Cyclospora* spp., have been detected in bivalves in other parts of Europe, Africa, and on the Atlantic coast of North America (Fayer et al., 1999, Freire-Santos et al., 2000; Gomez-Bautista et al., 2000; Negm, A.Y., 2003; Graczyk et al., 2004; Traversa et al., 2004). Factors associated with *Cryptosporidium* detection in bivalves have included sampling season and nearby livestock density (Fayer et al., 1999, 2002; Gomez-Bautista et al., 2000). Host-specific *C. hominis* and *C. baileyi*, as well as the anthropozoonotic *C. parvum* genotype 2 have been detected in bivalves in the Chesapeake Bay, implicating both human and animal fecal sources (Fayer et al., 1999, 2002). An important difference between testing water and testing bivalves is that while taking a water sample may indicate what pathogens are present in the water at the time of sampling, testing bivalve samples will provide insight as to what pathogens have been present in the water over the previous hours and days. In addition to the use of bivalves as bioindicators of fecal loading sources in the environment, bivalves are also of interest because humans and animals consuming raw bivalves may be ingesting significant doses of viable pathogens (Freire-Santos et al., 2000). In Chapters 2 and 3, mussels are evaluated as bioindicators of *Cryptosporidium* contamination in laboratory and nearshore marine field studies along

the California coast. In Chapter 4, clams are evaluated as bioindicators of *Cryptosporidium* contamination in freshwater ecosystems of California.

In California, the State Mussel Watch Program (SMWP, http://www.swrcb.ca.gov/programs/smw/) uses nearshore marine mussels and freshwater clams as bioindicators of organochlorine and metal contamination. In 2002, a preliminary study was conducted to determine whether *Cryptosporidium* DNA could be amplified from SMWP samples of pooled mussel homogenates. Fifteen samples from batches of sentinel mussels and clams outplanted in southern and central California coastal ecosystems were tested for *Cryptosporidium* DNA, and 2 of the 4 samples from mussel batches collected near San Diego tested positive for *Cryptosporidium* DNA (W. Miller et al., unpublished data). Sequence analysis of the 850 bp product amplified from the 18S rRNA gene showed that both samples were identical to each other but slightly different from reference *Cryptosporidium* spp. in GenBank. However, the *Cryptosporidium* genotype from the southern California mussels was the same as a novel genotype recently detected in marine mussels along the central California coast (see Chapter 3). These results suggest that environmental health agencies such as the State Mussel Watch Program could monitor for fecal pathogens in nearshore waters by using the same mussel samples for detection of organochlorines, metals, *Cryptosporidium* spp., and possibly other pathogens.

Another approach to monitoring ecosystem health is to consider marine mammals as sentinels because they are at the top of the food chain, and are therefore potentially exposed to all the organic and inorganic contaminants that are present in their prey items (Dierauf and Gulland, 2001). Sea otters may be uniquely qualified as nearshore sentinels

of fecal pollution because they spend the majority of their lives within 0.5 km of the coast, and they consume large volumes of filter feeding invertebrate prey species that may contain concentrated pollutants and pathogens (Reidman and Estes, 1990). Sea otters must consume approximately 25% of their body weight each day in prey items, including mussels, clams, crabs, and innkeeper worms, because in contrast to other marine mammals, sea otters have no blubber layer (Reidman and Estes, 1990).

In our recent studies, *Cryptosporidium* and *Giardia* have been detected in the southern sea otter (*Enhydra lutris nereis*) population along the California coast for the first time. In 2001, 2 sea otters tested positive for *Cryptosporidium* and one for *Giardia* by fecal floatation and/or DFA techniques (M. Miller et al., unpublished data). Over the past year, 113 sea otters have been tested for *Cryptosporidium* and *Giardia* by IMS concentration in combination with DFA detection, but no sea otters have tested positive (W. Miller et al., unpublished data). Interestingly, in a similar pattern to the sea otters, most of the *Cryptosporidium* genotypes detected in the wild mussels (see Chapter 3) were detected in the 2001-2002 year, not in the 2003-2004 year. Further study is needed to evaluate the relationship between pathogens present in nearshore waters, nearshore invertebrates, and the marine mammals that consume them.

Ecosystem Management

Innovative ecosystem management is becoming a necessity as the world population of humans, and the animals associated with them, keeps increasing. In the past, the health risks associated with fecal pathogens were often not recognized or were dealt with by simply transporting feces away from susceptible populations. Now that the complex web of relationships between humans, animals, and their environments is gaining

recognition (Fayer et al., 2004; Patz et al., 2004), management agencies must seek sustainable solutions to manage fecal waste and control fecal pathogens. The 2 main approaches to controlling *Cryptosporidium* spp. and other pathogens are to decrease 1) shedding in host species, and 2) the spread of oocysts in the environment (Atwill et al., 1999, 2002). Innovative studies are using a systems approach to evaluate various fecal loading units and a variety of Best Management Practices (BMPs) to provide management alternatives for improving water quality (Lewis et al., 2000, 2001; Atwill et al., 2002). These types of studies are applicable to point and non-point sources of fecal pollution, and also include public outreach components.

Point sources of fecal pollution, such as wastewater treatment plants and concentrated livestock operations, have been the main focus of management agencies because they are often the easiest to identify and have been known to discharge high concentrations of pathogens (Rose, 1997). *Cryptosporidium* spp. are being used as a protozoal indicator to evaluate possible solutions to minimize the spread of fecal pathogens. For wastewater treatment plants, filtration and ozonation have been the most effective at reducing the number of viable oocysts in treated water, but common treatment protocols for large volumes of water, such as chlorination and ultraviolet radiation, have not proven as effective (Rose, 1997; Hijnen et al., 2004). With regard to concentrated livestock operations, vaccination and probiotics have as yet proven ineffective in preventing cryptosporidiosis, but calves and calf hutches have been associated with large numbers of oocysts, suggesting that improved sanitation and herd management may lead to a reduced incidence of cryptosporidiosis (Harp et al., 1996; Atwill et al., 1998, 1999). To control the environmental spread of fecal pathogens throughout the farm, waste management and

water treatment systems have been implemented to minimize environment contamination (Craggs et al., 2003; Corkal et al., 2004). In addition to BMP studies, predictive modeling of point source discharges containing *Cryptosporidium* oocysts has begun and may provide insight into the flow of fecal pathogens in the environment (Medema and Schijven, 2001; Bonadonna et al., 2002).

Non-point sources of fecal pollution include storm runoff from rural and urban areas. The concentration of fecal pathogens may be lower in runoff from non-point sources than from point sources, but the volume of runoff is often higher from non-point sources, so the loading of *Cryptosporidium* oocysts into the environment may still be substantial (Rose, 1997; Lewis et al., 2000). Non-point source BMPs such as vegetative buffers reduce the volume of runoff by slowing down overland flow, so that microbes and runoff can settle and infiltrate into the ground (Coyne et al., 1998; Tyrrel and Quinton, 2003). While the settling velocity of free oocysts suggests that they will not sediment quickly in runoff, they have been shown to attach well to particulate matter in runoff and then take on the increased settling velocities of the particulate matter (Medema et al., 1998). Possibly due to the attachment and settling of oocysts and particulate matter in runoff, vegetated buffer strips have been shown to effectively reduce the load of *Cryptosporidium* oocysts in overland runoff (Atwill et al., 2002; Davies et al., 2004; Trask et al., 2004). Other BMPs that slow down overland flow and allow oocysts sedimentation time may also effectively reduce oocyst load but have not extensively studied. Chapter 5 evaluates a series of BMPs including vegetative buffer strips, mulch application to dry lots, water impoundment, and cattle exclusion for their ability to reduce the load of *Cryptosporidium* oocysts in storm runoff on California farms. Lastly, climate

is an important consideration when assessing the risk of exposure to viable oocysts in the environment and the effectiveness of BMP control strategies, because oocysts exposed to extreme temperatures or dessication have reduced viability (Kato et al., 2002). Thus, in climates where freezing occurs every winter, the likelihood of oocysts overwintering is lower than in the moderate climates of the California coast, which may allow for viable oocysts to survive throughout the year.

The transfer of knowledge from research studies to real-world implementation requires a significant outreach and public education effort (Corkal et al., 2004). Outreach efforts to improve ecosystem health and fecal management may be conducted on a large or small scale. For example, in the United Kingdom, the Safe Sludge Matrix (http://www.adas.co.uk/matrix/) involves the water industry, the food industry, and the government to implement changes in the way sewage sludge from wastewater treatment plants is managed (Tyrrel and Quinton, 2003). Changes promoted by the Safe Sludge Matrix include reduction in the use of treated sewage sludge on fruit and vegetable crops that are consumed raw, and elimination of the practice of spreading untreated sewage sludge on agricultural lands. On a smaller scale, the Tomales Bay Agricultural Group (TBAG) and Tomales Bay Watershed Council (http://www.tomalesbaywatershed.org) in California have brought together community members interested in finding sustainable solutions to local water quality issues. The participation of TBAG farms made possible the Chapter 5 evaluation of farm BMP practices to reduce the load of *Cryptosporidium* and *Giardia* in storm runoff.

In conclusion, this review demonstrates how *Cryptosporidium* spp. are being incorporated into innovative ecosystem monitoring and management studies. The

following chapters provide additional marine, riverine, and storm runoff examples in California, illustrating that *Cryptosporidium* spp. can be useful in the investigation and control of fecal pathogen pollution at the terrestrial-aquatic interface, where humans and animals must coexist.

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CHAPTER 2

Evaluation of Methods for Improved Detection of *Cryptosporidium* Species in Mussels (*Mytilus californianus*)

Evaluation of Methods for Improved Detection of *Cryptosporidium* Species in Mussels (*Mytilus californianus*)

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ABSTRACT

Bivalve molluscs concentrate *Cryptosporidium* oocysts from fecal-contaminated aquatic environments and are therefore useful in monitoring water quality. A real-time TaqMan polymerase chain reaction (PCR) system was developed to allow for large scale quantitative detection of *Cryptosporidium* spp. in mussels (*Mytilus californianus*). The TaqMan sensitivity and specificity were compared to conventional PCR and direct immunofluorescent antibody (DFA) methodologies, with and without immunomagnetic separation (IMS), to identify the best method for parasite detection in mussel hemolymph, gill washings and digestive glands. TaqMan PCR and two conventional PCR systems all detected 1 or more oocysts spiked into 1 ml hemolymph samples. The minimum oocyst detection limit in spiked 5 ml gill wash and 1 g digestive gland samples tested by TaqMan PCR and DFA was 100 oocysts, with a $\log_{10}$ improvement when samples were first processed by IMS. For tank exposed mussels, TaqMan and conventional PCR methods detected *C. parvum* in <5% of hemolymph samples. No gill washings from these same mussels tested positive by TaqMan PCR or DFA analysis even with IMS concentration. All methods detected the highest prevalence of *C. parvum*-positive samples in digestive gland tissues of exposed mussels. In conclusion, the most sensitive method for the detection of *C. parvum* in oocyst-exposed mussels was IMS concentration with DFA detection: 80% of individual and 100% of pooled digestive gland samples tested positive. TaqMan PCR was comparable to conventional PCR for detection of *C. parvum* oocysts in mussels and had the additional benefits of automated testing, high throughput, and quantitative results.

INTRODUCTION

Sensitive and specific detection of pathogenic protozoa such as *Cryptosporidium* spp. is of critical importance to public health authorities. Many outbreaks of cryptosporidiosis have been documented worldwide, including the 1993 contamination of a Milwaukee public water supply with *Cryptosporidium parvum* that infected over 400,000 people and caused over 100 deaths (28). Clinical disease in immunocompetent humans generally consists of self-limiting diarrhea resulting from either *Cryptosporidium hominis* or the anthropozoonotic *C. parvum* genotype 2 (33, 34). Immunocompromised humans can be chronically infected, sometimes fatally, with these as well as other *Cryptosporidium* species (4, 37). The oocyst stages of *Cryptosporidium* spp. are shed in the feces of animals and humans, which may then enter sewage treatment facilities via wastewater or persist in the environment. Depending on the type of sewage treatment, some but not all oocysts will be removed prior to environmental discharge of treated water (3, 35). *Cryptosporidium* parasites are endemic in many domestic and wild animal populations, with young animals often shedding over a million oocysts during initial infection, while the infective dose of *C. parvum* in humans can be as low as 10-100 oocysts (1, 8, 23, 30, 34).

Diagnosis of acute cryptosporidiosis has historically been based on acid fast stain, fecal flotation, or direct immunofluorescent antibody (DFA) detection methods. However, for these methods the limit of detection is approximately 600-1,000 oocysts/g feces, and DFA methods cannot determine *Cryptosporidium* genotype (36, 47). The analytical sensitivity of oocyst detection in feces can be increased by 1-2 $\log_{10}$ units using immunomagnetic separation (IMS), which concentrates oocysts and facilitates analysis of

a larger sample volume (36). When coupled with IMS, amplification of parasite DNA using conventional polymerase chain reaction (PCR) methods provides molecular data to determine the *Cryptosporidium* genotype, with a minimum detection limit of about 100 oocysts/g feces (6, 45). Unlike DFA, conventional PCR techniques do not provide quantitative data and do not allow for oocyst visualization. The real-time TaqMan PCR system described herein was designed to provide quantitative results, high throughput potential, an AmpErase UNG (uracil-N-glocosylase) system to prevent PCR product carry-over, and sensitive detection of all *Cryptosporidium* species. Other *Cryptosporidium* real-time PCR systems have been described (13, 21, 24, 25, 27), but their protocols were thought to lack the analytical sensitivity and specificity to identify the variety of *Cryptosporidium* genotypes and the low levels of oocyst contamination that are often expected in environmental samples (22, 38).

Environmental monitoring for *Cryptosporidium* spp. can be difficult, partly because of the dilution effect that occurs as oocysts are disseminated from terrestrial to aquatic ecosystems, and also because particulate matter can inhibit or interfere with *Cryptosporidium* detection methods (12, 22). Oocysts can be concentrated from large volumes of water mechanically, but expensive equipment and supplies are required (12, 22). Alternatively, filter feeding invertebrates such as bivalve molluscs, which can filter over 2 liters of water/hr/shellfish, can act as a natural concentration system (31). These bivalves can then be collected and tested for pathogens, providing an indication of water quality (15, 18, 41). Studies in North America and in Europe have shown that bivalves can act as indicators of aquatic fecal contamination with *Cryptosporidium* spp. and that molecular characterization can distinguish human from animal genotypes to assess

potential fecal loading sources (9, 10, 16, 17, 20). The IMS concentration technique is well accepted as an integral part of water testing methods (40, 43), but its potential to improve detection of *Cryptosporidium* spp. has not been critically evaluated on bivalve samples. The goal of this study was to evaluate a real-time TaqMan PCR system and compare it with conventional PCR and DFA methods, with and without IMS, for sensitive and specific detection of *Cryptosporidium* spp. in mussel tissues using laboratory spiked bivalve samples and samples from a tank exposure experiment.

MATERIALS AND METHODS

Experimental Design. Analytic sensitivity and specificity of *Cryptosporidium* detection techniques were first evaluated using oocyst dilutions and DNA samples without bivalve tissues. Sensitivity of the PCR and DFA techniques were established by testing serial dilutions ranging from 1-10,000 *Cryptosporidium* oocysts. Specificity testing for PCR systems was evaluated using protozoal DNA extracted from *C. andersoni, C. baileyi, C. canis, C. felis, C. hominis, C. meleagridis, C. parvum* genotype 2, *Giardia duodenalis* (synonymous with *G. lamblia* and *G. intestinalis*), *Neospora caninum, N. hughesi, Sarcocystis falcatula, S. neurona*, and *Toxoplasma gondii*, as well as non-protozoal DNA from marine mussels (*M. californianus* and *M. galloprovincialis*), freshwater clams (*Corbicula fluminea*), sandcrabs (*Emerita analoga*) and dinoflagellates (*Gymnodinium* spp.) Specificity testing for DFA utilized oocysts from *C. andersoni, C. felis, C. parvum, C. serpentis,* and cysts from *G. lamblia.*

Technique comparisons for *Cryptosporidium* detection in bivalve tissues were performed by spiking known oocyst numbers into mussel tissues. *Cryptosporidium*

dilutions containing 0, 1, 10, 100 or 500 oocysts were added to 6 sets of hemolymph, gills, and digestive gland tissues prior to any washes or processing, to allow the analytical sensitivity estimates to reflect any oocyst loss during tissue processing and analysis. TaqMan PCR was compared to 2 conventional PCR methods for hemolymph analysis. TaqMan PCR was also compared to DFA analysis, with and without IMS concentration, for evaluation of gill washings and digestive gland tissues. These two tissues are commonly tested by DFA in other laboratories, so DFA was used as the standard for comparison with TaqMan PCR in our study.

A tank experiment was then conducted to expose mussels (*Mytilus californianus*) to a known quantity of *C. parvum* genotype 2 oocysts. After an 8 hr oocyst exposure, mussel hemolymph, gill, and digestive gland tissues were harvested for technique comparison. Two tubs, each containing 70 mussels and 10 L of sea water, were inoculated with 1000 oocysts/L (mean, 142 oocysts/mussel) and left undisturbed for 8 hours. Each mussel tank was kept at 14°C with no additional water or nutrient supplementation. An airstone in each tank provided constant aeration and water mixing. A third tub with 70 mussels and 10 L sea water was maintained under the same conditions except that no oocysts were added to the water. After 8 hours, 60 mussels were removed from each tank: 30 were processed individually, and the remaining 30 were processed as 6 pools of 5 mussels each. Hemolymph, gill washings, and digestive gland were processed as described below. Hemolymph was analyzed by TaqMan PCR and two conventional PCR methods. Gill washings and sieved digestive gland were tested by TaqMan PCR and DFA analysis, with and without IMS concentration.

***Cryptosporidium* oocysts.** Wild-type oocysts of *C. parvum* genotype 2 used for sensitivity testing and the tank exposures were obtained from calves near the Veterinary Medical Teaching and Research Center, Tulare, California. Calf feces were sieved through a series of mesh strainers and then purified by sucrose flotation methods (2). Additional *Cryptosporidium* genotypes and other protozoa for specificity testing were obtained from the Veterinary Medical Teaching Hospital, University of California, Davis, the California Animal Health and Food Safety Laboratory, and Drs. Bruce Anderson in Idaho and Andrew Thompson in Australia. Oocyst concentrations for spiking experiments were determined using the mean of 8 hemacytometer counts and confirmed by DFA enumeration. Oocyst suspensions were kept at 4°C and used within one month of collection.

Mussels. Mussel spiking and tank exposure experiments were performed using wild surf mussels (*Mytilus californianus*) harvested outside Bodega Bay, California. Mussels 5-8 cm long were individually dissected to obtain hemolymph, gill, and digestive gland. Hemolymph was extracted by first filing a notch in the mussel shell and then aspirating 0.5-1.5 ml of hemolymph from the adductor muscle using a sterile syringe and a 22 gauge needle. The hemolymph was transferred to a microcentrifuge tube and centrifuged for 3 min at 14,000 rpm. The supernatant was removed and the cell pellet was storeed at -80°C for DNA extraction and PCR analysis. Next, the adductor muscle was cut to open the mussel, and the gills and digestive gland (0.2-2 g each) were excised. Gills from each mussel were vortexed in 5 ml sterile PBS as described (9). Gill tissue was removed and 200 µl of the gill wash were dried onto a slide for DFA testing. The remaining gill wash fluid was centrifuged for 10 min at 1000×g to obtain a pellet. The supernatant was

discarded and a 100 µl aliquot of the pellet was frozen at -80°C for TaqMan PCR analysis. The remaining gill wash pellet ≤ 0.5 ml was processed by IMS and the 100 µl product split for TaqMan and DFA analysis. Digestive gland samples were sieved through a 100 µm cell strainer and centrifuged for 15 min at 1000×g. A 100 mg aliquot was frozen for TaqMan PCR analysis, a 10 µl aliquot was dried onto a DFA slide, and then a 0.5 ml aliquot was processed by IMS for TaqMan PCR and DFA analysis.

TaqMan PCR. A real-time TaqMan PCR system was developed to detect most *Cryptosporidium* species based on 18S rRNA sequences deposited in GenBank. This TaqMan system was intentionally designed to recognize many *Cryptosporidium* genotypes, including *C. hominis* (GenBank accession number AF093489), *C. parvum* genotype 2 (AF093490), *C. muris* (AF090496), *C. felis* (AF108862), and *C. meleagridis* (AJ493549). The TaqMan probe was adapted from Limor et al. (25), and primers were newly designed to amplify a short PCR product to maximize analytical sensitivity. The sensitivity was 50 times greater when testing cDNA transcribed from mRNA as compared to testing genomic DNA (gDNA)(data not shown), so cDNA was used for all further analyses. In addition, a Bivalve TaqMan PCR system to be used for quality control was designed based on a conserved region of the 18S rRNA gene targeting bivalves including clams (*Corbicula fluminea* [AF305705]) and mussels (*Geukensia demissa* [L33450], *Mytilus californianus* [L33449], *M. edulis* [L78854], *M. galloprovincialis* [L33452], *M. trossulus* [L33453]). For each target gene, two primers and an internal, fluorescent labeled TaqMan probe (5′ end, reporter dye 6-FAM [6-carboxyfluorescein], 3′ end, quencher dye TAMRA [6-carboxytetramethylrhodamine]) were designed using Primer Express software (Applied Biosystems, Foster City, CA).

The length of the PCR products was short (between 110 and 182 bp) to enable high amplification efficiencies. TaqMan primer and probe sequences are listed in Table 2.1.

For nucleic acid extraction, tissue samples of ≤100 mg were loaded into 96 deep well plates prefilled with 500 µl 1X ABI lysis buffer (Applied Biosystems) and stored frozen until RNA extraction. Two grinding beads (4 mm diameter, SpexCertiprep, Metuchen, NJ) and Proteinase K (Invitrogen, Carlsbad, CA) were added and samples were homogenized in a GenoGrinder2000 (SpexCertiprep) for 2 min at 1000 strokes/min. After 30 min periods at 56°C and -20°C, total RNA was extracted from the tissue lysates using a 6700 automated nucleic acid (ANA) workstation (Applied Biosystems) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized using 100 units of SuperScript III (Invitrogen), 600 ng random hexadeoxyribonucleotide ($pd(N)_6$) primers (random hexamer primer) 10 U RNaseOut (RNase inhibitor), and 1 mM dNTPs (all Invitrogen) in a final volume of 40 µl. The reverse transcription reaction proceeded for 120 min at 50°C. After addition of 60 µl of water, the reaction was terminated by heating for 5 min to 95°C and cooling on ice.

Real-time TaqMan PCR reactions were conducted on 96 well plates in an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Each PCR reaction contained 400 nM of each primer, 80 nM of the TaqMan probe and commercially available PCR mastermix (TaqMan Universal PCR Mastermix, Applied Biosystems) containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 2.5 mM dNTPs, 0.625 U AmpliTaq Gold DNA polymerase per reaction, 0.25 U AmpErase UNG per reaction and 5 µl of the diluted cDNA sample in a final volume of 12 µl. Amplification conditions for the automated fluorometer were 2 min at 50°C, 10 min at 95°C, 40 cycles

of 15 s at 95°C and 60 s at 60°C. Cycle threshold (Ct) values less than 39.5 were considered positive based on the results of testing *C. parvum* exposed and unexposed mussels in the tank exposure experiment. For selected isolates, the PCR product was purified according to QIAGEN protocol (QIAGEN Inc., Valencia, CA), sequenced on an automated sequencer, and analyzed with Vector NTI (Informax, Frederick, MD) software for *Cryptosporidium* genotype identification.

Conventional PCR. Two conventional 18S rRNA PCR protocols (32, 48) were used for comparison with the TaqMan PCR system. Primers are listed in Table 2.1. For gDNA extraction of bivalve tissues, a 50 µl maximum pellet was suspended in 180 µl ATL buffer and suspended in liquid nitrogen for 4 min, then in boiling water for 4 min. Using the QIAGEN DNA Mini Kit tissue protocol (QIAGEN Inc.), mussel tissue was digested, bound to a QIAamp column, and washed according to manufacturer's instructions. The genomic DNA was eluted with 50 µl of 95°C PCR water and frozen at -80°C until PCR analysis.

Conventional PCR testing of mussel hemolymph utilized two protocols. The Xiao (48) 18S rRNA nested protocol first amplified a 1325 bp DNA segment and then an 850 bp DNA segment in the nested PCR step. The Morgan (32) 18S rRNA protocol amplified a 298 bp DNA fragment. The Xiao outer primer PCR reactions contained 5 µl Perkin-Elmer (Norwalk, CN) 10X PCR buffer, 6 mM $MgCl_2$, 200 µM each deoxynucleoside triphosphate (dNTP), 200 nm each primer, 1.5 U *Taq* polymerase, PCR-grade water, and 2 µl gDNA in a 50 ul total volume. The Xiao inner primer PCR reactions were the same except that the $MgCl_2$ was reduced to 3 mM. Amplification conditions for the outer and inner Xiao PCR reactions consisted of 1 cycle at 94°C for 3 min, followed by 35 cycles of

94°C for 45 s, 55°C for 45 s, and 72°C for 1 min, with a final extension at 72°C for 7 min. The Morgan PCR reactions contained 5 µl 10X PCR buffer, 3 mM $MgCl_2$, 200 µM each dNTP, 200 nm each primer, 1.5 U *Taq* polymerase, PCR-grade water, and 2 µl gDNA in a 50 µl total volume. Amplification conditions for the Morgan PCR reactions started with 2 min at 96°C, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 sec, with a final extension at 72°C for 7 min. Amplified DNA was run with a 100 bp ladder on a 2% agarose gel containing GelStar (Cambrex Co., East Rutherford, NJ) to determine product size and band intensity. For selected isolates, the PCR product was purified according to QIAGEN Qiaquick protocol and sequenced on an automated sequencer. The DNA sequences were analyzed with Chromas (Technelysium Pty Ltd, Tewantin, Qld, AU) and ClustalX software for *Cryptosporidium* genotype identification.[42]

Direct immunofluorescence. *Cryptosporidium* oocyst DFA detection was performed on 10 µl digestive gland or 200 µl gill wash dried onto 1 of the 3 wells on a Merifluor slide (Meridian Bioscience Inc., Cincinnati, OH). For IMS-DFA of gill washes and digestive gland, the 50 µl IMS product containing parasites was dried onto a DFA slide well, with 2 wells per tissue sample. The DFA slides were incubated with anti-*Cryptosporidium* and anti-*Giardia* antibodies and counterstained (Meridian Bioscience Inc.) for 30 minutes before a wash step and reading at 200X-400X magnification on a Zeiss Axioskop epifluorescent microscope. All slides were read by the same microscopist. *Cryptosporidium* parasites were identified as 5 µm diameter oocysts that were outlined in apple green, often with a midline seam. After parasite quantification, the slide well could then be scraped for DNA extraction, PCR, and DNA sequence

analysis. Hemolymph was not tested by DFA because preliminary tests showed that autofluorescence of mussel hemocytes interfered with the identification of oocysts.

Immunomagnetic separation. Selected gill wash and digestive gland pellets of up to 0.5 ml were concentrated and purified with IMS (Dynal Biotech, Oslo, Norway) as per the manufacturer's instructions, followed by DFA or TaqMan PCR for *Cryptosporidium* detection. Briefly, the tissue pellet was suspended in an L10 glass tube in 2 ml buffer and 10 ml deionized water. Next, 100 µl of *Cryptosporidium* IMS beads were added and the samples were rotated on the IMS mixer for 1 hour at setting 18. The IMS bead-parasite complexes were bound to a magnetic holder while the supernatant and debris were discarded. Bead-parasite complexes were resuspended in 1 ml buffer and transferred to 1.5 ml microcentrifuge tubes in a smaller magnetic holder to again bind the bead-parasite complexes while the supernatant was discarded. For parasite dissociation from the beads, 2 acid washes of 50 µl 0.1 N HCl were vortexed at the beginning and end of a 10 and 5 minute incubation. Tubes were again put in the small magnetic holder to attract the beads to the magnet, and the supernatant was transferred to a DFA slide or PCR tube containing 5 ul 1 N NaOH for neutralization. Hemolymph was not suitable for IMS because hemocytes attached to the glass tubes.

