New genotypes and factors associated with *Cryptosporidium* detection in mussels (*Mytilus* spp.) along the California coast[☆]

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Abstract

A 3 year study was conducted to evaluate mussels as bioindicators of faecal contamination in coastal ecosystems of California. Haemolymph samples from 4680 mussels (*Mytilus* spp.) were tested for *Cryptosporidium* genotypes using PCR amplification and DNA sequence analysis. Our hypotheses were that mussels collected from sites near livestock runoff or human sewage outflow would be more likely to contain the faecal pathogen *Cryptosporidium* than mussels collected distant to these sites, and that the prevalence would be greatest during the wet season when runoff into the nearshore marine environment was highest. To test these hypotheses, 156 batches of sentinel mussels were collected quarterly at nearshore marine sites considered at higher risk for exposure to livestock runoff, higher risk for exposure to human sewage, or lower risk for exposure to both faecal sources. *Cryptosporidium* genotypes detected in Haemolymph 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. Detection of *Cryptosporidium* spp. was not associated with higher or lower risk status for exposure to livestock faeces or human sewage sources. This study showed that mussels can be used to monitor water quality in California and suggests that humans and animals ingesting faecal-contaminated water and shellfish may be exposed to both host-specific and anthropozoonotic *Cryptosporidium* genotypes of public health significance.
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1. Introduction

The genus *Cryptosporidium* is comprised of host-specific and broadly infective genotypes (Fayer, 1997; Peng, 1997;

Morgan-Ryan et al., 2002; Xiao et al., 2004). For example, host-specific *Cryptosporidium hominis* oocysts are shed in the faeces of humans and are thought to be transmitted by the faecal-oral route primarily within the human population, while *Cryptosporidium parvum* genotype 2 (henceforth called *C. parvum*) has an anthropozoonotic cycle involving humans and many different animal species including ruminants, domestic pets, and wildlife (Peng, 1997; Rose, 1997; Morgan-Ryan et al., 2002). Other primarily host-specific species include *Cryptosporidium andersoni*, *Cryptosporidium felis*, *Cryptosporidium canis*, *Cryptosporidium baileyi*, and *Cryptosporidium muris* that are shed in

the faeces of cattle, cats, dogs, chickens, and rodents, respectively (Xiao et al., 2004). The oocysts from many of these species are morphologically identical, making molecular tools such as PCR and DNA sequence analysis invaluable for species identification and genotype characterisation (Morgan et al., 1997; Xiao et al., 1999; Morgan-Ryan et al., 2002; Atwill et al., 2004).

Cryptosporidium oocysts are environmentally resistant and have led to outbreaks of diarrhoeal disease after faecal contamination of surface, pool, and drinking waters (Gallaher et al., 1989; MacKenzie et al., 1994; McAnulty et al., 1994). Humans and livestock have served as sources of faecal contamination in outbreaks, with newly infected individuals shedding millions of oocysts during initial infection (Rush et al., 1990; Joce et al., 1991; Duke et al., 1996; Rose, 1997; Fayer et al., 1998). Spread of *Cryptosporidium* oocysts from humans and terrestrial animals into freshwater environments and downstream estuarine and marine ecosystems poses a health risk to humans and animals utilising these ecosystems (Fayer et al., 2004). Molecular tools such as PCR and DNA sequence analysis may be useful in studies investigating sources of faecal pathogen pollution and faecal loading into aquatic environments.

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

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

the wet season, when runoff into the nearshore environment is greatest.

