INDICATOR BACTERIA IN BEACH ENVIRONMENTS

by

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ABSTRACT

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Bacterial presence in the environment has become a growing concern in recent years. The exposure of the public to elevated levels of microbes in the environment has been linked with respiratory and gastrointestinal illnesses. It is important to understand the behavior of bacteria in the environment in order to address public health concerns. This project was intended to improve our understanding of bacteria in the environment that are used as indicators for fecal pollution. Using the standard indicator species *E. coli* and *Enterococci* data was collected at Bradford Beach in Milwaukee Wisconsin throughout the summer months of 2013 and 2014. In order to better understand enumeration of these species from sand samples an experiment was conducted comparing the effectiveness of PBS and DI water as eluents. This experiment found that on average DI water served as a more effective eluent for *E. coli* while PBS was somewhat better for eluting *Enterococci.* Another investigation included the impact of algae on bacteria levels. It was found that indicator bacteria are elevated in water and sand directly adjacent to algal mats but no significant differences were found at larger distances. Other factors investigated were the effects of rain, temperature, and time on indicator bacteria

levels in water. Moderate positive correlations were found for rain and temperature while no significant temporal correlations were found.

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1. Introduction

1.1 Motivation. The use of beaches for recreation is a widespread activity for many people globally. Locally located are the great lakes which contain many recreational beaches. Millions of people visit over 500 recreational beaches in the Great Lakes region every year[1]. Although there are many beaches in the great lakes area, the amount of people visiting beaches worldwide is much larger. For many years there have been concerns over public health for those visiting beaches. Bathing in coastal waters polluted with fecal contamination is estimated to cause more than 120 million cases of gastrointestinal illness and 50 million cases of respiratory disease around the world[2]. This large amount of contracted diseases is of great concern and creates motivation to study and understand the interactions between fecal bacteria, beach environments, and public health. This requires knowledge about and identification of sources, knowledge of bacterial behavior in the environment (including transportation, survival, and pathogenicity), and magnitude of influence bacterial presence can have on public health.

The issue of public health concerns for those going to beaches has been studied for decades and it has been shown that these systems are very complex. The variety of diseases associated with beaches contaminated with fecal pathogens can come from a large variety of pathogens. Pathogens include various types of bacteria, viruses, protozoan parasites, and other organisms [3]. Pathogen sources are also numerous and complex. Sources can be defined as point and non-point (diffuse) sources. Known sources include wastewater treatment facilities, combined sewer overflow, agricultural runoff, leaky septic tanks, urban runoff, boat discharge,

local animal populations, and others[4]. This variety of sources that can come from either a single point or a diffuse area cause difficulties in determining the source of contamination. Further adding to the complexity of the issue is the interactions between pathogens and the beach environments, especially concerning transport and survivability. It has been observed that many factors can influence the movement and enumeration of bacteria on beaches. These factors could include tidal rewetting, runoff, rain events, algae, and others[4]. The many components of the bacteria-environment-human interactions show a need for simplification of the system in order to assess the public health effects of pathogen presence in beach environments.

In order to estimate health outcomes of those exposed to beaches with pathogens present a system was developed in which "indicator" bacteria are used to predict health risks. Indicator bacteria are used due to it not being feasible to monitor all possible pathogens coming from fecal contamination. Previous research conducted at beaches around the world between 1953 and 1996 has shown a causal dose-related relationship between gastrointestinal symptoms and water quality using indicator bacteria[5]. This research has led to widespread use of certain fecal coliform bacteria as an indicator of the presence of and amount of fecal contamination. Due to the established links between these bacteria and public health risks the levels of fecal indicator bacteria are used to predict health risks and assess safety levels. In the United States safety thresholds for these bacteria have been established by the Environmental Protection Agency, which is the authoritative body on water quality for both consumption and human recreation. The EPA sets certain levels at which the risk of exposure to pathogenic bacteria is deemed unacceptable and causes beaches to be closed.

It is important to understand the costs and benefits of beach closures. The safety of the public is of paramount importance to public agencies in the United States such as the Environmental Protection Agency. It is obvious that beaches should be closed as a precaution in order to prevent disease and exposure to the public. However, these beach closures can have an economic and social impact as these areas can often be recreational areas. Many people may travel to beaches and spend money which can impact the local economy making unnecessary beach closure an unnecessary economic burden. Vacationers spend approximately $44 billion annually during coastal trips in the United States alone, showing that this is a very large an important economic sector[6]. It is important to weigh the risks of public health and environmental impact when deciding how often closures must be enacted. This calls for more research in order to more accurately predict public health outcomes on beaches. Due to Indicator bacteria being used as the current predictor of health outcomes it is important to understand all of the factors that impact enumeration of these bacteria and their link to pathogenic microbes.

Although the correlation between human health and indicator bacteria levels has been well established there is more research needed on the topic. Testing for these bacteria has become much easier than it was in the past. Older methods included plating of bacteria, waiting at least 24 hours and then counting plates by hand. Modern methods of measuring indicator bacteria can take less than two hours, maintaining the correlation between heightened indicator bacteria levels with increased health risks[7] . Despite the modern ease of measuring bacteria levels their accuracy in predicting human health risk is still not as accurate as desired[8]. It is important to understand the environmental factors that influence bacterial

counts so that the behavior of the bacteria can be better understood and improve predictive abilities for health outcomes.

1.2 Indicator Bacteria The most common indicator bacteria in use today are *E. coli* and Enterococci. It is important to distinguish the use of these two bacteria from other bacterial groups that could be used. Total coliforms is a widespread bacterial group that can be present in human and animal feces and in other places in the human body. Fecal coliforms is a subset of total coliforms but are distinguished by having a more fecal-specific origin. *E. coli* (EC) is a single species in the group of fecal coliforms that is specific to fecal material from humans and other warm-blooded animals. Fecal streptococci generally occur in the digestive system of humans and animals, but are not coliforms. *Enterococc*i (ENT) are a group within fecal streptococci, they can be distinguished by their ability to survive in salt water[9]. It was established in 1984 that *enterococcus faecium* and *enterococcus faecalis* could be considered a genus separate from streptococci, of which they previously belonged[10]. These two species that are used are familiar species that have been used in many biological experiments over the course of time. The amount of present knowledge available concerning these two species is helpful in explaining their behavior, yet their behavior in the environment is still lacking in knowledge and there is still much that is unknown.

Although fecal coliforms were previously used in the U.S. as a standard for water indicator this changed in 2004 when the EPA changed regulations to say that *Enterococci* would be used as the preferred indicator for saltwater beaches and *E. col*i for freshwater beaches[3]. The distinction between these bacteria for use freshwater and saltwater is due to the difference in correlated health outcomes in the two scenarios. Upon analysis of these species it

was found that *Enterococcus* is indicator bacteria best correlated with health outcomes in marine systems whereas *E. coli* are best correlated with health outcomes in fresh water systems.[5]. It has been shown that *Enterococcus* are able to survive in a variety of stressors such as varied pH, temperature, and salts[11]. This may be a factor in their better correlation with health outcomes in saltwater. These two species have inherent differences and evolutionary adaptations that causes their behavior, survivability, and transport properties to vary different scenarios.

The use of these two bacteria as indicators makes it simpler to study the behavior and environmental interactions as opposed to studying many different bacteria. This is due to their being much research on these bacteria having already been done in the past. Both species are well-known enteric bacteria that have been used in laboratory experiments for decades. There are similarities and differences between *E. coli* and *Enterococci* that are well known. Both of these are facultative anaerobes, meaning they can produce ATP aerobically or anaerobically. This gives the bacteria an ability to survive in various environments, including those with very little dissolved oxygen. These bacteria do differ in their external structure, which can impact their survivability and physical interactions with the environment. *Enterococc*i are gram positive bacteria and their outer layer consists of peptidoglycan. *E. coli* are a gram negative bacteria meaning they have an extra outer membrane (compared to gram negative bacteria) that is primarily lipopolysaccharides and protein. It has postulated that antibiotic resistance aided in the evolutionary development of the extra layers seen in gram-negative bacteria, which could aid in antibiotic resistance of these types of bacteria[12]. Differences in survivability, external

structure, size, shape and zeta potential are some of the factors influencing differences in behavior between these bacteria.

