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Determination of Survival Rates for Selected Bacterial and Protozoan Pathogens from Wet Weather Discharges

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ABSTRACT

Pathogenic organisms found in sewage can adversely impact public health when the sewage is discharged to waters that humans come in contact with through wading, swimming, fishing, drinking, etc. UAB is conducting research to develop a risk assessment methodology for evaluating varying degrees of risk related to human contact with pathogenic microorganisms found in sewage contaminated waters, especially those caused by separate sanitary sewer overflows (SSOs). One component of this research is to study the fate and transport of these microorganisms in the environment. The survivability, or die-off, rates for these organisms is critical to understanding their fate in the environment, e.g., from an SSO discharge through a receiving water.

Since there are a multitude of factors that contribute to microorganism survivability, the use of an *in-situ* method to characterize the rates of growth and death is necessary to account for variable environmental conditions. Hence, these experiments were conducted using a specially designed *in-situ* chamber.

This research was shown to be useful in obtaining survivability data based on the actual conditions present in an SSO polluted stream, and when combined with hydrologic data, can be used to predict the fate of pathogens downstream from a point source discharge of sanitary sewage. The information from these experiments can be used to construct a fitted-curve, which can then be used to determine a rate of decay for input into the computer model.

The results of this research could be applied to the construction of a fate and transport model, which could then be used by a community to target remediation efforts to those discharge points of highest public health concern. A model, tailored to a particular community, could be built, which would allow one to rank SSO discharge points from those of highest to lowest health risk. Therefore, use of this methodology would allow municipalities to apply their limited funds to the repair of discharges creating the greatest health risk first.

INTRODUCTION

Microorganisms have varying degrees of stability within the environment. Their numbers are dependent upon population dynamics, which is controlled by several criteria (McKinney 1962): 1) competition for food (limited food sources limit microbial numbers), 2) predator-prey relationships (some organisms consume others for food sources), 3) nature of organic matter (carbohydrates, organic acids, proteins all stimulate different organisms), and 4) environmental conditions (oxygen concentration, nutrient levels, temperature, pH, etc.). Since there are a multitude of factors that contribute to microorganism survivability, the use of an *in-situ* method to characterize the rates of growth and death is necessary to account for variable environmental conditions.

A dynamic water quality model, such as WASP5, will be used to simulate the fate and transport of the microorganisms. Laboratory and field studies to evaluate the survival characteristics of actual pathogens are being conducted at UAB. These studies are necessary to develop sufficient data to fulfill the input requirements of the computer model for evaluation of pathogens.

Traditional modeling of the fate and transport of biological contaminants has focused upon indicator bacteria; however, recent studies have questioned this tactic because many pathogens behave differently than the indicators, e.g., survive for longer periods of time. Historically, computer models built to simulate indicator bacteria fate and transport only took into account a first order die-off rate, which appears to be an oversimplification. In addition, previous studies of bacterial indicators were conducted for relatively short periods of time, approximately one to two days. However, pilot studies conducted at UAB have shown that the rate of die-off decreases substantially when experiments are conducted over longer periods (Newman, et al. 1998).

A literature review was conducted to evaluate which pathogenic microorganisms are currently of concern, i.e., those that are found in sewage and are of significant public health significance. Next, an evaluation of existing methods to detect and quantify these pathogens was conducted. The organisms finally selected were those that are of highest concern, and furthermore can be analyzed using existing or slightly modified methods. These are the indicator bacteria Total Coliforms and *E. coli* (for comparison to previous studies), the pathogenic bacteria *Enterococci,* and the protozoan pathogen *Giardia lamblia*. Future studies will include *Cryptosporidium parvum* and *E. coli* O157:H7.