Data analysis. Amplification efficiencies of the 18S rRNA *Cryptosporidium* and Bivalve TaqMan PCR systems were calculated using the formula: $E = 10^{1/-s}-1$, where E*100 is the % efficiency and s is the slope of the standard curve. The proportion of tissues positive per technique was calculated as the number of tissues that tested positive divided by the total number of tissue samples tested for each technique. Percent recovery values for DFA testing were calculated by dividing the number of oocysts counted on the

DFA slide by the number of oocysts expected per test aliquot. Expected oocysts per test aliquot were calculated by multiplying the number of oocysts spiked into a tissue by the proportion of spiked tissue analyzed. Paired t tests were used to compare the number of oocysts detected by DFA with IMS-DFA. McNemar's χ^2 test for paired data was used to compare the proportion of positive mussels detected by DFA with PCR. Statistical significance was defined as a p value <0.05.

RESULTS

Oocyst spiking experiments. The TaqMan PCR detection systems were validated using DNA and *Cryptosporidium* oocyst dilutions alone. The linearity of the TaqMan standard curve dilutions of cloned *C. parvum* plasmids and cDNA extracted from oocysts is shown in Figure 2.1. The amplification efficiencies were approximately 99% for both the *Cryptosporidium* TaqMan and Bivalve TaqMan systems that were designed to assess the RNA quality from extracted tissue.

The TaqMan and conventional PCR system specificity results are shown in Figure 2.2. The Bivalve TaqMan detection system detected invertebrate cDNA but not protozoal cDNA. The *Cryptosporidium* TaqMan detection system detected all *Cryptosporidium* and *Sarcocystis* species tested but not *Neospora* spp., *T. gondii*, *G. duodenalis*, dinoflagellates, or invertebrate DNA. The Xiao conventional PCR system was the most specific, detecting only *Cryptosporidium* species, while the Morgan conventional PCR system occasionally detected other protozoa, dinoflagellates, and invertebrate DNA in addition to *Cryptosporidium* spp. All three PCR systems were able to detect a single oocyst present in the 200 ul dilution without bivalve tissue.

The DFA technique detected 1 or more oocysts spiked onto a slide well, and was able to detect all *Cryptosporidium* genotypes tested, showing a strong apple green fluorescence outlining the *C. parvum* oocysts and more variable fluorescence with *C. andersoni, C. canis, C. felis,* and *C. serpentis.* As expected, *Giardia* cysts also showed strong fluorescence but could be easily distinguished by the larger oval cyst size of 10-14 μm diameter as compared to the 4-7 μm diameter size of *Cryptosporidium* oocysts.

Mussel tissue spiking experiments. *Cryptosporidium* detection techniques were next evaluated by spiking oocysts of *C. parvum* directly into mussel hemolymph, gill washings, and digestive gland, followed by tissue processing and analysis. First, the TaqMan PCR assay was compared with the Xiao and Morgan conventional PCR assays for the detection of *Cryptosporidium* DNA in spiked hemolymph samples (Table 2.2). All three techniques were able to detect the full range of 1-500 oocysts spiked into hemolymph, with some variation in the proportion of positive samples. For the high spike doses of 100 and 500 oocysts, all three techniques detected 83% or greater of spiked hemolymph samples. When 1 or 10 oocysts were spiked into a hemolymph sample, the TaqMan and Xiao techniques detected 33% of spiked samples while the Morgan technique detected 50% or more of positive samples. No false positives were detected in negative control samples.

Table 2.3 shows the proportion of spiked gill wash and digestive gland samples that tested positive for *Cryptosporidium* by TaqMan PCR or DFA, with and without IMS. All four detection methods were able to consistently detect gill wash or digestive samples spiked with 100 or 500 oocysts. For samples spiked with 100 oocysts, 50% of gill washings and 33% of digestive gland samples tested positive by TaqMan PCR. The

addition of immunomagnetic separation significantly improved *Cryptosporidium* detection in gill washings and digestive gland compared to TaqMan PCR or DFA detection methods alone ($p<0.01$). The oocyst detection limit in gill washings and digestive gland was improved by 1 $\log_{10}$ unit, from 100 to 10 oocysts per sample, when IMS concentration was used. For gill wash samples spiked with 10 *C. parvum* oocysts, IMS increased the proportion testing positive from 0% to 33% by TaqMan PCR and from 17% to 83% by DFA. Similarly, when 10 oocysts were spiked into digestive gland tissues, IMS increased the proportion of positive samples detected by TaqMan PCR from 0% to 17% and by DFA from 0% to 83%. Overall, TaqMan PCR was most successful in detecting low oocyst doses spiked into hemolymph as compared to gill wash or digestive gland samples. No negative control tissue samples tested positive.

Tank exposure experiment. *Cryptosporidium parvum* oocysts were detected in mussels that filtered oocysts during an 8 hour exposure to tank inoculated sea water. There was no mussel mortality during the experiment. Thirty *C. parvum*-exposed and 15 unexposed individual mussels had gill wash and digestive glands tested by DFA and TaqMan PCR, with and without IMS concentration. Thirty hemolymph samples from exposed mussels were tested with TaqMan PCR but only 28 were tested by each conventional PCR technique due to sample volume limitations. Hemolymph from 15 unexposed individual control mussels was tested with each PCR technique. For pooled samples of 5 mussels each, 12 exposed pools and 3 unexposed pools of each tissue type were tested by each detection technique. Pooled hemolymph samples were only tested by TaqMan PCR due to sample volume limitations. All mussels from the unexposed tank tested negative in all tissues with all techniques.

The proportion of *Cryptosporidium* positive mussels in the two oocyst-exposed tanks did not differ significantly ($p=1.0$), therefore data were combined for further analysis. Table 2.4 shows the tissue, detection method, and exposure status for individual and pooled mussels tested for *Cryptosporidium*. TaqMan PCR detected *Cryptosporidium* in 3% of hemolymph samples from exposed individual mussels, compared to 0% and 4% of hemolymph that tested positive by Xiao and Morgan conventional PCR, respectively. There was no significant difference between the TaqMan and conventional PCR techniques when testing hemolymph samples ($p=1.0$). TaqMan PCR did not detect *Cryptosporidium* cDNA in any of the exposed mussel pools, and these pools could not be tested by the two conventional PCR techniques due to sample volume limitations. None of the techniques detected *C. parvum* in individual or pooled gill wash samples. Immunomagnetic separation with TaqMan PCR detected *Cryptosporidium* in 7% of individual exposed digestive glands, compared to 23% detected by DFA and 80% by IMS-DFA. Because IMS-TaqMan PCR only detected *Cryptosporidium* in 7% of individual exposed mussel digestive glands and in none of the pooled mussel digestive glands, mussel samples without IMS concentration were not tested by TaqMan PCR. Pooled digestive gland tissues tested by DFA and IMS-DFA resulted in 33% and 100% positive, respectively. Immunomagnetic separation with TaqMan PCR was not significantly different from DFA alone ($p=0.18$), but detected significantly fewer positive individual mussel digestive glands than IMS concentration combined with DFA detection ($p<0.01$). The mean Ct value per positive digestive gland was 32 (range, 29-36 Ct), while the mean number of oocysts detected per mussel was 1.6 (range, 1-3 oocysts) by DFA and 19 (range, 1-150 oocysts) by IMS-DFA. IMS-TaqMan PCR was not

significantly different from DFA for pooled exposed mussel digestive glands ($p=0.13$) given the small sample size, and IMS-TaqMan PCR detected significantly fewer positive samples than IMS-DFA ($p<0.01$). The mean number of oocysts per positive digestive gland pool was 1.8 (range, 1-3 oocysts) by DFA and 9.8 (range, 2-24 oocysts) by IMS-DFA. When a duplicate set of pooled digestive gland samples stored 6 months at -20° C was tested, 0% and 83% tested positive by DFA and IMS-DFA, respectively. Many of the oocysts visualized in digestive gland that had been frozen were deformed or ruptured but still had a strong green fluorescence, suggesting that freezing does not destroy the antigens that bind antibodies in the IMS and DFA procedures, but that it does cause oocyst rupture that could make morphologic identification difficult.

Once preliminary spiking experiments establish test performance paramenters, quantitative detection techniques such as TaqMan PCR and DFA analysis provide data that can be used to estimate the parasite concentration in a sample. In the case of TaqMan PCR, the Ct value of an "unknown" sample can be correlated to the Ct values of known oocyst dilutions in bivalve tissues. For example, in Figure 2.3 the oocyst spike doses of 10-500 per mussel sample are shown for digestive gland concentrated by IMS. The regression line for oocysts spiked into digestive gland and concentrated by IMS is very similar to the regression line from dilutions of oocyst cDNA alone (Figure 2.1), suggesting that any residual mussel tissue does not inhibit the PCR reaction. The Ct values of the IMS-TaqMan-positive digestive gland samples from the tank experiment ranged from 29 to 36. The standard curve suggests that at least 100 oocysts may have been present in these digestive gland samples, which is consistent with the oocyst range quantified by IMS-DFA of up to 150 oocysts in a digestive gland.

In the case of DFA analysis, the number of oocysts in an unknown sample can be estimated by adjusting the data based on percent recovery estimates. Table 2.5 shows the mean recovery efficiency of *Cryptosporidium* oocysts spiked into 6 sets of gill wash and digestive tissues at doses ranging from 0-500 oocysts. A single oocyst spiked into gill washings or digestive gland was not detected, but when 10-500 oocysts were spiked into gill washings, the mean DFA percent recovery ranged from 17% to 42% per 10 ul test aliquot and 1% to 2% per total tissue spike. The use of IMS in combination with DFA on gill washes produced mean percent recoveries ranging from 40% to 44%, which reflected the individual test aliquot as well as the total tissue spike because all test aliquots were less than the recommended volume limit of 0.5 ml pellet for IMS concentration. Thus, for gill wash tissues, IMS-DFA data could be converted to estimate the true number of oocysts in an unknown sample by assuming that approximately 43% of oocysts were counted during IMS-DFA analysis. For digestive gland tissues spiked with 10-500 oocysts, mean percent recoveries ranged from 0% to 15% per 10 ul test and from 0% to 0.2% per whole tissue spike. The use of IMS on digestive gland produced mean percent recoveries that ranged from 58-66%, therefore raw IMS-DFA data could be adjusted to estimate the actual number of oocysts present in the sample by assuming that approximately 61% of actual oocysts were visualized during IMS-DFA analysis. The range of oocysts detected in the tank experiment by IMS-DFA ranged from 1-150 oocysts per digestive gland sample, and adjusting this range for 61% recovery efficiency suggests that there may have really been up to 246 oocysts per digestive gland sample. The mean percent of oocysts recovered by IMS-DFA was significantly higher than the mean percent recovered by DFA alone when comparing recovery per mussel ($p<0.01$). Mean percent

recovery per test was not significantly different between DFA and IMS-DFA for gill washes ($p=0.47$), but was significantly higher for IMS-DFA over DFA per test aliquot for digestive gland tissues ($p<0.01$). There was no significant difference in IMS-DFA percent recovery when spiked individual mussel digestive glands (0.2-2 gm before sieving) were compared to spiked pools of 5 mussel digestive glands (sieved pellet ≤ 0.5 ml) ($p=0.33$, data not shown), suggesting that testing pooled samples could decrease cost without significant loss of sensitivity.

DISCUSSION

In the present study, a real-time TaqMan PCR system was validated and compared to conventional PCR and DFA methodologies, with and without IMS concentration, for sensitive and specific detection of *Cryptosporidium* spp. in bivalve tissues. TaqMan PCR and the two conventional PCR systems were all able to detect 1 or more oocysts spiked into 1 ml hemolymph samples, but detected *C. parvum* in <5% of tank exposed mussel hemolymph samples. In the gill wash and digestive gland spiking experiments, IMS concentration in combination with TaqMan PCR or DFA detection improved the minimum detection limit by 1 $\log_{10}$ unit, from 100 to 10 oocysts per spiked mussel sample. Despite the success of gill wash methods in detecting *C. parvum* in the tissue spiking experiments, none of the tank exposed gill wash samples tested positive for *C. parvum* by any method. However, IMS concentration in combination with DFA testing detected *Cryptosporidium* in 80% of exposed individual and 100% of exposed pooled mussel digestive glands, suggesting that more oocysts are retained in the digestive gland than in the gills or hemolymph after the mussels have filtered oocysts from the

surrounding water. Both TaqMan PCR and DFA methods provided quantitative results that could be used to estimate the true number of oocysts present in a bivalve sample.

TaqMan PCR has several advantages over traditional *Cryptosporidium* detection methods. TaqMan PCR is an automated, rapid, high throughput system that allows investigators to submit fresh or frozen samples to a TaqMan service in batches for pathogen detection, reducing the need for investigators to invest in expensive equipment and expertise in their own laboratories. The Bivalve TaqMan PCR system that is run concurrently with the *Cryptosporidium* TaqMan system is useful in assessing tissue extraction efficiency and can be used as a quality control measure for invertebrate studies. Additionally, the *Cryptosporidium* TaqMan PCR system detects cDNA transcribed from total RNA, a nucleic acid that is more rapidly degraded than genomic DNA after cell death. This suggests that TaqMan results are more likely to correlate with oocyst viability than assays that target genomic DNA, which is more stable than RNA (14). A primary goal for developing a TaqMan PCR system was to maximize analytical sensitivity. For that reason we targeted the 18S rRNA at the RNA level, which should have a higher copy number per oocyst than targeting the 18S rRNA gene (5). TaqMan assays that target low copy number genes may correlate even better with oocyst viability than 18S rRNA systems (39, 46). Finally, the TaqMan PCR system provides quantitative results and detects many *Cryptosporidium* spp. The TaqMan and 2 conventional PCR systems detected all *Cryptosporidium* spp. evaluated in this study, but in the case of the Morgan PCR system, dinoflagellates and invertebrate DNA were also amplified. Other studies (40) have reported similar cross reactions with dinoflagellates when testing for *Cryptosporidium*, suggesting that false positive *Cryptosporidium* results are of concern in

field studies if DNA sequence analysis is not used in conjunction with PCR. The *Cryptosporidium* TaqMan system was more specific than the Morgan PCR system but also detected *Sarcocystis* species. Both TaqMan and conventional PCR techniques can be used in combination with DNA sequence analysis, the gold standard for obtaining detailed molecular data that can be deposited in GenBank for use in other molecular epidemiology studies.

Before this study, TaqMan PCR systems had not been applied to bivalve studies but had been used to test test water and fecal samples (13, 21, 24). Guy et al. (21) developed a quantitative multiplex real-time PCR system to detect *C. parvum* and *Giardia* spp. that was of comparable accuracy to immunofluorescent microscopy for testing water and sewage samples. Higgins et al. (24) developed a TaqMan PCR system that was able to quantitate *C. parvum* oocysts in 0.2 g calf diarrhea samples, but was not able to detect *C. parvum* in 0.2 gm adult cattle manure samples, possibly due to low oocyst numbers and the presence of substances inhibitory to PCR. Fontaine and Guillot (13) combined their TaqMan PCR system with IMS to detect 8 or more *C. parvum* oocysts in 100 L of tap water and 5 L of river water. Our TaqMan PCR system was developed for the analysis of bivalve samples; however, in conjunction with IMS concentration, it may also have valuable applications for fecal and water samples containing a variety of *Cryptosporidium* spp.

The *Cryptosporidium* DFA detection technique has been used to test fecal and water samples for over a decade, and more recently it has been applied to bivalve tissues (9, 19, 20, 29, 41, 47). A benefit of using the DFA technique is that oocysts can be identified visually using a combination of immunofluorescence, size, and morphology to

distinguish them from other organisms. Other benefits of DFA are that it provides quantitative data and can be used on formalin preserved samples, unlike conventional PCR. The limitations of the DFA technique are that it requires microscopy expertise and does not provide genotype data unless combined with PCR methods. Other studies have used DFA for testing hemolymph samples (19, 41). However, in our preliminary studies the autofluorescence and small size of the hemocytes often obscured *Cryptosporidium* oocysts and interfered with accurate oocyst visualization. Therefore this detection method was not included in the present comparative study. Before the availability of IMS, the small volume of sample analyzed was also a significant limitation in detecting low numbers of *Cryptosporidium* by DFA. For example, 100 oocysts spiked into a mussel digestive gland (mean wt, 0.6 gm; range, 0.2-2 gm) were detected in a 10 µl DFA aliquot 17% of the time. In contrast, 100% of the same samples were positive when IMS concentration was performed before DFA detection.

Immunomagnetic separation significantly increased the analytical sensitivity in gill wash and digestive gland samples in the tissue spiking experiments. The advantage of IMS is that a larger tissue volume can be analyzed (0.5 ml) as compared to <100 mg for PCR. Additionally, IMS decreases the amount of particulate matter and inhibitors in a sample. Although IMS increases the cost per test (up to $50 per test), it may also decrease the number of tests and time required to process a given sample volume. While IMS can concentrate the oocyst walls from fresh, frozen, or formalin samples, fresh tissues are often preferred for two reasons. First, because freezing can rupture oocysts, making DFA morphologic identification difficult and reducing the amount of concentrated oocyst DNA available for PCR amplification. Second, formalin is also a

problematic preservative because it can interfere with the DNA extraction and PCR detection. While gill wash and digestive glands were effectively concentrated by IMS, hemolymph was not extensively evaluated with IMS concentration because our preliminary studies showed that hemocytes adhered to the IMS glass tubes, thus interfering with the IMS concentration process. However, hemolymph was efficiently concentrated by centrifugation, allowing for sensitive PCR detection of a single oocyst spiked into 0.5 ml hemolymph without IMS concentration. Because oocyst spiking into hemolymph, gill washings, and digestive gland were done before tissue processing in this study, *Cryptosporidium* recovery efficiency estimates and analytical sensitivity may be more accurate than studies that have spiked oocysts after tissue processing (11, 20).

Immunomagnetic separation has occassionally been used in wild bivalve studies. An example is the Gomez-Bautista et al. (16) study that used immunofluorescent antibody testing in combination with IMS to increase assay sensitivity when testing wild mussel and cockle homogenates collected along the coast of Spain. The authors detected *C. parvum* genotype 2 oocysts in bivalves collected near the mouth of rivers draining watersheds with high ruminant stocking densities but not in bivalves collected from river mouths draining more pristine areas or from sites away from river mouths. Although the study did not assess the percent recovery and sensitivity limits of their assay, the technique of testing pooled bivalve tissues with IMS holds promise for cost-efficient environmental monitoring. Immunomagnetic separation has also been used to test shellfish for pathogenic *Vibrio* spp. and hepatitis A viruses (26, 44).

The mussel tank exposure experiment was designed to simulate an 8 hr pulse exposure to *Cryptosporidium*-contaminated nearshore waters. The exposure dose of

1,000 oocysts/L is within the range of surface water contamination levels reported near agricultural runoff.[38] Therefore, the results of this experiment may reflect the oocyst load to which wild shellfish can be exposed when located near fecal contaminated runoff. During an 8 hr exposure to 14°C water that originally contained 1000 oocysts/L, 10 L of salt water, and 70 mussels, each mussel was exposed to a mean of 142 oocysts. Based on the mean of 15 oocysts detected per mussel digestive gland (range from 1-150 oocysts) by IMS-DFA and adjusting the data based on the 61% recovery in spiked digestive gland tissues, at least 18% of the initial *Cryptosporidium* dose was likely to be retained in mussel tissues after 8 hours. Graczyk et al. (18) conducted a tank exposure experiment in which 100 *C. parvum* oocysts per day were inoculated for 31 days into tanks containing freshwater mussels and clams. The authors found that from 7% to 32% of oocysts could be recovered in homogenized bivalve tissues, with increasing numbers of oocysts accumulated in bivalve tissues as the oocyst concentration in the water increased over time. The results of the Graczyk study complement the findings of our 8 hr tank exposure study by demonstrating that bivalves can accumulate oocysts after repeated daily exposures to very low oocyst doses.

The most sensitive *Cryptosporidium* detection technique in our tank exposure experiment was to test digestive gland samples by DFA after IMS concentration. The majority of oocysts were detected in digestive gland samples, with 80% of exposed individual mussels and 100% of mussel pools testing positive by IMS-DFA, compared to <5% of hemolymph samples positive by PCR and 0% of gill washings positive by either DFA, IMS-DFA, or IMS-TaqMan PCR. The tissue spiking experiments had shown that low numbers of oocysts could be detected in hemolymph, gill wash, and digestive gland

samples, so the differences in detection success between the tissue spiking experiments and the tank exposure experiment are likely due to the varied tissue retention and low numbers of oocysts present in the exposed mussels after they filtered the oocysts during the tank experiment. Other tank exposure studies have yielded varied results, with some studies finding that digestive gland was the best tissue for oocyst detection (41) while other studies concluded that gills were more sensitive for detecting *Cryptosporidium* contaminated bivalves (7). Differences between studies could be attributable to experimental variables such as exposure time, dose, or detection techniques, or biologic variables such as bivalve species, filtration rate, or water characteristics.

In addition to determining the most sensitive *Cryptosporidium* detection method in mussels, the tank exposure experiment was used to define the TaqMan Ct cutoff for *Cryptosporidium*-positive bivalve samples. A TaqMan Ct value of <39.5 was defined as positive after comparing the TaqMan Ct values from *Cryptosporidium* exposed and unexposed mussels in the tank exposure experiment, to adjust for any background TaqMan signal. The lower the Ct value, the lower the number of TaqMan cycles required to produce detectable target DNA, with each cycle increasing the target DNA exponentially. Based on the relationship between TaqMan Ct values and standard *Cryptosporidium* dilutions, the concentration of oocysts in the test sample can be estimated. By comparing the range of Ct values (29-36) detected in the 2 positive IMS-TaqMan digestive glands in the tank experiment to the standard curve of oocyst spiked into digestive gland (Figure 3), over 100 oocysts were most likely present in those samples, which is similar to the IMS-DFA results for which 2 samples contained over 100 oocysts. This quantitation capability may be useful for future studies that aim to

measure changes in oocyst numbers based on environmental variables or *Cryptosporidium* treatment and inactivation regimens.

Further studies focused on the distribution and variation in *Cryptosporidium* genotypes in the environment are needed to gain a better understanding of the sources and flow of fecal pathogens from terrestrial to aquatic ecosystems. In addition to identifying the *Cryptosporidium* genotypes present, another goal of many epidemiologic investigations is to assess the magnitude of fecal contamination. The DFA and TaqMan PCR assays can provide a quantitative measure of *Cryptosporidium* load in a sample, though the results must be interpreted with an understanding of the dynamic ecosystem from which the sample came. For example, low numbers of *Cryptosporidium* in a shellfish sample could have been acquired from a large bolus of fecal contamination that was filtered a week before or from a small bolus filtered an hour before. Repeated sampling through time and space is needed to properly understand the relationships between bivalves, their aquatic environments, and terrestrial sources of fecal loading.

In conclusion, this study has shown that TaqMan PCR is similar in analytical sensitivity and specificity to conventional PCR methods, with the additional benefits of rapid turn around time, high throughput capacity, and quantitative results. TaqMan sensitivity was also on par with DFA, and for both methods the minimum detection limit was improve by 1 $\log_{10}$ unit when used in combination with IMS concentration. Both TaqMan and DFA detection methods provide quantitative results but only the TaqMan system can be expected to detect the variety of *Cryptosporidium* genotypes present in environmental samples. The most successful *C. parvum* detection method in the tank exposure experiment was IMS concentration of mussel digestive gland samples followed

by DFA detection, with 80% of oocyst-exposed individuals testing positive. TaqMan and conventional PCR were also able to detect *C. parvum* in up to 5% of oocyst-exposed individual hemolymph samples. This study provides the critical laboratory evaluations needed for selection of the most appropriate methods for detection and characterization of *Cryptosporidium* parasites in future studies.

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TABLE 2.1. Nucleotide sequence of PCR primers and TaqMan probes used to detect *Cryptosporidium* or bivalve 18S rRNA

Amplification target	Primer	Primer sequence (5'->3')	Length of PCR Product	Probe	TaqMan probe sequence (5'->3')[1]
TaqMan Bivalve spp.	Clam18-412f	CGGCTACCACATCCAAGGA	110	Clam18-434p	CAGCAGGCGCGCAAATTACCCACT
	Clam18-521r	CCAATTACGGGGCCTCGAA			
TaqMan *Cryptosporidium* spp.	Crypt-193f	GGAAGGGTTGTATTTATTAGATAAAGAACCA	182	Crypt-276p	CATTCAAGTTTCTGACCTATCAGCTTTAGACGG
	Crypt-374r	CTCCCTCTCCGGAATCGAA			
Xiao *Cryptosporidium* spp.[48]	C1F	TTCTAGAGCTAATACATGCG	1325		
	C1R	CCCTAATCTTTCGAAACAGGA			
	C2F	GGAAGGGTTGTATTTATTAGATAAAG	850		
	C2R	AAGGAGTAAGGAACAACCTCCA			
Morgan *Cryptosporidium* spp.[32]	18sif	AGTGACAAGAAATAACAATACAGG	298		
	18sir	CCTGCTTTAAGCACTCTAATTTTC			

[1] TaqMan probe oligonucleotides were labeled with 6-FAM at the 5' and the quencher TAMRA at the 3'.

TABLE 2.2. Proportion of spiked mussel hemolymph samples positive for *Cryptosporidium parvum* by different PCR protocols.[a]

No. oocysts spiked per hemolymph (1 ml)	TaqMan % Positive	Xiao % Positive	Morgan % Positive
1	33	33	67
10	33	33	50
100	100	83	100
500	100	100	100

[a] n=6 replicates per spiking dose.

TABLE 2.3. Proportion of spiked mussel tissues positive for *Cryptosporidium parvum* by TaqMan PCR and DFA, with and without IMS concentration[a]

Sample Type	No. oocysts spiked per mussel sample	TaqMan % Positive	IMS-TaqMan % Positive	DFA % Positive	IMS-DFA % Positive
Gill Wash (5 ml)	1	0	17	0	0
	10	0	33	17	83
	100	50	67	50	100
	500	100	100	67	100
Digestive Gland (1 g)	1	0	0	0	0
	10	17	17	0	83
	100	33	100	17	100
	500	50	100	67	100

[a] n=6 replicates per spiking dose and tissue type.

TABLE 2.4. Proportion of individual and pooled mussels (*Mytilus californianus*) testing positive for *Cryptosporidium parvum* after an 8 hr tank exposure to 1000 oocysts/L (142 oocysts/mussel).[a]

Mussel Tissue	Detection Method	Exposed Individuals % Positive (n=30)[b]	Unexposed Individuals % Positive (n=15)	Exposed Pools % Positive (n=12)[c]	Unexposed Pools % Positive (n=3)[c]
Hemolymph (1 ml/mussel)	TaqMan	3	0	0	0
	Xiao	0	0	ND	ND
	Morgan	4	0	ND	ND
Gill Wash (5 ml/mussel)	IMS-TaqMan	0	0	0	0
	DFA	0	0	0	0
	IMS-DFA	0	0	0	0
Digestive Gland (1 g/mussel)	IMS-TaqMan	7	0	0	0
	DFA	23	0	33	0
	IMS-DFA	80	0	100	0

[a] Exposed mussel tanks were inoculated *C. parvum* oocysts, unexposed mussels tanks were not inoculated.
[b] n=28 for Xiao and Morgan hemolymph testing.
[c] ND=not determined due to sample volume limitations.

