

**IDENTIFYING THE ORIGIN, AND IMPACT OF TEMPERATURE, ON  
*ENTEROCOCCUS* CONCENTRATIONS, PERSISTENCE, AND RE-GROWTH  
THROUGH MICROBIAL SOURCE TRACKING AND MONITORING AT A LONG  
ISLAND SOUND BATHING BEACH**

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**ABSTRACT**

An assessment of localized conditions of two creeks that flow into a downstream bathing beach within Silver Sands State Park in Milford, Connecticut was conducted in order to investigate factors contributing to high concentrations of indicator bacteria that may result in beach closures in the Long Island Sound coastal area. This study, funded by the Connecticut Department of Environmental Protection (CT DEP) Long Island Sound Fund Program, examined the impact of summer temperatures on the concentration, persistence and potential re-growth of indicator bacteria in sediments and the water column. The study concluded that there is no significant correlation between either sediment or water temperatures and *Enterococcus* levels. DNA markers were used to identify sources of fecal pollution and indicated that birds are major a contributor within the watershed. Another objective was to investigate if creek sediments serve as a source of *Enterococci* to overlying waters through resuspension and remobilization. Bacteria were isolated from sediments and analyzed by DNA fingerprinting to determine if they were re-growing or concentrating in the environment. The highly diverse population of *Enterococci* present in the sediment suggests that upstream creek sediments may serve as a sink, and act as a concentrating environment for indicator bacteria, but that they were not proliferating within the sediment.

**KEYWORDS**

*Enterococcus*, microbial source tracking, re-growth, DNA fingerprinting, bathing beach, Long Island Sound

## INTRODUCTION

In the summer of 2007, the Interstate Environmental Commission (IEC) conducted a study funded by the Connecticut Department of Environmental Protection (CT DEP) Long Island Sound Fund Program to analyze localized environmental conditions contributing to high concentrations of indicator bacteria that have caused beach closures in the Long Island Sound coastal area in the past, and could likely cause additional closures in the future. The study area, Silver Sands State Park in Milford, Connecticut, includes two creeks, Great Creek and Fletcher Creek, both of which empty into the Long Island Sound. This project was crucial in order to better predict and understand factors contributing to elevated bacteria levels in the study area and similar Long Island Sound coastal regions, and to improve overall water quality and promote safe recreational use of Long Island Sound bathing beaches.

Previous sampling, performed on a weekly basis by CT DEP at Silver Sands State Park, has revealed that *Enterococci* concentrations at the beach repeatedly exceeded bathing water criteria. Since CT DEP's surveys suggested that significant sources of human sewage might not be present, additional data was needed in order to enhance the understanding of estuarine processes and localized conditions that may contribute to elevated levels of indicator bacteria.

A number of physical factors affect survival, persistence, and re-growth of indicator bacteria in ambient waters. Various studies have confirmed that this includes temperature (Geldreich et al., 1968; Gameson and Gould, 1975; Howell et al., 1996; Esham and Sizemore, 1998; Noble et al., 2004). However, results have shown variable impact of temperature depending on the type of experiment conducted and the nature of water analyzed. Furthermore, much of the research that has been conducted concerning survival of indicator bacteria has focused on indicators such as fecal coliform that are not frequently used by environmental agencies to determine the sanitary quality of bathing beaches. Based upon the fact that the *Enterococci* group is a group of 20 different species (Torrell, 2003), the species present are likely to differ depending upon the source of fecal contamination. Therefore, there is a need to establish how particular environmental factors specifically affect persistence and re-growth of *Enterococcus sp.*, as this is the indicator used to determine sanitary quality in (marine) bathing waters as mandated by the United States Environmental Protection Agency (USEPA).

The role that aquatic sediments play as both a sink and a possible source of pollutants in marine systems is becoming widely recognized. Sediments serve as a surface for *Enterococci sp.* to bind to, and offer a suitable environment in which these indicator bacteria may survive and perhaps proliferate. Various studies have demonstrated that organisms associated with suspended particles and sediment contributes to larger concentrations than in the water column (Davies et al., 1995; Goulder, 1977; Obiri-Danso and Jones, 2000; Shiaris et al., 1997). Pathogenic microorganisms associated with sediment particles have the possibility of being resuspended back into the water column due to natural turbulence or human recreational activity (Irvine and Pettibone, 1993; Obiri-Danso and Jones, 2000).

Ferguson et al. (2005) studied levels and species distribution of *Enterococci* in intertidal and marine sediments and coastal waters at California bathing beaches, which were often in violation

of water-quality standards. The authors determined that the distribution of species present in water samples was comparable to those found in sediments. They also concluded that high levels of *Enterococci* in intertidal sediments indicated retention and possible re-growth in this environment. Furthermore, this study recognized that resuspension of *Enterococci* that are persistent in sediments may contribute to the levels of indicator bacteria that cause failure of meeting beach water quality standards.

The focus of this study was to determine the specific impact of summer temperatures, as well as pH, total suspended solids (TSS), turbidity and salinity, on the concentration, persistence and potential re-growth of indicator bacteria in sediments and the water column at the Silver Sands State Park Beach during summer months. In addition, this study also considered creek sediments and their ability to serve as a source of *Enterococci* to overlying waters through resuspension and remobilization. In order to investigate the dynamics of the *Enterococci sp.*, microbial source tracking methodologies were utilized to characterize indicator organisms. Water and sediment samples were probed for source-specific sequences using polymerase chain reaction in order to differentiate sources of *Enterococci* as being from human, bird, bovine, deer, or other wildlife origin. In addition, bacteria were isolated from sediments and analyzed by DNA fingerprinting to determine if indicator bacteria are re-growing or concentrating within this environment.

## METHODOLOGY

**Figure 1. Silver Sands State Park Sampling Locations**

### Study Area Description

The study area, Silver Sands State Park in Milford Connecticut, includes Great Creek (upstream 1 and downstream 1) and Fletcher Creek (upstream 2 and downstream 2), both of which empty into a Long Island Sound bathing beach. Figure 1. illustrates the sampling locations: Upstream 1, Downstream 1 (Great Creek); Upstream 2, Downstream 2 (Fletcher Creek); and the Beach location. At both upstream creek locations HOBO<sup>®</sup> H8 Pro Series loggers (Onset Corp, Bourne, MA) were mounted, sensors were placed in the creek sediment, and launched and



set to record continuous sediment temperature data at hourly intervals from May to November 2007.

