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IMPACT OF AQUATIC MACROPHYTES ON ESCHERICHIA COLI CONCENTRATIONS AT RECREATIONAL INLAND BEACHES

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ABSTRACT

IMPACT OF AQUATIC MACROPHYTES ON *ESCHERICHIA COLI* CONCENTRATIONS AT RECREATIONAL INLAND BEACHES

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Mary-Cathrine Christina Elaine Leewis

Vilas County, WI is home to more than 1300 inland lakes and recreational beach use is a large contributor to tourism dollars brought into the area. Thus, beach closures can be extremely costly in terms of lost tourism revenue. The indicator organism of fecal contamination used in beach monitoring in Wisconsin is *Escherichia coli*, as high levels of this organism have been correlated with recent fecal contamination events. It has been hypothesized that stands of aquatic macrophytes may harbor high concentrations of *E. coli*. The objective of this project was to assess the relationship between aquatic macrophytes and the persistence of the fecal indicator organism, *E. coli* in beach water using both field studies and laboratory studies. The laboratory study consisted of microcosms containing three different densities of *Sagittaria*, *Myriophyllum* or a plastic plant used in combination with lake water and a strain of environmental *E. coli*. Water from the microcosms was sampled to enumerate the *E. coli* concentrations in each of the microcosms for seven days. The laboratory study found that *E. coli* survival was not dependent on the presence of living plant matter. The field studies observed *E. coli* concentrations at four inland lakes with high tourist activity during the summers of 2005 and 2006. The field studies found that dense mats of aquatic macrophytes have an increased amount of *E. coli* when compared to two, five and ten meters from the mat.

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INTRODUCTION

The relationship between mats of freshwater aquatic plant matter and their ability to harbor water quality indicator bacteria has never been studied in detail. The idea was proposed by Whitman et al. (2003) in their paper on the relationship of the filamentous green algae *Cladophora* and the indicator organisms *Escherichia coli* (*E. coli*) and Entercocci in Lake Michigan, but work has yet to be conducted regarding the relationship of aquatic macrophytes and their interactions with *E. coli* and the more pathogenic bacteria for which *E. coli* is an indicator.

Mats of aquatic macrophytes are ubiquitous in water systems; these plants can be found wherever there is space and nutrients readily available. Macrophyte mats occur when several plants clump together to form a dense net of plant matter. This normally occurs near the shoreline in freshwater systems, although mats can also be found up-rooted and in deeper areas of the lake, depending on the wave action in the lake.

It has been hypothesized that mats of aquatic macrophytes provide an environment more suitable for bacterial growth than open water, due to properties associated with a mat of aquatic macrophytes. The majority of plants secrete amino acids and polysaccharides which can be used by associated bacteria as substrates for growth (Marsollier et al. 2004). Macrophyte mats also provide shelter from the sun and ultraviolet (UV) radiation that is normally damaging to bacteria. These mats are able to provide a stable pH, temperature and are resistant to desiccation when washed onto shore.

Fecal microorganisms found in water normally grow in the intestines of warm blooded animals and are excreted from the body in the form of feces (Madigan et al.

2002). Pollution of the water by these feces, called a fecal contamination event, may then occur. A fecal contamination event could be caused by agricultural runoff, faulty on site sewage treatment systems, broken sewage piping or partially treated sewage, or direct human fecal contamination (Kleinheinz et al. 2003). If the contamination is not identified and eliminated, a new host may consume the untreated water allowing a pathogen to colonize the intestines causing severe illness. Illnesses that can be associated with the consumption of contaminated water include gastroenteritis, dermatitis, and meningoencephalitis (Madigan et al. 2002). Two commonly used indicator organisms are *E. coli* and Enterococci. Both of these organisms show a strong relationship to the previously mentioned gastrointestinal diseases (US EPA, 1986). Enterococci are more effective in detecting contamination events in marine habitats. In freshwater systems, *E. coli* has been demonstrated to be the most specific indicator organism for human fecal contamination events (US EPA, 1986).

The current indicator organism for fecal contamination events in all Great Lakes States is *E. coli* (US EPA, 1986). For freshwater recreational systems, the current US EPA recommended limits are 235 *E. coli* per 100 mL of water (US EPA, 1976). Local agencies can increase or decrease this number as their own systems demand. In Wisconsin, water samples for beach monitoring are taken at a depth of 24 inches (60.96 centimeters) and in Michigan the water samples are taken at a depth of 30-60 inches (76.20-152.40 centimeters), depending on the local preferences (Kleinheinz et al. 2003).

The relationship between mats of the green filamentous algae *Cladophora* and *E. coli* is the subject of recent and developing research (Kleinheinz and Englebert 2005, Whitman et al. 2003, and Byappanahalli et al. 2003). These experiments have shown that

there are higher counts of *E. coli* in the mats of *Cladophora* than in the surrounding water. *Cladophora* is thought to promote bacterial growth by providing shelter from the sun, increased nutrients, and an environment that is warm with stable moisture.

The overall objective of this study was to assess and gain an understanding of the relationship between stands of aquatic macrophytes and the fecal indicator organism *E. coli.* These mats of macrophytes are normally dense enough to provide shelter for bacteria from the sun. Plants also naturally exude nutrients, which benefit bacterial growth. Finally they provide an environment that is less variable than open water with regard to temperature and pH.

This research had two main objectives. The first objective was to determine the relationship between *E. coli* and macrophytes mats in a northern freshwater lake environment (Chapter 2). The second objective of this research was to determine the survivability of an environmental strain of *E. coli* in a laboratory microcosm (Chapter 3).

CHAPTER 1
LITERATURE REVIEW

1.1 Beach Water Management

Tourism is a large source of income for many Midwestern states. The tourism industry in Wisconsin alone is worth $12 billion dollars annually (Kleinheinz and Englebert 2005). The summer months especially bring thousands of people to beaches seeking relief from the heat. If those beaches are closed due to microbial contamination, it is unlikely that tourists will return in the future. This necessitates finding an indicator organism that is a reliable and accurate indicator of fecal contamination events.

Recreational water systems are under constant threat of contamination. In rural areas, faulty septic systems can cause run off of partially treated or raw sewage. Run off from agricultural operations is also a potential problem (Griffin et al. 2001, Kleinheinz et al. 2003). In urban areas, broken sewage pipes or sewage overflow along with storm water run off are potential problems. In 1998, 729 beaches nationwide were closed for at least one day, resulting in more than 7000 days of closure (Rose and Grimes 2001). The need for routine monitoring of recreational water systems was especially apparent on June 11, 1998, when two children died as a result of playing in a water theme park that had been contaminated with a deadly strain of *E. coli* (Rose and Grimes 2001). The need for monitoring water for fecal contamination highlighted in September and October 2006 when at least 190 people were sickened and 3 people killed from eating spinach contaminated with the 0157:H7 pathogenic *E. coli* (Associated Press October 6, 2006). The spinach contamination was linked to irrigation water.

Detection of fecal indicator organisms, especially *E. coli*, has been used to monitor drinking water for over 100 years (Hanninen et al. 2003). In 1986, the US Environmental Protection Agency (US EPA) described water quality criteria for recreational water systems (US EPA 1986). The 1986 US EPA release was in conjunction with and superior to the previously studied Quality Criteria for Water (QCW) (US EPA 1976). The study showed that both *E. coli* and Enterococci are acceptable indicator organisms of fecal contamination events in recreational water systems. The US EPA described a "recreational water system" as a body of water or area of water where swimming and other recreation activities such as water skiing take place. The EPA also recommended sampling plans for each beach or body of water based on the frequency of usage. It was recommended that the most rigorous monitoring be done at designated swimming beaches where a life guard, parking lot, and other facilities are provided. The EPA limits for full body contact bathing in recreational waters for *E. coli* is 235 colony forming unites (CFU) per 100 mL and 61 CFU per mL for Enterococci. These criteria were designed to limit the risk of disease in humans to approximately 7 illnesses per 1000 swimmers. The EPA also reported that for marine systems Enterococci is the only reliable fecal coliform indicator, and for freshwater systems, both *E. coli* and Enterococci could be used as fecal coliform indicators. The term "coliform bacteria" describes a group of gram-negative, facultative anaerobic, rod-shaped bacteria commonly found in intestinal tracts of animals (Madigan et al. 2002). Because the role of *E. coli* as an indicator organism has been debated since it was first suggested as an indicator organism, many independent studies have been conducted to determine the effectiveness of *E. coli* as an indicator organism for viruses and pathogens associated with fecal contamination

events (Edberg 2000, Allwood et al. 2003). In a study completed by Allwood et al. (2003), other indicator organisms were tested, and *E. coli* was determined to have the highest specificity for enteric viral pathogens.

A "fecal contamination event" is an event that contaminates recreational water systems with pathogens normally associated with feces. These pathogenic organisms could be derived from either animal or human sources (Kleinheinz et al. 2003). Sources are varied and could be due to faulty septic systems, rain events causing run off from agricultural systems or contaminated pavement, animals in the water, or human related. The pathogenic organisms that are associated with fecal contamination events are wide ranging, but are normally found in the gastrointestinal tract of warm blooded animals. Examples of these pathogens are *Noroviruses, Salmonella, Shigella, Campylobacter, Giardia,* and *Cryptosporidium* (Kleinheinz and Englebert 2005). These pathogens can cause severe illnesses and gastrointestinal disorders which are particularly of concern for children and immunocompromised individuals who are most likely to suffer from even a small exposure to pathogens.

1.2 Methods of Beach Water Analysis.

A variety of methods are used to test for indicator organisms in recreational water systems. Two common methods are membrane filtration and defined substrate testing (Edberg 2000, Kleinheinz et al. 2003). Membrane filtration involves passing at least 100 mL of a water sample through a 0.45 µm sterile membrane filter (Madigan et al. 2002). This filter is then placed on the surface of a plate of highly differential and selective media. Eosin-methylene blue (EMB) and Modified membrane-Thermotolerant

Escherichia coli Agar (Modified mTEC) are two commonly used media for *E. coli* detection. These media allow for easy identification of *E. coli*; *E. coli* appears as a dark centered colony with a green metallic sheen on EMB and a purple colony on Modified mTEC (US EPA 2002). After incubation of the plate, the number of colonies is then counted to determine the number of *E. coli* in the original water sample. Defined substrate tests are much easier, less time consuming, and have more rapid results (18-24 hours) than the membrane filtration system (Kleinheinz et al. 2003). Defined substrate tests, such as the Colisure® and Quanti-tray system (IDEXX, Inc), also generally utilize 100 mL of the water of interest. The technology works by monitoring one of the defining reactions in coliform bacteria. The indicator, chlorophenol red (CPRG), binds to β–D-galactopyronoside and changes colour (yellow to red) after β–D-galactopyronoside has been acted upon by β-galactosidase which is produced by coliform bacteria (IDEXX, Inc). The colour change of yellow to red indicates that the test was positive and fecal coliforms were present in the sample. If there is no colour change, then β-galactosidase was not present to act on β–D-galactopyronoside and there were no coliforms present. To detect the presence of *E. coli* in the same test, both 4-methyl-umbeliferone (MUG) and CPRG are present in the medium that will be mixed with the water sample. The MUG reagent will bind with β–D-galactopyronoside, and if the β–D-galactopyronoside is acted upon by β-glucuronidase, the MUG will fluoresce under an ultraviolet light. If the sample fluoresces under an ultraviolet light, then *E. coli* was present in the test sample. The reaction that utilizes the enzyme β-glucuronidase is biochemically specific for *E. coli*, meaning that if there was no fluorescence then no *E. coli* was present. Although environmental strains of *E. coli* can vary genetically, the enzyme system of β-

glucuronidase is present in more than 95% of isolates. This method provides a cost effective method to easily monitor the *E. coli* levels in a water system and also requires little technical microbiological experience.

1.3 Primary Host Habitat of *E. coli*.

The primary host habitat of *E. coli* is the lower enteric tract of warm blooded animals (Winfield and Groismand 2003). The strains of *E. coli* present depend greatly on the numbers ingested, the host immune system, and the other resident microflora. The most common strains of *E. coli* are commensal organisms that are highly adapted to the conditions of the gut. The genome of *E. coli* encodes for proteins that are resistant to acidic pH and allows the bacteria to grow on lactose. The environment of the gastrointestinal tract is conducive to bacterial growth providing warm, constant temperatures of approximately 37 ^{o}C, anaerobic conditions, and high concentrations of nutrients, sugars, and amino acids. Due to these favorable conditions in the host environment, *E. coli* has a doubling time estimated at 12 to 48 hours. The cycle time of a strain of *E. coli* through a human host has been estimated to vary between 26 hours and 66 years. This, again, demonstrates the advantageous conditions of the gut for adapted bacteria (Winfield and Groismand 2003).

1.4 Secondary Non-Host *E. coli* Survival

The secondary habitat of *E. coli* consists of water, soil and sediments. A secondary habitat is a complex environment consisting of highly fluctuating temperature and light levels, predation, poor nutrient availability, water salinity, and other

microorganisms (Craig et al. 2004, Savageau 1983, Winfield and Groisman 2003). In this secondary habitat, *E. coli* has demonstrated a net negative growth rate, with a half life of approximately one day. In open water taken from natural systems, *E. coli* degrades to undetectable levels at a wide array of temperatures in a remarkably short period of six days (Bogosian 1996, Brettar and Hofle 1992). The secondary habitat is primarily aerobic, with pockets of anaerobic environments in sediment and soil. Concentrations of *E. coli* in the environment can vary depending on where sampling takes place (Kleinheinz et al. 2006, see Appendix A). In pristine waters, *E. coli* concentrations may be less than 1-10 CFU per mL. This concentration increases to approximately 10-100 CFU per mL in watersheds of un-grazed pastures and again increases to 100-1000 CFU per mL in grazed pastures and feedlots. In heavily polluted waters, *E. coli* values can exceed 10^4 CFU per mL (Savageau 1983). The concentrations of *E. coli* in sediments are highly related to those in the overlying water, but approximately 100-1000 fold greater than water. In a study completed by Brettar and Hofle (1992) that examined the survivability of *E. coli* in natural water systems, *E. coli* was added to two lake mesocosms in a manner similar to a hypothetical leakage of a production fermenter. Organic nutrients were also added to one lake mesocosm. Brettar and Hofle monitored bacterial decline in the water by immunofluorescence and culture techniques, and found that after four days of growth, 81% of bacterial cells were attached to floating particles. By the thirteenth day, cells were still detectable by immunoflourescence. The addition of organic nutrients displayed no initial effect, although after a week, bacteria in this mesocosm showed greater survivability. The greater survivability associated with bacterial attachment was postulated to have occurred

for two principle reasons: reduced predation of *E. coli* and increased availability of nutrients. The reduced predation was observed because flagellated protozoa were unable to graze on bacteria attached to particles. A succession of grazers was also seen over the course of the study, first flagellates, followed by rotifers, and finally macrozooplankton, mainly *Daphnia* species. The particles that *E. coli* attached to also functioned as sites of higher nutrient concentration. A greater survivability of *E. coli* in the lake mesocosm with added organic nutrients was thought to be more correlated with the particle production by organic nutrients than the actual nutrients. Bacteria populations associated with particles were also able to increase, although in small numbers. This again, demonstrates that bacteria had higher survivability when attached to larger particles. *E. coli* levels were undetectable in water 16 days after release. It has been postulated that non-host environments can provide *E. coil* with sufficient nutrients to sustain a dividing population (Edberg 2000). Many studies have been devoted to understanding the survival of *E. coli* in non-host water environments.

Brettar and Hofle (1992) and Craig et al. (2004) used laboratory microcosms and mescocosms to determine *E. coli* decline after contamination events. Both studies found that after an initial peak in bacteria, *E. coli* numbers quickly declined to almost undetectable levels in open water. The percentage of cells attached to particles was approximately 81% in the Brettar and Hofle study, and in both studies the only detectable *E. coli* was found attached after six days. These data mirror a study done in by Milne et al. (1991) who determined that the addition of nutrients and suspended solutes to seawater greatly increased survival times. *E. coli* has been postulated to survive longer in water that has solutes, because the solutes allow for increased nutrients, biofilm

formation, and protection from predation. Reduced predation may result from flagellated protozoa being unable to graze on the bacteria attached to particles and substrates. In water that had been filtered and sterilized, *E. coli* had much higher rates of survival, again pointing to predation as a possible factor of bacterial decrease (Bogosian et al. 1996, Banning et al. 2002). Particles are also potential sources of nutrients for environmental *E. coli* (Brettar and Hofle 1992). Biofilm formation would also allow for greater survival of bacteria by providing areas of protection, and a mechanism to trap nutrients for bacteria (Costerton et al. 1995, Martinko et al. 2002). It has been observed that bacteria associated with a biofilm have a doubling time approximately twice that of non-attached, or planktonic, bacteria in an aquatic environment (Costerton et al. 1995).

Water temperature has also been linked with bacterial survival (Bogosian et al. 1996, Brettar and Hofle 1992, and Smith et al. 1994). *E. coli* levels can be unexpectedly high in waters as cold as 4 ^{o}C (Sampson et al. 2006). In glass laboratory microcosms filled with lake water, it was found that *E. coli* had a lower rate of decline in water at 4 ^{o}C than water at 10, 14 and 25 ^{o}C (Sampson et al. 2006). In a laboratory microcosm with lake water and sand, survival rates of *E. coli* increased at each of the experimental temperatures, with water at 4 ^{o}C having the slowest rate of decline compared to water at 10, 14 and 25 ^{o}C. These findings indicate that water temperature can be a factor in beach water management.

1.5 *Cladophora* and *E. coli*.

The green filamentous algae *Cladophora* has recently reemerged as a nuisance in the Great Lakes. This unsightly and malodorous alga clumps to form mats that are

currently being studied for their ability to sustain the indicator organism *E. coli* as well as more pathogenic bacteria. Whitman et al. (2003) and Byappanahalli et al. (2003) determined that these mats of *Cladophora* may be an important source of environmental *E. coli*. The secondary habitat of open water leads to the rapid decline of bacterial survivability, yet Whitman et al. (2003) found evidence that *Cladophora* harbors both *E. coli* and Enterococci for extended lengths of time (over six months). Mats of *Cladophora* provide an environment extremely favorable for bacterial survival (Kleinheinz and Englebert 2005). Decaying mats of this alga both on shore and in the water provide nutrients, protection from the sun, and a stable temperature and pH for bacteria. It has been hypothesized that the environment within a mat of *Cladophora* is favorable enough that *E. coli* proliferate in these mats. This theory is currently being researched at the University of Wisconsin Oshkosh, University of Minnesota, and the United States Geological Survey (USGS) using genetic analysis to determine the clonality of *E. coli* in the mats, with the assumption that an increased clonality, or genetic relatedness, will correlate with the proliferation of bacteria (Englebert and Kleinheinz, unpublished data). Experiments comparing the survivability of pathogens in *Cladophora* and *E. coli* in *Cladophora* are also currently being performed (Englebert, unpublished data). Preliminary results show that *E. coli* persists much longer within the mat of *Cladophora* than do most of the pathogens for which *E. coli* acts as an indicator.

1.6 Aquatic Macrophytes as a Secondary Environment for *E. coli*.

Submerged aquatic macrophytes are ubiquitous in aquatic environments, and can frequently be noticed near beach and swimming areas. It has been repeatedly observed

that plants, both aquatic and terrestrial, are capable of exuding nutrients such as amino acids and polysaccharides which bacteria can use as substrates for growth (Baker and Orr 1986, Beattie and Lindow 1994, Eriksson and Weisner 1999, Kinkel et al. 2000, Marsollier et al. 2004, Morris et al. 1998, Muller et al. 2001). In addition to providing nutrients, aquatic macrophytes are able to provide a solid surface that aids in the formation of bacterial biofilms, which can increase bacterial survival. Dense stands of macrophytes can also provide bacteria with shade from the sun and UV light, decreased water flow and decreased predation. Bacteria that inhabit the surfaces of plants are termed epiphytic bacteria. The relationship between plants and bacteria can be highly variable, with varying amounts of bacteria on different leaves of the same plant and even varying concentrations of bacteria on the same leaf. Baker and Orr (1986) determined that submerged leaves of aquatic macrophytes had at least a 100-fold more bacteria than aerial leaves that were no longer submerged. Eriksson and Weisner (1999) noted that in shallow aquatic environments, dense stands of submerged macrophytes are capable of providing bacteria with an anaerobic environment which is favorable for *E. coli* growth. In addition to providing a habitat suitable for growth in the water environment, the sediment underneath rooted stands of aquatic macrophytes also provides a more favorable environment for bacterial growth than sediments that have no associated macrophytes (Eriksson and Weisner 1999). It is possible that conditions provided by mats of aquatic macrophytes can decrease the effects of the harsh secondary environment on the indicator *E. coli*. Due to wind or wave action, portions of aquatic macrophytes can detach from the stand or mat and wash up on shore. These clumps of aquatic macrophytes are thought to provide bacteria, in particular *E. coli*, with the same favorable

environment found in the main body of a stand of macrophytes in a manner similar to that of *Cladophora*. If these decaying clumps of aquatic macrophytes were to wash up onto a beach area and contained *E. coli* or other bacteria, this would potentially influence any beach water management that takes place at that lake.

