Title

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CRYPTOSPORIDIUM IN BIVALVES AS INDICATORS OF FECAL POLLUTION IN THE CALIFORNIA COASTAL ECOSYSTEM

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Abstract

Bivalve molluscs concentrate *Cryptosporidium* oocysts from fecal-contaminated aquatic environments and are useful for 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. The most sensitive method for the detection of *C. parvum* in tank-exposed mussels was IMS concentration with DFA detection: 80% of individual and 100% of pooled digestive gland samples tested positive.

The wild mussel study was conducted to evaluate estuarine and nearshore marine *Mytilus* spp. as bioindicators of fecal contamination in coastal ecosystems of California. Hemolymph samples from 4680 mussels were tested for *Cryptosporidium* genotypes using PCR amplification and DNA sequence analysis. *Cryptosporidium* genotypes detected in hemolymph samples from individual mussels included *Cryptosporidium parvum*, *Cryptosporidium felis, Cryptosporidium andersoni*, and two novel *Cryptosporidium* spp. Factors significantly associated with detection of *Cryptosporidium* spp. in mussel batches were exposure to freshwater outflow and mussel collection within a week following a precipitation event.

The freshwater clam study evaluated *Corbicula* spp. as bioindicators of fecal protozoan contamination using three approaches: 1) clam tissue spiking experiments to compare several detection techniques, 2) clam tank exposure experiments to evaluate clams that that had 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 three riverine systems in California. The spiking and tank experiments showed that DFA, IMS in combination with DFA, and PCR techniques could be used to detect *Cryptosporidium* in clam tissues. In the tank experiment, oocyst dose and collection time were significant predictors for detection of *C. parvum* oocysts in clams. In the wild clam study, *Cryptosporidium* and *Giardia* were detected in clams from all three study regions by IMS in combination with DFA analysis of clam digestive glands, with significant variation by sampling year and season. These bivalve studies have shown that clams and mussels can be used to monitor water quality in California and suggests 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.

Introduction and Problem Statement

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 (MacKenzie et al., 1994). Clinical disease in immunocompetent humans generally consists of self-limiting diarrhea resulting from either *Cryptosporidium hominis* or the anthropozoonotic *C. parvum* genotype 2 (Morgan-Ryan et al., 2002, Okhuysen et al., 1999). Immunocompromised humans can be chronically infected, sometimes fatally, with these as well as other *Cryptosporidium* species (Current et al., 1993; Pieniazek et al., 1999). 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 (Bonadonna et al., 2002; Payment et al., 2001). *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 (Atwill et al., 2001; Fayer et al., 1998; McGlade et al., 2003; Okhuysen et al., 1999).

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 (Pereira et al., 1999; Xiao and Herd, 1993). 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 (Pereira et al., 1999). 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 (Deng et al., 2000; Webster et al., 1996). Unlike DFA, conventional PCR techniques do not provide quantitative data and do not allow for oocyst visualization. The real-time TaqMan PCR system utilized in this project 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 (Fontaine and Guillot, 2003a; Guy et al., 2003; Higgins et al., 2001; Limor et al., 2002; MacDonald et al., 2002), 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 expected in environmental samples (Hallier-Soullier and Guillot, 2000; Rose, 1997).

Environmental monitoring for *Cryptosporidium* spp. can be problematic, 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 (Feng et al., 2003; Hallier-

Soullier and Guillot, 2000). Oocysts can be concentrated from large volumes of water mechanically, but expensive equipment and supplies are required. 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 (McMahon, 1991). These bivalves can then be collected and tested for pathogens, providing an indication of water quality (Freire-Santos et al., 2000; Graczyk et al., 2003; Miller et al., 2005; Tamburrini and Pozio, 1999). Studies on the Atlantic coast of 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 (Fayer et al., 1998b, 1999; Gomez-Bautista et al., 2000; Gomez-Couso et al., 2004, Graczyk et al., 1999). The IMS concentration technique is well accepted as an integral part of water testing methods (Sturbaum et al., 2002; US EPA, 2001), but its potential to improve detection of *Cryptosporidium* spp. in bivalve tissues has not been critically evaluated. In addition to evaluating innovative *Cryptosporidium* detection techniques in the laboratory, our research group is the first to utilize freshwater clams and estuarine/marine mussels as water quality monitoring tools on the Pacific coast of North America.

Objective

The objective of this study was to obtain critical data on the epidemiology of the fecal pathogen *Cryptosporidium* in freshwater, estuarine, and nearshore marine ecosystems along the California coast. We aimed to evaluate innovative *Cryptosporidium* detection techniques, to assess the magnitude and genotypes of *Cryptosporidium* flowing from land to sea, and to identify risk factors for fecal contamination in coastal California ecosystems. Our hypotheses were that:

1. A newly developed real-time TaqMan PCR system is as sensitive as the traditional conventional PCR and DFA *Cryptosporidium* detection techniques, with the added advantages of semi-quantitative results and increased sample throughput.

2. *Cryptosporidium* spp. are present in bivalve shellfish collected from sites exposed to fecal contamination in California, including sites near human sewage outfalls and livestock runoff.

3. *Cryptosporidium* genotypes detected in bivalves are associated with risk factors such as fecal risk status (near human sewage outfalls, near livestock runoff, or distant from both sources), season (wet or dry), and freshwater outflow exposure (high, medium, or low exposure to river/stream outflow).

Procedures

This study was conducted in three parts. First, innovative techniques were evaluated by comparing a TaqMan PCR system to traditional *Cryptosporidium* detection techniques using a series of mussel spiking and saltwater tank exposure experiments. Second, a multi-year wild mussel study was conducted by outplanting and collecting mussels at study sites along the central California coast

considered at higher risk or lower risk sites for fecal contamination based on distance from known sources of human and livestock sewage influence. Third, freshwater clams were evaluated as bioindicators of fecal contamination using spiking, tank exposure experiments, and outplanting in riverine systems that feed into the central California coastal waters.

Part 1: Evaluation of *Cryptosporidium* detection techniques in bivalves

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 0-10,000 *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. duodenalis*.