Of special interest are protozoa, such as *G. lamblia*, which form cysts that are excreted by infected hosts and transmitted to the environment. These cysts are extremely persistent. Protozoan cysts are resistant to dehydration, and other adverse environmental conditions similar to fungal spores. In addition, cysts do not metabolize significantly, like viruses, and therefore can survive for long periods outside of a host (McKinney 1962). For these reasons, it is expected that the die-off rates will be relatively slow.

MATERIALS AND METHODS

Several experiments were conducted to evaluate the rate of die-off, or decay, for the study microorganisms. These in-situ experiments were conducted in specially designed chambers, see Figure 1. These were designed to allow passage of water and nutrients between the inside of the chamber and the outside environment (five-mile creek in Jefferson County, AL), while sequestering the microorganisms inside to allow enumeration at various time points during the experiment.

These experiments included exposures over a twenty-one day period. Hence, a polyethersulfone (Supor®, Gelman Sciences) membrane, which is not susceptible to biological degradation, was used. This membrane material was clamped onto either end of a piece of acrylic tubing in a novel design devised by researchers at UAB, see Figure 2. The membrane pore size is 0.22 microns; consequently, the goals of allowing exchange and sequestering the microorganisms inside were achieved.

The methods used to enumerate the study organisms, given in Table 1, are relatively expensive. Therefore, care was taken to minimize the number of samples analyzed. For this reason, a composite sampling procedure was used (Gilbert 1987). Multiple chambers containing sewage samples were placed in the creek and removed after 0, 1, 3, 7, 10, 14, and 21 days. For each time point, three separate chambers were removed and composited for analysis. Therefore, instead of analyzing 3 samples × 7 time points = 21 samples, only 7 analyses were conducted per experiment. In order to calculate a first order die-off constant, a regression model was used to evaluate the change in microorganism concentration over time. The focus of the experiment was to determine a die-off rate constant, from the regression model, so evaluation of the variation at the various time points was secondary to evaluation of the variation in the regression model. Our compositing scheme therefore sacrificed description of the variation at individual time points, while still maintaining the ability to quantify the overall regression model variation. Once the samples were composited, they were blended (Warring blender for two minutes) to minimize agglomeration of the microorganisms.

Numerous factors can affect die-off of microorganisms in natural systems. Those of primary importance are sunlight, temperature, dissolved oxygen, level of inorganic salts, level of organic matter, and presence of toxins (Droste and Gupgupoglu 1982). Data for some of these factors will be collected throughout these in-situ experiments by deploying a YSI 6000UPG. The YSI 6000UPG is a continuous monitoring instrument capable of collecting data for depth, temperature, pH, conductivity, dissolved oxygen, oxidation-reduction potential and turbidity. This instrument will also be used to evaluate the frequency, magnitude and duration of wet weather events that occur during the 21 days of the in-situ experiment (Easton, et al. 1998).

The experiments conducted to evaluate degradation of *G. lamblia* were conducted *in-situ*. The sewage matrix was spiked with approximately 10,000 cysts per liter to enable detection of die-off. These cysts were formalinized in order not to risk releasing a potentially infectious pathogen into the environment. Since these organisms are in cyst form, i.e., relatively inert, it was hypothesized that the mechanism of die-off would be predation by other organisms and formalinized organisms would be a suitable surrogate for "live" ones.

RESULTS

The results of these experiments show a departure from the conventional wisdom, i.e., that microorganisms die off at a constant, rapid rate. In fact, this is correct for the initial die-off. However, as time progresses, the die-off rate slows as the microorganism levels approach equilibrium with the environment. Figure 3 shows the levels of Total coliforms, *E. coli*, and Enterococci versus time (Die-off plots). The dashed lines show the mean levels of these microorganisms in the creek during the experiment. It is interesting to note that the equilibrium levels reached in the chamber are approximately one to two log units higher than those in the creek. Recall, however, that the levels determined in the chamber include settled organisms (all material in the chambers was resuspended and blended prior to enumeration). The levels enumerated in the creek do not include those organisms that settle out. The creek samples were collected by grab sample in the water column only. One might speculate that the equilibrium levels in the creek and the chambers are approximately equal if one considers the effect of settling (i.e., if the creek samples had included some sediment from the stream bottom, then they would have been higher). Settling tests are being considered for future research.