TABLE 2.5. Recovery efficiency of *Cryptosporidium parvum* oocysts from spiked mussel tissues by DFA and IMS-DFA detection[a]

Sample Type	No. oocysts spiked per mussel sample	DFA mean % recovery per test[b] (%range)	DFA mean % recovery per mussel[c] (%range)	IMS-DFA mean % recovery per test and mussel[d] (%range)
Gill Wash (5 ml)	1	0	0	0
	10	42 (0-250)	2 (0-10)	40 (0-100)
	100	17 (0-50)	1 (0-2)	44 (10-89)
	500	37 (0-90)	2 (0-4)	44 (17-75)
Digestive Gland (1 g)	1	0	0	0
	10	0	0	58 (0-150)
	100	10 (0-57)	0.2 (0-1)	58 (43-83)
	500	15 (0-43)	0.2 (0-0.4)	66 (29-86)

[a] n=6 replicates per dose and tissue type.
[b] % recovery per test = No oocysts counted on a slide / No. oocysts expected in a test aliquot.
[c] % recovery per mussel = No. oocysts counted on a slide / No. oocysts spiked into the mussel sample.
[d] % recovery per test and mussel are the same because the whole sample could be analyzed in one IMS test aliquot.

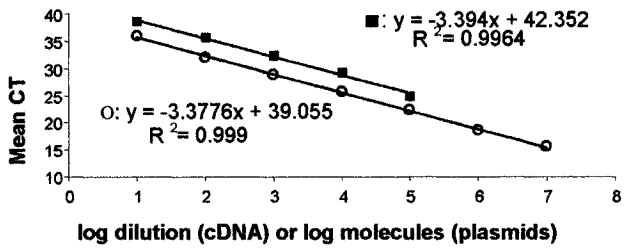

FIG. 2.1. TaqMan amplification of cloned *Cryptosporidium* PCR product (o) and cDNA generated on RNA extracted from 10-fold dilutions of *C. parvum* oocysts (■).Standard curves were determined in triplicate, standard deviations are too small to be visible.

PCR system	Protozoal DNA[a,c]																	Non-protozoal DNA[b,c]				
	Cryptosporidium andersoni (bovine)	*Cryptosporidium baileyi* (chicken)	*Cryptosporidium canis* (canine)	*Cryptosporidium felis* (feline)	*Cryptosporidium hominis* (human)	*Cryptosporidium meleagridis* (chicken)	*Cryptosporidium parvum* (bovine)	*Cryptosporidium parvum* (river otter)	*Cryptosporidium serpentis* (snake)	*Neospora caninum* (bovine)	*Neospora hughesi* (equine)	*Sarcocystis falcatula* (equine)	*Sarcocystis neurona* (equine)	*Sarcocystic neurona* (harbor seal)	*Sarcocystis neurona* (sea otter)	*Toxoplasma gondii* (human)	*Giardia duodenalis* (bovine)	*Gymnodinium* spp. (dinoflagellate)	*Mytilus californiamus* (surf mussel)	*Mytilus galloprovincialis* (hay mussel)	*Corbicula fluminea* (freshwater clam)	*Emerita analoga* (sand crab)
TaqMan Bivalve	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+
TaqMan *Cryptosporidium*	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	-	-	-	-	-	-	-
Xiao *Cryptosporidium*	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Morgan *Cryptosporidium*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-

[a] Protozoal spp. (host from which isolate obtained).

[b] Non-protozoal spp. (common name).

[c] + = PCR positive; - = PCR negative.

FIG. 2.2. Specificity testing of TaqMan and conventional PCR systems with protozoal, dinoflagellate, and invertebrate DNA samples.

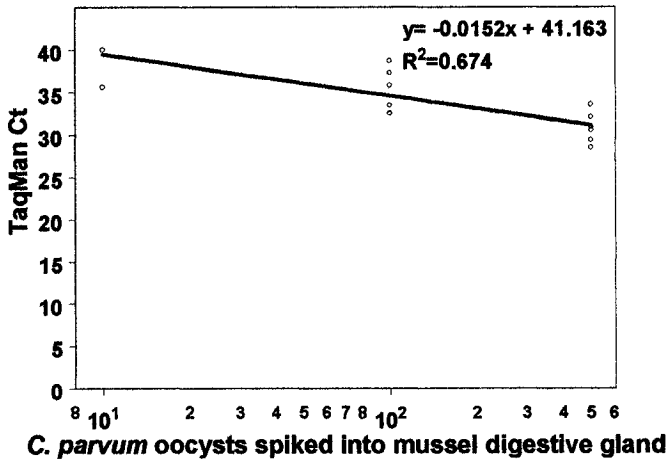

FIG. 2.3. TaqMan PCR detection of *Cryptosporidium parvum* oocysts spiked into mussel digestive gland and processed by immunomagnetic separation. Six replicates of 10, 100, and 500 oocysts per sample.

CHAPTER 3

Cryptosporidium Genotypes Detected in California Mussels (*Mytilus* Species)

Cryptosporidium Genotypes Detected in California Mussels (*Mytilus* Species)

This manuscript is formatted for submission to the International Journal for Parasitology.

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Abstract

A 3 year study was conducted to evaluate mussels as bioindicators of fecal contamination in nearshore California coastal ecosystems. Hemolymph samples from 4800 mussels (*Mytilus* spp.) were tested for *Cryptosporidium* genotypes using PCR amplification and DNA sequence analysis. Our hypotheses were that mussels collected from sites near livestock runoff or human sewage outflow would be more likely to contain the fecal pathogen *Cryptosporidium* than mussels collected distant to these sites, and that the prevalence would be greatest during the wet season when runoff into the nearshore marine environment is highest. To test these hypotheses, 156 batches of sentinel mussels were collected quarterly at nearshore marine sites considered at higher risk for exposure to livestock runoff, higher risk for exposure to human sewage, or lower risk for exposure to both fecal sources. *Cryptosporidium* genotypes detected in hemolymph samples from individual mussels included *C. parvum* genotype 2, *C. felis, C. andersoni*, and novel *Cryptosporidium* spp. Risk factors significantly associated with detecting *Cryptosporidium* spp. in mussel hemolymph were exposure to freshwater outflow and mussel collection within 7 day following a precipitation event. Detection of *Cryptosporidium* spp. was not associated with higher or lower risk status for exposure to livestock feces and human sewage sources. This study showed that mussels can be used to monitor water quality and that *Cryptosporidium* genotypes were found in a variety of polluted and relatively pristine environments.

Keywords: Cryptosporidium; mussel; bivalve; water quality; PCR; 18S rRNA

1. Introduction

The genus *Cryptosporidium* is comprised of host-specific species and species that are infective to a variety of human and animal hosts (Fayer, 1997; Peng, 1997; Morgan-Ryan et al., 2002; Xiao et al., 2004). For example, host-specific *C. hominis* oocysts are shed in the feces of humans and are thought to be transmitted by the fecal-oral route exclusively within the human population, while *C. parvum* genotype 2 has an anthropozoonotic cycle involving humans and many different animal species including ruminants, domestic pets, and wildlife (Peng, 1997; Rose, 1997; Morgan-Ryan et al., 2002). Other host-specific species include *C. andersoni, C. felis, C. canis, C. baileyi,* and *C. muris* that are shed in the feces of cattle, cats, dogs, chickens, and rodents, respectively (Xiao et al., 2004). The oocysts from many of these species are morphologically identical, making molecular tools such as PCR and DNA sequence analysis invaluable for species identification and genotype characterization (Morgan et al., 1997; Xiao et al., 1999; Morgan-Ryan et al., 2002).

Cryptosporidium oocysts are environmentally resistant and have caused outbreaks of diarrheal disease after fecal contamination of surface, pool, and drinking waters (Gallaher et al., 1989; Mackenzie et al., 1994; McAnulty et al., 1994). Humans and livestock have served as sources of fecal contamination in outbreaks, with newly infected individuals shedding millions of oocysts during initial infection (Rush et al., 1990; Joce et al., 1991; Duke et al., 1996; Rose, 1997; Fayer et al., 1998a). Spread of *Cryptosporidium* oocysts from humans and terrestrial animals into freshwater environments and downstream estuarine and marine ecosystems poses a health risk to humans and animals utilizing these ecosystems (Fayer et al., 2004). Molecular tools such as PCR and DNA sequence

analysis may be useful in studies investigating fecal pathogen pollution and fecal loading sources into aquatic environments.

In California, the coastal environment is highly valued and utilized by human populations. However, the sources and magnitude of fecal contamination into the nearshore marine environment are not well understood. Although most studies have utilized fecal coliform bacteria as indicators of fecal contamination (Boehm et al., 2002), *Cryptosporidium* spp. may be more useful as bioindicators because they are endemic in terrestrial animal and human populations, and detecting host-specific *Cryptosporidium* genotypes in environmental samples can help identify the sources of fecal contamination. *Cryptosporidium* spp. have been detected in terrestrial and marine mammals along the California coast (Atwill et al., 1997; Deng et al., 2000), but the prevalence of *Cryptosporidium* genotypes and factors influencing fecal contamination with *Cryptosporidium* spp. in coastal waters are unknown.

This study was designed to assess the presence and seasonal distribution of *Cryptosporidium* genotypes along the central California coast by using mussels as filter-feeding sentinels to concentrate *Cryptosporidium* oocysts from nearshore waters. Bivalves such as mussels, clams, and oysters have also been used as bioindicators of fecal contamination in studies on the Atlantic coast of the United States and in Europe (Fayer et al., 1999, 2002; Freire-Santos et al., 2000; Gomez-Bautista et al., 2000). Our hypotheses were 1) that *Cryptosporidium* would be detected more often in mussels collected from sites near livestock runoff or human sewage influence, as compared to mussels collected from sites distant to these fecal loading sources, and 2) that

Cryptosporidium spp. would be detected most often during the wet season, when runoff into the nearshore environment is greatest.

2. Materials and methods

2.1 Study Design

Mussel testing sites along the central California coast were chosen based on their designation as 'higher risk' for livestock fecal contamination, 'higher risk' for human sewage fecal contamination, or 'lower risk' for fecal contamination from both sources. Figure 3.1a shows the 9 site locations, with more detailed maps (3.1b-d) of subsites located within the northern, middle, and southern regions. The 3 sites considered at higher risk for livestock fecal contamination were located within 1 km of known sources of livestock runoff or freshwater outflow receiving such runoff. Three sites designated as higher risk for human sewage fecal contamination were located within 1 km of major municipal sewage outfalls or freshwater outflow with previously documented septic tank contamination. Three sites considered at lower risk for fecal contamination by humans or livestock were located at least 5 km from known sources of significant livestock runoff and major sewage outfalls. For sites located in estuarine regions, multiple subsites were sampled during the wet seasons to determine whether a spatial pattern in the distribution of *Cryptosporidium* spp. could be detected.

Sentinel mussels (*M. californianus*) were outplanted at coastal study sites based on the protocols of the California State Mussel Watch Program (http://www.swrcb.ca.gov/programs/smw/). Mussels were harvested at a lower risk site that had never tested positive for *Cryptosporidium*, and outplanted as batches of 40-50 mussels per mesh bag at each sentinel mussel site. After at least a month of water

filtration at the sentinel sites, the mussel batches were collected for *Cryptosporidium* testing. At sites where sentinel mussels could not be outplanted, 40 resident mussels (*M. californianus* or *M. galloprovincialis*) per batch were sampled at the same time points. In Year 1, mussel batches were sampled once during the wet and the dry seasons, while in Years 2 and 3, quarterly testing was completed in the early wet season (December-February), late wet season (March-May), early dry season (June-August), and late dry season (September-November, prior to precipitation events). Mussels were transported chilled within 2 days to the University of California, Davis for *Cryptosporidium* testing. All mussels were tested for *Cryptosporidium* using PCR to amplify the 18S rRNA gene from mussel hemolymph samples, with 30 mussels individually tested per batch.

In addition to the hemolymph PCR testing, mussel batches from 6 higher risk sites for fecal exposure were also tested with the most sensitive immunologic *C. parvum* detection techniques available. For 3 wet season mussel collections, 6 pools of 5 mussel digestive glands per mussel batch were tested with immunomagnetic separation (IMS) to concentrate *Cryptosporidium* oocysts and *Giardia* cysts, followed by direct immunofluorescent antibody (DFA) analysis for oocyst and cyst detection. For 1 wet season collection, gill wash samples were also concentrated with IMS followed by DFA detection of *Cryptosporidium* oocysts and *Giardia* cysts.

Statistical analyses were conducted to evaluate risk factors for *Cryptosporidium* detection in mussels. First, a phylogenetic analysis of novel *Cryptosporidium*-like sequences amplified from mussel hemolymph was performed, so that only recognized *Cryptosporidium* spp. or novel sequences that were classified within the *Cryptosporidium* clade would be included in the risk factor analysis. Next, logistic regression was used to

assess putative risk factors associated with detecting *Cryptosporidium* spp. in mussel batches including fecal risk category, season, exposure to freshwater outflow, recent precipitation, bivalve type, and water type.

2.2. Mussels

All sentinel mussels (*Mytilus californianus*) were 3-5 cm long when harvested near Bodega Bay and outplanted at coastal study sites. If mussels could not be outplanted immediately, they were held for up to 6 months in saltwater tanks at the Bodega Bay or Granite Canyon Marine Laboratory facilities. To ensure that testing sentinel mussels for *Cryptosporidium* spp. represented the water quality at the outplanted site, mussels were left for at least a month at study sites to allow for depuration of any *Cryptosporidium* oocysts that might have been present in sentinel mussels at the time of outplanting. In addition, for each round of mussel outplanting and collections, a mussel batch from the original Bodega Bay collection site was tested and found to be negative for *Cryptosporidium* DNA.

Mussels were collected and analyzed as batches of 30 per site and sampling time. Hemolymph was extracted first by filing a notch in the shell and aspirating 0.5-1.5 ml of hemolymph from the adductor muscle with a sterile syringe. The hemolymph was centrifuged and the concentrated cell pellet was stored at -80°C until DNA extraction and PCR analysis. Next, the adductor muscle was cut with a razor blade to open the mussel, and the gill tissues were excised. For selected samples, gill washes were performed based on the Fayer et al. (1998b) protocol, by vortexing the gills of 5 mussels per pool in 25 ml sterile saline for 45 sec and removing large pieces of gill. The gill wash was centrifuged for 15 min at 1000×g, and a 0.5 ml pellet of the sediment was then used for IMS

concentration and DFA analysis. Finally, the digestive gland was excised from the mussel using a new razor blade for each mussel. For selected samples, half of the digestive gland from 5 mussels were pooled, homogenized, and sieved through a 100 μm cell strainer. Tubes were centrifuged for 15 min at 1000×g and processed by IMS concentration and DFA analysis. All extra tissues were stored at -80°C.

2.3. Polymerase chain reaction

Two conventional 18S rRNA PCR protocols (Morgan et al., 1997; Xiao et al., 1999) were used for *Cryptosporidium* spp. detection in mussel hemolymph. For DNA extraction, a 20-50 μl pellet was suspended in 180 μl ATL buffer and placed in liquid nitrogen for 4 min, then in boiling water for 4 min. Using the QIAGEN DNA Mini Kit tissue protocol (QIAGEN Inc., Valencia, CA), mussel tissue was digested, bound to a QIAamp column, washed, and the DNA eluted with 50 μl of 95°C PCR water. Extracted DNA was frozen at -80°C until PCR analysis.

A direct PCR protocol was used to amplify a 300 bp segment of the *Cryptosporidium* 18S rRNA gene (Morgan et al., 1997). The *Cryptosporidium* primers consisted of 18sif: AGT GAC AAG AAA TAA CAA TAC AGG and 18sir: CCT GCT TTA AGC ACT CTA ATT TTC. The PCR reactions contained 5 μl 10X PCR buffer, 3 mM $MgCl_2$, 200 μM each dNTP, 200 nm each primer, 1.5 U Taq polymerase, PCR-grade water, and 2 μl DNA in a 50 μl total volume. Amplification conditions for the PCR reactions started with 2 min at 96°C, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, with a final extension of 72°C for 7 min.

A nested protocol was also used to amplify a larger 18S DNA segment. The Xiao (1999) 18S rRNA protocol used the outer primer set to amplify a 1325 bp DNA segment,

followed by amplification of an 850 bp DNA segment using the inner primer set. The outer primer sequences were C1F: TTC TAG AGC TAA TAC ATG CG and C1R: CCC TAA TCT TTC GAA ACA GGA, with inside primers C2F: GGA AGG GTT GTA TTT ATT AGA TAA AG and C2R: AAG GAG TAA GGA ACA ACC TCC A. The outer primer PCR reactions contained 5 µl Perkin-Elmer (Norwalk, CN) 10X PCR buffer, 6 mM $MgCl_2$, 200 µM each deoxynucleoside triphosphate (dNTP), 200 nm each primer, 1.5 U Taq polymerase, PCR-grade water, and 2 µl DNA in a 50 µl total volume. The inner primer PCR reactions were the same except that the $MgCl_2$ was reduced to 3 mM. Amplification conditions for the outer and inner PCR reactions consisted of hot start at 94°C for 3 min, followed by 35 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 1 min, with a final extension at 72°C for 7 min.

Amplified PCR products were separated by electrophoresis on a 2% agarose gel containing GelStar (Cambrex Co., East Rutherford, NJ). Positive and negative controls were included in each batch of PCR amplification. Size and band intensity of PCR products were evaluated compared to a 100 bp ladder included with each gel. For selected isolates, the PCR product was purified according to QIAGEN Qiaquick protocol, sequenced on an automated sequencer, and analyzed with Chromas (Technelysium Pty Ltd, Tewantin, Qld, AU) and ClustalX (Thompson et al., 1997) software for *Cryptosporidium* genotype identification.

2.4. Immunomagnetic separation

For selected samples, digestive gland pellets of up to 0.5 ml were concentrated and purified with IMS (Dynal Biotech, Oslo, Norway) per the manufacturer's instructions, followed by DFA analysis for parasite detection. Briefly, the tissue pellet was suspended

in a Dynal L10 glass tube with 2 ml buffer and 10 ml PCR water. Next, 100 µl *Cryptosporidium* and *Giardia* IMS beads were added, followed by sample mixing on the Dynal rotater for 1 hr at setting 18. The IMS bead-parasite complexes were then bound to a magnetic holder while the supernatant and debris were discarded. Bead-parasite complexes were resuspended in 1 ml buffer and transferred to 1.5 ml microcentrifuge tubes that were put into a smaller magnetic holder to bind the bead-parasite complexes while the supernatant was discarded. For parasite dissociation from the beads, 2 acid washes of 50 µl 0.1 N HCl each were vortexed at the beginning and end of the 10 min incubation. Tubes were then put in a small magnetic holder and the supernatant was transferred to a DFA slide containing 5 ul 1 N NaOH for neutralization, while the beads were held on the magnet. Hemolymph was not suitable for IMS because hemocytes were found to adhere to the glass tubes in our preliminary studies.

2.5. Direct immunofluorescence

Cryptosporidium oocyst and *Giardia* cyst DFA detection were performed on selected digestive and gill wash samples that were first concentrated by IMS. First, purified IMS products were dried onto 3-well Merifluor slides (Meridian Bioscience Inc., Cincinnati, OH). The DFA slides were incubated with anti-*Cryptosporidium* and anti-*Giardia* antibody, counterstained (Meridian Bioscience Inc.) for 30 min, and then read at 200X-400X magnification on a Zeiss Axioscope epi-fluorescent microscope with FITC as the fluorochrome. All slides were read by the senior author. *Cryptosporidium parvum*-like oocysts were identified as 5 µm diameter spheres that were outlined in apple green, often with a midline seam. *Giardia*-like parasites were also apple green but were elliptical, larger (10-14 µm diameter), and did not have a midline seam. Hemolymph DFA testing

was not done because preliminary tests showed that hemocytes autofluoresced, making oocyst identification difficult.

2.6. Phylogenetic analysis

All *Cryptosporidium* genotypes detected in mussel hemolymph were identified using DNA sequencing of purified PCR products, followed by sequence analysis with Chromas (Technelysium Pty Ltd), BLAST (http://www.ncbi.nlm.nih.gov/), ClustalX (Thompson et al., 1997), and Mega2 (http://www.megasoftware.net/) software. An initial BLAST search was performed to identify *Cryptosporidium*-like sequences by comparing the DNA sequences of our PCR products with reference sequences in GenBank. Next, our DNA sequences were aligned with GenBank reference sequences in ClustalX to determine whether our sequence was an exact match to a GenBank reference sequence or not. Any *Cryptosporidium*-like sequences that did not perfectly match the reference sequences were then included in a phylogenetic analysis along with related protozoa. All reference sequences were shortened to the 300 bp length of our novel *Cryptosporidium*-like sequences for phylogenetic analysis. Two approaches were used to evaluate the relationship between novel *Cryptosporidium*-like sequences from this study and GenBank reference sequences of *Cryptosporidium* spp. and related organisms. A neighbor-joining analysis inferred the phylogenetic relationships based on Tamura-Nei distances with 1000 bootstrap replicates. A maximum parsimony analysis analyzed the same data set and was based on the heuristic approach and 1000 bootstrap replicates. Reference sequences from GenBank included *C. parvum* genotype 2 (AF093490), *C. hominis* (AF093489), *C. wrairi* (AF115378), *C. meleagridis* (AF112574), *C. canis* (AJ493209), *C. felis* (AF108862), *C. baileyi* (AF093495), *C. serpentis* (AF093502), *C.*

andersoni (AF093496), and *C. muris* (AF093498), as well as the closely related neogregarine protozoa *Ophriocystis elektroscirrha* (AF129883), the coccidial protozoa *Toxoplasma gondii* (M97703), and the mesomycetezoan protozoa *Pseudoperkinsus tapetis* (AF192386).

2.7. Risk factor analysis

Covariate data on potential risk factors was collected for all mussel sites. Each site was categorized as higher risk for human feces if it was located within 1 km of known sewage outfalls or freshwater outflow receiving significant septic tank contamination, as higher risk for livestock feces when located within 1 km of livestock runoff or freshwater outflow receiving significant livestock runoff, or as lower risk for fecal contamination when located at least 5 km from these fecal loading sources. Each site was classified by season based on whether mussels were collected in the early wet season (December-February), late wet season (March-May), early dry season (June-August), or late dry season (September-November). A freshwater outflow category was assigned for each mussel site and sampling time by creating a model of the local precipitation and river gauge flow for the day preceding mussel collection, assuming exponential dilution once the freshwater started mixing with saltwater. Mussel sites were categorized based on whether they received low (<10 million L), medium (10-100 million L), or high (>100 million L) freshwater exposure in the day preceding mussel collection. All sites were also classified by water type based on location within estuaries or open ocean marine sites. Each mussel batch was categorized as to whether the mussels were transplanted as sentinel mussels during this study, or if they were resident bivalves growing at the sampling site. Each mussel batch was classified as to whether a precipitation event had

occurred in the preceding day, week, and month before mussel collections. Precipitation information was acquired from the California Department of Water Resources Division of Flood Management Data Exchange Center, and the University of California, Department of Agriculture and Natural Resources Integrated Pest Management Program and California Irrigation Management System.

All data were then modeled with logistic regression to evaluate the odds of detecting *Cryptosporidium* spp. in mussel batches collected during the 3 year study based on the putative risk factors. Univariate analysis was performed with each risk factor individually, with all odds ratios based on a referent category in the model. Then a multivariable model was created in a forward stepping process to simultaneously assess multiple risk factors. Two outcome variables were of interest: detecting all *Cryptosporidium* spp, and detecting *C. parvum* genotype 2 or *C. hominis*, the *Cryptosporidium* of highest public health significance. All statistical models were created using Stata software (Stata Corp., College Station, TX), using a cluster effect to adjust for repeated sampling of the same mussel sites over time. P-values <0.1 were considered significant.

3. Results

3.1. Mussel testing

Over the 3 year study, 156 batches of mussels were collected from coastal sites in central California. Occasionally, mussel batches could not be collected as planned because the sentinel bags were missing due to harsh weather, high seas, or unfavorable tides. Over 4800 mussels were tested by Morgan 18S PCR amplification of *Cryptosporidium* DNA from individual mussel hemolymph samples. *Cryptosporidium*

DNA was identified by PCR and confirmed by sequence analysis in 12% (19/156) of all mussel batches tested. Within *Cryptosporidium*-positive mussel batches, from 1-4 mussels tested positive by Morgan 18s PCR, and occasionally more than one genotype was detected in a mussel batch. However, only 2 of the strongest Morgan PCR-positive samples (~300 bp target) were also positive by Xiao 18s PCR (~850 bp target), possibly due to difficulty in amplifying large pieces of *Cryptosporidium* DNA from hemolymph samples. Therefore, all genotype results reported hereafter were obtained using the Morgan 18S PCR protocol in combination with DNA sequence analysis.

Table 3.1 shows *Cryptosporidium* mussel batch results from Year 1 of the study. The fecal risk category, site identification, and *Cryptosporidium* genotypes detected in mussel hemolymph are indicated for the dry and wet season mussel collections. *Cryptosporidium parvum* genotype 2 was detected in mussels collected from a higher risk site for human feces during the dry season, from a higher risk site for livestock feces during the wet season, and from a lower impact site during the wet season. At a higher risk site for livestock feces, *C. felis* was detected in mussel hemolymph during the dry and wet season. Additionally, a novel *Cryptosporidium*-like sequence based on the BLAST search results, designated New-1, was identified at a higher risk site for livestock feces during the dry season.

Cryptosporidium genotype results from Years 2 and 3 are shown in Table 3.2. Mussel batches were collected during the early and late wet and dry seasons for both years. *Cryptosporidium parvum* genotype 2 was detected in mussels from a higher risk site for human feces during the early dry season of Year 2, and in mussels at a lower risk site in the early dry season of Year 3. Novel *Cryptosporidium*-like DNA sequences

based on BLAST search results, designated New-2 and New-3, were detected in mussels from higher risk sites for livestock feces and human feces during the wet season samplings, and again from a livestock impacted site in the early dry season. A fourth unique *Cryptosporidium*-like DNA sequence based on BLAST search results, designated New-4, was detected in mussels from a higher risk site for human feces. The New-3 *Cryptosporidium*-like sequences were detected in mussels collected from 2 sites separated by over 200 km.

Table 3.3 shows the *Cryptosporidium* genotype results for the estuarine subsites in Tomales Bay (Fig. 3.1b), Elkhorn Slough (Fig. 3.1c), and Morro Bay (Fig. 3.1d). Table 3.3 lists the *Cryptosporidium* genotypes detected in mussels from the 3-4 subsites within each estuary and 1 site outside of the estuary. None of the Tomales Bay subsites (2A-2C) tested positive for *Cryptosporidium* spp., while *C. andersoni* and novel *Cryptosporidium* spp. were detected in mussels from the Elkhorn Slough and Morro Bay. The 4 subsites located within Elkhorn Slough (4A-4D) all tested positive for *Cryptosporidium* spp., but mussels collected from the site outside the slough (4E) were *Cryptosporidium*-negative at all sampling times. In contrast, only 1 of the sites inside Morro Bay (9C) tested *Cryptosporidium*-positive, and the site located outside Morro Bay (9A) was also positive but at a different sampling time.

In addition to PCR screening of mussel hemolymph, a subset of mussel batches was also analyzed with alternative *Cryptosporidium* detection methods to determine the most successful technique for pathogen detection. Table 3.4 shows the results of testing hemolymph for *Cryptosporidium* DNA by PCR and testing digestive gland and/or gill wash tissues from the same mussels for *Cryptosporidium* oocysts and *Giardia* cysts by

IMS concentration and DFA detection. *Cryptosporidium* spp. were detected by PCR of hemolymph tissues in mussel batches from the Year 2 sampling times but not by IMS processing in combination with DFA analysis of pooled digestive gland tissues. However, the *Cryptosporidium* spp. detected were *C. andersoni* and novel *Cryptosporidium* spp., not *C. parvum*, which is the species that IMS and DFA antibodies are designed to detect. In Year 3, no *Cryptosporidium* spp. were detected by PCR of hemolymph or by IMS concentration and DFA analysis of digestive gland or gill wash pools. Interestingly, *Giardia* cysts were detected in digestive gland pools from 3 of the mussel batches. Neither *Cryptosporidium* nor *Giardia* were detected in gill wash pools from the same mussels.

