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Corbicula fluminea as an Environmental Assay for *Cryptosporidium parvum* Oocysts in Surface Waters

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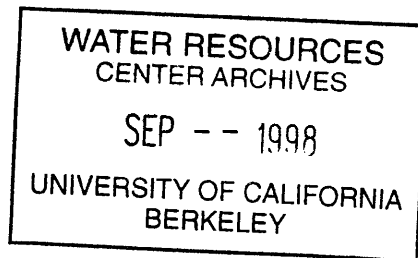

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ABSTRACT

Cryptosporidium parvum, a protozoal parasite, can be transmitted to humans through water and has become a major public health concern. The ability to detect the oocysts of this parasite has been hampered by techniques that are expensive, time-consuming and have low sensitivities. The goal of this research was to determine the applicability of using the resident filter-feeding bivalve, *Corbicula fluminea*, as a method of detecting *C. parvum* oocysts in surface waters. Oocysts were detected in the clam fecal bioassay for exposure concentrations of 10, 100, and 1,000 oocysts/L, with peak concentrations of oocysts in clam feces occurring just a few hours following exposure to oocysts. No oocysts were detected after 22 hours of depuration. There were significant differences in the number of oocysts excreted per hour per clam among exposure concentrations and among observation times within 100 and 1,000 oocysts/L exposure concentrations. Ninety percent of the total oocysts defecated by clams were defected within 6 hours post-exposure removal. Cooler ambient water temperature was a significant factor in decreasing defecation rates and thereby decreasing the sensitivity of the bioassay. Clam-passed oocysts remained fully infective and approximately 50% were viable as determined by excystation. The *Corbicula* assay had peak test sensitivities (probability of detecting one or more oocysts per assay) of 50%, 100%, and 100% for exposure concentrations of 10, 100, and 1,000 oocysts/L respectively. This research has shown that the filter-feeder, *Corbicula fluminea*, could be used as an inexpensive, sensitive test for detecting *C. parvum* oocysts in surface waters.

Key words: rivers, streams, watersheds, health, microorganisms, shellfish, disease, parasite, public health.

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PROBLEM AND RESEARCH OBJECTIVES

Human cryptosporidiosis is an enteric disease caused by the protozoan parasite *Cryptosporidium parvum* and is characterized as a self-limiting diarrheal illness lasting up to 2 weeks in healthy adults, and a chronic, life threatening illness in immunocompromised individuals. *C. parvum* is a coccidian parasite that invades then replicates within the microvilli of intestinal epithelial cells (Fayer and Unger 1986). The infective stage is a 5x5 μm oocyst shed from epithelial cells and during the course of an infection released in concentrations potentially exceeding several million oocysts per gram of feces. Transmission of *C. parvum* is by the fecal-oral route with modes involving drinking water, recreational water, foodborne, person-to-person, and animal-to-person exposures (Meinhardt et al 1996). *C. parvum* oocysts are resistant to most chemical disinfectants and many existing water treatment processes may not be effective against waterborne transmission (Casemore 1990). The waterborne route of infection has generated serious concern among public health, regulatory and water supply agencies, especially since the 1993 outbreak in Milwaukee that resulted in 400,000 cases of cryptosporidiosis and over 100 deaths (MacKenzie et. al. 1994).

The ability to detect *C. parvum* oocysts in surface water samples is key to identifying waterborne outbreaks and sources of oocysts in watersheds. However, current detection techniques for *Cryptosporidium* in surface waters are expensive, time-consuming, and have relatively low recovery rates, leading to poor sensitivity. Additional challenges are that the spatial variability of pathogens in a water body at one sampling time can be greater than the temporal variability at one sampling site (Jones and Simon 1980). Furthermore, the relatively small volumes of water evaluated relative to the entire volume of the waterbody of inference and the infrequency with which samples are taken hampers the interpretation of studies evaluating the causal

mechanisms driving the occurrence of *C. parvum* in surface waters (Meinhardt et al. 1996). It is probable that many surface waters have intermittently low background levels of *C. parvum* oocysts present which may be increased suddenly by the introduction of fecal wastes from domestic (Atwill et al., 1998), feral (Atwill et al., 1997) or wild animals or from municipal effluents discharged into the watershed (Smith and Rose 1990), especially following rainfall events (Hansen and Ongerth 1991).

The goal of this study was to determine the appropriateness of using a resident, filter feeding bivalve as a method of detecting *Cryptosporidium* in surface waters. *Corbicula fluminea* is an introduced freshwater clam that has become a common benthic invertebrate in many freshwater systems south of 40° latitude, with densities of more than 1000 clams/m^2 being common (McMahon 1983). Reported filtration rates of *Corbicula* vary widely, ranging from 11 to 600 ml/hr (Lauritsen 1986) to an average of 750 ml/hr and a filtrate size of <1- 10 µm (McMahon 1991). Populations of *Corbicula* have been shown to significantly affect seston removal in river systems (Cohen et. al. 1984). Previous studies using *C. fluminea* as a biomonitor concerned residue detection or long term growth effects of inorganic pollutants (Leard et al. 1980, McMahon 1991). The uptake and elimination rates of human pathogens by commercially important bivalves have been evaluated from a food safety perspective (Birkbeck and McHenery 1982, Canzonier 1971, Fayer et al. 1997, Martinez-Manzanares et al. 1991, Power and Collins 1990, Richards 1988, Rowse and Fleet 1982, Timoney and Abston 1984), however no laboratory studies were found which used environmentally representative concentrations of pathogens to evaluate the use of resident bivalves as a tool for water quality monitoring of pathogens. The enumeration of *C. parvum* oocysts in fecal samples was used in this project since the majority of filtered pathogens are found in the digestive tract (Power and Collins 1990, Graczyk et al. 1998).

The freshwater habitat, high filtration rates, and prolific populations of *Corbicula* may make these invertebrates suitable monitoring tools of *C. parvum* in resident surface waters.

The goals of this project were to determine:

1. Rates at which *C. parvum* oocysts are defecated from *Corbicula* after exposure to water containing known concentrations of oocysts.
2. The length of time that oocysts are detected in *Corbicula* feces after exposure.
3. The sensitivity of the *Corbicula* bioassay at varying exposure concentrations.
4. The viability and infectivity of *Corbicula*-passed oocysts.

REVIEW OF METHODOLOGY

C. parvum oocysts were purified from feces using a modified discontinuous sucrose gradient centrifugation (Arrowood and Sterling, 1987). One part calf feces to 3 parts Tween water (0.2% w/v Tween 20 to distilled water) were sieved sequentially through a 40, 100, 200 mesh, rinsing as needed. A 5 ml layer of fecal suspension was placed on top of 20 ml of sucrose solution (1.2 specific gravity) and centrifuged at 1200 × *g* for 10 minutes. Five ml of Tween water were carefully added to the top of the tube, water was stirred lightly so that the oocysts rose up off the interface, and 5 ml was aspirated off. Aspirates were pooled, centrifuged at 1200 × *g* for 10 minutes, supernatant discarded and the upper layer carefully removed. A hemocytometer was used to determine the concentration of oocysts.

3-Dose Experiment

Approximately 240 clams were collected from Putah Creek (at Road 98, Yolo County, California). Thirty randomly selected clams were measured (longest length from shell edges); all clams were rinsed with tap water and shells were gently cleaned of sediment or algae. Clams

were kept for 24 hours in unchlorinated, aerated well water.

Twelve, 18-gallon rubber containers were each filled with 50 liters of unchlorinated well water, two aeration tubes and a clam holding device. The clam holding device was made of a capped funnel held within a bucket weighted with gravel. Plastic mesh covered the funnel opening and the clams placed on the mesh so that clams were held in the center of the water column while feces dropped down into the capped funnel. Nine containers held 20 clams each. The three tubs without clams were used as positive controls. Three 5-gallon plastic buckets were each filled with well water, a clam holding device holding 20 clams, and an aeration tube. These chambers were used as negative controls.

Once all clams were in place, appropriate spiking of tubs with oocysts began on a 15 minute schedule, with spiking of each of three replicate tubs separated by 5 minutes. The dosing schedule was necessary to allow enough time to collect water and fecal samples at precise times later in the experiment.

Environmentally representative concentrations of 10, 100 and 1,000 oocysts/L were used as exposure concentrations. *Cryptosporidium* oocysts have been found in surface waters in concentrations averaging <1/liter to 63.5/liter (Hansen et al. 1991, Rose et al., 1997) to a maximum of 290/liter (Rose et al. 1991) and 5800/liter (Madore et al. 1987) in waters receiving sewage treatment plant and agricultural discharges. Exposure concentrations were also kept relatively low in order to reduce the production of pseudofeces by the clams (suspended particles that become entangled in gill mucus, transferred along rejection tracts to the mouth palps and ejected). Filter feeding bivalves respond to high concentrations of phytoplankton by rejecting excess material as pseudofeces, limiting intake to a maximum of 1×10^8 cells/hour for mussels (Birbeck and McHenry 1982). The three positive control tubs were each spiked with 50,000

oocysts (1,000 oocysts/liter). On fifteen minute intervals, test tubs were spiked with 500 oocysts (10 oocysts/liter), 5,000 oocysts (100 oocysts/liter), or 50,000 oocysts (1,000 oocysts/liter). Each tub was gently stirred after the oocysts were added. The 5-gallon buckets with clams (negative controls) were not spiked with oocysts.