2. Materials and methods

2.1. Study design

Mussel testing sites along the central California coast were chosen based on their designation as 'higher risk' for livestock faecal contamination, 'higher risk' for human sewage faecal contamination, or 'lower risk' for faecal contamination from both sources. Fig. 1a shows the nine site locations, with more detailed maps (Fig. 1b–d) of subsites located within the northern (38.114°N, 122.869°W), middle (36.815°N, 121.791°W), and southern (35.342°N, 120.813°W) regions. The three sites considered at higher risk for livestock faecal 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 faecal 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 faecal 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 Haemolymph 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
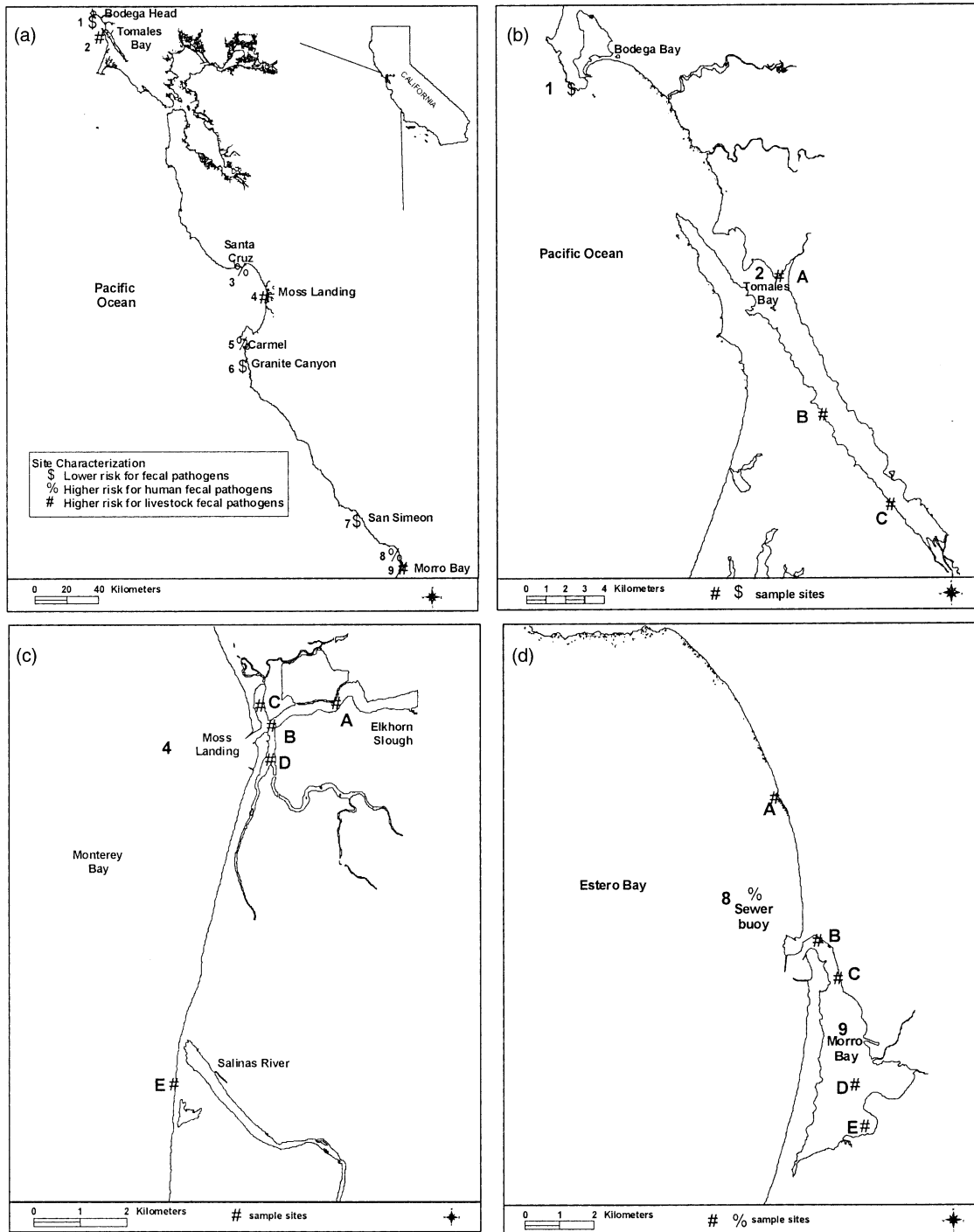
Fig. 1. Mussel collection sites located along the central California coast (a), with close up maps of the (b) northern (38.114°N, 122.869°W), (c) middle (36.815°N, 121.791°W), and (d) southern (35.342°N, 120.813°W) regions. Map numbers represent the core mussel sampling sites, while letters represent subsites within estuarine regions.

sequences amplified from mussel Haemolymph was performed, so that only recognised *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 faecal risk category, season, exposure to

freshwater outflow, recent precipitation, bivalve type, and water type.

2.2. 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 10,000 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 analysed with Chromas (Technelysium Pty Ltd, Tewantin, Qld, AU) and ClustalX (Thompson et al., 1997) software for *Cryptosporidium* genotype identification.

2.3. Mussels

All sentinel mussels (*Mytilus californianus*) were 3–5 cm long when harvested near Bodega Bay and outplanted at coastal study sites. If mussels could not be outplanted immediately, they were held for up to 6 months in saltwater tanks at the Bodega Bay or Granite Canyon Marine Laboratory facilities. To ensure that 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 analysed in batches of 30 at each site and sampling time. Haemolymph was extracted by filing a notch in the shell and aspirating 0.5–1.5 ml of Haemolymph from the adductor muscle with a sterile syringe. Haemolymph samples were centrifuged and the cell pellet DNA extracted as described (Miller et al., 2005).