3.2. Phylogenetic analyses

The Morgan 18S PCR was found to amplify segments of *Cryptosporidium* and non-*Cryptosporidium* DNA from the mussel samples. Of the 182 Morgan PCR products, 19 DNA sequences matched reference *Cryptosporidium* spp. from GenBank, 9 DNA sequences did not match reference *Cryptosporidium* sequences exactly but were most closely related to *Cryptosporidium* spp. based on BLAST search results, 64 DNA sequences were most closely related to non-*Cryptosporidium* dinoflagellate and aplicomplexan GenBank sequences in the BLAST search, 40 DNA sequences matched *Mytilus* spp. sequences from GenBank, and 50 DNA sequences were mixed or of poor quality and could not be identified. The 9 novel *Cryptosporidium*-like DNA sequences represented 4 unique sequences that were then included in a phylogenetic analysis to infer whether they were more closely related to reference *Cryptosporidium* spp. or to other organisms. Figure 3.2 shows the phylogenetic trees relating the 4 novel

Cryptosporidium-like sequences to reference *Cryptosporidium* spp. and other related protozoa. The same relationships were inferred using neighbor joining and maximum parsimony analysis with 1000 bootstrap replicates: the *Cryptosporidium*-like sequences New-2 and New-3 were classified within the *Cryptosporidium* clade, while New-1 and New-4 were classified outside the *Cryptosporidium* clade. Therefore, New-2 and New-3 were classified as *Cryptosporidium* spp. in the risk factor analysis, while New-1 and New-4 were not included.

3.3. Risk factor analysis

Table 3.5 shows the univariate analysis of risk factors for detection of *Cryptosporidium* spp. in mussel batches. Higher or lower risk status for fecal exposure was not significantly associated with detecting *Cryptosporidium* spp. in mussel batches ($P>0.5$). However, mussel batches collected in the late wet season ($P=0.01$), near medium or high freshwater outflow ($P\leq0.001$), or collected within a week of a precipitation event ($P=0.02$) had significantly increased odds for detection *Cryptosporidium* spp. Mussel batches collected near medium and high freshwater outflow were 9.7 and 20.8 times more likely to contain *Cryptosporidium* spp., respectively, than mussel batches collected near low freshwater outflow. Mussel batches collected in the late wet season were 3.2 times more likely to contain *Cryptosporidium* spp. than those collected in the early dry season. Mussel batches collected within 7 days following a precipitation event were 3.0 times more likely to contain *Cryptosporidium* spp. than mussel batches that were not collected within a week after a precipitation event. The odds of detecting *Cryptosporidium* spp. in batches of mussels were not significantly

associated with water type, mussel type, or having a precipitation event in the 30 days preceding mussel collection.

A multivariable logistic regression model was created to assess risk factors simultaneously. Table 3.6 shows the adjusted odds ratios for the 2 risk factors associated with detecting *Cryptosporidium* spp. in mussels. Freshwater outflow status was a significant risk factor ($P \leq 0.001$), with mussels collected near medium and high freshwater outflow being 10.8 and 14.9 times more likely to contain *Cryptosporidium* spp., respectively, than mussel batches collected near low freshwater outflow. Additionally, mussels collected within 7 days of a precipitation event were 2.6 times more likely to contain *Cryptosporidium* spp. than mussels collected when no precipitation event occurred within the week preceding collection ($P=0.04$). Other risk factors including fecal risk category, water type, bivalve type, and the occurrence of precipitation events within a day or month of mussel collection were not significantly associated with *Cryptosporidium* detection in mussel batches.

4. Discussion

This study was the first to assess the distribution of *Cryptosporidium* genotypes in bivalves along the Pacific coast of the United States. Multiple *Cryptosporidium* genotypes were detected, including species of public health importance, animal host-specific species, and novel *Cryptosporidium* genotypes. While *Cryptosporidium* spp. were detected in both the wet and dry seasons during this study, they were detected most often in mussel batches collected near high freshwater outflow, and in mussel batches collected within 1 week of a precipitation event. Fecal risk category was not significantly associated with detection of *Cryptosporidium* in mussel batches, nor was water type or

bivalve type. These findings support the concept that bivalves can be useful bioindicators of fecal pathogens in aquatic ecosystems, and suggest that either resident or transplanted sentinel bivalves can be used along the California coast in estuarine and marine sites for water quality studies.

Cryptosporidium parvum genotype 2 was detected in mussels from sites at higher risk for livestock fecal exposure, higher risk for human sewage exposure, and at lower risk sites. This finding is interesting because we had hypothesized that the higher risk sites receiving significant volumes of livestock and human feces would be more likely to contain *C. parvum* genotype 2 than lower risk sites. But in our study *C. parvum* genotype 2 was only detected once at a higher risk site for livestock feces, and was detected twice at the higher risk sites for human feces and twice at the lower risk sites. Two explanations for finding *C. parvum* genotype 2 at lower risk sites for fecal exposure are 1) that *C. parvum* genotype 2 is being shed in the feces of animals such as wildlife that were not included in the fecal risk categories as part of this study, and 2) that unrecognized loading of feces from livestock and human sources is reaching mussels at the lower risk sites, possibly through non-point source runoff, limited fecal sources such as septic tanks or boat bilges, or water currents carrying *Cryptosporidium* oocysts from distant locations. In other studies of *Cryptosporidium* spp. in bivalves on the Atlantic coast and in Europe, *Cryptosporidium parvum* genotype 2 has been the most widely reported genotype (Gomez-Bautista et al., 2000; Lowery et al., 2001; Fayer et al., 2002; Traversa et al., 2004). Other genotypes reported in these studies include *C. hominis* and *C. baileyi*, shed by humans and chickens, respectively, but neither of those genotypes was detected in mussels in the current study.

The 2 host-specific *Cryptosporidium* genotypes detected in mussels can provide clues to fecal loading sources in the adjacent or upstream terrestrial ecosystems. First, identifying *C. andersoni* in mussels suggests that cattle are contributing to the fecal loading of the nearshore environment. *Cryptosporidium andersoni* DNA was detected in mussels from livestock-impacted sites in Morro Bay and Elkhorn Slough in the early wet season, consistent with *C. andersoni* oocysts washing into the nearshore ecosystem in storm runoff. Additionally, *Cryptosporidium felis* was detected in mussels from Elkhorn Slough at 2 sampling times, suggesting that cats, the only known definitive host for *C. felis*, are contributing to the fecal load. The presence of a cat population in the Elkhorn Slough region is also supported by a recent study (Miller et al., 2002) that found that 79% of southern sea otters sampled in the Elkhorn Slough region were seropositive for *Toxoplasma gondi*, 1.5 times the odds of otters from other coastal locations being seropositive. Felid species are the only known definitive host for *T. gondii*, just as for *C. felis*, but whether domestic, feral, or wildlife felid species are significant contributors to fecal loading of these pathogenic protozoa in California is unknown.

Four novel *Cryptosporidium*-like DNA sequences were identified in this study based on the BLAST search results. However, only 2 genotypes fell within the *Cryptosporidium* clade in the more detailed phylogenetic analysis. These novel *Cryptosporidium* genotypes, New-2 and New-3, may represent as yet unrecognized species endemic in wildlife populations or other minimally studied animal populations. Further study into the *Cryptosporidium* genotypes present in terrestrial animal populations may provide additional DNA sequences for comparison with the novel sequences detected in nearshore mussels. Although the majority of *Cryptosporidium* spp.

have been reported in terrestrial animals, there have also been reports of *Cryptosporidium* in fish and marine mammals (Rush et al., 1990; Deng et al., 2000; Ryan et al, 2004), suggesting that a marine life cycle is possible. Interestingly, environmental studies of surface water quality have occasionally reported the detection of novel *Cryptosporidium* genotypes from unknown sources (Xiao et al., 2000; Ward et al., 2002). The New-1 DNA sequence that fell outside the *Cryptosporidium* clade was more closely related to the neogregarine *Ophriocystis elektroscirrha* than to the coccidial parasite *Toxoplasma gondii.* This finding is consistent with the Carreno et al. (1999) study that found *Cryptosporidium* spp. to be phylogenetically more similar to gregarine protozoa than to coccidial protozoa. The New-4 DNA sequence was not closely related to *Cryptosporidium* spp. or the gregarines, but may represent another eukaryotic marine or terrestrial organism.

The selection of 18S PCR with DNA sequence analysis as the *Cryptosporidium* detection method in mussel hemolymph enabled us to screen mussels for a wide variety of *Cryptosporidium* genotypes. An additional advantage of this method is the ability to concentrate hemolymph by centrifugation, a cost-effective procedure compared to the expense of IMS concentration that was used for digestive and gill wash tissues. A limitation of 18S PCR in our study, as in others (Sturbaum et al., 2002), is that non-*Cryptosporidium* DNA was also amplified, requiring DNA sequence analysis to identify *Cryptosporidium* genotypes that produced PCR products of the target 300 bp size. The alternative *Cryptosporidium* detection methods, IMS concentration with DFA detection on digestive gland and gill wash pools, can concentrate 0.5 ml pellets followed by visualization and quantification of *Cryptosporidium* oocysts and *Giardia* cysts.

However, these methods do not provide genotype data unless combined with PCR for molecular characterization. Hemolymph was not suitable for IMS concentration and DFA detection because, in our preliminary studies, hemocytes were found to adhere to the IMS glass tubes and to autofluoresce during DFA analysis, interfering with accurate oocyst identification.

In the subset of mussel samples tested by PCR of hemolymph and IMS concentration with DFA detection of digestive and gill wash pools, PCR of hemolymph was the only method that detected *Cryptosporidium* spp. in mussel batches. However, it is notable that *C. parvum* genotype 2 was not detected by PCR in the mussels tested with multiple *Cryptosporidium* detection methods. This finding might explain why a PCR protocol that could amplify DNA from a variety of genotypes was more successful than IMS and DFA methods that were designed to detect *C. parvum* genotype 2 oocysts. *Giardia* was detected by IMS concentration and DFA detection in digestive gland pools, but not in gill wash samples from the same mussels. This finding is consistent with bivalve studies that have detected *Cryptosporidium* more often in digestive than gill tissues (Fayer et al., 1997; Tamburrini and Pozio, 1999). Overall, the 12% of mussel batches positive in the California study is lower than studies from the Atlantic coast in which 81% of oyster batches in the Chesapeake Bay were positive (Fayer et al., 2002), in Ireland where 50% of mussel batches from the Shannon River were positive (Graczyk et al., 2004), and in Spain where 36% of estuarine and nearshore marine mussel batches were positive (Gomez-Bautista et al., 2000). These varying results may be attributable to differences in study design, to a larger dilution effect of the ocean dispersing fecal pathogens in this

study compared to the other riverine or estuarine studies, or to a smaller fecal load of *Cryptosporidium* flowing into the nearshore environment in California.

This study shows that bivalves can be used as a monitoring tool to assess fecal contamination with a variety of *Cryptosporidium* genotypes in estuarine and nearshore marine ecosystems in California. Advantages of using mussels as bioindicators in this study are that they are commonly found along the California coast, tolerate transplantation to outplanting sites, have minimal economic value, and are already used by water quality programs to monitor concentrations of pesticides and metals. The biggest disadvantage of using mussels as a monitoring tool in this study is the large cost and effort that was involved with field and laboratory components in order to sample and test 156 batches (4800 individual mussels), from which *Cryptosporidium* DNA was detected in 19 batches. One way that cost and effort might be reduced in future studies is to test pooled instead of individual mussel samples, as has been done in other studies (Gomez-Bautista et al., 2000; Graczyk et al., 2004), but it is possible that pooling would cause false-negative results if the load of *Cryptosporidium* is diluted by an uneven distribution of oocysts within the mussel batch. Another way to reduce the cost and effort would be to collaborate with monitoring agencies such as the California State Mussel Watch Program to layer *Cryptosporidium* testing on top of the contaminant testing that is already being done on mussels outplanted along the California coast. Based on our risk factor analysis, a third way to reduce cost and effort would be to use mussels as monitoring tools mainly during the wet season, when storm runoff is highest, with mussel collections occurring near maximal freshwater outflow and within a week of precipitation events to increase the odds of detecting *Cryptosporidium* spp. in mussel batches.

Associations between freshwater outflow, precipitation, season, and *Cryptosporidium* detection have been reported in previous studies (Bodley-Tickell et al., 2002; Fayer et al., 2002; Chai et al., 2001). In our study, the risk factors of collecting mussels near maximal freshwater outflow and within 1 week following a precipitation event were associated with increased odds of detecting *Cryptosporidium* spp. in mussel batches. These findings are similar to a study in the Chesapeake Bay (Fayer et al., 2002) that detected the most *Cryptosporidium*-positive oysters during the month with the most rainfall and the most freshwater stream flow into the bay. Our finding of freshwater outflow as a significant risk factor for detection of *Cryptosporidium* in mussels is also consistent with a recent study by Miller et al. (2002), which showed that sea otters located near high freshwater outflow exposure had 3 times the odds of being seropositive for the fecal pathogen *Toxoplasma gondii* compared to sea otters sampled near low freshwater outflow areas along the central California coast. In the Chai et al. (2001) study in Korea, season was associated with an increase in *Cryptosporidium* detection, with the prevalence of cryptosporidiosis in humans found to be significantly higher in the spring (March-May) than in other seasons, and was weakly correlated with rainfall. In contrast, the Bodley-Tickell et al. study (2002) in the United Kingdom detected the most *Cryptosporidium* oocysts in surface waters draining a livestock farm in the September-February time period as compared to the March-August time period. Further study will be required to clarify the associations between season, rainfall, freshwater outflow, and pathogen detection in various climates and parts of the world. In addition to modeling risk factors for detecting all *Cryptosporidium* spp. in mussel batches, a model was evaluated to analyze risk factors for detecting only *C. hominis* and *C. parvum* genotype 2, the

Cryptosporidium of public health importance. However, because *C. hominis* was not detected in this study and *C. parvum* genotype 2 was only detected in 5 mussel batches, the data were too sparse to create such a model.

In conclusion, this study was the first to investigate the distribution of *Cryptosporidium* genotypes and risk factors for *Cryptosporidium* detection in nearshore bivalves along the Pacific coast of the United States. In addition to illustrating how mussels can be used as bioindicators of fecal pollution in environmental monitoring programs, this study showed that humans and animals ingesting fecal contaminated water and shellfish may be exposed to both host-specific and anthropozoonotic *Cryptosporidium* genotypes of public health significance. Continued evaluation of sources of fecal loading and the application of management strategies to mitigate fecal contamination may help reduce pathogen pollution into the nearshore marine environment, an invaluable ecosystem and resource for people and animals, both wild and domestic.

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Table 3.1
Mussel testing for *Cryptosporidium* DNA in Year 1.

FECAL RISK CATEGORY	SITE ID	2001-2002	
		DRY SEASON	WET SEASON
Livestock Impacted	4B	*C. felis*	*C. felis*
	4D	- [a]	-
	9C	New-1 [b]	*C.parvum gen. 2*
Human Impacted	3	nm [c]	-
	5	nm	-
	9A	*C.parvum gen. 2*	-
Lower Impact	1	-	nm
	6	nm	*C.parvum gen. 2*
	7	nm	-

[a] - = all mussels in batch *Cryptosporidium*-negative by PCR.
[b] 'New' indicates a novel *Cryptosporidium*-like DNA sequence.
[c] nm = no mussels collected.

Table 3.2
Mussel testing for *Cryptosporidium* DNA in Years 2-3.

FECAL RISK CATEGORY	SITE ID	2002-2003				2003-2004			
		LATE DRY SEASON	EARLY WET SEASON	LATE WET SEASON	EARLY DRY SEASON	LATE DRY SEASON	EARLY WET SEASON	LATE WET SEASON	EARLY DRY SEASON
Livestock Impacted	2A	nm [a]	-	-	-	-	-	-	-
	4A	- [b]	New-2 [c]	New-3	-	nm	-	-	-
	9D	-	-	-	New-3	-	-	-	-
Human Impacted	3	-	-	-	*C.parvum gen.2*	-	-	-	-
	5	-	nm	-	-	-	New-4	-	-
	8	-	-	New-3	-	-	-	-	nm
Lower Impact	1	-	-	nm	-	-	-	-	-
	6	-	-	nm	-	-	-	-	*C.parvum gen. 2*
	7	-	-	-	nm	-	-	-	-

[a] nm = no mussels collected.
[b] - = all mussels in batch *Cryptosporidium* -negative by PCR.
[c] 'New' indicates a novel *Cryptosporidium* -like DNA sequence.

Table 3.3
Mussel testing for *Cryptosporidium* DNA at estuarine subsites.

SITE ID	SUBSITE ID	2002-2003		2003-2004	
		EARLY WET SEASON	LATE WET SEASON	EARLY WET SEASON	LATE WET SEASON
2 (Tomales Bay)	A	-[a]	-	-	-
	B	nm[b]	-	-	-
	C	nm	-	-	-
4 (Elkhorn Slough)	A	New-2	New-3	-	-
	B	*C.andersoni*, New-2	New-2	-	-
	C	-	New-2	-	-
	D	-	New-3	-	-
	E	-	-	-	-
9 (Morro Bay)	A	-	-	-	New-4
	B	-	nm	-	-
	C	*C.andersoni*	New-2	-	-
	D	-	-	-	-
	E	nm	-	-	nm

[a] - = all mussels in batch *Cryptosporidium* -negative by PCR.

[b] nm = no mussels collected.

[c] 'New' indicates a novel *Cryptosporidium* -like DNA sequence.

Table 3.4
Detection of *Cryptosporidium* DNA in mussel hemolymph, and *Cryptosporidium* oocysts and *Giardia* cysts in digestive gland from the same mussels.

	EARLY WET YEAR 2		LATE WET YEAR 2		EARLY WET YEAR 3		
SITE ID	Hemolymph PCR	Digestive IMS-DFA	Hemolymph PCR	Digestive IMS-DFA	Hemolymph PCR	Digestive IMS-DFA	Gill wash IMS-DFA
4A	New-2[a]	-[b]	New-3	-	-	-	-
4B	*C.andersoni*, New-2	-	New-2	-	-	-	-
4D	-	-	New-3	-	-	-	-
9A	-	-	-	-	-	*Giardia*	-
9B	-	-	nm[c]	nm	-	*Giardia*	-
9D	-	-	-	-	-	*Giardia*	-

[a] 'New' indicates a novel *Cryptosporidium*-like DNA sequence.

[b] - = all mussels in batch negative.

[c] nm = no mussels collected.

Table 3.5
Univariate logistic regression of risk factors for detection of *Cryptosporidium* spp. in mussels.

Risk factor	Group	Percent mussel batches positive	Odds ratio	95% CI	Odds ratio P-value
Fecal risk class	Lower	8 (n=24)	1.0	-	-
	Higher-Human	9 (n=34)	1.1	0.2-7.1	0.9
	Higher-Livestock	13 (n=98)	1.7	0.2-11.7	0.6
Season	Early Wet	9 (n=35)	1.0	-	-
	Late Wet	23 (n=43)	3.2	1.4-7.8	0.01*
	Early Dry	8 (n=38)	0.9	0.2-5.3	0.9
	Late Dry	5 (n=40)	0.6	0.1-2.5	0.4
Freshwater outflow	Low	2 (n=85)	1.0	-	-
	Medium	19 (n=53)	9.7	2.5-37.9	0.001*
	High	33 (n=18)	20.8	4.2-103.6	<0.001*
Precipitiation in past 1 day	No	10 (n=136)	1.0	-	-
	Yes	25 (n=20)	3.2	0.8-12.5	0.1
Precipitation in past 7 days	No	7 (n=97)	1.0	-	-
	Yes	19 (n=59)	3.0	1.2-7.0	0.02*
Precipitation in past 30 days	No	4 (n=45)	1.0	-	-
	Yes	14 (n=111)	3.6	0.6-20.5	0.2
Water	Estuarine	14 (n=90)	1.0	-	-
	Marine	8 (n=66)	0.5	0.2-1.5	0.2
Bivalves	Resident	14 (n=78)	1.0	-	-
	Transplant	9 (n=78)	0.6	0.2-1.9	0.4

* Significant P-values <0.10.

Table 3.6
Multivariable logistic regression model of significant risk factors for detection of *Cryptosporidium* spp. in mussels

Risk factor	Group	Adjusted odds ratio	95% CI	Odds ratio P-value
Freshwater outflow	Low	1.0	-	-
	Medium	10.8	2.5-46.2	0.001*
	High	14.9	3.3-66.6	<0.001*
Precipitation in past 7 days	No	1.0	-	-
	Yes	2.6	1.1-6.5	0.04*

* Significant P-values <0.10.

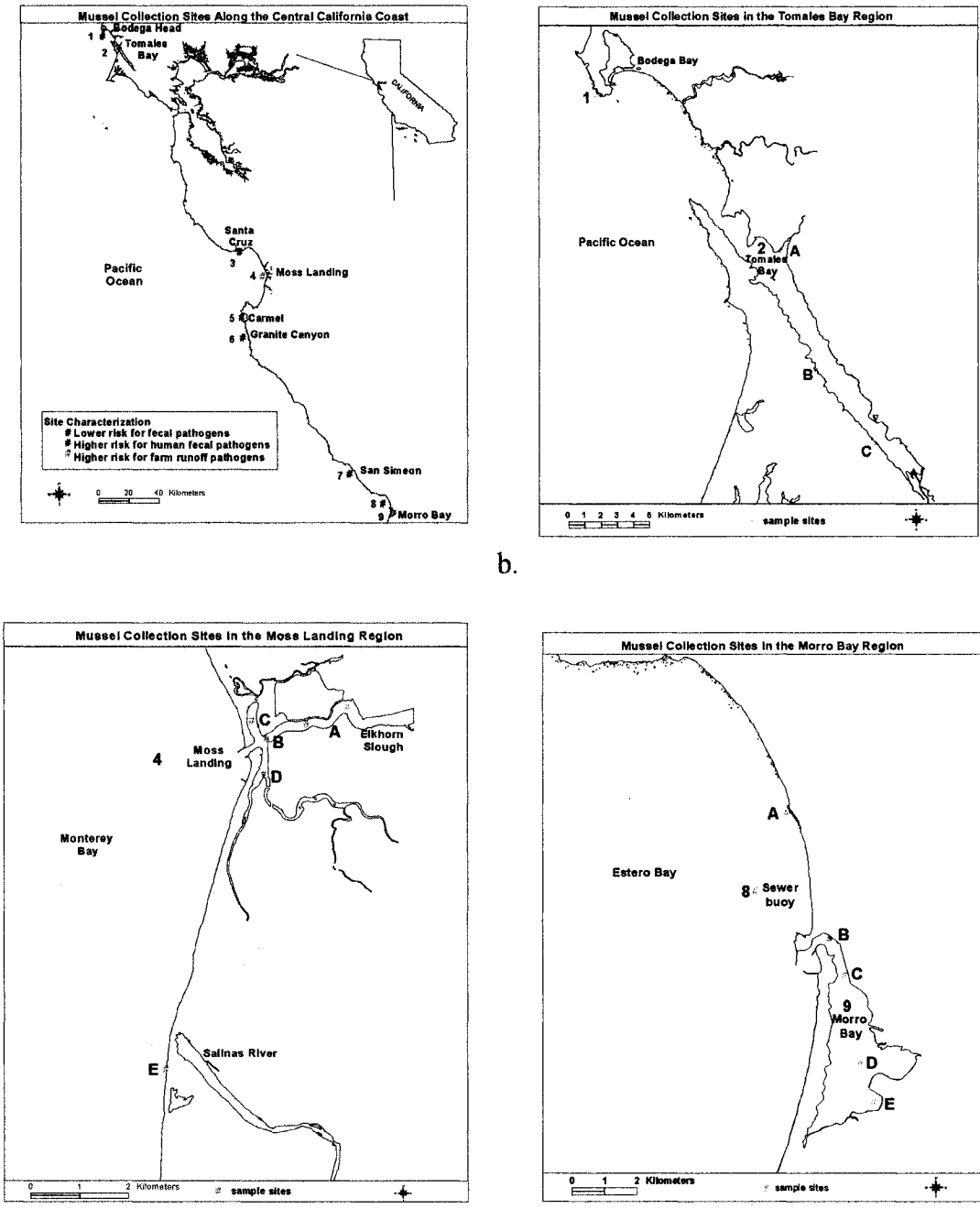

Fig. 3.1. Mussel collection sites located along the central California coast (a), with close up maps of the (b) northern, (c) middle, and (d) southern regions. Map numbers represent the core mussel sampling sites, while letters represent subsites within estuarine regions.

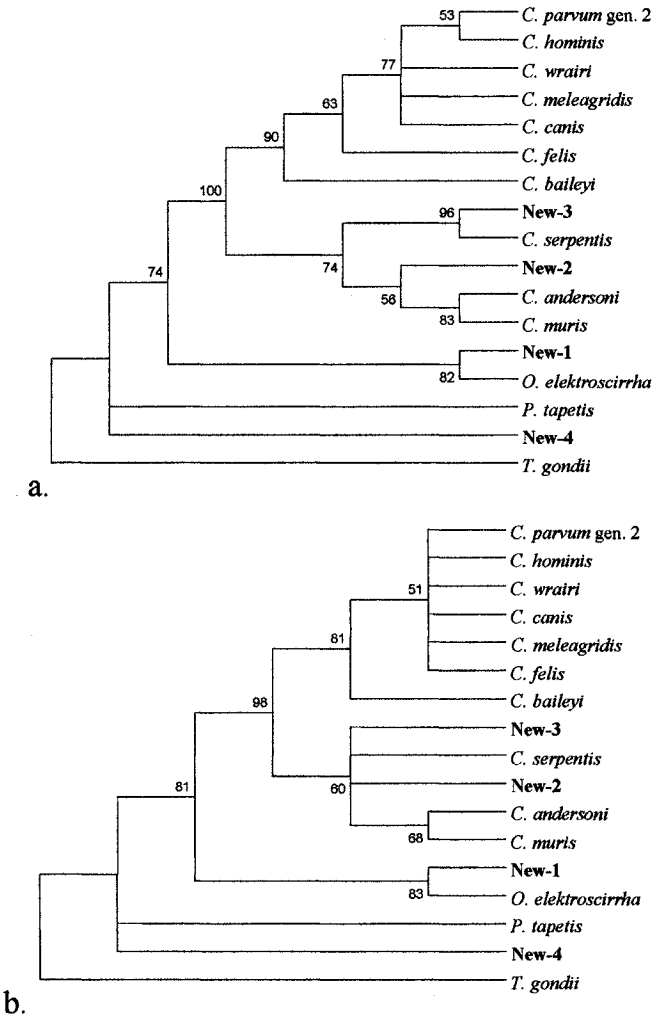

Fig. 3.2. Phylogenetic analysis of novel *Cryptosporidium*-like sequences detected in mussels, using (a) Neighbor Joining Tamura-Nei analysis, and (b) Maximum Parsimony Min-Mini Heuristic analysis, both with 1000 bootstrap replicates.

CHAPTER 4

Clams (*Corbicula fluminea*) as Bioindicators of Fecal Contamination with *Cryptosporidium* and *Giardia* Species in Freshwater Ecosystems in California

Clams (*Corbicula fluminea*) as Bioindicators of Fecal Contamination with *Cryptosporidium* and *Giardia* Species in Freshwater Ecosystems in California

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Abstract

This study evaluated clams as bioindicators of fecal protozoan contamination using 3 approaches: 1) clam tissue spiking experiments to compare several detection techniques, 2) clam tank exposure experiments to evaluate clams that that have filtered *Cryptosporidium* oocysts from inoculated water under a range of simulated environmental conditions, and 3) sentinel clam outplanting to assess the distribution and magnitude of fecal contamination in 3 riverine systems in California. Our spiking and tank experiments showed that direct fluorescent antibody (DFA), immunomagnetic separation (IMS) in combination with DFA, and polymerase chain reaction (PCR) techniques could be used to detect *Cryptosporidium* in clam tissues. The most analytically sensitive technique was IMS concentration with DFA detection of oocysts in clam digestive gland tissues, which detected 10 oocysts spiked into clam digestive glands 83% of the time. In the tank experiment, oocyst dose and collection time were significant predictors for detecting *C. parvum* oocysts in clams. In the wild clam study, *Cryptosporidium* and *Giardia* were detected in clams from all 3 study regions by IMS-DFA analysis of clam digestive glands, with significant variation by sampling year and season. The presence of *C. parvum* genotype 2 DNA in clams from riverine ecosystems was confirmed with PCR and DNA sequence analysis.