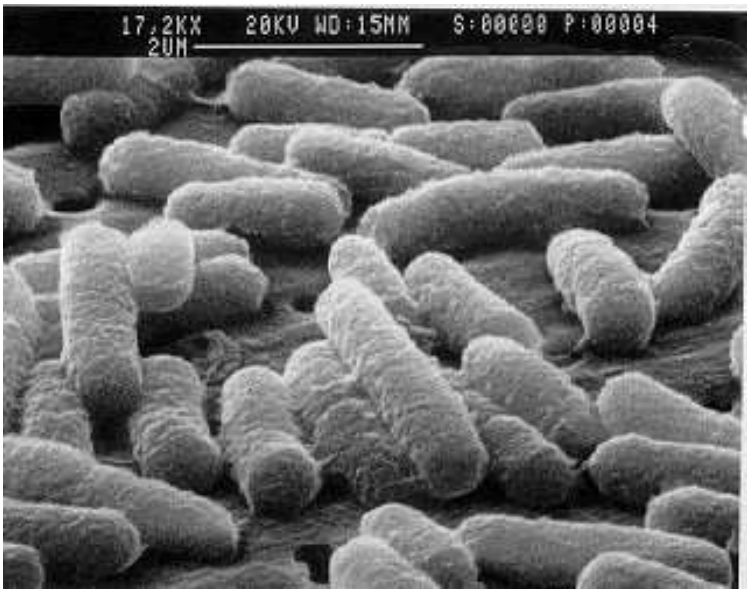


Figure 1 *E. Coli* under SEM. The rod-shape of the bacteria can be seen. (http://www.optics.rochester.edu/)

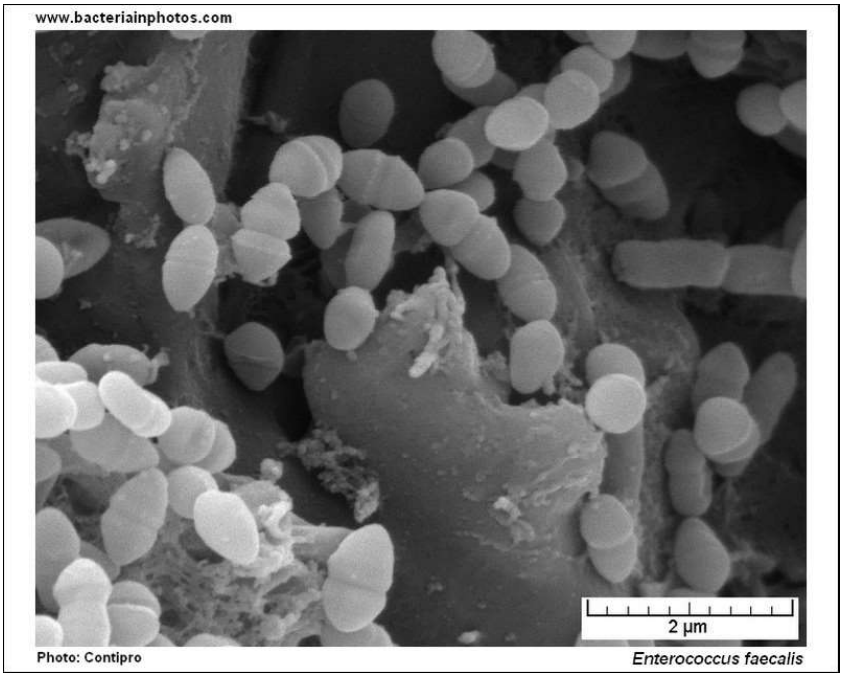


Figure 2. *Enterococcus* under SEM. When compared to EC images it can be seen that these two bacteria vary in both size and shape. (http://www.bacteriainphotos.com/bacterial-biofilm.html#)

The variable properties of bacteria is further complicated by the differences that occur within a single species of bacteria. It has been shown that single genes differences in both *E. coli* and *Enterococcus* can lead to differences in transport properties of the bacteria in sand. These single gene differences can lead to changes in surface properties and biofilm formation[13,14]. These differences indicate the importance of considering population genetics as well as species composition when analyzing behavior of bacteria in beach environment. In recent years advances in genetics have made it much easier to study these bacteria on the genetic level. Genetic variance and gene transfer can be concerning when dealing with antibiotic resistant genes. It was found that antibiotic-resistant *E. coli* were capable of growing in beach sand microcosms and were able to transfer a plasmid-encoded kanamycin-resistance gene in sand microcosms. Resistant phenotypes were stable in the sand environment even in the absence of the corresponding antibiotic[15]. This indicates an increased public health risk due to transfer of antibiotic resistant genes as well as a very genetically complicated population of bacteria in these environments. It follows basic biological rules that specific populations that are adapted to local environments would be the bacteria that are most common in that area over time. It is important to consider how local, persistent populations can impact the link between indicator bacteria and public health

1.3 EPA Regulations. In 1986, the United States Environmental Protection Agency produced guidelines recommending *Enterococci* and *E. coli* as appropriate bacterial indicators to monitor recreational waters[16]. A new set of recreational water quality standards were recommended by the EPA in 2012 .The EPA is no longer recommending the use of a single sample maximum

value. They now suggest using a combination of the geometric mean and statistical threshold value in order to determine the magnitude, duration and frequency of bacterial indicator presence. The geometric mean is determined by taking the log(10) of sample values, averaging those values, and then raising the average to the power of 10. The statistical threshold value is derived by estimating the percentile of the expected water quality distribution around the geometric mean criteria value. The new recommendations correspond to an estimated illness rate of 32-36 per 1000 primary contact recreators[17].

Table 1 (below) shows the most current (2012) EPA regulations put into place for bacterial sampling[17]

	Illness rate 36/1000		**Illness rate 32/1000**	
Indicator	GM (cfu/100mL)	STV (cfu/100mL)	GM (cfu/100mL)	STV (cfu/100mL)
Enterococci (marine & fresh)	35	130	30	110
E. coli	126	410	100	320

1.4 Environmental Factors Currently sampling efforts for recreational water quality are focused on the near shore waters. The monitoring of beaches has been limited to testing the water for indicator bacteria, however, a number of studies have suggested that beach sand may also be a significant source for fecal indicator bacteria[18-22]. An important consideration to make is that many beachgoers do not enter the water, yet still come into contact with beach sand. It has also been shown that exposure to sand (i.e. digging) is positively associated with gastrointestinal illness[23]. It was shown that culture based colony forming units in sand was correlated with gastrointestinal illness and diarrhea for those having contact with sand[24].

Several studies have reported pathogens, including those with microbial resistance, in beach sands[25,26]. This challenges the conventional manner of testing which is currently based on bacterial counts in water. Even though many people who go to the beach may not go into the water an increased contact with contaminated sand could increase health risks. It is therefore important to not only consider the water as the source of pathogens and danger to human health in beach environments.

The presence of bacteria in sand can be concerning not only due to increased pathogens but the sand environment must be considered when determining survivability of microbes in the environment. It has been observed that bacteria harbored in sand can survive longer than in water. This can be due to bacteria adhering more easily to sediment particles than the free particles in water[22]. It has also been shown that wet sands have cultivatable concentrations of ENT and EC that are 4–38 times higher and 3–17 times higher, respectively, than concentrations found in water[27]. The effects of sand on bacterial counts in water and bather health are not fully understood and there is a need for more research. Indicator bacteria in California beach sands were found to be mobilized by high tides and diffused into the water column [28]. The elevated levels of bacteria in near shore waters can be concerning as the mobilization of bacteria from the sand by tides or rainfall into the body of water can affect bacterial counts.

It has been observed that sands with similar conditions such as: grain size, organic carbon content, exposed to a similar wave climate, and having the same degree of anthropogenic influence, tended to have similar microbial communities[29]. This shows that the local environmental conditions have significant effects on the makeup of microbial

communities. The natural variation of local conditions could create very different communities based on local conditions. Given the ability of microbes to persist in the sand it is important to understand the complex environmental interactions. Column experiments identified microbes readily mobilized by seawater infiltrating through unsaturated intertidal sands. The ease with which microbes were mobilized suggests that intertidal sands may represent a reservoir of bacteria that seed the beach aquifer where they may partake in biogeochemical cycling[29]**.** The physical and chemical factors influencing the mobility of bacteria in porous media include solution chemistry, fluid velocity, surface roughness, charge heterogeneity, grain size, and saturation of porous media[30]. Due to the ability of bacteria to persist in certain environments and their capability of transport in between local environments such as sand or soil and water it is important to consider their behavior in all environments.

It is understood that interactions between measured indicator bacteria and environmental factors are very complex. It is important to understand how local environments can act as sources/sinks of these bacteria. Sandy environments are generally environments in which people are more likely to go to when visiting a body of water for swimming and recreation. Therefore it is important to understand the interactions between the sand, water, bacteria, bather health, and other environmental factors. The testing of water alone may be insufficient and further research is warranted to better understand microbial behavior in sandy environments. The scope of this project includes the testing of bacteria levels in both sand and water in order to better understand this issue.

1.5 Algae. The presence of *Cladophora*, a genus of nuisance green algae, has become an increasingly common occurrence on Great Lakes beaches. This is thought to be due to the

arrival of an invasive species called zebra mussels. Zebra mussels increase the water clarity and allow for photosynthetic activity to occur in deeper water than before. Another factor that is thought to cause growth of *Cladophora* is phosphorous accumulation from storm water runoff[31]. *Cladophora* is an algae that grows in strands or mats on hard substrates in lakes. Most of the *Cladophora* on beaches is due to it unlatching and washing into shallow waters. It is typically found in the near shore water but is also subject to washing up onshore during a high tide or storm event and can become stranded on the beach. *Cladophora* is known to cause unpleasant odors when mats are decaying. Some beaches have decided to take care of this problem by raking the algae onto the beach in order to allow it to decompose quicker. This process could lead to increased nutrient transfer into the sand, which allows the bacteria levels to grow even larger[32].

Cladophora mats have been shown to harbor high levels of *E. coli* and *Enterococci* in laboratory settings[33]. *E. coli* was detected 63 of 63 samples of *Cladophora* with average levels ranging from 2700 CFU/100 g to 7500 CFU/100 g[34] while it has also been observed that both *E. coli* and *Enterococci* survived over 6 months in sun dried *Cladophora* mats and could regrow upon rehydration[32]. Algae presence can allow for increased *E. coli* survival and sometimes replication by augmenting beneficial (nutrients, protection from predation, attachment sites) and reducing detrimental (ultraviolet light) environmental conditions[31]. It is important to understand the impact of algae not only on indicator species, but for pathogenic species as well. Given survivability and behavioral differences in species it is possible that the effects of algae could vary depending on individual bacteria or pathogen properties. It has been shown that *Cladophora* can serve as a reservoir for both indicator bacteria and pathogens[21]. Some

pathogens that have been observed in algae mats are pathogenic bacteria such as Shiga toxin-producing *Escherichia coli* (STEC), *Salmonella*, *Shigella*, and *Campylobacter*l[35]. Although it appears both indicator bacteria and pathogenic bacteria have the ability to survive in *Cladophora* it has been observed that *E. coli* can survive much longer than both *Salmonella* and *Shigella* in a laboratory microcosm[36].