Clams were removed from *C. parvum* oocyst exposure two hours after inoculation. Clams were removed from tubs, rinsed with distilled water, then placed into 5-gallon plastic buckets filled with clean well water, an aeration tube, and a clam holding device. This protocol was designed to approximate a short-term spike of oocysts in surface water, such as might occur subsequent to a runoff event or sewer overflow.

Water samples (5-liters) were collected from the three positive control tubs immediately and 2 hours after inoculation and from each test tub 2 hours after inoculation. All water samples were taken with five, 1-liter Nalgene sample bottles. Prior to filling each bottle, the tub water was mixed by rinsing each sample bottle 5 times with the water to be sampled. Fifty ml of 100% formalin were immediately added to each 1-liter sample to make an approximate 5% solution.

Fecal samples were collected from all test and negative control tubs 1, 2, 3, 4, 5, 6, 8, 12, 24, 26 and 48 hours after dosing with oocysts. All feces seen within the funnel or on clams at each sampling time was aspirated with a 10-ml plastic pipette and transferred to a 50 ml disposable centrifuge tube. Feces samples were refrigerated until processing the following day.

Fecal samples were centrifuged at $1,000 \times g$ for 10 minutes. The fecal pellet was then transferred to a labeled, pre-weighed (using a five digit scale) 1.5 ml centrifuge tube and centrifuged again at $1,000 \times g$ for 10 minutes. Supernatant was aspirated and the weight of fecal pellet (as is) determined. The density of clam feces had previously been determined to be approximately 1.23 grams/ml of centrifuged feces. Using this conversion factor, distilled water

was added to each fecal sample in order to attain a 1:5 v/v dilution of centrifuged feces to water. Fecal samples were diluted to aid in the detection of oocysts during microscopy. The diluted fecal sample was mixed thoroughly with a mechanical pipette, then 0.01 ml was transferred to a MERIFLUOR slide (Meridian Diagnostics, Inc., Cincinnati, Ohio) for IFA reading.

The processing of water samples began with adding 10 ml of 10% Tween 80 to each 1-liter sample bottle, capping it, then inverting and hand mixing the sample for 1 minute. Samples were transferred to four 250 ml plastic centrifuge bottles by pouring each through a 50 um Nytex cloth sieve (to separate out any flocculent feces which may have been inadvertently collected) into a plastic graduated cylinder. Samples were centrifuged for 20 minutes at 1,000 × *g*. The supernatant was aspirated, leaving approximately 2 ml. The pellet and remaining supernatant were then gently mixed with a plastic disposable hand pipette and transferred to a pair of 1.5 ml microcentrifuge tubes. Approximately 0.5 ml of distilled water was added to the 250 ml centrifuge bottle, mixed again to suspend any remaining oocysts, and added to the microcentrifuge tubes with the sample. Microcentrifuge tubes were centrifuged for 10 minutes at 1,000 × *g*. The supernatant was removed down to 0.1 ml. The pellet and supernatant were thoroughly mixed with a mechanical pipette before transferring 0.01 ml to a MERIFLUOR (MERIFLUOR *Cryptosporidium*/*Giardia* test kit, Meridian Diagnostics, In., Cincinnati, Ohio) slide well for IFA reading at ×400 magnification. Positive and negative controls were prepared and read along with each set of test slides.

<u>Low temperature test</u>

The effect of temperature on the *Corbicula* bioassay was determined by comparing the previously described test (May, 20°C) with a test conducted during January (10°C). The January test procedures were similar to those of the May test except that a single exposure of 1000

oocyst/l was used and fecal collection times were 1, 2, 3, 4, 5, 6, 8, 10, and 20 hours after inoculation.

Recovery percentages were determined for both fecal and water samples. Fecal samples were spiked with known quantities of *C. parvum* oocysts, processed as described, and enumerated. Recovery percentages for water samples were calculated by dividing the number recovered from initial samples (immediately after inoculation) by the number expected based on the inoculation. All analyses used transformed data which incorporated the appropriate recovery rate for water or fecal samples.

Mean filtration rate (FR) for each batch of 20 clams was calculated with the formula (Coughlan 1969):

$$FR = \frac{volume of water}{time} \ln \frac{[initial]}{[final]}$$

The filtration rate per clam was calculated as FR/20 clams.

Viability and infectivity

Eight 18-gal plastic tubs were filled with 50 liters of unchlorinated well water. Two aeration tubes, a clam holding device, and 20 clams (clams were collected 24 hours prior) were placed in each tub. A ninth 18-gallon plastic tub, a positive control, was filled with 50 liters of well water, two aeration tubes, and a clam holding device (no clams were added). All tubs were inoculated with 50,000 oocysts (1,000 oocysts/liter) three times daily. Prior to each inoculation and subsequent to the first one, tub water was replaced with fresh well water in order to avoid the concentration of oocysts to rise above 1000 oocysts/liter, as an environmentally realistic concentration of oocysts was desired. Clam feces were collected before each new inoculation. Feces was aspirated with a 10-ml disposable pipette and transferred to a 50-ml centrifuge tube.

Fecal collections were immediately refrigerated at 4°C. Daily fecal samples were centrifuged at 1000 × *g* for 10 minutes, pooled, and stored in potassium dichromate solution (2.5% $K_2Cr_2O_7$). After 7 days of collecting feces, all samples were pooled, centrifuged at 1000 × *g* for 10 minutes, and resuspended in 2.5% $K_2Cr_2O_7$. The pooled fecal solution was mixed well and oocysts were enumerated by placing 0.01 ml of solution on a MERIFLUOR slide for IFA reading. Five-liter water samples were collected from the positive control tub daily and processed as previously described, with the exception that no formalin was added to samples.

The pooled fecal solution and positive control were mailed via overnight carrier to the Veterinary Medicine Teaching and Research Center, School of Veterinary Medicine, UC Davis, Tuolare, California for viability determination and to the National Animal Disease Center (U.S.D.A.) in Ames Iowa for the mouse infectivity analysis of the clam-passed oocysts. For viability, the oocyst suspension was passed through a series of autoclaved sieves (#40, 100, 200 and 270 mesh) and then poured into a 15 ml centrifuge tube. Oocysts were purified using a discontinuous sucrose gradient whereby the sieved suspension was underlayed first with a sucrose solution of specific gravity 1.1.03 and secondly a sucrose solution of specific gravity 1.064. The gradient was centrifuged at 1500 × *g* for 30 minutes. Top and middle layers were transferred into a new 15ml tube and centrifuged at 1000 × *g* for 10 minutes. Final pellet was resuspended in 1 ml distilled water and triplicate 100 µl aliquots of purified clam-passed oocysts and positive controls (non-clam-passed) were transferred to three 1.5 microcentrifuge.
For excystation, we used a modification of Robertson et al., 1993. Briefly, to each 100 ml aliquot of oocysts we added 1ml of acidified PBS (pH 3.8) and incubated for 30 minutes at 37 °C. Tubes were centrifuged at 11,500 × g for 10 minutes, supernatant discarded and the pellet resuspended in 100 ml HBSS (Sigma). A volume of 200 ml of 1% bile (Sigma) and 50 ml of 0.44% NaHCO3

was added to the 100 ml oocyst aliquot and mixture was incubated for 1 hours. Using a light microscope equipped with DIC, approximately 100 ml of the resulting mixture were examined for oocysts. About 100 to 200 total oocysts were counted for all test and control triplicates, with each oocyst coded as complete (intact), partially excysted (1-3 sporozoites missing) and fully excysted (no visible sporozoites). We will count partially excysted oocysts as viable since they were capable of releasing at least 1 sporozoite. To determine the percent viable, we used the following ratio: ([number fully excysted + number of partial excysted] / [total oocysts counted]).

The infectivity of clam-passed and positive control oocysts were determined with a BALB/c *in vivo* neonatal mouse assay, the gold standard for measuring infectivity of *C. parvum* oocysts. To purify control and clam-passed oocysts, 1 ml of clam fecal material was diluted to 12 ml with 11 ml of PBS, pH 7.2. The 12 ml of diluted feces was overlain on a 2 part PBS, 1 part Sheather's sucrose solution (sp. gr. 1.254) in a 50 ml polypropylene tube and centrifuged at 600 × *g* for 15 minutes. The interface between the PBS and sucrose layers was harvested and diluted with PBS and centrifuged at 1500 × *g* for 15 minutes. The supernatant was decanted and the pellet resuspended in PBS and centrifuged at 750 × *g* for 15 minutes. The supernatant was again decanted and the pellet resuspended in PBS. The final centrifugation was done at 350 × *g* if there was a sufficient amount of sample (in order not to pellet some small particulate materials such as bacteria), otherwise it was centrifuged at 750 × *g*. Supernatant was decanted and the pellet was resuspended in a minimal amount of PBS. Oocyst concentrations were determined with a hemacytometer.