### Sampling Design and Collection

In order to best characterize variability of summer ambient temperatures during the bathing season, a total of five sampling events were completed between June and August 2007. Three sampling events had no rain in the 48 hours prior to the sampling and, according to rain gauge data from the Igor Sikorsky Memorial Airport in Bridgeport, Connecticut; the other two events had less than 0.08 inches of rain in the prior 48 hours. Events were conducted over the course of several hours spanning from early morning to afternoon. Samples were collected (at 4 time intervals approximately 1.5 hours apart) from each station, and overall included the field collection and laboratory analysis of 28 samples per run. Table 1 outlines the seven sampling stations: four (4) creek (two upstream and two downstream) surface water collection points, two (2) creek sediment collection points and one (1) beach surf zone collection point.

**Table 1. Sampling Stations**

Station	Sample Media Type	Location
U1	Water	Upstream 1 (Great Creek)
U2	Water	Upstream 2 (Fletcher Creek)
D1	Water	Downstream 1 (Great Creek)
D2	Water	Downstream2 (Fletcher Creek)
B	Water	Beach
U1S	Sediment	Upstream Creek 1
U2S	Sediment	Upstream Creek 2

Field measurements were taken for temperature, salinity, turbidity, and pH and laboratory analyses were performed for *Enterococci*, fecal coliform, TSS and turbidity. In addition, temperature data was supplemented with the use of the in-situ HOB0<sup>®</sup> continuous data loggers deployed at the upstream locations. Sediment temperature data was retrieved with BoxCar<sup>®</sup> Pro 4 software (Onset, Bourne, MA). During each event additional field parameters, including depth, velocity, and flow direction, were recorded at both downstream creek locations in conjunction with sample collection in order to assess tidal variability within both creeks.

One water sample from each of the two downstream locations was collected during four sampling events. Additionally, as an extension of the sampling plan, during the last sampling event one upstream water sample from Great Creek (U1) was also collected. These samples underwent molecular characterization using Host Specific PCR analyses to test for the presence or absence of specific DNA sequences associated with the human or particular animal source of bacterial pollution. During three of the sampling events, a small subset of *Enterococci* isolated from sediment samples from both upstream locations was collected and analyzed for sediment re-growth by ribotyping DNA fingerprinting.

## Analytical Methods

Water samples were analyzed for *Enterococci* and fecal coliforms using the MPN 3-tube, 4-dilution method. Sediment samples were prepared for indicator bacteria analyses using the method demonstrated by Peterson et al (2005) where 25 grams of each sediment sample was resuspended in 25 ml PBS, allowed to settle, and then the diluent was analyzed for *Enterococci* and fecal coliform by the methods referenced above.

## Molecular Characterization

Samples were analyzed for the presence of DNA markers that specifically indicate the source of fecal pollution in a watershed. These methods use polymerase chain reaction (PCR) to identify DNA targets within the bacterial chromosome that have been shown to be indicative of specific sources of fecal pollution (i.e., human, bird, dog, deer). The DNA markers approach investigates the presence or absence of exact DNA sequences. These sequences are associated with the presence of bacterial species present specifically in humans or a particular type of animal. For this project, samples were tested for the presence of bird, deer, and dog specific indicators.

Overall, the DNA marker methods exhibit a high degree of sensitivity and specificity. Confidence in results can be increased if the markers are detected in multiple sample events and if backup tests are also positive. A positive result is considered to be as highly specific due to little or no cross-reactivity. Negative results should be confirmed due to particle and target distribution, presence of inhibition, and low sample volumes.

The methods are highly specific for human fecal pollution. The Dog Bacteroides primer set has also shown specificity for all breeds of dogs tested, although many validation samples were collected from "dog parks" without knowing exact type of dog. The bird primer sets have been validated primarily on wading birds, shore birds, gulls and geese. Effectively, they are specific for flocking birds and would likely not detect an event from neighborhood sparrows or parakeets.

### Host specific *Enterococcus* PCR analysis

For each sample, 100 ml of water was filtered through a 0.45-micron membrane filter. The filter was placed on m*Enterococcus* media supplemented with indoxyl substrate (Becton Dickinson, MD) and the plate was incubated for 24 hours according to the protocol outlined in EPA Method 1600. Colonies exhibiting a blue halo were enumerated as *Enterococci*. Host specific PCR was carried out for respective targets using a modified version of the method described by Scott et al., (2005, 2007). DNA extraction was prepared using the Qiagen DNA extraction kit (Qiagen USA, CA), as per manufacturers instructions. Five micro-liter aliquots of purified DNA extract were used directly as template for subsequent PCR reactions. Amplification of PCR primers were carried out using HotStarTaq polymerase (Qiagen USA, CA) and master mix, which contained a final concentration of 1.5 mM MgCl<sub>2</sub>, 150 mM dNTP, and 0.3 mM of each primer. An Eppendorf Gradient Thermocycler (Eppendorf Inc., NY) was used with the following cycling parameters: 95°C for 15 minutes (to lyse cells and activate polymerase), followed by 35 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute and a final extension at 72°C for 5 minutes. PCR products were electrophoresed on 2% agarose gels, stained with GelStar nucleic acid stain (Cambrex Inc., NJ) and visualized under UV light.

### Specific Bacteroides spp. PCR analysis (used for dog analyses only)

For each sample, 100 ml of water was filtered through a 0.45-micron membrane filter. DNA was directly extracted from the membrane using the Qiagen DNA extraction kit (Qiagen USA, CA), as per manufacturers instructions. Five microliter aliquots of purified DNA extraction product were used directly as template for subsequent PCR reactions. Amplification of Bacteroides target sequence was carried out using HotStarTaq polymerase (Qiagen USA, CA), specific primers, and reaction master mix. The Master mix contained a final concentration of 1.5 mM MgCl<sub>2</sub>, 150 mM dNTP, and 0.3 mM of each primer. An Eppendorf Gradient Thermocycler (Eppendorf Inc., NY) was used with the following cycling parameters: 95°C for 15 minutes (to activate polymerase), followed by 35 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute, and a final extension at 72°C for 5 minutes. PCR products were electrophoresed on 2% agarose gels, stained with GelStar nucleic acid stain (Cambrex Inc., NJ) and visualized under UV light.

### **DNA Fingerprinting**

DNA fingerprinting is used to examine the genetic relatedness of organisms isolated from a particular location. Because bacteria reproduce by binary fission, each progeny is genetically identical to the parent organism and, therefore, produces an identical DNA fingerprint. Some watersheds contain reservoirs of fecal indicator bacteria (i.e. *Enterococci*, *E. coli*) that re-grow in the sediments. When these sediments are agitated (by wave action, rainfall, etc.) the organisms are resuspended into the water column and can lead to a false indication of recent fecal contamination. By fingerprinting the DNA of these suspended organisms, one can make presumptive determinations as to the nature of their presence (i.e. fecal source, environmental source). Highly clonal DNA would indicate regrowth, while highly heterogeneous fingerprints would initially indicate that the organisms are not genetically related. The latter result does not necessarily indicate the organisms are not re-growing nor does it indicate that they are not accumulating; however, it can be used as a tool to make decisions regarding future sampling and analyses. These tests reveal whether there is regrowth (identical DNA fingerprints) or new organisms being introduced to the area (different DNA fingerprints).

