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Clams (*Corbicula fluminea*) as bioindicators of fecal contamination with *Cryptosporidium* and *Giardia* spp. in freshwater ecosystems in California☆

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Abstract

This study evaluated clams as bioindicators of fecal protozoan contamination using three approaches: (i) clam tissue spiking experiments to compare several detection techniques; (ii) clam tank exposure experiments to evaluate clams that had filtered *Cryptosporidium* oocysts from inoculated water under a range of simulated environmental conditions; (iii) sentinel clam outplanting to assess the distribution and magnitude of fecal contamination in three riverine systems in California. Our spiking and tank experiments showed that direct fluorescent antibody (DFA), immunomagnetic separation (IMS) in combination with DFA, and 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 a clam digestive gland 83% of the time. In the tank experiment, oocyst dose and clam collection time were significant predictors for detecting *Cryptosporidium parvum* oocysts in clams. In the wild clam study, *Cryptosporidium* and *Giardia* were detected in clams from all three study regions by IMS-DFA analysis of clam digestive glands, with significant variation by sampling year and season. The presence of *C. parvum* DNA in clams from riverine ecosystems was confirmed with PCR and DNA sequence analysis.

Keywords: *Cryptosporidium*; *Giardia*; Bivalve; Clam; Fecal pollution; Waterborne pathogens

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 (http://wonder.cdc.gov/mmwr/mmwr_reps.asp?mmwr_table=2B&mmwr_year=2004&mmwr_week=30), 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 *Cryptosporidium 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 (Robertson et al., 1992; Ong et al., 1999). *Cryptosporidium* and *Giardia* spp. are shed by a variety of animals, including humans, livestock, domestic animals, and wildlife (Xiao et al., 1999; Heitman et al., 2002; McGlade et al., 2003; Zhou et al., 2004). 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 recognised worldwide as bioindicators of aquatic contamination with fecal origin bacteria, viruses,

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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 PCR and direct immunofluorescent antibody (DFA) assays (Graczyk et al., 1998; Gomez-Couso et al., 2003). An advantage of conventional PCR is that the DNA sequences can be amplified and analysed to identify the parasite genotype, but a limitation of PCR is that results are not quantitative. In contrast, detection methods utilising 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 analysed 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 per h per 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 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., 1998, 2003; Izumi et al., 2004), but these clams have not been evaluated as bioindicators of fecal contamination in natural ecosystems.

In this study we 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: (i) to evaluate DFA, IMS-DFA, and PCR methods for *Cryptosporidium* detection using spiked clam tissues; (ii) to evaluate these methods on clams that had filtered oocysts from inoculated waters under a range of simulated environmental conditions; (iii) to evaluate outplanted clams as bioindicators of fecal pathogen pollution in three 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. The 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 visualisation 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 six replicates per tissue and oocyst dose. Spiked digestive gland samples were homogenised, sieved, and centrifuged to create a pellet that was re-suspended and split for DFA and PCR analysis. A 10 µl digestive gland aliquot from each of the six replicates was dried onto a slidewell for DFA analysis, with the rest of the tissue then concentrated with IMS followed by DFA analysis. Six hemolymph replicates were each 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 two water temperatures (10 and 20 °C) and three oocyst concentrations (20, 200, and 2000 oocysts/L water), using two 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 h exposure the clams were moved to clean freshwater tubs for *Cryptosporidium* depuration over the next 3 weeks. Water samples (500 ml) from the inoculated and negative control clam tanks were processed by centrifugation for 10 min at 1000×*g*, followed by IMS concentration and DFA analysis to quantitate the remaining oocysts. Clams were collected at 3, 6, 9 h, 1, 3, 7, 14, and 21 days after initial oocyst exposure. The effect of water temperature, oocyst dose, and clam collection timepoint were analysed 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 fecal contamination problems and inclusion in the state list of impaired water quality areas (http://www.waterboards.ca.gov/tmdl/303d_lists.html). The San Lorenzo River feeds into the Monterey Bay near Santa Cruz, CA, utilises mainly 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, 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, has some sewer and some septic tank areas, and has significant crop and animal production industries.

