COASTAL ENVIRONMENTAL QUALITY INITIATIVE
STUDENT FELLOWSHIP AND RESEARCH SUPPORT
PROJECT COMPLETION REPORT

DATE 11/29/04
PREPARED BY Dr. Miller

1. **Institution:** University of California at Davis

2. **Title:** "*Cryptosporidium* in Bivalves as Indicators of Fecal Pollution in the California Coastal Ecosystem"

3. **Graduate Student:** Woutrina Miller (formerly Smith), DVM, MPVM, PhD
   Advisor: Patricia Conrad, DVM, PhD

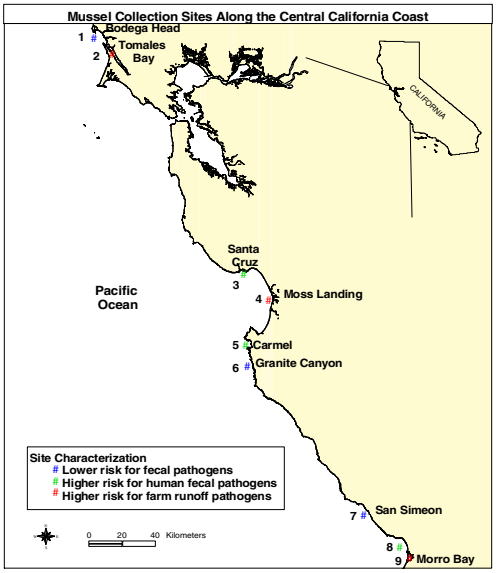
4. **Dates:** 1/1/02 - 12/31/03

5. **Hypotheses:**
   1. *Cryptosporidium* species are present in bivalves collected at sites exposed to fecal contamination, including selected sites near sewage outfalls and agricultural runoff.

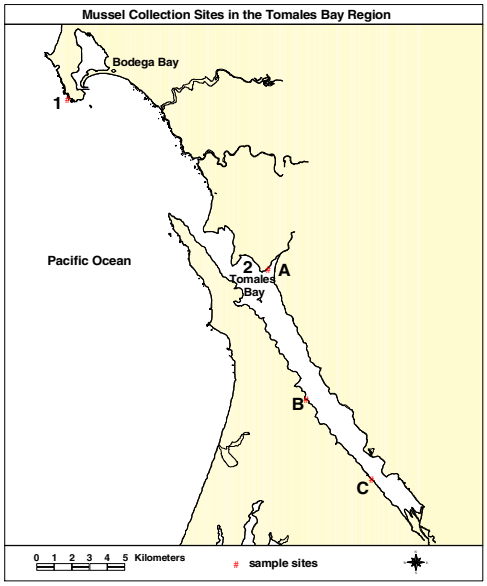
   2. *Cryptosporidium* genotypes will differ significantly in bivalves collected at sites exposed to human fecal contamination as compared to bivalves collected at sites contaminated with livestock feces.

6. **Objective:**
   Test mussels collected from up to 20 nearshore marine study sites designated as 'higher risk' or 'lower risk' for fecal pollution based on the proximity of known sources of livestock runoff and human sewage along the central California coast.

   Final Progress Report:
   In order to evaluate mussels as bivalve bioindicators of fecal pollution with *Cryptosporidium* spp., we compared molecular and immunologic *Cryptosporidium* detection methods for use on bivalve tissues in the laboratory before testing the 4800 mussels collected from coastal California study sites. In our laboratory evaluation of a new real-time TaqMan polymerase chain reaction (PCR) system to amplify *Cryptosporidium* DNA from mussel hemolymph compared to conventional PCR systems, all PCR assays were able to detect a single *Cryptosporidium* oocyst spiked into 1 ml hemolymph samples. For gill wash and digestive tissues, the analytic sensitivity of TaqMan PCR was similar to the direct immunofluorescent antibody (DFA) test, detecting 100 or more oocysts. The use of immunomagnetic separation (IMS) to concentrate oocysts from the tissues before DFA or PCR testing improved the sensitivity limit to 10 oocysts per sample. In a tank exposure experiment in which mussels filtered *C. parvum* oocysts from inoculated sea water, IMS concentration followed by DFA analysis detected the most *Cryptosporidium*-positive mussel samples. When wild mussels were tested with IMS-DFA and conventional PCR methods, the most *Cryptosporidium*-positive samples were detected by PCR of hemolymph samples. Because our goal was to screen wild mussels for *Cryptosporidium* spp., conventional PCR with DNA sequence analysis was used to identify *Cryptosporidium* genotypes in hemolymph from the 4800 wild mussels collected at the 'higher risk' and 'lower risk' coastal study sites for fecal pollution shown in Figure 1.



a.