2.4. Phylogenetic analysis

All *Cryptosporidium* genotypes detected in mussel haemolymph 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 neighbour-joining analysis inferred the phylogenetic relationships based on Tamura-Nei distances with 1000 bootstrap replicates. A maximum parsimony analysis utilised 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).

2.5. 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 categorised as higher risk for human faeces, higher risk for livestock faeces, or lower risk for these faecal inputs based on being < 1 km or > 5 km, respectively, from known faecal 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 categorised 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 categorised 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 modelled 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.

3. Results

3.1. 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 10,000 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 1000 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.

3.2. Mussel testing

Over the 3 year study, 156 batches of mussels were collected from coastal sites in central California. Occasionally, mussel batches could not be collected as planned because the sentinel bags were missing due to inclement weather, high seas, or unfavourable tides. In total, 4680 mussels were tested by PCR1 amplification of *Cryptosporidium* DNA from individual mussel Haemolymph 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 to 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 1 shows *Cryptosporidium* mussel batch results from Year 1 of the study. The faecal risk category, site identification, and *Cryptosporidium* genotypes detected in mussel haemolymph are indicated for the dry and wet season mussel collections. *Cryptosporidium parvum* was detected in mussels collected from a higher risk site for human faeces during the dry season, from a higher risk site for livestock faeces during the wet season, and from a lower risk site during the wet season. At a higher risk site for livestock faeces, *C. felis* was detected in mussel haemolymph 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 faeces during the dry season.

Cryptosporidium genotype results from Years 2 and 3 are shown in Table 2. 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 faeces during the early dry season of Year 2, and in mussels at a lower risk site in the early dry

Table 1
Cryptosporidium DNA detected in mussels in Year 1

Faecal risk category	Site id	Dry season (6/01–11/01)	Wet season (12/02–5/02)
Livestock impacted	4B	*C. felis*	*C. felis*
	4D	–[a]	–
	9C	Non-Crypto-1[b]	*C. parvum*
Human impacted	3	nm[c]	–
	5	nm	–
	9A	*C. parvum*	–
Lower impact	1	–	nm
	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 2
Cryptosporidium DNA detected in mussels in Years 2–3

Faecal 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 impacted	2A	nm[a]	–[b]	–	–	–	–	–	–
	4A	–	New-1[c]	New-2	–	nm	–	–	–
	9D	–	–	–	New-2	–	–	–	–
Human impacted	3	–	–	–	*C. parvum*	–	–	–	–
	5	–	nm	–	–	–	Non-Crypto-2[d]	–	–
	8	–	–	New-2	–	–	–	–	nm
Lower impact	1	–	–	nm	–	–	–	–	–
	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.

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 faeces and human faeces 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 faeces. 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 ($\leq 7\%$ prevalence per batch of 30 mussels).

Cryptosporidium spp. were detected in mussels collected within estuaries (Fig. 1) but not in the matched subsites located outside the estuaries. None of the Tomales Bay subsites in Fig. 1b tested positive for *Cryptosporidium* spp. at any timepoint. Estuarine mussel batches from the Moss Landing region in Fig. 1c (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 Fig. 1d (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.

3.3. 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 (Fig. 2) to further investigate their relationships to reference *Cryptosporidium* spp. and other protozoa. The same relationships were inferred using neighbour 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.