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 tissue processing, to allow the analytical sensitivity estimates to reflect any oocyst loss during 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^{o}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 (Arrowood and Sterling, 1987). 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^{o}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 stored at -80^{o}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 (Fayer et al., 1998b). 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^{o}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 a variety of *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. (Limor et al., 2002), and primers were newly designed to amplify a short PCR product to maximize analytical sensitivity. The sensitivity was 50 times greater when testing complementary DNA (cDNA) transcribed from

messenger RNA (mRNA) as compared to testing genomic DNA (gDNA), 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 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. The 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, henceforth called PCR1 (Xiao et al., 1999) and PCR2 (Morgan et al., 1997), respectively, were used for comparison with the TaqMan PCR system. Primers are listed in Table 1. The

PCR1 18S rRNA nested protocol first amplified a 1325 bp DNA segment and then an 850 bp DNA segment in the nested PCR step. The PCR2 18S rRNA protocol amplified a 298 bp DNA fragment. Extraction of gDNA and conventional PCR protocols were performed as described (Miller et al., 2005). For selected isolates, the PCR products were purified and the sequences analyzed as described above.

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) and analyzed as described (Miller et al., 2005). 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. All slides were read by the same microscopist. *Cryptosporidium* parasites were identified as 5 µm diameter oocysts outlined in apple green, often with a midline seam. 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 described (Miller et al., 2005), followed by DFA or TaqMan PCR for *Cryptosporidium* detection. During IMS concentration, the IMS bead-parasite complexes were bound to a magnetic holder while the supernatant and debris were discarded during a series of wash steps. For parasite dissociation from the beads, 2 acid washes of 50 µl 0.1 N HCl were vortexed at the beginning and end of two 10 minute incubations, with the IMS product then neutralized and transferred to a slidewell or PCR tube for parasite detection. Hemolymph was not suitable for IMS because hemocytes adhered 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.

Part 2: Marine and estuarine wild mussel studies

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. Fig. 4a shows the nine site locations, with more detailed maps (Fig. 4b-d) of subsites located within the northern (38.114^{o}N, 122.869^{o}W), middle (36.815^{o}N, 121.791^{o}W), and southern (35.342^{o}N, 120.813^{o}W) regions. The three 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 (*Mytilus 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 *Mytilus 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 two days to the University of California, Davis for *Cryptosporidium* testing. All mussels were tested using PCR to amplify *Cryptosporidium* DNA from hemolymph samples, with 30 mussels per batch individually tested.

Statistical analyses were conducted to evaluate factors associated with *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 regression analysis. Next, logistic regression was used to assess putative factors associated with detection of *Cryptosporidium* spp. in mussel batches including fecal risk category, season, exposure to freshwater outflow, recent precipitation, bivalve type, and water type.

Polymerase chain reaction

Three *Cryptosporidium* PCR protocols were initially compared to determine analytic sensitivity for detection of the low numbers of oocysts that may be expected in environmental samples. Serial dilutions of *C. parvum* were made from purified oocysts as described (Miller et al., 2005). For each PCR protocol, three replicate dilution sets were evaluated that contained 0, 1, 10, 100, 1000, or 10000 oocysts. A direct 18S rRNA PCR protocol (Morgan et al., 1997) and a nested 18S rRNA PCR protocol (Xiao et al., 1999), designated PCR1 and PCR2, respectively, were used for *Cryptosporidium* spp. detection as described (Miller et al., 2005). The PCR3 protocol amplified an alternative DNA locus from the *Cryptosporidium* Outer Wall Protein (COWP) as described (Spano et al., 1997). The PCR products were 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.

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 six months in saltwater tanks at the Bodega Bay or Granite Canyon Marine Laboratory facilities. To ensure that detecting *Cryptosporidium* in sentinel mussels 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 in batches of 30 at each site and sampling time. Hemolymph was extracted and processed as described above.

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, the DNA sequences were aligned with GenBank reference sequences in ClustalX to determine whether our sequence was an exact match to a GenBank reference sequence. 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* (AF093490), *C. hominis* (AF093489), *Cryptosporidium wrairi* (AF115378), *Cryptosporidium meleagridis* (AF112574), *C. canis* (AJ493209), *C. felis* (AF108862), *C. baileyi* (AF093495), *Cryptosporidium 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).

Factors associated with Cryptosporidium detection in mussels

Data on factors that may be associated with *Cryptosporidium* detection in mussels was collected for all mussel sites. Each site was categorized as higher risk for human feces, higher risk for livestock feces, or lower risk for these fecal inputs based on being <1 km or >5 km, respectively, from known 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 that assumed exponential dilution once 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 using logistic regression to evaluate the odds of detecting *Cryptosporidium* spp. in mussel batches collected during the three year study based on the putative factors. Univariate analysis was performed with each 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 factors. Two outcome variables were of interest: detecting all *Cryptosporidium* spp, and detecting *C. parvum* or *C. hominis*, the *Cryptosporidium* of greatest 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.

Part 3: Freshwater clam studies

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 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 six 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 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 ^{o}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 three 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 h, 6 h, 9 h, 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 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, utilizes 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 analyzed 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 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 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.

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, California and processed as described above.

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 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 acclimatize 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, 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 before DFA and IMS-DFA analysis.

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 as described above. 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 ^{o}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.

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 as described above. *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.

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 tissue as described above. 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 μm 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 cycles of 94 ^{o}C for 2 min, 56 ^{o}C for 1 min, and 72 ^{o}C for 2 min, followed by 55 cycles of 94 ^{o}C for 30 s, 56 ^{o}C for 20 s, and 72 ^{o}C for 45 s, with a final extension of 72 ^{o}C for 7 min. Amplified PCR products were separated by gel electophoresis and sequenced as described above.

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 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 (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.

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 (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 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.

Results

Part 1: Evaluation of *Cryptosporidium* detection techniques in bivalves

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 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. As expected, 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 µl 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 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). 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 (PCR1) techniques detected 33% of spiked samples while the Morgan (PCR2) technique detected 50% or more of positive samples. No false positives were detected in negative control samples.

Table 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 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^{o} 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 established test performance paramenters, quantitative detection techniques such as TaqMan PCR and DFA analysis provided data that could be used to estimate the parasite concentration per sample. In the case of TaqMan PCR, the Ct value of an "unknown" sample could be correlated to the Ct values of known oocyst dilutions in bivalve tissues. For example, in Figure 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 1), suggesting that any residual mussel tissue did 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 could be estimated by adjusting the data based on percent recovery estimates. Table 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 µl 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 adjusted 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 $\leq$ 0.5 ml) ($P=0.33$), suggesting that testing pooled samples could decrease cost without significant loss of sensitivity.

Part 2: Marine and estuarine wild mussel studies

Polymerase chain reaction

The 18S rRNA protocols PCR1 and PCR2 had lower minimum oocyst detection limits than the COWP protocol PCR3 based on testing three replicates of *C. parvum* serial dilutions that contained 0, 1, 100, 1000, or 10000 oocysts. The PCR1 protocol was the most sensitive overall, detecting 1-10 oocysts in all three trials. The PCR2 protocol also detected a single oocyst in two trials but only detected 1,000 or more oocysts in a third trial. The PCR3 protocol detected 100 oocysts in two trials and 1000 oocysts in the third trial. Based on the decreased sensitivity of PCR3, it was excluded from further use in this study. Based on our previous study (Miller et al., 2005) that showed that the PCR1 protocol could amplify non-*Cryptosporidium* DNA of the 300 bp target size from environmental samples, DNA sequence analysis was required and used in order to designate a sample as being *Cryptosporidium*-positive.

