Thesis

Entitled

Identification of Putative Geographic Sources of Bacterial Pollution in Lake Erie by Molecular Fingerprinting

By

Xixi Huang

Submitted as partial fulfillment of the requirements for

The Master of Science degree in Biology

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An Abstract of

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In 2005, roughly 20,000 days of closing or advisories impacted Great Lakes beaches, of which 75% were attributed to unknown source of fecal pollution. However, most municipalities have failed to identify and control sources of fecal pollution affecting beaches. Since fecal contamination is mainly responsible for swimming advisories at Lake Erie Beach (Oregon, OH), this study aimed to identify the putative ditches as geographic sources of bacterial pollution at Lake Erie Beach.

Previous research implicated Berger Ditch as a contributor of fecal pollution to the beach. However, we hypothesized that other nearby ditches might also

play a role in the pollution of Lake Erie Beach. Throughout one year, monthly and partial biweekly water samples collected from the beach and nearby tributaries, including Berger Ditch- (east end of beach), Tobias- and McHenry Ditches (west end), were analyzed for (i) *E. coli* density, and (ii) the genetic structure of whole *E. coli* communities assessed by PCR-denaturing gradient gel electrophoresis (DGGE) of the *β*-glucuronidase gene (*uidA*). Since this study represented the initial use of DGGE fingerprinting for bacterial source tracking, BOX-PCR, an established library-dependent method, was used to validate the results of DGGE analysis. The results showed that *E. coli* density and community structure in Lake Erie Beach and the three ditches were dynamic and seasonally variable during the year-long sampling period. The weak correlation between *E. coli* density in the ditches and the beach water demonstrated that monitoring bacterial densities is limited in its utility for bacterial source tracking and is best combined with complimentary methods. Community structure analysis as assessed by DGGE effectively described the relationship of *E. coli* communities in the ditches and the beach. While the results suggested that all three ditches sampled were involved in the bacterial pollution at some time, the contribution was likely seasonally-based. Tobias Ditch, in particular, was highly rainfall dependent, while Berger Ditch was the most consistent contributor of the three ditches. BOX-PCR of *E. coli* libraries validated the results of DGGE analysis, indicating that DGGE was a reliable method for rapidly screening the putative geographic sources of bacterial pollution in Lake Erie Beach.

Dedication

To my dear husband, Huai Wang

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Literature review

Section 1: Beach pathogens

Recreational use of fresh and marine waters is popular in the United States (USEPA, 2000a). However, water-based recreation and tourism can expose individuals to a variety of health hazards, including pathogenic microorganisms. From 1989 to 1999, approximately 170 waterborne disease outbreaks associated with recreational waters, including swimming pools, fountains, lakes, rivers, and oceans, were reported to the CDC (Centers for Disease Control and Prevention) (MMWR, 2000). One-half of these outbreaks resulted in reports of gastrointestinal illness, including such symptoms as stomach cramps, diarrhea and vomiting. In addition, Yoder et al (2004) reported that a total of 65 waterborne-disease outbreaks occurred from 2001-2002 causing illness among an estimated 2,536 persons, 8 of whom died. Of the 65 outbreaks, 30 (46.2%) involved gastroenteritis, 12 (18.5%) were caused by parasites, 6 (9.2%) were caused by bacteria, and 5 (7.7%) were due to viruses. Furthermore, in a study of 25,000 beach-goers exposed to bathing water at Hong Kong bathing beaches, it was reported that the incidence of swimming-related illness in 1992 was 4.1%, over 1% higher than was reported in 1987 (Kueh et al., 1995). The study also showed that symptoms involving the eyes, skin and respiratory tract, were between 2 and 20 times more prevalent in swimmers than in non-swimmers and that gastrointestinal symptoms were directly related to the pollution level and

indicator bacterial levels. From the above surveillance, it is reasonable to conclude that water quality is strongly associated with public health.

While most reported recreational water-related illnesses are the result of ingestion during swimming, non-swimmers are also at risk of exposure to waterborne pathogens (PrÜss, 1998). Non-swimmers are exposed to aerosolized pathogens (Feache et al., 1982) as well as pathogens present in the sand (WHO, 2003; CBC, 2005). To investigate several risk factors independently, PrÜss divided the results of 22 independent studies into two groups. To assess the risk of contact with water, the first group involved swimmers in unpolluted water versus non-swimmers. To estimate the risk of microbiological water quality, the second group included swimmers in polluted water versus those in non-polluted water. Data from 19 of the 22 studies indicated that the health risk of contact with water itself was lower than the risk of swimming in microbiological polluted water and further confirmed a significant relationship between the incidence of illness and the concentration of fecal indicator bacteria in recreational water (PrÜss, 1998).

Poor microbiological quality of beach water is not only a public health threat, but is also of economic concern. It is estimated that one third of all Americans visit coastal areas each year, resulting in a total of almost one billion visits and approximately $44 billion spent (USEPA, 2000a). Besides, the poor water quality could make potential visitor loss due to public perception of beach safety.

A survey about beach attitudes suggest that beach visitors are most interested in beach cleanliness and maintenance, with water quality appearing to play a strong role as well (Sohngen et al., 1999). Poor water quality causes economical loss not only as a result of lost beach and tourism revenue, but also the costs of treating waterborne illnesses. At beaches in Los Angeles and Orange Counties (CA), between 627,800 and 1,479,200 excess gastrointestinal illness occur every year, which corresponds to an annual economic loss of $21 to $51 million in the health cost of gastroenteritis (Given et al., 2006).

Pathogens related to recreational water quality

Pathogenic microorganisms associated with beaches include bacteria, parasites and viruses. Generally, waterborne pathogens enter the water from human and animal waste associated with runoff from impervious surfaces, groundwater drainage, releases of untreated sewage, faulty sewage pipes, or directly from the fecal material of animals (NRDC, 2006; Mallin, et al., 2000). Water polluted with fecal contamination has been shown to pose a variety of health risks including gastrointestinal, respiratory, dermatologic, and ear, nose, and throat infections (Henrickson, et al, 2001). Health risks are higher for young children, the elderly, pregnant women, and immunocompromized people, such as those weakened by AIDS, chemotherapy, recent surgery, and other chronic illness such as diabetes, or viral infection (NRDC, 2006).

BACTERIA

Pathogenic *Escherichia coli.* *E. coli* belong to the family Enterobacteriaceae, which also includes other pathogenic genera such as *Shigella*, *Salmonella* and *Klebsiella*. *E. coli* are a normal component in the intestinal microflora of warm-blooded organisms, including humans. However, while most strains of *E. coli* inhabiting human and animal intestines are harmless, commensal bacteria (Hartl and Dykhuizen,1984), a small percentage can cause disease in humans. Pathogenic *E. coli* can be broken into five broad classes, each based on the presence of different chromosomal- or plasmid-encoded virulence genes, as well as the bacterium's pattern of interaction with epithelial cells and tissue culture monolayers (Nataro and Kaper, 1998). These groups include (i) enterotoxigenic *E. coli* (ETEC), (ii) enteroinvasive *E. coli* (EIEC), (iii) enteropathogenic *E. coli* (EPEC), (iv)enterohemorrhagic *E. coli* (EHEC), and (v) enteroaggregative *E. coli* (EAEC). The pathogenic mechanism of EAEC is still unclear and is not discussed in detail here.