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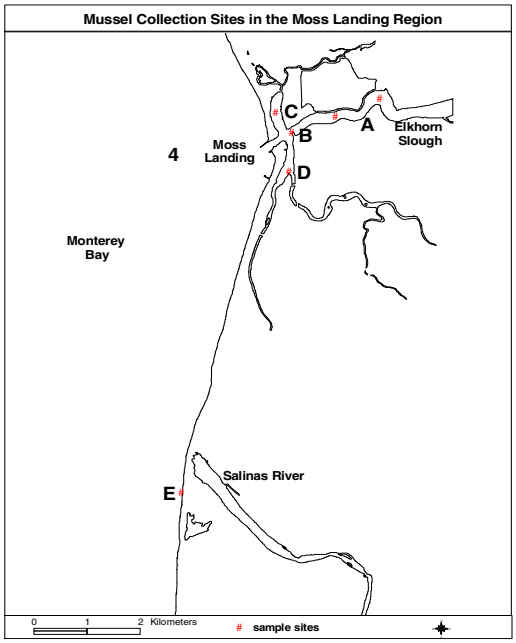
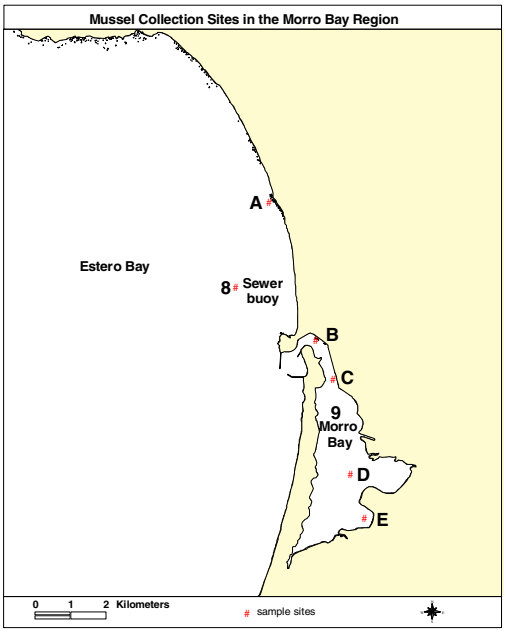


c.



d.

Fig. 1. Mussel collection sites located along the central California coast (a), with close up maps of the (b) northern, (c) middle, and (d) southern regions. Map numbers represent the core mussel sampling sites, while letters represent subsites within estuarine regions.

Over the 3 year study, 156 batches of 30 mussels per site were tested for *Cryptosporidium* DNA, with 12% of all mussel batches testing positive. Within *Cryptosporidium*-positive mussel batches, 1-4 mussels tested positive, and occasionally more than one genotype was detected in a mussel batch. Table 1 shows *Cryptosporidium* mussel batch results from Year 1 of the study. The fecal risk category, site identification, and *Cryptosporidium* genotypes detected in mussel hemolymph are indicated for the dry and wet season mussel collections. *Cryptosporidium*

parvum genotype 2 was detected in mussels from a higher risk site for human feces during the dry season, as well as in mussels collected from a higher risk site for livestock feces and from a lower impact site during the wet season. *Cryptosporidium parvum* genotype 2 is shed in the feces of humans and many animal species. At a higher risk site for livestock feces that is located in a nature reserve, *C. felis* was detected in mussel hemolymph during the dry and wet seasons. *Cryptosporidium felis* is shed in the feces of wild and domestic cats and has not been reported previously in bivalve samples. Additionally, a novel *Cryptosporidium*-like sequence, designated New-1, was identified at a higher risk site for livestock feces during the dry season. Novel *Cryptosporidium* spp. may represent as yet unrecognized genotypes shed in the feces of wildlife or other animals. The Year 1 *Cryptosporidium* genotype results were very exciting and gave strong preliminary evidence of fecal contamination present at higher risk and lower risk sites for human and animal fecal exposure along the California coast.