Mussel testing

Over the three 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 inclement weather, high seas, or unfavorable tides. In total, 4680 mussels were tested by PCR1 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 PCR1, and occasionally more than one genotype was detected in a mussel batch. However, only two of the strongest PCR1-positive samples (~300 bp target) were also positive by PCR2 (~850 bp target). Therefore, all genotype results reported hereafter were obtained using the PCR1 protocol in combination with DNA sequence analysis.

Table 6 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* 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 risk site during the wet season. At a higher risk site for livestock feces, *C. felis* was detected in mussel hemolymph during both the dry and wet seasons. Additionally, a novel sequence closely related to *Cryptosporidium* spp. based on the BLAST search results, designated Non-Crypto-1 (GenBank accession no. AY874869), was identified in two mussels from a higher risk site for livestock feces during the dry season.

Cryptosporidium genotype results from Years 2 and 3 are shown in Table 7. Mussel batches were collected during the early and late wet and dry seasons for both years. *Cryptosporidium parvum* 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* DNA sequences, designated New-1 and New-2 (GenBank accession nos. AY874868 and AY874867, respectively), were detected in mussels from higher risk sites for livestock feces and human feces during the wet season samplings, and again in mussels from a livestock impacted site in the early dry season. An additional sequence closely related to *Cryptosporidium* spp. based on BLAST search results, designated Non-Crypto-2 (GenBank accession no. AY874866), was detected in a mussel from a higher risk site for human feces. The New-1 *Cryptosporidium* sequences were detected in mussels collected from two sites separated by over 200 km. For all mussel batches in which novel *Cryptosporidium* DNA sequences were detected, only 1-2 mussels were positive (≤7% prevalence per batch of 30 mussels).

Cryptosporidium spp. were detected in mussels collected within estuaries (Fig. 4) but not in the matched subsites located outside the estuaries. None of the Tomales Bay subsites in Figure 4b tested positive for *Cryptosporidium* spp. at any timepoint. Estuarine mussel batches from the Moss Landing region in Figure 4c (subsites 4A-4D) tested positive for *C. andersoni* and novel *Cryptosporidium* sequences New-1 and New-2 during the Year 2 wet season but not during the Year 3 wet season. However, the Moss Landing mussel collection site outside the estuary (4E) was never positive for *Cryptosporidium* spp.. Similarly to the Moss

Landing region, estuarine mussel batches from the Morro Bay region in Figure 4d (subsite 9C) tested positive for *C. andersoni* and novel *Cryptosporidium* sequence New-1 in the Year 2 wet season, while no *Cryptosporidium* spp. were detected in the Year 3 wet season, and site 9A located outside Morro Bay was negative during both seasons.

Phylogenetic analyses

The PCR1 protocol amplified *Cryptosporidium* and non-*Cryptosporidium* segments of the 18S rRNA gene from bp 450-748 in mussel samples. Of the 189 PCR products, BLAST searches in GenBank revealed 19 DNA sequences matching reference *Cryptosporidium* spp., 16 DNA sequences that did not match reference *Cryptosporidium* sequences exactly but were most closely related to *Cryptosporidium* spp., 64 DNA sequences that were most closely related to non-*Cryptosporidium* dinoflagellates and aplicomplexans, 40 DNA sequences matching *Mytilus* spp., and 50 DNA sequences that were mixed or of poor quality and could not be identified. The 16 novel DNA sequences related to *Cryptosporidium* spp. represented four unique sequences (New-1, New-2, Non-Crypto-1, and Non-Crypto-2) that were then included in the phylogenetic analysis (Figure 5) to further investigate their relationships to reference *Cryptosporidium* spp. and other protozoa. The same relationships were inferred using neighbor joining and maximum parsimony analysis with 1000 bootstrap replicates: the *Cryptosporidium*-like sequences New-1 and New-2 were classified within the *Cryptosporidium* clade, while the Non-Crypto-1 and Non-Crypto-2 sequences were classified outside the *Cryptosporidium* clade. The New-1 sequence was most closely related to *C. andersoni*, with all 18 nucleotide changes found in the polymorphic region from bp 637-693. The New-2 sequence was most closely related to *C. serpentis*, with a single nucleotide addition at bp 515. New-1 and New-2 were classified as *Cryptosporidium* spp. for the regression analysis, while Non-Crypto-1 and Non-Crypto-2 were not included.

Factors associated with Cryptosporidium detection in mussels

Table 8 shows the univariate analysis of factors associated with the detection of *Cryptosporidium* spp. in mussel batches. Higher or lower risk status for fecal exposure was not significantly associated with detection of *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 of *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 seven 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 day or month preceding mussel collection.

A multivariable logistic regression model was created to assess multiple factors simultaneously. Table 9 shows the adjusted odds ratios for the two factors significantly associated with detecting *Cryptosporidium* spp. in mussels in the final model. Freshwater outflow status remained a significant 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 seven 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 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. A second multivariable logistic regression model was created that only considered one site within each of the three estuaries per time point, in addition to the non-estuarine sites. The previous finding of freshwater outflow as a factor significantly associated with *Cryptosporidium* detection in mussel batches was again significant ($P<0.01$) while the factor of precipitation in the week preceding mussel collection was not statistically significant ($P=0.12$), possibly due to the decreased power from the smaller data set.

Part 3: Freshwater clam studies

Clam tissue spiking

Table 10 shows the proportions of *Cryptosporidium*-positive samples from six clam digestive gland and six 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 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 1 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 11 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. Figure 6 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.

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 three 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 analyzed by DFA, IMS-DFA, and PCR methods to determine the best method for use on the rest of the tank experiment samples. Table 12 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 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 h, but low concentrations of oocysts continued to be detected in clams exposed to low or high doses for up to one and

three 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 13). 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 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 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 ^{o}C water temperature did not affect the number of oocysts detected with this assay (P=0.89).

Figure 7 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 three 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 oocysts detected while others had very low numbers, whereas at later timepoints oocysts were only detected at low numbers in exposed clam samples.

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). Figure 8 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* 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 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 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 14 shows the recovery efficiency of *Giardia* cysts spiked into clam digestive gland when using three 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). Figure 9 shows the prevalence of *Giardia* detected in clam digestive gland pools during the wet and dry seasons of 2003. 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.