Enterotoxigenic *E. coli* (ETEC) are the most common cause of travelers' diarrhea and have caused several foodborne outbreaks in the United States. There are an estimated 79,000 cases of ETEC infection in the United States each year (CDC, 2006b), but the number caused by recreational water contact is still not known. ETEC can cause watery diarrhea, abdominal cramps as a result of the production of several enterotoxins including the heat-stable (ST) and heat-labile (LT) toxins, which are commonly used as biomarkers to detect ETEC. While ETEC strains might express ST or LT only, or both enterotoxins

simultaneously (Nataro and Kaper, 1998), both toxins act in a similar manner, by permeating the epithelial cell membrane and causing excessive diarrhea and fluid loss (Percival et al., 2003). The minimum infective dose of ETEC that causes pathological infection is high, typically in the range of 10^6-10^9 organisms (Percival et al., 2003).

Enteroinvasive *E. coli* (EIEC) have the ability to invade and destroy colonic tissue, causing an invasive, dysenteric form of diarrhea in humans (DuPont, et al., 1971). The pathogenic features are identical to those of *Shigella spp.*, as both organisms invade the colonic epithelium, an activity mediated by both chromosomal and plasmid loci. EIEC express one or more enterotoxins that have a role in diarrheal pathogenesis (Nataro and Kaper, 1998). The invasion-associated locus (IAL) is located on the invasion plasmid of the EIEC (Rappelli et al., 2001), and is usually used as a biomarker to identify EIEC strains. The minimum infective dose of EIEC that causes pathogenic infection is at least 10^6 organisms (Percival et al., 2003).

The enteropathogenic *E. coli* (EPEC) are an important category of diarrheagenic *E. coli* related to infant diarrhea. High inocula (10^8 to 10^9) of EPEC have been shown to cause diarrhea in adult volunteers. Although the infectious dose for infants is not known, it is presumed to be much lower (Nataro and Kaper, 1998). Pathogenesis of EPEC involves an interaction between the bacterium and host that results in “attaching and effacing” (A/E) lesions in

intestinal epithelial cells (Batchelor, et al., 1999). This activity is mediated by intimin, a protein encoded by *eae* (Hicks, et al., 1998; Batchelor, et al., 1999). A/E lesions are characterized by attachment of the bacterium to the epithelial cell membrane followed by localized destruction (effacement) of the microvilli of the intestinal wall (Nataro and Kaper, 1998). Pathogenesis also involves an EPEC adherence factor (EAF), encoded by *eaf* (Tobe et al., 1999), which facilitates the formation of a bundle-forming pilus, thus enabling a strong and localized adherence of bacteria to intestinal cells (Tobe et al., 1999). However, the prevalence of the disease nationwide is unavailable (CDC, 2006b).

Enterohemorrhagic *E. coli* (EHEC) produce verocytotoxin or Shiga like toxin (encoded by *stx1 and stx2*), which causes hemorrhagic colitis or bloody diarrhea. If untreated or incorrectly treated, these symptoms can progress to the potentially fatal hemolytic uremic syndrome (HUS) in which the red blood cells are destroyed and the kidneys fail. In the United States, HUS is the principal cause of acute kidney failure in children while roughly 8% of people who seek medical attention due to diarrheal illness develop HUS (CDC, 2006a). Like EPEC, EHEC also produce characteristic attaching-and-effacing (A/E) lesions in epithelial cells. *E. coli* O157:H7 is the most important enterohemorrhagic *E. coli* associated with human disease. It is estimated that in the United States 73,000 *E. coli* O157:H7 infections are treated annually (Rangel et al., 2005). Compared with other infective types of *E. coli*, the minimum infective dose of EHEC is low, in particular for *E. coli* O157:H7, as between 10 and 100 cells will result in infection (Mead and

Griffin, 1998). From 1982 to 2002, 350 outbreaks in 49 states involving 8,598 cases of EHEC infection were caused by *E. coli* O157:H7 (Rangel et al., 2005). Of these outbreaks, 31 were waterborne. Contaminated food is another important source for *E. coli* O157:H7. In the fall of, 2006, 199 persons in 26 states who ate bagged spinach became infected with *E. coli* O157:H7. Among the infected individuals, 102 (51%) were hospitalized, 31 (16%) developed HUS, and three died (CDC, 2006a).

Shigella spp. *Shigella spp.*, like *E. coli*, are gram-negative, facultatively anaerobic, non-spore-forming, non-motile bacilli that belong to the family Enterobacteriaceae. Four *Shigella* species are recognized, including *S. dysenteriae*, which produces shiga toxin, *S. flexneri*, *S. boydii*, and *S. sonnei*, which represents the most mildly pathogenic species. All *Shigella spp*. are pathogenic, and since humans are their primary natural host, their common reservoir is the fecal material of infected persons (Baer et al., 1971). *Shigella spp.* cause gastrointestinal illness by invading the colonic epithelium, causing bacillary dysentery (shigellosis). The minimum infectious dose that causes shigellosis is between 10 and 100 bacteria (Jennison and Verma, 2004). Approximately 14,000 confirmed cases of shigellosis and an estimated 450,000 total cases (including unconfirmed cases by CDC), mostly due to *S. sonnei*, occur in the United States each year (CDC, 2006c). From 1999 to 2000, three waterborne outbreaks of *S. sonnei* infection were confirmed including one outbreak in Florida that affected 38 persons who visited a beach park. The other

two outbreaks occurred at swimming beaches in Minnesota and affected a total of 40 people (Lee et al., 2002).

Salmonella spp. *Salmonella spp*. are Gram-negative, rod shaped, facultatively anaerobic, motile bacteria and belong to the family Enterobacteriaceae. Two species of *Salmonella* are recognized: *S. bongori,* and *S. enterica*. *S. enterica* can be divided into several pathogenic subspecies including *S. typi, S. paratyphi* A and B, and *S. typhimurium* (Popoff et al., 2000). Salmonellosis is an infection with Salmonella bacteria. The most common Salmonellosis involves replication with phagocytic cells's response followed by cell lysis, escape and infection of other phagocytes, resulting in diarrhea, fever, or abdominal cramps (Gross, 2006). Like *Shigella spp.*, the common environmental reservoir of *Salmonella spp.* is the fecal material of infected persons, although they can be also found in asymptomatic persons. However the host range of *Salmonella spp.* extends also to animals such as horses and sheep, and contaminated food (Baer et al., 1971). The minimum infectious dose of *S. typhi* infection is about 10^5-10^7 cells (Musher and Musher, 2004). An estimated 40,000 cases of salmonellosis occur annually in the United States (CDC, 2006d), most related to food contamination. Since 1995, only one outbreak of salmonellosis (attributed to *S. java*, a variant of *S. paratyphi B* (Miko et al., 2002)) has been associated with recreational water. The ouotbreak involved three people who swam in a swimming pool (Levy et al., 1998).