3.4. Factors associated with Cryptosporidium detection in mussels

Table 3 shows the univariate analysis of factors associated with the detection of *Cryptosporidium* spp. in mussel batches. Higher or lower risk status for faecal exposure was not significantly associated with detection of *Cryptosporidium* spp. in mussel batches ($P > 0.5$). However,
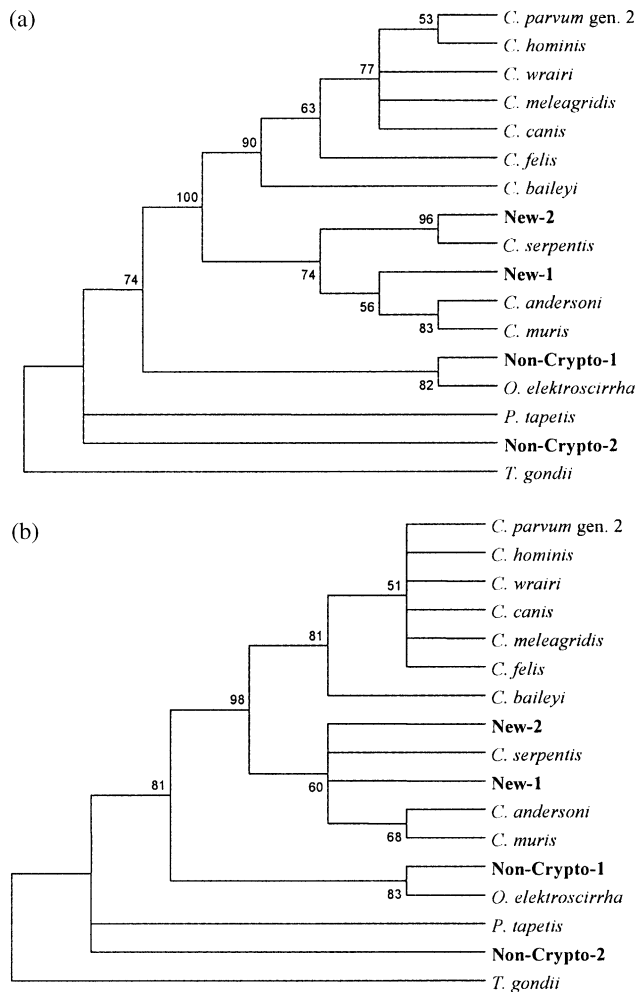
Fig. 2. Phylogenetic analysis of novel *Cryptosporidium*-like sequences detected in mussels, using (a) Neighbor Joining Tamura-Nei analysis, and (b) Maximum Parsimony Min-Mini Heuristic analysis, both with 1000 bootstrap replicates.

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 4 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 ($P\leq0.001$), 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 faecal 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.

4. Discussion

This study was the first to assess the distribution of *Cryptosporidium* genotypes in bivalves along the Pacific coast of the United States. Multiple *Cryptosporidium* genotypes were detected, including species of public health importance, animal host-specific species, and novel *Cryptosporidium* genotypes. While *Cryptosporidium* spp. were detected in both the wet and dry seasons during this study, they were detected most often in mussel batches collected near high freshwater outflow, and in mussel batches collected within a week of a precipitation event. Faecal risk category was not significantly associated with detection of *Cryptosporidium* in mussel batches, nor was water type or bivalve type. These findings support the concept that bivalves can be useful bioindicators of faecal pathogens in aquatic ecosystems, and suggest that both resident and transplanted sentinel bivalves can be used along the California coast in estuarine and marine sites for water quality studies.

Cryptosporidium parvum was detected in mussels from sites at higher risk for livestock faecal exposure, higher risk for human sewage exposure, and at lower risk sites. This finding does not support our hypothesis that the higher risk sites receiving significant volumes of livestock and human faeces are more likely to contain *C. parvum* than lower risk sites. In our study, *C. parvum* was only detected once at a higher risk site for livestock faeces, and was detected twice

Table 3
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
Faecal 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

Significant *P*-values <0.05.
[a] Higher risk sites <5 km from human sewage outflow or livestock runoff; lower risk sites >5 km.

at the higher risk sites for human faeces and twice at the lower risk sites. Two explanations for finding *C. parvum* at lower risk sites for faecal exposure are (i) that *C. parvum* is being shed in the faeces of animals such as wildlife that were not included in the faecal risk categories as part of this study and (ii) that unrecognised loading of faeces from livestock and human sources is reaching mussels at the lower risk sites, possibly through non-point source runoff, smaller faecal sources such as septic tanks or boat bilges, or water currents carrying *Cryptosporidium* oocysts from distant locations. In other studies of *Cryptosporidium* spp. in bivalves on the Atlantic coast and in Europe, *Cryptosporidium parvum* has been the most widely reported genotype (Gomez-Bautista et al., 2000; Lowery et al., 2001; Fayer et al., 2002; Traversa et al., 2004), though it is possible that novel genotypes were mistakenly identified as *C. parvum* when RFLP instead of DNA sequence analysis was used (Atwill et al., 2004). Other genotypes reported in those studies include *C. hominis* and *C. baileyi*, shed by humans and chickens, respectively, but neither of those genotypes was detected in mussels in the current study.