The relationships between *Cladophora*, indictor bacteria, and pathogenic bacteria needs to be further understood. It is clear that bacteria levels may be greatly affected by presence of algae on the beach. It is important to understand if this influence has an effect on the correlation between indicator bacteria and risks to human health. Algal presence could decrease accuracy of predictions for health outcomes. This is due to it being very difficult to understand the relationships between algae and all possible pathogenic microbes from enteric sources. This produces a complex issue as there is little data on the impact of algae on public health outcomes. Field observations from this project showed that throughout our sampling periods algae levels at Bradford Beach were highly variable quantitatively and temporally. Presence of algae on this and other recreational beaches is a concern.

1.6 Choice of Eluent One of the challenges in determining the amount of bacteria present in sand is to how to effectively enumerate the particles for testing. Various studies have used either deionized water (DI) or Phosphate buffered saline (PBS) to elute bacteria from sand samples. There has been an inconsistency among researchers as to what eluent should be used with sand samples to enumerate indicator bacteria. Many of the studies that used PBS as the eluent chose the EPA's formula which called for the following ratios; 0.32 grams of sodium dihydrogen phosphate, 1.1 grams of sodium monohydrogen phosphate, 8.5 grams of sodium

chloride, and 1 liter of distilled water[3]. There is a need to determine a preferred eluent type in sand bacteria testing in order to determine which eluent can produce the highest recovery of indicator bacteria as there is no uniform procedure currently in place. Furthermore, it could be convenient when looking at studies to be able to convert between values obtained using different eluents. Effect of eluent and eluent choice should be considered when looking at data concerning bacteria levels obtained from sand samples.

Eluent choice is a complex problem due to the various ways eluents could affect bacterial counts. The first issue is the amount of bacteria that becomes detached from sand particles when the eluent is added to the system. In order to explain this behavior DLVO theory, or modified versions of it, are often used. Standard DLVO theory is calculated by summing the van der walls forces and electrostatic double layer repulsion[37]. These forces are impacted by the external properties of the bacteria and sand particles as well as the solution chemistry. It has been shown that ionic strength of solution can have significant impact on deposition and is therefore important to consider the differences in ionic strength of eluents when using DLVO theory[30].

Along with the forces acting on bacteria and sand there are other factors to consider. The survivability of the species is important to consider as this would affect bacterial counts. PBS was first used in the 1950's for bacteriological purposes[38]. In general PBS is often used for bacteriological purposes as it mimics conditions inside the body and aids in bacterial survival. The effects of phosphate on adhesion has also shown to be significant[39]. The ability to further study bacterial behavior in the environment requires the development of standard procedures

so that there can be uniformity in the field. Choice of eluent is important as it is a complex issue that likely has significant effects on bacterial sampling.

2. Project Overview and General Methods

2.1 Project Overview. The primary objective of the project was to enhance knowledge of indicator bacteria enumeration in the environment in order to aid in development of methods to obtain more accurate predictions of public health outcomes from indicator bacteria data. It was planned that indicator bacteria would be sampled from the environment in order to obtain data. Sampling of bacteria from the natural environment was decided upon as the best way to obtain data as the goal of the project was to find methods of using field data to predict health outcomes, as opposed to laboratory experiments. Collected data and analysis of data was then used to analyze factors concerning sampled indicator bacteria levels, environmental factors, and public health outcomes.

2.2 General Sampling. Bacterial sampling occurred at Bradford Beach in Milwaukee Wisconsin throughout the summer months of both 2013 and 2014. Several analyses were performed on the data over time. Sampling methods were not the same for the two sampling periods, however they were uniform for each summer. Sampling methods were determined based on desired data as well as external collaboration.

Sampling methods throughout the summer of 2013 were uniform for each day sampled. Three different transects were used as sampling location. Transect location remained constant throughout the sampling period and local landmarks were used to ensure consistency of location. Each transect consisted of three samples; one water sample, one sand sample from

the swash zone, and one sand sample from 20 feet inland. The swash zone was defined as the area of the beach that is alternately wet and dry due to wave action. Sand samples from the swash zone were taken from the surface of the beach. The sand samples that were taken 20 feet up shore from the swash zone were extracted from the water table so that all sand samples were saturated. In order to do this a small trowel was used to dig down to the water table and once found sand samples were taken. The depth of the water table varied from 6-21 inches based on topography and recent rainfall amounts. Water samples were taken once sampler waded into the water to knee depth (around 1.5 feet) and the sample was taken from the top of the water with a bottle. All samples were placed into a cooler with ice for transport to the laboratory. Water samples were placed in 500ml plastic bottles while sand samples were in plastic Whirl-pak bags.

Figure 3 (above) shows the three transects that were used to sample in 2013. In 2014 only sites on transect 2 were used for sampling. The summer sampling schedule occurred in July and August, 2013. Samples were taken three days per week at approximately 9:15 am. There were no samples taken in June.

In the summer of 2014 samples were taken June through August. There were several changes to sampling techniques for this sample period. Throughout this sample period only one transect of the beach was used for sampling. Transect was taken at mid-beach at the same approximate location each sampling day. This transect was in the same location as transect 2 used for 2013 sampling (Fig. 3). For each transect three sand samples and one water sample was taken. Sand samples were taken at the swash zone (as defined previously), 10 feet inland from the swash sample and 20 feet inland from the swash sample. As in 2013 samplers first used a shovel to dig down to the water table before taking samples. In 2014 samples were not

simply taken from the top of the water table. A soil sampling kit was used to take a core sample from each location. Once the core was obtained it was taken out of the equipment and split up into three sections. The three sections were split horizontally with one being from the top, middle, and bottom third of the sample. Core samples generally produced a core of 9 inches in length meaning each sample size was representative of approximately 3 vertical inches of sand. In this manor three samples were taken for each sand sampling point leading to an overall of 9 sand samples and one water sample for each sampling event.

Figure 4 shows a typical core sample obtained in the field.

2.3 Laboratory Analysis. Water samples were using sterile 500ml plastic bottles. Sampler waded into the water until water reached knee depth (around 1.5 feet) and the water samples were collected form the surface of the water. At the sampling site samples were immediately put into a cooler filled with ice for preservation. Once transported to the lab 100 ml of water

was taken for each bacterial enumeration and tested using IDEXX Enterolert and Colilert methods. Water samples were put onto an Excella E24 Incubator Shaker Platform (New Brunswick Scientific, Enfield, CT) at 200 rpm for 5 minutes. The shaker platform was used for both water and sand samples and was meant to homogenize the samples. It was also expected to detach some of the bacteria from algae and sediment that may have been captured and suspend the bacteria in the water. The bottles were then left to settle for 5 minutes. Settling was conducted to allow larger particles to fall to the bottom so that they were not incorporated into the IDEXX quanti-trays and influence the bacterial counts. Two-100ml samples were then taken and put into two separate 100 ml IDEXX plastic bottles in order to analyze E. coli and Enterococci levels in the water.

For sand samples the analysis were similar. Each sand sample was thoroughly mixed to produce uniformity within the whirl-pak bag. A predetermined weight of sand was used for elution. The amount of sand used varied somewhat throughout the sampling period. Adjustments were made in order to keep data readings within the limits of testing equipment. This was not always achieved as some samples were above the threshold for the equipment being used. The appropriate mass of sand was then mixed with 200mL of eluent in a plastic bottle. Bottles were placed on an Excella E24 Incubator Shaker Platform (New Brunswick Scientific, Enfield, CT) at 200 rpm for 5 minutes. The shaker platform is used to aid in bacterial detachment through addition of energy and motion into the system. The bottles are then left for 5 minutes for settling. Settling used to allow the sand to settle on the bottom of the bottle in order to prevent interference of sand in bacterial counts of the eluent. It is possible that sand entering the tray could produce more positive bacterial results from biofilms instead of

suspended bacteria. This would affect overall bacterial counts. Two 100 mL samples of eluent were poured into two separate sterile plastic bottles. One of the bottles was used for *E. coli* measuring and the other was used for *Enterococci.*

Colilert indicator and the Quanti-Tray/2000 system (IDEXX Laboratories, Westbrook, ME) was used to measure the amounts of *E. coli* in units of "most probable number" (MPN). A packet of Colilert indicator was added to a bottle with 100 mL of water or eluent in it. . Samples were mixed by hand until the media was fully dissolved. After dissolution liquid samples were put into a sterile IDEXX Quanti-Tray/2000 packet. The Quanti-Tray/2000 packet is then put through the Quanti-Tray Sealer in order to seal the container in a manner which causes all compartmants of the tray to be separate from the others. This is important as each compartment is analyzed individually for bacterial presence when interpreting results. These steps were repeated for the *Enterococci* bottles with the exception that Enterolert media and indicator (IDEXX Laboratories) was used instead of Colilert indicator. Samples were left to incubate for 24 hours. Incubation temperatures were 35 deg. C for E. coli and 41 deg. C for Enterococci.