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 analysed for *Cryptosporidium* using IMS-DFA on six pools of five clam digestive glands from each batch of 30 clams. All *Cryptosporidium*-positive DFA slides were scraped, washed, amplified with PCR, and the sequences analysed to obtain parasite genotypes. Hemolymph was analysed for all clams individually. The pooling of clam digestive glands was possible because all the tissue could be analysed 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 µl 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 applied to all the wild clam samples collected in the second year of the study as described previously for *Cryptosporidium* testing.

2.2. Cryptosporidium oocysts

Wild-type *C. parvum* genotype 2 oocysts (synonymous with bovine genotype A), henceforth called *C. parvum*, 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, CA. 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 eight hemacytometer counts and confirmed with DFA enumeration. Oocyst suspensions were kept at 4 °C and used within 1 month of collection.

2.3. Clams

For the clam spiking and tank exposure experiments, *C. fluminea* were harvested near Oakley, CA, in cooperation with the California State Mussel Watch Program. Clams were transported on ice and held in freshwater flow-through tanks at the University of California, Davis, Aquatic Pathogen Facility. Clams were maintained for at least 3 weeks before being used in any experiments so they could acclimatise to their environment and depurate any residual oocysts they might have been exposed to in the wild. The tanks were constantly aerated and clams were fed Algamac (Aquafauna Biomarine Inc., Hawthorne, CA) every other day. Sentinel clams used for the outplanting studies were collected near Oakley or Davis, CA, 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* before 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 h 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 re-suspended 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, two 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 5 µl 1 N NaOH for neutralisation, 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, two IMS modifications were evaluated with six replicates per modification and spike dose. First, selected samples were processed using 50 µl of IMS beads instead of the usual 100 µl. 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 analysed 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 two wells per tissue sample. The DFA slides were incubated with anti-*Cryptosporidium* and anti-*Giardia* antibody, counterstained (Meridian Bioscience Inc.) for 30 min, and examined at 200–400× 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 5×7 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, designated PCR1 (Morgan et al., 1997) and PCR2 (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, PCR1, was used to amplify a 300 bp variable segment of the *Cryptosporidium* 18s rRNA gene. 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 10× 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 s, with a final cycle of 72 °C for 7 min.

The PCR2 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) 10× 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 one 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 *Giardia* PCR protocol was designed to amplify a 432 bp segment of the glutamate dehydrogenase gene from multiple genotypes of *Giardia 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 ρmol 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 two cycles 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 electrophoresis on a 2% agarose gel containing GelStar (Cambrex Co., East Rutherford, NJ). Product size and band intensity were 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 analysed with Chromas (Technelysium Pty Ltd, Tewantin, Qld, Australia) 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 three study regions along the San Lorenzo River, Salinas River, and Putah Creek, CA. Clam collection site data were categorised by study region and by upstream or downstream status. Information on sewage management was obtained from the Central Coast Regional Water Quality Control Board (http://www.waterboards.ca.gov/centralcoast/). 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 analysed. Second, the oocyst count data were modelled using Poisson regression for the DFA data and negative binomial regression for the IMS-DFA data (Pereira et al., 1999; Hardin and Hilbe, 2001; 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 modelling 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.

$$\text{Poisson } S(c_i) = 1 - e^{\beta x_i} \quad (1)$$

$$\text{Negative binomial } S(c_i) = 1 - [1/(1 + \alpha c_i W_i\, e^{\beta x_i})]^{1/\alpha} \quad (2)$$

The clam tank experiment IMS-DFA oocyst count data were also analysed using the negative binomial model 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 $P < 0.05$.

3. Results

3.1. Clam tissue spiking

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

Table 1
Percentage of *Cryptosporidium*-positive clams detected by direct fluorescent antibody (DFA), immunomagnetic separation (IMS) with DFA, and PCR methods for spiked hemolymph and digestive gland tissues

Oocysts	Digestive gland % positive				Hemolymph % positive	
	DFA	IMS-DFA	PCR1	PCR2	PCR1	PCR2
0	0	0	0	0	0	0
1	0	17	17	0	100	67
10	0	83	17	50	100	67
100	83	100	83	67	100	50
1000	nd[a]	nd	100	100	100	100

n = six replicates per technique and oocyst dose.
[a] nd, not done.