Keywords: *Cryptosporidium; Giardia;* bivalve; clam; IMS; PCR.

1. Introduction

In 2003, over 1200 human cases of cryptosporidiosis and 9100 cases of giardiasis were reported to the United States Centers for Disease Control and Prevention (CDC 2004), with many more cases going unreported or undiagnosed. *Cryptosporidium* and *Giardia* are protozoan pathogens transmitted by fecal-oral ingestion, and as few as 10-100 *C. parvum* oocysts have been shown to cause clinical disease in human volunteers (Okhuysen et al., 1999). These oocysts can survive for over a year in aquatic environments and have caused waterborne human diarrheal outbreaks worldwide (Ong et al., 1999; Tamburrini and Pozio, 1999). *Cryptosporidium* and *Giardia* spp. are shed by a variety of animals, including humans, livestock, domestic animals, and wildlife (Okhuysen et al., 1999; Heitman et al., 2002; McGlade et al., 2003). Some species of *Cryptosporidium* and *Giardia* are thought to be host-specific, while other species can infect many hosts (Monis and Thompson, 2003).

Bivalve shellfish have been used for decades as bioindicators of aquatic contamination with heavy metals and pesticides (O'Connor, 2002). In recent years, shellfish have also been recognized worldwide as bioindicators of aquatic contamination with fecal origin bacteria, viruses, and parasites (Fayer et al., 1998; Freire-Santos et al., 2000; Pommepuy et al., 2004). Filter feeding shellfish such as oysters, mussels, and clams have been shown to concentrate *Cryptosporidium* and *Giardia* spp in tank exposure experiments, with oocysts detected in shellfish even when none were detected in the surrounding waters (Atwill and Rose, 1998; Fayer et al., 1998; Graczyk et al., 1999; Tamburrini and Pozio, 1999). Multiple species of *Cryptosporidium* have been

detected in wild shellfish, and the oocysts were proven viable using mouse bioassays (Fayer et al., 1998, 1999; Gomez-Bautista et al., 2000).

Methods for detecting *Cryptosporidium* and *Giardia* spp. in bivalve shellfish include DNA amplification by polymerase chain reaction (PCR) and direct immunofluorescent antibody (DFA) assays (Graczyk et al., 1998; Gomez-Couso et al., 2004). An advantage of conventional PCR is that the DNA sequence can be amplifed and analyzed to identify the parasite genotype, but a limitation of PCR is that results are not quantitative. In contrast, detection methods utilizing DFA analysis provide quantitative data but these methods cannot distinguish between all parasite genotypes. Immunomagnetic separation (IMS) concentrates the parasites and thereby allows for a larger sample volume to be analyzed per test. Immunomagnetic separation can be used in combination with DFA or PCR detection methods (Sturbaum et. al., 2002; Ware et al, 2003). The use of IMS has increased the minimum oocyst detection limit by 1-2 logs when testing fecal samples (Pereira et al., 1999), and may be an important tool to improve the analytic sensitivity of bivalve testing methods.

Cryptosporidium and *Giardia* are endemic in animal and human populations in California, but we have insufficient understanding regarding the pathways and mechanisms of how these pathogens enter waterways from the terrestrial component of a watershed. Testing freshwater clams (*Corbicula fluminea*) may be a useful approach to detect fecal pathogens in freshwater ecosystems because these clams filter large volumes of water (up to 2.5 L/water/hr/clam), they survive well in polluted aquatic environments, and they are easily collected and transported to the laboratory for analysis (McMahon and Bogan, 2001). In addition to the practicality of testing clams as indicators of water

quality, they are also of interest because both humans and animals harvest them as a food source (unpublished observation), suggesting that bivalves might expose consumers to concentrated pathogens when eaten raw. In laboratory studies, *Cryptosporidium* and *Giardia* have been detected in *Corbicula* spp. after tank exposure to parasite-inoculated waters (Atwill and Rose, 1998; Graczyk et al., 2003; Izumi et al., 2004), but these clams have not been evaluated as bioindicators of fecal contamination in natural ecosystems.

This study evaluated methods for *Cryptosporidium* detection in *C. fluminea*, and then applied this methodology to evaluate experimentally-exposed and sentinel *C. fluminea* outplanted in riverine ecosystems in California. The study objectives were threefold: 1) to evaluate DFA, IMS-DFA, and PCR methods for *Cryptosporidium* detection using spiked clam tissues; 2) to evaluate these methods on clams that have filtered oocysts from inoculated waters under a range of simulated environmental conditions; and 3) to evaluate outplanted clams as bioindicators of fecal pathogen pollution in 3 riverine ecosystems in California.

2. Methods

2.1. Experimental design

Tissue spiking experiments were first conducted to determine which diagnostic methods would be sensitive enough to detect the low numbers of *Cryptosporidium* oocysts in clam (*C. fluminea*) tissues that might be expected in environmental samples. DFA, IMS-DFA, and PCR methods were evaluated using spiked digestive gland tissues. Two PCR methods (Morgan et al., 1997; Xiao et al., 1999) were also evaluated on spiked hemolymph tissues. Spiked hemolymph samples were not processed by DFA methods

because hemocytes were found to autofluoresce in our preliminary studies, making oocyst visualization difficult.

For the tissue spiking experiments, clam digestive gland and hemolymph were inoculated with *C. parvum* dilutions containing 0, 1, 10, 100, or 1000 oocysts, with 6 replicates per tissue and oocyst dose. Spiked digestive gland samples were homogenized, sieved, and centrifuged to create a pellet that was resuspended and split for DFA and PCR analysis. A 10 ul digestive gland aliquot was dried onto a slidewell for DFA analysis, with the rest of the tissue then concentrated with IMS followed by DFA analysis. Hemolymph was concentrated by centrifugation and the cell pellet frozen for PCR analysis. *Corbicula* gills were not assessed in this study due to their very small size.

The same *Cryptosporidium* detection methods used in the tissue spiking experiments were then evaluated on clam samples obtained from a tank exposure experiment. The experiment was designed to evaluate a range of environmental conditions, including 2 water temperatures (10° and 20° C) and 3 oocyst concentrations (20, 200, and 2000 oocysts/L water), using 2 different batches of *C. parvum* oocysts. During the tank exposure experiment, clams were kept in tubs containing 10 L of water, 80 clams, and an airstone that provided continuous aeration and water mixing. Two clam tubs were spiked for each treatment combination (oocyst dose, water temperature, and oocyst batch). In addition, negative control tubs contained water and clams but no oocysts, while positive control tubs contained water and oocysts but no clams. At the time of oocyst inoculation the clams were fed, and after a 6 hr exposure the clams were moved to clean freshwater tubs for *Cryptosporidium* depuration over the next 3 weeks. Water from the inoculated clam tanks was processed by IMS concentration and DFA analysis to quantitate

remaining oocysts. Clams were collected at 3 hr, 6 hr, 9 hr, 1 d, 3d, 7 d, 14 d, and 21 d after initial oocyst exposure. The effect of water temperature, oocyst dose, and clam collection timepoint were analyzed with a negative binomial regression model (Hardin and Hilbe, 2001).

The next step in evaluating clams as bioindicators of pathogen pollution was to test clams that may have filtered fecal pathogens from contaminated freshwater ecosystems in California. Three freshwater study regions were chosen based on water quality data that indicated a history of high coliform counts and fecal contamination problems. The San Lorenzo River feeds into the Monterey Bay near Santa Cruz, CA, and has had mean coliform counts over 13,000 MPN/100 ml in recent years (CCRWQCB, 2001). The watershed utilizes septic systems for sewage management and has minimal crop and animal production industries. The Salinas River feeds into the Monterey Bay near Moss Landing, CA and has had mean coliform counts over 8,000 MPN/100 ml in recent years (CCRWQCB, 2001). The Salinas watershed uses mainly sewer systems for sewage management and has significant crop and animal production industries. Putah Creek is a freshwater tributary that feeds into the California Delta and has had coliform counts over 1700 MPN/100 ml in recent years (CVRWQCB, 2002). The watershed has some sewer and some septic tank areas, along with significant crop and livestock activities.

Along each riverine region, sentinel clams were outplanted in mesh grids at an upstream and downstream site at least 5 km apart, left undisturbed to filter the water for at least 30 days, and collected as batches of 30 clams per site and timepoint. Clam outplanting and collections occurred in the wet and dry seasons of 2002 and 2003. Clams were dissected and analyzed for *Cryptosporidium* using IMS-DFA on 6 pools of 5 clam

digestive glands from each batch of 30 clams. All *Cryptosporidium*-positive DFA slides were scraped, washed, amplified with PCR, and the sequences analyzed to obtain parasite genotypes. Hemolymph was analyzed for all clams individually. The pooling of clam digestive glands was possible because all the tissue could be analyzed using the IMS concentration procedure before DFA detection, whereas the maximal PCR test volume was approximately one hemolymph sample. For each study site, information was gathered on environmental variables that could be used in a statistical risk factor analysis.

Giardia testing was added to the study in the second year. Based on our *Cryptosporidium* spiking and tank experiment findings that IMS-DFA of digestive gland was the most sensitive detection method, we focused on IMS-DFA methods for a *Giardia* tissue spiking experiment. In the spiking trial, the traditional IMS method of using a full dose of 100 ul IMS beads was compared with using a half dose of IMS beads, a modification that would significantly decrease the cost of each IMS test. The traditional method of acid dissociation was also compared with heat dissociation, based on a recent publication showing that heat dissociation could improve parasite recovery efficiency in spiked water samples (Ware et. al., 2003). Finally, negative binomial regression was used to compare the three methods, with the best method then applied to all the wild clam samples collected in year 2, as described previously for *Cryptosporidium* testing.

2.2 Cryptosporidium oocysts

Wild-type *C. parvum* genotype 2 oocysts (synonymous with bovine genotype A) were used for sensitivity experiments and the tank exposure experiment. Feces were obtained from infected calves from commercial dairies near the Veterinary Medical Teaching and

Research Center, Tulare, California. Calf feces were sieved through a series of mesh strainers and then purified by sucrose flotation methods (Arrowood and Sterling, 1987). Oocyst concentrations for spiking experiments were determined with 8 hemacytometer counts and confirmed with DFA enumeration. Oocyst suspensions were kept at 4°C and used within one month of collection.

2.3. Clams

For the clam spiking and tank exposure experiments, *C. fluminea* were harvested near Oakley, California, in cooperation with the California State Mussel Watch Program. Clams were transported on ice and depurated in freshwater flow-through tanks at the University of California, Davis Aquatic Pathogen Facility for at least 3 weeks before being used in any experiments. The tanks were constantly aerated and clams were fed Algamac (Aquafauna Biomarine Inc., Hawthorne, California) every other day. Sentinel clams used for the outplanting studies were collected near Oakley or Davis, California, with a batch of 30 clams pre-tested at the time of collection.

For all experiments, clams 2-3 cm long were dissected to obtain digestive gland and hemolymph samples. Hemolymph was extracted first by filing a notch in the clam shell and aspirating 0.5-1.5 ml of hemolymph from the adductor muscle with a sterile syringe. Hemolymph was centrifuged to concentrate the cell pellet that was stored frozen for later DNA extraction and PCR analysis. Next, the adductor muscle was cut to open the clam, and the digestive gland was excised. Half of the digestive gland was frozen for PCR and half was sieved through a 100 μm cell strainer and centrifuged for 15 min at 1000×g for DFA and IMS-DFA analysis.

2.4. Immunomagnetic separation

Digestive gland pellets of up to 0.5 ml were concentrated and purified with IMS (Dynal Biotech, Oslo, Norway) per manufacturer's instructions, followed by DFA analysis for parasite detection. Briefly, the tissue pellet was suspended in an L10 glass tube in 2 ml buffer and 10 ml millipore water. Next 100 µl IMS beads were added and the samples were rotated for 1 hr at setting 18. The IMS bead-parasite complexes were bound to a magnetic holder while the supernatant and debris were discarded. Bead-parasite complexes were resuspended in 1 ml buffer and transferred to 1.5 ml microcentrifuge tubes that were put into a smaller magnetic holder to again bind the bead-parasite complexes while the supernatant was discarded. For parasite dissociation from the beads, 2 acid washes of 50 µl 0.1 N HCl each were vortexed at the beginning and end of the 10 min incubation. Tubes were then put in a small magnetic holder and the supernatant was transferred to a DFA slide or PCR tube containing NaOH for neutralization, while the beads were held on the magnet. Hemolymph was not considered suitable for IMS because preliminary studies revealed that hemocytes adhered to the glass tubes.

In the *Giardia* IMS clam spiking experiment, 2 IMS modifications were evaluated with 6 replicates per modification and spike dose. First, selected samples were processed using 50 ul of IMS beads instead of the usual 100 ul. All other IMS parameters were the same as previously described. Second, selected samples were dissociated from the IMS beads in the final step using 95 °C sterile PBS instead of HCl, again without changing other IMS parameters. Cyst recovery data were then analyzed using negative binomial

regression to determine the most sensitive and cost effective protocol for use on the wild clam samples.

2.5. Direct immunofluorescence

Cryptosporidium oocyst DFA detection was performed on 10 µl digestive gland suspension dried onto a 3-well Merifluor slidewell (Meridian Bioscience Inc., Cincinnati, OH). For IMS-DFA analysis of digestive gland, the 50 µl IMS product containing parasites was dried onto a DFA well with 2 wells per tissue sample. The DFA slides were incubated with anti-*Cryptosporidium* and anti-*Giardia* antibody, counterstained (Meridian Bioscience Inc.) for 30 minutes, and examined at 200X-400X magnification on a Zeiss Axioscop epi-fluorescent microscope. All slides were examined by the same microscopist. *Cryptosporidium parvum*-like oocysts were identified as 5 µm diameter spheres that were outlined in apple green fluorescence, often with a midline seam, whereas *Cryptosporidium andersoni/muris*-like oocysts were 5x7 µm diameter and elliptical in shape. *Giardia*-like parasites were also elliptical and outlined in apple green, but were larger in size (10-14 µm diameter). After parasite quantification, the slide well was isolated from other wells with a grease pencil, scraped with a scalpel blade, and washed with sterile PBS into a microcentrifuge tube for DNA extraction, PCR amplification, and DNA sequence analysis. Testing of hemolymph by DFA was not done because preliminary studies revealed that hemocytes autofluoresced, making oocyst identification difficult.

2.6. Polymerase chain reaction

Two conventional 18S rRNA PCR protocols (Morgan et al., 1997; Xiao et al., 1999) were evaluated for *Cryptosporidium* spp. detection in clam tissues. For DNA extraction from hemolymph and digestive gland, a 50 µl maximum pellet was mixed in 180 µl ATL buffer in a microcentrifuge tube and suspended in liquid nitrogen for 4 min followed by boiling water for 4 min. Using the QIAGEN DNA Mini Kit tissue protocol (QIAGEN Inc., Valencia, CA), mussel tissue was digested, bound to a QIAamp column, washed, and the DNA eluted with 50 µl of 95°C PCR water. Extracted DNA was frozen until PCR analysis.

A direct PCR protocol was used to amplify a 300 bp variable segment of the *Cryptosporidium* 18s rRNA gene (Morgan et al., 1997). The *Cryptosporidium* primers consisted of 18sif: AGT GAC AAG AAA TAA CAA TAC AGG and 18sir: CCT GCT TTA AGC ACT CTA ATT TTC. The PCR reactions contained 5 µl 10X PCR buffer, 3 mM $MgCl_2$, 200 µM each dNTP, 200 nm each primer, 1.5 U Taq polymerase, and 2 µl DNA in a 50 µl total volume. Amplification conditions for the PCR reactions started with 2 min at 96°C, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 sec, with a final cycle of 72°C for 7 min.

The Xiao (1999) 18S rRNA protocol used an outer primer set to amplify a 1325 bp DNA segment, followed by amplification of an 850 bp DNA segment using the inner primer set. The outer primers sequences were C1F: TTC TAG AGC TAA TAC ATG CG and C1R: CCC TAA TCT TTC GAA ACA GGA, and the inner primer sequences were C2F: GGA AGG GTT GTA TTT ATT AGA TAA AG and C2R: AAG GAG TAA GGA ACA ACC TCC A. The outer primer PCR reactions contained 5 µl Perkin-Elmer

(Norwalk, CN) 10X PCR buffer, 6 mM $MgCl_2$, 200 µM each deoxynucleoside triphosphate (dNTP), 200 nm each primer, 1.5 U Taq polymerase, and 2 µl DNA in a 50 µl total volume. The inner primer PCR reactions were the same except that the $MgCl_2$ was reduced to 3 mM. Amplification conditions for the outer and inner PCR reactions consisted of hot start at 94°C for 3 min, followed by 35 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 1 min, with a final extension at 72°C for 7 min.

The *Giardia* PCR protocol was designed to amplify a 432 bp segment of the glutamate dehydrogenase gene from multiple genotypes of *G. duodenalis* (Read et al., 2004). Primers for the semi-nested protocol consisted of external forward primer GDHeF: TCA ACG TYA AYC GYG GYT TCC GT, internal forward primer GDHiF: CAG TAC AAC TCY GCT CTC GG and reverse primer GDHiR: GTT RTC CTT GCA CAT CTC C. PCR reactions contained 12.5 pmol of each primer, 200 um dNTPs, 1.5 mM $MgCl_2$, 0.5 U Taq polymerase, and 2 µl DNA in a 50 µl total reaction volume. Amplification conditions started with 2 cycle of 94 °C for 2 min, 56 °C for 1 min, and 72 °C for 2 min, followed by 55 cycles of 94 °C for 30 s, 56 °C for 20 s, and 72 °C for 45 s, with a final extension of 72 °C for 7 min.

Amplified PCR products were separated by gel electophoresis on a 2% agarose gel containing GelStar (Cambrex Co., East Rutherford, NJ). Product size and band intensity were evaluated compared to a 100 bp ladder included with each gel. For selected isolates, the PCR product was purified according to QIAGEN Qiaquick protocol, sequenced on an automated sequencer, and analyzed with Chromas (Technelysium Pty Ltd, Tewantin, Qld, AU) and ClustalX (Thompson et al., 1997) software for *Cryptosporidium* genotype identification.

2.7. Risk Factor Data

Covariate data on potential risk factors were collected for the 3 study regions along the San Lorenzo River, Salinas River, and Putah Creek, CA. Clam collection site data were categorized by study region and by upstream or downstream status. Information on sewage management was obtained from the Central Coast Regional Water Quality Control Board. The remaining risk factors were all coded as continuous variables. Precipitation data for the previous day, week, and month before each clam collection were obtained from the California Department of Water Resources (http://cdec.water.ca.gov). Livestock density data was collected from the 2002 agricultural census (http://www.nass.usda.gov/census/census02). Human population density data was collected from the Census 2000 (http://factfinder.census.gov). The risk factor data could then be evaluated as explanatory variables for detecting *Cryptosporidium* oocysts and *Giardia* cysts in clam tissues.

2.8. Data analysis

In the clam tissue spiking experiments, the proportion of tissues positive per technique was calculated as the number of tissues testing positive divided by the total number of tissue samples tested for each technique. Percent recovery values for DFA and IMS-DFA testing were calculated in two ways. First, by dividing the number of oocysts counted on the DFA slide by the number of oocysts expected per test aliquot. Expected oocysts per test aliquot were calculated by multiplying the number of oocysts spiked into a digestive gland sample by the proportion of spiked tissue analyzed. Second,

the oocyst count data were modeled using Poisson regression for the DFA data and negative binomial regression for the IMS-DFA data (Hardin and Hilbe, 2001; Pereira et al., 1999; Atwill et al., 2003). Both Poisson and negative binomial regression are designed to model count data, but negative binomial regression is more appropriate when the variance exceeds the mean (Hardin and Hilbe, 2001).

In addition to the percent recovery estimate, the assay sensitivity S(c), defined as the probability of detecting at least one oocyst per sample, was calculated as shown in equations (1) and (2), respectively, where $e^{\beta x}{}_i$ is the percent recovery of the assay, α is an ancillary parameter for modeling dispersion, c_i is the number of oocysts spiked per digestive gland, and W_i is the proportion of the digestive gland tested in the assay.

(1) $S(c_i) = 1 - e^{\beta x}{}_i$ (2) $S(c_i) = 1 - [1/(1 + \alpha c_i W_i e^{\beta x}{}_i)]^{1/\alpha}$

The clam tank experiment IMS-DFA oocyst count data were also analyzed using the negative binomial model (2) to assess the effect of water temperature, oocyst dose, and clam collection timepoint on the number of oocysts detected per digestive gland. For the wild sentinel clam study, each pool was first classified as positive or negative for each pathogen. Exact logistic regression was used to evaluate the association between the putative risk factors and the probability of predicting a pathogen-positive clam pool. Statistical significance was defined as a P-value <0.05.

3. Results

3.1. Clam tissue spiking

Table 4.1 shows the proportions of *Cryptosporidium*-positive samples from 6 clam digestive gland and 6 hemolymph samples spiked with 10 fold dilutions from 1-1000

oocysts and processed by DFA, IMS-DFA, and PCR methods. All negative control clams (not spiked with *C. parvum* oocysts) tested negative by all methods. The minimum oocyst detection limit for digestive gland tested by DFA was 100 oocysts. Concentrating the digestive gland samples with IMS before DFA analysis increased the minimum oocyst detection limit by 1-2 $\log_{10}$ units. All digestive glands spiked with 100 oocysts were detected by IMS-DFA, as well as 83% of samples spiked with 10 oocysts and 17% of samples spiked with 1 oocyst. For digestive gland samples tested by PCR, Xiao PCR detected 50% or more of samples spiked with at least 10 oocysts, but did not detect any samples spiked with a single oocyst. Morgan PCR detected 83% or more of all samples spiked at least 100 oocysts, and 17% of digestive samples spiked with 1 or 10 oocysts. For spiked hemolymph samples, Xiao PCR detected 50% or more of all samples spiked with 1 or more oocysts, and Morgan PCR detected 100% of all spiked samples. In conclusion, IMS-DFA and Morgan PCR were the only methods able to detect a single oocyst spiked into digestive gland samples, while a single oocyst spiked into clam hemolymph samples was detectable by both conventional PCR detection methods.

In order to estimate the true number of oocysts present in a digestive gland sample processed by our quantitative DFA methods, the recovery efficiency was determined from the spiking experiment data. Table 4.2 shows the percent of oocysts that were detected in *C. parvum* spiked digestive gland samples tested by DFA and IMS-DFA. For digestive gland samples spiked with 100 oocysts, a mean of 2 oocysts were counted per test using DFA alone, which represented 46% of the oocysts expected in a 10 ul test aliquot, but only 2% of the total oocyst dose spiked into the entire digestive gland sample. In contrast, for IMS-DFA analysis of clam digestive gland samples spiked with

100 oocysts, a mean of 69 oocysts were detected per test, which represented 69% recovery for both the test and total spiked digestive gland, because the whole digestive gland sample could be processed in the IMS-DFA test. The DFA method did not detect any of the clam tissues spiked with 1 or 10 oocysts. In contrast, the IMS-DFA method detected 50% of digestive gland samples spiked with 10 oocysts and 17% of samples spiked with 1 oocyst. By fitting a Poisson model to the DFA oocyst count data, the percent recovery per test was approximately 54%. The IMS-DFA count data was more variable, so a negative binomial model was used to regress the observed oocyst count data based on the known spike doses, leading to a percent recovery estimate of 58% per test. Figure 4.1 shows the sensitivity of DFA and IMS-DFA; the detection threshold where there was a 50% probability of detecting one or more oocysts (DT_{50}) in a digestive gland sample processed by DFA was 80 oocysts and DT_{90} was 200 oocysts. For IMS-DFA, the DT_{50} was 2 oocysts and DT_{90} was 8 oocysts per digestive gland sample.

3.2. Clam tank experiment

The DFA, IMS-DFA, and PCR *Cryptosporidium* detection methods were next applied to clams that had filtered oocysts from experimentally contaminated water. Clam mortality during the 3 week experiment was 12%. The oocyst concentration in clam tanks decreased by 55% over the first 6 hr after oocyst inoculation compared to positive control tanks that contained oocysts but no clams.

First a subset of tissue samples was analyzed by DFA, IMS-DFA, and PCR methods to determine the best method for use on the rest of the tank experiment samples. Table 4.3 shows the comparison of *Cryptosporidium* detection techniques on clam digestive

gland and hemolymph samples from oocyst exposed and unexposed clams. The IMS-DFA method was the most sensitive test evaluated, detecting *Cryptosporidium* in 52% of exposed clam digestive gland samples and none of the unexposed clams. For exposed clam digestive glands tested by other methods, 24% were positive by DFA and Morgan PCR, while all samples were negative by Xiao PCR. Hemolymph samples tested by Xiao PCR were all negative but 24% were positive by Morgan PCR. The Morgan PCR also resulted in some positive results from clams in the oocyst unexposed tanks. Sequence analysis of the PCR products confirmed that these were false-positive *Cryptosporidium* results and identified the sequences as clam and dinoflagellate DNA.

The most sensitive method, IMS-DFA of digestive gland samples, was then used to analyze all the tank experiment samples. The number of oocysts detected per digestive gland varied widely, ranging from 0-242 oocysts. The majority of oocysts were detected during the first 9 hr, but low concentrations of oocysts continued to be detected in clams exposed to low or high doses for up to 1 and 3 weeks, respectively. Dose and time since exposure of oocysts were highly associated with the ability of IMS-DFA to detect oocysts in clam digestive glands (Table 4.). Coefficients from the negative binomial model can be interpreted as the natural logarithm of the mean number of oocysts detected per assay for each treatment effect. For example, relative to the referent condition (low dose, time point at 3 hours post-exposure), the mean number of oocysts detected per assay for the middle dose and high dose at 3 hours post-exposure would be 5.1 and 36.2 additional oocysts ($e^{1.62}$, $e^{3.59}$), respectively. The number of oocysts detected per sample was not significantly different ($p=0.5$) at 6 hr compared to 3 hr post-exposure, but samples tested at >24 hours post-exposure had significantly fewer oocysts compared to 3 hr post-

exposure. Testing clam tissues at these latter times increases the likelihood of a false negative with this assay. Incubating clams at 10° compared to 20° C water temperature did not affect the number of oocysts detected with this assay ($p=0.89$).

Figure 4.2 illustrates the relationship between the mean observed oocysts per clam sample in relation to the predicted oocysts and 95% CI for the 3 oocyst exposure doses over the 3 wk experiment. The significant effect of oocyst dose is shown in the different y-axis values for the 3 graphs: the mean oocysts detected were 40 per clam in the high dose (250 oocysts/clam) tank at 3 hr post-exposure, whereas the mean observed oocysts were only 8 per clam at the middle dose (25 oocysts/clam), and 1 per clam at the low dose exposure (2.5 oocysts/clam). The confidence limits are widest at the early collection timepoints because some clams had over 100 oocysts detected while others had 0 or very low numbers, whereas at later timepoints oocysts were only detected at low numbers in exposed clam samples.

3.3 Wild clam testing

In 2002, all sentinel clam samples from the 3 riverine regions were negative by IMS-DFA of pooled digestive gland samples and PCR of individual hemolymph samples. However, in 2003, *Cryptosporidium* oocysts were detected in clams from all 3 riverine study regions, The mean number of *Cryptosporidium* detected per positive clam pool in 2003 was 2 oocysts (range, 1-7). Figure 4.3 shows the prevalence of *C. parvum*-like and *C. andersoni/ muris*-like oocysts detected in clam batches by IMS-DFA during the dry and wet seasons. Both *C. parvum*-like and *C. andersoni/muris*-like oocysts were detected significantly more often in the wet season than in the dry season ($p<0.005$). Using PCR

and DNA sequence analysis, *C. parvum* genotype 2 sequences were identified from DFA-positive slide scrapings from a San Lorenzo River clam and from a Salinas River clam. Individual hemolymph testing by PCR and DNA sequence analysis detected *C. parvum* genotype 2 in a Salinas River clam. No other *Cryptosporidium* genotypes were identified by PCR amplification of slide scrapings or hemolymph samples.