The two primarily host-specific *Cryptosporidium* genotypes detected in mussels may provide clues to faecal loading sources in the adjacent or upstream terrestrial ecosystems. First, identification of *C. andersoni* in mussels suggests that cattle are contributing to the faecal loading of the nearshore environment. *Cryptosporidium andersoni* DNA was detected in mussels from livestock-impacted sites in Morro Bay and Elkhorn Slough in the early wet season, consistent with *C. andersoni* oocysts washing into the nearshore ecosystem in storm runoff. Additionally,

Cryptosporidium felis was detected in mussels from Elkhorn Slough at two sampling times. This finding suggests that felid species, the major host for *C. felis*, may be contributing to the faecal load in that region, though *C. felis* has occasionally been reported in other hosts such as immunocompromised humans (Cama et al., 2003). The presence of a cat population in the Elkhorn Slough region is also supported by a recent study (Miller et al., 2002) that found that 79% of southern sea otters sampled in the Elkhorn Slough region were seropositive for *Toxoplasma gondii*, 1.5 times the odds of otters from other coastal locations testing positive. Felid species are the definitive host for *T. gondii*, just as for *C. felis*, but whether domestic, feral, or wildlife felid species are the most significant contributors to faecal loading of these pathogenic protozoa in California is unknown.

Four novel DNA sequences with a high degree of sequence homology to *Cryptosporidium* spp. were identified based on the BLAST search results. However, only

Table 4
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.

two of the four sequences fell within the *Cryptosporidium* clade in the more detailed phylogenetic analysis, which is the gold standard for relating novel DNA sequences to reference sequences. The novel *Cryptosporidium* genotypes, New-1 and New-2, may represent as yet unrecognised species endemic in wildlife populations or other minimally studied animal populations. Further study into the *Cryptosporidium* genotypes present in terrestrial animal populations may provide additional DNA sequences for comparison with the novel sequences detected in nearshore mussels. Although the majority of *Cryptosporidium* spp. have been reported in terrestrial animals, there have also been reports of *Cryptosporidium* in fish and marine mammals (Rush et al., 1990; Deng et al., 2000; Ryan et al, 2004), suggesting that a marine life cycle is possible. As in our study, environmental studies testing surface water quality directly have occasionally reported the detection of novel *Cryptosporidium* genotypes from unknown sources (Xiao et al., 2000; Ward et al., 2002). The Non-Crypto-1 DNA sequence that fell outside the *Cryptosporidium* clade was more closely related to the neogregarine *Ophriocystis elektroscirrha* than to the coccidial parasite *Toxoplasma gondii*. This finding is consistent with the Carreno et al. (1999) study that found *Cryptosporidium* spp. to be phylogenetically more similar to gregarine protozoa than to coccidial protozoa. The Non-Crypto-2 DNA sequence was not closely related to *Cryptosporidium* spp. or the gregarines, but may represent another eukaryotic marine or terrestrial organism.

The selection of 18S PCR with DNA sequence analysis as the *Cryptosporidium* detection method in mussel Haemolymph allowed for sensitive detection of a wide variety of *Cryptosporidium* genotypes in mussels. The PCR sensitivity experiments demonstrated that the 18S PCR1 protocol was more sensitive than the 18S PCR2 and COWP PCR3 protocols, which is consistent with the field study results where only the two haemolymph samples producing the strongest PCR1 signals were also positive by PCR2. The COWP PCR3 technique has been found to be as sensitive as 18S PCR techniques in some studies (Bialek et al., 2002) but not in others (Kato et al., 2003.) The nested PCR2 protocol was not as sensitive as the direct PCR1 protocol, possibly due to differences in primer selection, amplicon size, or PCR parameters that are known to affect the amplification efficiency and optimal reagent ratios within PCR reactions (Neumaier et al., 1998). One advantage of 18S PCR protocols is the high sensitivity attributed to multiple 18S copies per oocyst in *Cryptosporidium* spp. (LeBlancq et al., 1997; Piper et al., 1998). An additional advantage is that the amplicons can then be sequenced and analysed for comparison to known 18S sequences of characterised isolates in GenBank. This analysis allows for the identification of false positives as well as novel sequences when using primers to amplify highly conserved 18S regions that include polymorphic regions within the amplicon.