Conclusions

The study objective and aims are complete. This study was able to evaluate innovative *Cryptosporidium* detection techniques, to assess the distribution of *Cryptosporidium* genotypes in freshwater and marine shellfish, and to identify risk factors for fecal contamination in coastal California ecosystems. The major study accomplishments are as follows:

- Shellfish tissue spiking experiments showed that TaqMan PCR, conventional PCR, and fluorescent antibody techniques can all be used to detect *Cryptosporidium* in bivalves.

- Tank exposure experiments confirmed that mussels and clams concentrate *Cryptosporidium* oocysts from inoculated waters, and that oocysts are detectable for days to weeks post-exposure.

- The sentinel mussel study was the first multi-year *Cryptosporidium* assessment in bivalves on the Pacific coast, and detected zoonotic as well as host-specific *Cryptosporidium* genotypes in mussels.

- The sentinel clam study was the first report worldwide utilizing *Corbicula* clams to detect *Cryptosporidium* and *Giardia* in natural riverine ecosystems.

- Exposure to freshwater outflow and recent precipitation preceding bivalve collection were identified as significant risk factors associated

with protozoal detection in bivalve tissues. These findings are consistent with fecal contamination flowing from land to sea in California.

List of Accepted Publications

Miller, W.A., E.R. Atwill, M.A. Miller, A.C. Melli, R. Hedrick, S.S. Jang, H.M. Fritz, K. Worcester, and P.A. Conrad. Clams (*Corbicula fluminea*) as bioindicators of fecal contamination with *Cryptosporidium* and *Giardia* in freshwater ecosystems of California. *International Journal for Parasitology* 35(6): 673-684.

Miller, W.A., M.A. Miller, I.A. Gardner, E.R. Atwill, A.C. Melli, M. Harris, J. Ames, K. Worcester, N. Barnes, D. Jessup, and P.A. Conrad. New genotypes and factors associated with *Cryptosporidium* detection in mussels (*Mytilus* spp.) along the California coast. *International Journal for Parasitology* 35:1103-1113.

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Literature Cited

Atwill, E.R., Camargo, S.M., Phillips, R., Alonso, L.H., Tate, K.W., Jensen, W.A., Bennet, J., Little, S., Salmon, T.P., 2001. Quantitative shedding of two genotypes of *Cryptosporidium parvum* in ground squirrels (*Spermophilus beecheyi*). Appl. Environ. Microbiol. 67, 2840-2843.

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.

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.

Bonadonna, L., Rossella, B., Ottaviani, M., Veschetti, E., 2002. Occurrence of *Cryptosporidium* oocysts in sewage effluents and correlation with microbial, chemical and physical water variables. Environ. Monit. Assess. 75, 241-252.

Current, W.L., Reese, N.C., Ernst, J.V., Bailey, W.S., Heyman, M.B., Weinstein, W.M., 1993. Human cryptosporidiosis in immunocompetent and immunodeficient persons. Studies of an outbreak and experimental transmission. N. Engl. J. Med. 26, 1252-1257.

Deng, M.Q., Lam, K.M., Cliver, D.O., 2000. Immunomagnetic separation of *Cryptosporidium parvum* oocysts using MACS MicroBeads and high gradient separation columns. J. Microbiol. Methods 40, 11-17.

Fayer, R., Gasbarre, L., Pasquali, P., Canals, A., Almeria, S., Zarlenga, D., 1998a. *Cryptosporidium parvum* infection in bovine neonates: dynamic clinical, parasitic, and immunologic patterns. Int. J. Parasitol. 28, 49-56.

Fayer, R., Graczyk, T.K., Lewis, E.J., Trout, J.M., Farley, C.A., 1998b. 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.A., 1999. *Cryptosporidium parvum* in oysters from commercial harvesting sites in the Chesapeake Bay. Emerg. Inf. Dis. 5, 706-710.

Feng, Y.Y., Ong, S.L., Hu, J.Y., Song, L.F., Tan, X.L., Ng, W.G., 2003. Effect of particles on the recovery of *Cryptosporiduim* oocysts from source water samples of various turbidities. Appl. Environ. Micriobiol. 69, 1898-1903.

Fontaine, M., Guillot, E., 2003a. An immunomagnetic separation-real-time PCR method for quantification of *Cryptosporidium parvum* in water samples. J. Microbiol. Methods 54, 29-36.

Fontaine, M., Guillot, E., 2003b. Study of 18S rRNA and rDNA stability by real-time RT-PCR in heat-inactivated *Cryptosporidium parvum* oocysts. FEMS Microbiol. Lett. 226:237-243.

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.

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.

Guy, R.A., Payment, P., Krull, U.J., Horgen, P.A., 2003. Real-time PCR for quantification of *Giardia* and *Cryptosporidium* in environmental water samples and sewage. Appl. Environ. Microbiol. 69, 5178-5185.

Hallier-Soullier, S., Guillot, E., 2000. Detection of cryptosporidia and *Cryptosporidium parvum* oocysts in environmental water samples by immunomagnetic separation-polymerase chain reaction. J. Appl. Microbiol. 89, 5-10.

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

Higgins, J.A., Fayer, R., Trout, J.M., Xiao, L., Lal, A.A., Kerby, S., Jenkins, M.C., 2001. Real-time PCR for the detection of *Cryptosporidium parvum*. J. Microbiol. Methods 47, 323-337.

Limor, J.R., Lal, A.A., Xiao, L., 2002. Detection and differentiation of *Cryptosporidium* parasites that are pathogenic for humans by real-time PCR. J. Clin. Microbiol. 40, 2335-2338.

MacDonald, L.M., Sargent, K., Armson, A., Thompson, R.C.A., Reynoldson, J.A., 2002. The development of a real-time quantitative-PCR method for characterization of a *Cryptosporidium parvum* in vitro culturing system and assessment of drug efficacy. Mol. Biochem. Parasitol. 121, 279-282.

MacKenzie, W.R., Hoxie, N.J., Proctor, M.E., Gradus, M.S., Blair, K.A., Peterson, D.E., Kazmierczak, J.J., Addiss, D.G., Fox, K.R., Rose, J.B., 1994. A massive outbreak in Milwaukee of *Cryptosporidium* infection transmitted through the public water supply. N. Engl. J. Med. 331, 161-167.

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. 1991. Mollusca:Bivalvia. Ecology and classification of North American freshwater invertebrates, p. 315-401. In: Thorp, J.H., and A.P. Covich, (ed.), Academic Press, San Diego, Ca.

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.