Ribotyping of *Enterococcus* isolates was accomplished by the method described in Scott et al., (2004) and Scott et al., (2003a). The ribotyping was performed on a subset of three samples per event collected during three sampling events. Chromosomal DNA was extracted from *Enterococci* isolates and digested with Hind/III (Invitrogen, CA). Fragments were separated by agarose electrophoresis. The DNA was then transferred and fixed to a Zeta-probe membrane (BioRad, CA). A cDNA probe complementary to the *Enterococcus* 16S and 23S rDNA was labeled with digoxigenin-dUTP and was used to probe the membranes. The resulting genetic fingerprint was then analyzed using Bionumerics software (Applied Maths, NV) and compared for similarity to assess clonality.

### **Data Analysis and Interpretation**

Upon completion of sample analyses, data verification and validation, data interpretation and analyses were conducted. Concentrations of indicator bacteria were evaluated with respect to CT

DEP/CT Public Health Beach Closure Criteria. Also, the relationship of indicator bacteria in corresponding water and sediment samples at the upstream sampling locations was examined by comparing geometric means. Additionally, correlational analyses of temperature vs. *Enterococcus* were performed.

## RESULTS

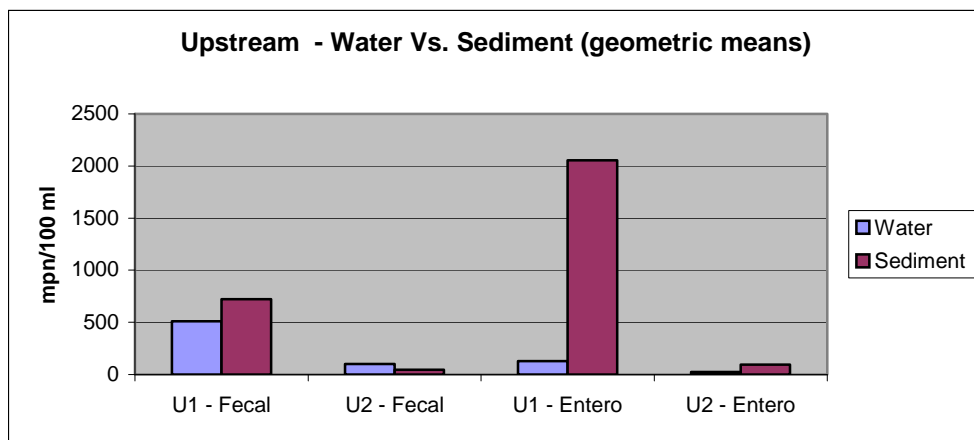
### Exceedences of Single Sample Maximum Criteria

Of the 20 samples taken from the beach, a total of four samples exceeded the Connecticut Beach Closure Criteria single sample maximum of 104 MPN per 100 ml. Connecticut Beach Closure Criteria for Single Sample Maximum requires resampling if *Enterococcus* is greater than this limit, and if the second result is also greater than 104 MPN/ 100 ml, then the beach is closed. Three of the four exceedences occurred on July 10, 2007. The other exceedence occurred on August 7, 2007. However, in that case the geometric mean of the four beach samples collected that day was 14 MPN per 100 ml, which is below the acceptable geometric mean limit of 35 MPN per 100 ml, which is used as the limitation that the geometric mean of five samples taken over a 30-day period should not exceed.

### Water vs. Sediment Samples

When comparing the geometric means of bacterial indicator results for the upstream (Station U1 and U2) water versus corresponding sediment samples, only the U1 *Enterococcus* results showed a great disparity between the two types of sample matrices. Figure 2. illustrates that the sediment sample levels was over one magnitude greater than the corresponding water levels.

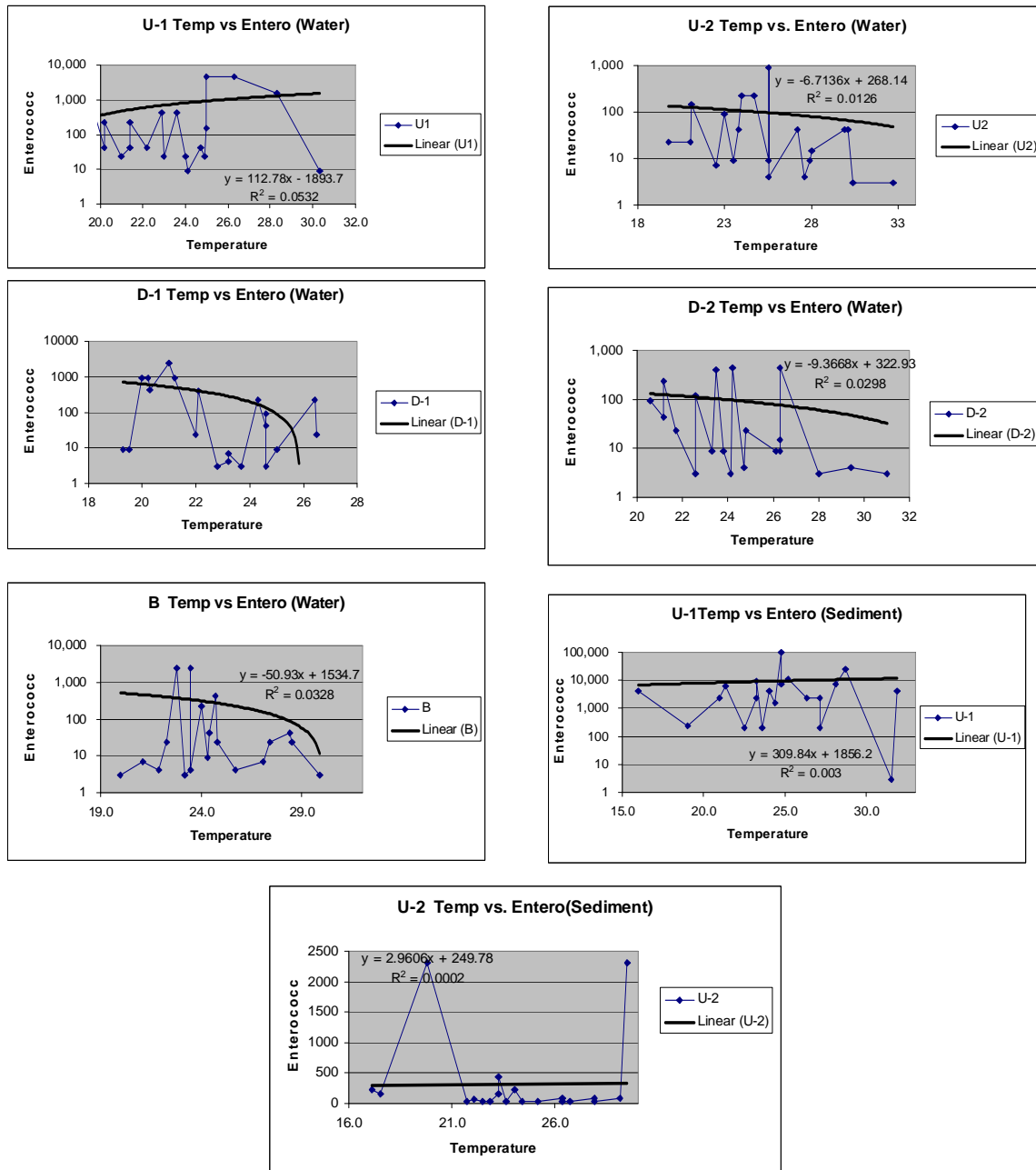
**Figure 2. Upstream Water vs. Sediment Indicator Bacteria Levels**