Maximising PCR sensitivity often comes at the expense of PCR specificity, resulting in amplification of non-target DNA as was seen in our study (<10% of PCR1 amplicons were confirmed as *Cryptosporidium* spp.) and others (Sturbaum et al., 2002). However, this problem can be overcome when amplification is followed by sequence analysis of the PCR products (Neumaier et al., 1998). Another limitation of low specificity assays is that competition with non-target DNA in the PCR reaction can result in false negative results that may lead to an underestimation of the true prevalence. Conservatively underestimating the true prevalence is scientifically and practically preferable to overestimating the prevalence based on false positive tests that can result in a cascade of regulatory management decisions (Rothman and Greenland, 1998). This study and others (Atwill et al., 2004) have successfully used DNA sequence analysis as the gold standard to identify novel *Cryptosporidium* genotypes in California. In future studies, the use of nested PCR assays that are highly specific for *Cryptosporidium* spp. and able to detect very low numbers of oocysts should be evaluated for use on bivalve tissues.

Overall, the 12% of mussel batches that were positive in this California study is lower than studies from the Atlantic coast in which 81% of oyster batches in the Chesapeake Bay were positive (Fayer et al., 2002), in Ireland where 50% of mussel batches from the Shannon River were positive (Graczyk et al., 2004), and in Spain where 36% of estuarine and nearshore marine mussel batches were positive (Gomez-Bautista et al., 2000). These varying results may be attributable to differences in study design, to differences in detection methods, to a larger dilution effect of the ocean dispersing faecal pathogens in this study compared to the other riverine or estuarine studies, or to a smaller faecal load of *Cryptosporidium* flowing into the nearshore environment in California. The speculation of smaller faecal loads of *C. parvum* flowing into nearshore California environment is supported in part by the Atwill et al. (2003) and Atwill and Pereira (2003) studies that have shown a substantially lower prevalence of *C. parvum* in adult beef and dairy cattle in California compared to studies conducted elsewhere in the United States, Canada, or Europe.

Associations between freshwater outflow, precipitation, season, and *Cryptosporidium* detection have been reported in previous studies (Sischo et al., 2000; Chai et al., 2001; Bodley-Tickell et al., 2002; Fayer et al., 2002; Pereira et al., 2002). In our study, collecting mussels near maximal freshwater outflow and within a week following a precipitation event were associated with increased odds for detection of *Cryptosporidium* spp. in mussel batches. These findings are similar to a study in the Chesapeake Bay (Fayer et al., 2002) that detected the most *Cryptosporidium*-positive oysters during the month with the most rainfall and the most freshwater stream flow into the bay. Our finding of freshwater outflow as a significant risk factor for detection of *Cryptosporidium* in mussels is also consistent with

a recent study by Miller et al. (2002), which showed that sea otters located near high freshwater outflow exposure had three times the odds of being seropositive for the faecal pathogen *Toxoplasma gondii* compared to sea otters sampled near low freshwater outflow areas along the central California coast. In the study by Chai et al. (2001) in Korea and by Pereira et al. (2002) in Brazil, the late rainy season was associated with an increase in human cryptosporidiosis cases. The study by Bodley-Tickell et al. (2002) in the United Kingdom detected more *Cryptosporidium* oocysts in surface waters draining a livestock farm between September and February than between March and August. In the Sischo et al. (2000) study of dairy cattle and associated runoff during the rainy season in the northeastern United States, the odds of *Cryptosporidium* oocyst detection in surface waters decreased as the total precipitation in the preceding 5 days increased. Further study will be required to clarify the strength of association between pathogen detection and risk factors such as season, rainfall, and freshwater outflow in various regions of the world.

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

References

Atwill, E.R., Pereira, M.D.G.C., 2003. Lack of detectable environmental loading of *Cryptosporidium parvum* in periparturient dairy cattle. J. Parasitol. 89, 1234–1236.

Atwill, E.R., Sweitzer, R.A., Pereira, M.G., Gardner, I.A., VanVuren, D., Boyce, W.M., 1997. Prevalence and associated risk factors for shedding *Cryptosporidium* oocysts and *Giardia* cysts within feral pig populations in California. Appl. Environ. Microbiol. 63, 3946–3949.

Atwill, E.R., Hoar, B., Pereira, M.D.G.C., Tate, K.W., Rulofson, F., Nader, G., 2003. Improved quantitative estimates of low environmental loading and sporadic periparturient shedding of *Cryptosporidium parvum* in adult beef cattle. Appl. Environ. Microbiol. 69, 4604–4610.

Atwill, E.R., Phillips, R., Pereira, M.D.G.C., Li, X., McCowan, B., 2004. Seasonal shedding of multiple *Cryptosporidium* genotypes in California ground squirrels (*Spermophilus beecheyi*). Appl. Environ. Microbiol. 70, 6748–6752.