Campylobacter spp. *Campylobacter spp.* are Gram-negative, non-spore forming, spiral-shaped bacilli, that belong to the family Campylobacteriaceae and represent the most common cause of acute gastroenteritis in developed countries (Moore et al., 2005). The most important members of the Campylobacateriaceae of human significance are *C. jejuni* and *C. coli*, which are known to produce nine different virulence factors (van Vliet and Ketley, 2001). Every year in the United States, an estimated 20 cases per 100,000 people (about 60,000 cases) suffer from *Campylobacter spp.* infection (CDC, 2006e), which results in diarrhea, cramping, and abdominal pain (Pericival et al., 2003). The environmental reservoir for *Campylobacter spp.* is the fecal material of warm-blooded animals (Baer et al., 1971) and gulls (Lévesque et al., 2000). *Camplylobacter spp.* exhibit a relatively low minimum infectious dose, as it is estimated that fewer than 1000 cells may cause infection (Black et al., 1988). Despite the increased frequency of *C. jejuni* isolation from freshwater (Lambert et al., 1998; Eyles et al., 2003; Arvanitidou et al., 1995) and marine waters (Arvanitidou et al.,1995), no case of *Campylobacter*-associated illness linked to contaminated recreational waters was recorded by the CDC surveillance system between 1986 and 2002 (Pond, 2005). However, a large outbreak of acute gastroenteritis associated with *Campylobacter*-contaminated drinking water occurred in SÖderhamn, Sweden, resulting in the infection of over 100 people (Martin et al., 2006).

Legionella spp. *Legionella spp.* are Gram negative, aerobic,

non-spore-forming rod-shaped bacteria that belong to the family Legionellaceae. Infection with *Legionella pneumophilia* can cause Legionnaire's disease, which is indicated by pneumonia and damage to the nervous, gastrointestinal and urinary systems (Cunha, 1998). *Legionella spp.* are naturally occurring, aquatic organisms that can be found in ponds and sewage-contaminated waters (Fewtrell et al., 1994), but are also inhabitants of less natural environments including cooling towers, domestic hot water systems and fountains (Grimes, 1991). The minimum infectious dose for human infection is thought to be as low as a single organism (Pond, 2005), which can be transmitted via aerosolization on water droplets (USEPA, 1985). It is estimated that between 8,000 and 18,000 cases of *Legionella infection* occur each year in the United States (CDC, 2006f), with most reported cases related to human contact with contaminated swimming pools and hot tubs (Tolentino et al., 1996; Benkel et al., 2000; Fields et al., 2001), as well as rivers, lakes and sewage (Tomov et al., 1981; Cherry et al., 1982).

Vibrio spp. *Vibrio spp*. are Gram-negative, facultatively anaerobic, non-sporulating, short rods that belong to the family Vibrionaceae. Several medically important species exist including *V. cholerae, V. alginolyticus, V. parahaemolyticus* and *V. vulnificus* (Ghinsberg et al., 1995). *V. cholerae* is the causal agent of cholera, and therefore the most important to public health. Infection results from the colonization of the epithelium of the small intestine and subsequent production of an enterotoxin called cholera toxin (Taylor et al., 1987). Following infection, *V. cholerae* causes watery diarrhea and a sudden onset of effortless vomiting, which combine to cause potentially deadly rapid and severe

dehydration (Percival et al., 2003). In healthy individuals, the minimum infectious dose of *V. chloreae* is relatively high, as more than 10^6 cells is required to induce infection (Levine et al., 1988). However, the presence of sodium bicarbonate can reduce the minimum infectious dose to less than 10^4 organisms (Cash et al., 1974; Levine et al., 1988), placing individuals who frequently ingest oral antacids at considerable risk. The primary reservoir of *V. cholerae* in the environment is the fecal material of human carriers (Percival et al., 2003) although *Vibrio spp.* are known to be natural inhabitants of marine waters worldwide (Oliver, 1995; Montanari et al., 1999). Infections by *Vibrio spp.* result from contact of wounds with contaminated seawater or ingestion of contaminated, raw seafood (Klontz et al., 1988; Hlady and Klontz, 1996; Patel et al., 2002). There is no national surveillance system for *Vibrio spp. infection*, but CDC collaborates with the states of Alabama, Florida, Louisiana, Texas, and Mississippi to monitor the number of cases of *V. vulnificu*s infection in the Gulf Coast region. Between 1988 and 1995, CDC received reports of over 300 *V. vulnificus* infections from the Gulf Coast states. These states also reported about 30-40 cases of *V. parahaemolyticus* infections occur each year (CDC, 2006g).

VIRUSES

Adenovirus. Adenoviruses belong to the family Adenoviridae and frequently cause viral pneumonia or bronchiolitis, (Pond, 2005), which inflames the tiny airways that lead to the lungs. Recreational water, primarily water in inadequately chlorinated swimming pools is a common source of adenoviruses

(Caldwell et al., 1974; Martone et al., 1980). Adenoviruses also exist in beach water. According to Jiang and coworkers (2001), adenoviruses were detected by PCR in water samples collected from beaches in southern California, with viral loads ranging from 880 to 7,500 particles per liter of water. Since it is estimated that less than 150 plaque forming units can cause infection (Pond, 2005), it can be concluded that water sampled in Jiang's study was highly polluted by adenoviruses, and posed a significant infectious risk. Adenoviruses are highly infectious, as calculations by Crabtree et al.(1997) showed the annual risks of infection from adenovirus in recreation water to be as high as 1/1000 for a single exposure.

Hepatitis A virus. Hepatitis B virus (HBV) and hepatitis C virus (HCV), which are transmitted primarily by blood and body fluid, can cause severe, chronic liver disease. Conversely, hepatitis A virus (HAV) does not have a chronic stage, and in general will not result in permanent liver damage. HAV is common in the United States and throughout the world. It is reported one-third of Americans have evidence of past infection (in the form of immunity) (CDC, 2006h). Humans, especially children are the known reservoir for the hepatitis A virus (HAV) (Armstrong and Bell, 2002), which is most often transmitted through the fecal-oral route. HAV transmission can occur as a result of blood exposures, but the frequency of blood borne transmission is rare because the presence of virus in the blood occurs with the onset of infection and is not thought to be present long. The symptoms of HAV infection are fever, diarrhea, nausea, fatigue, abdominal

pain and jaundice (Pond, 2005). In the U.S., since 1969, 60 cases of HAV associated with lake recreation, polluted streams and swimming pools have been reported (Bryan et al., 1974; Chapman, 1976; Mahoney et al., 1992). The minimum infectious dose for HAV is not known, however it is thought to be extremely low, possibly as low as a single infectious viral particle (Xu et al., 1992).