### Temperature versus *Enterococcus*

Figure 3. reflects the regression analyses that were conducted in order to correlate temperature and *Enterococcus* concentrations. Based on analyses for the seven sampling locations (twenty data points each), there was no significant correlation between temperature and *Enterococcus* at any of the seven locations.

**Figure 3. Regression Analyses: Temperature vs. *Enterococcus***



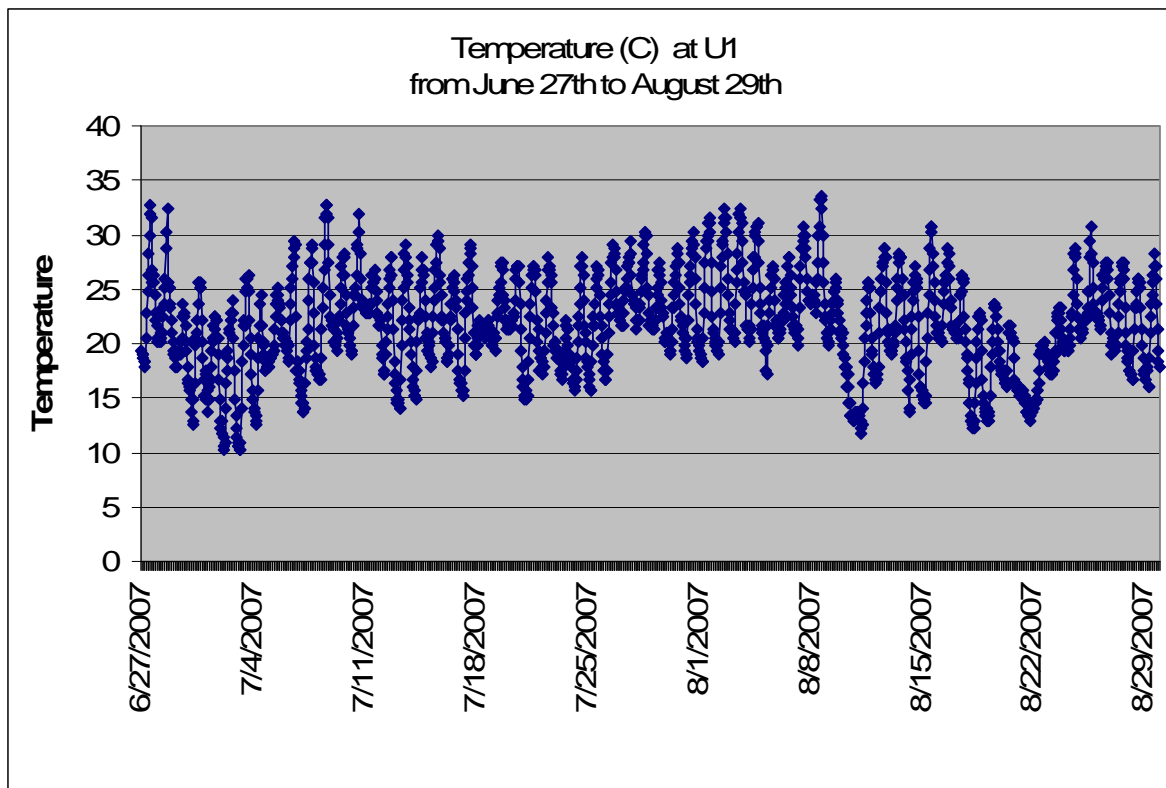


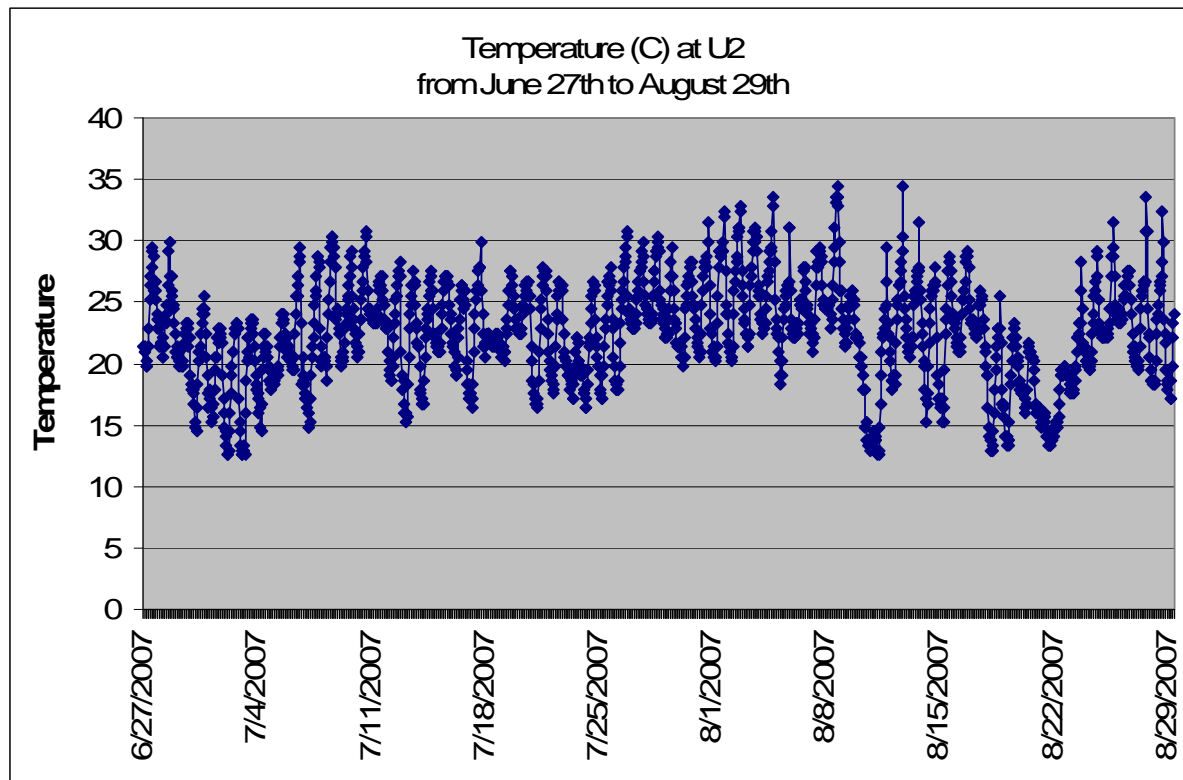
In addition to correlation analyses, daily plots of time vs. temperature and *Enterococcus* were examined (Appendix 1). Daily temperature fluctuations did not show any significant increase in *Enterococcus* results corresponding to an increase in temperature.