Figure 4 is a plot of the levels of *Giardia* cysts versus time. This plot is not called a "die-off", but rather a "degradation" plot as the cysts were formalinized and therefore not dying, but degrading. The method used to enumerate these organisms (EPA Method 1623) requires a presumptive test followed by a confirmed test. The presumptive test consists of identifying objects, of the correct size and shape, which are stained by a *Giardia*-specific antibody bound to a fluorescent probe. Next, the organisms are confirmed by identification of internal structures stained by the nuclear stain DAPI (4',6-Diamindino-2-phenylindole). Unfortunately, problems were encountered with the confirmation test in these experiments (the DAPI stain of the background was too intense to enable identification of internal structures. However, using the presumptive stain, which binds to the cyst cell wall, it was possible to detect differences in these presumptive *Giardia* cysts. Some cysts were intact (i.e., the stain covered the cell wall continuously), and some cysts were present, but degraded (i.e., the staining of the cell wall was less intense and not continuous). The levels of the former, "intact cysts", are plotted along with the levels of the latter, "degraded cysts" in Figure 4. An examination of this figure shows that the levels of intact cysts dropped rapidly, but the levels of degraded cysts rise before eventually dropping too. Consequently, one might speculate that this study is capturing an intermediate stage in the degradation of the cysts. Starting with an intact cyst, it becomes partially degraded, and then disappears entirely once it is completely degraded. The regression model, and subsequent determination of a die-off constant, was constructed using the intact cyst data.

It is interesting to note that the *Giardia* behaved similarly to the bacteria, i.e., these organisms are possibly not as persistent as would be expected. However, these data are inconclusive as the cysts were formalinized, and may not behave as live, intact cysts would.

The data used to construct the graph were used to perform a series of log-linear regressions in order to calculate a die-off rate constant, k. In addition the regression models were used to estimate the time required for 90 percent die-off (T_{90}), 99 percent die-off (T_{99}), and 99.9 percent die-off ($T_{99.9}$). These percentages: 90, 99, and 99.9 correspond to 1-log, 2-log, and 3-log removal, respectively. Since the microorganisms' rate of die-off seems to be decreasing over time, the regression model was applied step-wise, starting with the first three data points, and adding one additional point until the entire twenty-one day, or 7 point, data set was used. These results are presented in Table 2.

In general, the die-off rates decrease and T_x values correspondingly increase as data over longer time periods are included in the regression analyses. The T_{90} values for the indicator bacteria, total coliforms and *E. coli*, are in accordance with conventional wisdom. Many studies have shown T_{90} values for these organisms to be in the range of several hours to a few days (Droste and Gupgupoglu 1982, Geldreich, et al. 1968, Geldreich and Kenner 1969). Interestingly, the current results suggest that the rate of die-off is not constant. In fact, two or more log removal times can be predicted with a higher degree of accuracy by using significantly smaller die-off rates. Alternatively, and perhaps more appropriately, a second- or third-order equation should be used to model the die-off.

The initial, rapid die-off occurred, generally, within the first seven days of the experiment. Consequently, these data, which represent the region of the die-off curve exhibiting the best fit to the first-order die-off model, were analyzed further using additional statistical tests. These results are presented in Table 3. This table gives a first-order die-off constant, k ($days^{-1}$), and its associated ninety-five percent confidence interval, for each of the microorganisms. In

addition, the results of the Mann-Kendall Test (a non-parametric test for trend) are given. All of the die-off constants (slope of the regression line) are statistically significant except for Enterococci.