Similarities can be noted between mats of *Cladophora* and mats of aquatic macrophytes. Both types of mats provide a favorable secondary habitat consisting of increased nutrients, protection from the sun, an attachment point for growth and a stable temperature. If an increased amount of the indicator organism *E. coli* is found within and near macrophyte mats, this could lead to beach closures and the loss of tourism revenue. If increased amounts of pathogens are also found in association with macrophyte mats, these mats would become an area with increased human health risk. The objective of these studies was to determine the effect of mats of aquatic macrophytes on survivability of the indicator organism (*E. coli*) in both natural and artificial settings.

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CHAPTER 2
IMPACT OF AQUATIC MACROPHYTES ON *ESCHERICHIA COLI* CONCENTRATION IN NORTHERN WISCONSIN LAKES

ABSTRACT

Vilas County, WI is home to more than 1300 inland lakes where recreational beach use is a large contributor to tourism dollars brought into the area. Thus, beach closures can be extremely costly in terms of lost tourism revenue. The indicator organism used in beach monitoring in Wisconsin is *Escherichia coli (E. coli)*, as high levels of this organism have been correlated with recent fecal contamination events. These events may contain other, more pathogenic bacteria that pose a risk to human health. Another serious problem at many beaches in the state is stands of aquatic macrophytes (e.g. Eurasian water-milfoil). It has been recently hypothesized that these stands may harbor high concentrations of *E. coli*, in a similar manner to the green alga *Cladophora*, and allow for the proliferation of these indicator bacteria. Four inland lakes with high tourist activity were selected each summer during 2005 and 2006. A defined substrate test (Colisure®, IDEXX, Inc.) was used to count *E. coli* within the macrophyte mat and at different distances from the mat. The relative amount of aquatic macrophytes was also determined at each location via a subjective scale (low, medium, high). This study found that dense stands of aquatic macrophytes have more *E. coli* then two, five and ten meter distances from the mat.

INTRODUCTION

The summer months bring large numbers of swimmers to beaches across Wisconsin, seeking relief from the heat. As such, recreational beaches are a large part of tourism in many areas of Wisconsin, especially in the northern parts of the state. Therefore, beach closures can be very costly in terms of lost tourism revenue for the area as many people depend on the tourism income from the summer as their income for the entire year. The indicator organism for fecal contamination events most often used by local governments is *Escherichia coli* (*E. coli)* because high levels of this organism may indicate a recent contamination event or spill that may contain other, more pathogenic, bacteria (e.g., *Shigella*, *Salmonella*), viruses (e.g., Norovirus), and/or protozoans (e.g., *Giardia Cryptosporidium*) (Medical College of Wisconsin 7/31/02, Byappanahalli et al. 2003, Kinzelman et al. 2003). The Environmental Protection Agency (EPA) recommends that water samples should not exceed a single sample limit of 235 *E. coli* per 100 mL of water and/or a 5-day geometric mean of 126 *E. coli* per 100 mL of water (US EPA,1986).

Additional problems at many beaches in the state are thick stands or mats of native aquatic macrophytes (e.g. *Potamogeton* spp., *Elodea canadensis*), and some invasive species (e.g. *Myriophyllum spicatum*). Unfortunately, mats of these invasives are becoming increasingly common in waters across the state, and have become synonymous with recreational water issues. Macrophytes, both invasive and native species, grow near shorelines of lakes, and commonly occur in dense stands or mats. Invasive species of macrophytes are often transported to new lakes via recreational activities such as boating.

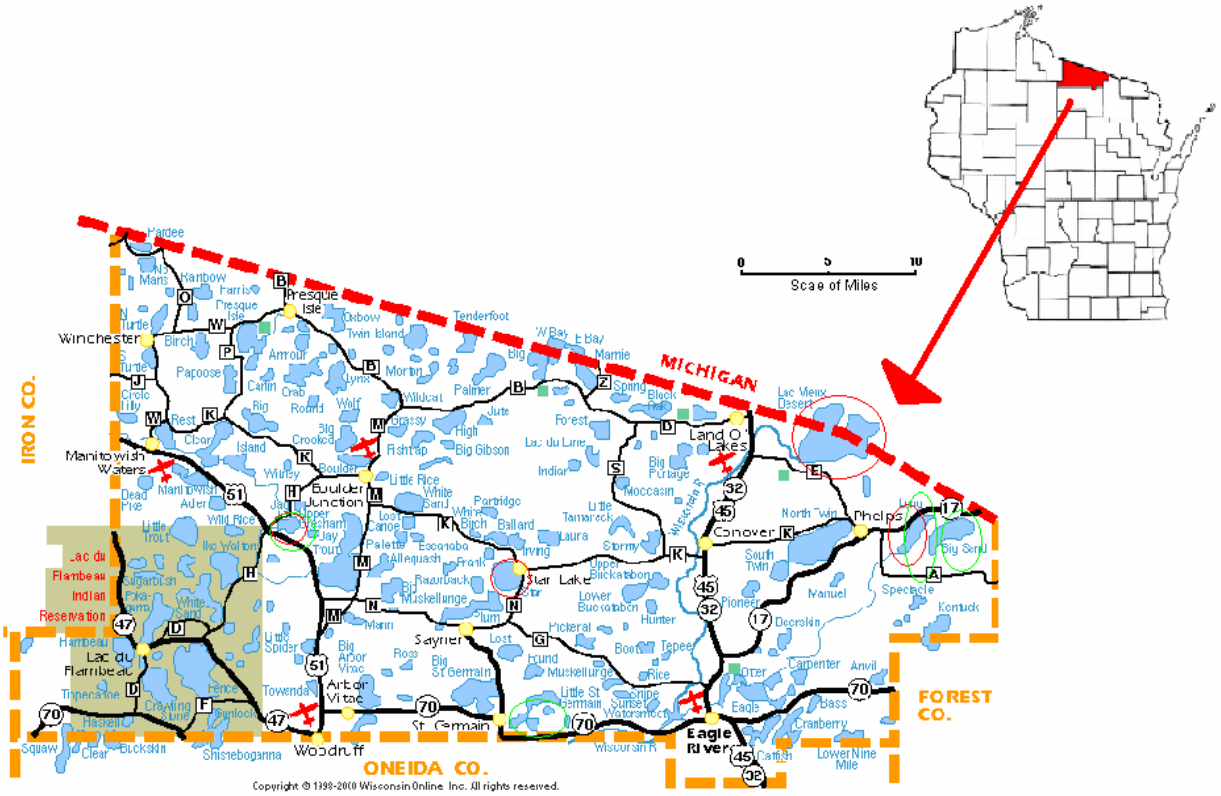
Recently, a separate but related body of work has emerged suggesting a link between the nuisance alga *Cladophora* and elevated levels of *E. coli* (Byappanahalli et al. 2003, Whitman et al. 2003). However, no published study draws a direct link between mats of *Cladophora* and beach closures at this time. It is believed that the mats of *Cladophora* offer a stable environment for bacteria, shielding them from such things as UV radiation, changes in pH, desiccation, and nutrient depletion (Byappanahalli et al. 2003, Whitman et al. 2003). Similarities can be noted between mats of *Cladophora* and mats of aquatic macrophytes (Baker and Orr 1986). Both mats provide a favorable secondary habitat consisting of increased nutrients, protection from the sun, an attachment point for growth and a stable temperature. An increased amount of the indicator organism *E. coli* within and near macrophyte mats could challenge the paradigm that *E. coli* is indicative of a recent fecal contamination event.

MATERIALS AND METHODS

Field Season 1, May-August 2005:

Four inland lakes in Vilas County, Wisconsin identified to have large macrophyte mats were sampled for *E. coli* concentrations over a ten-week period in the summer of 2005. All sample analyses were conducted at the University of Wisconsin-Oshkosh Water Testing Lab at the Vilas County Health Department, Eagle River, WI. Lakes sampled included Upper Gresham, Little St. Germain, Big Sand and Long Lake (Table 2.1 for plant species, Figure 2.1 for lake location). At each beach 10 water samples were collected in sterile, disposable 100 mL polystyrene bottles (IDEXX, Inc.). All samples were collected in duplicate and were collected as follows: two samples from within the macrophyte mat, two samples five meters from the mat (keeping beach to the left), two

samples ten meters from the mat (keeping beach to the left), two samples five meters from the mat (keeping beach to the right), and two samples ten meters from the mat (keeping beach to the right). Once collected, the samples remained on ice until analysis, and were processed within several hours of collection. In the lab, one packet of Colisure® (IDEXX, Inc.) and two drops of anti-foam agent were added to each sample and shaken vigorously until the sample and reagent were completely mixed. The contents of each bottle were then poured into a correspondingly marked Quanti-tray. The Quanti-trays were then sealed and incubated at 35 ^{o}C for 24 hours (Figure 2.2) (American Public Health Association 1998a).

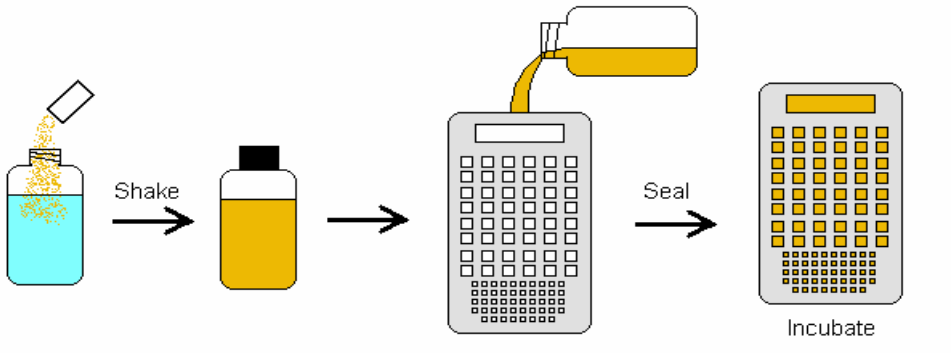


Map provided courtesy of Wisconline® www.wisconline.com. Used by permission.

Figure 2.1: Map showing location of Vilas County, Wisconsin and a detail map of the county showing the vast water resources within the county. The lakes sampled in 2005 are circled in green, and the lakes sampled in 2006 are circled in red. (Map courtesy of <http://www.wisconline.com/counties/vilas/map.html>)

Table 2.1: Dominant plant species found at each of the lakes sampled during the 2005 field season.

Lake (2005)	Dominant Species	Common Name
Upper Gresham	*Myriophyllum* spp.	Water Milfoil
	Eleocharis spp.	Spike Rush
	Potamogeton spp.	Pond Weed
	Nuphar advena	Yellow Water Lilly
	C.demersum	Coons tail
	Elodea canadensis	Canadian Waterweed
	Nymphaea odorata	American White Water Lilly
	Scirpus spp.	Bull rush
Little St. Germain	*Myriophyllum* spp.	Water Milfoil
	Potamogeton spp.	Pond Weed
	Elodea canadensis	Canadian Waterweed
	Ceratophyllum spp.	Coons tail
Big Sand	*Myriophyllum* spp.	Water Milfoil
	Potamogeton spp.	Pond Weed
	Elodea canadensis	Canadian Waterweed
Long Lake	*Potamogeton* spp.	Pond Weed
	Vallisneria americana	Water Celery
	Sparganium spp.	Burr Reed
	Typha spp.	Cattails
	Myriophyllum spp.	Water Milfoil
	Carex lacustris	Lake sedge
	Heteranthera dubia	Water Star Grass
	Juncus spp.	Common rush
	Ceratophyllum spp.	Coons tail



A **B** **C** **D**

Figure 2.2: Example of a defined substrate test (Colisure and/or Enterolert). **A.** Add reagent to 100 mL water sample. **B.** Shake sample to completely dissolve reagent. **C.** Pour the reagent/sample directly into the Quanti-Tray/2000. **D.** Seal Quanti-Tray with sealer and incubate for 24 hours at the required temperature (Colisure & Colilert=35 ^{o}C, Enterolert=41 ^{o}C).

After 24 hours, the samples were enumerated by counting the number of wells in the Quanti-trays (Quantitray 2000) that had changed to a magenta color, indicating coliform bacterial presence. The trays were also exposed to a UV light to determine the number of wells that fluoresced, indicating *E. coli* presence. These well counts were compared to a most probable number (MPN) table to determine the microbial concentration of each sample (American Public Health Association 1998a). Samples were all processed in the Eagle River Department of Public Health, a Wisconsin State Certified Laboratory with a Quality Assurance Plan on file with the Wisconsin State Environmental Protection Agency (License Number 105-455).

Prior to analyzing the data, duplicate samples taken from the same point were averaged for each distance class. Although the 5- and 10-m classes also had 2 "replicates" (one on either side of the mat), mean values were used in the analysis. These data were analyzed in two ways: (1) Analysis of Variance (ANOVA) with distance as a fixed factor and sample date treated as a block; and (2) Friedman's Rank Sum Test, which tested whether *E. coli* counts were consistently ranked mat > 5 m >10 m across the 10 weeks of sampling. All analyses were performed using SPSS, Statistical Software Package for Social Sciences Version 13, 2004 (SPSS Inc, Chicago Illinois).

Field Season 2 (May-August 2006):

Again, four inland lakes were chosen in Vilas County, Wisconsin and were sampled for *E. coli* concentrations over a ten-week period during the summer of 2006. Lakes sampled included Upper Gresham, Star Lake, Lac Vieux Desert and Long Lake (Table 2.2 for plant species listing, Figure 2.1 for lake location). Big Sand Lake was not

utilized in 2006 due to the high variability of the mat in 2005 and Little Saint Germain Lake was not used in 2006 because the Little Saint Germain Lake Association had used herbicide to decrease the macrophyte populations for the summer season of 2006. Star Lake and Lac Vieux Desert were chosen due to the proximity of a macrophyte mat to a beach area.

Table 2.2: Dominant plant species found at each of the lakes sampled during the 2006 field season.

Lake (2006)	Dominant Species	Common Name
Upper Gresham	*Myriophyllum spp.*	Water Milfoil
	Eleocharis spp.	Spike Rush
	Potamogeton spp.	Pond Weed
	Nuphar advena	Yellow Water Lilly
	Ceratophyllum spp.	Coonstail
	Elodea canadensis	Canadian Waterweed
	Nymphaea odorata	American White Water Lilly
	Schoenoplectus spp.	Bull rush
Star Lake	*Eleocharis spp.*	Spike Rush
	Myriophyllum spp.	Water Milfoil
Lac Vieux Desert	*Eleocharis spp.*	Spike Rush
	Myriophyllum spp.	Water Milfoil
	Schoenoplectus spp.	Bull rush
Long Lake	*Potamogeton spp.*	Pond Weed
	Vallisneria americana	Water Celery
	Sparganium spp.	Burr Reed
	Typha spp.	Cattails
	Myriophyllum spp.	Water Milfoil
	Carex lacustris	Lake sedge
	Heteranthera dubia	Water Star Grass
	Juncus spp.	Common rush
	Ceratophyllum spp.	Coonstail
	Schoenoplectus spp.	Bull rush

At each beach, 12 water samples were collected in sterile, disposable 100 mL polystyrene bottles (IDEXX, Inc.). The sample size increased from 2005 due to increasing the number of water samples taken from inside the mat. Also, the ten meters from the mat collection point was eliminated and a sample point at two meters from the

mat added. The change in sample size and sample collection location was a result of the data collected in 2005 which indicated that there was a significant difference between *E. coli* counts in the mat and five-meter point, but no significant difference between five and ten meters. All samples were collected in duplicate and were collected as follows: four samples from within the macrophyte mat, two samples two meters from the mat (keeping beach to the left), two samples five meters from the mat (keeping beach to the left), two samples two meters from the mat (keeping beach to the right), and two samples five meters from the mat (keeping beach to the right) (Figure 2.3). In addition, two standardized samples of submerged leaf matter within the mat were taken to determine the presence of attached (epiphytic) coliform bacteria. These samples were shaken for one minute to detach any epiphytic bacteria and processed using Colisure® and the Quanti-tray method (IDEXX, Inc.) (Figure 2.2). The density of macrophytes within the mat was recorded at the beginning and end of the summer (Table 2.3). The temperature, pH, and dissolved oxygen of the macrophyte mat were recorded at each lake using a Corning Meter (New York).

Table 2.3: Density of the macrophyte mat at each lake at the beginning (May) and end (August) of the summer sampling season 2006, measured subjectively. The densities ranged from zero with no macrophytes to four as a very dense macrophyte mat.

Lake	Density May 2006	Density August 2006
Upper Gresham	3	4
Star Lake	1	1
Lac Vieux Desert	1	2
Long Lake	3	4

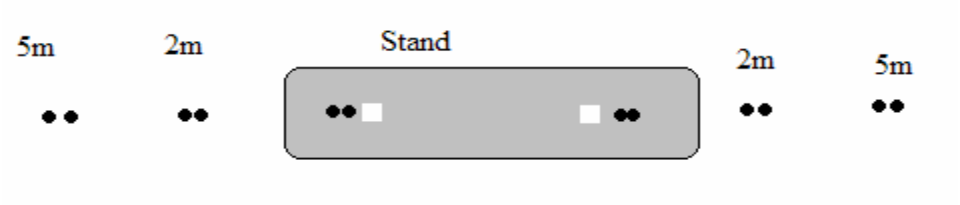


Figure 2.3: Design of the sampling scheme for Summer 2006. The grey box area represents the mat, black dots represent the sampling sites, and the white squares represent where plant matter was sampled to determine the attached coliform bacteria.

All sample protocols conducted during the 2006 sampling season were the same as the procedures followed in 2005.

After six weeks of sampling, it was determined that it would be possible to measure Enterococci in a manner similar to *E. coli*. The amount of available reagent allowed for four weeks of Enterococci monitoring in the same pattern as the *E. coli*, but in single samples, not in duplicate. The samples included five meters from the mat on both the left and right side, two meters from the mat on both the left and right side, and two samples from within the mat at either end of the mat. Also, two destructive leaf samples were taken to compare the epiphytic Enterococci to the planktonic Enterococci. The samples were processed using Enterolert, and the Quanti-tray method was used for determination of the most probable number of Enterococci (IDEXX, Inc)

Data were initially analyzed using a repeated measures ANOVA to compare the *E. coli* MPN among distance classes with repeat measurements taken weekly (10 total) for the same point. All four lakes were also included in this model as blocks. Duplicate samples from each sampling point were not independent, so an average value was computed. The repeated measures ANOVA turned out to have low power due to small sample sizes and high variability in *E. coli* counts over time and among lakes, so a

Friedman Rank test was used to further analyze the data. Because this test ranks the *E. coli* counts for mat, 5 m, and 10 m for a particular date, it is not sensitive to the variability problems; the test measures the consistency of ranking over the whole season. Data was analyzed using SPSS (Statistical Software Package for Social Sciences) Version 13, 2004 (SPSS Inc, Chicago Illinois).

RESULTS

Field Season 1 (2005):

Overall, *E. coli* concentrations were higher in samples collected from macrophyte mats, compared with other sample points of 5 and 10 meters from the mat (Figure 2.4 and Tables 2.4 and 2.5) (Appendix B for 2005 raw data). One-way ANOVA, with distances as a fixed factor and blocked by day was used to analyze the data collected in 2005. Sampling dates were assumed to be far enough apart to be considered somewhat independent. If the one-way ANOVA was found to be significant, then a Tukey's HSD test was run as a post-hoc test. *E. coli* counts among the three distance classes at Upper Gresham Lake was found to be marginally non-significant ($F=3.317$; d.f.=2, 18; $p=0.059$). *E. coli* counts among the three distance classes at Little St. Germain Lake were found to have statistical significance ($F=3.965$; d.f.=2, 18; $p=0.037$). The post-hoc analysis of the distance classes at Little St. Germain Lake determined a near significant difference between in mat *E. coli* counts and 10 m from the mat ($p=0.051$). *E. coli* counts among the three distance classes at Big Sand Lake were also determined to be significantly different ($F=10.550$; d.f.=2,6; $p=0.020$). The post-hoc Tukey analysis determined that *E. coli* counts in the mat were significantly different than 5 m and 10 m

distances from the mat (p=0.004, p=0.003 respectively). The Long Lake analysis determined a statistical difference between the *E. coli* counts among distance classes (F=6.784; d.f.=2, 18; p=0.006). The post-hoc Tukey analysis determined that the in mat *E. coli* counts were significantly different than *E. coli* counts at 5 m and 10 m distances from the mat (p=0.021, p=0.009 respectively).