### Sediment Temperature Variation

Data retrieved from the two HOBO<sup>®</sup> data loggers that were placed in the sediment at the two upstream locations was used to examine trends and variations in sediment temperatures during the course of the study period, which was used as a representative summer bathing season. Figure 4. reveals that the temperature in the sediment for the U1 sampling location ranged from 10.2° C to 33.6° C. Figure 5 shows sediment temperatures for U2 sampling location ranged from 12.6° C to 33.6° C.

**Figure 4. Sediment Temperature Variation at U1 During the Extent of Sampling Events**



**Figure 5. Sediment Temperature Variation at U2 During the Extent of Sampling Events**

### Bacterial Source Tracking

PCR results indicate that birds are significant contributors of bacterial pollution on-site. Table 2. outlines the finding that at the two downstream sampling locations (D1 and D2), two of the four samples contained a DNA marker specific for *Enterococcus* originating from birds. In addition, one of the four sediment samples at U2S (sediment) showed positive identification for *Enterococcus* from birds. The only water sample analyzed using PCR from U1 also showed a positive identification for *Enterococcus* from birds. This finding is significant, as the results from DNA markers suggests they do not persist or reproduce in the environment, and their presence indicates recent fecal pollution (explained further in DNA fingerprinting section, below). The fact that the marker was detected in the sediment suggests that at least some of the bacteria present there were deposited recently. One of the four water samples at D1 showed positive identification for *Enterococcus* from humans. The presence of human fecal pollution is always a significant finding from both a public health and remediation standpoint. This result, however, was not confirmed in subsequent assays or by additional tests specific for human fecal pollution. Therefore, the result should be confirmed before significant human fecal pollution is suspected. All of the analyses or examinations for bacteroides from dog and *Enterococcus* from deer came up negative.

**Table 2. DNA Markers from Most Specific DNA Tests**

DNA Markers from Most Specific DNA Tests

Location	Type	Bird Enterococcus				Human Enterococcus				Dog Bacteroides				Deer Enterococcus			
		6-Jun	10-Jul	14-Aug	29-Aug	27-Jun	10-Jul	14-Aug	29-Aug	27-Jun	10-Jul	14-Aug	29-Aug	27-Jun	10-Jul	14-Aug	29-Aug
U1	Sediment	ND	ND	ND		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
U2	Sediment	ND	Yes	ND		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
D1	Water	Yes	ND	ND	Yes	ND	ND	Yes	ND	ND	ND	ND	ND	ND	ND	ND	ND
D2	Water	ND	Yes	ND	Yes	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
U1	Water				Yes												

Notes:

"Yes" indicates a presence of the indicated fecal pollution source was detected

"ND", not detected, indicates no presence of the indicated pollution source was detected

U1 and U2 - Upstream sampling locations of Creeks #1 and #2, respectively

D1 and D2 - Downstream sampling locations of Creeks #1 and #2, respectively

- indicates no sample was taken (samples were included in original sampling design, based on the funds available)

## DNA Fingerprinting

DNA fingerprinting of *Enterococcus* isolated from the sediment samples revealed a highly heterogeneous genetic population. The heterogeneous genetic population (not related) indicates that re-growth is not occurring. While the analyses were not exhaustive, these results coupled with the presence of host specific DNA markers from birds, suggest that at least some of the sediment organisms were deposited recently and have a true fecal link. These results indicate that the sediments might serve as a limited fecal indicator reservoir that could potentially have a deleterious impact on water quality.

## DISCUSSION

The purpose of the project was to analyze localized conditions contributing to high concentrations of indicator bacteria that pose a general health hazard and prevent safe use of the Long Island Sound as a recreational resource. An examination of the relationship between summer temperatures and concentrations of *Enterococcus* was necessary in order to determine if elevated sediment temperatures enhance persistence or support re-growth of these microorganisms in both creek sediments and overlying waters. Enumeration and molecular characterization of *Enterococcus* in the creek sediments proved to be useful in determining how overlying waters compare with respect to *Enterococcus* concentrations in the sediment, and thereby helped to ascertain if the sediment is contributing to augmented levels of *Enterococcus* within the water column of the creeks, and ultimately, the downstream bathing beach. Samples analyzed for DNA markers were used to order to investigate sources of fecal pollution within the watershed, and DNA fingerprinting was employed to determine if indicator bacteria are re-growing or concentrating in the environment.

- The study results found there to be no significant correlation between either sediment or water temperatures and *Enterococcus* levels.

- The study showed that while upstream creek sediments may serve as a sink and act as a concentrating environment for indicator bacteria, there was a low re-growth of bacteria in the sediment, demonstrated by the high diversity of the samples. Therefore, at Silver Sands State Park Beach, sediments may have a certain, but most likely a limited, contribution to bacterial pollution in overlying creek and downstream waters.
- The results indicate that birds are significant contributors to bacterial pollution in beach waters. There is limited indication of humans as a possible source, and no indication of dogs or deer as sources of pollution.
- The results from the July 10, 2007, sampling at the beach sampling location exceeded Connecticut Beach Closure Criteria. This would have led to the beach being resampled and potentially being closed.

## CONCLUSIONS/RECOMMENDATIONS

This project was crucial in order to better predict and understand elevated bacteria levels in the study area and similar Long Island Sound coastal regions in order to improve overall water quality and promote safe recreational use of Long Island Sound bathing beaches. While the objectives of the study have been fully met and a fairly representative DNA-based set of analyses has been conducted for upstream sediment locations, we recommend having corresponding DNA-based water samples at upstream locations collected and analyzed in the future. Similarly, it would be beneficial to conduct a follow-up investigation to locate the specific source of human pollution discovered at the D1 downstream location.