These results show lower rates of die-off and longer removal times for pathogenic microorganisms, *G. lamblia* and Enterococci, which lends further support to the idea that use of traditional indicator bacteria may lead to inaccurate predictions regarding the fate of pathogenic microorganisms in receiving waters. In fact, the regression models for the indicator bacteria predict 3-log removal within two weeks, but regression models constructed using data from the pathogens themselves predict this does not occur until approximately one month after discharge.

DISCUSSION

The data generated by this study suggests that if one were using die-off constants from indicator bacteria studies, then one may tend to under predict the length of time or distance downstream in which adverse health effects due to pathogens in sewage are present. In addition, this data indicates that assumptions regarding the constancy of die-off rates may be invalid. There seems to be a modulation of the rate of die-off with increased time. Further data analyses must be conducted to evaluate whether simple log-linear regression is the best prediction model. It may be more accurate to use a more complex model, with additional terms, to better account for the curved relationship seen here.

All of the test organisms showed a pattern of leveling off toward some equilibrium level with increasing time. There are two possible reasons for this observed effect. One, the microorganisms die-off at a rapid rate until the carrying capacity of the environment, in this a case a stream, is reached. Then, the organisms are maintained at that level that is supported by the nutrients present. Or two, these studies show evidence of quorum sensing by these microorganisms. Quorum sensing is a biological characteristic whereby microorganisms have genetic programming that enables them to regulate their numbers (Strauss 1999). It is possible that the microorganisms are pre-programmed to die-off two or three log units, and then maintain that level. Future studies will be conducted to examine which hypothesis is correct. These studies will follow the same experimental protocol, but use lower numbers of microorganisms initially (dilute sewage in the chambers). Then, if the results were similar to these observations (i.e., the microorganisms die-off rapidly and then level off), then quorum sensing would be indicated. However, if these tests show that the organisms stay at this level (i.e., no rapid die-off), then the "carrying capacity" hypothesis would be more likely.

The Enterococci results are quite different from the others. There is no initial, rapid die-off, only a gradual decrease in numbers. One might speculate that because the levels of Enterococci in the chamber were barely above the equilibrium levels maintained in the creek, only the tail end of the die-off curve is being observed. Alternatively, these data may just indicate that Enterococci are extremely persistent (i.e., do not die-off rapidly), which agrees with the general characteristics of this genus (Facklam and Sahm 1995).

The *Giardia* results were not as expected. The descriptions of this organism found in the literature seem to predict that *Giardia* will persist for much longer. This study seems to show that *Giardia*, and perhaps other protozoan pathogens, exhibits die-off characteristics similar to the bacteria included in this study. However, as mentioned previously, these cysts were treated with formalin and therefore may have been less resistant to degradation in the environment. Future in-lab studies are planned to approximate these in-situ tests using live (unformalinized) cysts. These in-lab results can then be compared to the in-situ data.

The physical and chemical data acquired using the YSI 6000 has yet to be analyzed. It is expected, however, that one or more of these parameters may correlate with the die-off results and shed new light on these findings. In addition, the hypothesis was made that temperature change, i.e., from summer to winter, will significantly affect the die-off rate. Therefore, the experiment will be repeated in late summer of 1999 at a higher temperature to evaluate this effect.

Ultimately, the data from this study, and others, will be used to calibrate an existing water quality computer model (WASP5). This model could then be used to approximate the levels of pathogenic microorganisms in a given receiving water through space and time, downstream from the outfall and after discharge of sewage (i.e., from an SSO occurring during wet weather). These model results could then be used, in conjunction with knowledge regarding sites of probable human contact with the receiving water, to prioritize those sewage discharges

(overflows) that are of highest public health concern. This would enable communities with limited resources to target those resources at the discharges of highest concern first.

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Figure 1. Acrylic Parts of In-situ Chamber.