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

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

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

Cryptosporidium genotype results for Years 2 and 3 at the 9 higher risk and lower risk sites for fecal exposure are shown in Table 2. Mussel batches were collected during the early and late wet and dry seasons for both years. *Cryptosporidium parvum* genotype 2 was detected in mussels from a higher risk site for human feces during the early dry season of Year 2, and in mussels at a lower risk site in the early dry season of Year 3. Novel *Cryptosporidium*-like DNA sequences, designated New-2 and New-3, were detected in mussels from higher risk sites for livestock feces and human feces during the wet season samplings, and again from a livestock impacted site in the early dry season. A fourth unique DNA sequence, designated New-4, was detected in mussels from a higher risk site for human feces. The New-3 *Cryptosporidium*-like sequences were detected in mussels collected from 2 sites separated by over 200 km.

Table 3 shows the *Cryptosporidium* genotype results for the estuarine subsites in Tomales Bay (Fig. 1b), Elkhorn Slough (Fig. 1c), and Morro Bay (Fig. 1d). Table 3 lists the *Cryptosporidium* genotypes detected in mussels from the 3-4 subsites within each estuary, as well as 1 site outside of the estuary for comparison purposes. None of the Tomales Bay subsites (2A-2C) tested positive for *Cryptosporidium* spp. during the study. However, mussels collected from Elkhorn Slough and Morro Bay contained *C. andersoni*, shed in the feces of cattle, as well as novel *Cryptosporidium* spp. The 4 subsites located within Elkhorn Slough (4A-4D) all tested positive for *Cryptosporidium* spp., but mussels collected from the site outside the slough (4E) were *Cryptosporidium*-negative at all timepoints. In contrast, only 1 of the sites inside Morro Bay (9C) tested *Cryptosporidium*-positive, and the site located outside Morro Bay (9A) was also

positive but at a different timepoint. Detecting *Cryptosporidium* spp. in mussels within the estuaries but not outside the estuaries is consistent with fecal contamination entering the nearshore environment in the freshwater sources that feed into the estuaries, that are then further diluted in the open ocean outside the estuaries.

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

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

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

Table 3. Mussel testing for *Cryptosporidium* DNA at estuarine subsites.

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

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

This study was the first to evaluate bivalves on the Pacific coast for *Cryptosporidium* spp. In an effort to better understand the advantages and limitations of using bivalves as bioindicators in nearshore waters, a risk factor analysis was performed to evaluate whether fecal risk category (higher or lower risk for fecal pollution), bivalve sampling season (early wet = December-February, late wet = March-May, early dry = June-August, late dry = September-November), freshwater outflow exposure (low, medium, high), recent precipitation (in preceding 1, 7, or 30 days before bivalve collections), water type (estuarine or marine), and bivalve type (resident or transplanted mussels) were associated with detection of *Cryptosporidium*-positive mussel batches. Table 4 shows the univariate analysis that evaluated the strength of association for each putative risk factor alone with the odds of detecting *Cryptosporidium* spp. in a mussel batch. Fecal risk status was not associated with increased odds of detecting *Cryptosporidium* spp. in mussel batches. However, mussel batches sampled in the late wet season, near medium or heavy freshwater outflow, and within 1 or 7 days of a precipitation event were more likely to contain *Cryptosporidium* spp. than mussel batches sampled at other times. Water type and bivalve type

were not significant explanatory variables for *Cryptosporidium* detection in mussels, suggesting that resident or transplanted mussels could be used to monitor for *Cryptosporidium* spp. in estuarine or marine environments.

Table 4. Univariate logistic regression of risk factors for detection of *Cryptosporidium* spp. in mussels