After the incubation period the pouches were analyzed to obtain the Most Probable Number (MPN) of Colony Forming Units (CFU) of the bacteria being analyzed. Recorded values of MPN/100mL were the values used in analysis of bacterial enumeration data. The quanti-tray system uses a tray with many separate pouches in which the liquid media flows into. Once sealed these pouches are separate. Each pouch in analyzed as positive or negative for bacterial presence. The number of large and small capsules that returned positive results are counted for

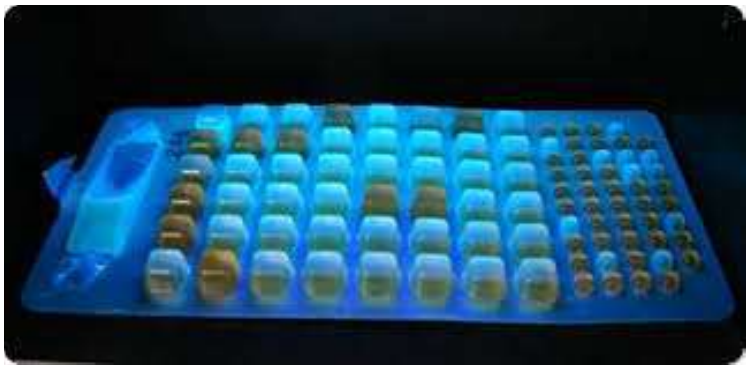
each tray and these numbers are used to predict the most probable number of colony forming units using a statistical approximation developed by IDEXX.

For *Enterococci* samples it was determined whether or not a sample was positive (after incubation) was if it had a blue fluorescence. Blue fluorescent samples indicate a positive result for Enterococci and a lack of said fluorescence indicates a negative result. The blue fluorescence is determined by analysis with a UV lamp. The *E. coli* samples can be analyzed for bacterial presence for both total coliforms and E. coli. Samples determined positive for total coliforms if they present a yellow color in normal lighting conditions. E.coli samples are measured similarly to Enterococci samples in that a UV-lamp is used to observe fluorescence. In this case fluorescence also indicates a positive result. If a compartment has both a yellow color and fluorescence then it shown to be positive for *E. coli*. Based on the number of large and small positive compartments for each sample, an MPN value was obtained from the chart per 100 mL of eluent or water. The MPN value is equivalent to CFU (colony forming units), which is the conventional bacteria measuring value. The MPN/100 mL of eluent for the sand samples was then standardized to be MPN/100 grams of sand for comparison purposes.

2.4 IDEXX Information. IDEXX describes the system used thusly: "The Quanti-Tray/2000 is based on the same statistical model as the traditional 15-tube serial dilution. With the Quanti-Tray/2000, the sample is automatically divided into the proper portions when sealed by the Quanti-Tray Sealer PLUS. The Quanti-Tray system does not require the use of test tubes, Durham tubes or any dilutions. By automatically distributing the sample into 97 wells of 2 different sizes, Quanti-Tray/2000 yields a counting range of 1–2,419 with a far better 95% confidence limit than a 15-tube serial dilution[40]." This system has shown to be a reliable

replacement for standard methods that include much more tedious methods. The ability to rapidly analyze samples using this method allowed us to process more samples than the standard colony counting vacuum filtration method.

In 2007 the Quanti-Tray system gained EPA approval for compliance testing of wastewater[41]. It has also been approved for use in drinking water testing in several countries[40]. One study conducted over a 2-year period compared the IDEXX method and an approved USEPA membrane filtration method (m-Tec agar) for the quantitative determination of *E. coli* in split samples from fresh water beaches. Results indicated in an ANOVA analysis of regression relation there was no significant difference between the data sets obtained using these methodologies[42]. It has also been shown that this system can produce false positives in samples based on the absence of target terminal restriction fragments in individual positive wells[43]. Despite the possible lack of specificity of these tests it appears that on a large scale they can be accurate predictors of indicator species. The goals of this project were to analyze the various factors affecting indicator bacteria levels. Due to a high number of data points required for the analysis it was necessary to use a system in which multiple samples can be processed in a short period of time. The quanti-tray system was decided to be the best option for our purposes due to its ability to rapidly process larger quantities of data and maintain a high level of accuracy in predicting the amount of colonies that would be counted using the vacuum filtration method.



Figures 5, 6 (above) show Quanti-tray sealer as well as the Quanti-tray with both positive and negative results. Positive results show fluorescence.

3. Eluent Comparison

3.1 Methods. Two eluents were used to elute sand samples of bacteria in order to determine the most effective eluent. It was assumed that the eluent producing higher bacterial counts is the more effective and desirable eluent. Eluents used were Deionized Water (DI) and Phosphate Buffered Saline (PBS). The PBS solution was made according to EPA standards; 0.32 grams of sodium dihydrogen phosphate, 1.1 grams of sodium monohydrogen phosphate, 8.5 grams of sodium chloride, and 1 liter of distilled water. The ionic strength of PBS is approximately 165 mM while distilled is approximately 0 mM.

Eluents were only used for sand samples, no water samples were used in the eluent analysis. Once sand samples had been collected from the beach as previously discussed they were transported to the lab in a cooler on ice. Two appropriate sand mass samples were taken (after mixing) and one was eluted with 200ml DI water while the other was eluted with 200ml PBS. 100 ml from each of these samples was taken and used for analysis for *E. coli* and *Enterococc*i as described previously.

All sampling and laboratory procedures were identical to the general sampling methods with the exception of eluent. Samples were mixed, weighed(adjusted according to expected bacteria levels), combined with the proper eluent, shaken for 5 minutes, left to settle for 5 minutes, and then mixed with the proper bacterial indicator/media before incubation and analysis. Results for each sample were separated for each of the eluents to create a paired set of data. Analysis was conducted using Excel spreadsheets and the free software Deducer, and add-on to R.

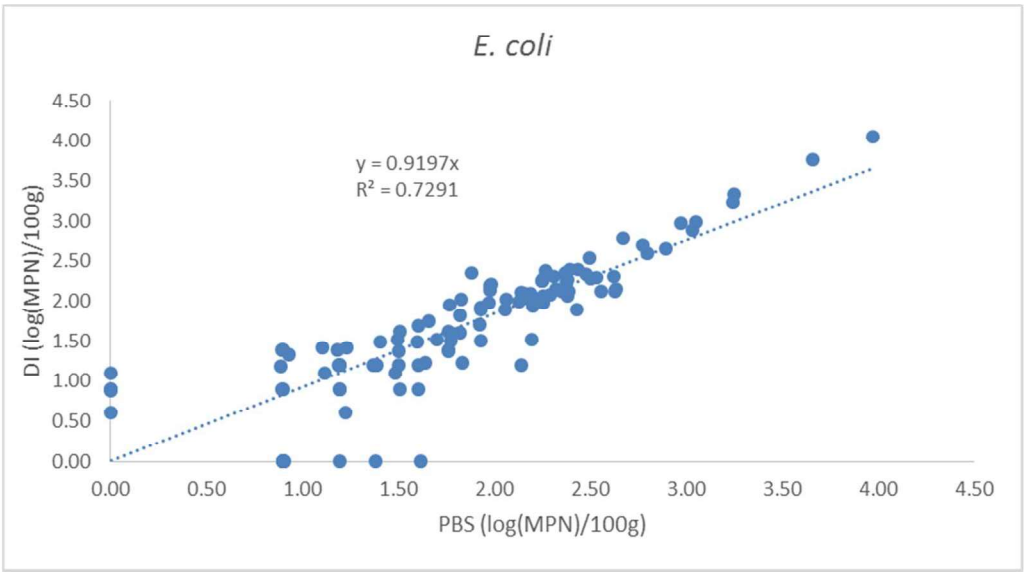
3.2 Results.

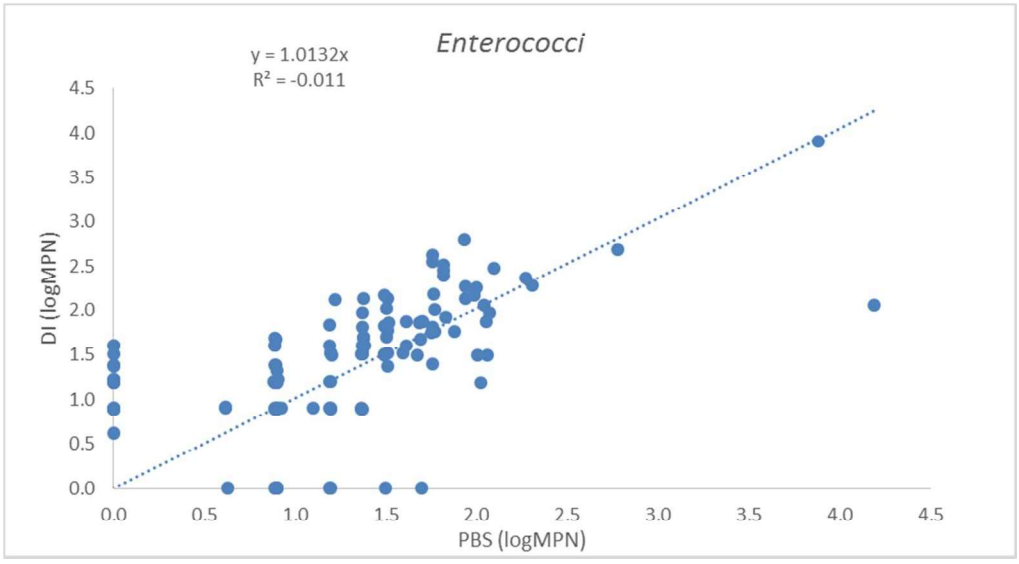
Tables 2, 3 (below) show paired t-test results. Units used were log(MPN/100g sand). The difference in the number of samples in the two comparisons is due to all values in which no bacteria were present being thrown out for the comparison.