## ACKNOWLEDGMENTS

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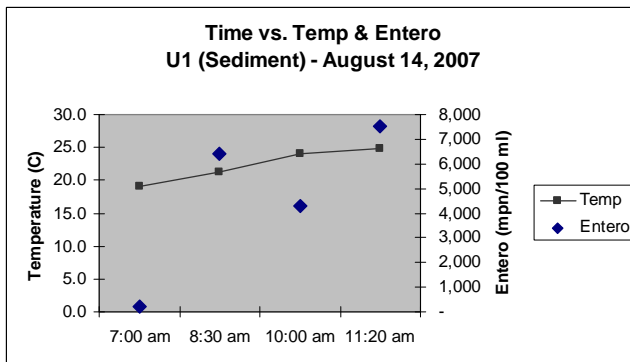
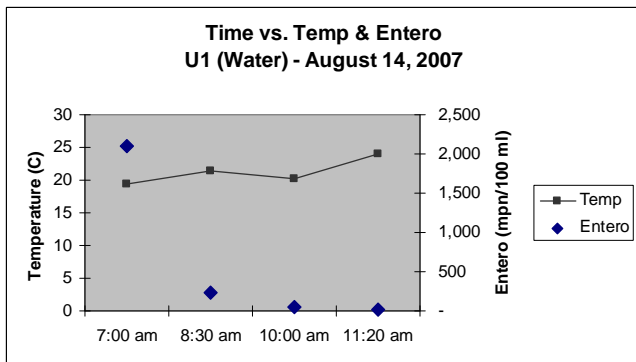
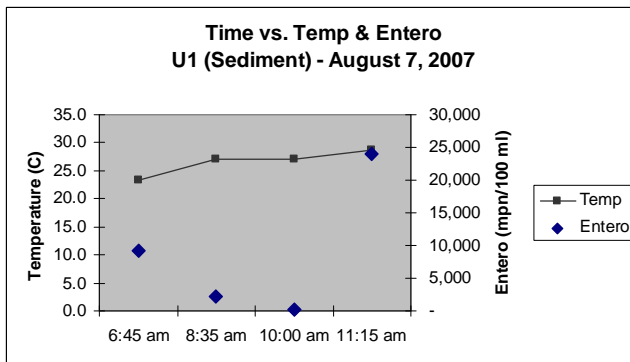
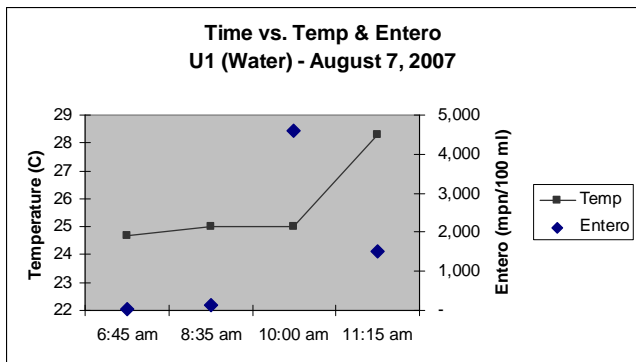
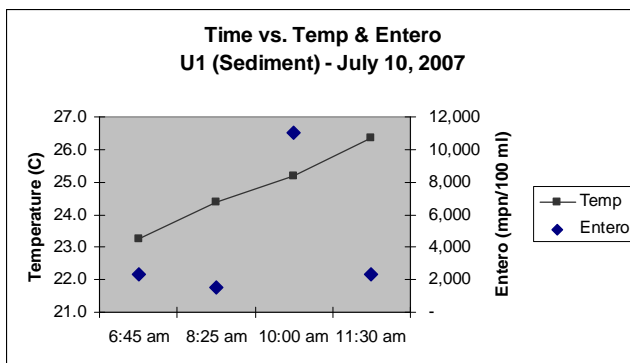
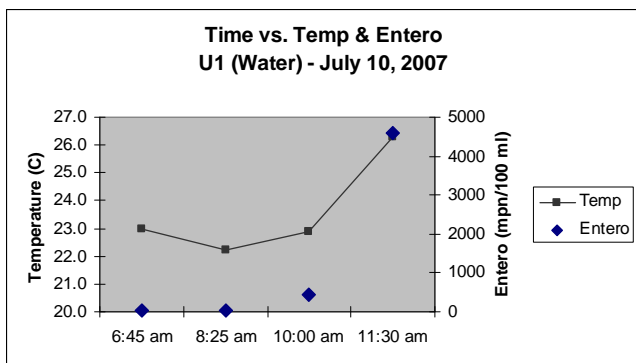
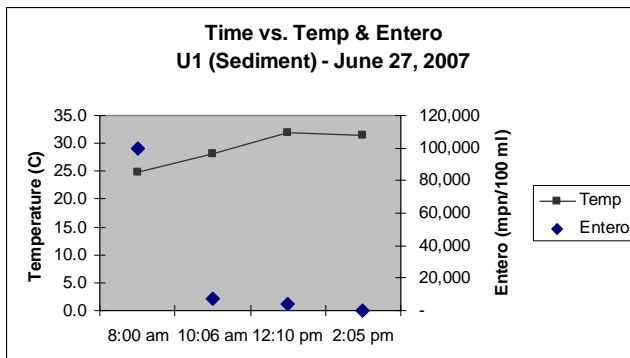
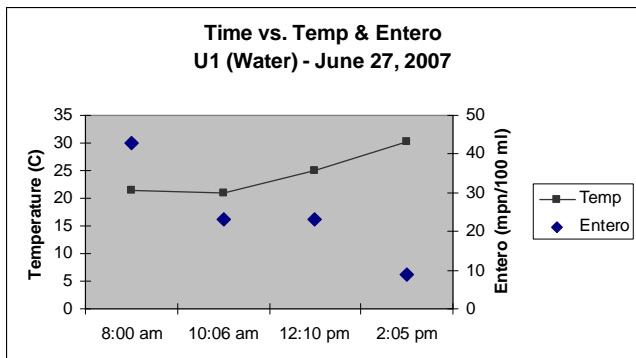
U.S. EPA (1998) Bacterial Water Quality Standards for Recreational Waters (Freshwater and Marine Waters). U.S. Environmental Protection Agency, Office of Water: Washington, D.C.

Wade, T.J.; Pai, N.; Eisenberg, J.N.; Colford, J.M. (2003) Do US EPA water quality guidelines for recreational waters prevent gastrointestinal illness? A systematic review and meta-analysis. *Environ. Health Perspect.*, 111, 1102-1109.