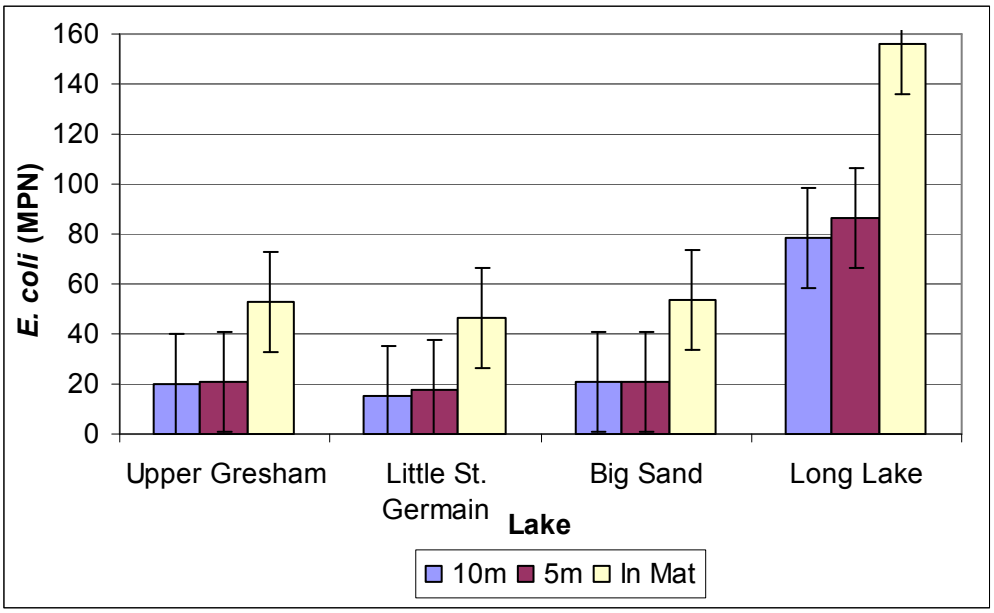


Figure 2.4: Mean *E. coli* concentrations found at the four sampling sites during the 2005 summer sampling season (Note scale) (Bars represent standard error).

Table 2.4: Summary of statistical significance (p-values) between mean *E. coli* concentrations at the three sampling locations at each study site (α=0.05).

Lake	Overall	M vs 5 m	M vs 10 m	5m vs 10 m
Upper Gresham	0.059	0.099	0.088	0.998
Little St. Germain	0.037	0.079	0.051	0.972
Big Sand	0.020	0.004	0.003	1.000
Long Lake	0.006	0.021	0.009	0.925

Table 2.5: *E. coli* MPN means for the summer 2005 sampling season.

Lake	Mat	5 m	10 m
Upper Gresham	52.81	20.69	19.74
Little St. Germain	46.16	17.67	14.89
Big Sand	53.31	20.60	20.46
Long Lake	155.88	86.78	78.08

A Friedman Rank test was used to further analyze the 2005 field season data to determine the overall ranking of *E. coli* counts at the measured distance classes. The Friedman Rank test revealed significant differences for all four lakes (Table 2.XXX).

Table 2.6: Friedman Rank test statistical output for the 2005 sampling season.

Lake	Mean Rank			Significance	X^2
	10m	5m	In Mat		
Upper Gresham	1.70	1.60	2.70	0.03	7.40
Little St. Germain	1.50	1.80	2.70	0.02	7.80
Big Sand	1.38	1.63	3.00	0.04	6.53
Long Lake	1.70	1.40	2.90	0.00	12.60

Upper Gresham Lake *E. coli* showed consistently more *E. coli* within the mat than at any distance, mat>5 m and 10 m, 5 m> 2 m (Friedman X^2=7.4; d.f.=2; p=0.025). Little St. Germain Lake *E. coli* counts were consistently mat>5 m>10 m (Friedman test X^2=7.8; d.f.=2; p=0.020). Big Sand Lake *E. coli* counts were also consistently mat>5 m> 10 m (Friedman test X^2=6.533; d.f.=2; p=0.038). Long Lake *E. coli* counts were consistently higher within the mat than at any distance: mat>5 m and 10 m, but 10 m>5 m (Friedman test X^2=12.6; d.f.=2; p=0.002).

Field Season 2 (2006):

The 2006 field season data collected displayed a similar pattern to the 2005 data collected in that *E. coli* concentrations tended to be higher in samples collected from the macrophyte mat compared with other sample points of two and five meters from the mat

(Figure 2.5, Table 2.7) (Appendix C for 2006 raw data). The amount of *E. coli* at each sampling site, as well as between each sampling site, however, was highly variable over the course of the summer (Figures 2.6, 2.7, 2.8, 2.9). Overall, the amount of *E. coli* found within the mat was higher than the amount found at varying distances from the mat. The repeated measures ANOVA revealed no significant distance effect (p=0.302), but the sphericity assumption of the model was not met. The corrected statistics indicated significant day (Sphericity Assumed F=10.970; d.f.=9; p=0.000; Greenhouse-Geisser F=10.970; d.f.=1.188; p=0.004; Huynh-Feldt F=10.970; d.f.=2.453; p=0.000; Lower-bound F=10.970; d.f.=1.000; p=0.006), and day*lake effects (Sphericity Assumed F=11.752; d.f.=27; p=0.000; Greenhouse-Geisser F=11.852; d.f.=3.565; p=0.000; Huynh-Feldt F=11.752; d.f.=7.359; p=0.000; Lower-bound F=11.752; d.f.=3; p=0.001).

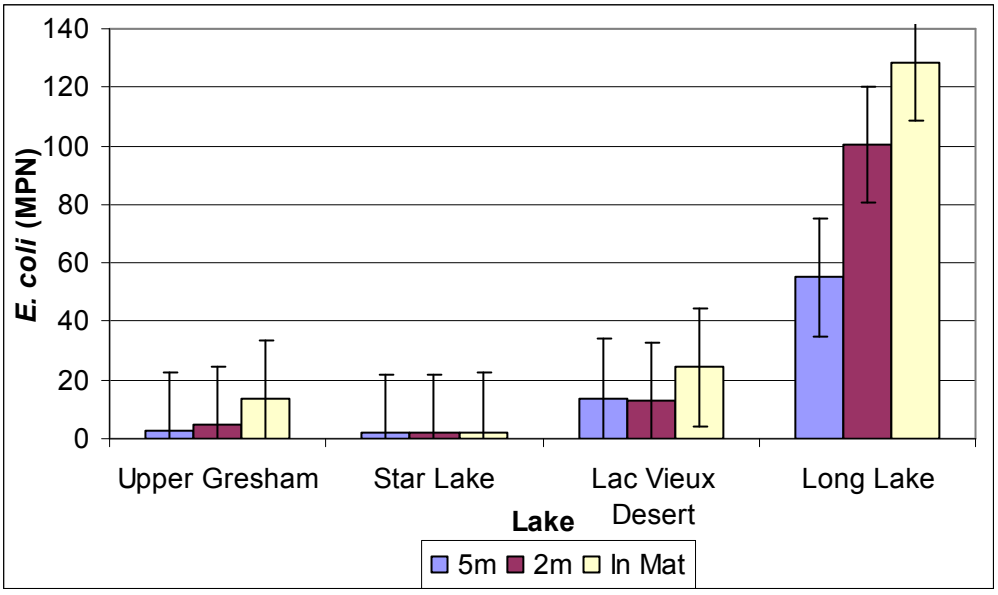


Figure 2.5: Mean *E. coli* concentrations found at the four sampling sites during the 2006 summer sampling season (Note scale) (Bars represent standard error).

Table 2.7: *E. coli* MPN means for the summer 2006 sampling season.

Lake	5 m	2 m	Mat
Upper Gresham	2.44	4.89	13.63
Star Lake	1.80	1.83	2.32
Lac Vieux Desert	13.96	12.96	24.38
Long Lake	55.12	100.33	128.54

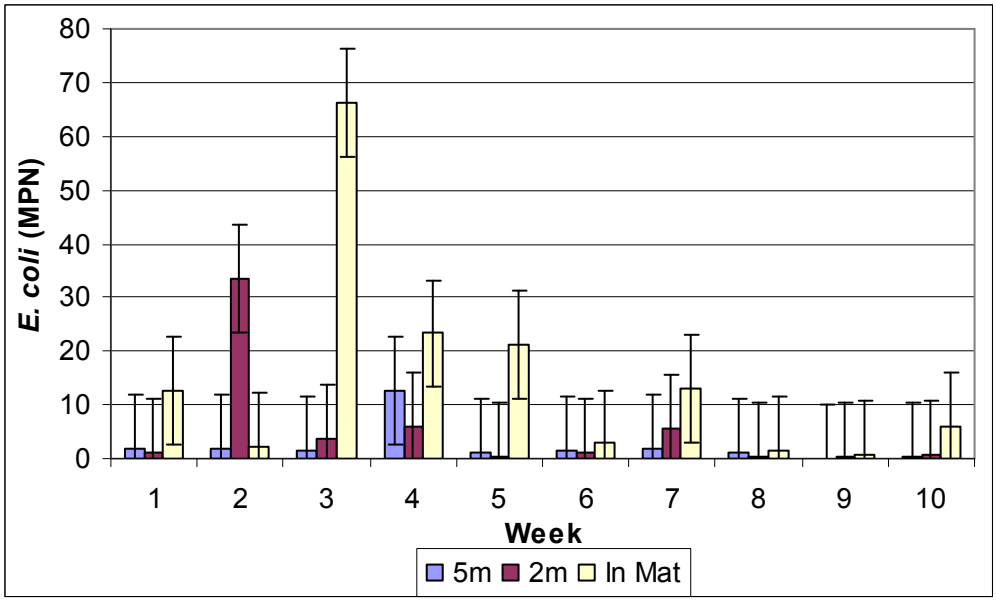


Figure 2.6: *E. coli* MPN at Upper Gresham Lake over the 2006 field sampling season (Note scale) (Bars represent standard error).

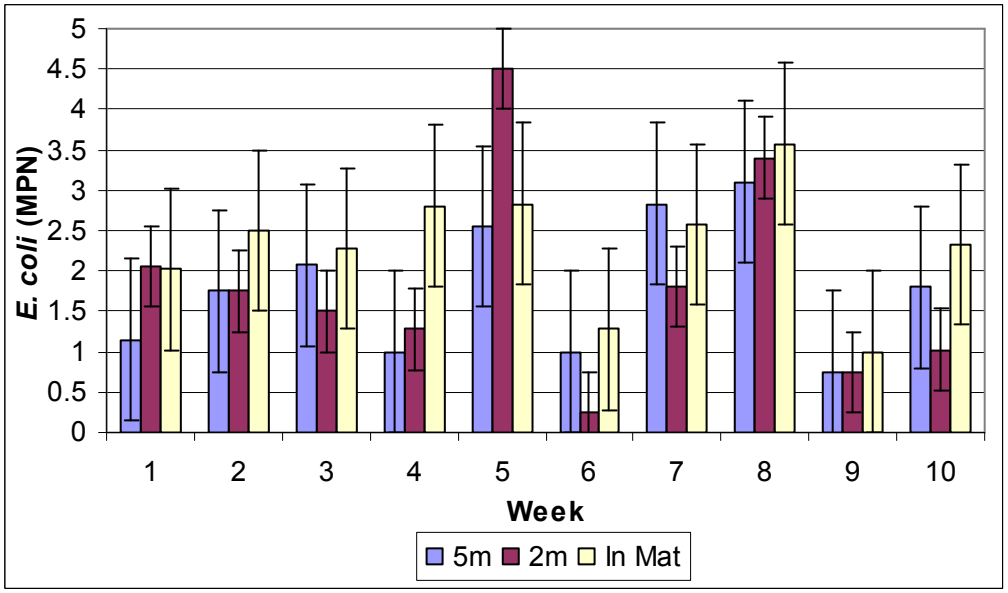


Figure 2.7: *E. coli* MPN at Star Lake over the 2006 field sampling season (Note scale) (Bars represent standard error).

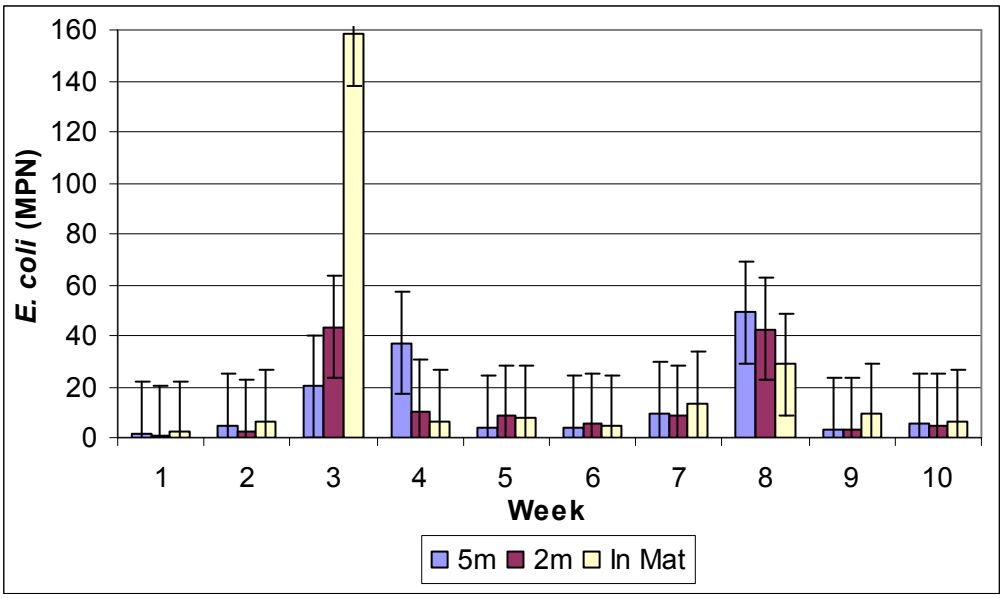


Figure 2.8: *E. coli* MPN at Lac Vieux Desert over the 2006 field sampling season (Note scale) (Bars represent standard error).

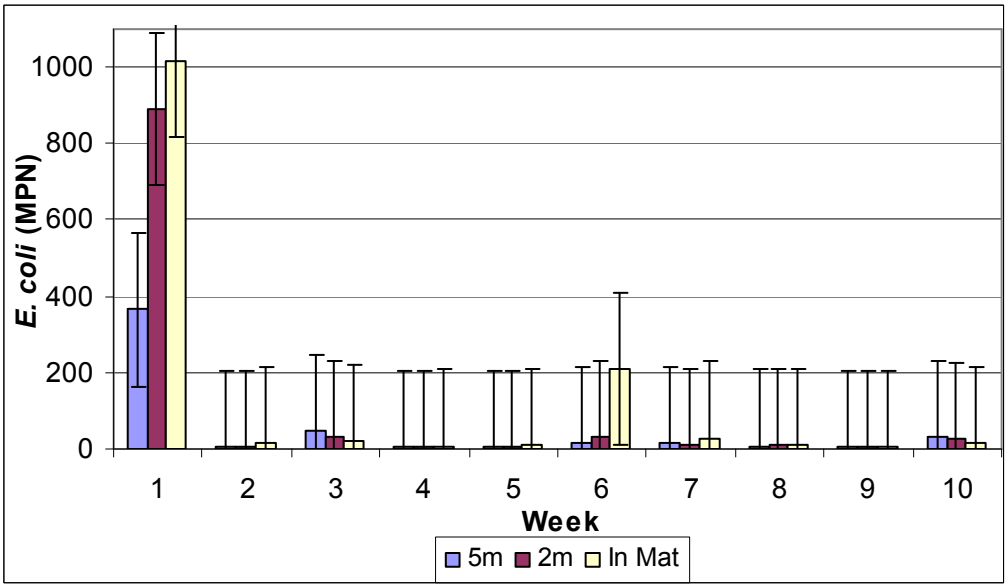


Figure 2.9: *E. coli* MPN at Long Lake over the 2006 field sampling season (Note scale) (Bars represent standard error).

A Friedman Rank test was used to further analyze the data because the test relies on ranks of the data, and is not sensitive to the high variability in the amount of *E. coli* between lakes and sampling events. The Friedman Rank test statistically analyzed the

hypothesis that there was more *E. coli* within the mat versus two and five meters from the mat (Table 2.8).

Table 2.8: Friedman Rank test statistical output for the 2006 sampling season.

Lake	Mean Rank			Sig.	X^2
	5m	2m	In Mat		
Upper Gresham	1.50	1.60	2.90	0.00	12.20
Star Lake	1.60	1.70	2.70	0.02	7.79
Lac Vieux Desert	1.90	1.70	2.40	0.27	0.27
Long Lake	1.70	1.70	2.60	0.07	5.40

The Friedman Rank test determined that Upper Gresham and Star Lakes were significant ($p<0.020$), Long Lake was nearly significant ($p<0.067$), and Lac Vieux Desert was not significant ($p<0.273$). Thus, for three of the four lakes *E. coli* counts were consistently higher in the mat compared to 2 m and 5 m over the entire 2006 sampling season. Upper Gresham *E. coli* counts were consistently mat>2 m>5 m (Friedman test $X^2=12.2$; d.f.=2; $p=0.002$). Star Lake *E. coli* counts were consistently mat>2 m>5 m (Friedman test $X^2=7.789$; d.f.=2; $p=0.020$). Lac Vieux Desert *E. coli* showed consistently more *E. coli* within the mat than at any distance, mat>2 m and 5 m, 5 m>2 m (Friedman test $X^2=2.6$; d.f.=2; $p=0.273$). Long Lake *E. coli* counts were also consistent for having higher *E. coli* within the mat than at any distance from the mat, mat>2 m and 5 m, however, 2 m and 5 m displayed no difference (Friedman $X^2=5.4$; d.f.=2; $p=0.067$).

Abiotic Factors:

Abiotic factors of pH, dissolved oxygen (DO), and temperature were measured in the macrophyte mat at the four measured lakes (Appendix D for raw abiotic data) (Table 2.9).

Table 2.9: The averaged abiotic factors measured at each of the lakes during the summer 2006 sampling season.

Lake	pH	DO (mg/L)	Temp (oC)
Upper Gresham	7.26	0.73	22.74
Star Lake	7.18	0.73	24.48
Lac Vieux Desert	7.17	0.64	26.40
Long Lake	7.17	0.68	25.74

Using stepwise multiple regression, temperature was the only independent variable that was a significant predictor of *E. coli* numbers (Norusis 2004). Scatter diagrams and analyses of residuals, however, indicated that the relationship between temperature and *E. coli* was non-linear, so the data was reanalyzed using an exponential model. Three of the four lakes examined had no significant interaction between the abiotic factors and in mat *E. coli* measured. At Upper Gresham Lake, an exponential model was highly significant ($F=22.012$, $df=9$, $p=0.002$, $R^2=0.733$, Adjusted $R^2= 0.700$) (See Figure 2.10).

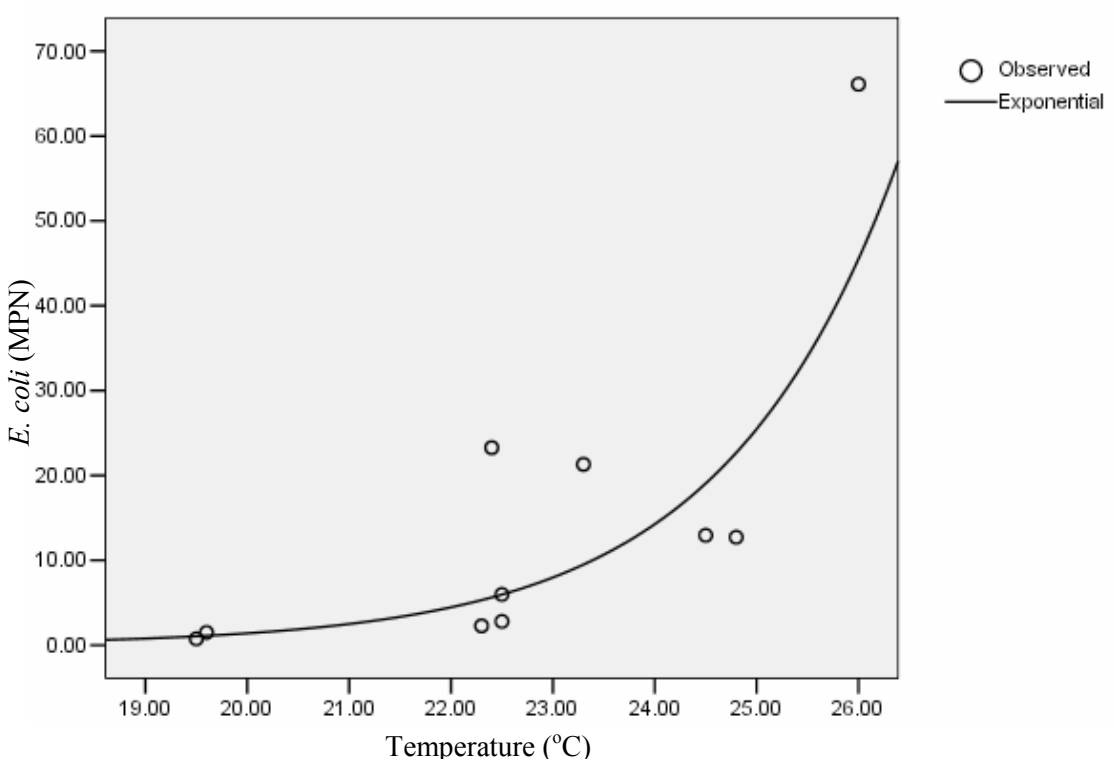


Figure 2.10: The relationship between temperature (X-axis) and in mat *E. coli* MPN (Y-axis) as determined by an exponential model at Upper Gresham Lake.