Clam-passed and control oocysts were administered by gavage to 1-week old BALB/c mice. Concentrations of 20, 300, 600, and 1000 oocysts per mouse pup were tested. Four days after gavage with the oocysts, mice were killed and ilea removed and fixed for histology. Tissue

was paraffin-embedded, cut and stained with hematoxylin and eosin. Slides were then examined for colonization with *C. parvum*.

Statistical methods

Statistical analyses were done with BMDP (BMDP Statistical Software, Inc.) Repeated measures analyses were used to test effect of dose (concentration of oocysts in water) on defecation rate (number of oocysts/hr/clam) over time among the three exposure concentrations and between temperatures at the same exposure concentration. The global level of significance was set at α=0.05. Additional ANOVA analyses were conducted to determine which observation times had significant differences in defecation rates among the three doses tested. Multiple comparisons were done for each observation time with a significant dose or temperature effect, using the Bonferonni technique with an overall α=0.05. A repeated measures ANOVA analysis was also conducted within each dose to determine if there was a time effect for oocyst defecation rates. Multiple comparisons were done within each dose with a significant time effect, using the Bonferonni technique with an overall α=0.05. The Huynh-Feldt adjusted p-value was used when the assumption of sphericity was violated.

Test Sensitivity

Statistical modeling of the sensitivity (probability to detect one or more oocysts per assay) of the bioassay will allow use to determine the probability of detecting one ore more oocysts given a specified concentration of oocysts in water. The Poisson distribution has been previously utilized to model the sensitivity of detecting oocysts in cow feces using the MERIFLUOR test kit (Jones and Atwill, 1998). Under the assumption of random spatial distribution of oocysts in sieved clam feces, the number of oocysts detected per smear of clam feces (X) may be described by the Poisson distribution with mean rcW, where r=the recovery rate of the test (using

Corbicula, the MERIFLUOR detection kit, and a 1:5 fecal dilution), c=the concentration of oocysts per unit volume of feces, and W=volume of feces tested. If a sample is declared test-positive whenever one or more oocysts are detected, then the mean probability of a positive test result can be approximated as:

$$P(X > 0) = 1 - P(X = 0) = 1 - e^{-rcW}$$

In this analysis, rcW=mean number of oocysts counted per 10 µl clam feces placed on the IFA slide. The probability of a positive test result given that *Corbicula* were exposed to *C. parvum* oocysts for 2 hours was calculated for all observation times and exposure concentrations from the 10°C and 20°C tests.

This model predicts that the test has perfect specificity (no false positives). Binding of antibody to antigen is in general quite specific. There can be false positives if other species of *Cryptosporidium* are present, the monoclonal antibody cross-reacts with the non-*C. parvum* species, and the microscopist is unable to differentiate the oocyst morphology. Nevertheless, since the inoculations were experimentally controlled and we used only *C. parvum* in these experiments, there was little chance of cross-reactions during this set of applications. We found no *C. parvum*-like particles to be present among our negative clam fecal controls.

DISCUSSION OF RESULTS AND THEIR SIGNIFICANCE

The estimated recovery rate for detecting *Cryptosporidium* oocysts in clam feces with the MERIFLUOR IFA kit was 55%. The estimated recovery rate for oocysts in water samples was 29%.

Three dose concentrations, one temperature

Oocysts were detected in clam feces from all exposure concentrations tested. The peak defecation rates (oocysts/hr/clam) occurred 3 hours after exposure began (i.e., 1 hour after removing the clams from the 2 hour exposure) for all exposures tested. No oocysts were detected in clam feces after 24 hour post-inoculation (22 hours after removal from exposure) for all exposures tested. No oocysts were detected in negative control clam feces.

Corbicula excreted oocysts for a shorter period of time after the removal of an exposure in this study than that found by Graczyk et al. (1998). They exposed *Corbicula* to $1x10^6$ oocysts/L ($1.9x10^5$ oocysts/clam) for 24 hours and detected low amounts of oocysts in feces 14 days post inoculation (13 days after the exposure was removed). There are several possible reasons for this discrepancy. They reported a relatively high mortality rate of 10% during the test, indicating that the clams were stressed. The efficacy of depuration is related to the rates of pumping and feeding, which are affected by environmental conditions, such as water quality and temperature (Rowse and Fleet 1984, Lauritsen 1986, Jorgensen 1996). If bivalves are held under sub-optimal conditions, they will filter feed and defecate at lower levels, if at all, relative to those under optimal conditions. If the organisms were siphoning sporadically due to environmental factors, *C. parvum* oocysts could have remained in the digestive systems for prolonged periods. A second explanation is the effect of dose on the depuration time. Marinez-Manzanares et al. (1991) found that the depuration time was proportional to inoculation concentrations. Longer

depuration times were required for more heavily contaminated shellfish. An exposure of 1×10^6 oocysts/L is 3 orders of magnitude higher than the highest exposure used in this study and over 2.5 orders of magnitude higher than the highest levels found in surface waters. The results of this study are more applicable to environmental levels of *C. parvum* oocysts that those of previous studies looking at the fate of waterborne oocysts in *Corbicula*.

The global test for exposure effect showed a statistically significant (p=0.0005) difference among the three exposure defection rates over time. Defecation rates increased with increasing exposure concentration. There was also a significant interaction between dose and time (p= 0.0230). The results for the dose-time interaction and multiple comparison analysis are presented in table 1.

There were no significant differences in defecation rates between the 10 and 100 oocysts/L exposures for all observation times at the 5% level. The 1,000 oocysts/L exposure had a significantly higher defecation rates than the 10 and 100 oocysts/L exposure for the 4-, 6-, and 8-hour post inoculation observation times. The mean defecation rate for the 1,000 oocysts/L exposure, 5-hour post-inoculation observation time had high variance, thus was not significantly different than the 10 or 100 oocysts/L exposure (though the 5-hour means followed the temporal trend apparent in the data). Figure 1 shows the mean defecation rate per clam responses for each observation time graphically. The interaction between time and dose is apparent as the mean defecation rates are highest between 2 and 6 hours post-inoculation for the 100 and 1,000 oocysts/L exposures.

Table 1

Summary of Dose Effect on Mean Defecation Rate (oocysts/hr/clam)

for Each Observation Time (* no ANOVA could be computed since there was at most one group with variance greater than zero. Within each observation time, mean defecation rates with a superscript in common were not significantly different with a level of significant of 5% over all comparisons).

Observation Time (hours after dosing)	Exposure Concentration (oocysts/L) 10	100	1000	p-value of ANOVA for each time period
1	0	0	1.75	*
2	1.03[a]	5.39[a]	6.70[a]	0.08
3	1.89[a]	10.49[a,b]	26.50[b]	0.03
4	0.96[a]	6.33[a]	17.36	0.003
5	1.28[a]	2.88[a]	12.61[a]	0.34
6	0.56[a]	2.54[a]	7.89	0.02
8	0.24[a]	0.80[a]	2.72	0.02
12	0.17[a]	0.25[a]	0.64[a]	0.58
24	0.00[a]	0.043[a]	0.147[a]	0.38
26	0.00[a]	0.00[a]	0.00[a]	*
48	0.00[a]	0.00[a]	0.00[a]	*