Risk factor	Group	Percent mussel batches positive	Odds ratio	95% CI	Odds ratio P-value
Fecal risk class	Lower	8 (n=24)	1.00	-	-
	Higher-Human	9 (n=34)	1.06	0.16-7.12	0.95
	Higher-Livestock	13 (n=98)	1.68	0.24-11.69	0.60
Season	Early Wet	9 (n=35)	1.00	-	-
	Late Wet	23 (n=43)	3.23	1.35-7.76	0.01*
	Early Dry	8 (n=38)	0.91	0.16-5.28	0.92
	Late Dry	5 (n=40)	0.56	0.13-2.46	0.44
Freshwater outflow	Low	2 (n=85)	1.00	-	-
	Medium	19 (n=53)	9.65	2.46-37.88	0.001*
	High	33 (n=18)	20.75	4.16-103.57	<0.001*
Precipitiation in past 1 day	No	10 (n=136)	1.00	-	-
	Yes	25 (n=20)	3.15	0.80-12.47	0.10*
Precipitation in past 7 days	No	7 (n=97)	1.00	-	-
	Yes	19 (n=59)	2.95	1.23-7.02	0.02*
Precipitation in past 30 days	No	4 (n=45)	1.00	-	-
	Yes	14 (n=111)	3.62	0.64-20.51	0.15
Water	Estuarine	14 (n=90)	1.00	-	-
	Marine	8 (n=66)	0.49	0.16-1.46	0.20
Bivalves	Resident	14 (n=78)	1.00	-	-
	Transplant	9 (n=78)	0.60	0.19-1.91	0.39

* Significant P-values <0.10.

Table 5. Logistic regression model of significant risk factors for detection of *Cryptosporidium* spp. in mussels.

Risk factor	Group	Adjusted odds ratio	95% CI	Odds ratio P-value
Freshwater outflow	Low	1.00	-	-
	Medium	10.84	2.54-46.22	0.001*
	High	14.85	3.30-66.64	<0.001*
Precipitation in past 7 days	No	1.00	-	-
	Yes	2.63	1.07-6.49	0.04*

* Significant P-values <0.10.

After univariate analysis was complete, a multivariable model was created to simultaneously evaluate the risk factors associated with detection of *Cryptosporidium* spp. in mussel batches (Table 5). Mussel batches exposed to medium and high freshwater outflow were 10.84 and 14.85 times as likely to contain *Cryptosporidium* spp. as mussel batches exposed to low freshwater outflow in the day preceding mussel collection. Similarly, mussel batches collected within 7 days of a precipitation event were 2.63 times as likely to contain *Cryptosporidium* DNA as mussels collected at other timepoints. These findings suggest that mussels will be the most useful in monitoring for fecal pollution when outplanted near areas of high freshwater outflow and when collected with a week of a precipitation event.

In conclusion, this study evaluated novel approaches for ecosystem monitoring using molecular *Cryptosporidium* detection techniques on bivalves that had filtered fecal pathogens from nearshore waters along the California coast . *Cryptosporidium* spp. were detected in bivalves collected from sites considered at higher risk for livestock fecal contamination, at higher risk for human sewage contamination, and sites more than 5 km from these known fecal sources. These findings show that *Cryptosporidium* spp. and fecal contamination are widespread in nearshore ecosystems in California. The genotypes of *Cryptosporidium* detected suggest that both cats and cattle are significant terrestrial sources of fecal loading. This study demonstrates the potential for water quality monitoring agencies to use bivalves as bioindicators of fecal contamination, in addition to their current use as bioindicators of pesticide and metal contamination in aquatic ecosystems.

MAJOR ACCOMPLISHMENTS:

- Development and validation of a real-time TaqMan polymerase chain reaction (PCR) system for quantitative detection of *Cryptosporidium* DNA in bivalve tissues.

- Comparative stidy of the TaqMan PCR system with conventional PCR and direct immunofluorescent antibody (DFA) techniques, with and without immunomagnetic concentration (IMS) for sensitive and specific detection of *Cryptosporidium* spp. in bivalve tissues.

- First evaluation of *Cryptosporidium* spp. present in wild mussels along the Pacific coast of the United States, as an indicator of fecal pollution in coastal ecosystems.

- First report of *C. felis* and *C. andersoni* detected in bivalve tissues.

- Detection of previously unreported novel *Cryptosporidium* spp. in wild bivalves.

- First report of freshwater outflow and recent precipitation as significant risk factors for detection of *Cryptosporidium* in wild mussels along the Pacific coast.

- This study shows that bivalves can be used as monitoring tools for fecal pathogens in estuarine and marine waters, which is consistent with our upstream studies that have detected *Cryptosporidium* and *Giardia* spp. in freshwater clams living in rivers that flow into the Monterey Bay.

7. Changes in income and personnel:

No changes in income or personnel were made during the final year of the project. Dr. Miller is now completing her dissertation and will receive her Ph.D. in December, 2004.