Epiphytic Bacteria:

The destructive assay displayed a high amount of variability between each of the sampling events at each of the lakes (Figures 2.11-2.14). When represented graphically, there appears to be a large difference between the epiphytic and planktonic bacteria. However, an overall repeated measures ANOVA (SPSS, Inc.) conducted for each of the lakes revealed no statistically significant difference between the epiphytic and planktonic *E. coli* at any of the lakes (data not shown). A one way ANOVA (SPSS, Inc.) was conducted for each sampling event at each lake to determine if any single event displayed a significant difference between epiphytic and planktonic *E. coli*. The analysis on Upper Gresham Lake revealed a p-value near the significance threshold on week eight which indicated that there was more epiphytic *E. coli* than planktonic *E. coli* ($p<0.058$). The

Star Lake analysis determined that week three had significantly more planktonic than epiphytic *E. coli* ($p<0.026$). The analysis on Lac Vieux Desert determined that sampling weeks two and seven had significantly more planktonic than epiphytic *E. coli* ($p<0.040$, $p<0.032$ respectively). Long Lake had no statistically significant difference between epiphytic and planktonic *E. coli* ($p<0.076$). The graphical representation of this data displays a different overall trend. Upper Gresham Lake had seven of the nine and Long Lake had eight of the nine sampling events with a higher amount of epiphytic than planktonic *E. coli*. Conversely, Star Lake and Lac Vieux Desert both showed four of the nine sampling events had more planktonic than epiphytic *E. coli*.

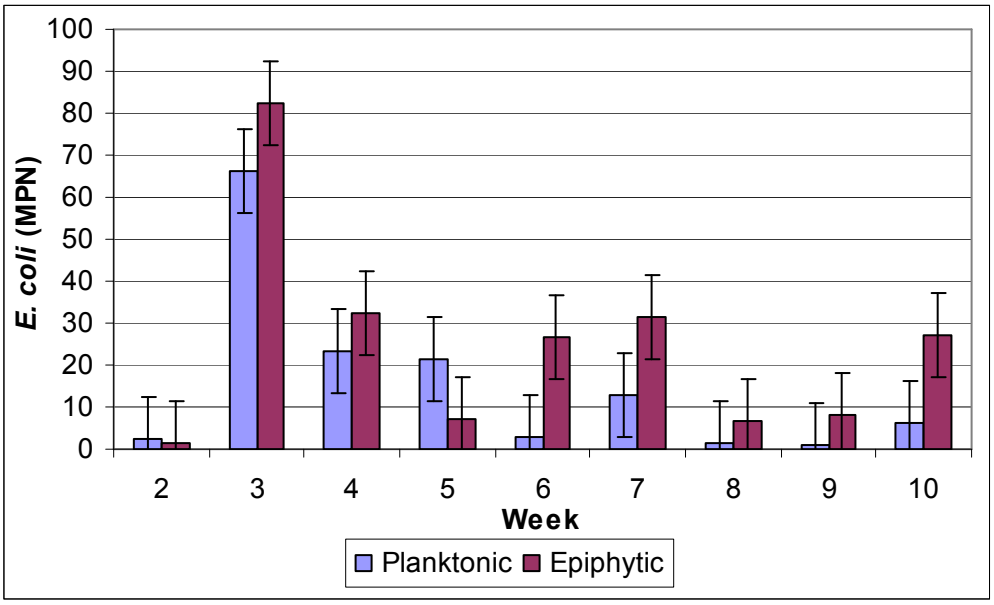


Figure 2.11: The comparison of epiphytic and planktonic *E. coli* over the 2006 field season at Upper Gresham Lake (Note scale) (Bars represent standard error).

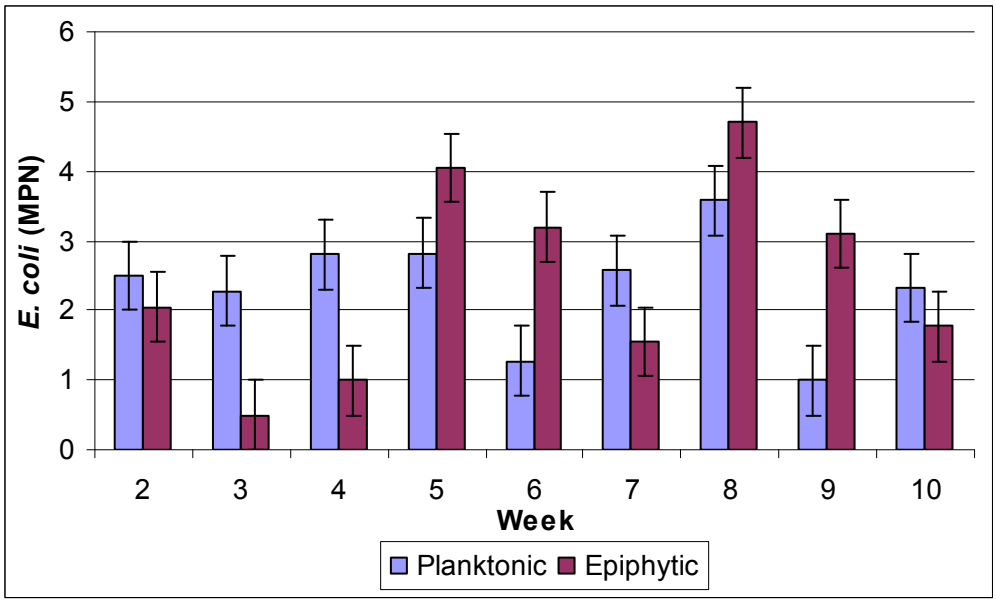


Figure 2.12: Comparison of epiphytic to planktonic *E. coli* in Star Lake (Note scale) (Bars represent standard error).

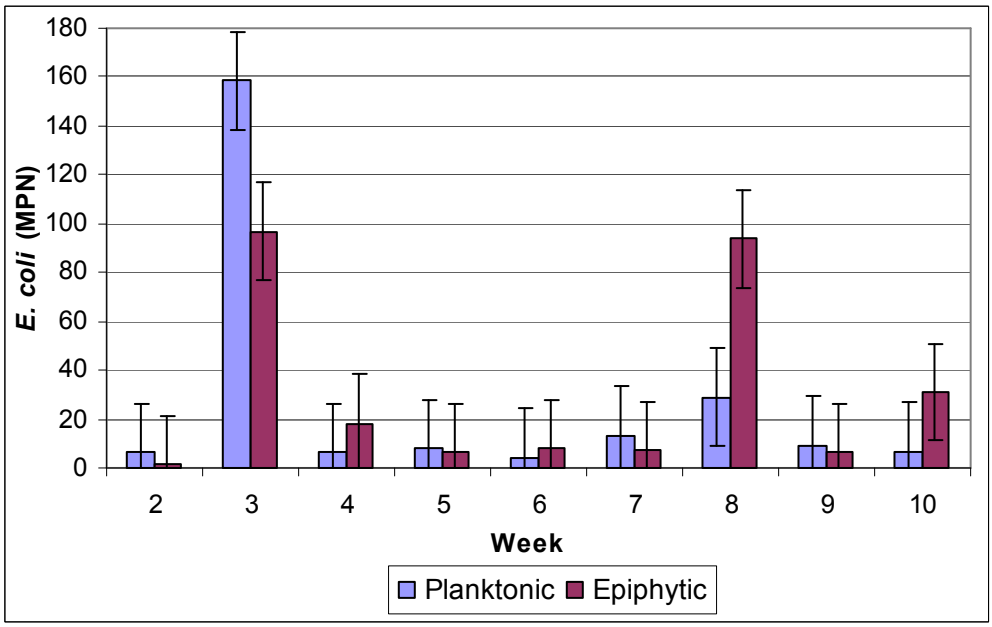


Figure 2.13: Comparison of epiphytic to planktonic *E. coli* in Lac Vieux Desert (Note scale) (Bars represent standard error).

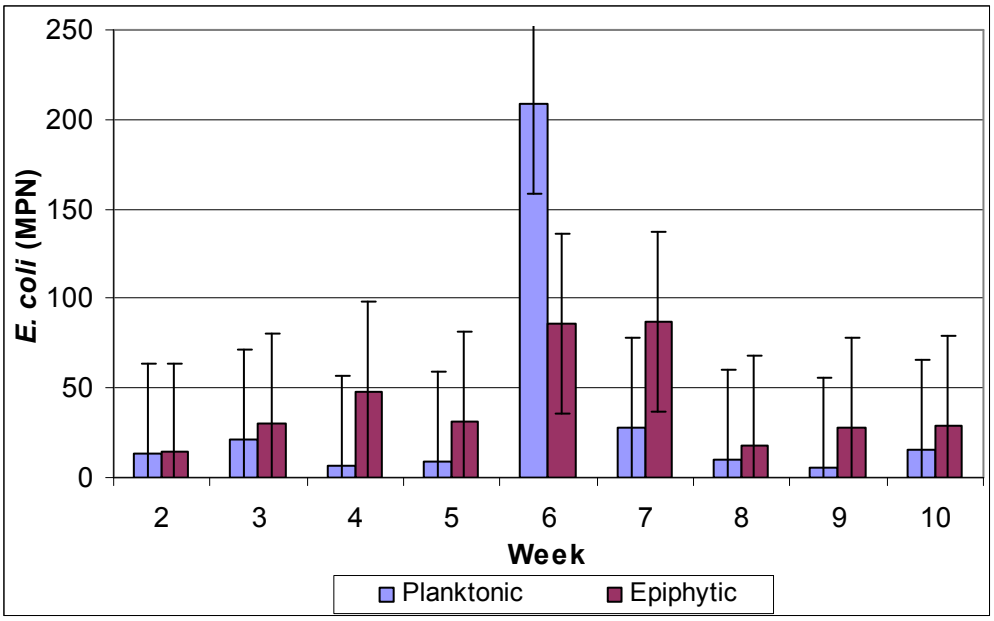


Figure 2.14: Comparison of epiphytic to planktonic *E. coli* in Long Lake (Note scale) (Bars represent standard error).

Enterococci:

Enterococci levels at each of the lakes were highly variable between each of the sampling events and there was no overall trend (Figures 2.15-2.18). Upper Gresham and Star Lakes both displayed Enterococci levels that were higher than the *E. coli* levels. The mat at Upper Gresham Lake had the highest levels of Enterococci compared to Enterococci levels at other distances and to the overall *E. coli* numbers. Star Lake also had higher levels of Enterococci than *E. coli*, but the amount of both species of bacteria was highly variable between sampling events. Overall, Lac Vieux Desert and Long Lake both displayed a higher amount *E. coli* than Enterococci. At Lac Vieux Desert, all distances measured were highly variable for both Enterococci and *E. coli*, although, overall, more *E. coli* were observed than Enterococci. Long Lake displayed the most difference between Enterococci and *E. coli* levels. Three of the four sampling events displayed more *E. coli* within the mat than two and five meters from the mat, and all

sampling events displayed more *E. coli* present than Enterococci. There was no statistical analysis completed because samples were not collected in duplicate due to reagent restrictions.

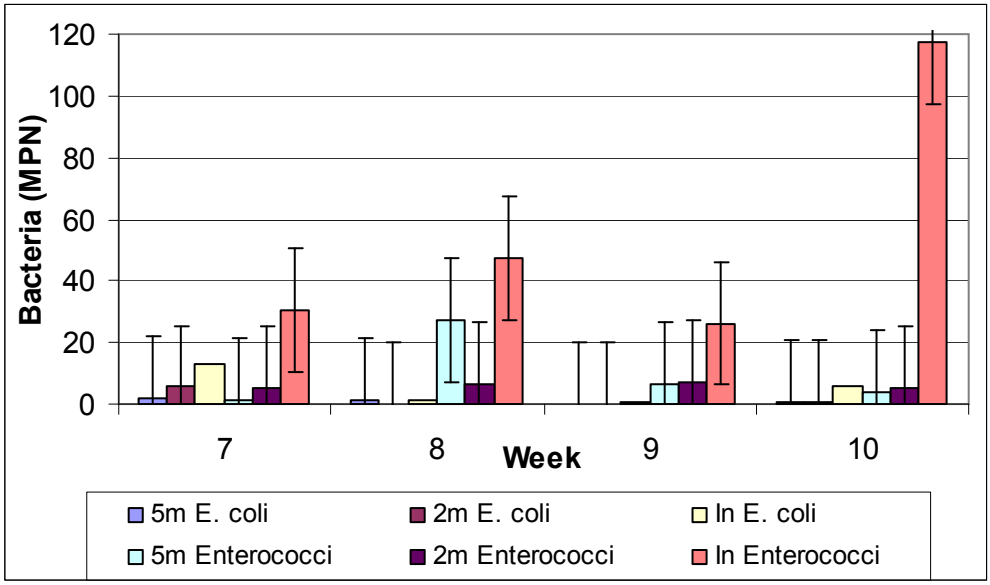


Figure 2.15: Comparison of *E. coli* and Enterococci during the last four weeks of the 2006 sampling season in Upper Gresham Lake (Note scale) (Bars represent standard error).

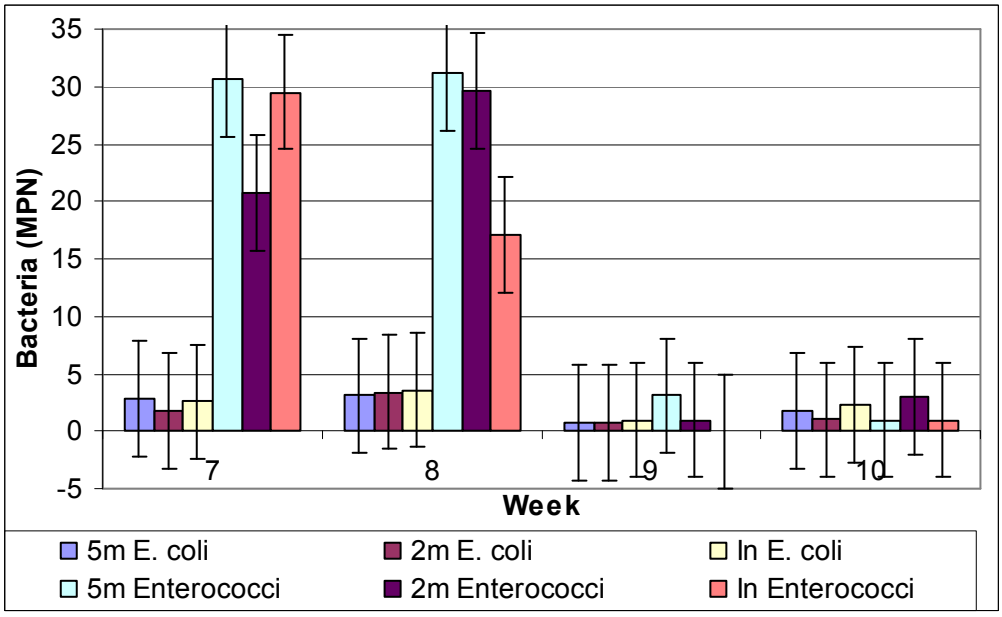


Figure 2.16: Comparison of *E. coli* and Enterococci in Star Lake (Note scale) (Bars represent standard error).

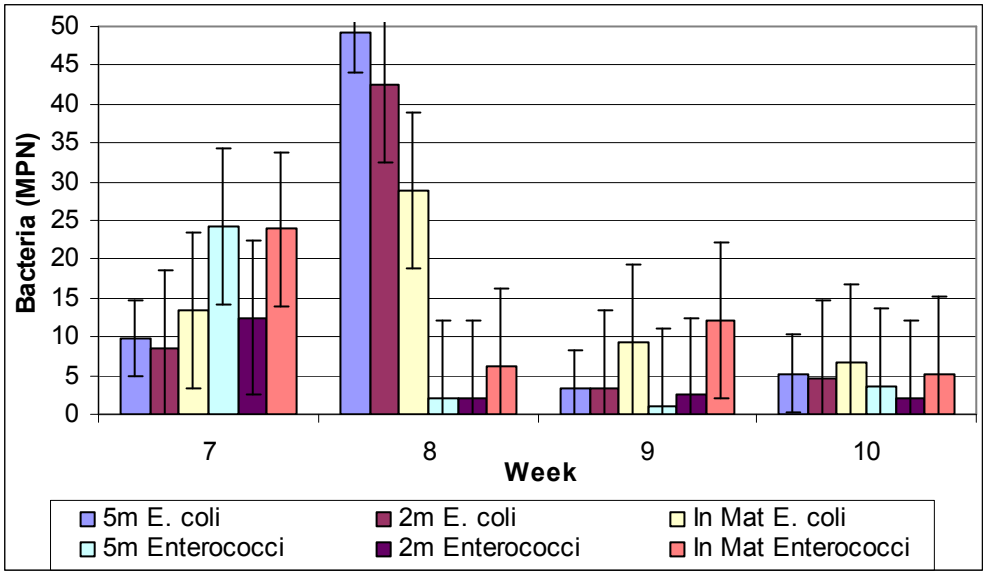


Figure 2.17: Comparison of *E. coli* and Enterococci in Lac Vieux Desert (Note scale) (Bars represent standard error).

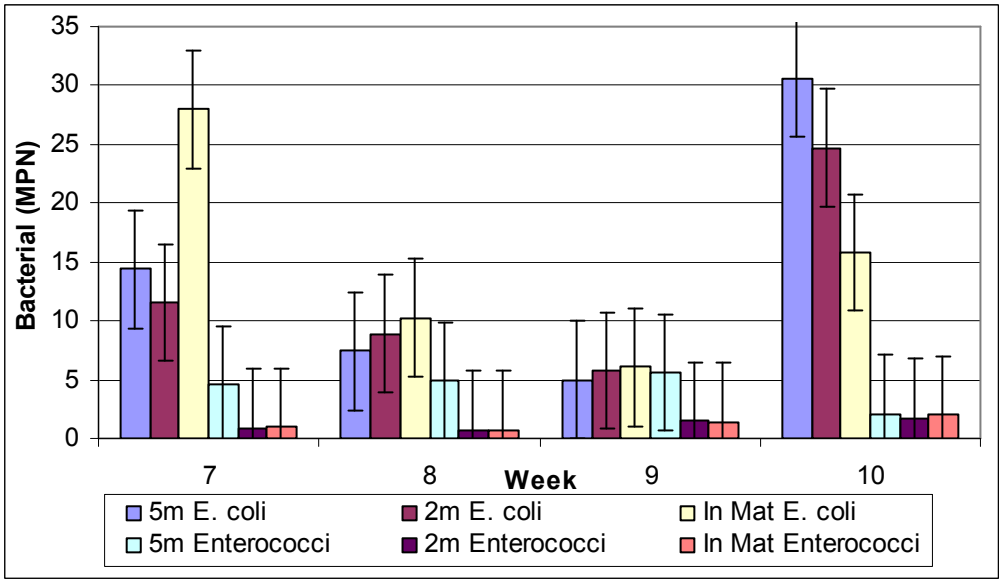


Figure 2.18: Comparison of *E. coli* and Enterococci in Long Lake (Note scale) (Bars represent standard error).

In summary, the compilation of results from both Field Seasons One and Two indicate that *E. coli* are found at increased numbers in mats of aquatic macrophytes (Figure 2.19).

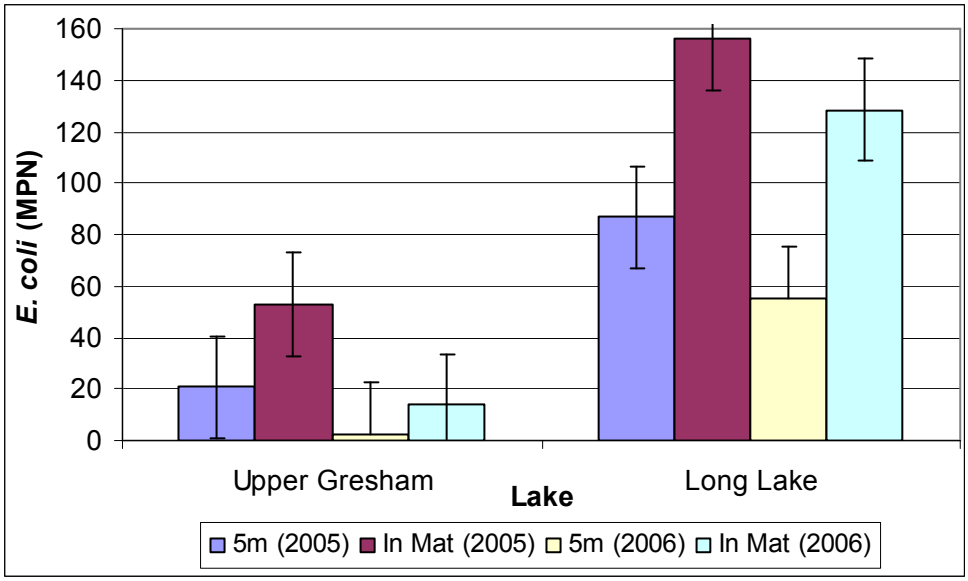


Figure 2.19: Compilation of the 2005 and 2006 data from the two lakes that were measured over both sampling seasons (Note scale) (Bars represent standard error).

