

Report for 2003NY21B: Validity Assessment of Methods to Distinguish Between Ruminant and Human Sources of Fecal Contamination in Watersheds

- Other Publications:
 - Braun-Howland, E, 2005, Assessment of methods to distinguish between human and ruminant sources of fecal pollution, 29th Annual meeting of the New England Association of Environmental Biologists, Fort William Henry, Resorts & Conference Center, Lake George, NY, March 2005.
- unclassified:
 - Lendrum, Jacqueline, E Braun-Howland, 2005, Assessment of methods to distinguish between human and ruminant sources of fecal pollution, State University of New York, School of Public Health, Student Poster Day, April 2005.

Report Follows

Title: Validity Assessment of Methods to Distinguish Between Ruminant and Human Sources of Fecal Contamination in Watersheds

Problem & Research Objectives:

Nonpoint source (NPS) pollution, including runoff from agricultural operations and failing or improperly sited septic systems can have a substantial detrimental impact on source water quality. In addition to environmental concerns, significant public health effects have been attributed to NPS fecal contamination of both drinking and recreational waters. Pathogens of concern include *Giardia* and *Cryptosporidium*, pathogenic strains of *Escherichia coli*, *Salmonella* sp., and *Listeria monocytogenes*.

Because indicator organisms are commonly used to ascertain the safety of a water supply, it is important to understand the behavior of these organisms under varying environmental conditions. The studies presented herein examined fecal suspensions *in situ* to concomitantly measure seasonal and temporal effects on the survival of organisms indicative of fecal contamination. Numbers of traditional indicator organisms, including total coliforms, *E. coli* and enterococci were monitored over time using approved culture techniques. These results are compared with the survival of *Bacteroides*, determined using the PCR-based method developed by Bernhard and Field (2000). In contrast to detection methods based on culturing, this alternative technique identifies fecal contamination through the amplification of *Bacteroides* DNA. Previous studies in our laboratory have shown that the *Bacteroides* test is able to detect species-specific markers of fecal pollution in surface waters impacted by CAFO operations and in septage samples. Finally, the use of rRNA-based *in situ* hybridizations for the identification of *Bacteroides*, would permit the detection of potentially viable organisms, rather than dead cells. The proposed studies are important because they will determine whether the *Bacteroides* method detects recent fecal contamination, or whether the organisms detected could have originated at a spatially or temporally distant site. These results are critical to the validation of the *Bacteroides* method as an appropriate technique for unambiguously discriminating between recent human and agricultural sources of fecal pollution.

The original objectives of the project were:

- To measure the die-off kinetics of fecally-derived indicator bacteria including: *Bacteroides*, *E.coli*, total coliforms, and enterococci *in situ* under varying environmental conditions;
- To determine the effects of fecally-impacted and non-impacted stream conditions on the *in situ* survival of the various indicator organisms; and
- To confirm that the molecular-based method for the identification of *Bacteroides* results in the detection of DNA from viable organisms, rather than dead cells, using whole cell, *in situ* hybridizations.

Four significant project adjustments were made. The first is that the studies were carried out in surface water environments on private property, rather than streams, due to potential public health consequences associated with breakage of the chamber

membranes during incubation. Secondly, *in situ* incubations were carried out cross-seasonally due to the unexpected duration of survival of the test organisms. Thirdly, the calf feces used in these studies were naturally infected with *Cryptosporidium parvum* oocysts, permitting concomitant evaluation of the detection of this important protozoan pathogen over the course of these studies. Lastly, detection of *Bacteroides* using *in situ* hybridizations was unsuccessful due to an unacceptable amount of autofluorescence associated with the fecal samples.

Methodology:

Fecal samples obtained as a composite mixture from several calves were a kind gift from Dr. Dwight Bowman, Cornell University. Diffusion chambers (McFeters and Stuart, 1972) containing 30 ml of bovine fecal suspensions at two concentrations were incubated, in triplicate, in a pond environment minimally impacted by fecal contamination and in a separate, fecally-impacted pond. The diffusion chambers were sampled over a period of 223 days, beginning with weekly sampling during the month of May, then biweekly sampling during June and July, followed by monthly sampling from August to December. Diffusion chambers were removed from the environment in December, prior to deep-freezing of the ponds. Diffusion of molecules through chamber membranes was ascertained after their removal from the environment by spectrophotometrically demonstrating the transfer of FITC dissolved in an outside aqueous environment to the inside of the chambers. Also, the extent of biofilm formation on chamber membranes was assessed using scanning confocal laser microscopy.

Samples were analyzed for total coliforms, *E. coli*, and enterococci using EPA-approved Colilert/Enterolert methodologies until the October sampling date, when limits of detection for organisms remaining in some of the test chambers required the addition of a membrane filtration format. For detection of *E. coli* SM 9222D (APHA) was used, followed by incubation on NA-MUG medium. Enterococci were detected using mE agar and confirmed on EIA agar according to EPA Method 1106.1.

Polymerase chain reaction (PCR) amplifications for detection of *E. coli*, enterococci, *Bacteroides* and *Cryptosporidium* were used to evaluate the presence of specific DNA sequences during the sampling period. Because *E. coli*, enterococci and, in many cases, *Cryptosporidium* oocysts were detectable using standard techniques after 223 days incubation, amplifications were performed on samples from selected, rather than each, sampling date. Amplification conditions and primers used were based on the following protocols: *Cryptosporidium* (LeChevallier et al., 2003); enterococci (Haugland et al., 2005); *E. coli* (Williams and Braun-Howland, 2003, Lane et al., 1985) and *Bacteroides* (Bernhard and Field, 2000).

Cryptosporidium oocysts were microscopically detected and enumerated, in duplicate, at each time point using standard immunofluorescence staining techniques on 10-50 μ l aliquots of sample (or dilution thereof) that had been spotted and dried on a multiwell slide coated with poly-L-lysine. Inclusion of the fluorochrome, DAPI, aided in the

identification of oocysts containing sporozoite nuclei. Because the results of previous studies indicated that sample drying temperature affects the percentage of oocysts that are DAPI positive, detection of *Cryptosporidium* oocysts was carried out using drying temperatures of both 41 °C and 65 °C.

Water quality parameters including water temperature, pH, dissolved oxygen, and conductivity were measured in both pond locations at the time of sampling. Nutrient samples including total phosphorus, TKN, nitrate, ammonia and TOC for both pond environments were collected.