Risk factor data were then analyzed to look for variables that might affect the likelihood of detecting *Cryptosporidium* in sentinel clam batches. The covariates for precipitation accumulation, human density, and animal density across the 3 study sites did not vary and so were excluded from further analysis. Using Exact logistic regression, the effect of season ($p<0.001$) and year ($p<0.005$) were significantly associated with the probability of detecting *Cryptosporidium*-positive clam pools. The clam collection region, upstream versus downstream site location, and sewage management practices were not significantly associated with *Cryptosporidium* detection in this study ($p>0.2$).

Giardia testing was initiated in the second year of the study. Table 4.5 shows the recovery efficiency of *Giardia* cysts spiked into clam digestive gland when using 3 variations of the IMS-DFA protocol. There was no significant difference between using a full and half dose of IMS-beads with acid dissociation ($p=0.126$), while using acid dissociation was significantly better than heat dissociation ($p<0.001$). The most sensitive and cost efficient method, a half dose of IMS beads with acid dissociation, detected a mean of 65% of 100 oocysts and 66% of 10 oocysts spiked into digestive gland samples.

This IMS-DFA method was then applied to the sentinel clams in year 2 of the study. *Giardia* was detected in clams from all 3 riverine study regions. The mean number of cysts detected per clam pool was 3 (range, 1-26). Using the same risk factor analyses that

were applied for *Cryptosporidium*, *Giardia*-positive clam pools were significantly associated with wet season sampling ($p=0.015$) and downstream location ($p<0.001$). Clam region and sewage management practices were not significantly associated with *Giardia*-positive clams. Figure 4.4 shows the prevalence of *Giardia* detected in clam digestive gland pools during the wet and dry seasons of 2003. No positive PCR results were obtained from amplifying DNA from hemolymph samples and *Giardia*-positive IMS-DFA slide scrapings.

4. Discussion

This study showed that IMS, DFA and PCR methods can be used to detect *Cryptosporidium* in spiked clam tissues, clams that have filtered oocysts from water inoculated with environmentally-plausible oocyst doses, and clams outplanted in naturally-contaminated freshwater ecosystems in California, USA. The most analytically sensitive technique for oocyst detection in digestive gland was IMS concentration followed by DFA analysis, which detected oocysts in 17% and 83% of the samples exposed to 1 and 10 oocysts, respectively. For oocyst detection in hemolymph, both PCR techniques were able to detect a single oocyst in a 1 ml hemolymph aliquot at least 67% of the time. In the tank experiment, the most sensitive *Cryptosporidium* detection technique was IMS concentration of digestive gland tissues followed by DFA analysis for parasite quantification, while PCR did not perform as well. The greatest number of oocysts were detected in clams exposed to high oocyst doses and collected during the first day after oocyst exposure, though low numbers of oocysts were detected as long as 3 weeks post-exposure. The wild clam study used IMS-DFA and PCR to detect

Cryptosporidium and *Giardia* spp. in sentinel clams from 3 riverine study regions in California. Risk factor analysis showed that clams sampled during the wet season were more likely to contain *Cryptosporidium* oocysts and *Giardia* cysts. *Cryptosporidium parvum* animal genotype was confirmed in outplanted riverine clams using PCR and DNA sequence analysis.

This study is the first to evaluate the utility of IMS concentration for improving the analytical sensitivity of DFA techniques on clam digestive gland samples. Pathogen detection in fecal and environmental samples is challenging due to the small sample volume that can be analyzed per test, and the presence of substances inhibitory to PCR assays. The IMS concentration method overcomes these problems in 2 ways. First, the volume of processed sample is increased from 10 ul per DFA test or 50 ul per PCR test to 500 ul per IMS test. Second, the IMS procedure removes inhibitors by using several wash steps while the parasites are bound to a magnetic holder via the immunomagnetic beads. Our finding that IMS concentration improved the minimum oocyst detection limit in digestive glands by 1-2 $\log_{10}$ units is similar to the findings in another study that applied IMS to bovine fecal samples (Pereira et al., 1999). The $\log_{10}$ increase in IMS-DFA sensitivity from 100 to10 oocysts per clam digestive sample is important for accurate detection of the low numbers of oocysts that may be expected in environmental samples. One other study (Gomez-Bautista et al., 2000) used IMS to improve assay sensitivity by concentrating bivalve homogenates and water samples by IMS when samples had tested negative by fluorescent antibody testing alone. This approach has merit because the investigators were able to maximize their assay sensitivity using IMS

concentration, while minimizing the number of assays run by testing pooled bivalve homogenates.

Two modifications of the IMS technique were evaluated in this study. First, to reduce the cost of reagents of IMS testing (approximately $50/test), a half dose of IMS beads was compared to using the full dose of beads. In the *Giardia* spiking trial, we found that there was no significant difference in the cyst recovery efficiency when using a half or full dose of IMS beads, suggesting that the cost per IMS test could be halved by using a half dose of beads per sample in future studies. The second IMS modification assessed in the *Giardia* spiking trial was acid dissociation compared to heat dissociation of oocyst-bead complexes, based on a recent publication that found increased parasite recovery efficiencies in water samples using heat dissociation (Ware et al., 2003). In our spiking experiment, cyst recovery efficiencies for samples with acid dissociation of oocyst-bead complexes during the IMS protocol were significantly better than recovery efficiencies for samples with heat dissociation. Possible explanations for this discrepancy include differences in the study samples analyzed (clam tissues vs water samples), parasite characteristics (*Cryptosporidium* vs *Giardia*), or study design. Most IMS studies have used acid dissociation, including studies based on the Environmental Protection Agency Method 1623 for detection of *Cryptosporidium* and *Giardia* in water samples (Sturbaum et al., 2002; USEPA 2001), and in our hands the heat dissociation did not improve assay sensitivity for oocyst detection in bivalve digestive glands.

Whether or not IMS is used for sample concentration, all samples must still be analyzed using a parasite detection method such as DFA or PCR. The most widespread detection methods used in bivalve studies to date have been fluorescent antibody

techniques (Fayer et al., 1998; Graczyk et al., 1998; Gomez-Bautista et al., 2000). The advantages of these methods are that results are quantitative and that fresh or formalin-preserved samples can be analyzed. However, frozen tissues are not suitable for DFA analysis because oocysts and cysts often rupture during the freeze-thaw process. Another disadvantage of DFA is that it cannot distinguish between the many *Cryptosporidium* genotypes that look morphologically identical. In contrast, PCR methods can be used for genotype identification and can be used on fresh or frozen tissues, but formalin-preserved samples are problematic. In addition, a limitation of conventional PCR is that results are not quantitative, though the increasing accuracy of quantitative PCR techniques such as TaqMan PCR may solve this problem. The ultimate choice between DFA and PCR detection methods may then depend on whether the goal is parasite quantification or genotype identification, or both.

PCR has been used in wild bivalve studies for parasite genotype identification, and in some cases the *Cryptosporidium* genotypes detected in wild bivalves have provided clues about the fecal loading sources (Fayer et. al., 1998; Gomez-Bautista et. al., 2000). For example, a study in the Chesapeake Bay detected *C. hominis*, *C. baileyi*, and *C. parvum* animal genotype in oysters (Fayer et. al., 1998). *Cryptosporidium hominis* and *C. baileyi* oocysts are normally only shed by humans and chickens, respectively, while *C. parvum* animal genotype oocysts are shed by both humans and animal hosts. After detecting the host-specific genotypes *C. hominis* and *C. baileyi* in bivalves, nearby sources of human and poultry feces could then be identified for further investigation into the sources of fecal pollution and possible remediation strategies to reduce fecal pollution. For non-host specific genotypes such as *C. parvum* animal genotype, only the geographic location of

contaminated bivalves could provide clues as to sources of fecal pollution. In the case of riverine ecosystems, water flow is unidirectional and so fecal pollution sources will usually be found upstream. However, in estuarine and marine waters there is continuous mixing, making identification of pollution source locations more difficult. Another advantage of testing bivalves from riverine systems is that the pathogen load may be more concentrated than in the larger estuarine or marine bodies of water that tend to dilute the incoming freshwater pollutants.

In an effort to simulate a riverine clam environment, our tank exposure experiment was designed to evaluate a range of environmentally plausible water temperatures and oocyst doses that may affect oocyst detection in individual clams over time (Rose, 1997). Several important findings became apparent from the tank experiment data. First, there was substantial variability in the number of oocysts detected in individual clams (range, 1-242 oocysts/clam). This suggests that sampling a larger number of clams by testing pooled samples will be more likely to represent the true *Cryptosporidium* status within a clam batch than testing a smaller number of clams individually. Second, there was no significant difference in the number of oocysts detected between clams held at 10 °C and 20 °C, so water temperatures in this range should not limit the utility of clams as bioindicators of fecal protozoal contamination in freshwater ecosytems. Third, the greatest number of oocysts were detected within the first day post-exposure but at very low levels for as long as 3 weeks post-exposure, suggesting that clams collected within a day of oocyst exposure events such as storm runoff may be the best bioindicators of *Cryptosporidium* load in freshwater ecosystems. Finally, oocysts were detected at all three exposure doses ranging from 20-2000 oocysts/L (3-300 oocysts/clam), and more

oocysts were detected in clams exposed to the high dose tanks than in clams from the medium or low dose tanks. This suggests that clams may accumulate oocysts in proportion to the exposure dose, a finding that is consistent with a recent study (Graczyk et al., 2003) in which the authors exposed clams to daily low oocyst doses for a month. They found that the number of oocysts detected in clams increased over the exposure month and decreased over the following 3 weeks post-exposure.

Our wild clam study used the best methods from the spiking and tank exposure experiments and applied them to clams that had filtered fecal pathogens from naturally contaminated waters. The 3 riverine study regions were carefully selected sites where *C. fluminea* clams were already in existence, because these clams are an aggressive non-native species that we did not want to introduce into new watersheds. *Corbicula* clams have been used as bioindicators of metals and pesticide contamination by the California State Mussel Watch Program for decades, and there was interest in determining whether clams would also be useful bioindicators of fecal pathogens for water quality monitoring purposes in California. During the 2002 dry and wet season sentinel clam testing, all 3 study regions were negative for *Cryptosporidium* spp. by IMS-DFA and PCR testing. However, during the 2003 dry and wet season testing, both *Cryptosporidium* and *Giardia* spp. were detected in clams from the 3 study regions by IMS-DFA. The year-to-year variability in results is not surprising considering the dynamic ecosystems in which clams are exposed to a variety of water quality events over time. Because clams slowly depurate the pathogens they filter, any one clam batch represents a snapshot of water quality over the past days to weeks, thus repeated sampling will be needed to better understand how fecal pathogens flow through riverine ecosystems.

Risk factor analysis evaluated the association between environmental variables and pathogen prevalence in clam batches. *Cryptosporidium* was detected more often in the wet season than the dry season, while *Giardia* prevalence was not significantly different between the wet and dry seasons. There are several explanations for the differences in seasonal detection for *Cryptosporidium* as compared to *Giardia.* First, it is possible that *Cryptosporidium* oocysts enter the riverine systems primarily via overland runoff during storm events, while *Giardia* oocysts could be entering the riverine systems directly via animals and humans that utilize the waterways. Another explanation is that *Giardia* might survive better than *Cryptosporidium* in the environment, though this seems unlikely since, in our experience, *Giardia* cysts usually rupture more easily than *Cryptosporidium* oocysts. A third explanation is that the difference in *Cryptosporidium* and *Giardia* prevalence during the dry season was a stochastic process that might not be related to season at all. Further clam testing over time would be necessary to evaluate this explanation. Some studies have found seasonality in *Cryptosporidium* detection in environmental bivalve and water samples (Fayer et al., 2002; Tsushima et al., 2003), while others have not (Robertson and Gjerde, 2001; Gomez-Couso et al., 2003), and this variability in outcomes might be attributable to the different environmental factors and study designs used.

Multiple organisms were detected by PCR analysis in the sentinel clam tissues. *Cryptosporidium parvum* animal genotype was detected using Morgan PCR and DNA sequence analysis of 2 digestive gland slide scrapings and a hemolymph sample from the Salinas and San Lorenzo Rivers. This species of *Cryptosporidium* is shed by both humans and animals, so the contributing fecal source in this case cannot be determined.

The Morgan PCR protocol also amplified clam and dinoflagellate DNA from slide scrapings and hemolymph samples, as determined with DNA sequence analysis. This cross reaction between a *Cryptosporidium* PCR system and dinoflagellate DNA has been reported by others (Sturbaum et al., 2002), and emphasizes the importance of DNA sequence analysis to confirm PCR positive results.

More parasite types were detected by IMS-DFA than by PCR analysis of sentinel clam tissues. In addition to the 5 μm round *C. parvum*-like oocysts detected by IMS-DFA, 5x7 μm *C. andersoni/muris*-like oocysts and 10x14 μm *Giardia*-like cysts were detected, all elliptical with bright green walls. However, no DNA sequences were obtained to confirm genotypes other than *C. parvum*. The reason for not detecting other *Cryptospordium* or *Giardia* genotypes by PCR is not adequately explained by a lack of PCR specificity, as both the *Cryptosporidium* and *Giardia* PCR protocols were selected because they have been shown to amplify a wide variety of parasite genotypes (Morgan et al., 1997; Xiao et al., 1999). It is more likely that the low numbers of oocysts and cysts present in the clam samples were below the sensitivity limit of these PCR methods when used on environmental samples. If this is the case, future studies that amplify parasites in cell culture before attempting genotype identification may be more successful. Without genotype identification of the *C. andersoni/muris*-like and *Giardia*-like parasites, no conclusions can be drawn as to contributing fecal sources. This is because *C. muris*-like oocysts can be shed by a variety of rodent species and are morphologically identical to the *C. andersoni* oocysts shed by cattle. In *Giardia* as well, there are both host-specific and zoonotic genotypes that share a similar morphology.

This sentinel clam study was the first to utilize clams as bioindicators of fecal protozoal pollution with *Cryptosporidium* and *Giardia* in natural riverine ecosystems. In future studies, bivalves could be used as part of ambient water quality monitoring programs focused on understanding the dynamics of fecal pathogen loading in recreational waters and evaluating whether mitigation of terrestrial fecal loading sources will decrease the pathogen load in downstream waterways. Bivalves could also be used to monitor for other fecal pathogens such as *Toxoplasma gondii,* a parasite that causes neurologic disease in humans and animals (Miller et al., 2004; Montoya and Liesenfeld, 2004). A recent study of risk factors for *Toxoplasma* infection in southern sea otters (*Enhydra lutris nereis*) found a significant correlation with freshwater outflow (Miller et al., 2002), and this finding is consistent with our clam study that documented the presence of fecal protozoal pathogens in 2 of the major freshwater tributaries in the sea otter home range. Like *Cryptosporidium* and *Giardia* spp, *T. gondii* oocysts have been shown to accumulate in bivalves during tank exposure experiments (Lindsay et al., 2001; Arkush et al., 2003), but no studies have yet determined whether bivalves are important in the transmission of protozoal pathogens to animals and humans that eat raw shellfish.

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Table 4.1
Proportion of *Cryptosporidium* positive clams detected by PCR, DFA, and IMS-DFA methods for spiked hemolymph and digestive gland tissues[a].

	Digestive gland % positive				Hemolymph % positive	
Oocysts	DFA	IMS-DFA	Xiao PCR	Morgan PCR	Xiao PCR	Morgan PCR
1	0	17	0	17	67	100
10	0	83	50	17	67	100
100	83	100	67	83	50	100
1000	nd[b]	nd	100	100	100	100

[a]n = 6 replicates per technique and oocyst dose.
[b]nd = not done.

Table 4.2
Recovery efficiency of DFA and IMS-DFA methods for *Cryptosporidium* oocysts spiked into clam digestive glands[a].

	DFA			IMS-DFA	
C. parvum oocysts spiked per clam	Mean no. oocysts detected (range)	Mean % recovery per test (range)	Mean % recovery per clam (range)	Mean no. oocysts detected (range)	Mean % recovery (range)[b]
1	0	0	0	0.17 (0-1)	17 (0-100)
10	0	0	0	5 (0-9)	50 (0-90)
100	2 (0-6)	46 (0-71)	2 (0-6)	69 (43-75)	69 (43-75)

[a]n=6 replicates per technique and oocyst dose.
[b]IMS-DFA mean % recovery represents the recovery per test and per clam because the whole clam digestive gland can be processed in one test with this method.

Table 4.3
Proportion of *Cryptosporidium* positive clams detected after a 6 hr tank exposure to 250 oocysts/clam.

Clam *Cryptosporidium* oocyst exposure status	Digestive gland % positive				Hemolymph % positive	
	DFA	IMS-DFA	Xiao PCR	Morgan PCR	Xiao PCR	Morgan PCR
Exposed (n=21)	24	52	0	24	0	24
Unexposed (n=3)	0	0	0	33[a]	0	66[a]

[a]DNA sequence analysis of PCR products showed that dinoflagellate and clam DNA were amplified, not *Cryptosporidium* DNA.

Table 4.4
Estimated maximum likelihood coefficients of the negative binomial regression model fitted to oocyst recovery data from the clam *Cryptosporidium parvum* oocyst tank exposure experiment[a].

Parameter	Coefficient	95% Confidence Interval		P value
Intercept	0.012	-0.88	0.91	0.98
Oocyst dose/clam				
2.5 oocysts[b]	0.0	-	-	-
25 oocysts	1.62	0.73	2.52	<0.001
250 oocysts	3.59	2.61	4.57	<0.001
Time post-exposure to oocysts				
3 hr[b]	0.0	-	-	-
6 hr	-0.57	-2.24	1.1	0.5
9 hr	-1.69	-2.76	-0.62	0.002
1 d	-1.99	-3.31	-0.66	0.003
3 d	-6.28	-8.59	-3.98	<0.001
7 d	-3.37	-5.61	-1.12	0.003
14 d	-18.9	-20.08	-17.74	<0.001
21 d	-6.28	-8.56	-4.01	<0.001

[a]Oocyst dose and timepoint coefficients are in relation to the low dose and 3 hr timepoint as reference values in the negative binomial model.
[b]Referent condition for the negative binomial regression model.

Table 4.5
Recovery efficiency of *Giardia* cysts spiked into clam digestive gland and processed by 3 IMS protocols[a].

Giardia cysts spiked per clam	Full dose IMS beads + acid dissociation [a]	Half dose IMS beads + acid dissociation [a]	Half dose IMS beads + heat dissociation [a]
10	60 (50-75)	66 (38-88)	2 (0-6)
100	55 (44-64)	65 (57-71)	41 (26-63)

[a]Mean % recovery for 6 replicates (% recovery range)

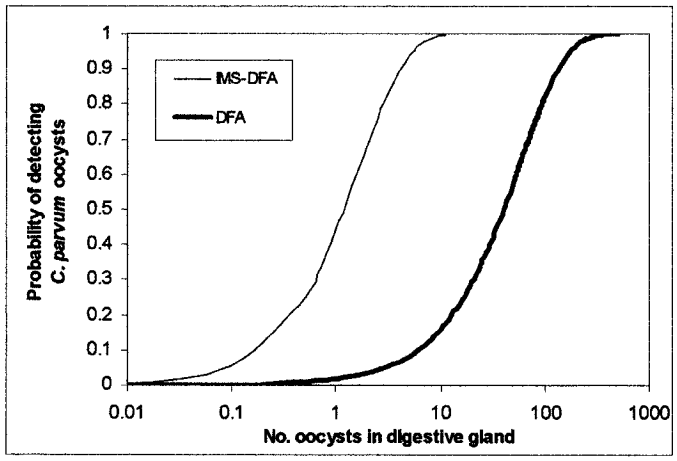

Fig. 4.1. Sensitivity of DFA and IMS-DFA for detection of *C. parvum* oocysts spiked into clam digestive gland tissues, using Poisson and negative binomial regression, respectively.

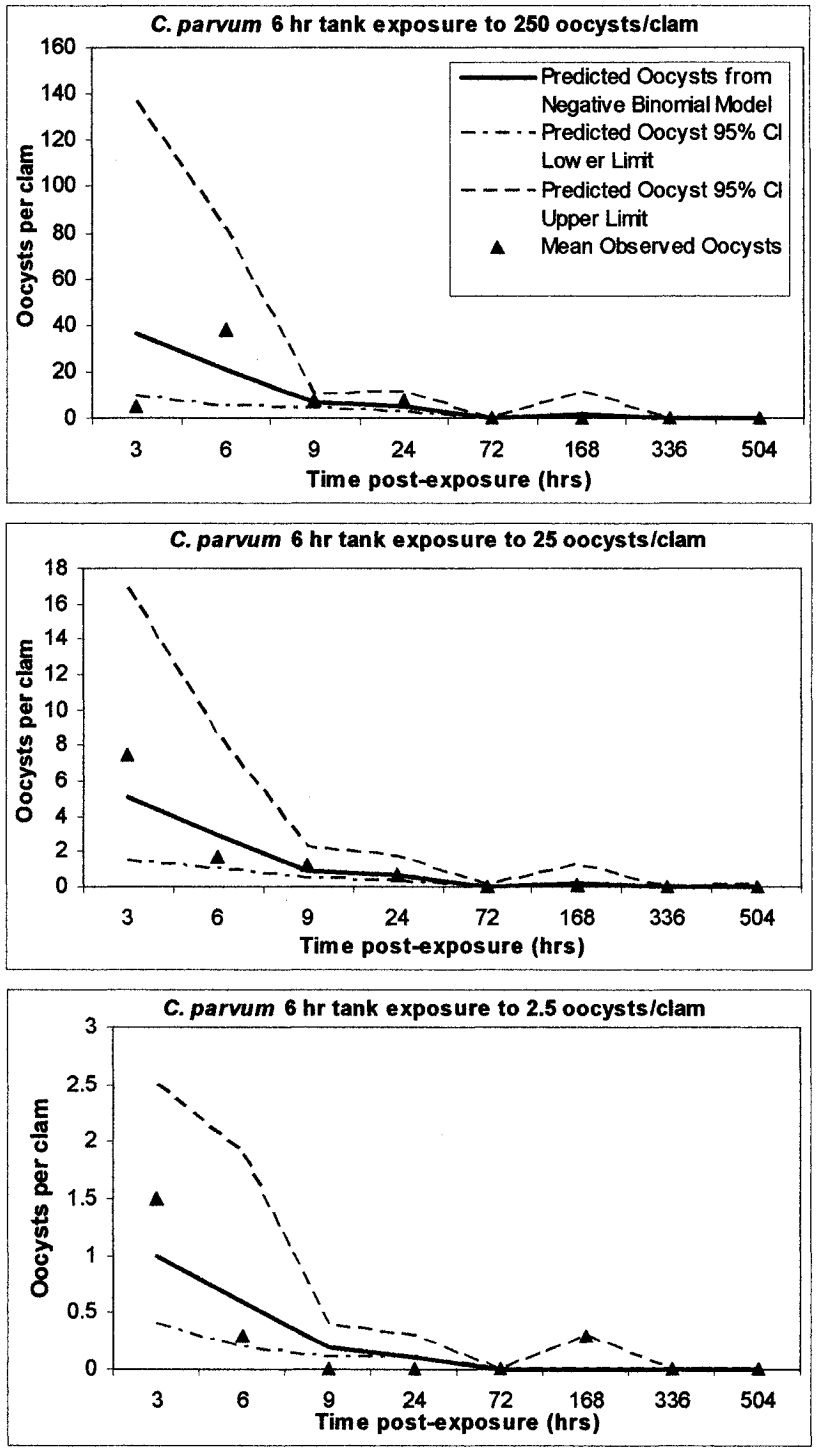

Fig. 4.2. *C. parvum* oocysts detected in clam digestive gland by IMS-DFA for three oocyst exposure doses used in a 6 hr clam tank exposure experiment. Clams were tested at 3h, 6h, 9h, 1d, 3d, 7d, 14d, and 21d post-exposure.

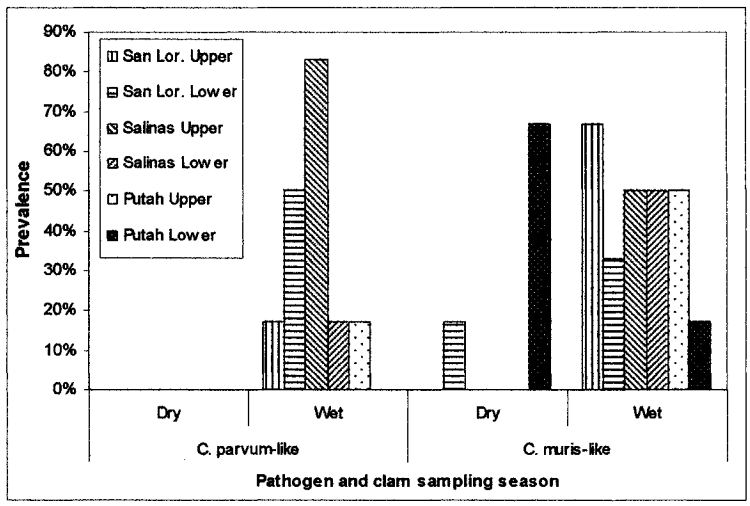

Fig. 4.3. *Cryptosporidium* spp. prevalence detected by IMS-DFA in clams collected from 3 California rivers in 2003.

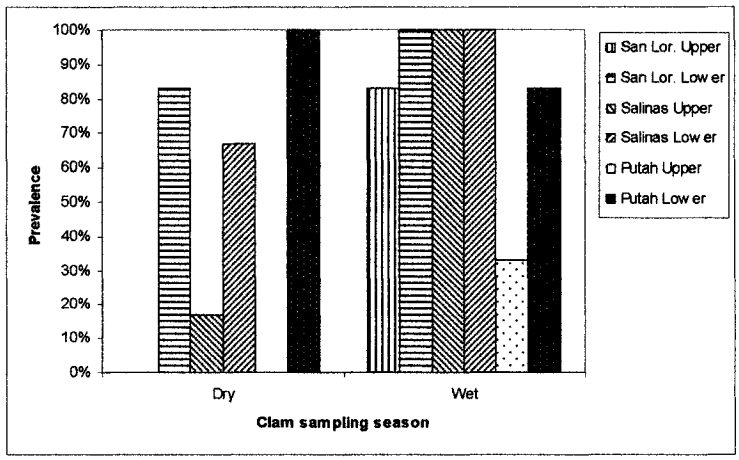

Fig. 4.4. *Giardia* spp. prevalence detected by IMS-DFA in clams collected from 3 California rivers in 2003.

CHAPTER 5

Cryptosporidium and *Giardia* in Dairy Storm Runoff: Evaluation of Risk Factors and Efficacy of Best Management Practices to Reduce Protozoal Loads

Cryptosporidium and *Giardia* in Dairy Storm Runoff: Evaluation of Risk Factors and Efficacy of Best Management Practices to Reduce Protozoal Loads

This manuscript has been formatted for Applied and Environmental Microbiology

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ABSTRACT

Cryptosporidium and *Giardia* distribution and loading in storm runoff, as well as the efficacy of selected Best Management Practices (BMPs) for reducing the load of oocysts and cysts, were evaluated on 5 coastal California dairies over 3 storm seasons. Immunomagnetic separation and/or direct immunofluorescent antibody analysis was used to test 620 storm runoff samples. *Cryptosporidium* oocysts and *Giardia* cysts were detected in runoff from <10% of pasture samples, <20% of road and dry lot samples, and >60% of waste management system samples. Oocysts and cysts were detected in 16-31% of runoff samples collected near cattle less than 6 months old and in <10% of runoff samples collected near cattle over 6 months old. Risk factors associated with environmental loading of *Cryptosporidium* and *Giardia* included farm loading unit, cattle age, cumulative seasonal precipitation, and water turbidity. The most effective BMPs included vegetative buffers and water impoundments that reduced the load of *Cryptosporidium* oocysts and *Giardia* cysts in storm runoff by at least 90% compared to control sites in the majority of samples.