DISCUSSION

There is a long and consistently documented relationship between plants and bacteria (Baker and Orr 1986, Beattie and Lindow 1994, Eriksson and Weisner 1999, Kinkel et al. 2000, Marsollier et al. 2004, Morris et al. 1998, Muller et al. 2001). Bacterial survival in aquatic systems has been well researched, and it is known that there is a direct relationship between bacterial survival and the presence of particulate matter for bacterial attachment (Bogosian et al. 1996, Banning et al. 2002, Brettar and Hofle 1992). It is also documented that mats of the filamentous alga *Cladophora* provide a favorable habitat for bacterial survival in aquatic systems by providing a site for attachment and protection from factors such as UV light and predation (Whitman et al.

2003, Byappanahalli et al. 2003, Kleinheinz and Englebert 2005). However, the association between mats of aquatic macrophytes and *E. coli* survival has not previously been studied. The primary objective of this study was to determine the relationship, if any, between mats of aquatic macrophytes and *E. coli* concentrations.

Results from the first field season found an increased amount of *E. coli* in macrophyte mats when compared to five and ten meters from the macrophyte mat, but the results showed no significant differences between the distances five and ten meters (Figure 2.4, Table 2.4, 2.6). Five meters was generally found to have higher *E. coli* concentrations than ten meters but was not always higher than ten meters. This indicated the spatial effect of the mats on *E. coli* concentrations may be in the zero to five meter area. This variability may be due to changing conditions (i.e., wind direction) and mat densities. Some of the macrophyte mats were found to be exceedingly delicate and could change density due to wind and wave conditions, and animal movement. In fact, sampling of Big Sand Lake was discontinued after three weeks, because the mat there dissipated due to a strong wind storm the previous week.

The second field season provided similar results to the first, with *E. coli* levels found higher in the macrophyte mat than two and five meters from the mat (Figure 2.5, Table 2.7). The repeated measures ANOVA determined a higher amount of *E. coli* in the mat than any distance from the macrophyte mat. The Friedman Rank test also found higher amounts of *E. coli* in the mat than any distance from the mat. These results, when compiled with the previous field season, provide a strong argument that *E. coli* are harbored within a macrophyte mat (Figure 2.19). Despite the large daily and yearly

variation in *E. coli* numbers, the overall trend in these data is that *E. coli* levels are increased within dense mats of aquatic macrophytes.

The epiphytic bacteria study revealed a statistical difference between very few of the sampling events (Figures 2.11 - 2.14). Three of the four significant sampling events revealed that there was more planktonic than epiphytic *E. coli*. These findings are unexpected, as other studies have revealed that there are typically more bacteria in association with a substrate of any type (Bogosian et al. 1996, Banning et al. 2002, Brettar and Hofle 1992, Sampson et al. 2006). Although not significantly different, seven of the nine sampling events at Upper Gresham and eight of nine of the sampling events at Long Lake had higher levels of epiphytic *E. coli* than planktonic *E coli*. At Lac Vieux Desert and Star Lake, both lakes with macrophyte mats that are less dense, almost half of the sampling events (four of the nine) had more epiphytic *E. coli* than planktonic *E. coli*. These discrepancies reveal that more study is needed to determine the true relationship between epiphytic and planktonic *E. coli* in a macrophyte mat. If *E. coli* is found to have higher numbers in association with aquatic macrophytes, it is likely that epiphytic *E. coli* could slough into the water (Whitman and Nevers 2003).

Results from the Enterococci study should be interpreted with caution because of the low sample number and the lack of statistical analysis. This study observed that each lake was highly variable for Enterococci levels, and that there was no observable trend between the macrophyte mat and distances measured. More studies should be completed to discover the relationship between Enterococci and mats of aquatic macrophytes.

The abiotic factors regression analysis introduces another factor to the macrophyte mat and *E. coli* relationship (Table 2.9, Figure 2.10). The analysis revealed a

statistically significant relationship between increasing temperature and increasing amounts of *E. coli* at Upper Gresham Lake (p=0.002). These findings agree with a relationship observed by Whitman and Nevers (2003). Whitman and Nevers (2003) determined that *E. coli* counts in sand were significantly correlated to water temperature, and as the water temperature increased, the counts of *E. coli* increased due to higher growth rates associated with warmer temperatures. The observed mat environment at Upper Gresham Lake provided an average temperature of 22.73 ^{o}C (standard deviation of 2.08), which is within the optimal range for *E. coli* replication of 21 to 48 ^{o}C (Madigan et al. 2002). More studies are needed to determine the relationship of *E. coli* to the water temperature not just in a macrophyte mat, but at different distances from the mat.

The findings of this study are in accord with similar previous studies completed which observed the dynamic between *Cladophora* and *E. coli* (Whitman et al. 2003, Byappanahalli et al. 2003, Kleinheinz and Englebert 2005). Such studies suggest that *E. coli* can persist and replicate in mats of *Cladophora* due to the nutrients and protection from predation, UV light and other facilitating factors for *E. coli* survival that the filamentous algae provide. Detached *Cladophora* also can clump in thick mats along shoreline beach areas and can get buried in sand; both sand and *Cladophora* can act as a secondary habitat for *E. coli* survival (Sampson et al. 2006, Whitman et al. 2003). Studies that have measured *E. coli* levels in association with *Cladophora* typically observe levels that are near or above the beach closure levels (Englebert, unpublished data), whereas the *E. coli* levels found in association with aquatic macrophytes in this study were nowhere near the beach closure levels; this could be because mats of aquatic macrophytes are generally not as dense or large scale as mats of *Cladophora*. Both mats

or stands of aquatic macrophytes and detached masses of *Cladophora* can release *E. coli* into the water, which would cause elevated numbers of the indicator organism in the water.

Overall, the numbers of *E. coli* measured during both field seasons were below the Wisconsin DNR advisory level of 235 *E. coli* per 100 mL and well below the closure level of 1000 *E. coli* per 100 mL, indicating that beaches in Vilas County, Wisconsin are not a public health risk. During the 2005 and 2006 field seasons, there was one beach sample that exceeded the warning level of 235 *E. coli* per 100 mL. While these data indicate that beaches in Vilas County are near pristine, the higher *E. coli* counts found in and near macrophyte mats are still a plausible cause for alarm.

While the factors that contribute to increased levels of *E. coli* in macrophyte mats should be further studied, this study shows that substantial mats of aquatic macrophytes are a reservoir for *E. coli* in a northern freshwater lake environment. The implications of this study include that beach water management planning should include factors other than the simple presence or absence of a bacteria to determine water quality. Planning for the presence of macrophytes near a beach area could include raking any loose plant material from beach areas or the use of herbicides or mowing to limit the amount of macrophytes in a beach area.

Laboratory studies are needed to further validate and expand on the findings of this study. A microcosm study to determine the length of *E. coli* survival in differing densities of macrophyte mats would aid in determining the effectiveness of using macrophyte mat size as a predictor of beach water contamination. Also, a microcosm study determining the survivability of the pathogens for which *E. coli* acts as an indicator

organism (eg. *Salmonella, Shigella, Campylobacter*) in macrophyte mats would aid in determining the effectiveness of *E. coli* as an indicator organism in situations where there are mats of aquatic macrophytes. Genetic testing to determine the relatedness of *E. coli* found in mats of aquatic macrophytes would also be beneficial. This data would help to determine the sources of *E. coli* contamination as well as if the *E. coli* measured is replicating and if their presence is the result of a contamination event.

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CHAPTER 3
DETERMINATION OF *ESCHERICHIA COLI* SURVIVAL IN LABORATORY MICROCOSMS

ABSTRACT

Recreational beach use is a large contributor to tourism dollars brought into areas around the Great Lakes region, and beach closures can be extremely costly in terms of lost tourism revenue. The indicator organism used in beach monitoring in Wisconsin is *Escherichia coli*, as high levels of this organism have been correlated with recent fecal contamination events. These events may contain other, more pathogenic, bacteria and pose a risk to human health. Another serious problem at many beaches in the state is stands of aquatic macrophytes (e.g. Eurasian Water-Milfoil). The objective of this project was to asses any relationship between densities of aquatic macrophytes and the persistence of the fecal indicator organism, *E. coli* in a laboratory microcosm. Three different densities of *Sagittaria*, *Myriophyllum* or a plastic plant were used in combination with lake water and placed in a greenhouse on a shaking platform to simulate wave actions. Water from the microcosms was sampled to enumerate the *E. coli* concentrations in each of the microcosms for seven days. *E. coli* was measured using a standard dilutions protocol and spread plated onto EMB agar; plates were incubated for 24 hours at 28 ^{o}C. The experiment determined that there were no statistically significant differences between the different densities of plant matter and *E. coli* concentrations, although there was a significant difference between *E. coli* concentration in the living plant microcosms and *E. coli* concentrations in the plastic plant microcosm.

INTRODUCTION

Tourism is a large source of income for many Midwestern states. The tourism industry in Wisconsin alone is worth $12 billion dollars annually (Kleinheinz and Englebert 2005). The summer months especially bring thousands of people to beaches seeking relief from the heat. If those beaches are closed due to microbial contamination, it is unlikely that tourists will return in the future. This necessitates finding an indicator organism that is a reliable and accurate indicator of fecal contamination events.

Detection of fecal indicator organisms, especially *E. coli*, has been used to monitor drinking water for over 100 years (Hanninen et al. 2003). In 1986, the U.S. Environmental Protection Agency (US EPA) described water quality criteria for recreational water systems (US EPA, 1986). The study showed that both *E. coli* and Enterococci are good indicator organisms of fecal contamination events in recreational water systems. The EPA warning threshold for full body contact bathing in recreational waters for *E. coli* is no more than 235 cells per 100 mL and 61 cells per mL for Enterococci. These criteria were designed to limit the risk of disease in humans to approximately seven illnesses per 1000 swimmers.

A fecal contamination event is an event that contaminates recreational water systems with pathogens normally associated with feces. These pathogenic organisms could be derived from either animal or human sources (Kleinheinz et al. 2003). Sources are varied and could be due to faulty septic systems, rain events causing run off from agricultural systems or contaminated pavement, animals in the water, or human related. The pathogenic organisms that are associated with fecal contamination events are wide ranging, but are normally found in the gastrointestinal tract of warm blooded animals.

Examples of these pathogens are Norovirus*, Salmonella, Shigella, Campylobacter, Giardia,* and *Cryptosporidium* (Kleinheinz and Englebert 2005). These pathogens can cause severe illnesses and gastrointestinal disorders; this is particularly of concern for children and immunocompromised individuals who are most likely to suffer from even a small exposure to pathogens.

The primary host habitat of *E. coli* is the lower enteric tract of warm blooded animals (Winfield and Groisman 2003). The most common strains of *E. coli* are commensal organisms that are highly adapted to the conditions of the gut. The genome of *E. coli* encodes proteins that are resistant to acidic pH and allows the bacteria to grow on lactose. The environment of the gastrointestinal tract is conducive to bacterial growth providing warm, constant temperatures and high concentrations of nutrients. Due to these favorable conditions in the host environment, *E. coli* has a doubling time estimated at 12 to 48 hours (Winfield and Groisman 2003).

The secondary environment, outside of the host, provides harsh conditions for *E. coli* (Winfield and Groisman 2003), and the organism has been shown to have a half life of only one day. In open water taken from natural systems, *E. coli* declines to undetectable levels at a wide range of temperatures in a remarkably short period of six days (Bogosian 1996, Brettar and Hofle 1992). Brettar and Hofle (1992) experimentally examined the survivability of *E. coli* in natural water systems. *Escherichia coli* was added to two lake mesocosms in a manner similar to a hypothetical leakage of a production fermenter. Organic nutrients were also added to one lake mesocosm. Brettar and Hofle (1992) monitored bacterial decline in the water by immunofluorescence and culture techniques, and found that after four days of growth, 81% of bacterial cells were

attached to floating particles. By the thirteenth day, cells were still detectable by immunofluorescence. The greater survivability associated with bacterial attachment was postulated to have occurred for two principle reasons: reduced predation of *E. coli* and increased availability of nutrients. The reduced predation was observed because flagellated protozoa were unable to graze on bacteria attached to particles. A greater survivability of *E. coli* in the lake mesocosm with added organic nutrients was thought to be more correlated with the particle production by organic nutrients than the actual nutrients. Bacteria associated with particles were also able to replicate, although in small numbers. *Escherichia coli* levels were undetectable in water 16 days after release.

Recently, the green filamentous algae *Cladophora* has become a reemerging nuisance in the Great Lakes. These unsightly and malodorous algae clump to form mats that are currently being studied for their ability to sustain the indicator organism *E. coli* as well as more pathogenic bacteria. Whitman et al. (2003) determined that these mats of *Cladophora* may be an important source of environmental *E. coli*. Open water habitat leads to the rapid decline of bacterial survivability, yet Whitman et al. (2003) found evidence that *Cladophora* harbors both *E. coli* and Enterococci for extended lengths of time (over six months). Mats of *Cladophora* provide an environment extremely favorable for bacterial survival (Kleinheinz and Englebert 2005). Decaying mats of this alga both on shore and in the water provide nutrients, protection from the sun, and a stable temperature and pH for bacteria. Preliminary results show that *E. coli* persist much longer within the mat of *Cladophora* than do the pathogens for which *E. coli* acts as an indicator (Kleinheinz, G.T. personal communication). A study by Whitman et al. (2003) also indicated that mats of *Cladophora* at least 6 mm thick allow for *E. coli* survival in

the environment up to 6 months and that any bacteria in dried algal mats is able to multiply upon the addition of water.

Similarities can be noted between mats of *Cladophora* and mats of aquatic macrophytes (Baker and Orr 1986). Both mats provide a favorable secondary habitat consisting of increased nutrients, protection from the sun, an attachment point for growth and a stable temperature. Higher *E. coli* counts within and near macrophyte mats could lead to beach closures and the loss of tourism revenue. The objective of this study was to determine the effect of mats of aquatic macrophytes on survivability of the indicator organism (*E. coli*) in a laboratory microcosm.

MATERIALS AND METHODS

Bacteria Strain and Culture:

An environmental strain of *Escherichia coli* was used in this study. The strain was isolated from the Siskiwit Beach on Lake Superior near Ashland, Wisconsin in the summer of 2005 by University of Wisconsin – Oshkosh Environmental Microbiology Laboratory (Wisconsin State Certification Number 105-445). The bacterium was determined to be *E. coli* through culture on Modified membrane-Thermotolerant *Escherichia coli* (modified mTEC) agar. *E. coli* stock cultures were prepared using a 20% glycerol solution (ICN Biomedicals Inc. Lot# R11451) and stored in sterile cryotubes at -30 ^{o}C until needed for use. *E. coli* for use in the microcosm was grown by pipetting 100 µL of the stock culture into 45 mL of nutrient broth (Difco, Inc). This culture was then incubated for 24 hours at 37 ^{o}C, on an orbital shaking incubator at 110 rpm.

Microcosm Experiment

Design of Microcosm Experiment:

Water for use in the microcosms was recovered from Lake Superior Lower Harbor Park in Marquette, MI. The water was not sterilized or filtered in order for the microcosms to mimic a natural environment. The water used was plated on eosin methylene blue (EMB, Difco) agar to enumerate natural bacteria found in the water. For the experiment, 199 mL of lake water was placed into sterile Magenta Vessels (6.5 cm x 6.5 cm x 9.6 cm made by Magenta Corp.). The Magenta Vessels were capped with a lid that had a 10 mm diameter filter with 0.22 μm pore size to allow for the natural passage of air and water vapor, and to eliminate the number of extraneous contaminants allowed into the system. Each Magenta cube was then inoculated with 1 mL of the 24 h broth culture of the environmental *E. coli*.

Six classes of microcosms were used: three controls and three treatments, each with five replicates. Control one (named treatment C) was water only with *E. coli*, Control two (named treatment P) was water and the plant at a medium density, with no added *E. coli*, Control three (named treatment E) consisted of water, *E. coli* and a plastic plant at the medium density (2 g). The three treatments were categorized as low, medium, and high densities of plants. The low (L) treatment boxes contained 1 gram of plant matter in each microcosm. The medium (M) treatment contained 2 grams of plant matter in each microcosm, and the high treatment (H) contained 3 grams of plant material in each microcosm. Plant species used were *Sagittaria* or *Myriophyllum* (Carolina Biological Supply Company), the plastic plant mimicked the *Sagittaria* in appearance.

Plants were sterilized in a 10% bleach solution for 5 min, then rinsed thoroughly in distilled water for 5 min.

After inoculation, the 30 magenta cubes were placed in the research side of the NMU Greenhouse, on an orbiting shaker (Eberbach Corporation) and shaken continuously 4 hours each day at 110 rpm. The greenhouse temperature was set between 21 and 24 ^{o}C during the day, and 18 and 21 ^{o}C at night. The greenhouse was lit for ten hours each day between 8 am and 6 pm.

Bacterial Detection:

The water was tested every 24 hours to determine bacterial survivability in each treatment. One hundred µL of treatment water was sampled and placed into 9.90 mL sterile phosphate buffered water (PBW). Numbers of *E. coli* were determined through spread plating serial dilutions onto EMB in duplicate, and agar plates were incubated at 28 ^{o}C for 24 hours before each plate was read. Colonies appearing to be *E. coli* were tested using Simmons Citrate agar as a means of quality control. The water testing continued until no *E. coli* was detected for 48 hours. (For detailed sampling protocol see Appendix E.)

Biolog Assay

In addition to the *E. coli* enumeration, a Biolog Assay was utilized to metabolically profile the strain of *E. coli* used in the laboratory experiment (LSSK) (Biolog, Inc Hayward, CA). The GN2 microplate was used for the Biolog Assay and run in duplicate. Carbon utilization was determined by the presence of a purple colour in each well. The purple colour is indicative of the reduction of tetrazolium dye which results in the presence of a purple colour to indicate that a carbon source was utilized. A

carbon source was determined to be utilized if the well was purple in both replicates. (A detailed sampling scheme for the Biolog Assay can be found in Appendix F.)

Statistical Analysis

A univariate analysis of variance (ANOVA) with a post-hoc 2-sided Dunnett analysis was completed using the GLM Univariate module in SPSS (Statistical Software Package for Social Sciences Version 13, 2004 SPSS Inc, Chicago Illinois) with the log – transformed average data compiled from the laboratory experiment (Appendix H for data, Appendix I for output). The statistical significance level was set at a P of 0.05 (Zar 1984).

RESULTS

Microcosm Experiment

E. coli survival in laboratory microcosms was found to be partially dependent on the presence of living plant material (Table 3.1, Figure 3.1, Raw Data Appendix G).

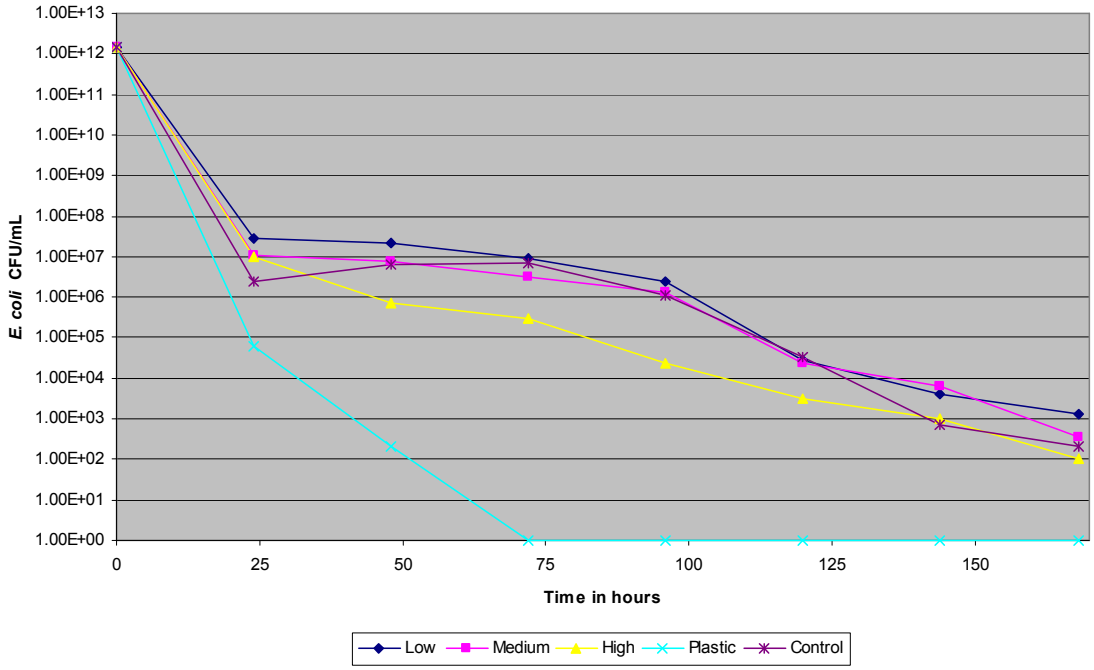

Figure 3.1: Survival of *E. coli* in lab microcosms as measured over 168 hours at three densities of plant matter (Low, Medium, and High), a plastic plant, and the control of open water.

Table 3.1: Mean *E. coli* CFU as measured over 168 hours in each treatment.