Bialek, R., Binder, N., Dietz, K., Joachim, A., Knobloch, J., Zelck, U., 2002. Comparison of fluorescence, antigen, and PCR assays to detect *Cryptosporidium parvum* in faecal specimens. Diagn. Microbiol. Infect. Dis. 43, 283–288.

Bodley-Tickell, A.T., Kitchen, S.E., Sturdee, A.P., 1886. Occurence of *Cryptosporidium* in agricultural surface waters during an annual farming cycle in lowland UK. Water Res. 36, 1880–1886.

Boehm, A.B., Grant, S.B., Kim, J.H., Mowbray, S.L., McGee, C.D., Clark, C.D., Foley, D.M., Wellman, D.E., 2002. Decadal and shorter period variability of surf zone water quality at Huntington Beach, California. Environ. Sci. Technol. 36, 3885–3892.

Cama, V.A., Bern, C., Sulaiman, I.M., Gilman, R.H., Ticona, E., Vivar, A., Kawai, V., Vargas, D., Zhou, L., Xiao, L., 2003. *Cryptosporidium* species and genotypes in HIV-positive patients in Lima. Peru. J. Eukaryot. Microbiol. 50S, 531–533.

Carreno, R.A., Martin, D.S., Barta, J.R., 1999. *Cryptosporidium* is more closely related to the gregarines than to coccidian as shown by phylogenetic analysis of apicomplexan parasites inferred using small-subunit ribosomal RNA gene sequences. Parasitol. Res. 85, 899–904.

Chai, J., Kim, N., Guk, S., Park, Y., Seo, M., Han, E., Lee, S., 2001. High prevalence and seasonality of cryptosporidiosis in a small rural village occupied predominantly by aged people in the Republic of Korea. Am. J. Trop. Med. Hyg. 65, 518–522.

Deng, M., Peterson, R.P., Cliver, D.O., 2000. First findings of *Cryptosporidium* and *Giardia* in California sea lions (*Zalophus californianus*). J. Parasitol. 86, 490–494.

Duke, La., Breathnach, A.S., Jenkins, D.R., Harkis, B.A., Codd, A.W., 1996. A mixed outbreak of *Cryptosporidium* and *Campylobacter* infection associated with a private water supply. Epidemiol. Infect. 116, 303–308.

Fayer, R., Farley, C.A., Lewis, E.J., Trout, J.M., Graczyk, T.K., 1997. Potential role of the Eastern oyster, *Crassotrea virginica*, in the epidemiology of *Cryptosporidium parvum*. Appl. Environ. Microbiol. 63, 2086–2088.

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

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.

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

Fayer, R., Dubey, J.P., Lindsay, D.S., 2004. Zoonotic protozoa: from land to sea. Trends Parasitol. 20, 531–536.

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* oocsts in bivalve molluscs destined for human consumption. J. Parasitol. 86, 853–854.

Gallaher, M.M., Herndon, J.L., Nims, L.J., Sterling, C.R., Grabowski, D.J., Hull, H.F., 1989. Cryptosporidiosis and surface water. Am. J. Public Health 79, 39–42.

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

Graczyk, T.K., Conn, D.B., Lucy, F., Minchin, D., Tamang, L., Moura, L.N.S., DaSilva, A.J., 2004. Human waterborne parasites in zebra mussels (*Dreissena polymorpha*) from the Shannon River drainage area. Ireland Parasitol. Res. 93, 385–391.

Joce, R.E., Bruce, J., Kiely, D., Noah, N.D., Dempster, W.B., Stalker, R., Gumsley, P., Chapman, P.A., Norman, P., Watkins, J., et al., 1991. An outbreak of cryptosporidiosis associated with a swimming pool. Epidemiol. Infect. 107, 497–508.

Kato, S., Lindergard, G., Mohommed, H.O., 2003. Utility of the *Cryptosporidium* oocyst wall protein (COWP) gene in a nested PCR approach for detection infection in cattle. Vet. Parasitol. 111, 153–159.

LeBlancq, S.M., Khramtsov, N.V., Zamani, F., Upton, S.J., Wu, T.W., 1997. Ribosomal RNA gene organization in *Cryptosporidium parvum*. Mol. Biochem. Parasitol. 90, 463–478.

Lowery, C.J., Nugent, P., Moore, J.E., Millar, B.C., Xiru, X., Dooley, J.S., 2001. PCR-IMS detection and molecular typing of *Cryptosporidium parvum* recovered from a recreational river source and an associated mussel (*Mytilus edulis*) bed in Northern Ireland. Epidemiol. Infect. 127, 545–553.