8. Changes in scientific goals and anticipated schedule for project completion:

No changes in scientific goals were made. Project objectives were completed.

9. a. Publications:

1. Conrad, P.A., Miller, M., Kjemtrup, A.J., Smith, W., Gardner, I.A. The human-wildlife-domestic animal interface provides exciting research opportunities for parasitologists. *Journal of Parasitology* 89(Suppl.): S27-S36. 2003.

2. Miller, W.A., Gardner, I.A., Atwill, E.R., Leutenegger, C.M., Miller, M.A., Hedrick, R.P, Melli, A.C., Barnes, N.M., Conrad, P.A. Evaluation of Methods for Improved Detection of *Cryptosporidium* Species in Mussels. Submitted to *Applied and Environmental Microbiology*, 11/04.

3. Miller, W.A., Miller, M.A., Gardner, I.A., Atwill, E.R., Harris, M., Ames, J., Jessup, D.A., Melli, A.C., Paradies, D., Worcester, K., Olin, P., Barnes, N.M., Conrad, P.A. *Cryptosporidium* Genotypes Detected in California Mussels (*Mytilus* Species). In preparation to submit to the *International Journal for Parasitology* in 12/04.

4. Miller, W.A. *Cryptosporidium* Species in Coastal California Ecosystems. Dissertation, University of California, Davis. 12/2004.

b. Conference Presentations:

1. Miller, W.A., Miller, M.A., Gardner, I.A., Atwill, E.R., Harris, M., Ames, J., Jessup, D.A., Worcester, K., Paradies, D., Olin, P., Melli, A.C., Barnes, N.M., Conrad, P.A. Mussels (*Mytilus* spp.) as bioindicators of fecal pollution with *Cryptosporidium* spp. in coastal California ecosystems. Western Society for Naturalists Conference, Rohnert Park, California, 2004.

2. Miller, W.A., Miller, M.A., Gardner, I.A., Atwill, E.R., Harris, M., Ames, J., Jessup, D.A., Worcester, K., Paradies, D., Melli, A.C., Barnes, N.M., Conrad, P.A. *Cryptosporidium* epidemiology in fecal impacted coastal California ecosystems, using mussels (*Mytilus* species) as bioindicators. International *Giardia* and *Cryptosporidium* Congress, Amsterdam, Netherlands, 2004.

3. Conrad, P.A. Exciting Protozoological Discoveries at the Human-Wildlife-Domestic Animal Interface – American Veterinary Medical Association Conference, Colorado; Society for Tropical Veterinary Medicine, Brazil; Institute for Parasitology, School of Veterinary Medicine, Bern, Switzerland, 2003.

4. Conrad, P.A. Adventures and New Discoveries at the Wildlife-Domestic Animal-Human Interface. Schalm Lectureship, School of Veterinary Medicine, Davis, California, 2003.

5. Smith, W.A., Atwill, E.R., Gardner, I.A., Miller, M.A., Ames, J., Jang, S., Melli, A., Jessup, D., Conrad, P.A. Sea otters, shellfish, and fecal pathogens: can we connect the dots? Defenders of Wildlife Carnivore Conference, Monterey, California, 2002.

6. Smith, W.A., Miller, M.A., Gardner, I.A., Atwill, E.R., Jang, S., Jessup, D., Leutenegger, C., Melli, A., Conrad, P.A. Shellfish as indicators of fecal borne protozoa and bacteria in the California marine ecosystem. Wildlife Disease Association Annual Meeting, Humboldt, California, 2002.

10. Public relations success story potential:

In this study, bivalves collected along the California coast were shown to contain *Cryptosporidium* parasites for the first time. The study was undertaken for 2 main reasons: 1) to evaluate mussels as bivalve bioindicators of fecal contamination for water quality and watershed management purposes; and 2) to evaluate the possibility that humans and animals consuming raw bivalve shellfish along the California coast may be ingesting significant doses of fecal pathogens known to cause clinical disease. Laboratory and field studies demonstrated that mussels can be used as bioindicators of fecal pollution along the California coast and could be incorporated in water quality monitoring programs. The detection of both host-specific and zoonotic *Cryptosporidium* genotypes helps to identify contributing sources of fecal contamination in coastal ecosystems, and supports the concept that humans and animals consuming raw bivalve shellfish may be ingesting significant doses of fecal pathogens.