Time (h)	Low	Medium	High	Plastic	Control	No *E. coli*
0	1.4E+12	1.4E+12	1.4E+12	1.4E+12	1.4E+12	2.79E+03
24	2.84E+07	1.09E+07	9.78E+06	6.30E+04	2.40E+06	2.79E+03
48	2.10E+07	7.87E+06	6.90E+05	2.00E+02	6.25E+06	0
72	8.88E+06	3.30E+06	3.00E+05	0	6.66E+06	0
96	2.36E+06	1.32E+06	2.31E+04	0	1.14E+06	0
120	2.90E+04	2.36E+04	3.27E+03	0	3.26E+04	0
144	4.08E+03	6.10E+03	1.01E+03	0	7.30E+02	0
168	1.34E+03	3.60E+02	1.00E+02	0	2.00E+02	0

An ANOVA revealed that there were significant differences among the treatments ($p=0.000$, $F=7.503$, d.f.=4). The post-hoc Dunnett test revealed that there was no significant difference between the densities of live plants and the control ($p=0.90$). The

post hoc test also determined a significant difference between the plastic treatment and the control ($p=0.001$).

Surprisingly, the low-density treatment macrophyte microcosms had the highest final *E. coli* counts. The high density of plant matter had the lowest counts of *E. coli*. The treatment with plastic plants and no living matter displayed a sharp decline in population at 24 hours and no bacteria were measured at 72 hours. The control treatment of open water with no plant matter continued to have medium levels of *E. coli* present throughout the experiment and had more surviving bacteria at the end of the measurement period than the high density of plant matter. The negative control of the sterilized plant in lake water was determined to have a minimal level of *E. coli* present at the beginning of the experiment; the levels of *E. coli* then sharply declined and no *E. coli* were measured at 48 hours. The presence of *E. coli* in the negative control was accounted for in the determination and interpretation of the data, but the negative control was not included in the statistical analyses.

The Biolog Assay results were interpreted using a presence or absence of purple colour to indicate the use of a carbon source (Appendix J) (Biolog, Inc, Konopka et al. 1998). That a carbon source was more readily used was shown by a dark purple well, and if a carbon source was not as well suited for use by the *E. coli,* then the well was light purple. Of the 96 available carbon sources, LSSK *E. coli* utilized 21 or 21.9% (Table 3.2).

Table 3.2: The usage of carbon sources by LSSK *E. coli* as determined by a replicated Biolog GN2 Microplate Assay. Usage was determined by the presence of a purple colour in both of the replicates.

Well	Carbon Source	Low Usage	High Usage
B2	D-Fructose		X
H2	Inosine		X
A3	Dextrin		X
B3	L-Fucose	X	
C3	D-Psicose	X	
H3	Uridine	X	
H4	Thymidine	X	
C5	L-Rhamnose	X	
B6	α-D-Glucose	X	
C6	D-Sorbitol	X	
D6	D-Galacturonic Acid		X
E6	D,L-Lactic Acid		X
C7	Sucrose	X	
D7	D-Gluconic Acid		X
A8	N-Acetyl-D-Glucosamine	X	
C8	D-Trehalose	X	
B10	Maltose	X	
B11	D-Mannitol	X	
H11	α-D-Glucose-1-Phosphate	X	
B12	D-Mannose		X
H12	D-Glucose-6-Phosphate	X	

DISCUSSION

The results indicate that living plant material is not required for *E. coli* survival over long periods of time. It has been observed many times that bacterial survival in aquatic habitats is greatly increased when attached to solid matter (Byanappanahalli et al. 2003, Brettar and Hofle 1992, Baker and Orr 1986, Marsollier et al. 2004). Biofilm production and bacterial typing have been studied in detail in association with living plants and results similar to those measured in this experiment were determined (Byanappanahalli et al. 2003, Brettar and Hofle 1992, Baker and Orr 1986, Marsollier et al. 2004, Muller et al. 2001).

Of particular note in this experiment is the rapid decline in *E. coli* levels observed in the microcosm containing only a plastic plant mimic, with observed *E. coli* decreasing to zero after two days of inoculation (Figure 3.1 and Table 3.1). This experiment utilizing microcosms expanded on the experiment of Bogosian (1996) where it was observed that *E. coli* levels dropped below observable levels in non-sterilized water microcosms that had no substrate available for bacterial attachment. In a similar experiment, Brettar and Hofle (1992) utilized lake water mesocosms to determine the persistence of *E. coli*, and again the data support the findings of this present microcosm study where non-sterilized lake water with living substrate contained a higher amount of *E. coli* than the microcosm with non-sterilized lake water with plastic substrate.

There was no statistical difference observed between the different densities (low, medium, and high) of plant matter ($p<0.90$). The increased *E. coli* survival in association with any density of living plant matter can be attributed to biofilm formation. Submerged aquatic macrophytes provide a large accessible surface for microbial attachment and may create a favorable environment for bacterial attachment and eventual biofilm formation (Costerton et al. 1995, Eriksson and Weisner 1999). Many bacteria produce biofilms in natural situation, especially when in the presence of high amounts of nutrients similar to those produced by aquatic macrophytes (Costerton et al. 1995). Biofilm creation provides participating bacteria with a physiologically stable environment when compared to planktonic bacteria as well as protection from predation by protozoa and other naturally occurring bacterial predators; both of these factors lead to increased bacterial survival over a period of time (Costerton et al. 1995, Brettar and Hofle 1992). By providing a larger area for bacterial attachment and biofilm formation, the high density of

plant matter would perhaps have a decreased amount of planktonic *E. coli* but an increased amount of epiphytic *E. coli* in comparison to the low and medium plant densities. This would help to explain why the observed *E. coli* levels were opposite of those expected with the high density having the lowest amount of bacteria.

Also of note is the survival of *E. coli* in the control (open water) treatment. This result is curious and can perhaps be attributed to the water that was used in the experiment. The water was taken from Lake Superior and therefore may have provided the experimental *E. coli* increased particulate matter in the water which would allow for *E. coli* attachment and growth. Biofilm production on the sides of the Magenta Vessel was observed at the end of the experiment and the presence of this biofilm may have influenced the amount of *E. coli* observed. This result is especially curious when compared to the plastic treatment which had a severely low survival rate in comparison to the other treatments. Because the plastic plant provided a larger area for biofilm production, the experimenter would expect that the plastic treatment would result in an increased rate of *E. coli* survival when compared to the open water control. One explanation would be that the plastic plant provided a surface that would be detrimental to bacterial attachment due to an inhibitory chemical or other commercial treatment. This was attempted to be controlled for by cleaning and disinfecting the plastic plants prior to their use in the experiment. Another possible explanation of this discrepancy is that the plastic plant provided a surface for attachment and biofilm formation that was excellent enough that there were no bacteria sloughed that could be measured by sampling of the water.

The Biolog Assay determined that 21 of 96, or 21.9%, of possible carbon sources were utilized. These data indicate a narrow usage of carbon sources by the environmental *E. coli* strain LSSK. This finding was surprising because intuitively, an environmental strain of *E. coli* would be expected to have a broad usage of carbon sources to have a higher probability of survival (Button et al. 1993). However, Konopka et al. (1998) found that bacteria adapted to oligotrophic conditions grow too slowly to be accurately represented by a Biolog assay and LSSK *E. coli* was collected at Lake Superior Siskiwit Beach, an oligotrophic area. One experiment found that of 31 environmental isolates, the median usage of carbon sources in a Biolog GN Microplate was only two (Upton et al. 1989). These findings indicate that the LSSK *E. coli* are perhaps adapted to an environment with carbon sources that are not present in the Biolog Assay.

In conclusion, there was no significant association of the survivability of *E. coli* and a certain density of plant matter. In the future, it is recommended to measure *E. coli* attachment to plant matter in addition to the planktonic *E. coli* found in water for each day. In addition, Modified mTEC should be used instead of EMB, as Modified mTEC agar is highly selective for *E. coli* and this would decrease the amount of error and variability in sample analysis. Future research should also be completed to determine and compare the metabolic profiles of a variety of environmental *E. coli* strains.

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CONCLUSION

It is hypothesized that mats and stands of aquatic macrophytes provide a stable environment for *E. coli*, shielding them from such things as UV radiation, changes in pH, desiccation, and nutrient depletion. Mats of aquatic macrophytes can also provide a site of attachment and biofilm formation for *E. coli*, thus allowing persistence. It is believed that this was the first study conducted that tested the effects of mats or stands of aquatic macrophytes on *E. coli* concentrations. Laboratory and field studies were conducted to help understand the relationship between aquatic macrophytes and *E. coli*.

The laboratory microcosm experiments indicated that living plant material is not required for survival of the LSSK strain of *E. coli* over periods of time. No statistical difference was observed between the different densities of living plant matter (low, medium, high). The microcosm containing a plastic plant mimic displayed a rapid decline in *E. coli* levels. These data indicate that survival of the LSSK *E. coli* is not dependent on the presence of living substrate.

Results from the first field season found an increased amount of *E. coli* in macrophyte mats when compared to five and ten meters from the macrophyte mat, but the results showed no significant differences between the distances of five and ten meters. The second field season provided similar results to the first, with *E. coli* levels found higher in the macrophyte mat than two and five meters from the mat. Higher amounts of *E. coli* were also consistently observed in lakes with a higher density of plants. These results provide a strong argument that *E. coli* are harbored within a macrophyte mat.

Overall, the results of the field and laboratory microcosm studies found that there is a relationship between aquatic macrophytes and *E. coli*.

APPENDIX A
Influence of sampling depth on *Escherichia coli* concentrations in beach monitoring.

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Influence of sampling depth on *Escherichia coli* concentrations in beach monitoring

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ABSTRACT

While the US Environmental Protection Agency's (EPA) Beaches Environmental Assessment and Coastal Health (BEACH) Act requires coastal and Great Lakes' states to implement plans for monitoring bacterial contamination of recreational beach water, exactly how this monitoring should occur has not been regulated. This study examined differences in concentration of *Escherichia coli* in water collected from different depths and from different horizontal locations across the beach. *E. coli* concentrations were significantly different ($p<0.05$), however, when water from different depths was compared. Sampling water at depths of 30, 60, and 120 cm resulted in significantly greater *E. coli* concentrations as depth increased. Had the State of Wisconsin chosen to collect beach water monitoring samples at a shallower or deeper depth, numbers of beach closures and the potential risk to public health would have changed substantially. These data imply that a revised and standardized protocol for monitoring beach water should be adopted by all states of a monitoring region to better compare microbial contamination of beaches and protect public health.

1. Introduction

In 2000, the US Congress passed the Beaches Environmental Assessment and Coastal Health Act (BEACH Act) (US Environmental Protection Agency, 2002). The BEACH Act calls for microbial monitoring of public beaches on a routine basis in order to help protect the public health. Each US state with coastal recreational beaches established standards for allowable indicator organism concentrations in beach water and for other implementation logistics within the framework of the BEACH Act. Both Great Lake and ocean coasts are included in this monitoring effort, but for the sake of clarity only Great Lakes beaches will be discussed in this paper.

The indicator organism, *Escherichia coli* (*E. coli*), is used in Wisconsin (USA), with sampling occurring 30 cm below the surface in water with a depth of approximately 60 cm (Wisconsin DNR, 2001). *E. coli* concentrations from 0 to 234 *E. coli*/100 mL of water indicate that the water is "Good" for swimming and the beach is open. *E. coli* concentrations between 235 and 999/100 mL of water result in a "Caution" advisory, indicating that there is an increased risk of encountering disease-causing organisms when "full body submersion" occurs in beach water. Concentrations above 1000 *E. coli*/100 mL of water warrant a closure of the beach to prevent swimmer contact with potential pathogens (Wisconsin DNR, 2001).

While the BEACH Act has advanced our knowledge of the degree of microbial contamination at beaches and potentially protects the public from microbial hazards, it has been applied very differently in each of the Great Lake states that have implemented it (Table 1). For example, each state has different *E. coli* concentration closure benchmarks, and some states (MI, WI) have an additional 'advisory' *E. coli* limit (beach not closed but swimmers are cautioned). In addition to these

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Table 1 – Sampling depths and advisory and closure levels used by Great Lake states for implementation of the US EPA BEACH Act

State	Sampling depth[c] (cm)	Number of samples required per beach	Critical *E. coli* levels[a,b]	
			Advisories	Closures
Illionis	60	1 or more	n/a	235
Indiana	46	1 or more	n/a	235
Michigan	90–180	At least 3	300	1000
Mennestota	60	1 or more	n/a	235
New York	60	1 or more	n/a	235
Ohio	90	1 or more	n/a	235[d]
Pensylvania	76	1 or more	n/a	235
Wisconisn	60	1 or more	235	1000

[a] Units = colony-forming units/100 mL (CFU/100 mL) or most probable number (MPN/100 mL).
[b] Only single sample monitoring criteria are listed. Some states also allow individual locations to use the geometric mean of some number of *E. coli* results.
[c] Illinois, Minnesota, and New York specify a depth of "knee deep"–Ee have estimated this to be 60 cm.
[d] Ten percent of the samples must to be over 235 CFU/100 mL in a 30 day period for a closure.

variations in *E. coli* concentration required to trigger public notification, each state has different recommendations on sampling procedures, including the depth of the water from which the sample can be collected and how many samples should be collected during a single sampling event.

The variability in sampling methods and allowable *E. coli* concentration limits raises two important issues when making beach management decisions. First, it is difficult to assess the significance of *E. coli* concentrations in terms of actual health risk to swimmers (i.e., cases of gastroenteritis per 100 swimmers). The original epidemiological studies conducted by the United States Environmental Protection Agency (DuFour, 1984) to determine *E. coli* concentrations for closure recommendations were conducted in 'chest high' water during the 1970s. As other studies have shown, the depth of sample collection may influence the results obtained in that sample (Sampson et al., 2005). Second, differences in sampling procedures and closure limits make it difficult for groups such as the National Resources Defense Council (NRDC) and others to compare beach-monitoring statistics from one state to another. The NRDC does detailed reporting of all samples collected, locations, and percent of beach days with beach closures, but does not take into consideration sample collection depth in discussions of each state's beach monitoring program (NRDC, 2005). This poses a problem because sampling methodologies and closure limits imposed by each state may greatly influence the percent of days a beach is closed, and may be more or less protective of public health than the original US EPA recommendations. Thus, this type of comparison of percentage of days a beach is closed, or percent of samples resulting in beach closures is not a reliable method for assessing "beach health".

The overarching objective of this study was to investigate how spatial variability (depth and horizontal location) during water sample collection can influence the number of days a beach would be closed due to elevated *E. coli* concentrations. Specifically, we evaluated five Lake Superior and five Lake Michigan beaches in 2004 and 10 Lake Michigan beaches in 2005 to determine if location of sample collection played a significant role in the amount of bacterial contamination detected during a beach-monitoring season.

2. Materials and methods

2.1. *Sample sites*

During 2004, five northern Lake Michigan beaches, located in Door County, WI, USA, were utilized for our sampling study. These included Ephraim, Fish Creek, Otumba Park, Sister Bay, and Whitefish Dunes beaches (Fig. 1). In addition, five beaches along the Lake Superior shoreline of Wisconsin were studied. These included Bay View, Big Bay, Maslowski Park, Siskiwit, and Oronto Park beaches (Fig. 1). During 2005 the five Door County beaches again were studied, and five additional (Anclam, Bailey's Harbor, Ellison Bay, Lakeside, and Sunset Park beaches) were added (Fig. 1).

2.2. *Sample collection*

2.2.1. *Depth sampling*

Water samples were collected three times each week for the 14 week beach season for the Lake Michigan beaches (2004, 2005), and two times each week for Lake Superior beaches. Samples (unless otherwise noted) were collected from the center of the beach (Wisconsin DNR, 2001) in water with depths of 30, 60, and 120 cm (12, 24, and 48 inches). All beach shorelines measured less than 500 m. All collections occurred approximately 15–30 cm (6–12 in) below the water surface following US Environmental Protection Agency, (1986) standard protocols. All samples were collected into sterile, polystyrene 100 mL collection bottles (IDEXX Corp., Portland, ME). Samples were immediately placed in a cooler at 4 °C until analysis. All sites were sampled at approximately the same time each day to attempt to standardize effects such as

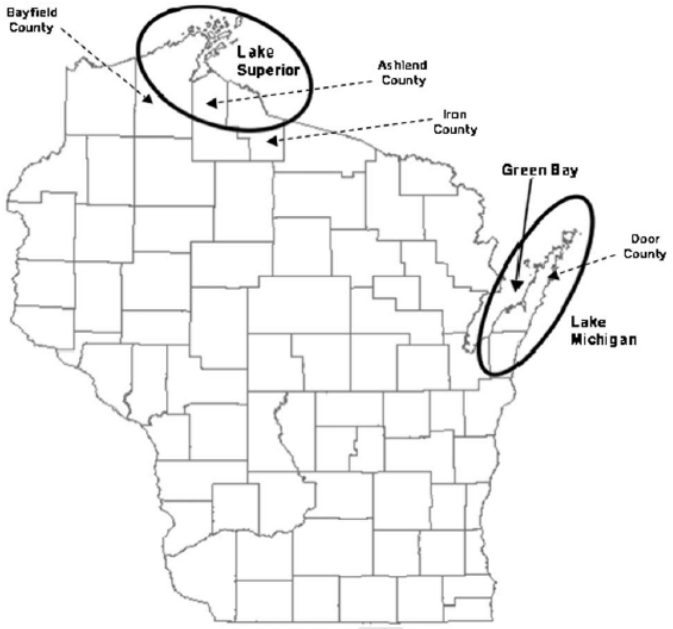

Fig. 1 – Map of the State of Wisconsin and the two areas that contained the beaches discussed in this study. Counties along the Lake Superior shore were Ashland, Bayfield, and Iron. Door County is a peninsula that is bordered by Lake Michigan on the east and Green Bay on the west.

sunlight, and bather and waterfowl numbers. *E. coli* analysis of samples was conducted within 4 h of sample collection. At all times, great care was taken to collect samples in a uniform and non-intrusive way, as not to alter water samples by disruption of sediments.

2.2.1. *Horizontal sampling*

In addition to water samples collected in the center of each beach, samples also were collected on the left and right boundaries. Fig. 2 illustrates the sampling scheme utilized for one Lake Michigan beach (Lakeside Park) during the 2005 beach season.

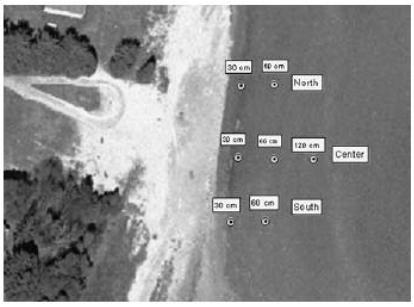

Fig. 2 – Arial view of Lakeside Park (Lake Michigan) showing the spatial sampling scheme utilized in 2005. Similar sample collection schemes were used at other Lake Michigan and Lake Superior locations.

2.3. *Sample analysis*

The defined substrate test, Colisure® with Quantitray 2000 format (IDEXX Corp., Portland, ME), was used to analyze all samples for *E. coli* (American Public Health Association, 1999). Incubation, data collection, and data analysis were conducted following the manufacturer's recommendations. The University of Wisconsin-Oshkosh laboratory utilized for all analyses is a Wisconsin State Certified Laboratory with a Quality Assurance/Quality Control plan on file with the Wisconsin Department of Agriculture, Trade and Consumer Protection. All results were reported as most probable number (MPN) of *E. coli* per 100 mL of water.

2.4. *Statistical analysis*

Statistical analysis was performed using SYSTAT 11.0. Paired *t*-tests were used to evaluate differences between sample

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sites (center, left of center, right of center) at the same depth. Analysis of Variance (ANOVA) was utilized to determine differences between sample depths (within and between transects). The null hypothesis is that there is no difference in the mean *E. coli* concentrations from different depths or from different horizontal locations across the beach and the alternate hypothesis is that there is a difference in these means. Alpha was set at 0.05 (Zar, 1984).

Table 2 – Analysis of variance (ANOVA) for differences in *E. coli* concentrations from water with depths of 30, 60, and 120 cm in Lakes Michigan and Superior

Lake	Beach	Year	n[a]	p-Value	Seasonal *E. coli* mean (60 cm)
Michigan	Anclaim	2005	28	0.044	57
Michigan	Bailey's harbor	2005	28	0.128	14
Michigan	Ellison bay	2005	28	0.570	45
Michigan	Ephraim	2004/2005	34/28	0.001/0.173	30/96
Michigan	Fish creek	2004/2005	34/28	0.001/0.016	124/162
Michigan	Lakeside	2005	28	0.005	125
Michigan	Otumba	2004/2005	34/28	0.001/0.001	134/154
Michigan	Sister bay	2004/2005	34/28	0.096/0.567	40/34
Michigan	Sunset	2005	28	0.023	259
Michigan	Whitefish dunes	2004/2005	34/28	0.132/0.009	55/66
Superior	Bayview	2004	14	0.981	42
Superior	Big bay	2004	14	0.870	12
Superior	Maslowski A	2004	28	0.000	111
Superior	Maslowski B	2004	28	0.003	121
Superior	Oronto bay	2004	14	0.921	7
Superior	Siskiwit	2004	14	0.162	49

[a] n = number of sampling events.