Principal Findings & Significance:

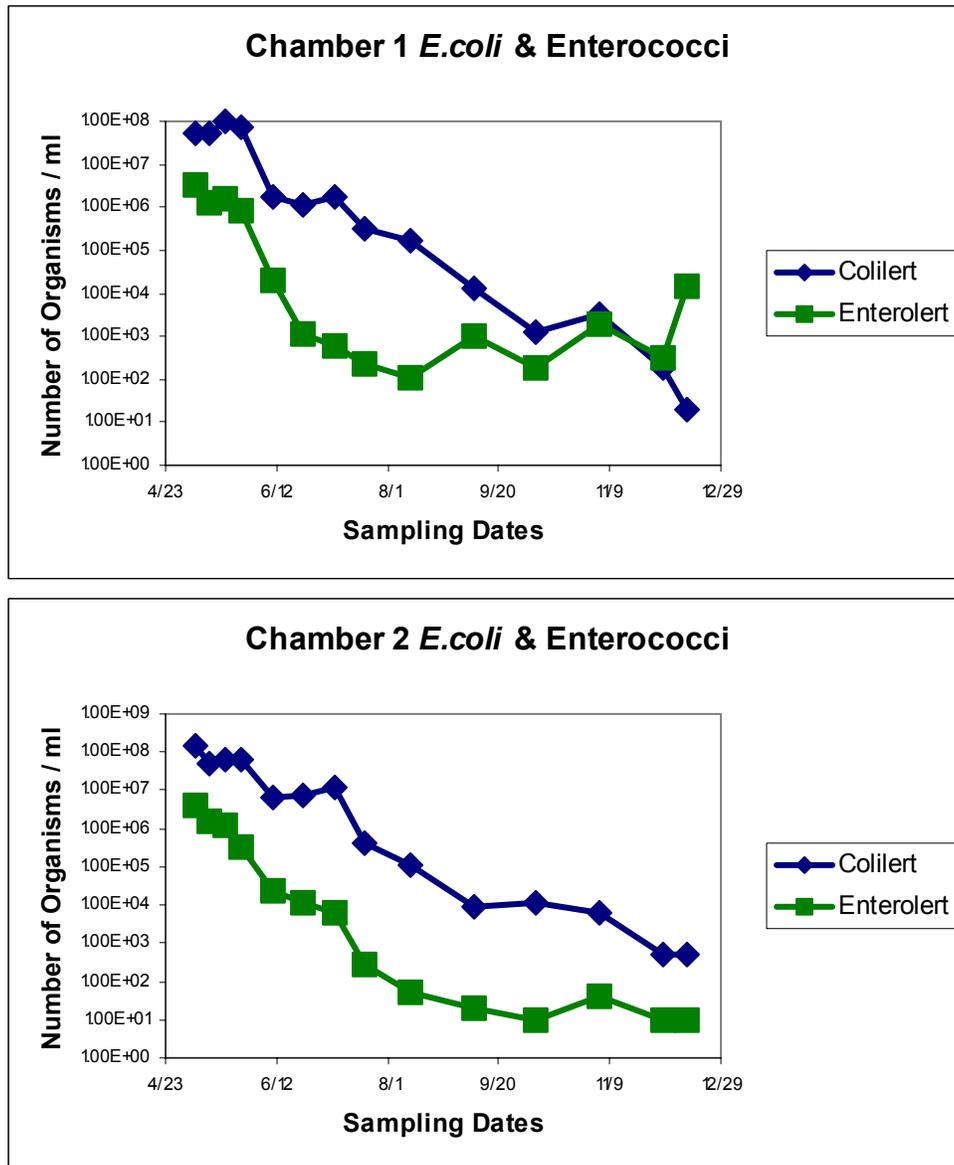
To our knowledge, this is the first study to concomitantly examine *in situ* survival rates of *E. coli*, enterococci, *Bacteroides* and *Cryptosporidium parvum* in unadulterated calf feces. All organisms were detectable in at least one of the triplicate diffusion chambers examined over a 223 day incubation period. Therefore, under the conditions employed, none of the organisms evaluated was a good indicator of recent fecal pollution. These results do, however, suggest that *E. coli* may be an appropriate indicator for the presence of agriculturally-derived *C. parvum*.

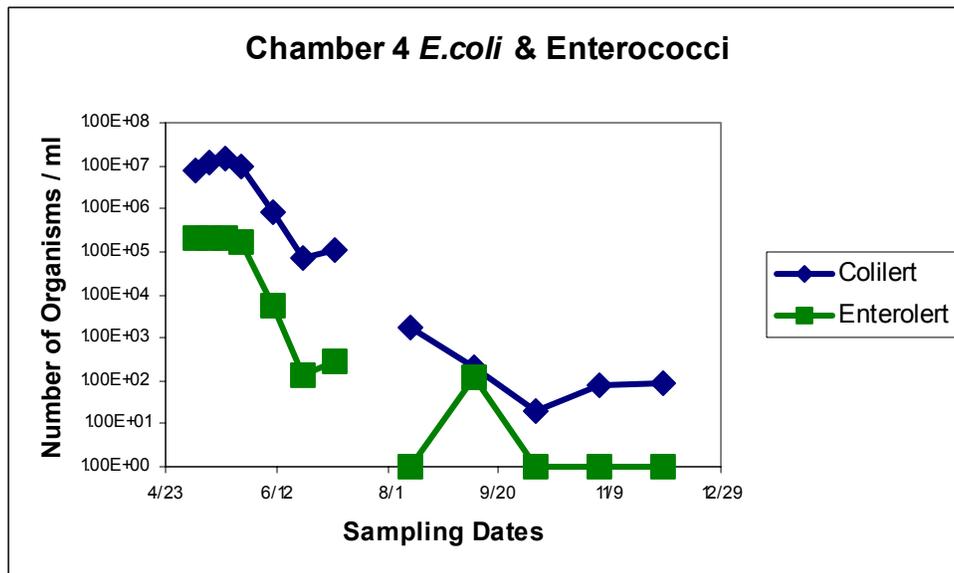
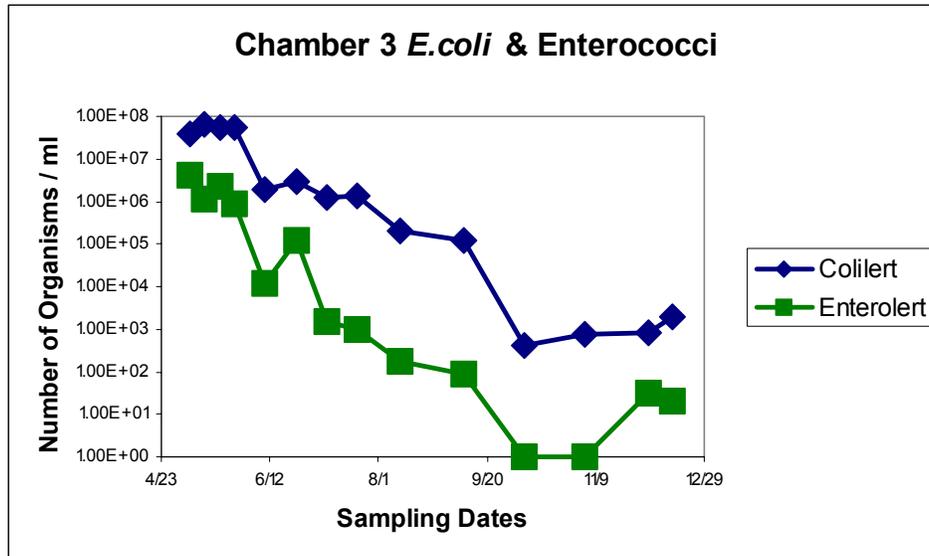
One original premise of these studies was that bacteriological indicators of fecal pollution would become undetectable during the incubation period using growth on bacteriological medium as the criterion. At that point, molecular techniques would be used to ascertain the presence of the various indicator organisms. Therefore, PCR amplification-based detection of *E. coli* and enterococci could be compared to the PCR-based detection of *Bacteroides*. However, the results of these studies demonstrated that, using standard techniques, measurable amounts of *E. coli* and enterococci remained in diffusion chambers after 223 days incubation in the environment