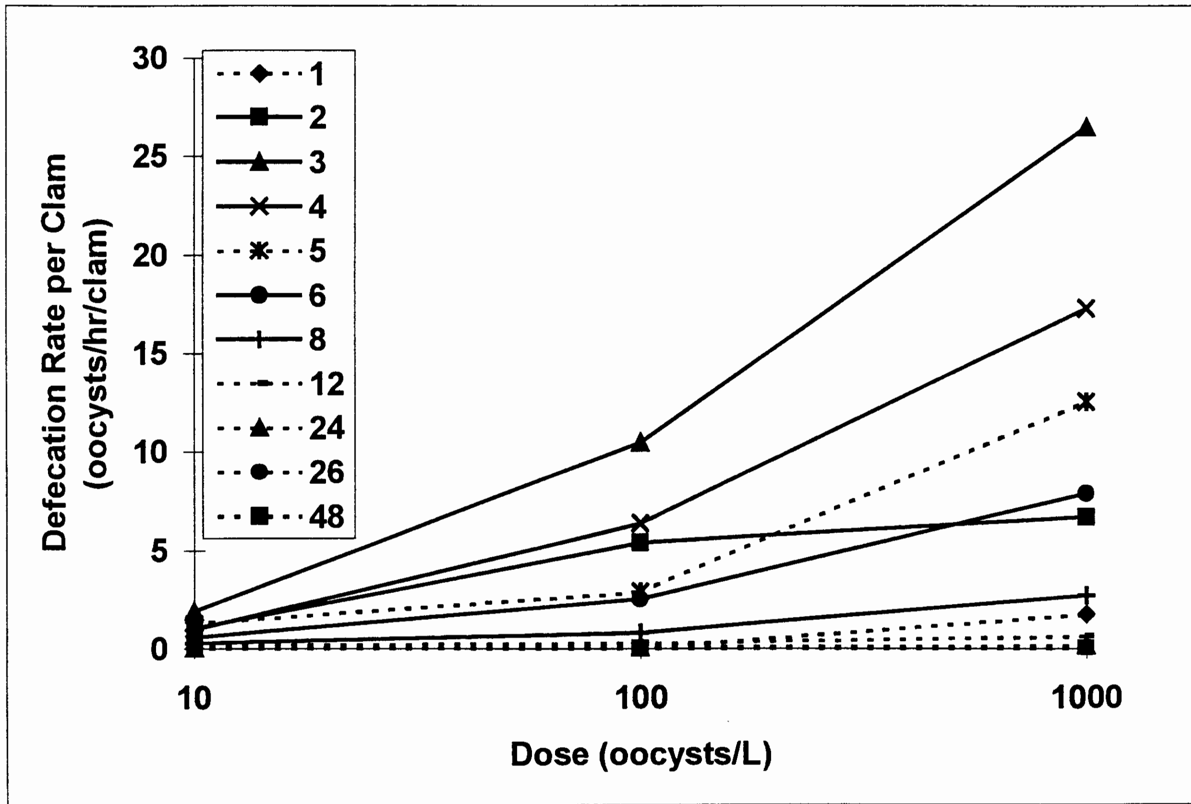

Figure 1. Graphical Display of Mean Defecation Rate per Clam at Each Observation Time (solid lines indicate significant differences for the mean defecation rate across the 3 dosages for the indicated times, with a *P*-value of ≤0.05 used for significance)

A repeated measures ANOVA procedure was also run for each of the 3 exposure concentrations to determine if defecation rates per clam differed significantly over subsequent observation times. The results for the time effect per dose and multiple comparison analysis are presented in table 2. Figure 2 shows the effect of time on mean defecation rate per clam for each dose graphically. No comparisons of defecation rates among observation time within the 10 oocysts/L dose were significantly different at the 5% level. There were significant differences in defecation rates over time for the 100 oocysts/liter exposure ($p=0.004$). The mean defecation rate 3 hours after inoculation was significantly higher than rates at 1 and rates at more than 5 hours after dosing. The peak defecation rates at 3, 4, and 5 hours post-inoculation were not statistically different from each other.

Table 2

Summary of Time Effect on Mean Defecation Rate per Clam After Inoculation with *C. parvum* Oocysts (within each dose concentration, mean defecation rates with a superscript in common were not significantly different with a level of significant of 5% over all comparisons).

Measurement Time (hours after dosing)	Exposure Concentration (oocysts/L) 10	100	1000
1	0.00[a]	0.00[a]	1.75[a]
2	1.03[a]	5.39[a,b]	6.70[a]
3	1.89[a]	10.49[b]	26.50[b]
4	0.96[a]	6.33[a,b]	17.36[a,b]
5	1.28[a]	2.88[a,b]	12.61[a,b]
6	0.56[a]	2.54[a]	7.89[a,b]
8	0.24[a]	0.80[a]	2.72[a]
12	0.17[a]	0.25[a]	0.64[a]
24	0.00[a]	0.04[a]	0.15[a]
26	0.00[a]	0.00[a]	0.00[a]
48	0[a]	0[a]	0[a]
p-value of ANOVA for each dose	.38	.004	.04

The 1000 oocysts/liter dose also had significant differences among defecation rates over time (p=0.04). The mean defecation rate 3 hours after inoculation was significantly higher than

those at 1,2, and those greater than 6 hours after dosing. The mean defecation rates 3, 4, 5 and 6 hours after inoculation were not significantly different from each other.

The data show that *Corbicula* defecate oocysts in highest concentrations 2 to 6 hours after being exposed to *C. parvum* oocysts, and defecation peaks at 3 and 4 hours after initial exposure. Further, there is the potential use of *Corbicula* as a quantitative assay of *C. parvum* oocysts in water, as shown by the significant dose effect between the 100 and 1000 oocysts/L exposures. If *Corbicula* were used in laboratory situation to test water (expose experimentally-housed *Corbicula* to a water sample of unknown oocyst concentration), the feces generated 2 to 6 hours post-exposure generate the highest probability for oocyst detection. A rough estimate of the oocyst concentration in the source water could be made, especially if the concentration were at least 100 oocysts/L. *Corbicula* could also be used to monitor for elevated concentrations of oocysts in natural surface waters by collecting resident clams and allowing them to defecate for several hours in a controlled environment. This technique of testing fecal material from clams collected from the field would provide an assessment of the presence of *C. parvum* from surface water which had passed over the clam during the preceding 1 to 24 hours.

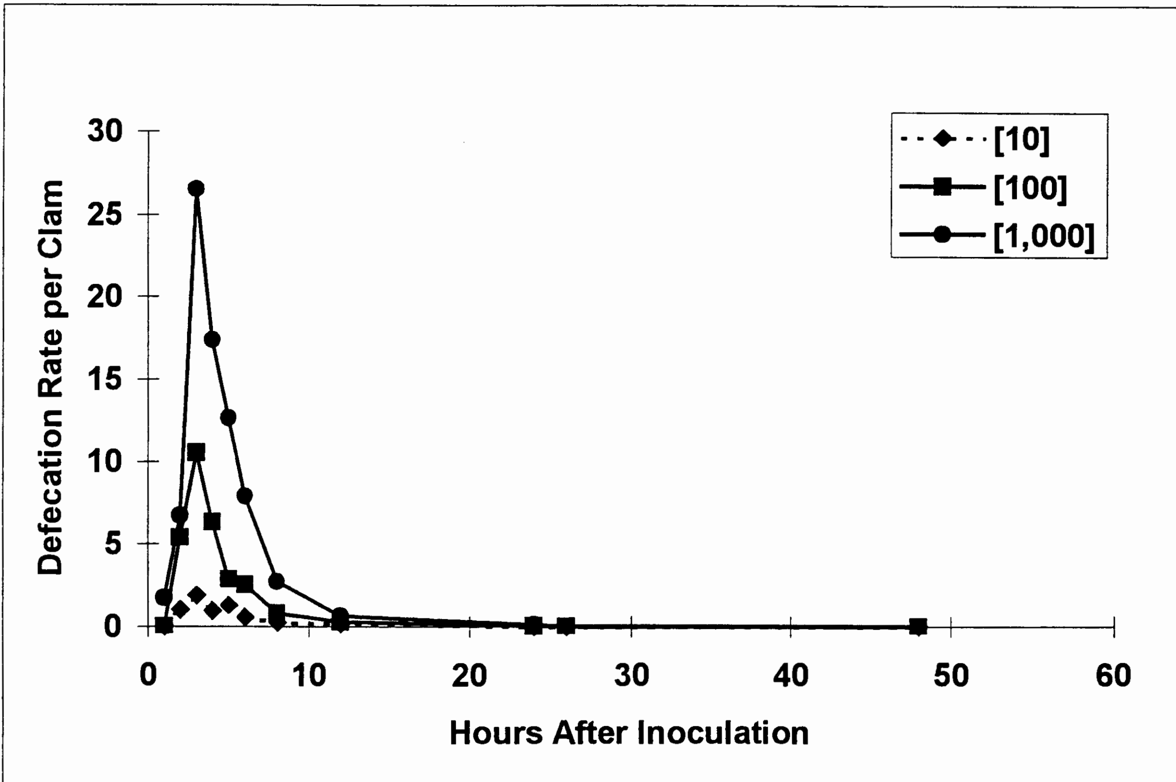

Figure 2. Graphical Display of Mean Defecation Rate per Clam for Each Dose (solid lines indicate significant time effects for that exposure concentration at the 5% level; dashed lines indicate the time effects for that exposure concentration exceeded the 5% level)

The cumulative total of oocysts defecated over the 48 hour test period (figure 3) increased asymptotically, reaching at least 90% of the total defecated within 8 hours post dosing (6 hours after removal from exposure). The mean total number of oocysts collected from three replicates of 20 clams per dose was 138, 614, and 1652 oocysts from the 10, 100, and 1000 oocyst/liter dose concentrations respectively. The majority of oocysts ingested by the clams within the 2-hour exposure period were subsequently excreted within the next 8 hours. The percentage of total oocysts excreted relative to the total number of oocysts spiked into in the 50 L water column was 28% (138/500) for 10 oocysts/L, 12% (614/5,000) for 100 oocysts/L, and 3% (1,652/5,000) for 1,000 oocysts/L.