MacKenzie, W., Neil, M., Hoxie, N., Proctor, M., Gradus, M., Blair, K., Peterson, D., Kazmierczak, J., Addiss, D., Fox, K., 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.

McAnulty, J.M., Fleming, D.W., Gonzalez, A.H., 1994. A community-wide outbreak of cryptosporidiosis associated with swimming at a wave pool. J. Am. Med. Assoc. 272, 1597–1600.

Miller, M., Gardner, I.A., Kreuder, C., Paradies, D.M., Worcester, K.R., Jessup, D., 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 of southern sea otters (*Enhydra lutris nereis*). Int. J. Parasitol. 32, 997–1006.

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 faecal contamination with *Cryptosporidium* and *Giardia* spp. in freshwater ecosystems in California. Int. J. Parasitol., 35, 673–684.

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

Morgan-Ryan, U.M., Fall, A., Ward, L.A., Hijjawi, N., Sulaiman, I., Fayer, R., Thompson, R.C., Olson, M., Lal, A., Xiao, L., 2002. *Cryptosporidium hominis* n. sp. (Apicomplexa: Cryptosporidiidae) from *Homo sapiens*. J. Eukaryot. Microbiol. 49, 433–440.

Neumaier, M., Braun, A., Wagener, C., 1998. Fundamentals of quality assessment of molecular amplification methods in clinical diagnostics. Clin. Chem. 44, 12–26.

Peng, M.M., Xiao, L., Freeman, A.R., Arrowood, M.J., Escalante, A.A., Weltman, A.C., Ong, C.S., MacKenzie, W.R., Lal, A.A., Beard, C.B.,

1997. Genetic polymorphism among *Cryptosporidium parvum* isolates: evidence of two distinct human transmission cycles. Emerg. Infect. Dis. 3, 567–573.

Pereira, M.D.G.C., Atwill, E.R., Barbosa, A.P., Silva, S.A.E., Garcia-Zapata, M.T.A., 2002. Intra- and estra-familial risk factos associated with *Cryptosporidium parvum* infection among children hospitalized for diarrhea in Goiania, State of Goias, Brazil. Am. J. Trop. Med. Hyg. 66, 787–793.

Piper, M.B., Bankier, A.T., Dear, P.H., 1998. A HAPPY map of *Cryptosporidium parvum*. Genome Res. 8, 1299–1307.

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

Rothman, K.J., Greenland, S., 1998. Approaches to statistical analysis, Modern Epidemiology. Lippincott-Raven Publishers, Philadelphia pp. 183–199.

Rush, B.A., Chapman, P.A., Ineson, R.W., 1990. A probable waterborne outbreak of cryptosporidiosis in the Sheffield area. J. Med. Microbiol. 32, 239–242.

Ryan, U., O'Hara, A., Xiao, L., 2004. Molecular and biological characterisation of a *Cryptosporidium molnari*-like isolate from a guppy (*Poecilia reticulata*). Appl. Environ. Microbiol. 70, 3761–3765.

Sischo, W.M., Atwill, E.R., George, J., Lanyon, L.E., 2000. *Cryptosporidia* on dairy farms and the role these farms may have in contaminating surface water supplies in the northeastern United States. Prev. Vet. Med. 43, 253–267.

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.

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.

Traversa, D., Giangaspero, A., Molini, U., Iorio, R., Paoletti, B., Otranto, D., Giansante, C., 2004. Genotyping of *Cryptosporidium* isolates from *Chamelea gallina* clams in Italy. Appl. Environ. Microbiol. 70, 4367–4370.

Ward, P.I., DePlazes, P., Regli, W., Rinder, H., Mathis, A., 2002. Detection of eight *Cryptosporidium* genotypes in surface and waste waters in Europe. Parasitology 124, 359–368.

Xiao, L., Morgan, U.M., Limor, J., Escalante, A., Arrowood, M., Shulaw, M., 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.

Xiao, L., Alderisio, K., Limor, J., Royer, M., Lal, A., 2000. Identification of species and sources of *Cryptosporidium* oocysts in storm waters with a small-subunit rRNA-based diagnostic and genotyping tool. Appl. Environ. Microbiol. 66, 5492–5498.

Xiao, L., Fayer, R., Morgan, U., Upton, S.J., 2004. *Cryptosporidium* taxonomy; recent advances and implications for public health. Clin. Microbiol. Rev. 17, 72–97.