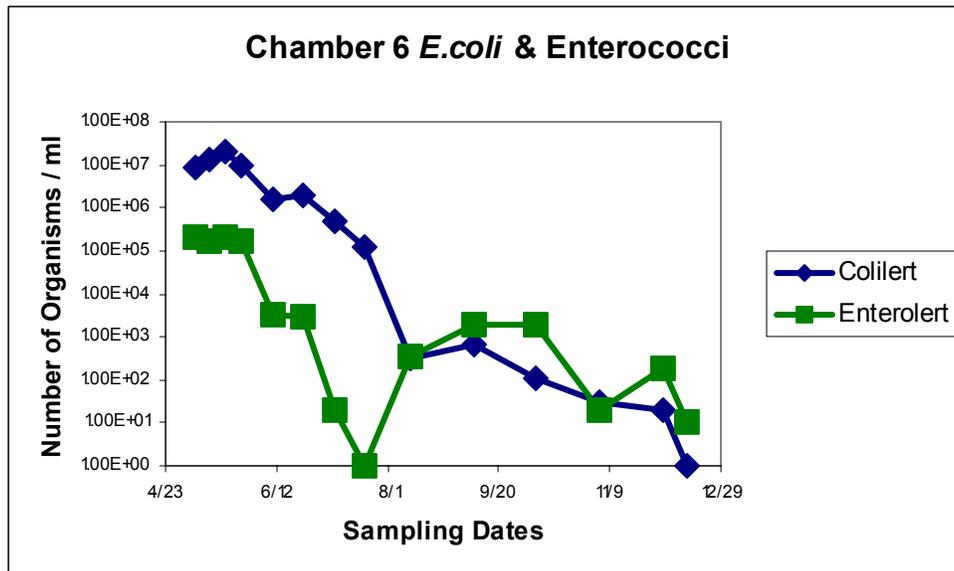
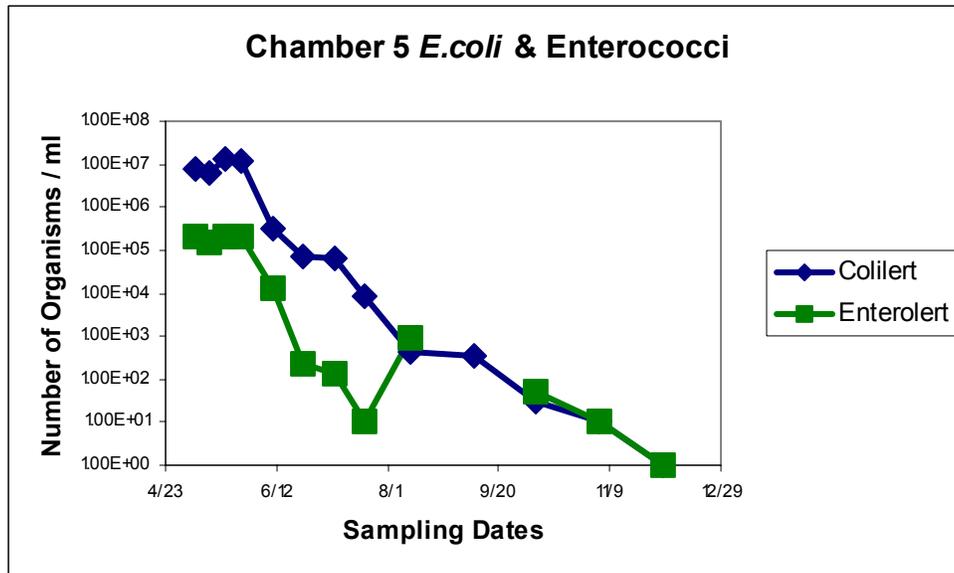
Numbers of *E. coli* and enterococci detected in diffusion chamber samples over time are presented in Figure 1. Chambers 1-6 were incubated in the low nutrient environment, where as 7-12 were incubated in a pond moderately impacted by farming activities. Membranes on two of the three chambers containing undiluted sample (chambers 7,9) were compromised during the study due to parasite infestation.

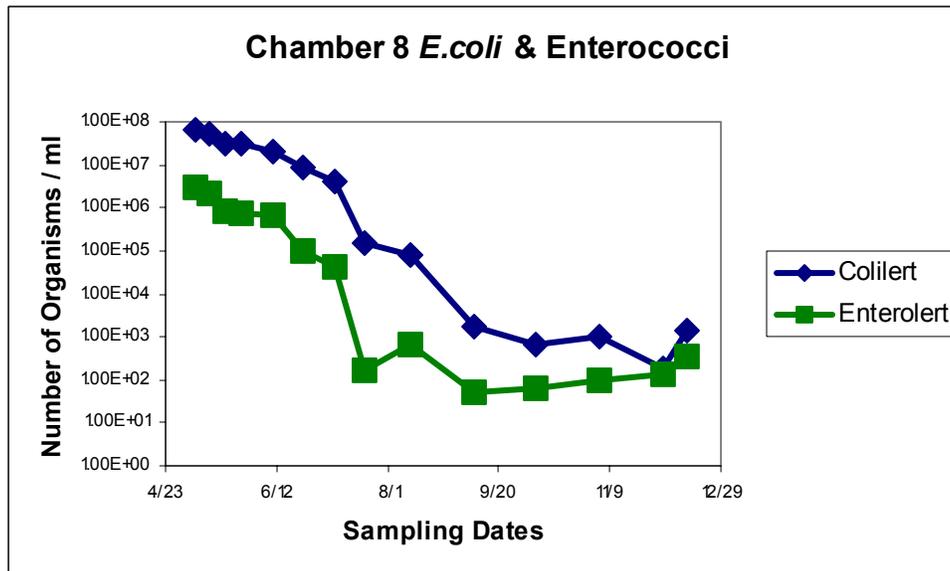
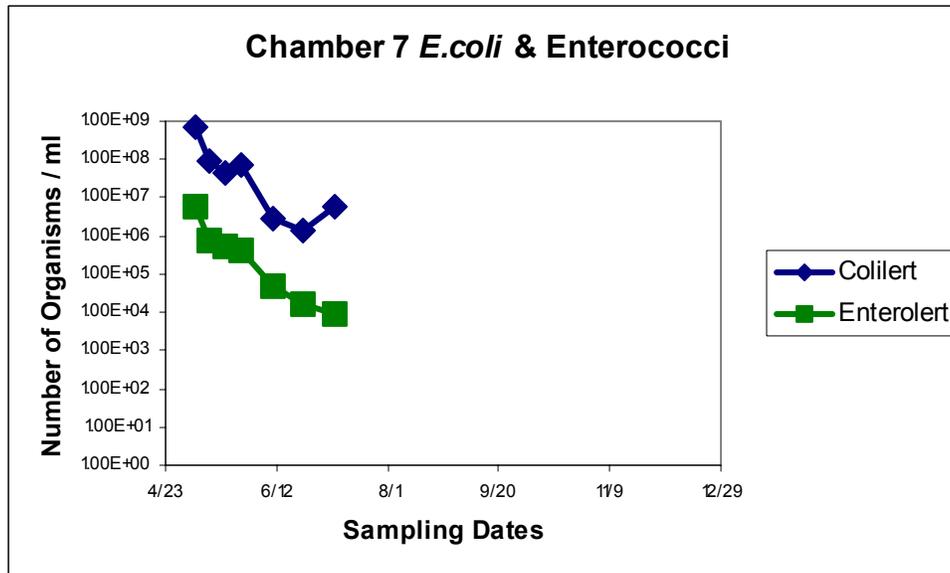
Relative survival of organisms was apparently unimpacted by incubation conditions (chambers 1-3 versus 8; 4-6 versus 10-12). Numbers of *E. coli*, approximating 10^8 organisms/ml in undiluted chambers, were stable for about one month in both pond environments; low numbers of *E. coli* (10^0 - 10^1 organisms/ml) were still detectable on bacteriological media at the end of the incubation period. Survival of *E. coli* and enterococci was apparently affected by initial concentration of organisms (chambers 1-3 vs. 4-6; 8 versus 10-12): a more rapid reduction in bacterial numbers was observed in diluted samples. Similar results were observed for numbers of enterococci in the twelve chambers, although the numbers were much more variable between the triplicate chambers.