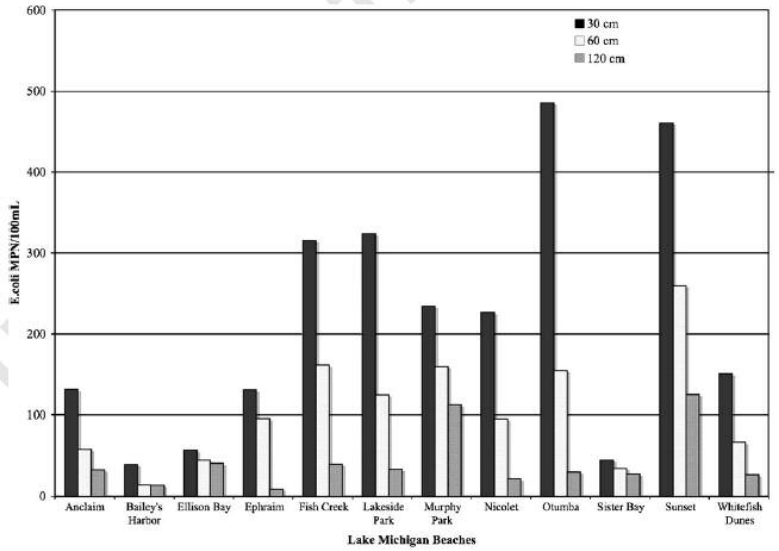

Fig. 3 – Mean seasonal *E. coli* concentrations measured in water with a depth of 30, 60, and 120 cm at ten Lake Michigan beaches. N = 28 per location and depth.

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3. Results and discussion

3.1. Horizontal sampling

Water samples were collected from three locations (center, left-most border, and right-most border) horizontally across beaches in Door County, WI (Lake Michigan) in 2004 ($n = 5$ beaches) and 2005 ($n = 10$ beaches). Samples were collected in water with a depth of 30, 60, or 120 cm and were analyzed for *E. coli* concentration. Data were analyzed with a paired *t*-test. Regardless of year (or combination of both years), there is no significant difference in *E. coli* concentration measured at different horizontal locations ($p < 0.05$) (data not shown). For these beaches, it appears that there is no substantial run-off input from storm sewers, streams, or other conveyances on the periphery of the beaches.

3.2. Depth sampling

E. coli concentrations measured in waters with three different depths (30, 60, 120 cm) were analyzed for both Lake Michigan (2004, 2005) and Lake Superior (2004) beaches. Using analysis of variance (Zar, 1984), differences in *E. coli* concentrations at these depths were determined (Table 2). For individual Lake Michigan beaches in 2004 and 2005, 60% showed a significant difference between measured *E. coli* concentrations at the three depths ($p \leq 0.05$). When these beaches are looked at as a county-wide aggregate for either 2004 or 2005, however, there is a very strong significant difference in *E. coli* concentration across depths ($p < 0.01$).

At Lake Superior beaches these differences were less pronounced; only 20% of beaches showed a significant difference ($p < 0.05$) in *E. coli* concentrations measured at the three depths (Table 2). Again, however, if the beaches are analyzed as an aggregate, a strong statistical difference is observed ($p < 0.01$).

For all beaches tested on Lake Michigan, the seasonal average *E. coli* concentration from water with a depth of 30 cm was greater than that measured in water with a depth of 60 cm, and average *E. coli* concentration from water with a depth of 60 cm was always greater than from 120 cm (Fig. 3). Similar results were obtained from Lake Superior beach seasonal *E. coli* concentrations (data not shown).

When seasonal *E. coli* concentration averages are compared statistically with a paired *t*-test, some differences between depths show significance, while others do not (Tables 3 and 4). When examining 2004 data separately from 2005, five of ten beaches tested in 2005 showed statistical differences in *E. coli* concentrations between water of 30 and 120 cm ($p < 0.05$). When data from 2004 and 2005 are combined (Table 5), five of five beaches showed statistical differences between *E. coli* concentrations detected from water with 30 vs. 120 cm depths, and three of five from waters with 60 vs. 120 cm depths,

Table 3 – Comparison of *E. coli* concentrations at specific sampling depths for 2004 Lake Michigan sampling data, using paired *t*-tests

Beach	Depth comparison (cm)	*p*-Value
Ephraim	30 vs. 60	0.01
	60 vs. 120	0.05
	30 vs. 120	0.00
Fish creek	30 vs. 60	0.01
	60 vs. 120	0.11
	30 vs. 120	0.00
Otumba	30 vs. 60	0.61
	60 vs. 120	0.16
	30 vs. 120	0.05
Sister bay	30 vs. 60	0.02
	60 vs. 120	0.60
	30 vs. 120	0.12
Whitefish dunes	30 vs. 60	0.74
	60 vs. 120	0.50
	30 vs. 120	0.28

Table 4 – Comparison of *E. coli* concentrations at specific depths for 2005 Lake Michigan sampling data, using paired *t*-tests

Beach	Depth comparison (cm)	*p*-Value
Anclaim	30 vs. 60	0.143
	60 vs. 120	0.191
	30 vs. 120	0.068
Bailey's harbor	30 vs. 60	0.170
	60 vs. 120	0.788
	30 vs. 120	0.127
Ellison bay	30 vs. 60	0.907
	60 vs. 120	0.662
	30 vs. 120	0.566
Ephraim	30 vs. 60	0.432
	60 vs. 120	0.082
	30 vs. 120	0.050
Fish creek	30 vs. 60	0.137
	60 vs. 120	0.060
	30 vs. 120	0.025
Lakeside	30 vs. 60	0.346
	60 vs. 120	0.180
	30 vs. 120	0.041
Otumba	30 vs. 60	0.082
	60 vs. 120	0.032
	30 vs. 120	0.002
Sister bay	30 vs. 60	0.566
	60 vs. 120	0.408
	30 vs. 120	0.231
Sunset	30 vs. 60	0.488
	60 vs. 120	0.094
	30 vs. 120	0.030
Whitefish dunes	30 vs. 60	0.070
	60 vs. 120	0.032
	30 vs. 120	0.011

depths. Differences in *E. coli* concentrations in water with 30 and 60 cm depths were less frequently seen (one of five beaches).

At Lake Superior beaches seasonal average *E. coli* concentrations were greater from water with a depth of 30 cm, than from deeper water. Only one individual beach ($n = 5$) showed statistically significant differences, however, between *E. coli* concentrations measured at 30 vs. 60 cm, 60 vs. 120 cm, or 30 vs. 120 cm (data not shown). Again, relatively low *E. coli* concentrations were measured at all depths for these beaches, and differences become less distinct.

Table 5 – Comparison of *E. coli* concentrations at specific depths for combined 2004 and 2005 Lake Michigan sampling data, using paired *t*-tests

Beach	Depth comparison (cm)	*p*-Value
Ephraim	30 vs. 60	0.1104
	60 vs. 120	0.0434
	30 vs. 120	0.0037
Fish creek	30 vs. 60	0.0046
	60 vs. 120	0.0150
	30 vs. 120	0.0003
Otumba	30 vs. 60	0.0785
	60 vs. 120	0.0090
	30 vs. 120	0.0003
Sister bay	30 vs. 60	0.1585
	60 vs. 120	0.3550
	30 vs. 120	0.0518
Whitefish dunes	30 vs. 60	0.2873
	60 vs. 120	0.2680
	30 vs. 120	0.0248

3.3. *Beach management implications*

From a beach management standpoint these depth-dependent differences in *E. coli* concentration become even more important. Wisconsin's two-tiered beach notification system includes a "Beach Advisory" (Caution) when concentrations exceed 235 *E. coli*/100 mL water and a "Beach Closure" when concentrations exceed 1000 *E. coli*/100 mL water. The Lake Michigan beaches examined in this study had 109 advisories and 20 closures during the combined 2004 and 2005 seasons, with samples collected in water with depth of 60 cm. If Wisconsin had chosen to collect samples from water with a depth of 120 cm (as Michigan does), these same beaches would have had 19 advisories and only 2 closures for the same time period (Table 6). Beaches from Lake Superior would have had no advisories or closures, using the 120 cm depth for water collection (Table 6). The aforementioned differences illustrate the difficulties in comparing beach monitoring results (i.e., percent of samples resulting in closures, or total number of beach days with a closure, etc.) from state-to-state when collection protocols are different.

Water collection from shallow depths (30 cm) has not been implemented by any Great Lake states (Table 1). There are

Table 6 – Potential closures and advisories for each beach location for 2004 and 2005, had *E. coli* concentrations from water samples from other depths been utilized Note that Wisconsin uses *E. coli* concentrations from samples collected at 60 to issue advisories or closures

Beach	30 cm		60 cm		120 cm	
	Advisories	Closures	Advisories	Closures	Advisories	Closures
Lake Michigan						
Anclam	10 (12.0)	3 (3.6)	2 (2.4)	0 (0)	1 (1.2)	0 (0)
Baileys harbor	3 (3.6)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Ellison bay	5 (6.0)	0 (0)	4 (4.8)	0 (0)	0 (0)	0 (0)
Ephraim	27 (14.5)	6 (3.4)	10 (5.4)	3 (1.6)	0 (0)	0 (0)
Fish creek	63 (33.7)	22 (11.8)	31 (16.7)	3 (1.6)	0 (0)	0 (0)
Lakeside	21 (22.6)	9 (10.7)	8 (9.5)	3 (3.6)	1 (1.2)	0 (0)
Otumba	54 (29.0)	16 (8.6)	21 (11.3)	4 (2.2)	6 (4.6)	0 (0)
Sister bay	14 (7.5)	0 (0)	7 (3.8)	0 (0)	5 (3.9)	0 (0)
Sunset	31 (36.9)	13 (15.5)	19 (22.6)	6 (7.1)	3 (10.7)	1 (3.6)
White fish	30 (16.1)	5 (2.7)	7 (3.8)	1 (0.5)	3 (2.3)	1 (0.8)
Total	258	74	109	20	19	2
Lake superior (2004 only)						
Big bay	0 (0)	0(0)	0 (0)	0 (0)	0 (0)	0 (0)
Bayview	1 (3.9)	0(0)	0 (0)	0 (0)	0 (0)	0 (0)
Maslowski	11 (23.9)	3(6.5)	5 (11.1)	2 (4.4)	0 (0)	0 (0)
Oronto bay	0 (0)	0(0)	0 (0)	0 (0)	0 (0)	0 (0)
Siskiwit	2 (8.7)	0(0)	1 (4.2)	0 (0)	0 (0)	0 (0)
Total	13	3	6	2	0	0

Values in parenthesis indicate the percent of samples collected at that depth that would have resulted in an advisor.

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many problems associated with collection at this depth, such as frequent disturbance of beach sediments and contamination of water with sand or algae. This portion of the beach, however, is frequented by children who are more likely to consume beach water and have been shown to be at greater risk for recreational water-acquired gastrointestinal disease (Proctor and Davis, 2000; Paunio et al., 1999) and to suffer more severe consequences from illness. The depth-dependent differences in *E. coli* concentrations presented here would indicate that whatever the *E. coli* concentration measured from water with a depth of 60 cm, the concentration of this organism in more shallow water will be greater (and the risk to public health likely increased). These findings suggest that new epidemiologic studies for determination of the real risk to bathers at Great Lakes beaches may be necessary, and include risk of gastrointestinal illness when full-body exposure occurs at different depths of water, when water is ingested vs. simply contacting the skin, and when the bather body size is small. Standardization of sampling protocols across states is crucial to the accurate comparison of results across state borders.

4. Conclusions

The following conclusions can be drawn from the data presented in this paper:

1. For the beaches investigated in this study, sampling locations horizontally spaced across beaches do not significantly change *E. coli* concentrations measured from recreational water.
2. At many locations, the depth of water from which samples are collected does have a significant impact on *E. coli* concentrations measured in beach water, with samples collected in shallower water having greater *E. coli* concentrations than samples collected from deeper water.
3. In Wisconsin, changing the depth of water from which samples were collected (from 60 to 120 cm) in 2005 would have resulted in approximately six times fewer advisory and ten times fewer closures issued.
4. Not all states of the Great Lakes region collect water samples for beach monitoring from water with the same depth, or use the same criteria for beach closures or advisories.
5. Standardization of sampling depths used by the Great Lakes states is crucial for making comparisons of microbial contamination between states, and for ensuring better protection of public health.

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APPENDIX B
Raw Data from 2005 Field Season.

Table B.1: List of abbreviations for 2005 field sampling season.

Abbreviation	Meaning
UG	Upper Gresham Lake
LS	Little St. Germain Lake
BS	Big Sand Lake
LL	Long Lake
IMA	In mat sample 1
IMB	In mat sample 2
5RA	5 m right of mat sample 1
5RB	5 m right of mat sample 2
5LA	5 m left of mat sample 1
5LB	5 m left of mat sample 2
10RA	10 m right of mat sample 1
10RB	10 m right of mat sample 2
10LA	10 m left of mat sample 1
10LB	10 m left of mat sample 2

Table B.2: Raw *E. coli* numbers from Upper Gresham Lake Summer 2005 (see table B.1 for abbreviation key).

Day	UG IMA	UG IMB	UG 5RA	UG 5RB	UG 5LA	UG 5LB	UG 10RA	UG 10RB	UG 10LA	UG 10LB
1	83.3	71.2	5.2	1	90.7	30.5	4.1	2	1	2
2	61.3	41.9		47.9	119.8	131.3	93.2	56.5	78.8	172.3
3	18.9	101.7	8.6	6.3	6.3	30.7	12.2	17.3	91	7.2
4	16.1	3	3.1	11	13.4	9.7	7.4	7.4	19.9	14.6
5	151.5	35.5	1	1	2	5.2	2	1	4.1	4.1
6	24.3	38.9	6.3	3	29.5	6.3	12.1	2	17.5	14.6
7	5.2	2	1	0	3	1	0	0	2	1
8	186	193.5	20.1	21.6	73.3	66.3	11	13.2	66.3	39.3
9	4.1	5.2	4.1	0	4.1	1	0	0	1	0
10	6.2	6.3	3.1	3	3	5.1	3.1	1	3.1	4.1

Table B.3: Raw *E. coli* numbers from Little Saint Germain Lake, Summer 2005 (see table B.1 for abbreviation key).

Day	LS IMA	LS IMB	LS 5RA	LS 5RB	LS 5LA	LS 5LB	LS 10RA	LS 10RB	LS 10LA	LS 10LB
1	24.6	13.2	6.3	11	7.4	5.2	4.1	1	3.1	6.3
2	48.8	71.9	2	4.1	18.5	16	9.7	2	12.2	10.9
3	40.2	14.3	18.7	9.8	3	4.1	10.9	5.2	3.1	1
4	49.5	93.3	117.8	116.9	5.2	1	44.1	49.5	1	8.4
5	31.3	10.9	3.1	24.6	3	2	7.4	6.3	1	3
6	387.3	71.7	78.9	46	59.9	76.8	35	40.4	143	125
7	10.8	6.3	4.1	7.4	12.1	7.3	3.1	2	6.3	4
8	2	5.1	0	6.3	6.3	8.6	0	2	6.3	6.3
9	25.6	13.4	0	2	5.2	4.1	7.4	12.1	0	5.2
10	1	2	0	0	1	1	0	0	3.1	4.1

Table B.4: Raw *E. coli* numbers from Big Sand Lake, Summer 2005 (see table B.1 for abbreviation key).

Day	BS IMA	BS IMB	BS 5RA	BS 5RB	BS 5LA	BS 5LB	BS 10RA	BS 10RB	BS 10LA	BS 10LB
1	8.6	71.2	32.3	24.1	7.4	4.1	14.8	25.3	1	1
2		26.9	10.8	6.3	7.4	18.7	3.1	8.6	9.7	7.4
3	71.2	12.2	1	2	2	1	1	1	2	2
4	141.4	68.1	47.1	65	41.6	58.8	88.2	86	30.5	45.7

Table B.5: Raw *E. coli* numbers from Long Lake, Summer 2005 (see table B.1 for abbreviation key).

Day	LL IMA	LL IMB	LL 5RA	LL 5RB	LL 5LA	LL 5LB	LL 10RA	LL 10RB	LL 10LA	LL 10LB
1	151.5	307.6	48	25.9	137.6	117.8	16.1	21.6	42.5	49.5
2	39.9	28.5	11	10.8	17.3	16.1	16.1	37.3	19.9	17.5
3	58.3	9.7	6.3	8.4	20.3	5.2	4.1	6.2	31	8.5
4	31.8	24.3	6.3	4.1	38.2	36.8	9	16.9	18.3	22.3
5	198.9	38.2	14.8	26.5	17.3	22.8	62	40.4	13.2	12.2
6	980.4	816.4	435.2	461.1	866.4	920.8	517.2	517.2	648.8	686.7
7	18.7	47.4	4.1	2	7.4	10.9	17.3	1	2	1
8	34.5	25.3	21.3	7.5	37.9	51.2	7.3	18.7	67.7	78
9	118.7	45.7	12.2	11	10.9	7.4	17.3	23.3	10.8	21.3
10	62.4	79.4	2	5.2	2	3.1	5.2	5.2	7.4	5.2

APPENDIX C
Raw Data from 2006 Field Season.

Table C.1: List of abbreviations for 2006 field sampling season.

Abbreviation	Meaning
UG	Upper Gresham
SL	Star Lake
LVD	Lac Vieux Desert
LL	Long Lake
IRA	In mat right sample 1
IRB	In mat right sample 2
ILA	In mat left sample 1
ILB	In mat left sample 2
2RA	2 m right sample 1
2RB	2 m right sample 2
2LA	2 m left sample 1
2LB	2 m left sample 2
5RA	5 m right sample 1
5RB	5 m right sample 2
5LA	5 m left sample 1
5LB	5 m left sample 2

Table C.2: Raw *E. coli* numbers from Upper Gresham Lake, Summer 2006 (see table C.1 for list of abbreviations).

Day	UG 5Ra	UG 5Rb	UG 2Ra	UG 2Rb	UG IRa	UG IRb	UG ILa	UG ILb	UG 2La	UG 2Lb	UG 5La	UG 5Lb
1	0	0	0	1	1	0	5.1	44.8	1	3.1	5.2	2
2	1	1	18.9	109.8	3	1	3.1	2	2	3.1	4.1	2
3	2	0	5.1	5.2	14.8	28.2	93.3	128.1	3.1	2	3.1	1
4	1	1	5.1	10.9	64	16	6	7.1	1	7.5	19.7	28.8
5	0	2	1	0	3.1	5.2	3.1	73.8	0	0	0	2
6	1	1	1	3.1	2	2	3.1	4.1	1	0	0	4
7	0	4.1	11	6.3	22.6	19.7	1	8.5	1	4.1	0	3
8	3.1	2	0	0	1	2	2	1	0	1	0	0
9	0	0	0	0	2	1	0	0	1	0	0	0
10	2	0	1	2	7.5	16.4	0	0	0	0	0	0

Table C.3: Raw *E. coli* numbers from Star Lake, Summer 2006 (see table C.1 for list of abbreviations).

Day	SL 5Ra	SL 5Rb	SL 2Ra	SL 2Rb	SL IRa	SL IRb	SL ILa	SL ILb	SL 2La	SL 2Lb	SL 5La	SL 5Lb
1	0	4.6	1	3.1	0	3	4.1	1	4.1	0	0	0
2	1	1	1	0	2	2	2	4	1	5	4	1
3	0	0	0	1	3.1	2	2	2	2	3	3.1	5.2
4	2	2	4.1	0	2	5.2	3	1	1	0	0	0
5	1	2	1	4.1	1	2	5.2	3.1	9.8	3.1	5.2	2
6	2	0	1	0	1	0	3.1	1	0	0	0	2
7	1	1	0	3.1	0	2	3.1	5.2	0	4.1	4.1	5.2
8	5.2	1	1	2	4.1	5.2	2	3	7.5	3.1	3.1	3.1
9	1	0	0	0	0	2	0	2	1	2	2	0
10	3.1	3.1	0	1	2	6.3	1	0	0	3.1	1	0

Table C.4: Raw *E. coli* numbers from Lac Vieux Desert, Summer 2006 (see table C.1 for list of abbreviations).

Day	LVD 5Ra	LVD 5Rb	LVD 2Ra	LVD 2Rb	LVD IRa	LVD IRb	LVD ILa	LVD ILb	LVD 2La	LVD 2Lb	LVD 5La	LVD 5Lb
1	3.1	1	1	1	1	1	2	4.1	0	0	2	1
2	0	2	4	1	6	5	9.2	6	3	2	6	11.1
3	4.1	3.1	11	7.5	20.3	24.3	201.4	387.3	66.3	88.4	38.4	34.5
4	95.9	46.4	9.7	27.5	12.2	6.3	4.1	3.1	3.1	1	X	3
5	4.1	8.5	7.5	9.7	3	11	13.5	4.1	8.6	7.5	X	2
6	4.1	3.1	6.3	4.1	4.1	6.2	3.1	4.1	6.3	5.2	3.1	6.3
7	12.2	6.3	7.5	9.7	9.8	13.4	15.8	14.6	8.5	8.4	11	9.7
8	14.8	9.8	34.5	37.3	27.9	42.6	20.1	24.6	63.8	34.5	81.3	90.8
9	8.4	2	3	3.1	5.2	21.6	3.1	7.4	3.1	4.1	1	2
10	8.6	1	3.1	1	9.7	4.1	7.5	5.2	8.6	6.3	6.3	5.2

Table C.5: Raw *E. coli* numbers from Long Lake, Summer 2006 (see table C.1 for list of abbreviations).