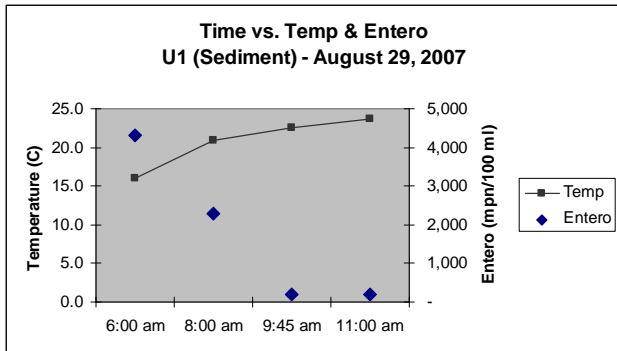
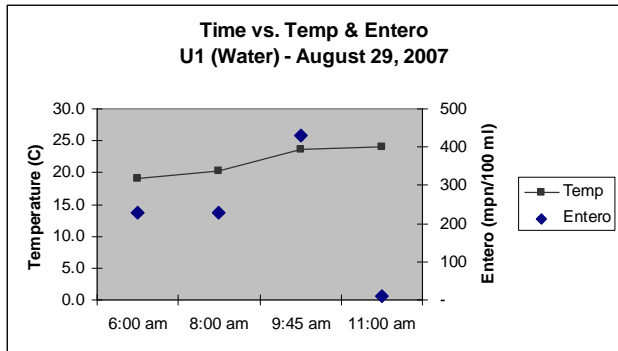
**APPENDIX 1. Time vs. Daily Temperature and *Enterococcus* Graphs**

**U1 Water**

**U1 Sediment**

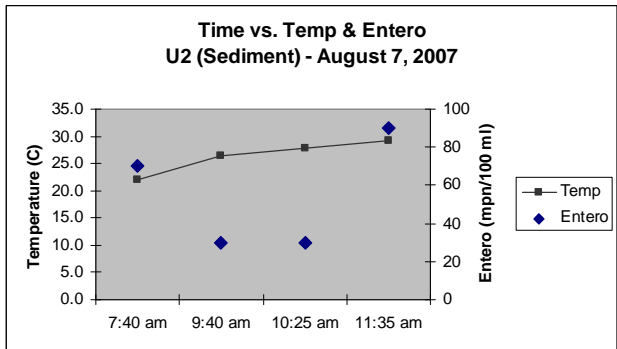
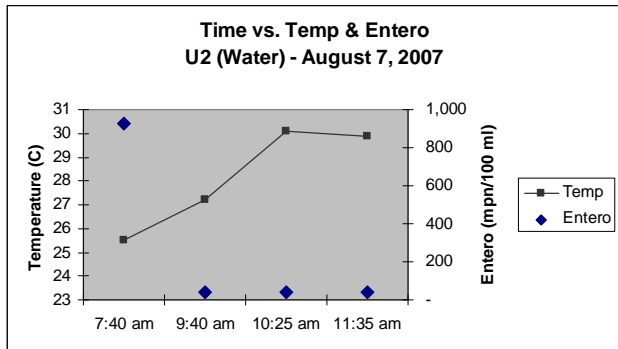
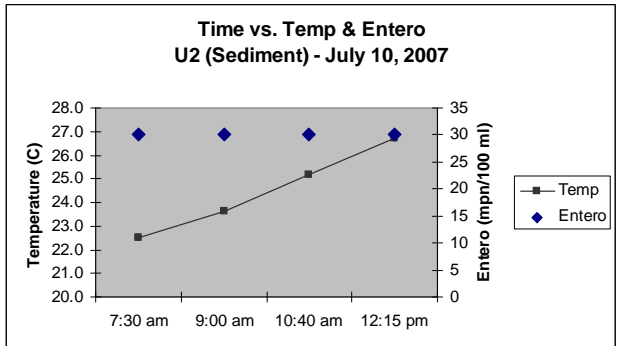
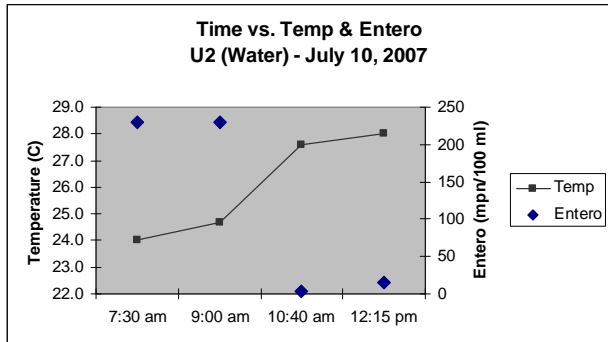
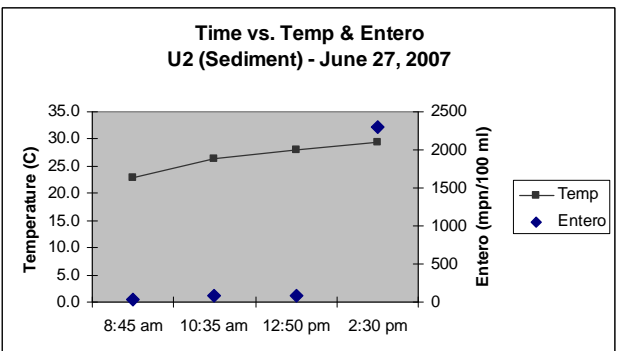
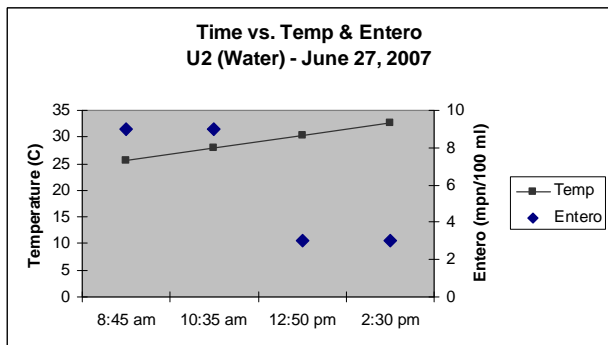


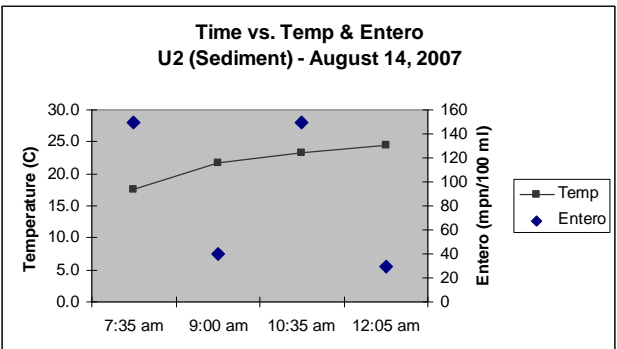
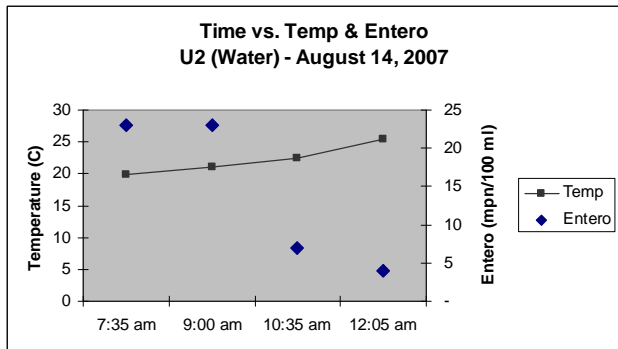