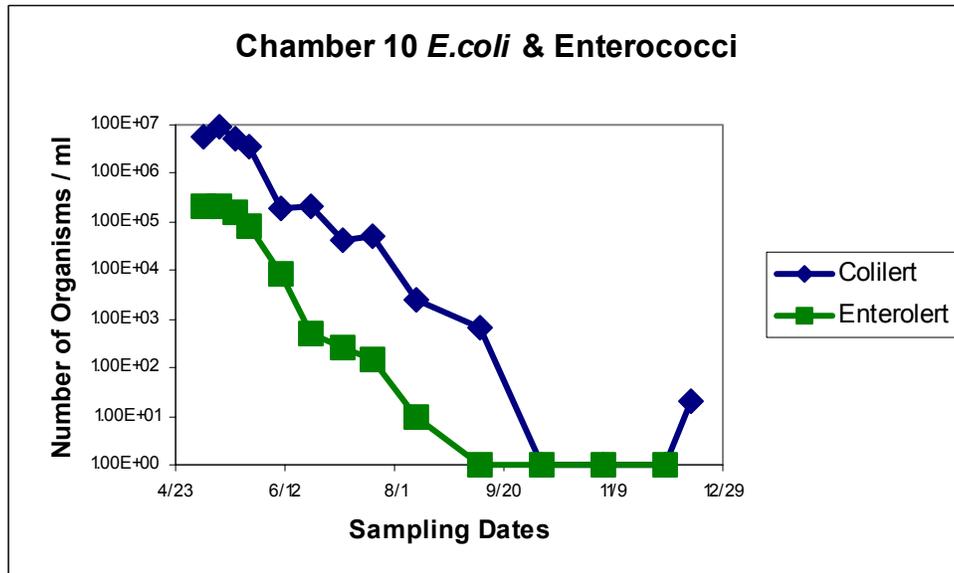
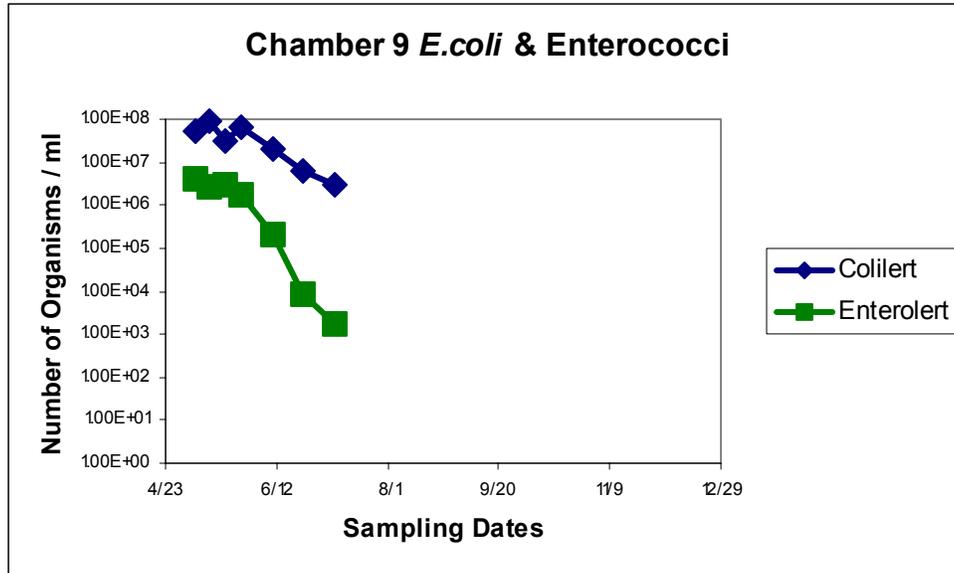
Figure 1. Detection of *E. coli* and enterococci over time in diffusion chambers. Chambers 1-3 and 7-9 contained undiluted calf feces; contents of chambers 4-6 and 10-12 were diluted ten-fold. Chambers 1-6 were incubated in a pond relatively unimpacted by human or agricultural waste, whereas chambers 7-12 were incubated in a pond moderately impacted by farming activities.