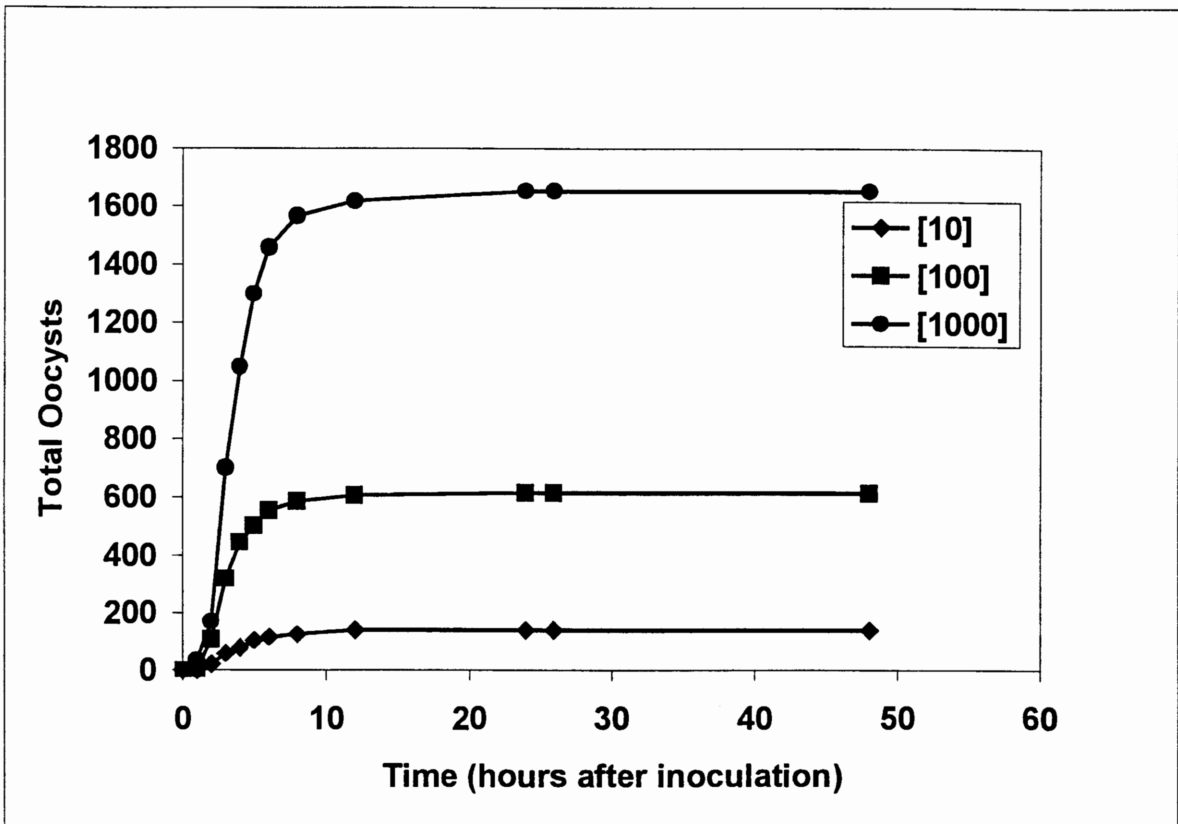

Figure 3. Cumulative Total Oocysts in Feces.

There was no significant difference in mean filtration rates per clam among exposures ($p=0.51$). Mean filtration rates and statistical significance are shown in table 3. There are conflicting studies as to whether filtration rates are affected by quantity and quality of suspended matter in the water column. The proponents of this idea (Bayne et al. 1988) argue that feeding is physiologically regulated based partially on food concentration in water. Mattice (1979), Lauritsen (1986) and Jorgensen (1996) concluded that no such concentration affect on filtration rate exists. Lauritsen (1986) also showed that measurement technique can lead to vastly different filtration rate calculations and resulting conclusions. *C. parvum* oocysts are not a desirable organism to use to test this hypothesis because of the difficulty in measuring their concentrations in water samples (low recoveries and high variance), as experienced in this study. If filtration rates by *Corbicula* are not affected by phytoplankton or other suspended particulate concentrations (within typical environmental ranges), the *Corbicula* test could be used on surface water samples with varying turbidity, unlike standard water testing techniques which are highly impaired by water with even moderate turbidity.

Table 3

Summary of Mean Filtration Rates (L/hr/clam) Among Clams in Three Exposure Concentrations

Exposure (oocysts/L)			p-value of
10	100	1,000	ANOVA
0.17	0.77	0.55	0.51

One dose concentration, two temperatures

The ambient water temperature had a significant effect on oocyst defecation rates (p=0.03). The rate of oocyst excretion in clam feces from the 10°C experiment was significantly lower than that of the 20°C test. No oocysts were detected in clam feces after 10 hours post-inoculation (8 hours post exposure removal) in the 10°C test. There was also a significant time effect (p=.08) and dose-time interaction in the 10°C test. The results for the multiple comparison analysis for the time effect are presented in table 4.

Table 4

Summary of Temperature Effect on Mean Defecation Rate (oocysts/hr) per Clam for Common Observation Times to Both Tests (within each observation time, mean defecation rates with a superscript in common were not significantly different with a level of significant of 5% over all comparisons).

Measurement Time	Test Temperature		p-value of ANOVA for
(hours after dosing)	10°C	20°C	each time period
1	0.64[a]	1.75[a]	0.57
2	3.91[a]	6.70[a]	0.34
3	2.06	26.50	0.04
4	1.61	17.36	0.01
5	1.18[a]	12.61[a]	0.29
6	0.70	7.89	0.03
8	0.14	2.72	0.01

Defecation rates per clam were not significantly different until 3 hours post-inoculation. After that observation time, all but the 5-hour defecation rate were significantly higher in the 20°C test than the 10°C test (the 20°C, 5-hour observation had a high variance, though the mean defecation rate followed the temporal trend apparent in the data). These data are consistent with previous studies that describe increasing filtration rates with increasing temperature (within the tolerance range of the filter feeder). Mattice (1979) found the highest filtration rate for *Corbicula* at 24°C while Foe and Knight (1986) found the filtration rate to increase through 30°C. Lauritsen (1986) found a significantly lower filtration rate at a winter temperature of 8°C in *Corbicula* and a non-significant difference in rates between tests at 20°C and 31°C. Thus the potential use of Corbicula as an assay for pathogens appears to depend on ambient water temperature. This could limit the use of the assay seasonally and/or geographically and the comparability of the assay over spatial and temporal scales.

The 10°C test had no significant differences in defecation rates among observation times ($p=0.36$). There was no significant peak in oocyst excretion, so the efficiency of testing clam feces only during a designated window of high oocyst defecation doesn't appear to apply for low temperature.

Sensitivity analysis

Given the time-dependency of oocyst defecation rates across the 42 hours post exposure, the expected sensitivity (probability of detecting 1 or more oocysts) of the *Corbicula* bioassay likewise varied across time for each dose and temperature tested (figures 4-6 and 8). The 10 oocysts/L exposure had a peak expected sensitivity of 50%, and retained this level of sensitivity from 3 to 5 hours post-inoculation. The 100 and 1,000 oocyst/L exposures had peak expected sensitivities of 100%, with an expected sensitivity of at least 80% for a duration of 4 and 10

hours, respectively. These high expected sensitivities demonstrate the potential utility of using this affordable bioassay for the detection of *C. parvum* in surface waters.

To further show the utility of the *Corbicula* test, figure 7 shows the number of batches of 20 clams per test that would be required in order to gain an overall expected sensitivity of at least 90%, i.e. having a ≥90% probability of detecting at least one *C. parvum* oocyst in contaminated water having a concentration of 10, 100 or 1000 oocysts/L. The overall sensitivity of using a test multiple times, assuming independence between tests, is calculated as:

$$\text{Overall Sensitivity} = 1\text{-}(1\text{-Individual Test Sensitivity})^n$$

where n is the number of times the test is applied. For the time periods following exosure in which oocysts were routinely detected by the bioassay, the maximum number of 20-clam required to generate a ≥90% probability of detecting at least one *C. parvum* oocyst in water containing at least 10 oocysts/L was 7 (figure 7). Seven *Corbicula* bioassays may still be less expensive than traditional water testing for *Cryptosporidium* and have a higher test sensitivity.