Miller, W.A., Atwill, E.R., Gardner, I.A., Miller, M.A., Fritz, H.M., Hedrick, R.P., Melli, A.C., Barnes, N.M., Conrad, P.A., 2005. Clams (*Corbicula fluminea*) as bioindicators of fecal contamination with *Cryptosporidium* and *Giardia* spp. in freshwater ecosystems in California. Int. J. Parasitol. 35, 673-684.

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

Morgan, U.M., Constantine, 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.

Morgan-Ryan, U.M., Fall, A., Ward, L.A., Hijjawi, N., Sulaiman, I., Fayer, R., Thompson, R.C.A., Olson, M., Lal, A., Xiao, L., 2002. *Cryptosporidium hominis* n. sp. (Apicomplexa:Cryptosporidiidae) from *Homo sapiens*. J. Eukaryot. Microbiol. 49, 443-440.

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.

Payment, P., Plante, R., Cejka, P., 2001. Removal of indicator bacteria, human enteric viruses, *Giardia* cysts, and *Cryptosporidium* oocysts at a large wastewater primary treatment facility. Can. J. Microbiol. 47, 188-193.

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.

Pieniazek, N.J., Bornay-Llinares, F.J., Slemenda, S.B., da Silva, A.J., Moura, I.N.S., Arrowood, M.J., Ditrich, O., Addiss, D.G., 1999. New *Cryptosporidium* genotypes in HIV-infected persons. Emerg. Inf. Dis. 5, 444-449.

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.

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

Spano, F., Putignani, L., McLauchlin, J., Casemore, D.P., Crisanti, A., 1997. PCR-RFLP analysis of the *Cryptosporidium* oocyst wall protein (COWP) gene discriminates between *C. wrairi* and *C. parvum*, and between *C. parvum* isolates of human and animal origin. FEMS Microbiol. Lett. 150, 209-217.

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. Nucleic Acids Res. 25, 4876-4882.

US EPA, 2001. Method 1623: *Giardia* and *Cryptosporidium* in water by filtration/IMS/FA. United States Environmental Protection Agency. 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.

Webster, K.A., Smith, H.V., Giles, M., Dawson, L., Robertson, L.J., 1996. Detection of *Cryptosporidium parvum* oocysts in feces: comparison of conventional coproscopical methods and the polymerase chain reaction. Vet. Parasitol. 61, 5-13.

Xiao, L., Herd, R.P., 1993. Quantitation of *Giardia* cysts and *Cryptosporidium* oocysts in fecal samples by direct immunofluorescent assay. J. Clin. Microbiol. 31, 2944-2946.

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.

Table 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			
PCR1 *Cryptosporidium* spp.[48]	C1F	TTCTAGAGCTAATACATGCG	1325		
	C1R	CCCTAATCTTTCGAAACAGGA			
	C2F	GGAAGGGTTGTATTTATTAGATAAAG	850		
	C2R	AAGGAGTAAGGAACAACCTCCA			
PCR2 *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
Proportion of spiked mussel hemolymph samples positive for *Cryptosporidium parvum* by different PCR protocols.[a]

No. oocysts spiked per hemolymph (1 ml)	TaqMan % Positive	PCR1 % Positive	PCR2 % Positive
0	0	0	0
1	33	33	67
10	33	33	50
100	100	83	100
500	100	100	100

[a] n=6 replicates per spiking dose.

Table 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)	0	0	0	0	0
	1	0	17	0	0
	10	0	33	17	83
	100	50	67	50	100
	500	100	100	67	100
Digestive Gland (1 g)	0	0	0	0	0
	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 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
	PCR1	0	0	ND	ND
	PCR2	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.
[b] n=28 for Xiao and Morgan hemolymph testing.
[c] ND=not determined due to sample volume limitations.

Table 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.

Table 6
Cryptosporidium DNA detected in mussels in Year 1.

FECAL RISK	SITE ID	DRY SEASON (6/01-11/01)	WET SEASON (12/02-5/02)
Livestock	**4B**	***C. felis***	***C. felis***
Impacted	**4D**	**-** [a]	**-**
	9C	**Non-Crypto-1** [b]	***C.parvum***
Human	**3**	**nm** [c]	-
Impacted	**5**	**nm**	-
	9A	***C. parvum***	-
Lower	**1**	**-**	**nm**
Impact	**6**	**nm**	***C.parvum***
	7	**nm**	**-**

[a] **- = all mussel batches *Cryptosporidium* -negative by PCR.**
[b] **'Non-Crypto' = a sequence similar to *Cryptosporidium* spp.**
[c] **nm = no mussels collected.**

Table 7
Cryptosporidium DNA detected in mussels in Years 2-3.

FECAL RISK CATEGORY	SITE ID	LATE DRY SEASON (9/02-11/02)	EARLY WET SEASON (12/02-2/03)	LATE WET SEASON (3/03-5/03)	EARLY DRY SEASON (6/03-8/03)	LATE DRY SEASON (9/03-11/03)	EARLY WET SEASON (12/03-2/04)	LATE WET SEASON (3/04-5/04)	EARLY DRY SEASON (6/04-8/04)
Livestock	**2A**	**nm** [a]	**-** [b]	**-**	**-**	**-**	**-**	**-**	**-**
Impacted	**4A**	**-**	**New-1** [c]	**New-2**	**-**	**nm**	**-**	**-**	**-**
	9D	**-**	**-**	**-**	**New-2**	**-**	**-**	**-**	**-**
Human	**3**	**-**	**-**	**-**	***C. parvum***	**-**	**-**	**-**	**-**
Impacted	**5**	**-**	**nm**	**-**	**-**	**-**	**Non-Crypto-2** [d]	**-**	**-**
	8	**-**	**-**	**New-2**	**-**	**-**	**-**	**-**	**nm**
Lower	**1**	**-**	**-**	**nm**	**-**	**-**	**-**	**-**	**-**
Impact	**6**	**-**	**-**	**nm**	**-**	**-**	**-**	**-**	***C. parvum***
	7	**-**	**-**	**-**	**nm**	**-**	**-**	**-**	**-**

[a] **nm = no mussels collected.**
[b] **- = all mussel batches *Cryptosporidium* -negative by PCR.**
[c] **'New' = a novel *Cryptosporidium* DNA sequence.**
[d] **= 'Non-Crypto' = a sequence similar to *Cryptosporidium* spp.**

Table 8
Univariate logistic regression of factors associated with *Cryptosporidium* detection in mussels.

Factor	Group	Percent mussel batches positive	Odds ratio	95% CI	Odds ratio *P*-value
Fecal risk class[a]	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

[a] Higher risk sites <5 km from human sewage outflow or livestock runoff; Lower risk sites > 5 km.