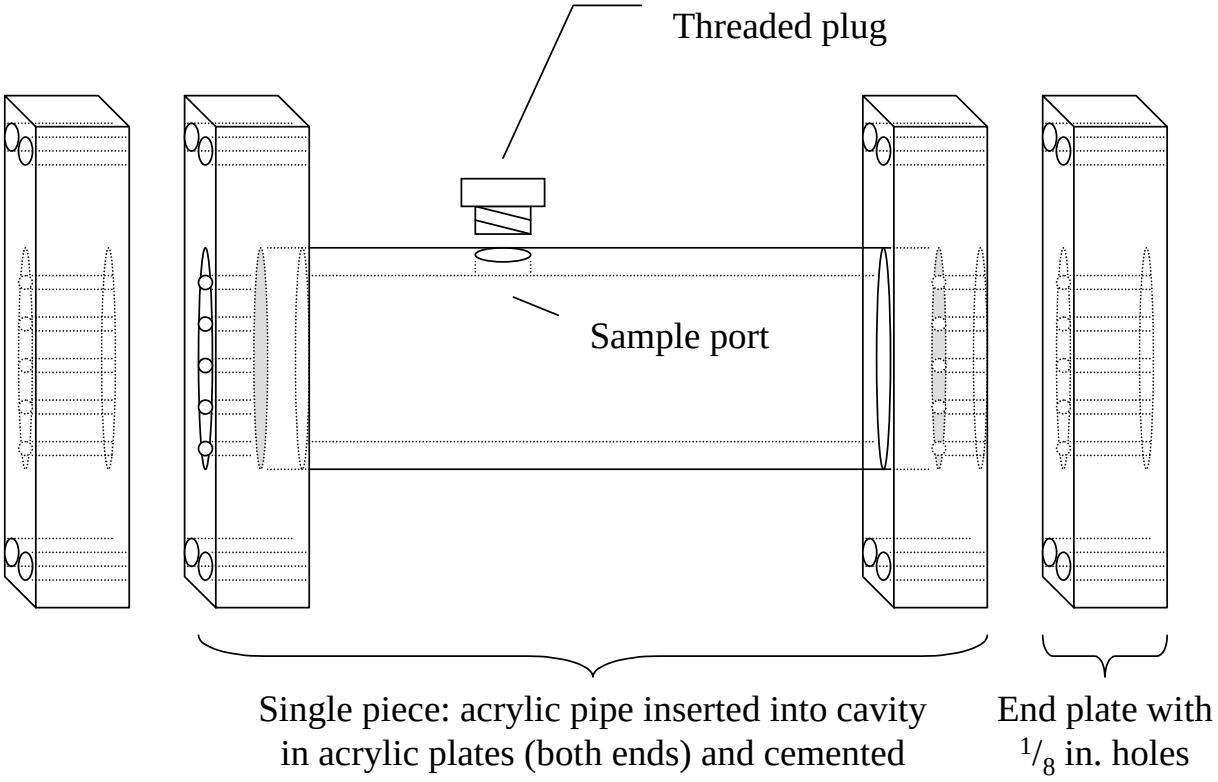

Figure 2. End-plate of In-situ chamber Showing the location of membrane filter.