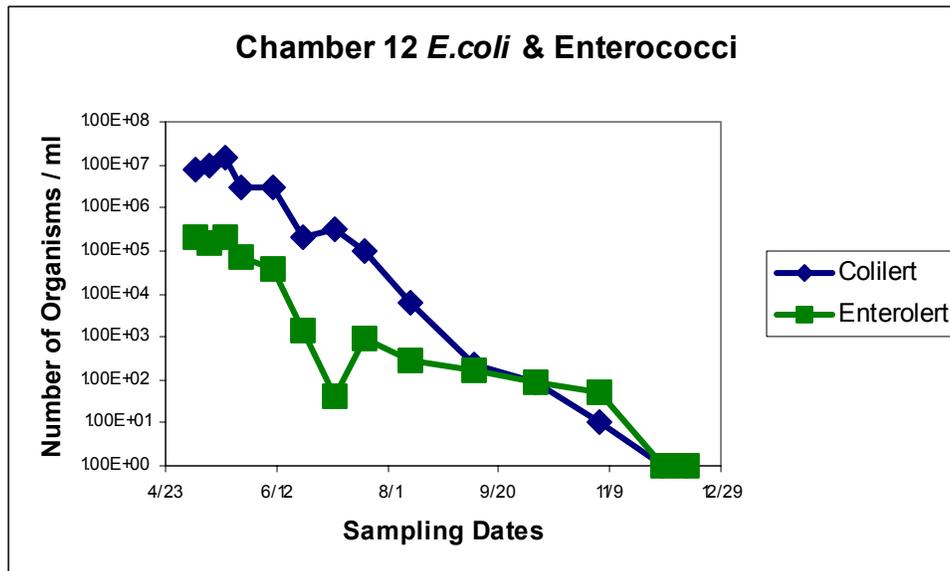
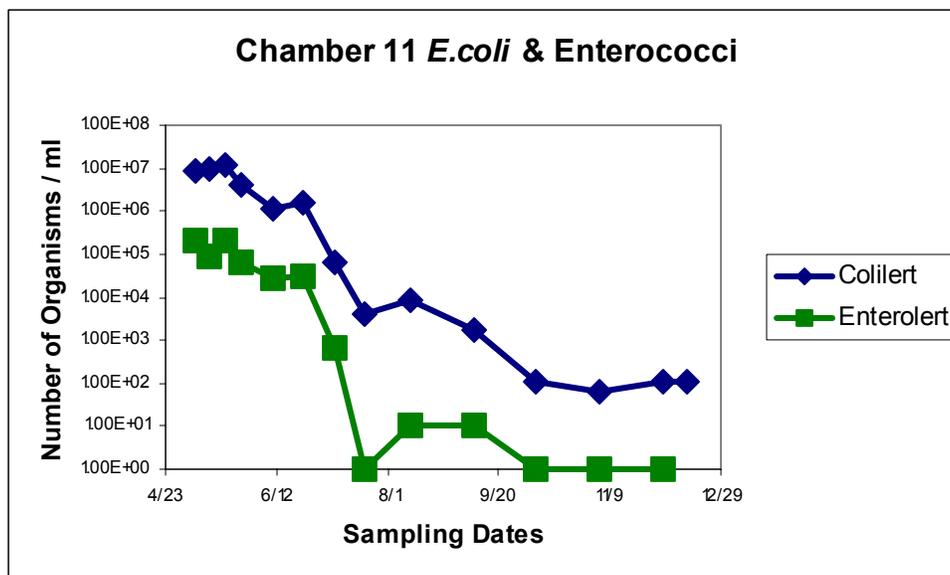