* Significant *P*-values <0.05.

Table 9
Multivariable logistic regression model of significant factors associated with the detection of *Cryptosporidium* spp. in mussels.

Factor	Group	Odds ratio	95% CI	*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 <0.05.

Table 10
Proportion of *Cryptosporidium*-positive clams detected by direct fluorescent antibody (DFA), Immunomagnetic separation (IMS) with DFA, and polymerase chain reaction (PCR) methods for spiked hemolymph and digestive gland tissues[a].

	Digestive gland % positive				Hemolymph % positive	
Oocysts	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[b]	nd	100	100	100	100

[a]n = six replicates per technique and oocyst dose.

[b]nd = not done.

Table 11
Recovery efficiency of direct fluorescent antibody (DFA) and immunomagnetic separation (IMS) with 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]
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)

[a]n = six 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 12
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 13
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
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
Clam collection time post-oocyst-exposure				
3 h[b]	0.0	-	-	-
6 h	-0.57	-2.24	1.1	0.5
9 h	-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
Intercept	0.012	-0.88	0.91	0.98

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

Table 14
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)

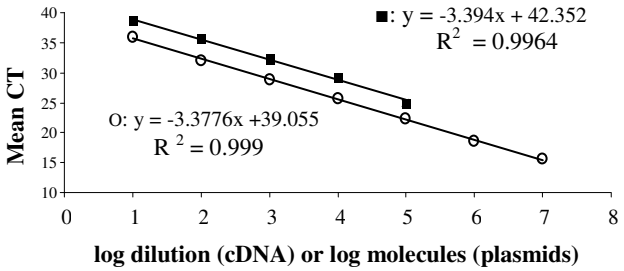

Fig. 1. TaqMan amplification of cloned *Cryptosporidium* PCR product (O) and cDNA generated on RNA extracted from 10-fold dilutions of *C. parvum* oocysts (■).Standard curves were determined in triplicate, standard deviationsare too small to be visible.

	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 californianus* (surf mussel)	*Mytilus galloprovincialis* (bay mussel)	*Corbicula fluminea* (freshwater clam)	*Emerita analoga* (sand crab)
TaqMan Bivalve	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+
TaqMan *Cryptosporidium*	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	-	-	-	-	-	-	-
PCR1 *Cryptosporidium*	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
PCR2 *Cryptosporidium*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-

a Protozoal spp. (host from which isolate obtained).
b Non-protozoal spp. (common name).
c + = PCR positive; - = PCR negative.

Fig. 2. Specificity testing of TaqMan and conventional PCR systems with protozoal, dinoflagellate, and invertebrate DNA samples.

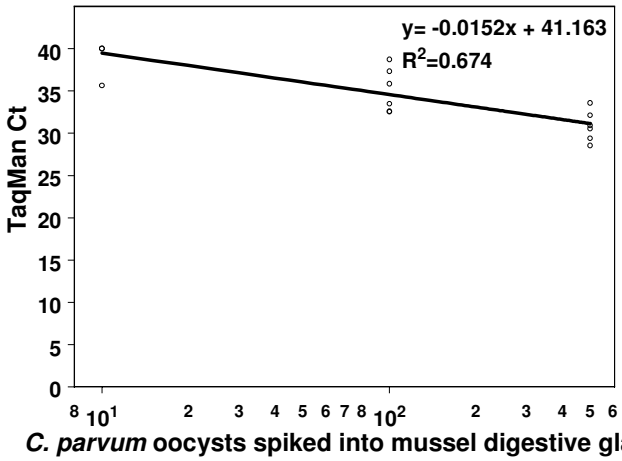

Fig. 3: TaqMan PCR detection of *Cryptosporidium parvum* oocysts spiked into mussel digestive gland and processed by immunomagnetic separation. Six replicates of 10, 100, and 500 oocysts per sample.