PARASITES

Giardia duodenalis. *G. duodenalis* is a one-celled, binucleated, flagellated protozoan parasite that normally lives the upper small intestine of humans and other large mammals and releases cysts in the infectious stage of its life cycle. Most outbreaks of *Giardia spp.* have been related to the consumption of water contaminated by human fecal material (Thompson et al., 2000), resulting in acute, self-limited or chronic diarrhea, stomach cramps, bloating, weight loss and dehydration (Pond, 2005). Infection by *G. duodenalis* is the most frequent cause of protozoan intestinal disease worldwide causing an estimated 280 million cases per year (Lane and Lloyd, 2002). The cysts of *Giardia spp.* are protected by an outer shell that allows persistance in the secondary environment for long periods of time (Pond, 2005). Infection by one or more cysts can cause disease (Lane and Lloyd, 2002). *Giardia spp*.-related waterborne outbreaks are usually associated with ingestion of both drinking and recreational water, including water from lakes, rivers, or swimming pools (Kramer et al., 1996, Furness et al., 2000).

Cryptosporidium spp. *Cryptosporidium* is an obligate enteric parasite that infects the gastrointestinal tract of humans and livestock, where it grows and reproduces (Pond, 2005). The majority of *Cryptosporidium spp*.-related human disease is caused by *C. parvum*, which can also infect animals, and *C. hominis*, which is largely restricted to human hosts (Fayer et al., 2000; Chalmers et al., 2002). The predominant features of *Cryptosporidium spp*. infection are watery diarrhea, abdominal pain, fever, weight loss, and vomiting (Percival, 2003). Oocysts are the primary transmissible form of *Cryptosporidium spp*. through the fecal-oral route and are ubiquitous in lakes and streams. The minimum infectious dose of *Cryptosporidium spp*. oocysts/cysts for humans is unknown, but is probably less than 10 oocysts (Dillingham et al., 2002). No national or state monitoring programs exist, but the annual incidence of infection in the U.S. was estimated as 1.17 per 100,000 people, or roughly 3500 cases per year (Groseclose et al., 2002). Exposure to contaminated recreational water is a common route of *Cryptosporidium spp*. infection (MMWR, 1990; Fayer et al., 2000; Rose et al., 2002). For example, in 1994, a four week long *Cryptosporidium spp*. outbreak in New Jersey resulted in the treatment of more than 2000 people following exposure to contaminated lake water (Kramer et al., 1998).

Pathogens related to beach sand quality

The impact of the microorganisms of beach sand on human health has been largely overlooked. Since beach-goers tend to spend more time on the beach (in

contact with sand) than in the water (CBC, 2005), beach sand could represent a significant pathway of bacterial infection in humans. Although epidemiological studies reporting the health impacts of recreational water exposure are numerous, studies of the risks associated with beach sand pollution are limited. Additionally, it has been stressed that epidemiological and microbiological studies should focus on wet beach sand and sediments in an effort to couple recreational water quality with health impacts (WHO/UNEP 1992, 1994). A variety of potential pathogenic bacteria, fungi and parasites have been isolated from beach sand (Mendes et al., 1997; Sato et al., 2005) and found to comprise a significant component of the beach sand biota. Beach sands might actually represent a more hospitable environment for microorganisms than water. Since little information exists concerning the ecology of viruses and parasites in beach sands, in this review I shall focus primarily on bacteria known to inhabit beach sand.

Fecal indicator bacteria. Fecal indicator bacteria, including enterococci and *E. coli*, have been enumerated in beach sand (Whitman and Nevers, 2003; Kinzelman et al., 2004). Whitman and Nevers (2003) found *E. coli* densities in beach sand range between 10^3 and 10^4 CFU/100 cm^3 of sand. It has also been shown that enterococci and *E. coli* were more prevalent in sand than in the water column at freshwater bathing beaches. Densities of these indicators were found to be between 3 and 38 times higher (up to 900 CFU/100 g dry wt) in the top 20 cm of wet sand cores (Alm et al., 2003), as compared with densities in water.

Staphylococci. Staphylococci are Gram-positive cocci belonging to the family Micrococcaceae. *S. aureus* produces at least five enterotoxins (enterotoxins A through E) that can cause soft tissue infections, bloodstream infections and pneumonia. It has been estimated that primary contact with as few as 100 cells/mL of water might induce infection in wounded skin (Percival et al., 2003). Although no outbreaks related to *S. aureus* in recreational water and sand have been reported, it has been detected in beach sand. Prado et al. (1994) studied two popular beaches in Chile. In that study, it was shown that 24% of the staphylococci were *S. aureus*. The presence of *S. aureus* in the beach was probably derived from the swimmers as Papadakis (1997) found *S. aureus* counts in sand samples were correlated with the number of swimmers present on the beach.

Aeromonas spp. *Aeromonas spp.* are Gram-negative, rod-shaped, non-spore forming, facultatively anaerobic bacteria that belong to the family Aeromonadaceae. The aeromonads have been divided into two groups, the non-motile psychrophilic aeromonads, which tend to be pathogenic to fish; and the motile mesophilic aeromonads, which are significant to human health and include *A. hydrophila, A. caviae* and *A. veronii* biovar *sobria* (Joseph, 1996). *Aeromonas spp.* are widespread in the environment and are commonly found in fresh- and marine waters, soil, and agricultural produce (Percival et al., 2003) as well as in marine waters and sand (Ghinsberg et al., 1995). *Aeromonas spp*. pathogenesis primarily results in gastrointestinal distress. While the disease

conditions are usually self-limiting, *Aeromonas spp*. has been shown to produce severe, life-threatening cholera-like symptoms (Joseph, 1996). *Aeromonas spp.* have also been associated with human wounds, although the pathogenesis of opportunistic infection by *Aeromonas spp*. is still not completely understood (Gosling, 1996). The minimum infectious dose of *Aeromonas spp*. is not known with certainty, however Morgan et al. (1985) indicated that between 10^7 and 10^9 organisms of *A. hydrophila* can cause mild diarrhea. The prevalence of *Aeromonas spp.* related infections is not available.

Vibrio spp. While primarily known as a waterborne pathogen, *Vibiro spp.* have also been found in both wet and dry beach sand (Ghinsberg et al., 1995; Vieira et al., 2001). Although it was shown that the density of *Vibiro sp.* in sand can be low (less than 10 cells per 80 to 100 g of wet sand), it was also reported that people with open skin wounds were at increased risk of infection by seawater (Hlady and Klontz, 1996).

Campylobacter and Salmonella. Bolton and coworkers (1999) found sand samples from 82 (45%) and 10 (6%) beaches (182 total) in the United Kingdom contained *Campylobacter spp.* and *Salmonella spp.*, respectively. Furthermore, both pathogenic bacteria were detected in wet and dry sand.

Although there is no report of outbreaks related to the pathogens from the beach sand, to date no effort has been made to discount the possibility that

outbreaks assumed to be of aquatic origin were not associated with beach sand contamination. The prevalence of pathogens in beach sand could result from the presence of bird fecal material (Lévesque et al., 1993; 2000), untreated sewage (Ghinsberg et al., 1994), and residues or detritus from beach users (Mendes et al., 1998).