Table 2
Recovery efficiency of direct fluorescent antibody (DFA) and immunomagnetic separation (IMS) with DFA methods for *Cryptosporidium* oocysts spiked into clam digestive glands

C. parvum oocysts spiked per clam	DFA			IMS-DFA	
	Mean no. oocysts detected (range)	Mean % recovery per test (range)	Mean % recovery per clam (range)	Mean no. oocysts detected (range)	Mean % recovery (range)[a]
0	0	0	0	0	0
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)

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

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 one oocyst. For digestive gland samples tested by PCR1, 83% or more of all samples spiked with at least 100 oocysts, and 17% of digestive samples spiked with one or 10 oocysts were detected. In contrast, when tested by PCR2, 50% or more of samples spiked with 10 or more oocysts were detected, but no samples spiked with a single oocyst were detected. For spiked hemolymph samples, PCR1 detected 100% and PCR2 detected at least 50% of all samples spiked with one or more oocysts. In conclusion, IMS-DFA and PCR1 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 protocols.

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 2 shows the percentage 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 two oocysts were counted per test using DFA alone, which represented 46% of the oocysts expected in a 10 µl 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 one 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 one 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. Fig. 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 two oocysts and DT_{90} was eight 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 h after oocyst inoculation compared to positive control tanks that contained oocysts but no clams. All water and clam samples from the negative control clam tanks that contained water and clams but were not inoculated with oocysts tested negative at all timepoints.

First a subset of tissue samples was analysed by DFA, IMS-DFA, and PCR methods to determine the best method for use on the rest of the tank experiment samples.

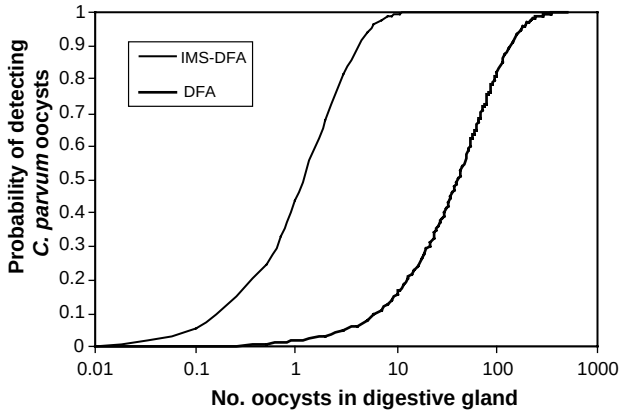

Fig. 1. Sensitivity of direct fluorescent antibody (DFA) and immunomagnetic separation (IMS) with DFA for detection of *Cryptosporidium* oocysts spiked into clam digestive glands, using Poisson and negative binomial regression, respectively.

Table 3
Proportion of *Cryptosporidium*-positive clams detected after a 6 h tank exposure to 250 oocysts/clam

Clam *Cryptosporidium* oocyst exposure status	Digestive gland % positive				Hemolymph % positive	
	DFA	IMS-DFA	PCR1	PCR2	PCR1	PCR2
Exposed ($n=21$)	24	52	24	0	24	0
Unexposed ($n=3$)	0	0	33[a]	0	66[a]	0

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

Table 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 PCR1, while all samples were negative by PCR2. Hemolymph samples tested by PCR2 were all negative but 24% were again positive by PCR1. Several samples tested by PCR1 from clams in the oocyst-unexposed tanks also produced gel bands of the appropriate size. Sequence analysis of the PCR1 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 analyse all the tank experiment samples. The number of oocysts detected per digestive gland varied widely, ranging from 0 to 242 oocysts. The majority of oocysts were detected during the first 9 h, 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 h post-exposure), the mean number of oocysts detected per assay for the middle dose and high dose at 3 h 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 h compared to 3 h post-exposure, but samples tested at >24 h post-exposure had significantly fewer oocysts compared to 3 h post-exposure. Testing clam tissues at these latter times increased 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 ($P=0.89$).