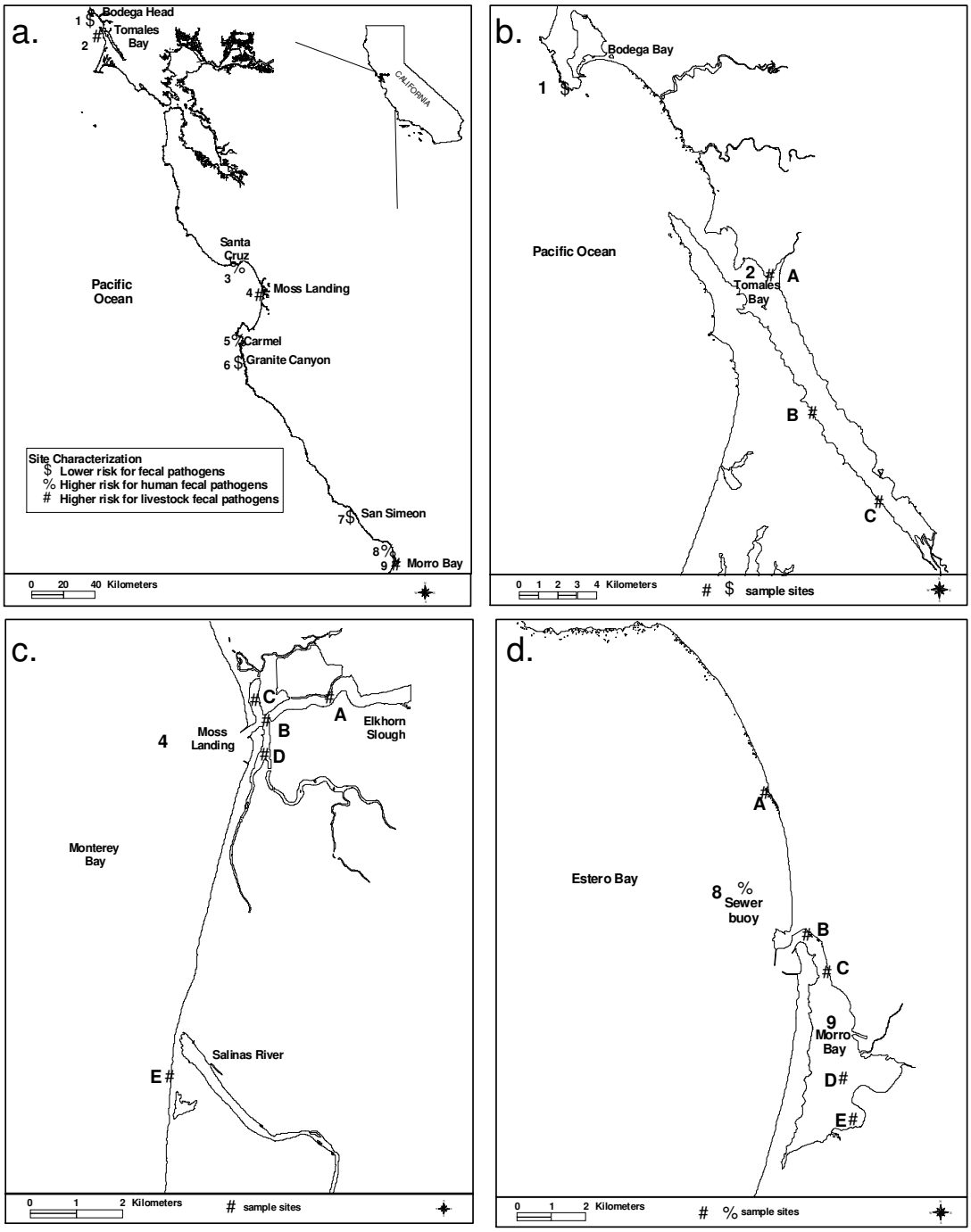

Fig. 4. Mussel collection sites located along the central California coast (a), with close up maps of the (b) northern (38.114°N, 122.869°W), (c) middle (36.815°N, 121.791°W), and (d) southern (35.342°N, 120.813°W) regions. Map numbers represent the core mussel sampling sites, while letters represent subsites within estuarine regions.

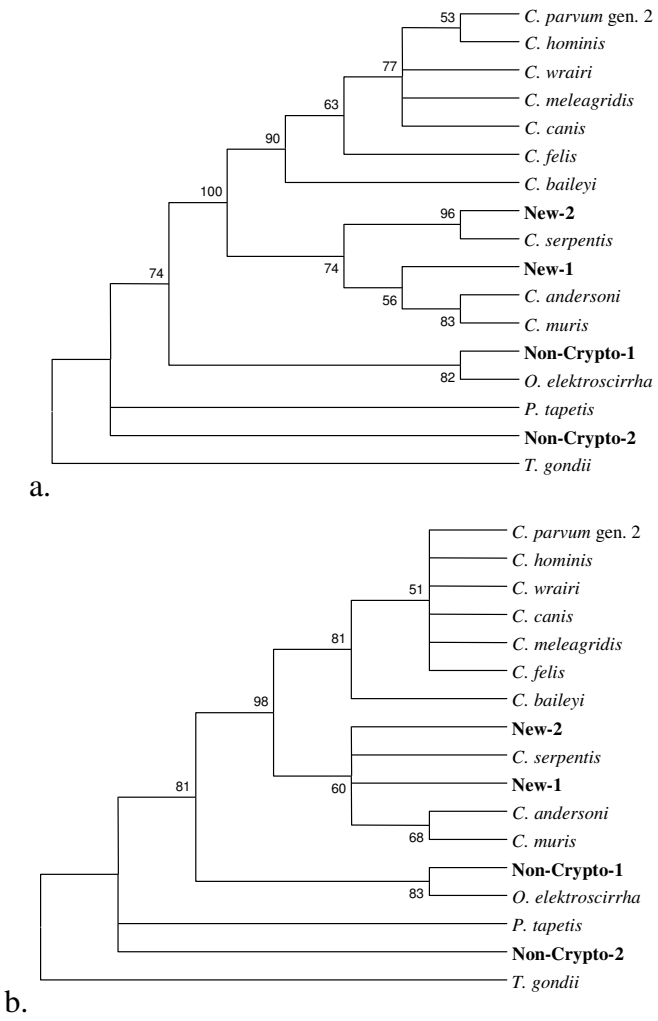

Fig. 5. Phylogenetic analysis of novel *Cryptosporidium*-like sequences detected in mussels, using (a) Neighbor Joining Tamura-Nei analysis, and (b) Maximum Parsimony Min-Mini Heuristic analysis, both with 1000 bootstrap replicates.

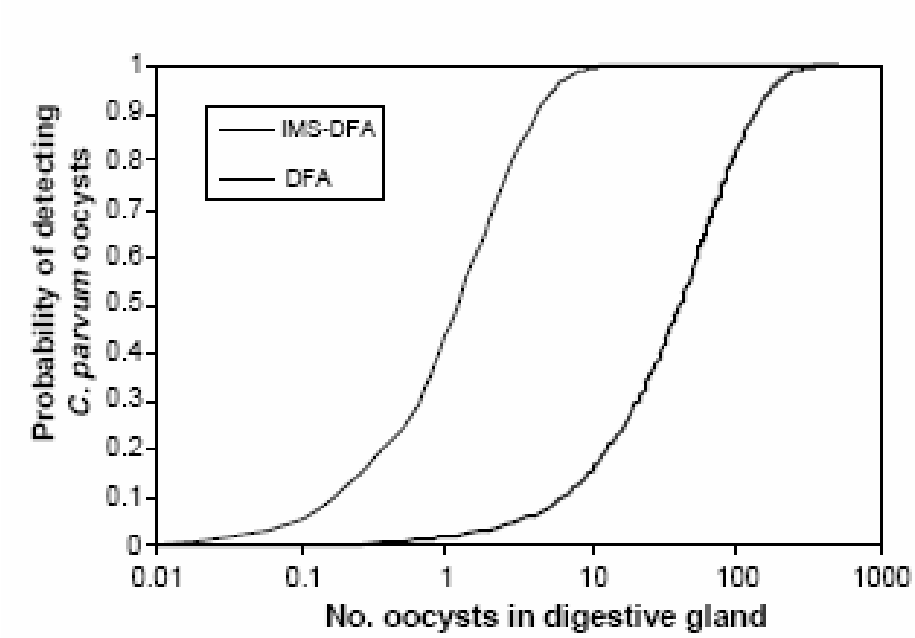

Fig. 6. Sensitivity of direct fluorescent antibody (DFA) and immunomagnetic separation (IMS) with DFA for detection of *Cryptosporidium* oocysts spiked into clam digestive glands, using Poisson and negative binomial regression, respectively.