Pathogens, such as *S. aureus*, anthropogenic yeasts, and moulds have been recovered from sand but not water samples, indicating beach sand provides better protection, as these pathogens can adhere to sediment particles and take advantage of organic nutrients in beach sand (Papadakis et al., 1997). These data indicate that sand represents a significant, but understudied reservoir of environmental pathogens. Taken together, these data highlight the need to address the pathogen ecology in beach sands, as the increased presence and survival of pathogen indicators might signal a significant public health concern.

Section 2: Bacterial Source Tracking

Water quality issues at beaches

The Clean Water Act was passed in 1972, with the goal of creating "fishable and swimmable" public waters established in 1983 (Copeland, 2002). However, many lakes, rivers, and streams in the U.S. are impaired and thus still do not meet this goal. For example, 40% of monitored streams, 45% of lakes and 50% of estuaries are not clean enough to support recreational uses such as fishing and swimming (USEPA, 2002). Furthermore, nearly 20,000 days of posted closings and advisories at ocean, bay and Great Lakes beaches occurred throughout the U.S. in 2005 (NRDC, 2006). It is reported over 2,500 of these advisory days impacted Great Lake Beaches, an increase of more than five percent from 2004; and 85% of beach closings/advisories in 2004 were the result of detected indicator bacteria levels that exceeded water quality standards (NRDC, 2005). Unfortunately, most municipalities have failed to identify and control sources of bacteria and other pollution impacting natural waters (NRDC, 2005).

Escherichia coli as bacterial indicator

It is difficult and time-consuming to detect all of the pathogens that might be present in a given water sample. The great potential diversity of pathogens and their growth requirements often preclude their rapid and accurate detection and identification. Therefore, easily detected organisms that can signal the presence of pathogens are used as indicators, limiting the resource intensive exercise of assaying a water sample for the numerous pathogens that might be present.

Ideally, indicator bacteria should be nonpathogenic, rapidly detected, easily enumerated, have similar survival ability as the pathogens of concern, and numerically correlate with the presence of pathogenic microorganisms (Scott et al., 2002). Since the bulk of the concern over bacterial pollution in recreational waters surrounds the presence of fecal pathogens, the most common indicators include total coliforms and fecal coliforms (not commonly used nowadays), *Escherichia coli (E. coli)* and *Enterococcus* spp., which are common members of human and animal fecal material (Meays et al., 2004).

Total coliforms and fecal coliforms have been used to determine water quality for a long time. The total coliform group includes *Escherichia spp*., *Enterobacter spp*., *Citrobacter spp.* and *Klebsiella spp*., etc. They are generally harmless, and appear in great quantities in the intestines and fecal material of warm-blooded animals. Although total coliforms are easy to detect and quantify with common growth media, it was shown that the correlation of total coliform number with the presence of fecal contamination was questionable, as some coliforms are commonly found in natural environmental samples, including soil, water or vegetation (Caplenas and Kanarek, 1984). The fecal coliform bacteria are a sub-group of the total coliform group. However, *Klebsiella spp.* also belonging to fecal coliform, can ubiquitously exist in the environment, such as water and soil (Bagley, 1985; Podschun et al., 2001), which could falsely indicate fecal pollution for source tracking. Therefore, the utility of both total coliforms and fecal coliforms as indicators of fecal pollution was limited. In 1986, USEPA decided to

use *E. coli* as freshwater fecal indicator bacteria instead of total coliforms and fecal coliforms (USEPA, 1986).

E. coli is regarded as the standard indicator of fecal pollution in freshwater, as *E. coli* was shown to correlate best with swimming related illness among swimmers in freshwater, compared with other indicators, such as fecal coliforms, total coliforms and staphylococci (Prüss, 1998). It was also reported that *E. coli* better correlated with the incidence of highly credible gastrointestinal illness (occurring gastrointestinal symptoms during a single 24-hour period) and total gastrointestinal illness with swimming in fresh water as correlation coefficients were 0.80 and 0.53, respectively, comparing with fecal coliforms, which were 0.08 and 0.24, respectively (Dufour, 1984). In Ohio, *E. coli* densities have been used to monitor beach water quality since 1996, (NRDC, 2005). In 2006, the Ohio EPA adopted the use of the single sample maximum standard to replace the previous system which monitored the geometric mean of *E. coli* density over five evenly spaced samplings in a 30 day period (USEPA, 1998). Under the new standard, beach will close or go under advisory if the *E. coli* density exceeds 235 CFU/100 mL of a single daily water sample (ODH, 2006).

The enterococcus group is a subgroup of the fecal streptococci. Enterococci are reliable indicators of fecal bacteria pollution in marine waters (Cabelli, et al., 1983) and are sometimes used as an alternative indicator of fecal pollution for fresh water (USEPA, 1986). This is not without some controversy,

as it has been shown that the enterococci cannot accurately predict the presence of nonbacterial pathogens such as infectious enteric viruses, *Cryptosporidium spp*., and *Giardia spp*. (Harwood, 2005). Despite this limitation, Jin et al. (2004) found that enterococci were a more stable indicator of water quality than *E. coli* and fecal coliforms.

The major flaws of the indicator system is that indicator bacteria could persist in the environment (such as soil or sediment) for a long time (up to one year) (Whitman et al., 2006) and have potential growth in soil or sediment (Davies et al, 1995) that it may limit the function of signaling the current fecal contamination.

Transport of fecal pollution into public waters

Rain/storm water run-off. Storm water known to carry pollution is the largest known cause of beach closures or advisories, responsible for 13% (about 4,000 days) of the total advisory days in 2004 (NRDC, 2005). Runoff from agricultural lands, municipal point sources (sewage treatment plants), and hydrologic modifications (flow regulation, and dredging) are the primary causes of impairment for lakes and streams. Storm water washing over impervious surfaces (roads, rooftops, parking lots) (Mallin, et al., 2000) and industrial or commercial sites can become contaminated with oil, heavy metals, pesticides, litter, and pollutants from vehicle exhaust (NRDC, 2006). Fecal materials from dogs, cats, pigeons, birds could be picked up by stormwater when entering stormdrains (NRDC, 2006). Farmland leaching of waters associated with liquid manure or municipal biosolids land applications can also contribute to bacterial

contamination of surface waters. Evans and Owens (1972) found that the concentration of bacteria in drainage water increased by 30 to 900 fold within two hours of liquid swine manure spreading to farmland. Densities of *E. coli* in drain water could increase by 85 fold from 100 to approximately 8,500 CFU/100 mL after biosolid application even after three months (Scarbro, 2006). It was also shown that by blocking the drainage outlets to surface water bodies prior to application of liquid manure to farmland, the bacterial load was reduced by a factor of between 2 and 500 (McLellan et al., 1993).