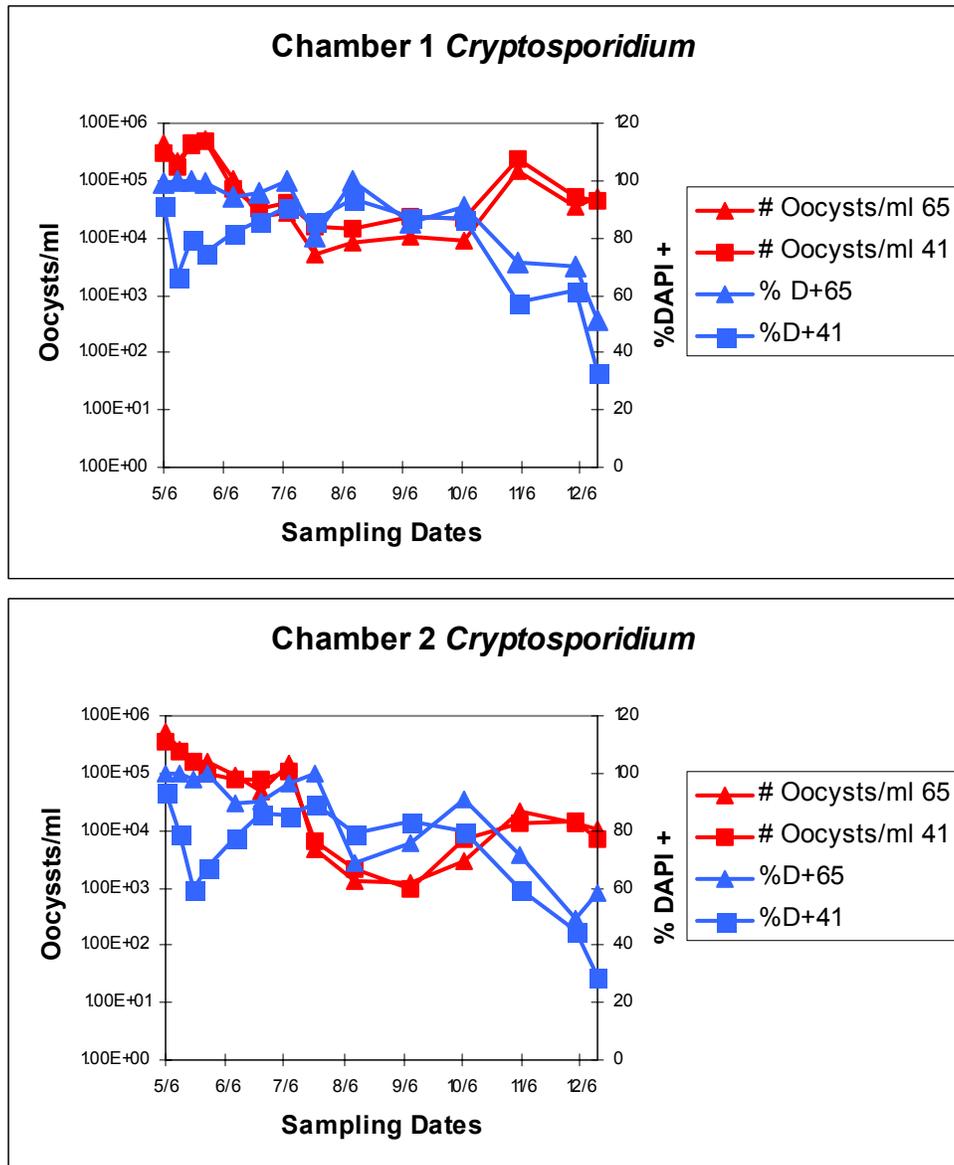


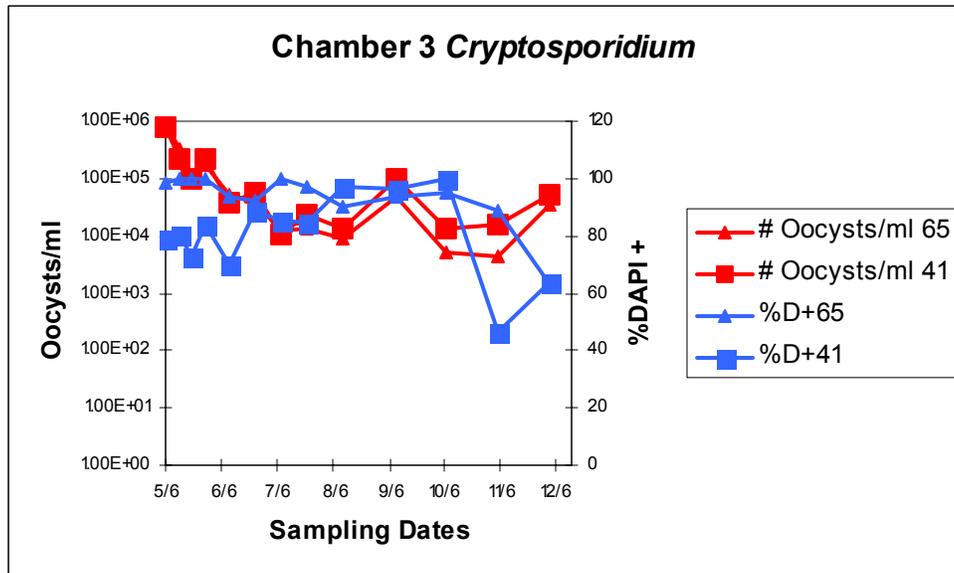


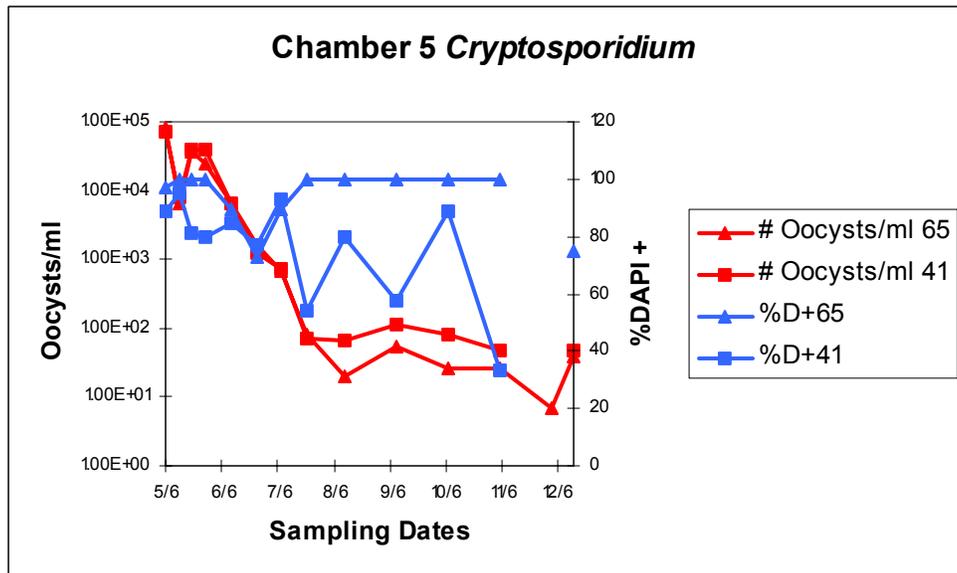
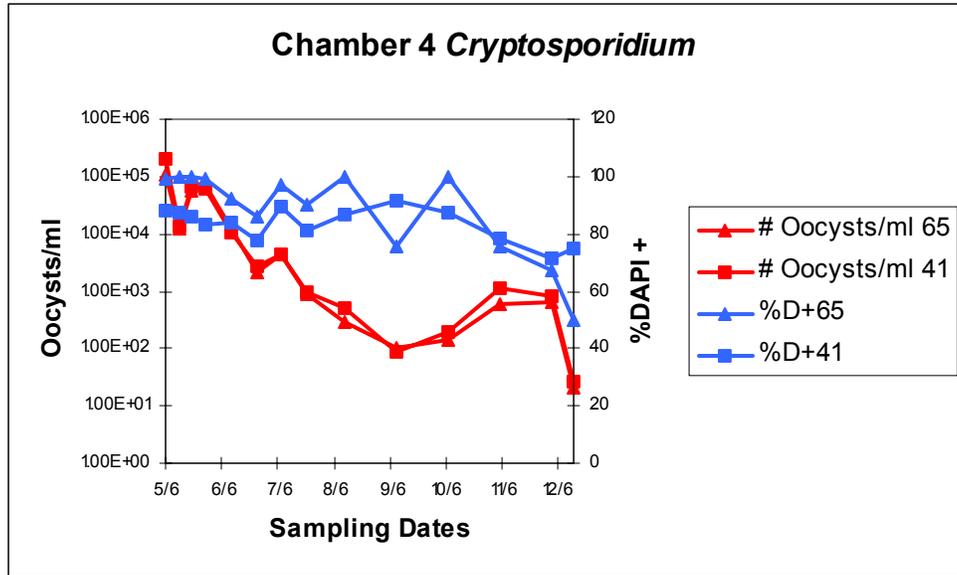
Numbers of *Cryptosporidium parvum* oocysts detected at 41 °C and 65 °C, and the relative percentages of DAPI positive oocysts, are presented in Figure 2. Numbers of DAPI positive oocysts were low, but detectable, at the end of the incubation period. Excystation assays performed on a sample removed in December, 2004 demonstrated the presence of oocysts capable of excystation. However, limited infectivity assays performed using the cell culture-focus detection method (DiGiovanni et al., 1999) indicated that few, if any, of the oocysts were infectious.

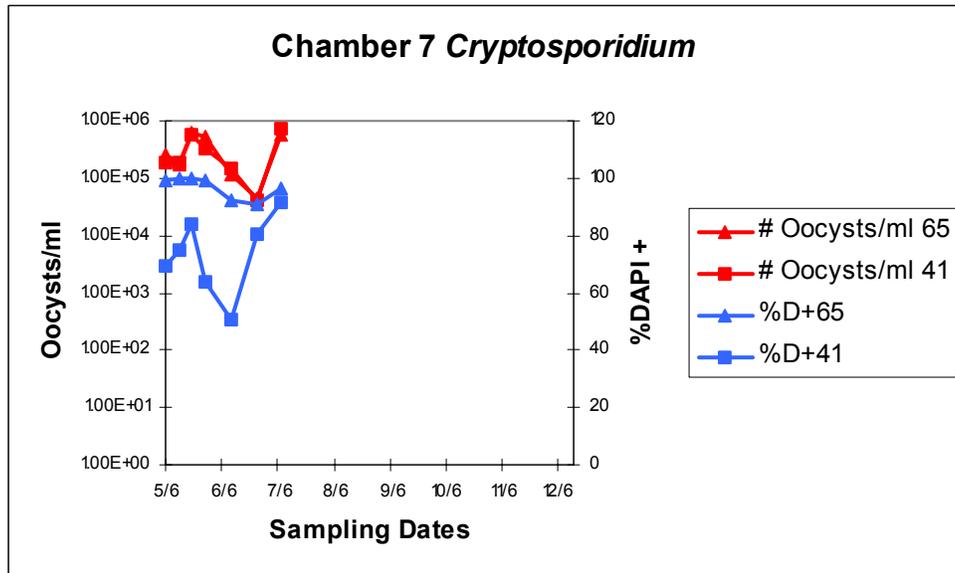
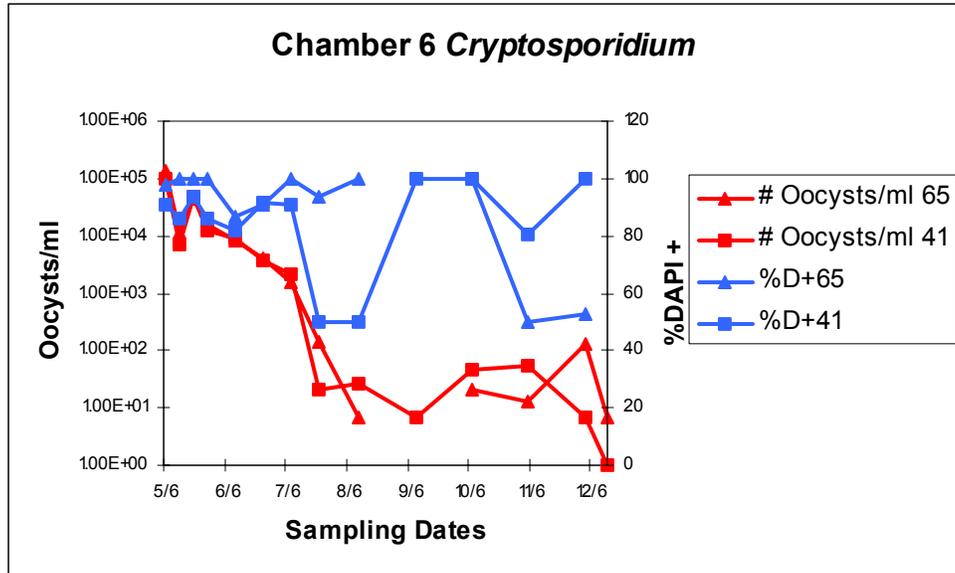
Relative numbers of *E. coli* observed inside and outside of the incubation chambers, together with microscopic data showing the presence of unique algal genera inside, versus outside, of the chambers (data not shown), indicated that the chamber membranes remained intact throughout the sampling period.