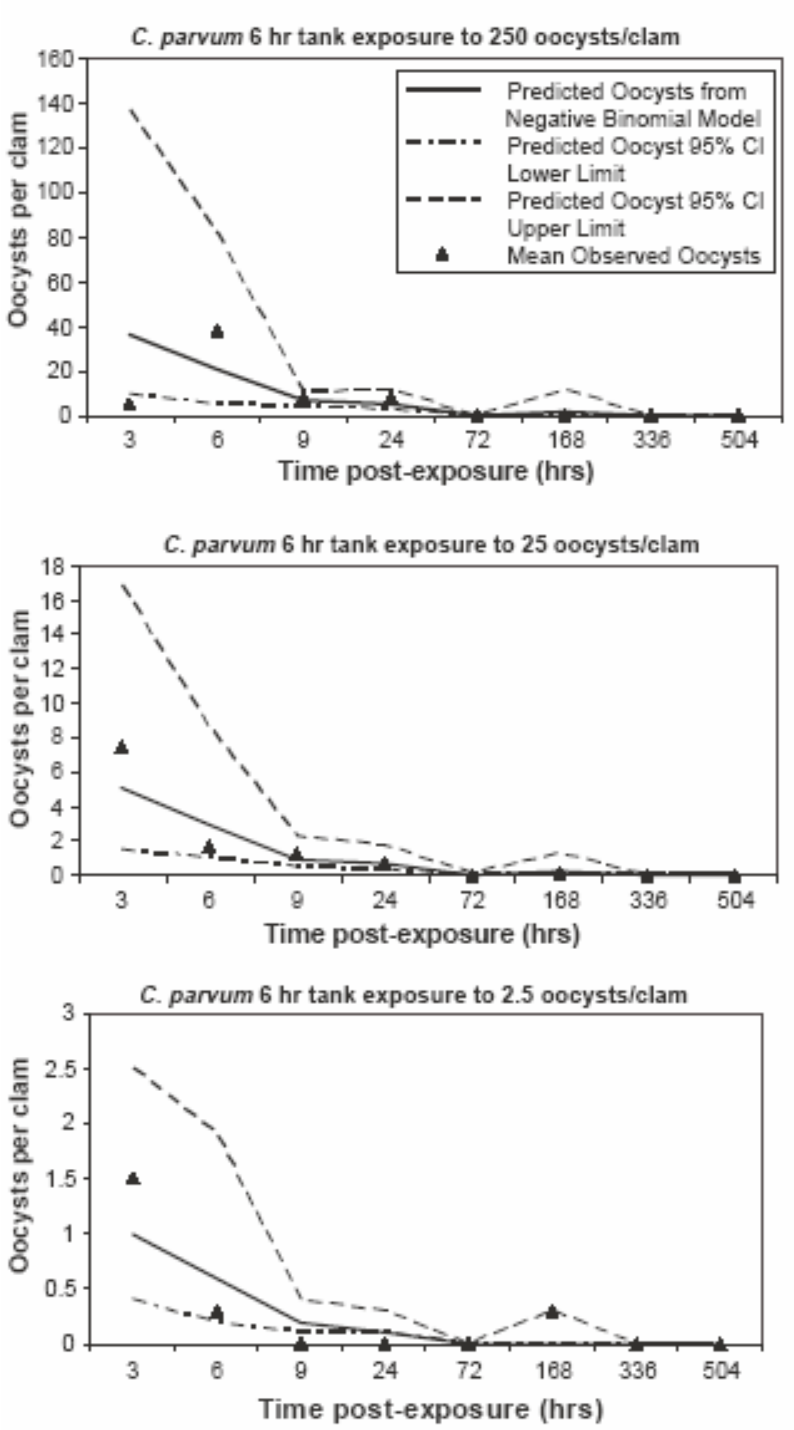

Fig. 7. *Cryptosporidium* oocysts detected in clam digestive gland by immunomagnetic separation with direct fluorescent antibody testing for three oocyst exposure doses used in a 6 h clam tank exposure experiment. Clams were tested at 3h, 6h, 9h, 1d, 3d, 7d, 14d, and 21d post-exposure.

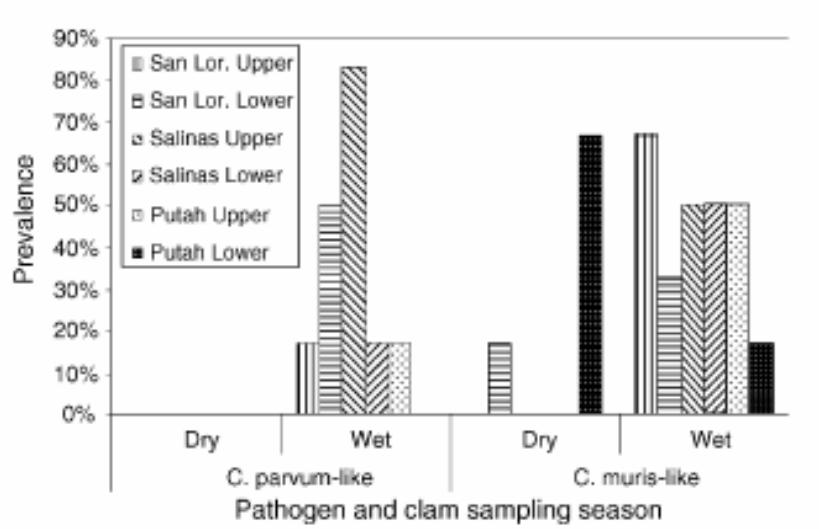

Fig. 8. *Cryptosporidium* spp. prevalence detected by immunomagnetic separation with direct fluorescent antibody testing of clam batches collected from three California rivers in 2003.

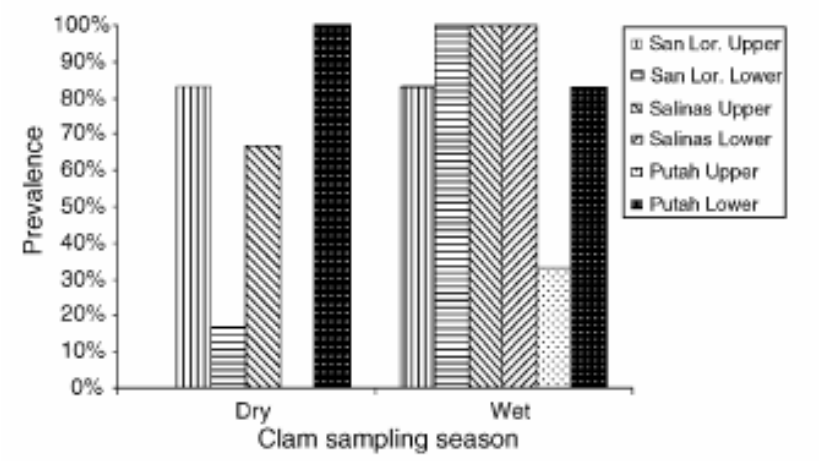

Fig. 9. *Giardia* spp. prevalence detected by immunomagnetic separation with direct fluorescent antibody testing of clam batches collected from three California rivers in 2003.