INTRODUCTION

Cryptosporidium parvum and *Giardia duodenalis* are leading parasitic causes of waterborne gastroenteritis outbreaks in the United States (15). Both of these protozoal parasites are shed in the feces of a wide variety of animals, including humans and livestock, and are found worldwide (18). As few as 10-100 *Cryptosporidium* oocysts and *Giardia* cysts have been shown to cause diarrheal disease in healthy human volunteers (23, 27). In animals, the highest prevalence of cryptosporidiosis is in newly infected young animals who may shed millions of oocysts during initial infection (9). Reducing

the incidence of cryptosporidiosis and giardiasis in animals and humans requires a better understanding of the environmental ecology of these parasites.

Waterborne *Cryptosporidium* outbreaks have been attributed to fecal contamination from human and livestock sources (30). Laboratory studies have evaluated water treatment methods for disinfection at sewage and water treatment plants, with filtration and ozonation proving the most effective (30). However, these methods are not practical for livestock operations, partially due to the cost involved and the non-point source nature of livestock runoff. In the case of farm and environmental runoff, an alternative proposed strategy to improve water quality is the implementation of Best Management Practices (BMPs) that can be incorporated into farm and environmental management plans (13). The BMPs can include a variety of infrastructure or management practices that function to improve farm attributes such as water quality or animal production. Regulatory agencies currently rely on the concentration of indicators such as coliform bacteria or *Cryptosporidium* oocysts for water quality assessments, but the indicator load, calculated as the concentration multiplied by flow, is an alternative measure that also considers the volume of runoff.

The most common BMPs evaluated for reducing the bacterial or protozoal load in storm runoff have been vegetative buffer strips (6, 7, 37). These buffers provide a grass barrier to slow down runoff and allow for infiltration and sedimentation before the runoff reaches surface water tributaries. Vegetative buffers have had variable efficacy in reducing the load of bacteria, nutrients and pesticide loads in runoff (6, 21, 32, 34). However, in recent laboratory studies evaluating the capacity of vegetative buffers to

reduce the load of *Cryptosporidium* oocysts in storm runoff, a 1-3 $\log_{10}$ reduction/m was observed (3, 7).

This study extends the previous evaluation of vegetative buffers for reduction of *Cryptosporidium* load in storm runoff from the laboratory to a field setting. Five dairy farms near Tomales Bay, California, participated in the 3 year study. The goals were to evaluate 1) the distribution of *Cryptosporidium* and *Giardia* parasites and associated risk factors in storm runoff collected around dairy farms, and 2) the efficacy of BMPs to reduce the load of *Cryptosporidium* and *Giardia* in storm runoff compared to adjacent control sites. In addition to vegetative buffers, other BMPs evaluated included mulch application, runoff impoundment, cattle exclusion and manure spreading. The hypotheses being tested were: 1) that the most *Cryptosporidium* and *Giardia* would be found in runoff draining calf areas, and 2) that BMPs such as vegetated buffers would be able to reduce the *Cryptosporidium* and *Giardia* load in storm runoff by at least 90%.

MATERIALS AND METHODS

Study design. The 5 dairy farms were selected based on voluntary participation and location within the Tomales Bay region. Using a systems approach, stormwater samples were collected from dry lot and pasture BMP and adjacent control sites, as well as from roads and the waste management systems on the farms. Stormwater samples from these loading units were collected over 3 consecutive storm seasons in 500 ml volumes per site and timepoint. The water samples were concentrated by centrifugation and then analyzed for *Cryptosporidium* oocysts and *Giardia* cysts with immunomagnetic separation (IMS) and/or direct fluorescent antibody (DFA) analysis for parasite quantification. Randomly selected samples were amplified with PCR and genotyped by DNA sequence analysis.

In addition to the farm stormwater sampling, a series of water spiking experiments were performed to evaluate the analytic sensitivity of the water processing protocols. By creating stormwater samples in the laboratory that represented a range of oocyst doses and water turbidities, the total water processing assay performance could be statistically modeled. Percent recovery estimates could then be used to extrapolate from the raw parasite count data to the estimated true protozoal load in stormwater samples for further statistical analyses.

Finally, statistical analyses were performed to determine the distribution of parasites among stormwater sampling sites, associated risk factors, and the reduction in parasites in stormwater collected from BMP treated sites compared to control sites. Stormwater samples were classifed by loading unit (pastures, roads, dry lots, or waste management systems), predominant cattle age (1-2 months, 3-6 months, 6-12 months, or >1 year), and BMP status (control site, impoundment, vegetative buffer, dry lot mulch application, manure spread on pastures, irrigation of pastures, soil aeration, and winter cattle exclusion). All sampling sites were also characterized with respect to the continuous variables surface-adjusted flow, turbidity, cumulative precipitation, slope, and animal density. Pastures were considered non-point sources of contamination while dry lots were considered point-sources. Waste management samples were not included in the point vs non-point categories because the waste systems are designed to retain the fecal material instead of allowing it to flow off the farm in runoff.

Protozoa for laboratory spiking trials. *Cryptosporidium parvum* genotype 2 oocysts (synonymous with bovine genotype A) and *Giardia duodenalis* (synonymous with *G. lamblia* and *G. intestinalis*) cysts were used for the water spiking experiments.

Cryptosporidium oocysts were obtained from commercial dairies near the Veterinary Medical Teaching and Research Center in Tulare, California. *Giardia* cysts were obtained from calves submitting fecal samples to the Veterinary Medical Teaching Hospital in Davis, California. Calf feces were sieved through a series of mesh strainers and then purified by sucrose flotation methods (1). Oocyst concentrations for spiking experiments were determined from the mean of 8 hemacytometer counts and confirmed with DFA enumeration. Oocyst suspensions were kept at 4°C and used within one month of collection.

Stormwater collection and processing. All stormwater samples for protozoal analysis were collected in 500 ml plastic bottles. Surface-adjusted flow at each sampling site was measured with a Global Waters flow meter (Global Waters Inc., Gold River, California) or the area-velocity method and multiplied by 0.8 to adjust for surface interactions (20). Water samples were transported chilled within 72 hr of collection, from the farm study sites to the University of California, Davis for laboratory analysis. For each 500 ml water sample, 125 ul 0.2% Tween 80 and 25 mls MOPS extraction solution were added. Samples were briefly hand mixed and decanted in 250 ml aliquots into 2 spin bottles. Bottles were agitated for 5 min on a hand-wrist shaker set at speed 7. Next the bottles were centrifuged at 1100 × *g* for 10 min. Supernatant was aspirated to leave a 1:1 pellet:liquid ratio, and the volume of the pellet was recorded. For *Giardia* quantification, a 10 ul aliquot was dried onto a 3-well Merifluor (Meridian Bioscience Inc., Cincinnati, Ohio) slide for DFA analysis. *Giardia* IMS concentration was not done due to financial constraints. For *Cryptosporidium* quantitation, if the total water pellet was <50 ul, then the pellet was analyzed by DFA without IMS concentration. For larger

pellets, up to 0.5 ml of the water pellet was processed by *Cryptosporidium* IMS and then DFA analysis. In addition to protozoal analysis, stormwater turbidity in nephlometric turbidity units (ntu) was determined using a turbidmeter (Orbeco Analytical Systems, Farmingdale, New York).

Immunomagnetic separation. Water pellet samples of up to 0.5 ml were concentrated and purified with IMS (Dynal Biotech, Oslo, Norway) per manufacturer's instructions, followed by DFA analysis for parasite detection (25). Briefly, the pellet was suspended in an L10 glass tube in 2 ml buffer and 10 ml PCR water. Then 100 µl IMS beads were added and the samples were rotated for 1 hr at setting 18. The IMS bead-parasite complexes were bound to a magnetic holder while the supernatant and debris were discarded. Bead-parasite complexes were resuspended in 1 ml buffer and transferred to 1.5 ml microcentrifuge tubes that were put into a smaller magnetic holder to again bind the bead-parasite complexes while the supernatant was discarded. For parasite dissociation from the beads, 2 acid washes of 50 µl 0.1 N HCl each were vortexed at the beginning and end of the 10 minute incubation. Tubes were then put in a small magnetic holder and the supernatant was transferred to a Merifluor DFA slide containing 3 ul of 0.1 N NaOH for neutralization.

Quantitative immunofluorescent microscopy. Slides processed by DFA for *Cryptosporidium* and *Giardia* quantification followed the recommended Merifluor protocol. Briefly, the DFA slides were incubated with anti-*Cryptosporidium* and anti-*Giardia* antibody, counterstained (Meridian Bioscience Inc.) for 30 min, and read at 200X-400X magnification on a Zeiss Axioskop epifluorescent microscope. All slides were read by the same microscopist. *Cryptosporidium parvum*-like oocysts were

identified as 5 μm diameter spheres that were outlined in apple green, often with a midline seam. *Cryptosporidium andersoni*-like oocysts were 5x7 μm diameter ellipses with a green outline. *Giardia*-like parasites were also elliptical and outlined in apple green but were larger in size, 10-14 μm in diameter. After parasite quantification, selected slides were scraped with a razor blade and washed with sterile PBS into a microcentrifuge tube for subsequent DNA extraction, PCR amplification, and DNA sequence analysis.

Polymerase chain reaction. Two conventional 18S rRNA PCR protocols (19, 42) were used to genotype selected *Cryptosporidium* stormwater samples. For DNA extraction, a 50 μl maximum pellet was suspended in 180 μl ATL buffer and suspended in liquid nitrogen for 4 min, then boiling water for 4 min. Using the QIAGEN DNA Mini Kit tissue protocol (QIAGEN Inc., Valencia, CA), the sample was digested, bound to a QIAamp column, washed, and the DNA eluted with 50 μl of 95°C PCR water. Extracted DNA was frozen until PCR analysis.

The Xiao (42) 18S rRNA nested protocol was used because it has been widely used to amplify DNA from a variety of *Cryptosporidium* spp. The outer primer set first amplifies a 1325 bp DNA segment, followed by a nested amplification of an 850 bp DNA segment. The Xiao outer primers used were C1F: TTC TAG AGC TAA TAC ATG CG and C1R: CCC TAA TCT TTC GAA ACA GGA, with inside primers C2F: GGA AGG GTT GTA TTT ATT AGA TAA AG and C2R: AAG GAG TAA GGA ACA ACC TCC A. The Xiao outer primer PCR reactions contained 5 μl Perkin-Elmer (Norwalk, CN) 10X PCR buffer, 6 mM $MgCl_2$, 200 μM each deoxynucleoside triphosphate (dNTP), 200 nm each primer, 1.5 U Taq polymerase, PCR-grade water, and 2 μl DNA in a 50 μl total volume.

The Xiao inner primer PCR reactions were the same except that the $MgCl_2$ was reduced to 3 mM. Amplification conditions for the outer and inner Xiao PCR reactions consisted of hot start at 94°C for 3 min, followed by 35 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 1 min, with a final extension at 72°C for 7 min.

A second, unnested 18S rRNA protocol Morgan (19) was also used to amplify a 300 bp piece of DNA from various *Cryptosporidium* spp. The Morgan *Cryptosporidium* primers consisted of 18sif: AGT GAC AAG AAA TAA CAA TAC AGG and 18sir: CCT GCT TTA AGC ACT CTA ATT TTC. The PCR reactions contained 5 µl 10X PCR buffer, 3 mM $MgCl_2$, 200 µM each dNTP, 200 nm each primer, 1.5 U Taq polymerase, PCR-grade water, and 2 µl DNA in a 50 µl total volume. Amplification conditions for the Morgan PCR reactions started with 2 min at 96°C, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 sec, with a final extension of 72°C for 7 min.

The *Giardia* nested PCR protocol (16) was designed to amplify a 130 bp piece of the 18S rRNA gene of a variety of *G. duodenalis* genotypes. The McGlade protocol utilized outside primers RH11: CAT CCG GTC GAT CCT GCC and RH4: AGT CGA ACC CTG ATT CTC CGC CAG G (12), and inside primers consisted of GiarF: GAC GCT CTC CCC AAG GAC and GiarR: CTG CGT CAC GCT GCT CG (26). PCR reactions were performed in a 25 ul final volume, and contained 2.5 ul 10X PCR Buffer, 200 uM dNTP's, 5 pmol forward and reverse primers, 1.25 ul dimethyl sulfoxide, 0.5 U of AmpliTaq, PCR-grade water, and 2 ul template DNA. Thermocycler conditions consisted of 3 cycles of 94° C for 2 min, 53° C for 1 min, and 72° C for 2 min, followed by 50 cycles of 94° C for 30s, 53° C for 20s, 72° C for 30s, and finally extension of 72° C for 7 min.

Amplified PCR products were run with a 100 bp ladder on a 2% agarose gel containing GelStar (Cambrex Co., East Rutherford, NJ) to determine product size and band intensity. For selected isolates, the PCR product was purified according to QIAGEN Qiaquick protocol, sequenced on an automated sequencer, and analyzed with Chromas (Technelysium Pty Ltd, Tewantin, Qld, AU) and ClustalX (36) software for *Cryptosporidium* genotype identification.

Statistical analyses. For the water spiking experiments, percent recovery values for DFA and IMS-DFA testing were calculated using negative binomial regression to model the raw oocyst count data (4,10). In addition to the percent recovery estimate, the assay sensitivity $S(c_i)$, defined as the probability of detecting at least one oocyst per sample, was calculated as shown in Equation [1], where $e^{\beta x_i}$ is the percent recovery of the assay, α is an ancillary parameter for modeling dispersion, c_i is the number of oocysts spiked per digestive gland, and W_i is the proportion of the digestive gland tested in the assay.

$$S(c_i) = 1 - [1/(1 + \alpha c_i W_i e^{\beta x_i})]^{1/\alpha} \qquad [1]$$

In the field study, water samples were first classified as positive or negative for *Cryptosporidium* and *Giardia* for use in the prevalence calculations. Next the percent recovery estimates were used to adjust the oocyst and cyst count data from all the water samples, in order to estimate the true concentration of parasites in each sample. The adjusted parasite concentrations were then used to calculate instantaneous parasite load by multiplying the concentration by the flow for each sample in Equation [2]:

$$\text{Load (oocysts/min)} = \text{Oocyst concentration (oocysts/L)*Flow (L/min)} \qquad [2]$$

For risk factor analysis, univariate and multivariable mixed effects linear regression was used to evaluate the association between farm covariates and detection of

Cryptosporidium oocysts and *Giardia* cysts in storm runoff (3). For comparative purposes, the natural log of both the parasite concentrations and parasite loads were analyzed as outcome variables. The group effect of water sampling site was used in the regression models to adjust for repeated sampling at the same sites over time. Lastly, the differences between groups such as *Cryptosporidium* or *Giardia* load in runoff from BMP treated and control sites were analyzed using t-tests for paired samples. Statistical significance was defined as a P-value <0.10.

RESULTS

Over the 3 year study, a total of 620 water samples were collected on the 5 dairy farms. *Cryptosporidium parvum*-like and *C. andersoni*-like oocysts were detected by IMS in combination with DFA in runoff from 4 of the 5 dairy farms. DNA amplification and sequence analysis of up to 5 randomly selected DFA-positive samples per farm confirmed that *C. parvum* genotype 2 and *C. andersoni* were present on 4 of the 5 dairies. *Giardia* cysts were detected by IMS concentration with DFA detection on all 5 farms. Amplification of *Giardia* DNA by PCR was successful, but no clean *Giardia* sequences were obtained after DNA sequence analysis, possibly due to mixed *Giardia* infections or amplification of non-*Giardia* DNA in the water samples. Results and statistical analyses focus on *C. parvum* (henceforth called *Cryptosporidium)*, and *Giardia*, not *C. andersoni*, due to the difficulty in quantifying *C. andersoni* oocysts by DFA and because *C. andersoni* is not of significant public health importance.

Overall, *Cryptosporidium* were detected in 16% and *Giardia* in 12% of storm runoff samples throughout the study. Table 5.1 demonstrates that *Cryptosporidium* oocysts and *Giardia* cysts were detected in storm runoff from all 4 farm loading units.

Cryptosporidium and *Giardia* were present in <10% of pasture runoff samples, <20% of dry lot samples, and 63% of samples from the waste management systems. Table 5.2 shows the prevalence of *Cryptosporidium* oocysts and *Giardia* cysts detected in runoff collected near cattle of various ages. Overall, the lowest prevalences of *Cryptosporidium* and *Giardia* were 3-8%, in runoff samples influenced by cattle 6 months of age or older. The highest prevalences of 31% and 28% of samples positive for *Cryptosporidium* and *Giardia*, respectively, were found in runoff collected near calves 1-2 months old. *Cryptosporidium* and *Giardia* were also detected in 25% and 16% of storm runoff samples collected near 3-6 month old calves, respectively.

A series of water spiking experiments were performed to evaluate the recovery efficiencies of the *Cryptosporidium* and *Giardia* detection methods in 500 ml water samples to which a known quantity of oocysts and cysts had been added. Table 5.3 shows the percent recovery and 95% confidence intervals for the 3 detection methods that were generated using a negative binomial regression model. The highest percent recovery of 47% was calculated for the IMS-DFA detection of *Cryptosporidium* oocysts, whereas the recovery efficiencies by DFA alone for *Cryptosporidium* and *Giardia* were 21% and 27%, respectively. These values were then used to adjust the raw oocyst and cyst count data from the field samples so that the true parasite concentrations in the storm runoff samples could be estimated for further epidemiologic analysis.

Figure 5.1 illustrates the different assay sensitivity curves for *Cryptosporidium* oocysts and *Giardia* cysts detected by DFA in storm water samples, as well as for *Cryptosporidium* oocysts detected by IMS concentration in combination with DFA detection. The IMS-DFA analytic sensitivity improvement of 1-2 $\log_{10}$ units is shown by

the IMS-DFA curve shifted far to the left of the DFA curves. The 50% probability detection threshold (DT_{50}) improved from 100 to 10 oocysts per 0.5 ml water pellet with IMS concentration, and the DT_{90} improved from approximately 500 to 10 oocysts.

The recovery-adjusted parasite concentrations for each field sample, multiplied by the instantaneous flow at the sampling site, approximated the instantaneous load of *Cryptosporidium* oocysts and *Giardia* cysts in overland flow during the storm event. Figure 5.2 shows the mean instantaneous flows (L/min), parasite concentrations (oocysts or cysts/L) and resulting parasite loads (oocysts or cysts/min) in storm runoff from dry lot point sources as compared to pasture non-point sources. Significantly lower parasite concentrations ($p<0.05$) and higher flow rates ($p<0.01$) were detected at the non-point source pasture sites compared to the point-source dry lot sites. While the parasite concentration and flow rates were significantly different for non-point and point sources, there were no significant differences between the 2 sources in loading of *Cryptosporidium* ($p=0.51$) or *Giardia* ($p=0.11$).

The concentrations and instantaneous loads of *Cryptosporidium* and *Giardia* in storm runoff were next used in a mixed effects linear regression model for risk factor analysis, using a group effect of site to account for repeated sampling at the same sites during the 3 year study. Table 5.4 shows the univariate analysis of the putative risk factors loading unit, cattle age, animal density, total seasonal precipitation, water turbidity, and slope with the dependent variables as the natural log of the oocyst or cyst concentrations and loads. For both *Cryptosporidium* and *Giardia*, the significant risk factors ($P<0.1$) were the same for the concentration and load outcome variables. Significantly more *Cryptosporidium* and *Giardia* were detected in runoff samples from the dry lot areas and

waste management system compared to the referent pasture samples. Similarly, the most *Cryptosporidium* and *Giardia* were detected in cattle under 6 months of age compared to the referent category of cattle over 1 year of age. The continuous variables animal density and water turbidity were positively associated with detecting *Cryptosporidium* and *Giardia* in storm runoff samples. In contrast, as slope increased, the concentration and load of both parasites decreased. The only difference between risk factors for *Cryptosporidium* and *Giardia* was that as total accumulated seasonal precipitation increased, the concentration and load of *Cryptosporidium* oocysts decreased, while no significant relationship was detected between *Giardia* concentration and loads.

Table 5.5 shows the significant risk factors that are apparent in the multivariable model that considers all the risk factors simultaneously while adjusting for the repeated sampling at the same sites over time. Loading units were analyzed in the multivariable model and dry lots were not found to be significantly different than the referent pasture samples, so the factor loading unit was excluded from the final model in a similar manner to the variables slope and animal density. As with the univariate analysis, the same risk factors were significant when comparing the concentration and load models for each parasite. Cattle age less than 6 months and total seasonal precipitation were the significant risk factors in determining the concentration and load of *Cryptosporidium* oocysts in a water sample ($P<0.0001$). *Giardia* concentration and load were significantly associated with cattle less than 6 months of age and with water turbidity ($P<0.02$). Regression coefficients can be interpreted with regard to the natural logarithm of the parasite concentration or load. As an example in Table 5.5 of a continous variable, for each inch increase in the total seasonal precipitation, the natural log of the concentration

of *Cryptosporidium* oocysts decreased by 0.87, suggesting that the fewer oocysts will be detected as the storm season progresses. For the categorical variable of cattle age, compared to the referenent category of cattle over 1 year in age, the natural log of the concentration of *Cryptosporidium* oocysts detected in storm runoff samples would increase by 3 and 5 times in samples collected near 3-6 month old and 1-2 month old cattle, respectively.

The efficacy of BMPs in reducing the load of *Cryptosporidium* and *Giardia* in storm runoff was evaluated using adjacent paired control and BMP sites. Figure 5.3 shows an example of the *Cryptosporidium* and *Giardia* raw loading data at a paired control and BMP grass buffer site during a single storm event. At the three sampling timepoints, up to 16,000 *Cryptosporidium* oocysts and 5,000 *Giardia* cysts per min were loading from the control site, whereas less than 500 oocysts or cysts per min were loaded from the grass buffer BMP site. This represents a 1-2 $\log_{10}$ reduction of the parasites between the buffer and control sites.

Because the number of paired sites where *Cryptosporidium* or *Giardia* were detected at the same storm collection timepoints was low, the *Cryptosporidium* and *Giardia* data were pooled for further analysis. Table 5.6 shows how often the parasite load in storm runoff was reduced by at least 90% or 50% for each type of BMP site compared to the paired control site. For example, at all 4 samplings (100%) of the water impoundment pairs and for 30 of 44 (68%) of the vegetative buffer pair samplings, the load of oocysts and cysts was reduced by at least 90% in the BMP treated site compared to the load in the runoff sample from the adjacent control site. Up to a 7 $\log_{10}$ reduction in parasite load was detected in some cases. Surprisingly, the load was also decreased by at least 90% in

the majority of pastures with manure spread on them compared to their control site that did not have manure spread. A parasite reduction of >90% was detected in less than half of BMP sites where mulch was applied to dry lots, lots were scraped, pastures were irrigated, or cattle were excluded during the winter.

An alternative assessment of BMP efficacy data is given in Figure 5.4, in which the mean parasite loads are shown for the matched control and BMP sites. The reduction in storm loading was statistically significant for vegetative buffers (p=0.06), pastures spread with manure (p=0.04), and sites where cattle were excluded during the winter (p=0.08) compared to the paired control sites. A reduction in parasite loads at water impoundments (p=0.18), mulch treated dry lots (p=0.14), scraped lots (p=0.14), and irrigated pastures (p=0.28) was not statistically significant.

DISCUSSION

This is the first multi-year epidemiologic study to evaluate the distribution and magnitude of *Cryptosporidium* and *Giardia* loading in storm runoff from various farm loading units on dairies in California. Significant risk factors for *Cryptosporidium* and *Giardia* loading in storm runoff included cattle age, water turbidity, and cumulative seasonal precipitation. This study is also the first field study to evaluate the efficacy of various BMP practices in reducing the protozoal load in storm runoff by working with the farmers to implement sustainable BMPs into their daily farm management practices. Vegetative buffers established on dairy farms were effective at reducing the load of *Cryptosporidium* oocysts and *Giardia* cysts by at least 90% in the majority of samples, supporting the findings of the previous controlled studies (3, 7).

While *Cryptosporidium* and *Giardia* spp. were detected on most farms, they were only detected in a small proportion of the total water samples tested. *Cryptosporidium parvum* and *Giardia* were detected on 80-100% of dairies but only in 12%-16% of all storm runoff samples from dairies. These findings are similar to another study (33) that detected *Cryptosporidium* on 91% of 11 dairy farms tested in the northeastern United States, but in only 9% of the farm associated stream samples. These findings are in contrast to a Japanese study which detected *Cryptosporidium* spp. in 88% of dairy associated river samples but none of the equine farm associated river samples (39), and a study in the United Kingdom that found *Cryptosporidium* in 79% of surface water samples collected near a farming site (5). The previous studies all used flocculation and/or flotation to concentrate oocysts instead of performing the US EPA recommended IMS concentration, which may explain the lower (<20% recovery) reported assay sensitivity (5, 40), compared to our 47% recovery with IMS concentration. Our study demonstrates how even when most farms in a region may have endemic *Cryptosporidium* infections, the environmental loading of oocysts may vary, depending on both biotic animal factors and abiotic environmental factors.

Multiple species of *Cryptosporidium* were detected on dairy farms by morphologic and molecular methods. The presence of *C. parvum* and *C. andersoni* on California farms is consistent with other studies that have genotyped *Cryptosporidium* in fecal and farm runoff samples (24, 31). *Cryptosporidium andersoni* is thought to be less of a pathogen because while it infects the abomasums of cattle, it does not usually cause the enteritis and associated diarrhea that may occur with *C. parvum* infections in cattle and humans. In a similar manner, *Giardia* genotyping studies in cattle have detected multiple

genotypes of differing public health significance (22, 38). While *G. lamblia* Assemblage A is thought to be zoonotic, *G. lamblia* Assemblage E is thought to be specific to hoofed livestock, and therefore of little public health importance (22). Unfortunately, no *Giardia* genotyping information could be obtained in the current study, because purified PCR products did not yield clean DNA sequences that could be analyzed. Possible explanations for the *Giardia* sequencing problem include amplification of multiple *Giardia* genotypes in a sample, and amplification of non-*Giardia* DNA due to lack of PCR specificity.

The water spiking trials were critical to accurately assessing the water processing and detection assay recovery efficiencies, in order to then adjust the raw oocyst counts in the field samples to estimate the true parasite concentration in each sample. The overall percent recovery of 47% for *Cryptosporidium* IMS-DFA analysis, and 21% and 27% for *C. parvum* and *Giardia* DFA analysis without IMS, respectively, is similar to what has been found in other studies (3, 4, 25). The Pereira study (25) reported a mean percent recovery of 28%-34% for DFA when 1,000 or more oocysts were spiked into a fecal sample and analyzed by DFA alone. The same study found an improved percent recovery of up to 48% when 100 or more oocysts were spiked into a fecal sample and concentrated with IMS before DFA analysis. Likewise, the Atwill study (4) reported an IMS-DFA recovery of 32%-40% when 10-500 oocysts were spiked into a fecal sample. Finally, the Atwill study (3) reported 63%-81% oocyst recovery in water samples from soil box flow experiments that were simulations of the field conditions such as those tested in the current study.