Sewer network failures and overflows. Sewage systems are sometimes designed to discharge untreated sewage into local waterways when systems are overloaded during a heavy rainfall event. For example, combined sewer overflow (CSO) is a design that combines untreated sewage from residences and industrial sites with stormwater runoff and during high volume rainfall events, will limit the contamination of public and private properties by discharging combined stormwater and sewage into natural waters. The USEPA estimates that approximately 43,000 CSO events occur annually nationwide (USEPA, 2004). Similarly, sanitary sewer systems are designed to carry only human and industrial waste from buildings to sewage-treatment plants. However, the EPA estimates that between 23,000 and 75,000 SS overflows occur every year (USPEA, 2004). Alternatively, sewage contamination of natural waters can occur when a system breaks down due to blockage, breakage, or pump failure. Under high volume rainfall event, it has been shown that as little as one-quarter of an inch of rain can

overload a combined wastewater system (NRDC, 2005). During an overflow event, nutrients, pathogens, organic toxicants and metals, including heavy metals, enter the water way which empties to the receiving waters, and can cause significant environmental harm and affect ecological health.

Improperly maintained septic systems. Coastal populations increased by 33 million people (28 %) between the years 1980 and 2003, and now account for 53% of the total population in the United States (Crossett, et al., 2004). Many people build their houses near the coast, bay, or Great Lakes with underground septic systems. With no federal regulatory program to control waste from septic systems (NRDC, 2006), and rare inspection by local governments or states, improperly sited, built, or maintained septic systems are subject to failure, such as malfunctioning or overloading. Many pathogens have been detected in septic sewage (Gerba and Smith, 2005), including *Salmonella spp., Shigella spp., Campylobacter jejuni*, and pathogenic strains of *E. coli*. According to the 2001 American Housing Home Survey, 6% of septic systems fail annually, resulting in improper treatment, or the potential release of between 66 to 144 billion gallons of sewage (NRDC, 2005), allowing pathogens to enter and contaminate receiving waters.

Natural animal inputs. Animal waste can be a reservoir of many pathogens. For example, dogs were identified as hosts of *Giardia spp*. and *Salmonella spp*. (Pitt, 1998). Pet waste can accumulate on surfaces such as

streets, sidewalks and lawns. It has been shown that one gram of dog fecal material can contain 23 million fecal coliforms (van der Wel, 1995). Furthermore, on average, a dog produces about 227 gram of waste per day, resulting in over 10 billion fecal coliforms per dog. Farm animals also harbor pathogens such as *E. coli* O157:H7 (Hancock, 1997). Poultry are a primary reservoir of *Salmonella spp.*, as are swine, which can also carry *Shigella spp.* (Krumperman, 1983). Animal waste material can be transported offsite during rainfall events and can eventually contaminate public waters. For example, fecal coliform density in runoff from cattle-grazed land was approached 10^6 CFU/100 mL, and were five to ten times more than the fenced, ungrazed areas, indicating the potential risk to public health associated with contaminated runoff (Doran and Linn, 1979). Thus, fecal bacteria and pathogens detected in public waters can occur as a result of direct excretion of waste from domestic and wild animals into waterways as well as overland runoff and leaching of bacteria associated with animal fecal material.

Birds fecal material. In the beach environment, large numbers of waterfowl can represent a significant source of fecal pollution that results in elevated indicator densities in beach sand and water. For example, gulls and geese harbor large numbers of fecal coliforms, ranging from 10^3 to 10^9 bacteria per gram of fecal materials (Alderisio and DeLuca, 1999) as well as other pathogens including *Salmonella spp.* (Quessy and Messier, 1992; Fenlon, 1981), *Campylobacter spp.* (Kapperud and Rosef, 1983; Quessy and Messier, 1992), and *Shigella spp.* (Karaguzel et al., 1993). Lévesque et al (1993) studied the

number of fecal coliforms in beach water before and after birds were attracted to the beach with food, and found gull droppings could increase bacterial concentrations upto 5,000 CFU/100 mL in the beach surface water, indicating gulls can contribute to the bacteriological degradation of recreational water.

Methods to identify sources of fecal pollution

In 2005, more than 20,000 days of closings and advisories were reported in U.S. ocean, bay and Great Lakes beaches (NRDC, 2006). The majority (75%) of the closing and advisory days were the result of elevated levels of bacteria associated with fecal contamination. Although the cause of the impairments was identified as bacterial pollution, the origin of the pollution remained unknown. Identifying the source of fecal pollution could make management and remediation of water pollution more effective, and reduce the public health risk associated with recreational water use. However, most municipalities have failed to identify and control sources of bacteria pollution in waters that are associated with adjacent beaches (NRDC, 2006).

Two classes of bacterial pollution sources exist: those defined by a specific animal host, and those defined by a geographic source. **Host source identification** aims to identify the animal host serving as the source of fecal pollution and is commonly performed by matching phenotypic or genotypic characteristics in bacteria isolate libraries or bulk bacteria communities isolated from both the polluted site and the suspected animal host. A positive match will

help determine which animal is the pollution source.

In **geographic source identification**, specific locations (ditches, creeks, known runoff areas) are treated as bacterial sources. Identifying a geographic source relies on the assumption that the community structure of bacteria populations differs depending on geographic location. It is assumed that the structure of the bacterial communities in two ditches suspected of contributing bacterial pollution to a known sink will be different, and that the difference will be detectable. Therefore, similarity among communities isolated from suspected sources and the sink might implicate the suspected source as pollution contributor.

Increased interest in identifying the sources of fecal contamination has resulted in two broad categories of bacterial source tracking (BST) methodology that can be used in host- and geographic source identification: library-*dependent* and library-*independent* methods (reviewed in Simpson et al., 2002; Scott et al., 2002).

Library-dependent methods

Library-dependent methods are based on the construction of reference libraries of bacterial isolates of target microorganisms (most often indicator bacteria) collected from suspected animal hosts or potential source locations. Common methodologies associated with library-dependent techniques include

antibiotic resistance analysis (ARA), carbon utilization profiling, repetitive element PCR (rep-PCR), ribotyping and pulse field gel electrophoresis (PFGE). In the broadest sense, these methods classify an isolate to a given host (humans, animal, etc.) or location (ditch, stream, etc.) by generating a metabolic or genetic characterization of the isolate. Once the libraries are complete, characters that define isolates collected from the pollution sink can be compared to those in the known host/location libraries to identify the sources of pollution to the sink. In general, library dependent methods are discriminative and reproducible (Louws et al., 1994; Carson et al., 2003; McLellan et al., 2001, 2003). However, isolate based libraries require a comprehensive isolate library from hundreds to thousands from each potential source and thus result in significant expenditure of time and money (Johnson et al., 2004; Wiggins et al, 2003).