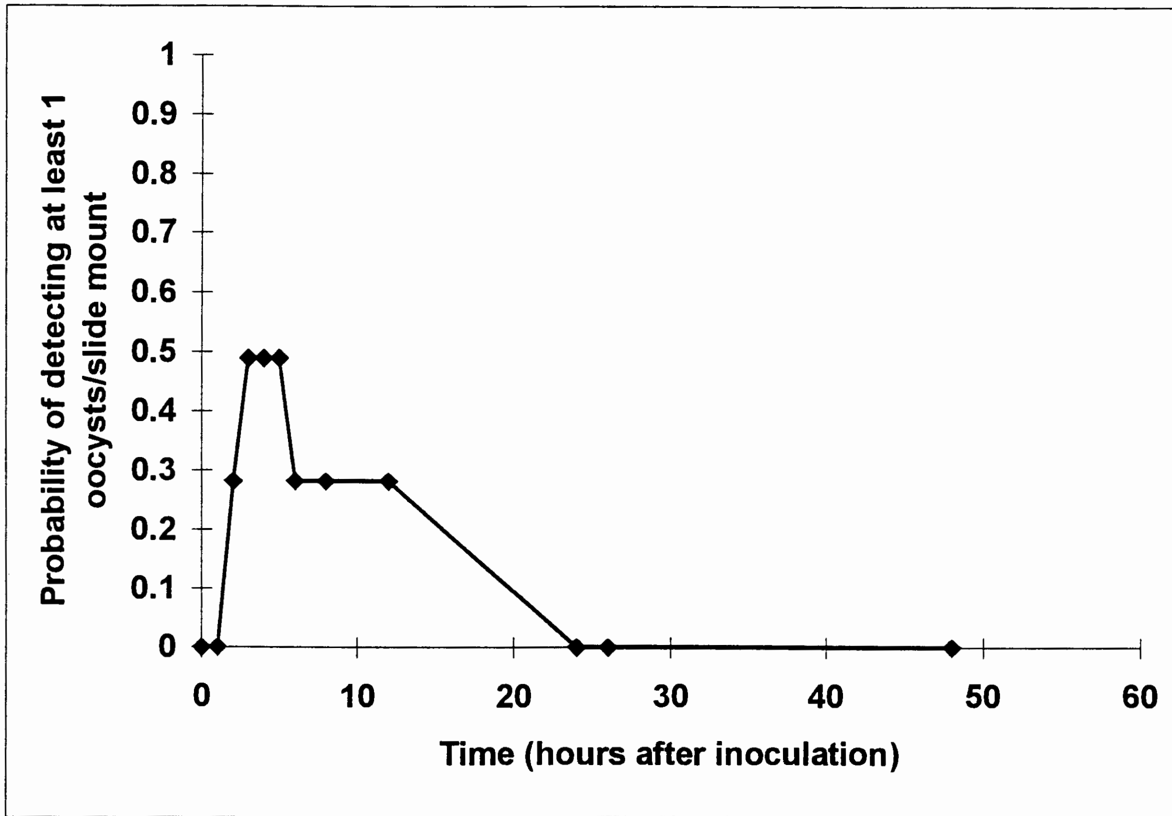

Figure 4. Expected Sensitivity of the *Corbicula* Bioassay at 20°C for Detecting One or More Ooycsts in Water Containing 10 oocysts/L

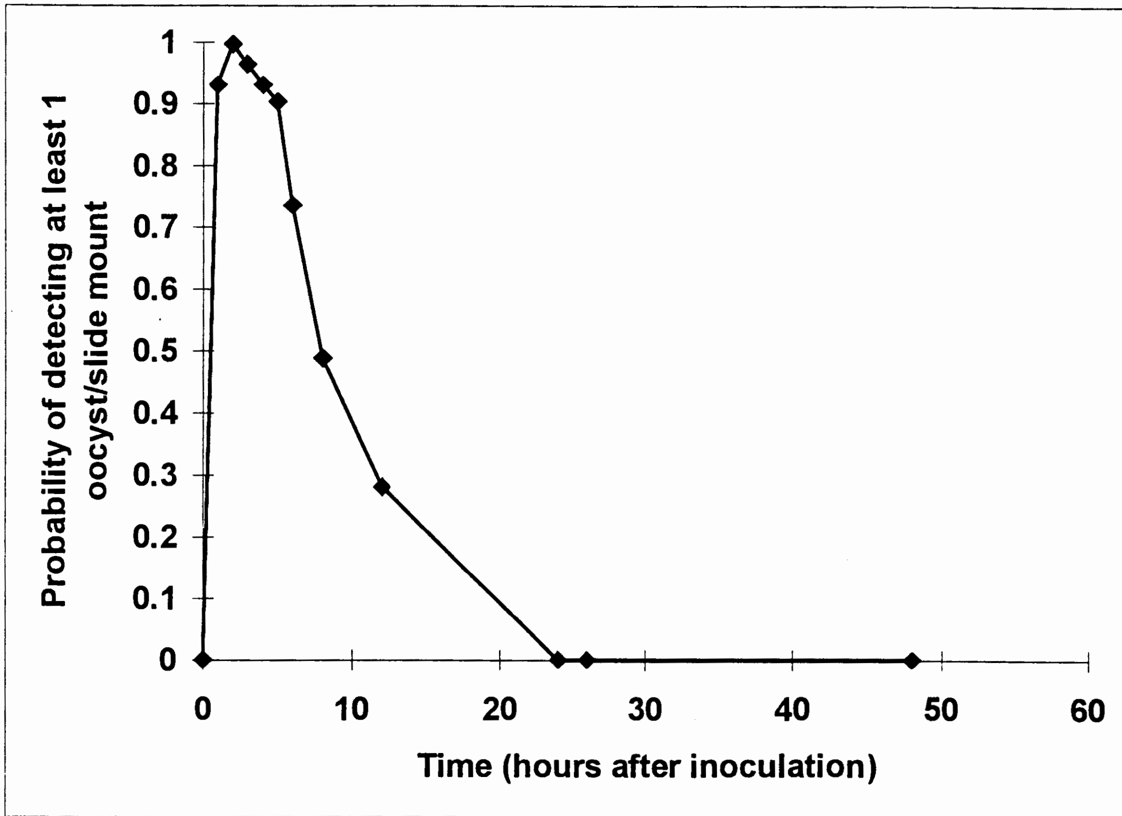

Figure 5. Expected Sensitivity of the *Corbicula* Bioassay at 20°C for Detecting One or More Oocysts in Water Containing 100 oocysts/L

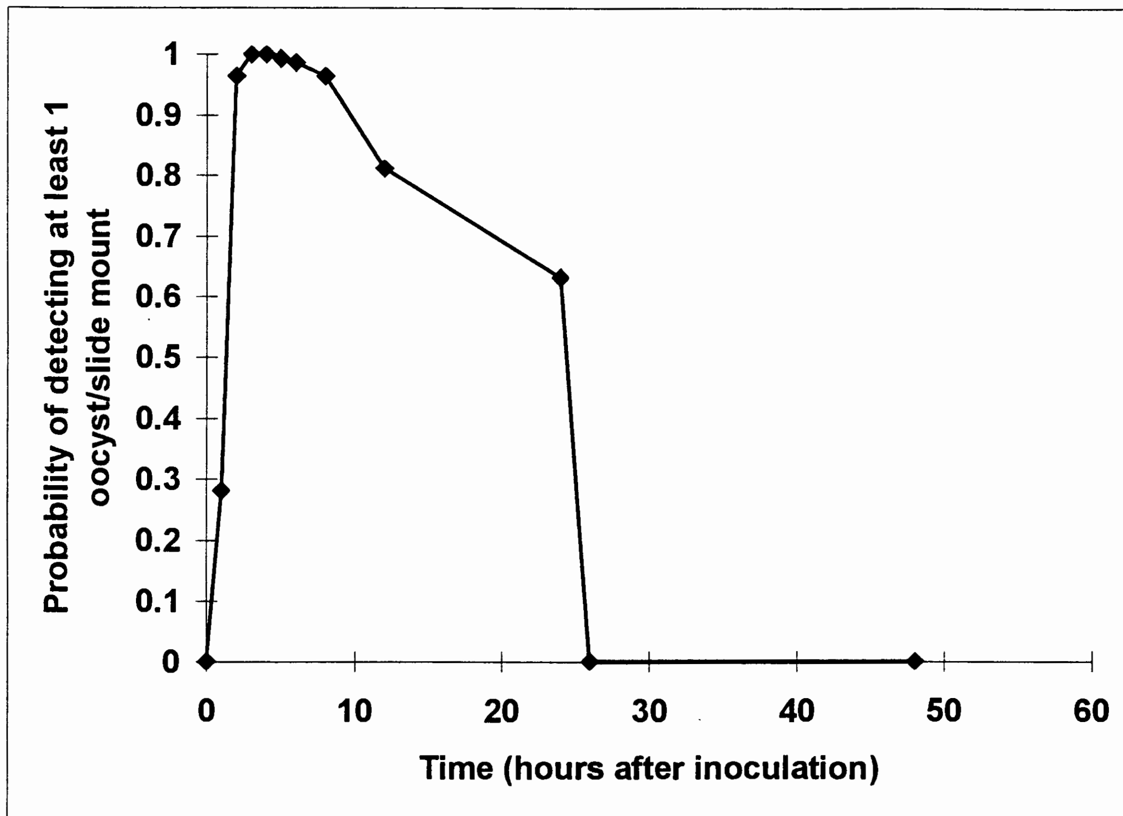

Figure 6. Expected Sensitivity of the 20°C *Corbicula* Bioassay for Detecting One or More Oocysts in Water Containing 1,000 oocyst/L