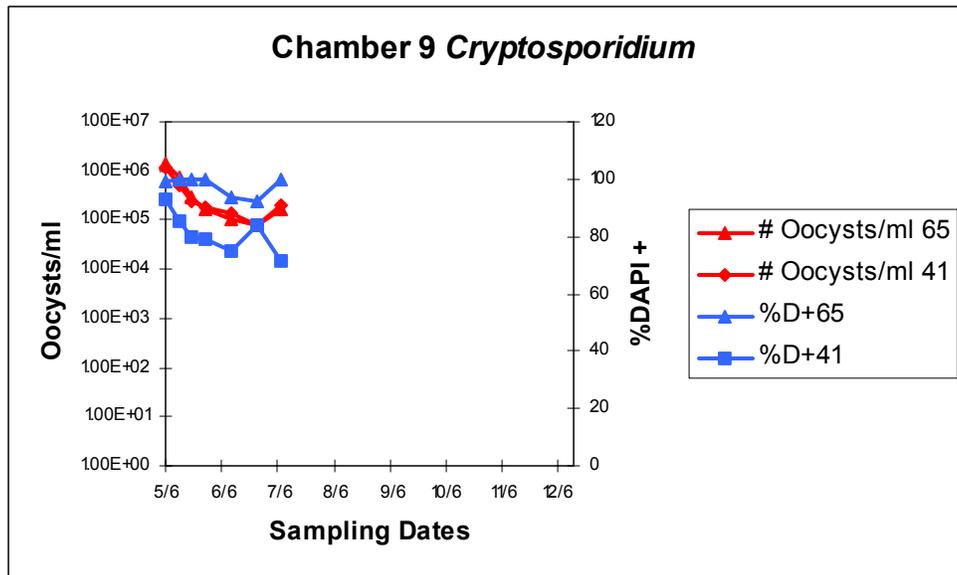
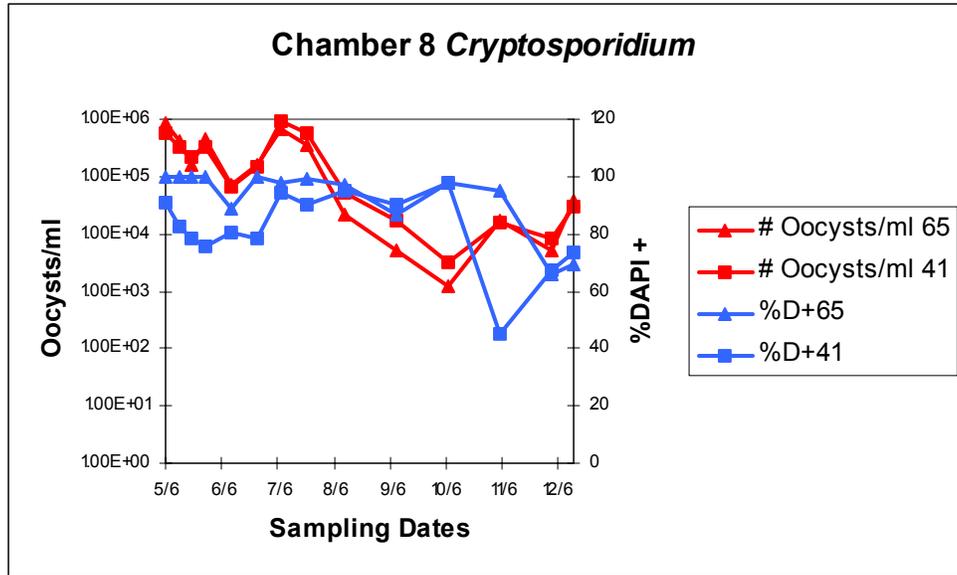
Figure 2. Detection of *Cryptosporidium parvum* oocysts over time in diffusion chambers using immunofluorescent staining. Sporozoite nuclei were visualized using DAPI (D+). Samples were dried at either 65 or 41°C.

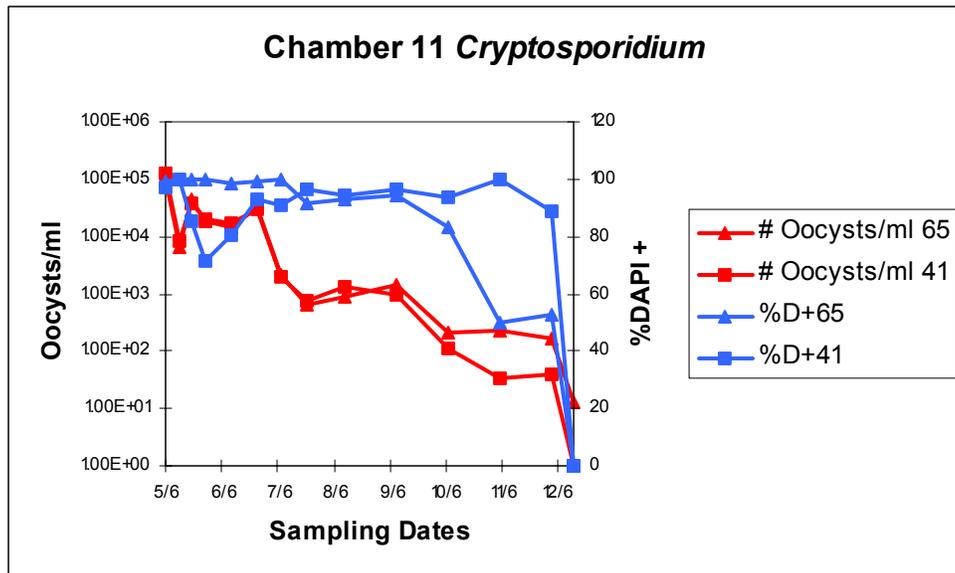
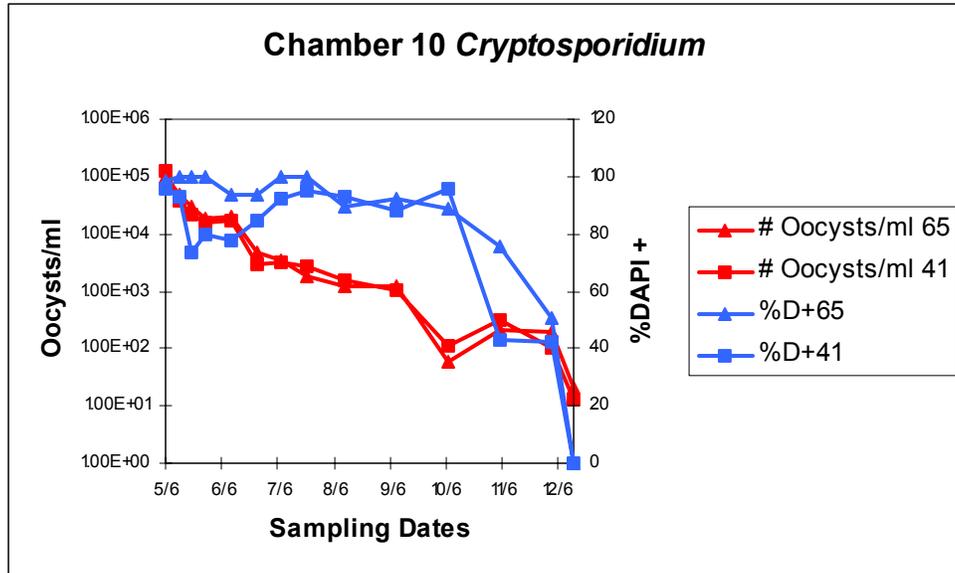