Fig. 2 illustrates the relationship between the mean observed oocysts per clam sample in relation to the predicted oocysts and 95% confidence interval for the three oocyst exposure doses over the 3 week experiment. The significant effect of oocyst dose is shown in the different *y*-axis values for the three graphs: the mean oocysts detected were 40 per clam in the high dose (250 oocysts/clam) tank at 3 h post-exposure, whereas the mean observed oocysts were only eight per clam at the middle dose (25 oocysts/clam), and one 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

Table 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

Parameter	Coefficient	95% Confidence interval		*P*-value
Oocyst dose/clam				
2.5 oocysts[a]	0.0	–	–	–
25 oocysts	1.62	0.73	2.52	<0.001
250 oocysts	3.59	2.61	4.57	<0.001
Clam collection time post-oocyst exposure				
3 h[a]	0.0	–	–	–
6 h	−0.57	−2.24	1.1	0.5
9 h	−1.69	−2.76	−0.62	0.002
1 day	−1.99	−3.31	−0.66	0.003
3 days	−6.28	−8.59	−3.98	<0.001
7 days	−3.37	−5.61	−1.12	0.003
14 days	−18.9	−20.08	−17.74	<0.001
21 days	−6.28	−8.56	−4.01	<0.001
Intercept	0.012	−0.88	0.91	0.98

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

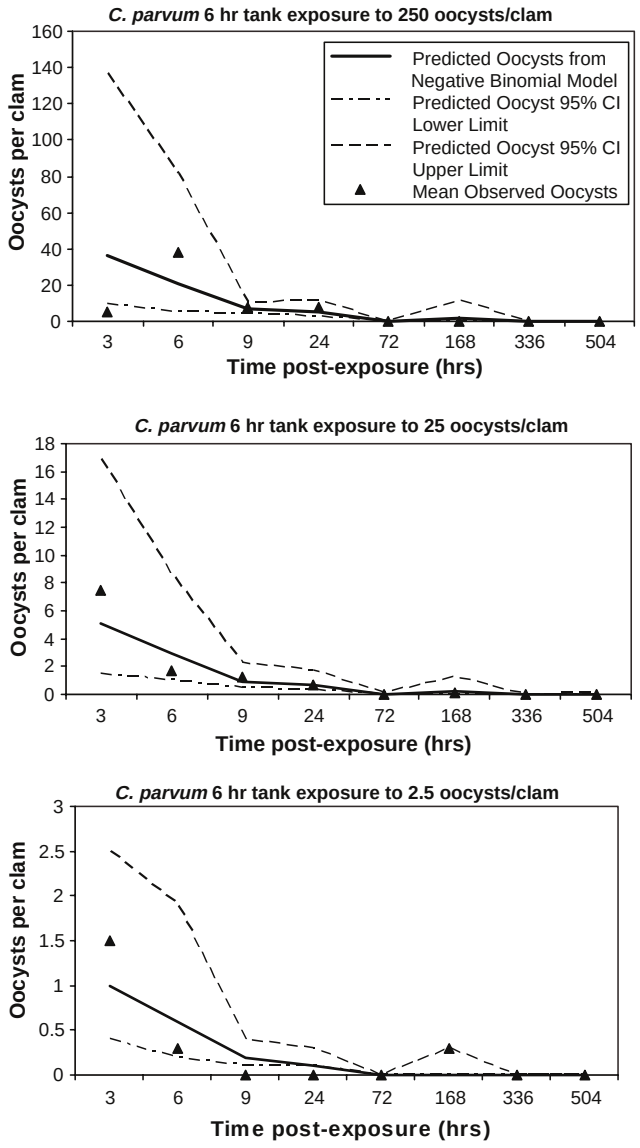

Fig. 2. *Cryptosporidium* oocysts detected in clam digestive gland by immunomagnetic separation with direct fluorescent antibody testing for three oocyst exposure doses used in a 6 h clam tank exposure experiment. Clams were tested at 3, 6, 9 h, 1, 3, 7, 14, and 21 days post-exposure.