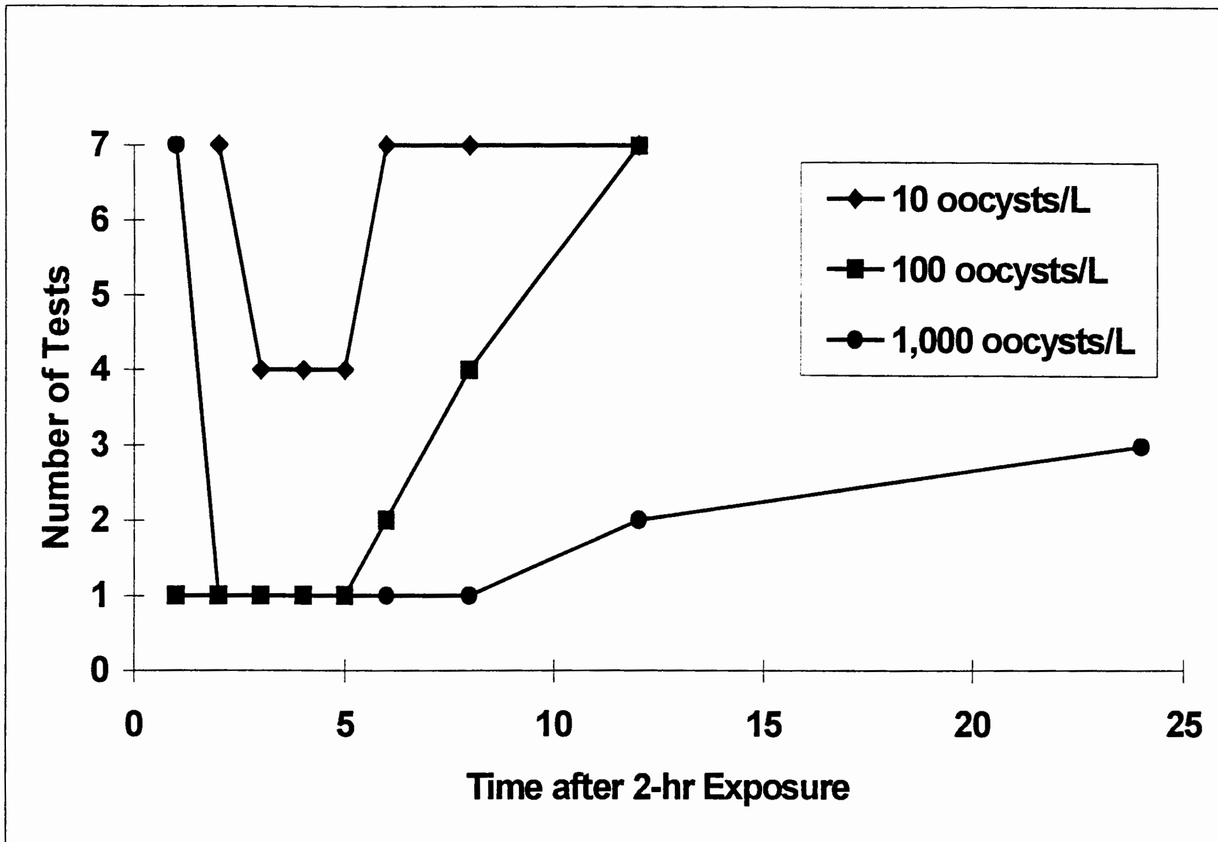

Figure 7. Number of Batches of 20 Clams per 20°C Bioassay Required to Generate Overall Expected Test Sensitivity of at Least 90%

The sensitivity of the *Corbicula* assay at 10°C over all observation times is shown in figure 8. The sensitivity of the test can be seen to be quite high, peaking at 100% and remaining over 80% for between 1 and 6 hours after inoculation (4 hours after removal of exposure). The predicted number of *Corbicula* assays required for an overall test sensitivity of 90% is shown in figure 9. Even with what appeared to be a small defecation rate of oocysts in the 10°C test compared with the 20°C test, the assay is quite powerful. With the individual 20-clam test sensitivity being so high, the total number of tests required to attain an overall sensitivity of 90% is only 2. Again, when no oocysts were detected in the assay, the number of tests required is infinite and is not shown.

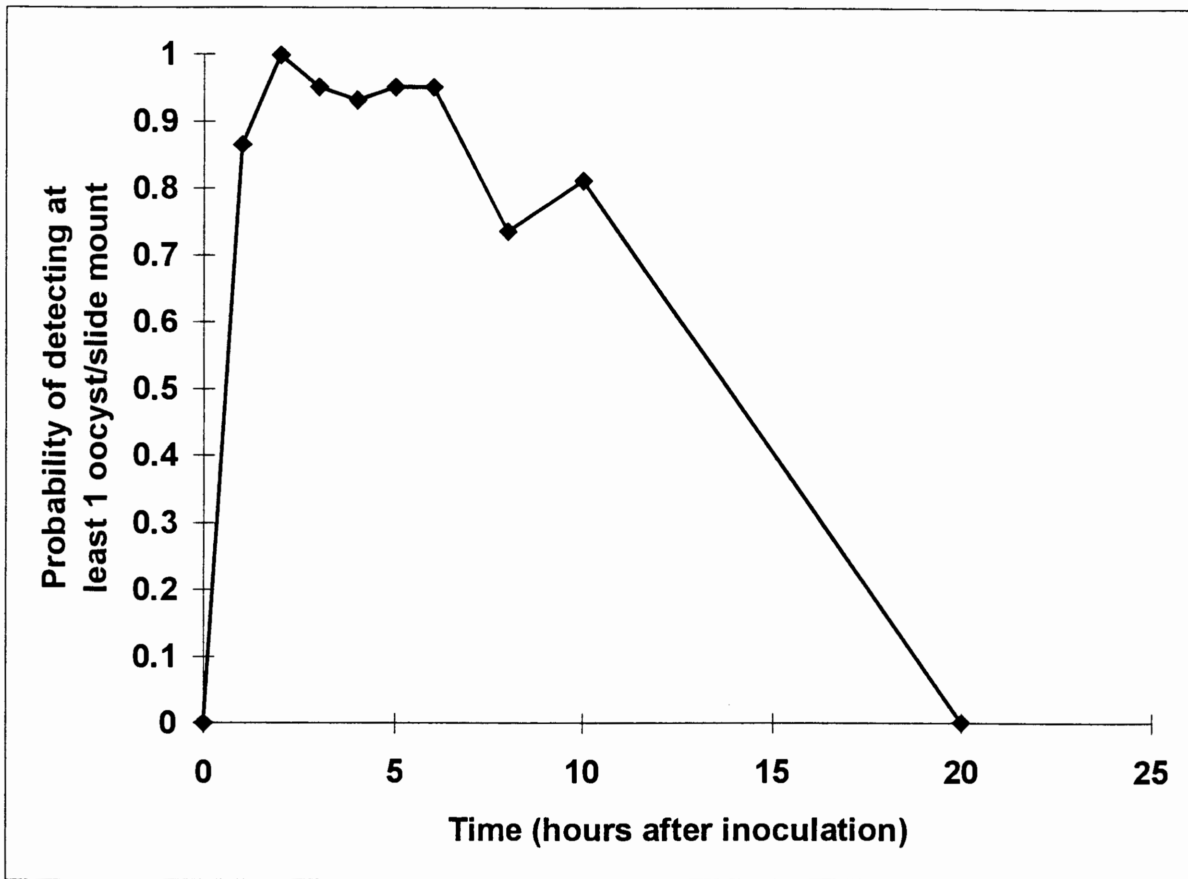

Figure 8. Expected Sensitivity of the 10°C *Corbicula* Bioassay for Detecting One or More Oocysts in Water Containing 1,000 oocysts/L