Antibiotic Resistance Analysis (ARA). Antibiotics are used to treat infection in humans and domestic animals. They are also commonly used in commercial handwashing and household cleaning products, such as detergent, soap, mouthwash and toothpaste (Perencevich et al., 2001). It has been shown that bacteria shed from animals and humans treated with antibiotics exhibit increased resistance to those antibiotics (Austin et al., 1999). It has also been shown that human fecal bacteria exhibit resistance to a greater array of antibiotics than do bacteria from livestock and wildlife (Hager, 2001). In antibiotic resistance assays, the bacterium of interest is exposed to various antibiotics at various concentrations, and then scored based on the growth in the presence of

the different antibiotics, thereby generating an antibiotic resistance profile. Resistance profiles expressed by a bacteria isolate can be matched to existing library profiles in an effort to identify from which source it originated. ARA is commonly used for source identification because it is rapid and relatively simple. For example, analysis of antibiotic resistance patterns in 892 fecal streptococci isolates differentiated between human, beef cows, deer and waterfowl sources of fecal pollution in natural waters (Hagedorn et al., 1999). In the same study, it was shown that the average rate of correct classification of the entire database was 88%, and ranged from 85% for beef cows isolates to 93% for human isolates.

Antibiotic resistance assays require expenditure time and labor for constructing a large reference database (Hagedorn et al.,1999; (Wiggins et al., 2003). Furthermore, the ARA profile may be geographically or temporally specific (Wiggins et al., 1999). In addition, antibiotic resistance genes are often carried by plasmids and mobile genetic elements, horizontal transfer to the same or other different fecal bacteria (such as between *E. coli* strains and *Salmonella* strains) (Bass et al., 1999; Kruse et al., 1994). Acquisition or loss of a plasmid can change the resistance profile of an isolate, thereby introducing bias to the host identification.

Ribotyping. Ribotyping is based on characterizing a bacterium isolate by DNA fingerprinting its 16S rRNA gene. Briefly, following restriction endonuclease digestion, genomic DNA is separated by electrophoresis and

hybridized with labeled oligonucleotide probes that target the conserved regions of the 16S rRNA gene (Farber, 1996). The genetic fingerprints of bacterial isolates from the sink can then be compared to those of bacteria from the suspected animal source. The source(s) of fecal pollution is determined if the fingerprints of bacteria isolates from the source and the sink match. Ribotyping was reported to be an effective method to track human and nonhuman sources of pollution (Parveen et al., 1999; Hartel et al., 2002). Despite the successes in source identification associated with ribotyping, several disadvantages limit the method. Ribotyping is resource intensive, requires a reference database and multiple steps to complete the analysis. Additionally, the variations of the ribotyping procedure, including different enzymic digestion, different detection methods during the southern blotting procedure, could result in difference in ability to discriminate between bacteria from various animal hosts (Carson et al., 2001; Hartel et al., 2002; Parveen et al., 1999).

Repetitive Element PCR (rep-PCR). Repetitive element PCR is a broad class of PCR methods that are performed with a primer(s) that targets randomly spaced, repetitive and conserved regions in the genome of a diverse range of bacterial genera. rep-PCR results in the amplification of differently sized DNA fragments that can be size-fractionated by electrophoresis, generating DNA fingerprint patterns specific for individual bacterial strains (Versalovic et al., 1991, 1994). The resulting DNA banding pattern (fingerprint) can be used as a tool to differentiate bacteria and potentially classify them to discrete hosts, assuming that

a given host consistently harbors microflora (e.g. indicator bacteria) of specific rep-PCR profile. Three rep-PCR methods commonly used for bacterial source tracking are (i) repetitive extragenic palindromic PCR (REP-PCR) (Stern et al., 1984), (ii) enterobacterial repetitive intergenic consensus sequences PCR (ERIC-PCR) (Hulton et al., 1991), and (iii) extragenic repeating elements PCR (BOX-PCR) (Martin et al., 1992). BOX-PCR has proven the most effective in distinguishing environmental isolates of *E. coli*, when compared to other rep-PCR protocols (Dombek et al., 2000). This superiority is due to the ability of BOX-PCR to generate robust and reproducible band patterns (Lanoot et al., 2004). Carson et al. (2003) reported that BOX-PCR fingerprinting resulted in an average rate of correct classification of 96.6% for human and nonhuman *E. coli* isolates, while McLellan et al. (2003) reported an average rate of correct classification of 79.3% for *E. coli* from sewage (human), gull and cattle analyzed using REP primers. Although rep-PCR techniques are comparatively rapid, simple, and do not require specialized equipment (Dombek et al., 2000), as with all library-dependent methods, these methods require a large library of isolates (Johnson et al., 2004). Furthermore, interlaboratory reproducibility can be limited (Deplano et al., 2000).

Pulse Field Gel Electrophoresis (PFGE). PFGE creates fingerprints of bacterial isolates by separating large pieces of genomic DNA (30 to 2,000 kb) generated by digesting with a rare-cutting enzyme (Schwartz and Cantor, 1984). Following digestion, the resulting DNA fragments are generally too large to be

separated by conventional electrophoresis. PFGE overcomes this limitation by using multiple orientations of the electric field to redirect the large DNA molecules, forcing them through the pores in the gel based on the angle of the current, pulse time and voltage (USEPA, 2005). PFGE has been shown to be more discriminative than rep-PCR for differentiating environmental *E. coli* isolates (McLellan et al., 2001), and ribotyping for discriminating strains of *Lactobacillus spp*. (*L. rhamnosus* and *L. casei*) (Tynkkynen et al., 1999) and strains of *Staphylococcus aureus* (Prevost et al., 1992). However, the PFGE technique is time consuming, requiring from two to four days (Olive and Bean, 1999; Van Belkum et al., 2001), requires specialized equipment, and considerable expertise to achieve consistent results.

Library-independent methods

Library-independent methods are based on the presence or absence of genes, in an environmental sample, associated with a target organism or function. Since genes can be isolated from a given environment (fecal material, water, soil, etc.) in the form of directly isolated DNA, an isolate library is not necessary. Library-independent methods can be used to identify either specific hosts of bacterial pollution (animals, humans, etc.) or geographic sources, such as streams, straight pipes, or drainage ditches.

PCR-based host specific markers. Specific human and animal DNA markers can be used to discriminate human and ruminant feces (Bernhard and

Field, 2000 a, b), and to differentiate *E. coli* isolates originating from geese and ducks (Hamilton et al., 2006), thereby providing effective tools to identify the animal source of bacterial pollution. Griffith et al. (2003) found host-specific PCR performed best in discriminating fecal indicators from human and non-human sources in samples containing one to three of five possible fecal sources (human, dog, cattle, seagull or sewage) (Griffith et al., 2003). PCR with a host specific marker is relatively rapid and inexpensive. However, it requires extensive field testing to determine the geographic distribution of the host-specific markers before the marker can be confidently used for fecal source identification (Bernhard and Field, 2000b).

Community fingerprinting. Community structure analysis can be used to identify geographic sources of fecal pollution. This is a relatively new application of a standard community fingerprinting method known as denaturing gradient gel electrophoresis (DGGE) or temperature gradient gel electrophoresis (TGGE), which generates band-based fingerprints that can be used to characterize mixed bacterial assemblages (Muyzer and Smalla, 1998).