oocysts detected while others had 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 three 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 three riverine study regions. The mean number of *Cryptosporidium* detected per positive clam pool in 2003 was two oocysts (range, 1–7). Fig. 3 shows the prevalence of *C. parvum* and *C. andersoni/muris*-like oocysts detected in clam batches by IMS-DFA during

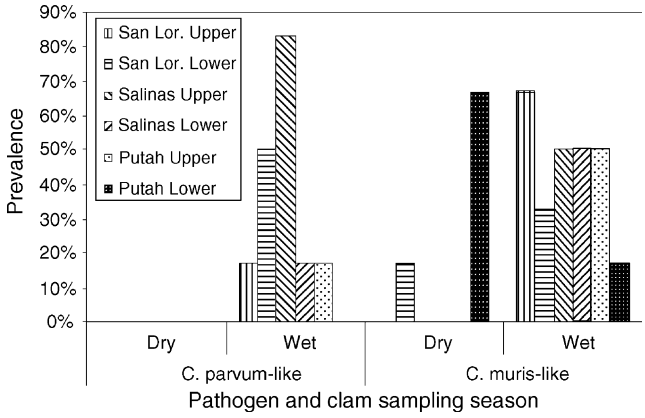

Fig. 3. *Cryptosporidium* spp. prevalence detected by immunomagnetic separation with direct fluorescent antibody testing of clam batches collected from three California rivers in 2003.

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* sequences were identified from DFA-positive slide scrapings from a San Lorenzo River clam and from a Salinas River clam (Genbank Accession Nos. AY864316 and AY864317). Individual hemolymph testing by PCR and DNA sequence analysis detected the same *C. parvum* sequences 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 analysed 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 three 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 5 shows the recovery efficiency of *Giardia* cysts spiked into clam digestive gland when using three

Table 5
Recovery efficiency of *Giardia* cysts spiked into clam digestive gland and processed by three immunomagnetic separation (IMS) protocols

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]
0	0	0	0
10	60 (50–75)	66 (38–88)	2 (0–6)
100	55 (44–64)	65 (57–71)	41 (26–63)

[a] Mean % recovery for six replicates (% recovery range).

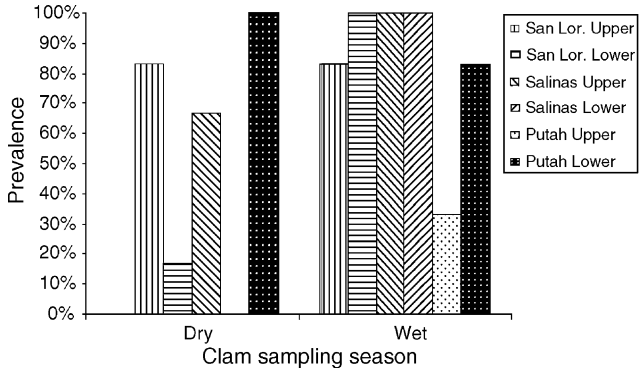

Fig. 4. *Giardia* spp. prevalence detected by immunomagnetic separation with direct fluorescent antibody testing of clam batches collected from three California rivers in 2003.

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 cysts spiked into digestive gland samples.

This IMS-DFA method was then applied to the 2003 sentinel clams. *Giardia* was detected in clams from all three riverine study regions. The mean number of cysts detected per clam pool was three (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. Fig. 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 three 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* 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 analysed per test, and the presence of substances inhibitory to PCR assays. The IMS concentration method overcomes these problems in two ways. First, the volume of processed sample is increased from 10 µl per DFA test or 50 µl per PCR test to 500 µl 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 to 10 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 maximise their assay sensitivity using IMS concentration, while minimising 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 $US 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 analysed (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 (United States Environmental Protection Agency, 2001; Sturbaum et al., 2002), 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 analysed 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 analysed. 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. 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. As with our study, Sturbaum et al. (2002) observed that false positives can occur with PCR detection if DNA sequence analysis is not performed. 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 *Cryptosporidium hominis*, *Cryptosporidium baileyi*, and *C. parvum* in oysters (Fayer et al., 1998). *Cryptosporidium hominis* and *C. baileyi* oocysts are primarily shed by humans and chickens, respectively, while *C. parvum* oocysts are shed by both human 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*, 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/digestive gland sample). Each of these samples represented the total number of oocysts detected by IMS-DFA in half a clam digestive gland, unadjusted for the IMS 58% oocyst recovery efficiency. Because the whole tissue pellet could be concentrated by IMS for each clam sample, the variability is more likely attributable to biologic variability in clams filtering oocysts than assay limitations that occur when only a sub-sample of the tissue pellet can be analysed. 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 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 was 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 to 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 three weeks post-exposure.