	E. Coli	
	DI	PBS
Mean	1.84	1.69
St. Dev.	0.75	0.81
2 tailed, paired t-test		
P value	0.00041	
n	113	

	Enterococci	
	DI	PBS
Mean	1.24	1.46
St. Dev.	0.72	0.66
2 tailed, paired t-test		
P value	0.00019	
n	127	

Above figures shows the results of paired t-tests for both bacteria comparing DI and PBS as eluents. Results show a slight difference in mean MPN/100g for both bacteria. Interestingly the eluent which produced higher bacterial counts is not the same for both species. DI is shown to produce higher E. coli counts while it can be seen that PBS produced higher Enterococci counts.

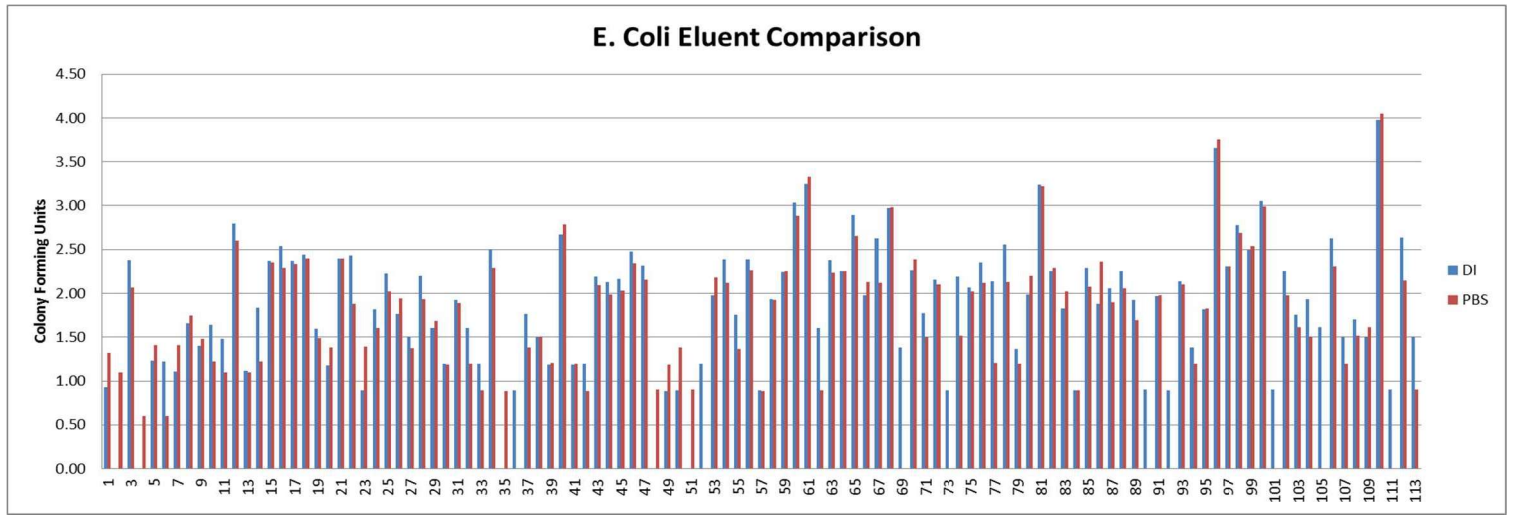


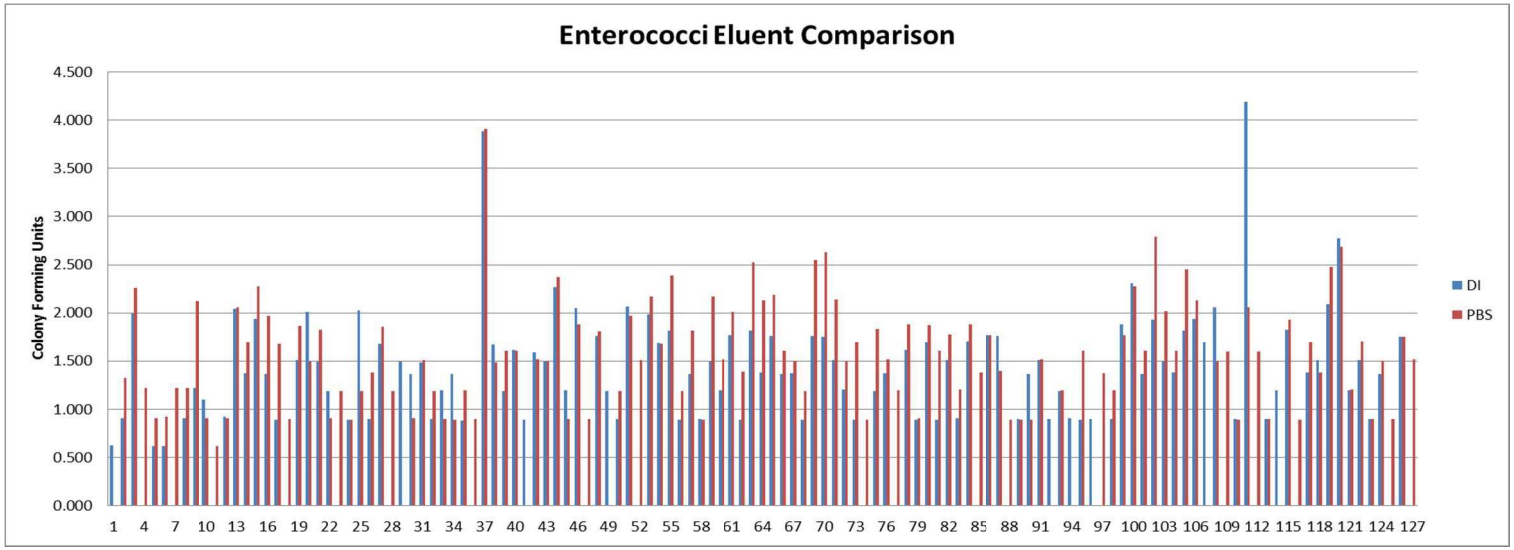


Figures 7,8 (above) show a graphical comparison of counts produced by each eluent for a single sample. This analysis was done for both species.

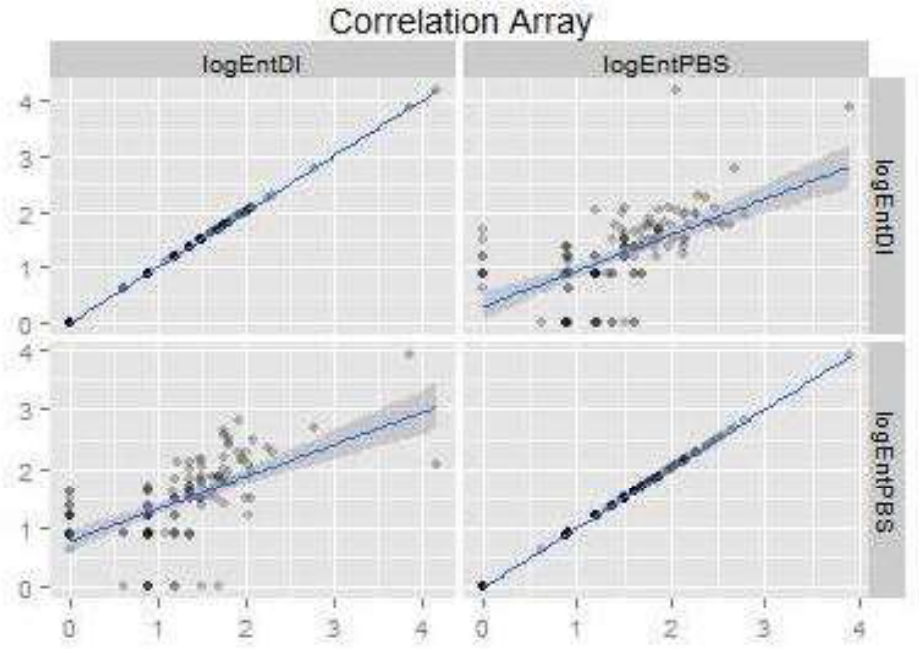
Although the t-test data shows that there are significant differences in mean values of counts for each eluent it is also valuable to establish a mathematical relationship between eluents and bacteria counts. The establishment of a formula to accurately predict the relationship between the two eluents for a bacteria would allow for studies using different eluents to be more easily compared. This would aid in producing more uniformity of data and

add to the accuracy of data analysis. In these figures it can be seen that there is a relatively strong (R^2=0.73) linear relationship between the two eluent s for E. coli. This relationship has a slope of nearly 1 (0.92) showing that data from these two eluents could be compared to each other without a large discrepancy being expected due to the influence of the eluent. The data for *Enterococc*i showed a much more varied difference between the eluents. The ratio between the eluents was much less consistent and a linear relationship was not established. It should be noted that E. coli is generally used as the indicator of choice for freshwater environments and for the sampling location in this case, this data may be more valuable. This data shows that there are significant differences in the behavior of these species.