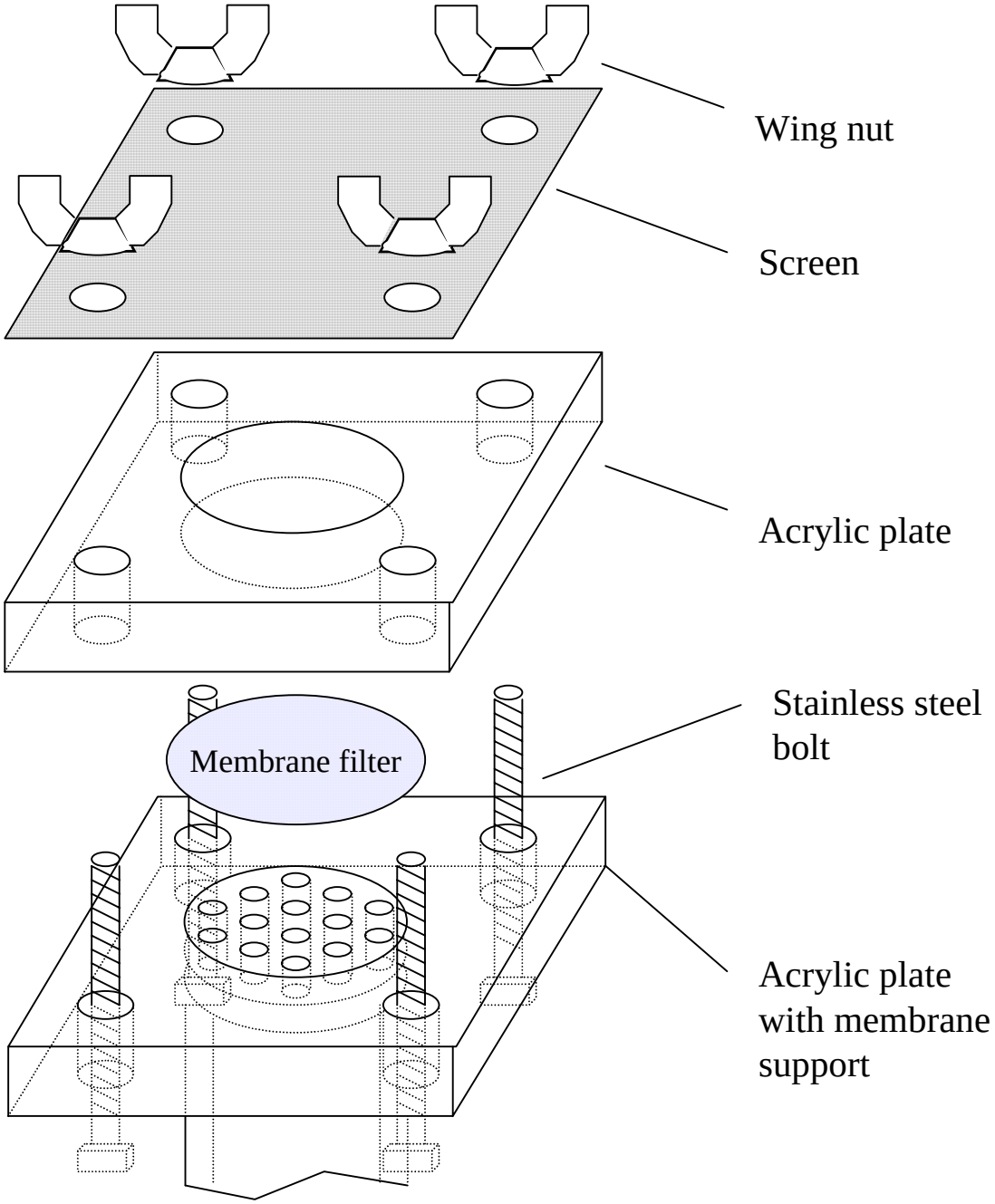

Table 1. Laboratory Methods.

Organism	Quantification Method	Reference
Total Coliforms	IDDEX Colilert 18™	(Edberg, et al. 1990)
E. coli	IDDEX Colilert 18™	"
Enterococci	IDDEX Enterolert™	(Eckner 1998)
Giardia lamblia	Immunomagnetic separation/fluorescent antibody	(U.S. EPA 1997)

Figure 3. Die-off Plots for Total Coliforms, *E. coli*, and Enterococci. Closed symbols with lines represent levels of microorganisms in the in-situ chambers. Open symbols with dashed lines represent mean levels of microorganisms in stream water.