Our wild clam study utilised 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 three 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. During the 2002 dry and wet season

sentinel clam testing, all three 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 three 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, and 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* and *Giardia* were detected more often in the wet than the dry seasons in this study. An explanation for the observed seasonality is that *Cryptosporidium* oocysts and *Giardia* cysts enter the riverine systems primarily via overland runoff during storm events. Another explanation is that oocyst and cyst shedding may occur more often or at a greater magnitude during the wet season (November–April) than the dry season (May–October) in California. 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). This variability in outcomes may be attributable to the different environmental factors and study designs used.

This sentinel clam study was the first to utilise 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 the fecal protozoal pathogens *Cryptosporidium* and *Giardia* in two of the major freshwater tributaries that feed into the southern sea otter home range.

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References

Arrowood, M.J., Sterling, C.R., 1987. Isolation of *Cryptosporidium* oocysts and sporozoites using discontinuous sucrose and isopycnic Percoll gradients. J. Parasitol. 73, 314–319.

Atwill, E.R., Rose, T.L., 1998. *Corbicula fluminea* as an environmental assay for detection of *Cryptosporidium parvum* in surface waters. Technical Completion Report, Water Resources Center, University of California, pp. 1–46.

Atwill, E.R., Hoar, B.R., Pereira, M.D.G., Tate, K.W., Rulofson, F., Nader, R., 2003. Improved quantitative estimates of low environmental loading and sporadic periparturient shedding of *Cryptosporidium parvum* in beef cattle. Appl. Environ. Microbiol. 89, 4604–4610.

Fayer, R., Graczyk, T.K., Lewis, E.J., Trout, J.M., Farley, C.A., 1998. Survival of infectious *Cryptosporidium parvum* oocysts in seawater and eastern oysters (*Crassotrea virginica*) in the Chesapeake Bay. Appl. Environ. Microbiol. 64, 1070–1074.

Fayer, R., Lewis, E.J., Trout, J.M., Graczyk, T.K., Jenkins, M.C., Higgins, J., Xiao, L., Lal, A., 1999. *Cryptosporidium parvum* in oysters from commercial harvesting sites in the Chesapeake Bay. Emerg. Infect. Dis. 5, 706–710.

Fayer, R., Trout, J.M., Lewis, E.J., Xiao, L., Lal, A., Jenkins, M.C., Graczyk, T.K., 2002. Temporal variability of *Cryptosporidium* in the Chesapeake Bay. Parasitol. Res. 88, 998–1003.

Freire-Santos, F., Oteiza-Lopez, A.M., Vergara-Castiblanco, C.A., Arees-Mazas, M.E., Alvarez-Suarez, E., Garcia-Martin, O., 2000. Detection of *Cryptosporidium* oocysts in bivalve molluscs destined for human consumption. J. Parasitol. 86, 853–854.

Gomez-Bautista, M., Ortega-Mora, L.M., Tabares, E., Lopez-Roda, V., Costas, E., 2000. Detection of infectious *Cryptosporidium parvum* oocysts in mussels (*Mytilus galloprovincialis*) and cockles (*Cerastoderma edule*). Appl. Environ. Microbiol. 66, 1866–1870.

Gomez-Couso, H., Freire-Santos, F., Martinez-Urtaza, J., Garcia-Martin, O., Mazas, M.E., 2003. Contamination of bivalve molluscs by *Cryptosporidium* oocysts: need for new quality control standards. Int. J. Food Microbiol. 87, 97–105.