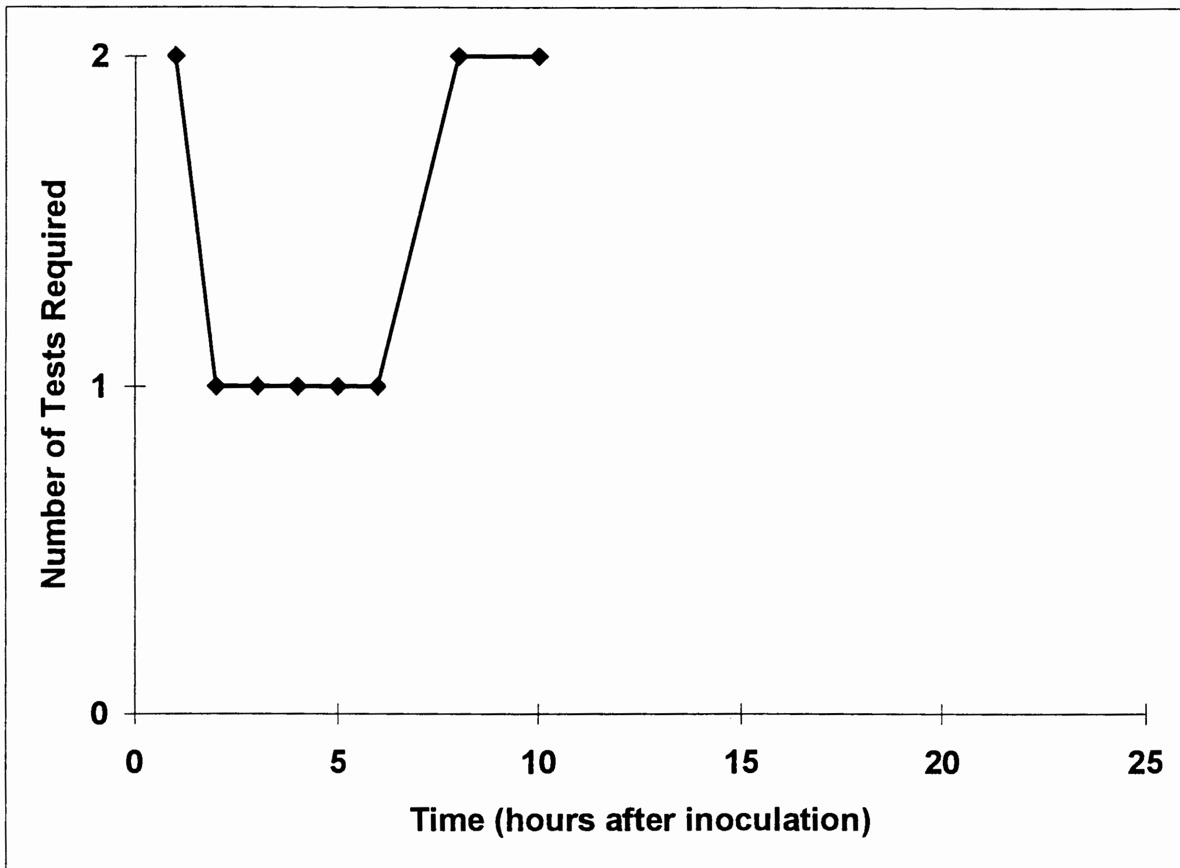

Figure 9. Number of Batches of 20 Clams per 10°C Bioassay Required to Generate Overall Expected Test Sensitivity of at Least 90%

Viability and infectivity

The viability trials, as measured by percent excystation, are presented in table 5. The average percent viable for positive control and clam-passed oocysts are 93.5% and 49.5%, respectively. The processing of *C. parvum* oocysts by *Corbicula* reduced the viability by 53%. We defined viability as the ability of an oocyst to excyst whereby the suture line is opened and 1 or more sporozoites are released. Presumably all oocysts need to excyst to be infectious, but not all excysted sporozoites retain the necessary cellular functions to initiate an infectious cycle. Hence, percent viable is typically an overestimate of the percent infectious (Jenkins et al, 1997).

Table 5

Mean Viability Percentages of Clam-Passed Oocysts Positive Control

	Test Date	
	4/98	5/98
+ Control oocysts	95%	92%
Clam-passed oocysts	37%	62%

Table 6 summarizes the dose per animal, the total number inoculated and the total number infected as determined by histology. A logistic regression model was developed from the dose-response data (Collett 1991) for the clam-passed and the positive control oocysts and was used to calculate the respective 50% infective doses (ID_{50}). The resulting models and estimated ID_{50}s are shown in table 7. The probability that neonatal mice became infected with experimentally inoculated *C. parvum* was not statistically different between control oocysts (no clam passage) compared to oocysts which had been filtered, ingested and excreted by *Corbicula* (*P*-value =

0.14).

Table 6

Summary of *C. parvum* Oocysts Infectivity for 9-day Old BALB/c Mice

	Clam-passed		Control (no clam passage)	
Dose (oocysts per mouse)	No. of mice dosed	No. of mice infected	No. of mice dosed	No. of mice infected
20	9	0	11	1
100	10	0	11	1
300	12	6	11	1
600	9	2	8	1
1000	9	8	4	4

The calculated ID_{50} for both the clam-passed and positive control oocysts are higher than those reported for *C. parvum* oocysts in other studies. Jenkins et al. (1997) and Finch et al. (1993) reported ID_{50}s of 81.5 and 79 respectively for CD-1 mice (using 6- and 4-day-old mouse pups respectively). Ernest et al. (1986) reported an ID_{50} of between 100 and 500 for neonatal Swiss-Webster mice and Korich et al. (1990) an ID_{50} of 60 for BALB/c mice. Animal model systems contain several sources of variation, including the strain of mouse and *C. parvum* used, the age of the mouse pup, and the infectivity of the oocysts (Finch et al. 1993). A logistic regression model was developed for the reported Finch et al. (1993) data. The resulting ID_{50} 90% confidence interval was calculated as (32.2, 477.1), showing the degree of variation in the mouse model system in other studies.

Table 7

Summary of Dose-Response Models and ID_{50} for *C. parvum* Oocysts to 9-day Old BALB/c Mice

$(\log it(p) = \left[\ln\left(\frac{p}{1-p}\right)\right]$ where p is the proportion of infected mice for a given inoculation)

Treatment of Oocysts	Model	ID_{50} (logit=0)
Clam feces	$\text{logit}(\hat{p}) = -9.446 + 3.273[\log_{10}(Dose) + 0.831(Txt = 1)]$	428 oocysts
Control	$\text{logit}(\hat{p}) = -9.446 + 3.273[\log_{10}(Dose) + 0.831(Txt = 0)]$	769 oocysts

The calculated ID_{50} for both the clam-passed and positive control oocysts were higher than those reported for *C. parvum* oocysts in other studies. Jenkins et al. (1997) and Finch et al. (1993) reported ID_{50}s of 81.5 and 79 respectively for CD-1 mice (using 6- and 4-day-old mouse pups respectively). Ernest et al. (1986) reported an ID_{50} of between 100 and 500 for neonatal Swiss-Webster mice and Korich et al. (1990) an ID_{50} of 60 for BALB/c mice. Animal model systems contain several sources of variation, including the strain of mouse and *C. parvum* used, the age of the mouse pup, and the infectivity of the oocysts (Finch et al. 1993). A logistic regression model was developed for the reported Finch et al. (1993) data. The resulting ID_{50} 90% confidence interval was calculated as (32.2, 477.1), showing the degree of variation in the mouse model system in that study.

PRINCIPLE FINDINGS AND THEIR SIGNIFICANCE

C. parvum oocysts were detected in *Corbicula* feces after exposure to a wide range of environmentally representative exposure concentrations. Oocysts were defecated in increasing rates with increasing exposure concentrations, with the peak defecation occurring 3-4 hours after exposures were initiated. No oocysts were detected in clam feces after 22 hours of depuration for all exposure concentrations tested, and 90% of total detected oocysts were defecated within 8 hours after exposure.

Defecation rates decreased with decreasing ambient water temperatures. Even with lower defecation rates in low temperature experiments, no oocysts were detected after 8 hour of depuration.

Peak sensitivities for the *Corbicula* test to detecting oocysts in surrounding water ranged from 50% for the 10 oocysts/L exposure concentration to 100% for the 100 and 1,000 oocysts/L exposure concentrations. With these high test sensitivities, the number of replicate, 20-clam *Corbicula* tests needed to detect *C. parvum* oocysts in contaminated surface waters 90% of the time would range from 1 to 7 (depending on oocysts concentration) according to this study.

The Corbicula assay used in this study proved to be a sensitive test for C. parvum oocysts in its surrounding waters. The technique of testing for oocysts in feces provides an easy, inexpensive and non-invasive method of utilizing a resident filter feeder to concentrate oocysts from surrounding surface waters.

Clam-passed oocysts from environmentally representative exposure concentrations retain approximately 50% of their viability and remain infectious. This has serious public health applications, as certain minority groups have been witnessed to collect *Corbicula* for consumption, and to eat clams raw in the process (T. Rose).

Ambient water temperatures were found to significantly affect *Corbicula* defecation rates of *C. parvum* oocysts. There may be other confounding factors for the detection of oocysts in *Corbicula* feces not found in this study. Similar tests as those in this study should be run over a range of geographic and seasonal parameters to ensure similar results for other areas of the country which may have different species or strains of Corbicula, different temperature regimes, and possibly other factors which may affect the detection of oocysts in *Corbicula* feces after exposure to *C. parvum*.

SUMMARY

This study has shown that a prolific, resident filter-feeding bivalve, Corbicula fluminea, can effectively be used in the detection of *C. parvum* oocysts in surrounding surface water. These clams filter, concentrate and defecate oocysts, allowing the detection of which with IFA staining techniques. The *Corbicula* assay had high test sensitivities, even in low oocyst concentrations and in low ambient water temperature.

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