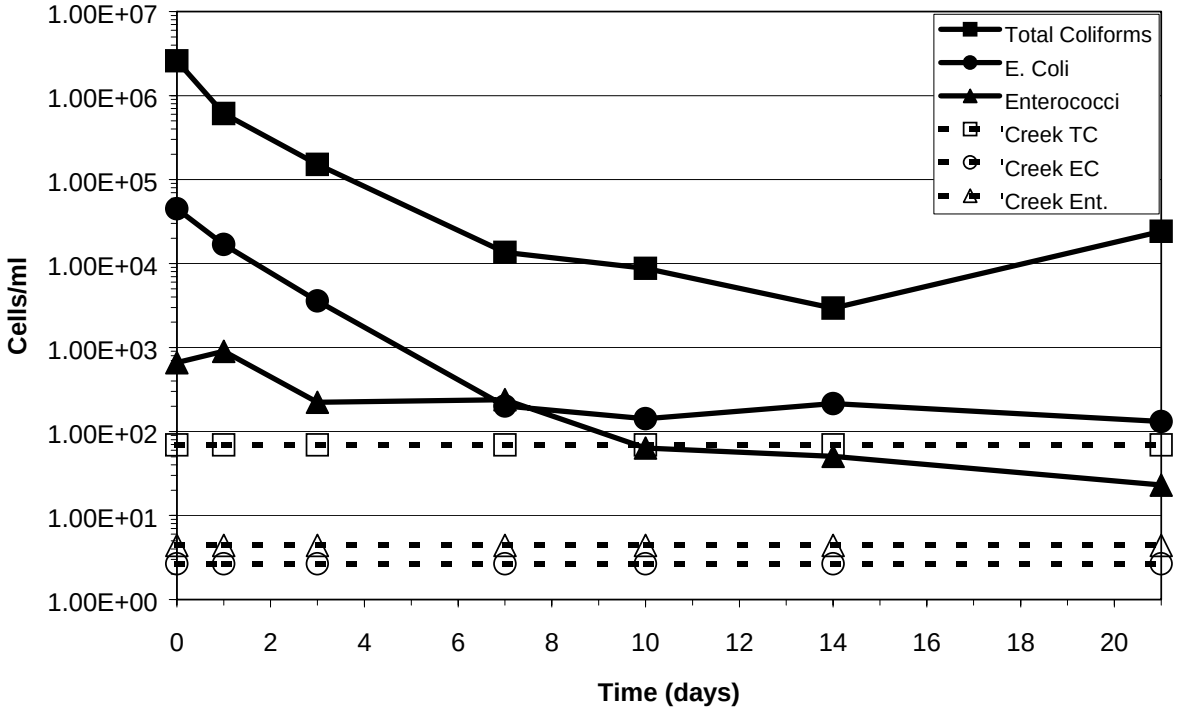

Figure 4. Degradation Plot of *Giardia* Cysts.