Graczyk, T.K., Fayer, R., Cranfield, M.R., Conn, D.B., 1998. Recovery of waterborne *Cryptosporidium parvum* oocysts by freshwater benthic clams (*Corbicula fluminea*). Appl. Environ. Microbiol. 64, 427–430.

Graczyk, T.K., Fayer, R., Lewis, E.J., Trout, J.M., Farley, C.A., 1999. *Cryptosporidium parvum* in bent mussels (*Ischadium recurvum*) in the Chesapeake Bay. Parasitol. Res. 85, 518–521.

Graczyk, T.K., Conn, D.B., Marcogliese, D.J., Graczyk, H., de Lafontaine, Y., 2003. Accumulation of human waterborne parasites by zebra mussels (*Dreissena polymorpha*) and Asian freshwater clams (*Corbicula fluminea*). Parasitol. Res. 89, 107–112.

Hardin, J., Hilbe, J., 2001. Negative binomial family. In: Generalized Linear Models and Extensions. Stata Press, College Station, TX, pp. 141–158.

Heitman, T.L., Frederick, L.M., Viste, J.R., Guselle, N.J., Morgan, U.M., Thompson, R.C.A., Olson, M.E., 2002. Prevalence of *Giardia* and *Cryptosporidium* and characterization of *Cryptosporidium* spp. isolated from wildlife, human, and agricultural sources in the North Saskatchewan River Basin in Alberta, Canada. Can. J. Microbiol. 48, 530–541.

Izumi, T., Itoh, Y., Yagita, K., Endod, T., Ohyama, T., 2004. Brackish water benthic shellfish (*Corbicula japonica*) as a biological indicator for *Cryptosporidium parvum* oocysts in river water. Bull. Environ. Contam. Toxicol. 72, 29–37.

McGlade, T.R., Robertson, I.D., Elliot, A.D., Read, C., Thompson, R.C.A., 2003. Gastrointestinal parasites of domestic cats in Perth, Western Australia. Vet. Parasitol. 117, 251–262.

McMahon, R.B., Bogan, A.E., 2001. Mollusca:Bivalvia. In: Thorp, J.H., Covich, A.P. (Eds.), Ecology and Classification of North American Freshwater Invertebrates. Academic Press, San Diego, CA, pp. 331–397.

Miller, M.A., Gardner, I.A., Kreuder, C., Paradies, D.M., Worcester, K.R., Jessup, D.A., Dodd, E., Harris, M.D., Ames, J.A., Packham, A.E., Conrad, P.A., 2002. Coastal freshwater runoff is a risk factor for *Toxoplasma gondii* infection in southern sea otters (*Enhydra lutris nereis*). Int. J. Parasitol. 32, 997–1006.

Miller, M.A., Grigg, M.E., Kreuder, C., James, E.R., Melli, A.C., Crosbie, P.R., Jessup, D.A., Boothroyd, J.C., Brownstein, D., Conrad, P.A., 2004. An unusual genotype of *Toxoplasma gondii* is common in California sea otters (*Enhydra lutris nereis*) and is a cause of mortality. Int. J. Parasitol. 34, 275–284.

Monis, P.T., Thompson, R.C.A., 2003. *Cryptosporidium* and *Giardia*-zoonoses: fact or fiction? Infect. Genet. Evol. 3, 233–244.

Montoya, J.G., Liesenfeld, O., 2004. Toxoplasmosis. Lancet 363, 1965–1976.

Morgan, U.M., Constantine, C.C., Forbes, D.A., Thompson, R.C.A., 1997. Differentiation between human and animal isolates of *Cryptosporidium parvum* using rDNA sequencing and direct PCR analysis. J. Parasitol. 83, 825–830.

O'Connor, T.P., 2002. National distribution of chemical concentrations in mussels and oysters in the USA. Mar. Environ. Res. 53, 117–143.

Okhuysen, P.C., Chappell, C.L., Crabb, J.H., Sterling, C.R., DuPont, H.L., 1999. Virulence of three distinct *Cryptosporidium parvum* isolates from healthy adults. J. Infect. Dis. 180, 1275–1281.