Figures 9,10 above show the log MPN values for all samples analyzed. It can be seen that the ratio for each sample between the two eluents is highly variable.



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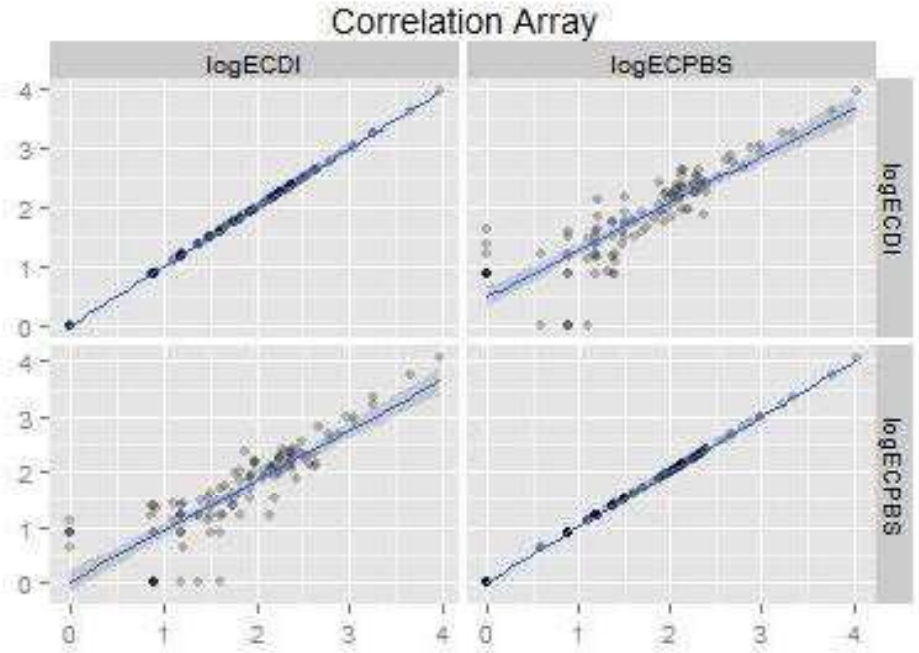


Figures 11, 12 Pearson correlation value for DI and PBS in ENT samples was found to be 0.5904 Pearson correlation value for DI and PBS in EC samples was found to be 0.8539.

Correlation values were calculated in R software. Correlation values indicate positive correlation for both bacteria. This correlation is quite strong for EC while it is quite weak for ENT. These values further demonstrate that the EC data provides a well-correlated dataset which can accurately demonstrate the difference between the eluents. It is expected that the correlation coefficient would be quite high due to both eluents being used on a single sample. The data for ENT shows this is a much more variable dataset and it is difficult to develop a relationship between the two eluents for this species.

3.3 Discussion. The results showing that DI water produced slightly larger *E. coli* counts while PBS produced slightly larger *Enterococcus* counts shows that the interactions between solution chemistry and bacterial adhesion are complex. DLVO theory calculations predict electrostatic interactions to be an important factor in bacterial adhesion. Under most conditions quartz sand, *E. coli,* and *Enterococcus* are electronegative with Enterococcus generally being the more electronegative species, although *E. coli* has been shown to have a positive zeta potential in solutions with elevated ionic strength[30]. Calculated DLVO energy profiles predict a much larger energy barrier to attachment for *Enterococci* when compared to *E. coli*.

Solution ionic strength can have significant effects on bacterial adhesion to surfaces due to the impact on electrostatic interactions. Deposition rates of several bacteria were found to increase at higher ionic strengths of solutions[30,44]. Electrostatic forces play a significant role in the interactions between bacteria and quartz sand. With an increase in the ionic strength of the background solution, the repulsive force between the two electronegative forces is lessened[45]. Column experiments have shown that solutions with increased ionic strength can aid in deposition of Enterococci. These results are in accordance with DLVO predictions. Similar

results for *E. coli* have produced opposing results (decreased deposition), which is not in accordance with DLVO theory[30].

Although the eluent ionic strength is likely to play a large role in bacterial adhesion to sand, the effects of phosphate also appear to be significant. Phosphate has been found to decrease the zeta potential of both sand and bacteria. In one case the addition of phosphate caused the release of attached *E. coli* from quartz sand[39]. It is also thought that phosphate can compete with bacterial cells for binding space on surfaces therefore limiting bacterial adhesion.

Variation in surface properties has not only been observed between these two species but also within each of them. Among several E. coli strains zeta potentials were observed to vary between -3.5 to -49mV. Other surface properties such as hydrophobicity, surface charge density, total protein content of the EPS and cell size were variable among the strains as well[46] . This natural variation significantly altered transport behavior between strains. Presence of a specific Enterococcal surface protein has shown to affect transport behavior of Enterococci[14] . Variation in surface properties within species could aid in explaining the high variability of enumerated bacteria between samples. Outside factors such as contamination source, temperature, rain events could also affect the genetic makeup of the bacterial community. Selective changes in the genetic makeup of the bacterial community due to environmental conditions could affect bacterial adhesion and transport.

Although the attachment of bacteria to sand is an important factor the survivability of the bacteria in particular eluents may also have an effect. Distilled water can cause an increase in osmotic pressure on a gram-negative bacteria cell like *E. coli*[47]. Despite the possible increase

in cell death among *E. coli* in DI water when compared to PBS, DI was found to be a more effective eluent. This appears to indicate that for *E. coli* the eluent effects on sticking efficiency are more influential than possible cell death when comparing DI water to PBS. *Enterococci* are expected to be largely unaffected by the osmotic stress in DI water due to the ability of gram-negative bacteria to endure significant osmotic stress[48,49].

During elution procedure bottles with the saturated sand and eluent were put onto a shake table for five minutes. This procedure introduces forces into the system that are not accounted for by DLVO theory. Mixing of sand in this manor causes the sand to not be static. Sand particles are suspended and can physically collide due to mixing. This introduces another force unaccounted for in DLVO or XDLVO theory.

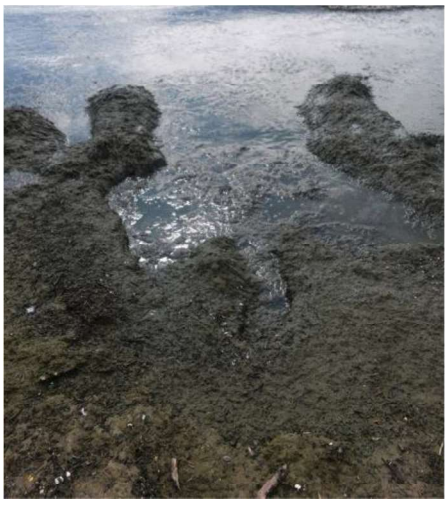
Due to testing limitations the sand samples that are being compared between the two eluents are not completely uniform or equivalent. Sand samples were put into a whirl-pak bag, mixed, and two separate samples were taken from the bag to be used for each eluent. Incomplete mixing is likely a cause of much variation in samples. Non-uniform sand and organic material concentration levels were found in some samples. These natural variations are also a likely cause of variation in data.

4. Algae Comparison

4.1 Methods Although two eluents were used to elute each sand sample in 2013 in order to maintain uniformity only data for sand samples eluted with DI water were used for algae comparison. DI water was the only eluent used in 2014. Both water samples and sand sample results were used in the algae comparison. The purpose of this experiment was to determine

the impact of algae on bacteria levels both in sand and water. Sand and water samples were processed in the same manner as described in section 2.3. MPN values for samples were used and analyzed.

In order to see the impact of algae levels on bacteria levels it was important to first develop a system to define algal presence on the beach. A visual classification system was used to determine the levels of algae each sampling day. The rating scale was as follows: 0 for no algae, 1 for low algal presence on the beach, 2 for moderate presence, and 3 for high. A “3” rating was used when there was no wave action onto the beach due to the thick algal covering in the near shore water which led to stagnation (*Figure15)*. A “2” rating had wave action on beach but large amounts of algae in the water (*Figure 14*). A “1” rating had small amounts of algae visible in the water or beach(*Figure 13*).



Figures 13-15 show various algae classification levels. Top being 1, middle being 2, and the bottom being level 3 algae. It can be seen there is little algae present in a level 1 algae classification while the level three has enough algae present to hinder wave action on the beach. Several feet of thick algal mats were observed on several occasions in 2013.

This algae classification system was used and algae levels were recorded when samples were taken. In 2013 where three transects were used algae levels were recorded for each transect whereas 2014 had one transect and one algal classification. Data for bacterial enumeration was taken and pooled into groups based on algae level and sample type (sand, water, distance from shore etc). Data analysis was performed using Excel spreadsheets and a free software add-on to R called Deducer.

4.2 Results. Data for water and sand samples was collected for each transect in order to analyze the impact of algae. For 2013 data each transect was analyzed independently for bacterial counts and algae levels. This applies to the data analysis but not for figure which shows average bacteria levels in the water and averages algae levels across the three transects.

Table 4 (below) shows the differences between average MPN/100ml or MPN/100g for EC and ENT in the water and swash zone.

Average		0	1	2	3
EC	Water	25	102	262	612
	Swash	126	232	257	618
ENT	Water	7	115	57	507
	Swash	15	50	81	1045

Averages indicate a positive correlation between algae presence and bacterial counts. It also appears incrementally increasing algae levels may be positively correlated with bacterial counts as all bacterial averages increase with an increase in algae. The one exception to this is the averages for algae levels 1 and to in ENT levels in water. Below figure shows the sample sizes acquired for each category. It can be seen that there are 10 or fewer samples for algae level 0 in each scenario. This sample size is less than desirable however other algae levels were

found more frequently. It is worth noting that this is reflective of the amount of transects sampled being much more likely to have some (or a lot of) algae present with the most frequent algae levels being 1 and 3.