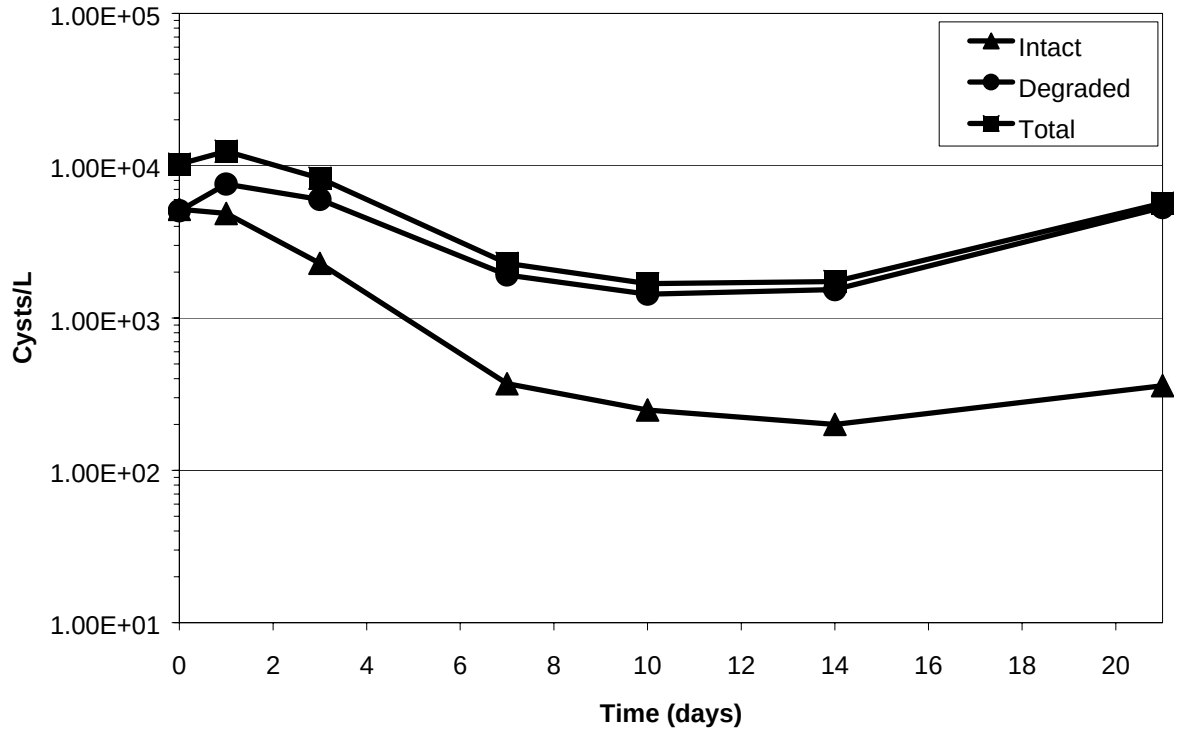

Table 2. Regression Results: Die-off Rates; Correlation Coefficients; and Time for 90, 99, and 99.9% Removal.

Last data point included	k (day^{-1})	R^2	T_{90} (days)	T_{99} (days)	$T_{99.9}$ (days)
Total Coliforms					
Thru day 3	0.395	0.961	2.29	4.82	7.35
Thru day 7	0.309	0.975	2.64	5.87	9.10
Thru day 10	0.243	0.937	2.88	6.99	11.10
Thru day 14	0.202	0.923	2.90	7.86	12.81
Thru day 21	0.104	0.550	1.70	11.33	20.96
E. coli					
Thru day 3	0.362	0.997	2.70	5.46	8.23
Thru day 7	0.331	0.998	2.85	5.87	9.10
Thru day 10	0.260	0.950	3.15	7.00	10.85
Thru day 14	0.179	0.809	3.33	8.93	14.53
Thru day 21	0.118	0.696	2.89	11.37	19.85
Enterococci					
Thru day 3	0.178	0.729	6.31	11.92	17.53
Thru day 7	0.077	0.607	13.15	26.05	38.95
Thru day 10	0.099	0.838	10.70	20.82	30.95
Thru day 14	0.087	0.884	11.74	23.20	34.67
Thru day 21	0.074	0.906	13.09	26.59	40.09
Giardia lamblia					
Thru day 3	0.125	0.931	8.33	16.35	24.36
Thru day 7	0.171	0.980	6.37	12.23	18.09
Thru day 10	0.146	0.970	7.15	14.00	20.85
Thru day 14	0.114	0.916	8.38	17.13	25.89
Thru day 21	0.066	0.658	11.42	26.52	41.61

Table 3. Die-off Rates. Determined Using Day 0 to Day 7 Data.

Organism	Die-off Rate (day^{-1})	95% CI	Mann-Kendall Trend *
Total Coliforms	-0.310	± 0.152	p = 0.042
E. coli	-0.331	± 0.049	p = 0.042
Enterococci	-0.078	± 0.189	p = 0.375 †
Giardia	-0.171	± 0.074	p = 0.042

* $p < 0.05$ indicates significant downward trend

† Not significant, no trend (die-off)