Day	LL 5Ra	LL 5Rb	LL 2Ra	LL 2Rb	LL IRa	LL IRb	LL ILa	LL ILb	LL 2La	LL 2Lb	LL 5La	LL 5Lb
1	648.8	547.5	1046	2419.6	488	435	1733	1414	548	920.8	142.1	119
2	8	8.1	6	4	14.2	20.6	13.2	6	6	5	6	5
3	83.9	45.7	32.7	36.9	31.8	36.9	8.5	7.5	22.6	23.1	32.3	18.7
4	6.3	5.2	2	5.2	7.4	2	12.1	6.3	2	3.1	1	8.5
5	0	0	2	2	3.1	5.2	16	13.5	6.3	6.3	6.3	6.3
6	21.7	17.3	17.5	14.5	12	22	435.2	365.4	39.3	47.3	12.2	13.4
7	17.5	21.6	14.6	12.1	24.3	27.2	34.1	26.2	8.5	11	5.2	13.4
8	4.1	0	5.2	9.8	7.5	8.5	17.5	7.4	12.1	8.5	13.4	12.2
9	4.1	4.1	2	4.1	4.1	3.1	8.5	8.5	7.4	9.6	2	9.8
10	6.3	5.2	10	4.1	9.5	10.7	19.7	23.3	41.7	42.8	54.6	56.3

APPENDIX D
Raw abiotic data from 2006 field season

Table D.1: Abiotic factors measured at Upper Gresham Lake 2006. pH, dissolved oxygen (DO), and temperature (temp) were measured.

Day	pH	DO (mg/L)	temp (oC)
1	7.18	x	24.8
2	x	x	22.3
3	7.19	0.78	26
4	7.19	0.02	22.4
5	7.8	0.1	23.3
6	7.17	0.4	22.5
7	7.17	0.72	24.5
8	7.19	1.16	19.6
9	7.22	1.3	19.5
10	7.19	1.35	22.5

Table D.2: Abiotic factors measured at Star Lake 2006. pH, dissolved oxygen (DO), and temperature (temp) were measured.

Day	pH	DO (mg/L)	Temp (oC)
1	7.19	x	24.7
2	x	x	21.7
3	7.2	0.85	26.5
4	7.18	0.06	23.8
5	7.17	0.09	25
6	7.17	0.4	27.6
7	7.16	0.73	24.8
8	7.16	1.16	23.8
9	7.19	1.24	22.3
10	7.17	1.3	24.6

Table D.3: Abiotic factors measured at Lac Vieux Desert 2006. pH, dissolved oxygen (DO), and temperature (temp) were measured.

Day	pH	DO (mg/L)	Temp (oC)
1	x	x	21.8
2	x	x	23
3	7.21	0.57	26.9
4	7.17	0.05	28.1
5	7.18	0.08	30.6
6	7.18	0.36	30.6
7	7.18	0.7	26.9
8	7.15	0.98	25.4
9	7.16	1.11	24.1
10	7.14	1.3	26.6

Table D.4: Abiotic factors measured at Long Lake 2006. pH, dissolved oxygen (DO), and temperature (temp) were measured.

Day	pH	DO (mg/L)	temp (oC)
1	x	x	21.8
2	x	x	22.9
3	7.21	0.55	27
4	7.18	0.08	29.2
5	7.18	0.08	25
6	7.18	0.37	30
7	7.17	0.69	26.5
8	7.15	1.16	24.7
9	7.16	1.16	24.8
10	7.14	1.31	25.5

APPENDIX E
Detailed Laboratory Sampling Procedure for *E. coli* Enumeration.

Aquatic Macrophyte and *E. coli* Laboratory Experiment (Survivability and Attachment)

1. Label 16 sterile, empty, test tubes with the appropriate sample name

2. Bring with you to the Green House:
   14-5 mL pipettes
   Pipette aid
   Gloves
   Bag for the used pipettes to go in
   Long Forceps

3. In the Green House:
   Turn off the shaker if it is running.
   Sample 2 mL from each flask and place into the appropriate test tube. From each of the "mat" flasks remove a small bit of plant material (approx. 1 g).

4. Back up to Dr. Becker's Lab (2001 NSF), follow the Serial dilutions protocol:

5. Turn on and disinfect the flow hood. Set up 3 plate spreaders (hockey sticks) in a flask filled half way with 70% Ethanol, turn on flame.

6. Gather and place into the fume hood test tubes filled with 9.90 mL PBW
   (tt 9.99 mL = 4 x # samples collected)

7. $10^{-2:}$ Pipette 100 µL of the sample into test tube containing 9.90 mL PBW. Discard pipette tip. This first dilution is 10^{-2}. Vortex 10^{-2} solution (see picture).

8. $10^{-4:}$ Pipette 100 µL of this 10^{-2} solution into a test tube containing 9.90 mL PBW. Discard pipette tip. This second dilution is $10^{-4.}$ Vortex the 10^{-4} solution.

9. 10^{-6}: Pipette 100 µL of this 10^{-4} solution into a test tube containing 9.90 mL PBW.

10. $10^{-8:}$ Pipette 100 µL of this 10^{-6} solution into a test tube containing 9.90 mL PBW.

11. Repeat steps 9-12 for each of the test tubes filled with water collected from the Green House (14x).

12. Plant matter samples: weigh out 1 g (discard any excess), place in a blue capped conical tube (containing 9 mL PBW) and shake well for 2 min. Using the supernatant solution, follow the serial dilution protocol (steps 9-12) being careful

to get none of the plant matter when you pipette.

13. Label agar (EMB) plates with each dilution (initials, date, bacteria, sample name).

14. To plate the samples make sure you vortex the test tubes. For the appropriate dilutions and amounts to put on each plate see the key below (or the picture). After the sample is on the appropriate plate, discard the pipette tip. Pull the spreader from the EtOH and flame. Wait 30s for the glass to cool. With the plate on the spinner, open the lid of the plate, place the glass on the sample and spin the plate. After the sample is spread close the plate lid, flame the hockey stick and place it back into the EtOH. Repeat with each of the dilutions.

    10^{-2}: pipette 1 mL of the 10^{-2} tt onto the Petri plate.
    10^{-3}: pipette 0.1 mL (100 µL) of the 10^{-2} tt onto the Petri plate.
    10^{-4}: pipette 1 mL of the 10^{-4} tt onto the plate
    10^{-5}: pipette 0.1 mL of the 10^{-4} onto the plate
    10^{-6}: pipette 1 mL of the 10^{-6} onto the plate
    10^{-7}: pipette 0.1 mL of the 10^{-6} onto the plate
    10^{-8}: pipette 1mL of the 10^{-8} onto the plate
    10^{-9}: pipette 0.1 mL of the 10^{-8} sample onto the plate.

15. Clean up hood and place contaminated PBW in appropriate area, etc.

16. Invert the spread plates, tape them and label them with your initials, the date and time, the bacteria and when they should be read (48hrs).

17. Turn off hood and spray down with 70% ethanol.

18. Finish any clean up, check if the pipette tips need to be refilled (refill if needed) and notify lab manager if any more agar/PBW/test tubes need to be made.

APPENDIX F
Instructions for use of the Biolog GN2 Microplate

Protocol for GN2 MicroPlate

1) Grow cells on BUG agar.
    a. 37°C for 24 hours
2) Turbidity Range:
    a. Set 100% transmittance using un inoculated GN/GP-IF tube
    b. Read transmittance using Turbidity Standard (61%) (can vary) ____71.1____ (% transmittance).
    c. Blank turbidimeter (100% transmittance) with blank GN/GP-IF
    d. Add 3 drops of sodium thioglycolate to the GN/GP-IF tube to be used.
3) Prepare Suspension of Bacteria:
    a. Remove cells from plate with sterile swab transfer to the GN/GP-IF tubes (make sure no clumps, break any above the liquid line).
    b. Adjust density until the % transmittance is what the turbidity standard was +/- 2% (see 2b).
        i. Lower density by adding more GN/GP-IF
        ii. Raise density by adding more cells
4) Immediately inoculate the Micro Plate
    a. 150 µL per well
    b. Cover when done
5) Incubate Micro Plate under same conditions as #1
    a. Place Micro Plate in a plastic container with wet paper towel when incubating to prevent drying out.
    b. Incubate for 4-6 hours.
6) Read plate using A-1 as reference
    a. Wells with same transmittance as A-1 are (-) (no purple) (Carbon source not utilized).
    b. Wells with difference transmittance as A-1 are (+) (purple) (Carbon source utilized).

Adapted from "GN2 MicroPlate Instructions for Use" Biolog, Inc. 21124 Cabot Blvd. Hayward, CA 94545 www.biolog.com Copyright September 2004.

APPENDIX G
Raw data from the Determination of *Escherichia coli* Survival in Laboratory Microcosm Experiment.

Key:

1 P: Plant + H20 only
2 E: Plastic Plant + E. coli
3 C: H20 + E. coli
4 L: Low density plant +E. coli
5 M: Medium density plant + E. coli
6 H: High density plant + E. coli

Hours after initial	Sample Name	CFU	Notes
0	broth	1.4E+12	
24	P1a	4200	Citrate +
	P1b	4400	no E. coli!
	P2a	6500	
	P2b	2100	
	P3a	1200	
	P3b	800	
	P4a	1000	
	P4b	1100	
	P5a	3500	
	P5b	3100	
	E1a	0	
	E1b	0	
	E2a	0	
	E2b	0	
	E3a	0	
	E3b	630000	
	E4a	0	
	E4b	0	
	E5a	0	
	E5b	0	
	C1a	2180000	RH
	C1b	tntc	contam
	C2a	2260000	
	C2b	2340000	
	C3a	2450000	cluster
	C3b	3160000	
	C4a	2440000	
	C4b	2270000	cluster
	C5a	1920000	
	C5b	2550000	cluster
	L1a	8800000	
	L1b	9800000	
	L2a	94000000	

	L2b	99000000		
	L3a	11700000		
	L3b	10300000		
	L4a	13400000		
	L4b	11400000		
	L5a	13300000		
	L5b	12400000		
	M1a	12100000		
	M1b	10600000		
	M2a	11600000		
	M2b	11800000		
	M3a	10300000		
	M3b	11100000		
	M4a	8900000		
	M4b	10600000		
	M5a	11400000		
	M5b	10200000		
	H1a	6900000		
	H1b	6400000		
	H2a	11500000		
	H2b	9600000		
	H3a	9100000		
	H3b	9100000		
	H4a	8600000		
	H4b	9600000		
	H5a	14700000		
	H5b	12300000		
48	E1a	0		
	E1b	0		
	E2a	200		
	E2b	0		
	E3a	0		
	E3b	0		
	E4a	0		
	E4b	0		
	E5a	0		
	E5b	0		
	C1a	6400000		
	C1b	9400000		
	C2a	6100000		
	C2b	7400000		
	C3a	4100000	large cluster	
	C3b	2900000		
	C4a	3700000		
	C4b	6300000		
	C5a	8000000		
	C5b	8200000		
	L1a	tntc		
	L1b	41000000		cluster
	L2a	14900000		

	L2b	15500000	
	L3a	18200000	
	L3b	15400000	
	L4a	tntc	
	L4b	tntc	
	L5a	tntc	
	L5b	tntc	Citrate -
	M1a	12400000	
	M1b	16800000	
	M2a	8600000	
	M2b	7400000	
	M3a	1070000	
	M3b	1360000	
	M4a	2230000	
	M4b		problems with M and H due to spreading contamination, counting only E. coli (Citrate -)
	M5a	13100000	at least 4 different spp of bacteria
	M5b		
	H1a		
	H1b		
	H2a	930000	
	H2b	1000000	
	H3a		
	H3b	410000	
	H4a		
	H4b		
	H5a	390000	
	H5b	720000	
72	E1a		no more #'s due to spreading bacteria, not E. coli (citrate +)
	E1b		not sure where came from (Aeromonas in H2O?)
	E2a		
	E2b		
	E3a		
	E3b		
	E4a		
	E4b		
	E5a		
	E5b		
	C1a	5700000	controls all have contam, but E. coli is most prevalent (id from the ref plates).
	C1b	5500000	
	C2a	7000000	
	C2b	5200000	
	C3a	8500000	
	C3b	6900000	
	C4a	9000000	
	C4b	7100000	
	C5a	3300000	
	C5b	8400000	
	L1a	141000	

	L1b	11100000	citrate -
	L2a	7300000	
	L2b	14500000	
	L3a	16400000	
	L3b	10500000	
	L4a	9900000	
	L4b	13800000	
	L5a	3900000	
	L5b	1300000	
	M1a	3600000	GB
	M1b	3200000	
	M2a	3300000	
	M2b	4300000	
	M3a	2400000	
	M3b	2700000	
	M4a	3900000	
	M4b	2500000	
	M5a	4000000	
	M5b	3100000	
	H1a		sparse e. coli, mostly contam.
	H1b		
	H2a	200000	
	H2b		
	H3a	100000	
	H3b		
	H4a		atleast 5 other species present.
	H4b		
	H5a	400000	
	H5b	500000	
96	P	no E. coli	
	E	TNTC, spreading contam	
	C1a	1180000	citrate -
	C1b	4600000	
	C2a	50000	
	C2b	150000	
	C3a	180000	
	C3b	500000	
	C4a	110000	
	C4b	700000	
	C5a	2200000	
	C5b	1700000	
	L1a	6300000	
	L1b	4900000	
	L2a		nothing, only spreading contam
	L2b	400000	
	L3a	10000	
	L3b		clusters (not E. coli)
	L4a		spreading contam only@!
	L4b		
	L5a	200000	

	L5b		
	M1a	140000	
	M1b	100000	
	M2a	3900000	
	M2b	2300000	
	M3a	500000	
	M3b	1000000	
	M4a		no distinct colonies, only sheeing on plates
	M4b		
	M5a		
	M5b		
	H1a	7000	
	H1b	10000	many problems with accuracy due to contamination taking over (atleast 5 spp see)
	H2a	43000	
	H2b	45000	
	H3a	10000	
	H3b	60000	
	H4a	2000	
	H4b		
	H5a	14000	
	H5b	17000	
120	P		
	E	still lawn with citrate + colonies	
	C1a	83000	
	C1b	106000	
	C2a	1000	
	C2b	2000	
	C3a	6000	
	C3b	12000	
	C4a	13000	
	C4b	10000	
	C5a	43000	
	C5b	50000	
	L1a	50000	
	L1b	160000	
	L2a	16000	fungal contam
	L2b	14000	
	L3a	9000	
	L3b	4000	
	L4a	0	
	L4b	12000	
	L5a	15000	
	L5b	10000	
	M1a	30000	fungal contam
	M1b	21000	
	M2a	43000	
	M2b	23000	
	M3a	35000	
	M3b	45000	
	M4a	5000	

	M4b	13000		
	M5a	12000		
	M5b	9000		
	H1a	1100		
	H1b	900		
	H2a	2100		
	H2b	1200		
	H3a	1600		
	H3b	900	spreading contam	
	H4a	2500		clump
	H4b	400		
	H5a	9000		
	H5b	13000		
144	P		fungal contam. Many colonies, but definite decrease	
	E	10-3 still full lawn		
	C1a	400		
	C1b	400		
	C2a	5000		
	C2b	1000		
	C3a	300		
	C3b	0	spreading contam	
	C4a	0		
	C4b	0		
	C5a	200		
	C5b	0		
	L1a	2000		
	L1b	5000		
	L2a	5000		
	L2b	2000		
	L3a	18000		fungus
	L3b	5000		
	L4a	2000		
	L4b	1500		
	L5a	200		fungus
	L5b	100		
	M1a	800		
	M1b	200		
	M2a	13000		
	M2b	7000		
	M3a	10000		
	M3b	10000		
	M4a	9000		
	M4b	8000		fungus
	M5a	1000	spreading cluster	
	M5b	2000		
	H1a	1100		
	H1b	1600		
	H2a	1600		
	H2b	600		
	H3a	0		

	H3b	600	
	H4a	1000	
	H4b	900	
	H5a	800	spreading mucoid colonies
	H5b	1900	"
168	P	fungi present in all and lower number of colonies in all	
	E	still no E. coli	
	C1a	0	
	C1b	0	
	C2a	200	
	C2b	0	
	C3a	0	
	C3b	0	
	C4a	0	
	C4b	0	
	C5a	0	
	C5b	0	
	L1a	2100	
	L1b	900	
	L2a	800	
	L2b	600	
	L3a	500	
	L3b	1300	
	L4a	900	
	L4b	800	
	L5a	4400	
	L5b	1100	
	M1a	0	taken over by fungus
	M1b	0	"
	M2a	400	
	M2b	0	
	M3a	500	
	M3b	400	
	M4a	0	
	M4b	400	fungus
	M5a	100	
	M5b	0	
	H1a	0	all H plates clean of any bacteria (other than noted)
	H1b	0	
	H2a	0	
	H2b	0	
	H3a	100	contam as well
	H3b	0	
	H4a	100	contam as well
	H4b	0	
	H5a	0	fungus
	H5b	0	

APPENDIX H

Data used for the statistical analysis of the Determination of *Escherichia coli* Survival in Laboratory Microcosm Experiment.

Key:

1 C: H20 + E. coli
2 E: Plastic Plant + E. coli
3 L: Low density plant +E. coli
4 M: Medium density plant + E. coli
5 H: High density plant + E. coli

day	time	trtmt (new)	CFU	logCFU	
1	24	1	2396667	6.379608	C
1		2	63000	4.799341	E
1		3	28410000	7.453471	L
1		4	10860000	7.03583	M
1		5	9780000	6.990339	H
2	48	1	6250000	6.79588	C
2		2	200	2.30103	E
2		3	21000000	7.322219	L
2		4	7870000	6.895975	M
2		5	690000	5.838849	H
3	72	1	6660000	6.823474	C
3		2	0	0	E
3		3	8884100	6.948613	L
3		4	3300000	6.518514	M
3		5	300000	5.477121	H
4	96	1	1137000	6.05576	C
4		2	0	0	E
4		3	2362000	6.37328	L
4		4	1323333	6.121669	M
4		5	23111.11	4.363821	H
5	120	1	32600	4.513218	C
5		2	0	0	E
5		3	29000	4.462398	L
5		4	23600	4.372912	M
5		5	3270	3.514548	H
6	144	1	730	2.863323	C
6		2	0	0	E
6		3	4080	3.61066	L
6		4	6100	3.78533	M
6		5	1010	3.004321	H
7	168	1	200	2.30103	C
7		2	0	0	E
7		3	1340	3.127105	L
7		4	360	2.556303	M
7		5	100	2	H

APPENDIX I
SPSS output for Univariate ANOVA with post-hoc Dunnett analysis.

Number key:

number	treatment
1	control
2	plastic
3	low
4	medium
5	high

Descriptive Statistics

Dependent Variable: logCFU

treatment	Mean	Std. Deviation	N
1.00	5.1046	1.89642	7
2.00	1.0143	1.87644	7
3.00	5.6140	1.83410	7
4.00	5.3266	1.75104	7
5.00	4.4556	1.75176	7
Total	4.3030	2.42190	35

Levene's Test of Equality of Error Variances(a)

Dependent Variable: logCFU

F	df1	df2	Sig.
.094	4	30	.984

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

A Design: Intercept+treatment

Tests of Between-Subjects Effects

Dependent Variable: logCFU

Source	Type I Sum of Squares	df	Mean Square	F	Sig.	Noncent. Parameter	Observed Power(a)
Corrected Model	99.733(b)	4	24.933	7.503	.000	30.011	.991
Intercept	648.061	1	648.061	195.009	.000	195.009	1.000
treatment	99.733	4	24.933	7.503	.000	30.011	.991
Error	99.697	30	3.323				
Total	847.492	35					
Corrected Total	199.430	34					

a Computed using alpha = .05

b R Squared = .500 (Adjusted R Squared = .433)

Multiple Comparisons

Dependent Variable: logCFU
Dunnett t (2-sided)

(I) treatment	(J) treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
2.00	1.00	-4.0903(*)	.97442	.001	-6.6025	-1.5781
3.00	1.00	.5094	.97442	.958	-2.0028	3.0215
4.00	1.00	.2220	.97442	.998	-2.2901	2.7342
5.00	1.00	-.6490	.97442	.907	-3.1612	1.8631

Based on observed means.
* The mean difference is significant at the .05 level.
A Dunnett t-tests treat one group as a control, and compare all other groups against it.

APPENDIX J
Raw Data from the Biolog GN2 Microplate Assay.

Biolog Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A			++					+	++			
B		++	+			+			+	++	++	++
C			++		+	++	+	++				
D					+	++	+					
E						++						
F												
G							+					
H		+++	+	++				++		++	++	+

Biolog Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A			+ +				+	++		++		
B		++	+			+				+	+	++
C			+		+	+	+	+				
D						++	++					
E						++						
F												
G								+				
H		++	+	+					+		+	+