Ong, C.S., Eisler, D.L., Goh, S.H., Tomblin, J., Awad-El-Kariem, F.M., Beard, C.B., Xiao, L., Sulaiman, I., Lal, A., Fyfe, M., King, A., Bowie, W.R., Isaac-Renton, J.L., 1999. Molecular epidemiology of cryptosporidiosis outbreaks and transmission in British Columbia, Canada. Am. J. Trop. Med. Hyg. 61, 63–69.

Pereira, M.D.G., Atwill, E.R., Jones, T., 1999. Comparison of sensitivity of immunofluorescent microscopy to that of a combination of immunofluorescent microscopy and immunomagnetic separation for detection of *Cryptosporidium parvum* oocysts in adult bovine feces. Appl. Environ. Microbiol. 65, 3226–3239.

Pommepuy, M., Dumans, F., Caprais, M.P., Camus, P., LeMennec, C., Parnaudeau, S., Haugarreau, L., Sarrette, B., Vilagines, P., Pothier, P., Kholi, E., LeGuyader, F., 2004. Sewage impact on shellfish microbial contamination. Water Sci. Technol. 50, 117–124.

Read, C.M., Monis, P.T., Thompson, R.C.A., 2004. Discrimination of all genotypes of *Giardia duodenalis* at the glutamate dehydrogenase locus using PCR-RFLP. Infect. Genet. Evol. 4, 125–130.

Robertson, L.J., Gjerde, B., 2001. Occurrence of *Cryptosporidium* oocysts and *Giardia* cysts in raw waters in Norway. Scand. J. Public Health 29, 200–207.

Robertson, L.J., Campbell, A.T., Smith, H.V., 1992. Survival of *Cryptosporidium parvum* oocysts under various environmental pressures. Appl. Environ. Microbiol. 58, 3494–3500.

Rose, J.B., 1997. Environmental ecology of *Cryptosporidium* and public health implications. Ann. Rev. Public Health 18, 135–161.

Sturbaum, G.D., Klonicki, P.T., Marshall, M.M., Jost, B.H., Clay, B.L., Sterling, C.R., 2002. Immunomagnetic separation (IMS)-fluorescent antibody detection and IMS-PCR detection of seeded *Cryptosporidium parvum* oocysts in natural waters and their limitations. Appl. Environ. Microbiol. 68, 2991–2996.

Tamburrini, A., Pozio, E., 1999. Long-term survival of *Cryptosporidium parvum* oocysts in seawater and in experimentally infected mussels (*Mytilus galloprovincialis*). Int. J. Parasitol. 29, 711–715.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucl. Acids Res. 25, 4876–4882.

Tsushima, Y., Karanis, P., Kamada, T., Makala, l., Xuan, X., Tohya, Y., Akashi, A., Nagasawa, H., 2003. Seasonal change in the number of *Cryptosporidium parvum* oocysts in water samples from the rivers in Hokkaido, Japan, detected by ferric sulfate flocculation method. J. Vet. Med. Sci. 65, 121–123.

United States Environmental Protection Agency, 2001. Method 1623: *Giardia* and *Cryptosporidium* in water by filtration/IMS/FA. Office of Water, Washington, DC, EPA 821-R01-025.

Ware, M.W., Wymer, L., Lindquist, H.D., Schaefer, F.W., 2003. Evaluation of an alternative IMS dissociation procedure for use with Method 1622: detection of *Cryptosporidium* in water. J. Microbiol. Methods 55, 575–583.

Xiao, L., Morgan, U.M., Limor, J., Escalante, A., Arrowood, M., Shulaw, W., Thompson, R.C.A., Fayer, R., Lal, A.A., 1999. Genetic diversity within *Cryptosporidium parvum* and related *Cryptosporidium* species. Appl. Environ. Microbiol. 65, 3386–3391.

Zhou, L., Fayer, R., Trout, J.M., Ryan, U.M., Schaefer, F.W., Xiao, L., 2004. Genotypes of *Cryptosporidium* species infecting fur-bearing mammals differ from those of species infecting humans. Appl. Environ. Microbiol. 70, 7574–7577.