Table 5 shows the number of samples acquired for each category over the sampling period

Samples (n)		0	1	2	3
EC	Water	7	26	16	24
	Swash	10	25	18	23
ENT	Water	8	26	18	22
	Swash	10	23	17	23

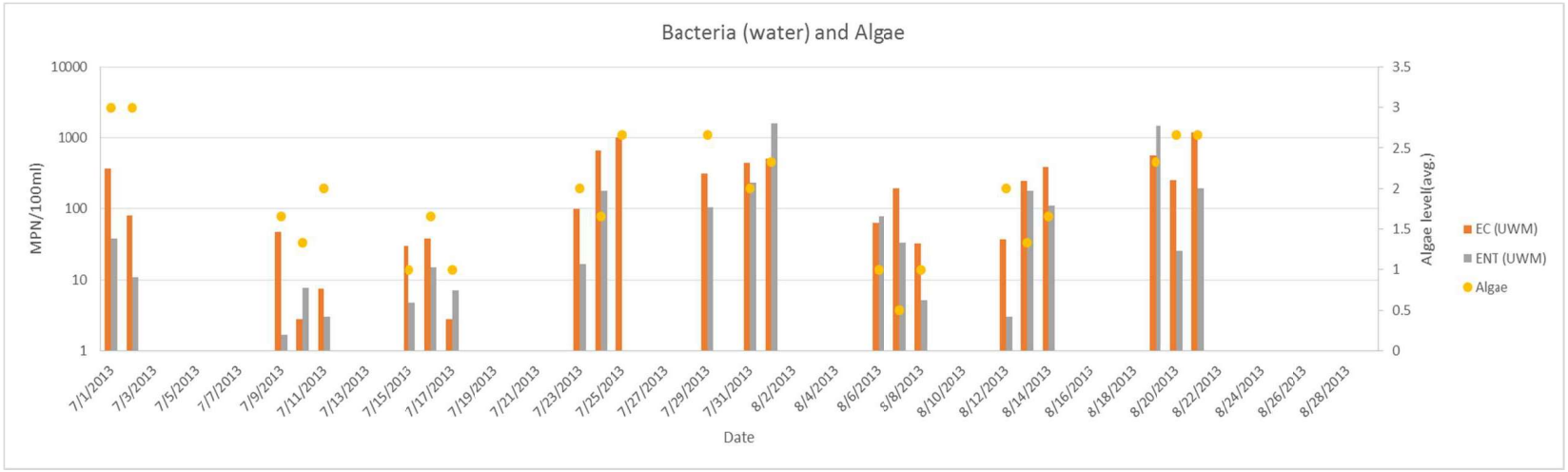
Table 6 shows results for T-tests when comparing average results for each category of algae level.

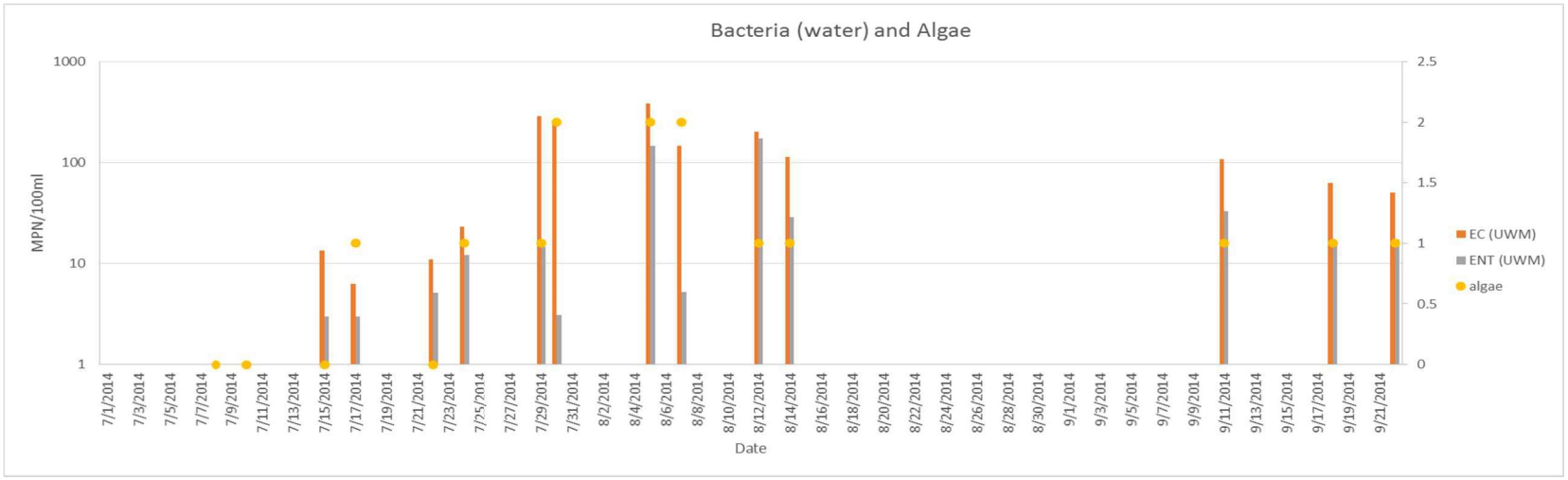
		1,3	0,3	0,1	0,2	1,2	2,3
EC	Water	0.001	0.000	0.003	0.004	0.033	0.018
	Swash	0.178	0.119	0.113	0.022	0.381	0.191
ENT	Water	0.033	0.007	0.104	0.018	0.254	0.013
	Swash	0.094	0.087	0.002	0.033	0.188	0.101

T-tests were conducted between each algae level for the bacteria in water and sand. Results show that for EC there is a clear positive correlation between algae presence and bacteria levels as well as algae quantity and bacteria levels. This trend is less visible with ENT but this may be the case here as well. Results for the swash zone are much less convincing. It does appear that algae levels have an impact on bacterial counts but these results are not statistically significant.

Graphical results indicate a correlation between algal presence and bacterial counts as well. Data for 2013 show that the average values for bacteria in the water and average algae levels across all transects. This data appears to indicate some correlation between algae levels

and bacterial counts as the events with the highest bacterial counts often occur on days with elevated algal presence. This trend also appears in the plot for 2014 data. It can also be seen that the two bacteria are not always present in equal ratios. In most cases E. coli are shown to produce higher counts than enterococci but there are many sampling event where this is not the case.





Figures 16,17 show the EC and ENT levels found in water sampled over time compared with algae.

4.3 Discussion. One factor that appears to contribute to higher bacteria levels is the presence of *Cladophora*. For the water and swash zone samples, the data shows an increase in bacteria levels during higher degrees of algae presence. This correlation between algae levels and bacterial counts was found to be significant in the water, somewhat significant in the swash zone and not significant at locations further up the beach. This is likely due to the complex interactions between transport and survival of bacteria in sand and water. It has been clearly shown in other experiments that algae can harbor indicator bacteria but the link between algae presence, indicator bacteria levels, and overall health risk are still very much unknown.

Algal mats are full of nutrients that allow bacteria to grow and their presence near beaches has been shown to lead to higher bacteria counts in the near shore area[32,34]. As a result of wind and wave action, these microorganisms can detach and be released to surrounding waters[50]. As stated previously, the link between indicator bacteria and pathogenic microbe presence with regards to algae is not fully understood. It appears to be the case that algae has a limited circle of influence for increasing indicator bacteria presence. This influence may not be large enough to affect the health risks of those at beaches as a whole, yet it appears that it may be wise to advise the public to avoid regions very near to decaying algae.

5. Temporal and Environmental Factors

5.1 Methods. Beach testing data at Bradford Beach was obtained from a secondary source. Data on EC levels in water and water temp were taken from Wisconsin Department of Natural Resources Wisconsin Beaches site. Data for rain was also obtained, and was taken from the