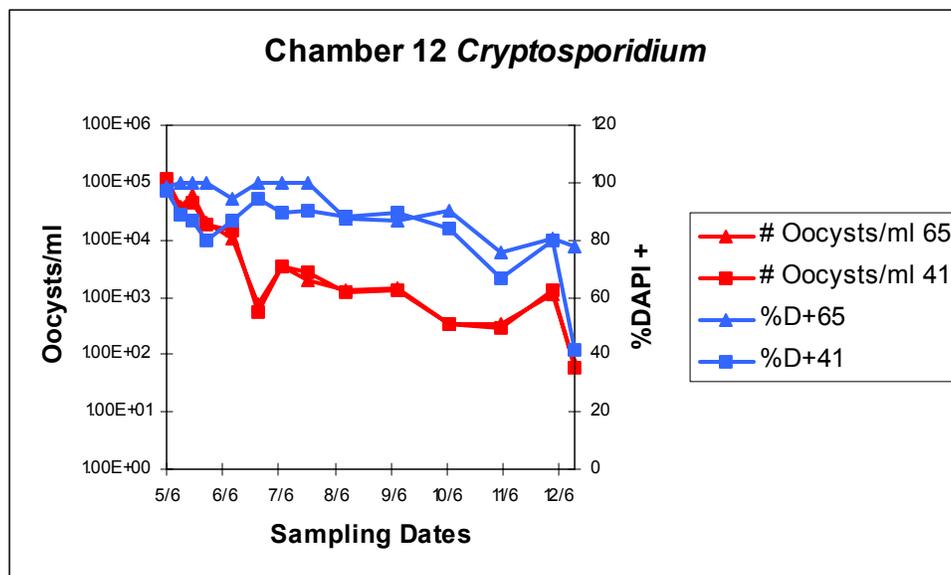












The results of PCR amplifications performed on samples obtained during May, July and December are presented in Table 1. At study onset in May, all samples in all chambers were PCR-positive for *E.coli*, enterococci, *Bacteroides* and *Cryptosporidium parvum*, except one repeatedly negative *Cryptosporidium* result in chamber 6. In July, PCR amplifications performed on diluted fecal samples incubated in the unimpacted pond (chambers 4-6) were repeatedly negative for *Cryptosporidium* and *Bacteroides*. However, *Bacteroides* DNA was detected in two of these three chambers in December. *Cryptosporidium* was not detected using PCR amplification results in chambers 4,5, and 6 during the December sampling. By the final sampling date in December, *E. coli* was no longer detectable using the PCR in any of the chambers containing diluted sample (4-6, 10-12), as well as chamber 8. Chambers 10-12 and 6 were additionally PCR-negative for enterococci. It should be noted, however, that negative PCR amplification results are commonly attributable to the presence of inhibitors in DNA preparations. Alternatively, the assay may have reached its limit of detection under the conditions used.

Table 1. Detection of indicator organisms and *Cryptosporidium parvum* over time using polymerase chain reaction amplifications. Negative results were subjected to additional DNA purification steps, as well as alternate DNA concentrations and sources of polymerase.

Chamber number	1	2	3	4	5	6	7	8	9	10	11	12
Date	May											
<i>Cryptosporidium</i>	+	+	+	+	+	neg	+	+	+	+	+	+
<i>E. coli</i>	+	+	+	+	+	+	+	+	+	+	+	+
<i>Enterococcus</i>	+	+	+	+	+	+	+	+	+	+	+	+
<i>Bacteroides</i>	+	+	+	+	+	+	+	+	+	+	+	+
	July											
<i>Cryptosporidium</i>	+	+	+	+	neg	neg		+		+	+	+
<i>E. coli</i>	+	+	+	+	+	+		+		+	+	+
<i>Enterococcus</i>	+	+	+	+	+	+		+		+	+	+
<i>Bacteroides</i>	+	+	+	neg	neg	neg		+		+	+	+
	Dec											
<i>Cryptosporidium</i>	+	+	+	neg	neg	neg		+		+	+	+
<i>E. coli</i>	+	+	+	neg	neg	neg		neg		neg	neg	neg
<i>Enterococcus</i>	+	+	+	+	+	neg		+		neg	neg	neg
<i>Bacteroides</i>	+	+	+	+	neg	+		+		+	+	neg

A variety of statistical measures will be used to assess the significance of these findings. Cross correlation plots are being utilized to determine “differences” in microbiological data between incubation locations, chamber triplicates and the two fecal sample concentrations. A linear regression model is being developed to better understand the impact of incubation temperature, algal numbers and dissolved oxygen on organism detection. A mass balance must also be examined to explore the potential effect of dilution due to sample removal on experimental results.

Notable achievements:

2004. Excellence in Research Award presented to Jacqueline Lendrum, University at Albany, School of Public Health for a poster describing this work.

Poster presentations:

“Assessment of Methods to Distinguish Between Human and Ruminant Sources of Fecal Pollution.” 29th Annual Meeting of the New England Association of Environmental Biologists. Fort William Henry, Resorts & Conference Center, Lake George, NY. March, 2005.

“Assessment of Methods to Distinguish Between Human and Ruminant Sources of Fecal Pollution.” State University of New York, School of Public Health, Student Poster Day. April, 2005.

Student support: Jacqueline Lendrum, University at Albany, School of Public Health, Department of Environmental Health and Toxicology. Ph.D. expected December, 2005.

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