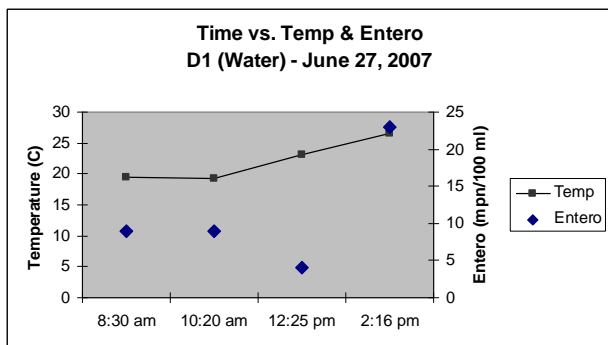
**U2 Water**

**U2 Sediment**

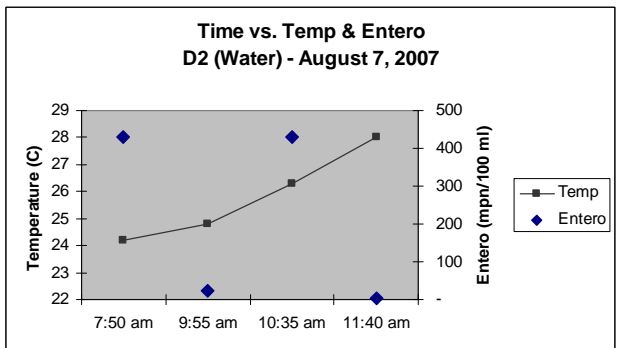
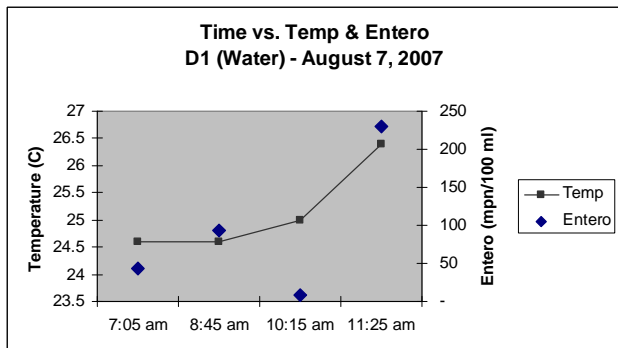
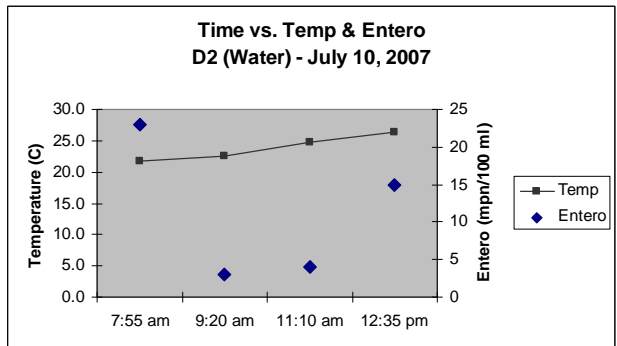
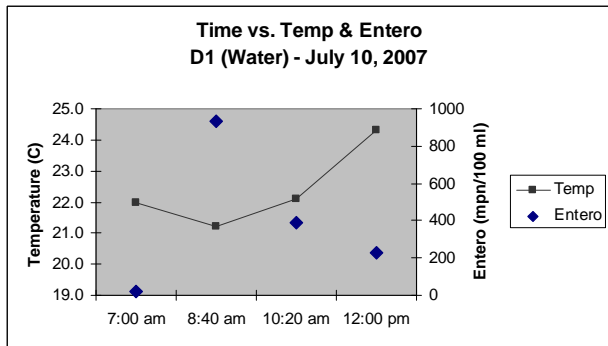
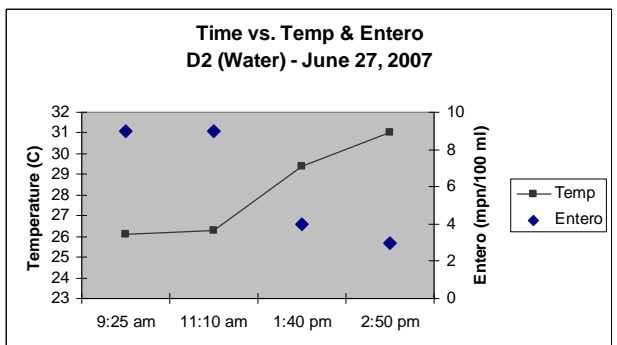


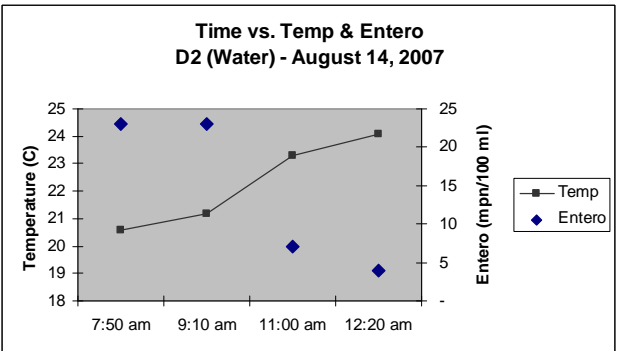
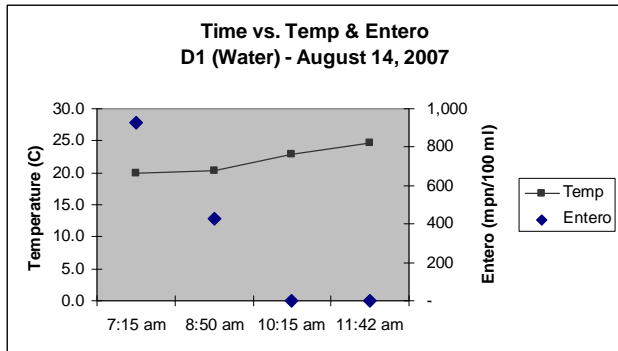


**D1 Water**



**D2 Water**





**Beach Water**