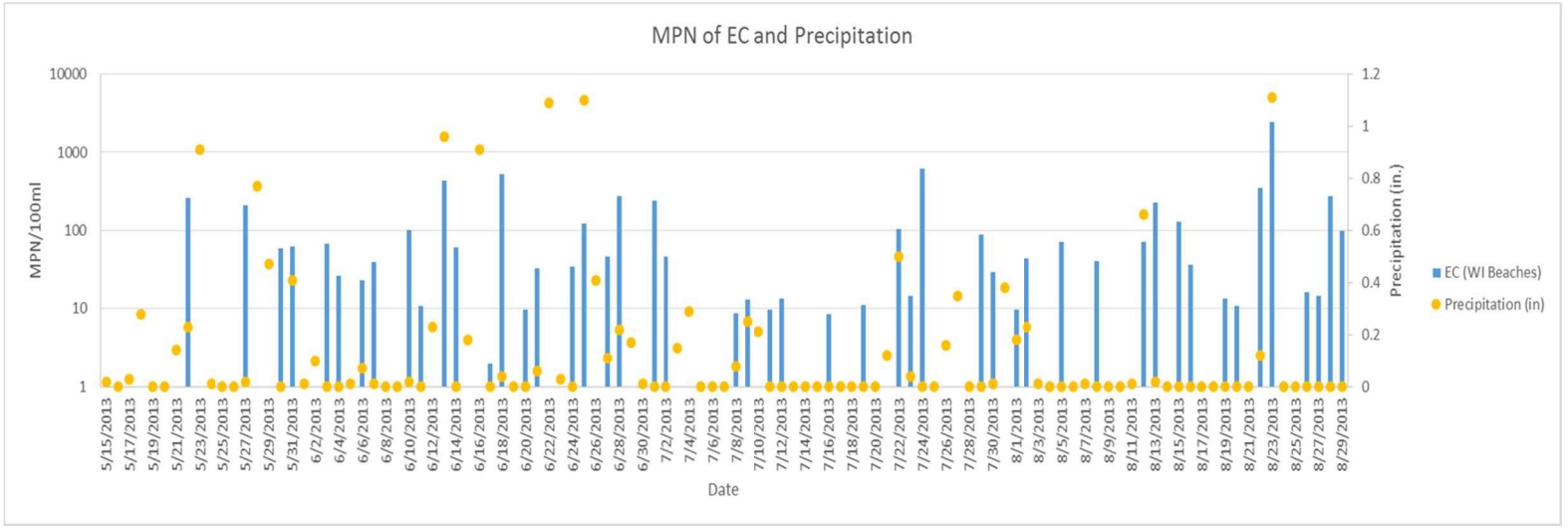
CoCoRaHS network (sponsored by NOAA and the NSF). Analyses were performed to determine the impact of environmental factors on bacterial counts.

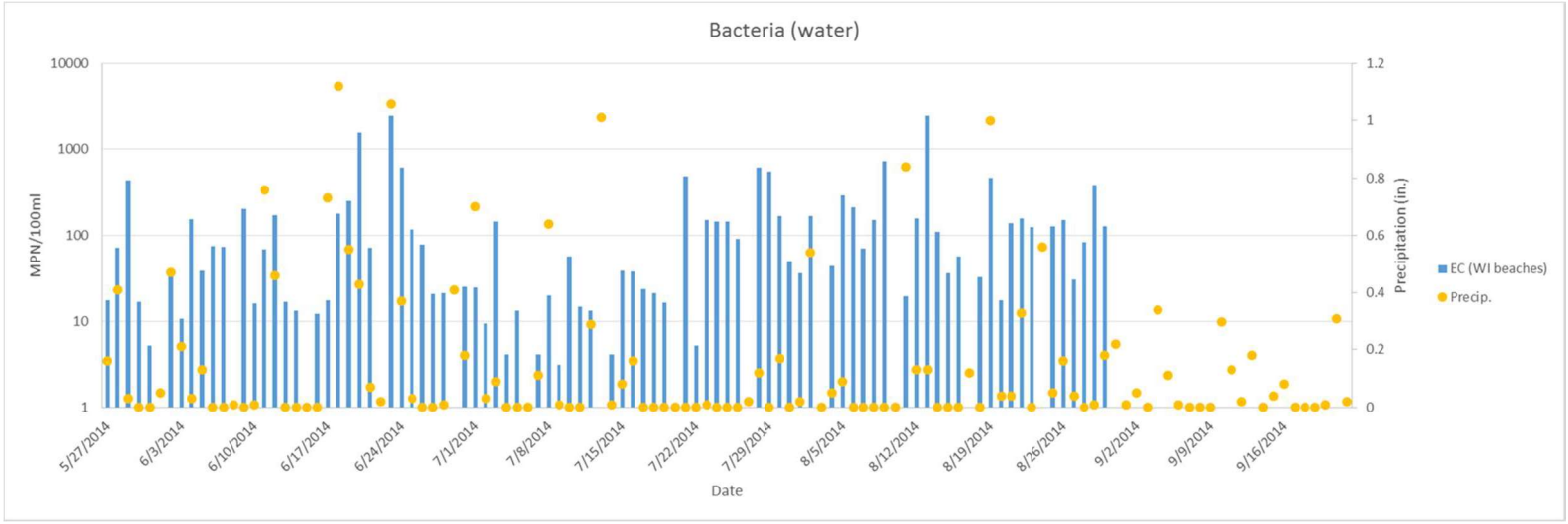
5.2 Results.

Table 7 (below) shows WI beaches EC data from 2008 to 2014.

	2008	2009	2010	2011	2012	2013	2014
mean	151	115	189	147	123	145	193
median	55	36	63	23	39	44	70
max	866	980	1300	1414	1986	2419	2419
st. dev	189	203	313	301	275	352	417

Data was analyzed for each year. Results indicate that the maximum value bacterial count increased in this time period. The mean and median values fluctuated through these years but remained within an order of magnitude of other values.





Figures 18,19 show a comparison between EC levels in water and precipitation

. Table 8 (below) shows correlation coefficients for EC levels, algae, and rain for 2013 and 2014. The EC levels in the swash zone and algae were also analyzed for 2013.

	Rain EC	Algae EC(water)	Algae EC(swash)
2013	0.552	0.4867	0.3032
2014	0.2906	0.2906	

Tables 9, 10(below) show averages for EC and ENT in water and sand samples. Water samples are recorded in MPN/100ml with sand samples being MPN/100g sand

2013	EC Water	ENT Water	EC Swash	ENT Swash	EC 20 ft.	ENT 20 ft.
Average	255	194	184	46	193	21
St. Dev.	397	543	272	48	645	36

2014	EC Water	ENT Water	EC Swash	ENT Swash	EC 10 ft.	ENT 10 ft.	EC 20 ft.	ENT 20 ft.
Average	128	14	261	48	588	128	677	41
St. Dev.	122	10	199	53	1110	380	1269	80

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Tables 11, 12 (below) show average ratios of ENT/EC across all samples in water and sand.

Average ratios for ENT/EC 2013

Water	Swash	20 ft.
0.55	0.68	0.59

Average ratios for ENT/EC 2014

Water	Swash	10 ft.	20 ft.
0.25	0.23	0.25	0.44

5.3 Discussion. One study made an attempt to correlate bacteria counts with various environmental factors. It was found that in contrast to dry sand, daily mean ENT abundance in wet sand was not correlated to tidal range, and only weakly related to air temperature ($r = -0.31$, $p = 0.08$) and previous 48 h precipitation ($r = 0.32$, $p = 0.07$). Temperature of the water sample ($r = 0.45$, $p = 0.01$) and the air at the time of sampling ($r = 0.36$, $p = 0.03$) were also significantly correlated to ENT abundance[51]. These results indicate weak correlations with temperature and precipitation. Data for the years 2008-2014 was also analyzed to see if there was a significant correlation between the date and bacteria levels. Bacteria levels were found to not have a significant correlation with the passing of time.

Results from analysis of Wisconsin Beaches and NOAA data that were calculated were found to be similar. It was found that rain in the 48 hours previous to sampling and EC counts had a weak positive correlation. Figure below shows the correlation coefficients for bacteria levels, rain, and algae. The weak correlations between temperature, precipitation, and bacteria levels shows the complexity of environmental factors and bacterial counts. Many factors influence the survivability and growth of bacteria, of which temperature and moisture are important. However, each factor alone does not determine growth or death of bacterial communities.

Data collected in this study shown in tables 9-12 show the differences in levels between the two species tested in 2013 and 2014. Data show that on average bacteria levels in sand can be much higher that levels in water. The differences in these values being MPN/100g sand and MPN/100ml water meaning they are comparable yet not equivalent. Even though these numbers are not wholly equivalent it remains evident that comparable amounts of sand and

water can harbor very different levels of bacteria. It is also evident that the ratio between these two bacteria can vary. It is likely that different sources can harbor different levels of these bacteria. Environmental factors affecting survivability of the species differently is likely to affect this as well.

6. Conclusions

Through various analyses in this project and elsewhere it has been demonstrated that the issue of indicator bacteria use as a predictor for public health risks is quite complex. Due to these bacteria being present at high levels in the sand and the demonstrated elevation in health risks it is important to consider this environment[52,53]. Laboratory procedure for enumeration of bacteria in sand should be standardized. Evidence presented here shows that for *E. coli* the eluents PBS and DI water may be reasonably comparable, yet the eluent of choice for *Enterococci* is still unclear. Transport of these bacteria in beach environments is also a concern. The interactions between microbes in sand, water, and their sources is obviously complex and bacteria levels are highly variable over time. Future testing methods should consider the role of bacteria in environments nearby waters and further research should be conducted in order to understand their impact on public health.

The impact of other environmental factors such as algae, temperature, and rain are further considerations that should be made in this system. These issues further complicate the issue as these factors can have variable impacts on indicator bacteria as well as pathogenic microbes. It is likely that other factors can influence these bacteria. Due to the large variety of sources and the factors that can affect these forces it can be difficult to determine the cause of

elevated bacteria levels. The links between bacterial sources, indicator bacteria levels, environmental factors, and pathogenicity must be further understood in order to develop methods that can more accurately predict public health risks in beach environments. It is also important to consider economic impacts of these closures so that informed decisions can be made.

Future research on the subject has many areas in which knowledge can be improved. Due to the amount of evidence showing that bacterial communities can adapt and survive for long period of time the local population genetics is of concern. Due to the increased availability of genetic identification techniques it has become much easier to determine the genetics of a population through genetic markers and other characteristics. Further research on the make-up of populations will aid in determining if bacterial presence is due to persistent and adapted populations or recent pollution events which would contain a genetically different microbial community. The ability to differentiate between these two communities could be very useful in the future in order to form a stronger link between test results and public health risks.

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