The risk factors of cattle age, water turbidity, and total seasonal precipitation were significant in the *Cryptosporidium* and/or *Giardia* mixed effects linear regression models. Cattle age is a well recognized risk factor for *Cryptosporidium* and *Giardia* infection (31, 33, 38), with the highest prevalence and oocyst or cyst shedding often found in cattle less than 2 months of age. Similar to our findings, a Norwegian study of surface waters found that turbidity was a significant risk factor for detection of *Cryptosporidium* oocysts (28). Season or sampling month has been a significant risk factor for environmental loading with fecal pathogens in some studies (5, 40) but not others (11, 28). The differing results among studies may be attributable to variation in study designs or confounding factors, such as choosing month as the outcome variable when it might be more appropriate to choose cumulative precipitation if runoff is really what is hypothesized to influence the study outcome. Month may be confusing when comparing studies because monthly precipitation patterns vary with the location and climate of the study region. Cumulative seasonal precipitation was a significant risk factor for *Cryptosporidium* loading in storm runoff in our study, with decreasing oocyst loads detected as precipitation accumulated between the months November and March each season. Pasture and lot loading units were significantly different in the univariate analysis but not when considered with other risk factors in the multivariable regression model. This discrepancy may occur because confounding variables such as cattle age are not considered in the univariate loading unit regression, but are accounted for in the multiple regression model, suggesting that farm management practices that focus on controlling the spread of pathogens in calf feces should be a high priority.

Most of the BMPs demonstrated their potential to significantly reduce the load of *Cryptosporidium* and *Giardia* in storm runoff. Water impoundments work by damming and retaining water, allowing for increased sedimentation of particulate matter. Similarly, vegetative buffers and mulch application may reduce the parasite load in runoff by slowing the overland flow and allowing for increased infiltration and sedimentation (3, 37). Based on the very slow settling velocity of oocysts and cysts, it is likely that the majority of *Cryptosporidium* and *Giardia* that are removed from overland flow by sedimentation are adhered to particulate matter (17, 41). Other studies have shown that vegetative buffers can reduce the load of pathogens, nutrients, particulate, and/or pesticides in runoff (3, 7, 21, 32, 34, 37). The Atwill study (3) that evaluated the reduction in *C. parvum* oocysts in runoff flowing through grass buffer strips showed a 1-3 $\log_{10}$ reduction per meter, a similar finding to the current study where up to 7 $\log_{10}$ reductions were detected in vegetative buffers up to 33 meters long. The Atwill study also found that as the soil bulk density decreases, the capacity for vegetative buffers to retain oocysts increases, possibly by providing more area for infiltration in combination with the vegetation slowing down the overland runoff. Interestingly, the Davies study (7) found significantly reduced oocyst loads in runoff from vegetated soil plots compared to recently devegetated plots. This finding suggests that it is not just the available infiltration channels and macropores that lead to oocyst reduction from overland flow, but also that the vegetation functions to slow down the overland flow.

Other BMPs reduced the oocyst and cyst load in storm runoff in different ways. Excluding cattle during the winter significantly decreased the parasite storm loading, likely due to the decreased volume of feces and parasites deposited on the sampling sites

during the storm season. Spreading manure on pastures also significantly decreased the mean load of parasites compared to control sites, an unexpected result when considering that spreading manure would most likely increase the volume of feces and pathogens present on the fields. Further evaluation is needed to determine whether this finding is real or if there are other confounding factors that are as yet undetermined. The mean parasite loads for scraped lots and irrigated pastures were not significantly different from the respective values for matched control sites. Therefore, with respect to protozoal water quality, the farm resources used to scrape dry lots and irrigate pastures might be better focused on the more effective BMPs such as vegetative buffers near calf areas or winter cattle exclusion from riparian corridors.

Although the farm BMPs evaluated in this study do not directly kill *Cryptosporidium* oocysts or *Giardia* cysts, they do increase the time that oocysts and cyst are exposed to potentially harsh environmental conditions. Studies have shown that oocysts and cysts exposed to extreme temperatures or dessication are more quickly inactivated than those kept in moist conditions and moderate temperatures (8, 14, 29), whereas oocysts have remained viable in water under laboratory conditions for at least 1 year (35). It seems likely that oocysts and cysts may undergo more physical damage or dessication when adhered to blade of grass in a cattle pasture compared to being washed immediately downstream, since viability is more easily maintained in aquatic environments.

In conclusion, the investigation of risk factors for protozoal loading in storm runoff and the BMP evaluations in this study provided important information to better understand the environmental ecology and control of *Cryptosporidium* and *Giardia* on coastal California farms. The BMPs evaluated in this study represent practical on-farm

solutions to improve water quality and decrease pathogen loading in storm runoff. The systems approach to evaluate various farm loading units such as pastures, roads, dry lots, and waste management systems will help farmers and regulatory agencies prioritize their management efforts in the future.

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TABLE 5.1. Prevalence of *Cryptosporidium* and *Giardia*-positive storm runoff samples collected from various loading units on 5 dairies.

Loading Unit	*Cryptosporidium*[a] (%)	*Giardia*[b] (%)	n
Pasture	8	3	196
Road Runoff	17	17	6
Dry Lot	19	15	410
Waste System	63	63	8

[a] Percent positive samples for *C. parvum*-like oocysts identified morphologically by DFA or IMS-DFA.
[b] Percent positive samples for *Giardia* cysts identified morphologically by DFA.

TABLE 5.2. Prevalence of *Cryptosporidium* and *Giardia*-positive storm runoff samples collected near dairy cattle of various ages.

Cattle Age	*Cryptosporidium*[a] (%)	*Giardia*[b] (%)	n
>1 year	4	5	190
6-12 months	8	3	63
3-6 months	25	16	151
1-2 months	31	28	80

[a] Percent positive samples for *C. parvum*-like oocysts identified morphologically by DFA or IMS-DFA.
[b] Percent positive samples for *Giardia* cysts identified morphologically by DFA.

TABLE 5.3. Estimated maximum likelihood coefficients of the negative binomial regression model fitted to the *Cryptosporidium* and *Giardia* percent recovery data

Detection method	Percent recovery	95% Confidence interval
IMS-DFA (*C.parvum*)[a]	47.3	(38.6, 58.0)
DFA (*C.parvum*)[b]	21.0	(16.5, 26.6)
DFA (*G.duodenalis*)[b]	27.5	(21.5, 35.2)

[a] n=48 replicates of spiked water samples.
[b] n=60 replicates of spiked water samples.

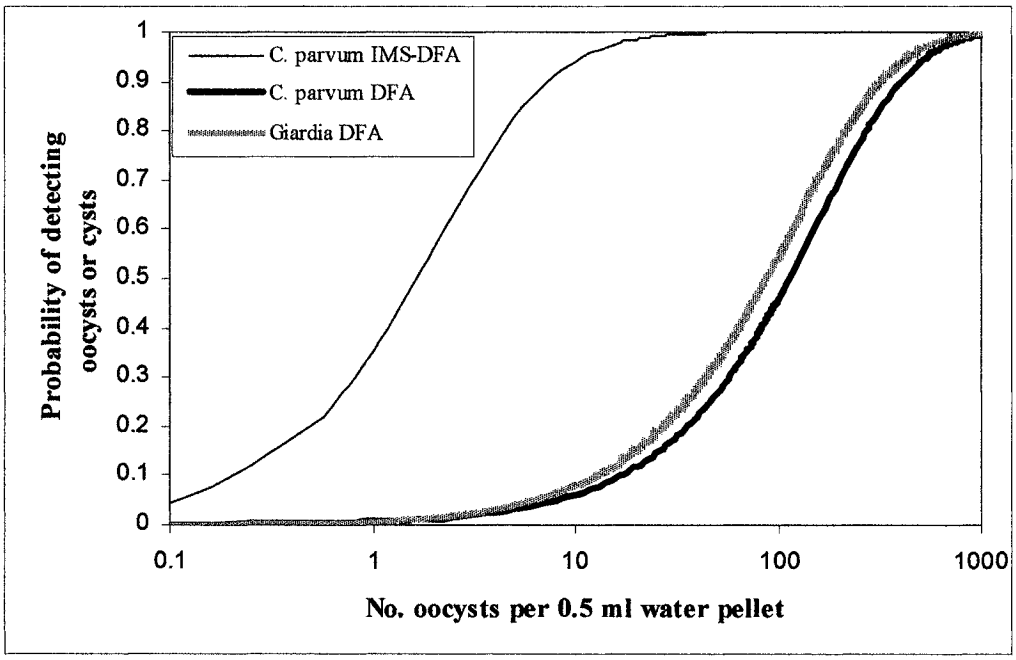
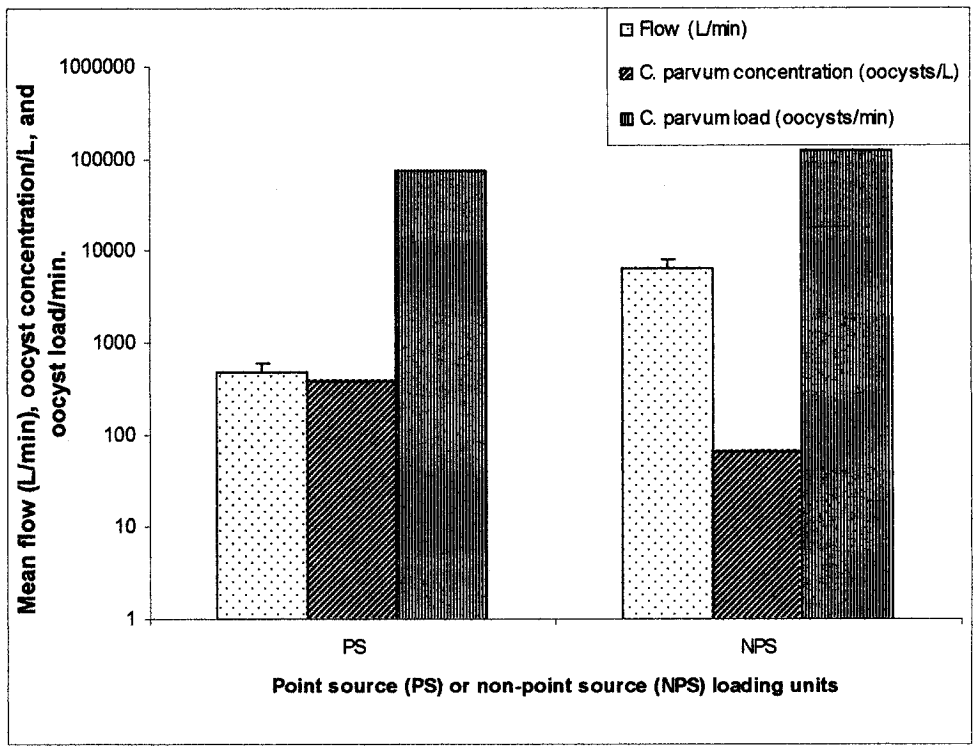
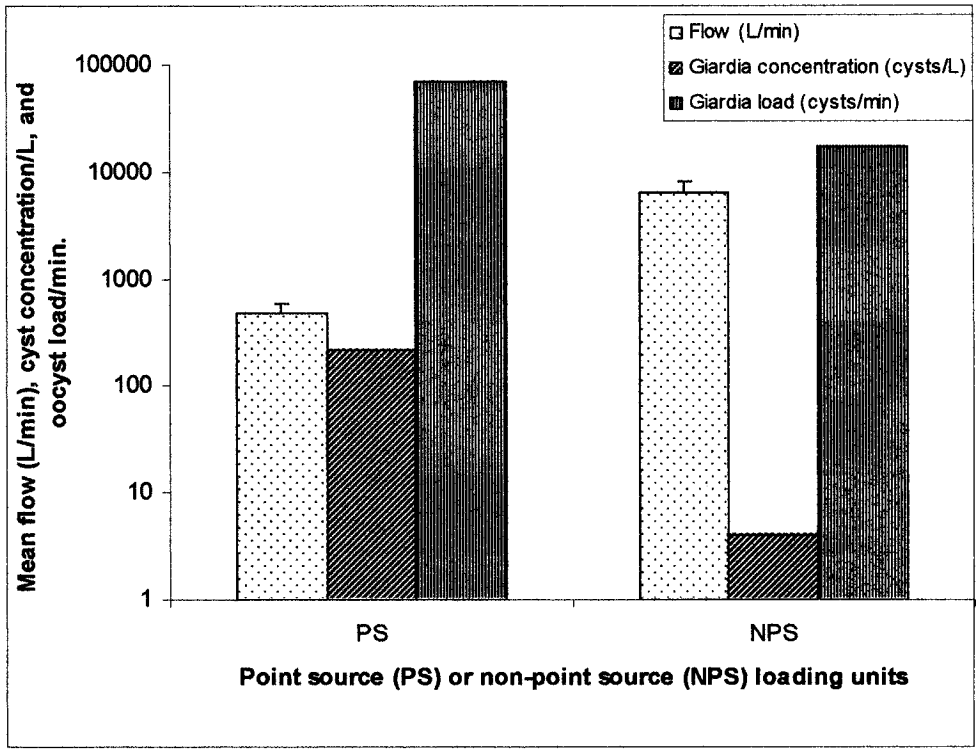


FIG 5.1. Sensitivity of DFA and IMS-DFA for detection of *Cryptosporidium* oocysts and *Giardia* cysts spiked into stormwater samples using negative binomial regression.



a.



b.

FIG. 5.2. Point source (PS) and non-point source (NPS) storm runoff flow, *Cryptosporidium* (a) and *Giardia* (b) concentrations, and loads on 5 dairies. Significantly higher flow rates ($p<0.01$) and lower pathogen concentrations ($p<0.05$) were detected from NPS as compared to PS loading units, but pathogen loads were not significantly different.

TABLE 5.4. Univariate mixed effect linear regression of risk factors associated with detection of *Cryptosporidium* oocysts and *Giardia* cysts in storm runoff from 5 dairies (natural log transformation used to stabilize variance).

	Cryptosporidium				*Giardia*			
	Runoff Concentration (oocysts/L)		Runoff Load (oocysts/min)		Runoff Concentration (oocysts/L)		Runoff Load (oocysts/min)	
Risk Factor	Regression Coefficient	P-value	Regression Coefficient	P-value	Regression Coefficient	P-value	Regression Coefficient	P-value
LOADING UNIT								
Pasture [a]	-	-	-	-	-	-	-	-
Road Runoff	0.90	0.61	1.43	0.57	1.37	0.41	2.10	0.36
Dry Lot	1.21	0.034[b]	1.67	0.035[b]	1.66	0.0026[b]	2.24	0.0022[b]
Waste System	7.49	<0.0001[b]	11.80	<0.0001[b]	8.00	<0.0001[b]	12.61	<0.0001[b]
CATTLE AGE								
>1 year [a]	-	-	-	-	-	-	-	-
6-12 months	0.30	0.69	0.49	0.63	-0.69	0.34	-0.91	0.33
3-6 months	3.14	<0.0001[b]	4.50	<0.0001[b]	1.76	0.0024	2.30	0.0021
1-2 months	4.93	<0.0001[b]	6.74	<0.0001[b]	3.61	<0.0001[b]	4.95	<0.0001[b]
ANIMAL DENSITY (animals per acre)	0.025	0.0004[b]	0.037	0.0002[b]	0.03	<0.0001[b]	0.047	<0.0001[b]
TOTAL SEASONAL PRECIPITATION (inches)	-0.053	0.0023[b]	-0.077	0.0028[b]	0.012	0.46	0.02	0.39
WATER TURBIDITY (ntu)	0.00019	<0.0001[b]	0.0003	<0.0001[b]	0.000098	0.025[b]	0.00016	0.010[b]
SLOPE (degrees)	-0.097	0.0056[b]	-0.14	0.0054[b]	-0.07	0.037[b]	-0.098	0.035[b]

[a] Referent category for regression comparisons.

[b] Significant if P<0.1.

TABLE 5.5. Multivariable mixed effects linear regression of risk factors associated with the detection of *Cryptosporidium* oocysts and *Giardia* cysts in storm runoff from 5 dairies (natural log transformation used to stabilize variance).

	Cryptosporidium				*Giardia*			
	Runoff Concentration (oocysts/L)		Runoff Load (oocysts/min)		Runoff Concentration (oocysts/L)		Runoff Load (oocysts/min)	
Risk Factor	Regression Coefficient	P-value	Regression Coefficient	P-value	Regression Coefficient	P-value	Regression Coefficient	P-value
CATTLE AGE								
>1 year [a]	-	-	-	-	-	-	-	-
6-12 months	0.39	0.63	0.6	0.58	-0.64	0.43	-0.86	0.42
3-6 months	3.12	<0.0001[b]	4.42	<0.0001[b]	1.89	0.0037[b]	2.48	0.0037[b]
1-2 months	5.11	<0.0001[b]	7.18	<0.0001[b]	3.51	0.0002[b]	4.72	0.0001[b]
TOTAL SEASONAL PRECIPITATION (inches)	-0.087	0.0001[b]	-0.12	0.0001[b]	-0.0059	0.78	-0.0051	0.86
WATER TURBIDITY (ntu)	0.00007	0.27	0.0001	0.26	0.00014	0.02[b]	0.0002	0.018[b]
INTERCEPT	-5.42	<0.0001	-4.8	<0.0001	-6.21	<0.0001	-5.98	<0.0001

[a] Referent category for regression comparisons.

[b] Significant if P<0.1.

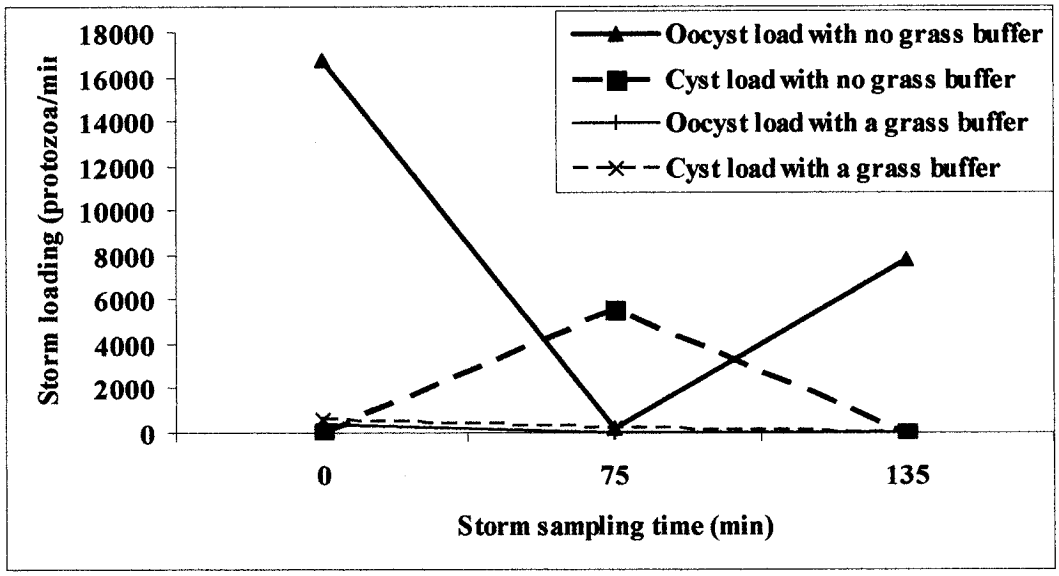


FIG. 5.3. Raw data example of *Cryptosporidium parvum* and *Giardia* loads in storm runoff from a calf dry lot site, with and without a grass buffer.

TABLE 5.6. Prevalence of >90% or >50% reduction in storm runoff load of *Cryptosporidium parvum* oocysts or *Giardia* cysts for BMP sites compared to adjacent control sites.

BMP	>90% parasite load reduction	>50% parasite load reduction
Water impoundment	100% (4/4)	100% (4/4)
Vegetative buffer	68% (30/44)	77% (34/44)
Mulch applied to lots	48% (12/25)	56% (14/25)
Lots scraped	29% (4/14)	29% (4/14)
Manure spread on pastures	77% (10/13)	85% (11/13)
Pastures irrigated	43% (3/7)	43% (3/7)
Cattle excluded	43% (4/5)	43% (4/5)

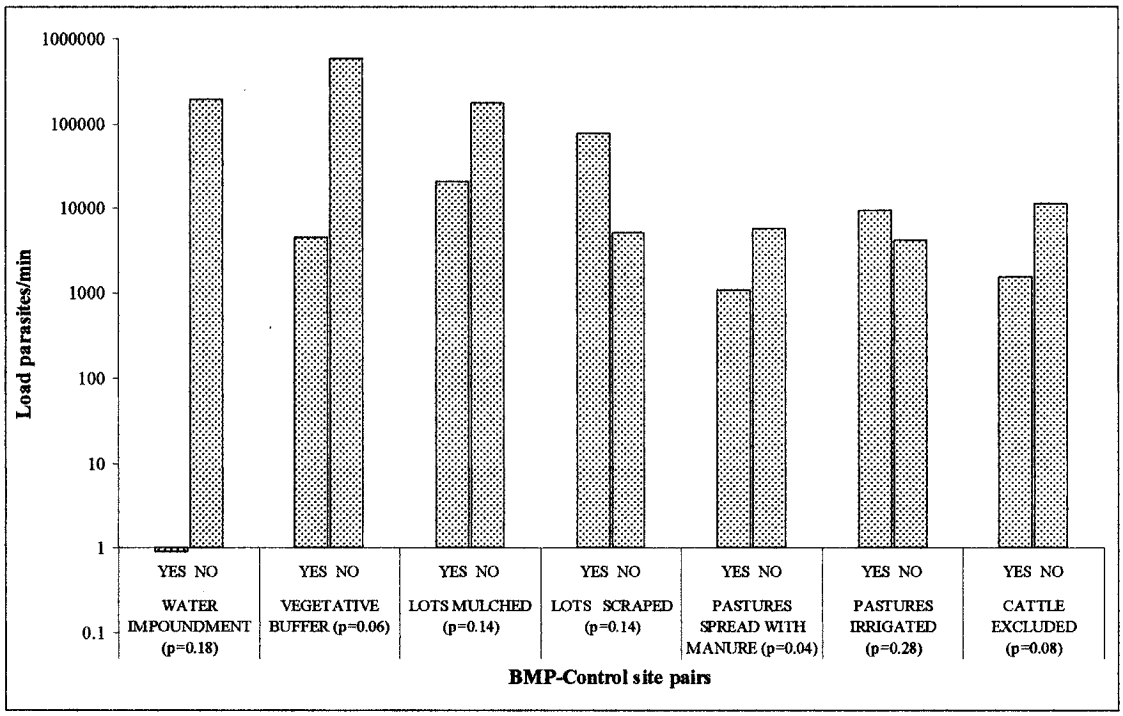


FIG. 5.4. Mean load of *Cryptosporidium* and *Giardia* in dairy runoff from sites treated with Best Management Practices (BMPs) and their matched control sites. Significantly lower parasite loads between BMP and control sites are indicated by p-values <0.1.

CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

CONCLUSIONS AND FUTURE DIRECTIONS

These studies evaluated innovative methods for detection of *Cryptosporidium* spp. in environmental samples, a historically challenging medium to work with when using molecular methods. The insights gained with regard to pathogen detection in water and shellfish samples will be of value to environmental monitoring agencies and public health agencies interested in sensitive and quantitative detection of zoonotic agents in water and food. Regardless of whether monitoring agencies such as the California State Mussel Watch Program or academic researchers at universities build on the work herein, important steps have been taken toward understanding and managing fecal pollution in aquatic ecosystems. As was the case with my dissertation research projects, a collaborative and interdisciplinary team effort may make the quickest progress towards understanding and controlling fecal pollution at the human-animal-environment interface.

Cryptosporidium spp. were detected in marine bivalves, freshwater bivalves, and storm runoff, though not in the majority of samples in any of the field studies. Over 4800 mussels, 700 clams, and 600 storm water samples were processed, and <20% were positive for *Cryptosporidium* spp. This is in contrast to other studies that have detected *Cryptosporidium* in the majority of bivalve and water samples tested (Tsushima et al., 2001, Bodley-Tickell et al., 2002; Graczyk et al., 2002, 2004). These differences could be attributable to a lesser degree of fecal contamination present along the California coast as compared to the other study areas on the Atlantic coast and in Japan. However, the differences could also be attributable to different study designs, in which *Cryptosporidium* detection may be affected by the sampling season, the number of samples collected, the assay sensitivities, the fecal loading sources, and the hydrologic

mixing of the aquatic ecosystems. It is also important to remember that an absence of evidence does not necessarily indicate an evidence of absence, such as when pathogens in the environment are diluted to concentrations beneath our assay detection limits, or when undersampling reduces our power to detect the pathogen during the study.

The *Cryptosporidium* genotypes identified in California bivalves were useful in identifying contributing fecal sources, such as *C. felis* and *C. andersoni* associated with cat and cattle feces, respectively. No conclusions about loading sources can be drawn based on the detection of the non-host specific *C. parvum* genotype 2 in bivalves collected from both higher and lower risk sites for fecal pollution. *Cryptosporidium* could be entering nearshore waters at the lower risk sites for fecal pollution if terrestrial non-point sources or unidentified point sources are loading oocysts, or if hydrologic mixing is carrying oocysts to lower risk sites from higher risk areas further along the coast. Additional investigation is required to further our understanding of the dynamics of fecal loading at the terrestrial-aquatic interface.

As with many studies, more work was done than is reported in this dissertation. Sometimes the seed projects that are generated as a sidebar to funded projects provide completely new avenues to explore, with unexpected but exciting sequelae. There is opportunity to build on the bivalve studies by changing the study designs to incorporate increased bivalve and water sampling to further investigate whether season and spatial associations will persist through time. There are opportunities to build on the storm runoff studies by extending the *Cryptosporidium* evaluation from the farm to the watershed scale. Laboratory and field studies are also needed to further evaluate these and other Beneficial Management Practices (BMPs) that have potential for improving

fecal waste management plans. The BMPs could focus on the animal aspects in efforts to reduce infection and shedding of *Cryptosporidium* oocysts, or on environmental aspects to minimize the spread of fecal pathogens on and around fecal loading sources.

Cryptosporidium spp. are one of many microbes that could be considered as bioindicators in ecosystem monitoring and management of fecal pollution. Other protozoal, bacterial, and viral indicators would have their own advantages and limitations. The ideal fecal bioindicator would be present in every fecal sample but not present in pristine environments, be easily detectable, survive as long as the most environmentally resistant pathogen of interest, and have genotypes unique to the animal host species. Because there may be no ideal fecal bioindicator, the best approach to ecosystem monitoring for fecal pollution may then be to screen for a suite of bioindicators that together can help identify polluted areas and contributing fecal sources. For example, *Giardia* seems to be even more widespread than *Cryptosporidium* spp. in the environment (Monis and Thompson, 2003; Becher et al., 2004; Horman et al., 2004), and the molecular tools are now becoming available to screen for host-specific genotypes in a similar manner to *Cryptosporidium* spp. Neither *Cryptosporidium* nor *Giardia* are as environmentally resistant as *Toxoplasma* oocysts, another pathogen of public health concern, but *Toxoplasma* oocysts are only shed by felid species and so aren't as useful in identifying sources of fecal pathogen pollution. Some bacterial species are present in all fecal samples, and studies have found that enterococci correlate better with fecal loading than the coliform bacteria that have traditionally been used as fecal bioindicators (Simpson et al., 2002).

As with the choice of fecal indicator microbes, there is also a choice as to the best bioindicator invertebrate. Mussels and clams were used in these studies because they are filter feeding invertebrates widely distributed in the ecosystems of interest, but it is possible that other invertebrates would also be useful bioindicators of fecal pollution. For example, a recent study of domoic acid in a variety of invertebrates in the Monterey Bay found that in contrast to mussels, innkeeper worms (*Urechis caupo*) retained much higher levels of domoic acid and for longer periods of time after the algal bloom had ceased (Goldberg, 2003). Further study is needed to evaluate whether innkeeper worms or other filter-feeding invertebrates are better environmental monitoring organisms than mussels. If other invertebrates are found to be more effective concentrators of biologic pathogens, the distribution and availability of the invertebrates in the ecosystems of interest will also need to be considered before embarking on large scale environmental monitoring projects.

In conclusion, this dissertation research has been both challenging and fulfilling. I made many new connections, with people, ideas, and within myself. I truly enjoyed experiencing both the laboratory and the field aspects of this research, and I learned many new ways of analyzing data and testing hypotheses. I see that there are many ways to approach a problem and assess solutions, and I look forward to applying my skills to new and interesting projects, in work and in life.

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