DGGE and TGGE fingerprints result from the separation of PCR products of similar length, but of differing sequences. DNA fragments with sequence variations possess varying stability under denaturing conditions (Muyzer et al., 1993). Therefore, separation of DNA fragments by DGGE or TGGE is based on denaturing conditions in the gel matrix that cause double stranded DNA to “melt”,

thereby creating a sequence dependent alteration in the electrophoretic mobility of the DNA molecule. DGGE and TGGE assays are commonly performed in polyacrylamide gels containing a linear gradient of denaturants (including urea and formamide, for DGGE) or a linear temperature gradient (for TGGE), respectively (Muyzer et al.,1993; Muyzer and Smalla, 1998). Electrophoresis results in a pattern of bands corresponding to different species in the bacterial communities. Furthermore, the similarity among banding patterns that represent differing microbial communities can shed light on how closely the communities are related (or conversely, how dissimilar they are).

Community fingerprinting techniques, especially DGGE, have become important molecular tools in microbial ecology. DGGE of PCR-amplified 16S rDNA fragments were first used to study community complexity of microbial mats as well as bacterial biofilms (Muyzer et al., 1993) and have more recently been used to investigate microbial communities in soil and water environments (Sigler and Zeyer, 2004; Sekiguchi et al., 2002). Although DGGE analysis was originally used to study the overall complexity of diverse bacterial communities, this technique can also characterize and differentiate fecal bacteria. For example, Farnleitner et al. (2000 a, b) used DGGE of the *β*-D-glucuronidase gene (*uidA*) to differentiate *E. coli* isolates from freshwater. He found a strong correlation of the sequence variations detected by PCR-DGGE of *uidA* with those detected by PCR followed by direct sequencing, and suggested PCR-DGGE of *β*-glucuronidase gene could be used for the characterization of fecal pollution in

the aquatic environment. *uidA* is expressed in approximately 95% of environmental *E. coli* (Martins et al., 1993); it codes for the enzyme, *β*-glucuronidase, which has been shown to be an appropriate molecular target for *E. coli* detection (Bej et al.,1991). Most recently, Sigler and Pasutti (2006) showed that PCR-DGGE of *uidA* could differentiate mixed assemblages of environmental *E. coli* isolates and provide an effective tool to characterize *E. coli* dynamics in the secondary environment.

DGGE does possess some limitations. For example, it was reported that multiple sequence differences could migrate to the same position (Buchholz-Cleven et al., 1997; Jackson et al., 2000). It is likely because the melting properties of a DNA sequence are determined not only by its nucleotide composition but also by the interactions between different nucleotides within the molecule (Breslauer et al., 1986). Thus, even the nucleotide composition of DNA fragment is the same, nucleotide change in different position can result in different melting properties.

In summary, while library-dependent methods utilize the power of a comprehensive isolate database of hundreds to thousands of isolates from the sink site as well as each potential source (Wiggins et al., 2003), it has been shown that library-independent methods outperformed library-based methods in their ability to identify or exclude samples related to human fecal contamination (Griffith et al., 2003). Furthermore, library-independent methods have been proven

effective in differentiating *E. coli* populations from environmental freshwaters (Sigler and Pasutti, 2006; Farnleitner et al., 2000a) without the prerequisite development of isolate libraries. Library independent methods, in particular DGGE, might be a useful and effective tool for bacterial source tracking.

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Introduction

Lake Erie Beach (LEB), within Maumee Bay State Park (MBSP), is a popular destination in northwest Ohio for water recreation including swimming, fishing, boating, windsurfing, as well as non-water-contact activities, such as sunbathing, and recreating in the sand. MBSP, along with LEB, is a vital economic resource for northwest Ohio.

Pathogens originating from the fecal material of warm blooded animals, are frequently detected in recreational waters and pose a variety of health hazards, including gastrointestinal, respiratory, dermatologic, and ear, nose, and throat infections (Henrickson, et al, 2001). To protect beachgoers from exposure to these pathogens, the monitoring of the microbiological quality of beach water in coastal and Great Lakes states is required (USEPA, 2000b). Indicator bacteria, such as *E. coli* or Enterococci, were used to signal the presence of pathogens without intensive exercise of assaying a water sample for the numerous pathogens that might be present. If the indicator bacteria level exceeds EPA standards, an advisory is posted to warn the public of the health risk associated with primary contact with the water. While the role of advisory posting is to protect public health, it can also create an extensive economic impact. LEB is partially responsible for tourism that contributes $6.2 million to the local economy annually. Of total 1.4 million visitors annually to MBSP, 17% (238,000) go to

specifically swim at the beach (Sohngen et al., 1999). In 2003, LEB was closed for a total of 40 days (ODH, 2006), resulting in an estimated loss of $347,800 to the local economy (assuming a 120 day swimming season).

According to the Ohio Department of Health (ODH, 2006), the total advisory days for LEB were 42, 20 and 8 days during the swimming season (June through August) for 2003, 2004 and 2005, respectively. Until 2005 an advisory was issued whenever the geometric mean density of *E. coli* for any five samples collected within a 30-day period exceeded 126 *E. coli* CFU/100 mL of water. In 2006, the Ohio EPA initiated the use of the single sample maximum standard. This new standard dictated that a beach will be closed or posted under an advisory if the *E. coli* density exceeds 235 CFU/100 mL in a single water sample (ODH, 2006). Under the new standard, posted advisories at LEB increased to 17 days in 2006, from 8 days in 2005. While common causes for beach advisories can include various contaminants including elevated bacterial levels of unknown origin, stormwater runoff, sewage spills and overflows, algal blooms, dredging, and chemical contamination (NRDC, 2005), all LEB advisories from 2003 to 2005 were attributed to bacteria levels that exceeded established beach water quality standards (ODH, 2006).

Ingestion of pathogens associated with poor water quality is a significant risk to the health of people visiting a beach (Kueh et al., 1995). It was reported that on average, children and adults recreating in water swallow 37 and 16 ml of water,

respectively, per every 45 minutes of activity (Dufour et al., 2006). Since the *E. coli* density in beach water can be as high as 2 X 10^4 CFU/100 mL (McLellan and Jensen, 2005), 7400 and 3200 *E. coli* cells could theoretically be consumed by children and adults, respectively during less than one hour of activity. Ingested bacteria loads in this range can pose a significant health risk. For example, it has been established that as few as ten *E. coli* O157:H7 cells can cause gastrointestinal illness in humans (Mead and Griffin, 1998). Therefore, even small volumes of ingested water might pose a risk to beachgoers, in particular young children, the elderly, pregnant women, and immunocompromised people (NRDC, 2006).

Although it has been established that bacteria greatly impact water quality, most municipalities have failed to identify sources of bacterial pollution to recreational waters (NRDC, 2006). There are two classes of bacterial pollution sources: host sources (identifying animals or human as sources) and geographic sources (identifying ditch, stream, or creek as sources). Receiving waters can be either directly contaminated by host animal shedding, or indirectly by farmland leaching, septic tank leaking or ditch water transport. For example, geographic source identification involves identifying the specific location, such as streams, ditches, or agricultural land as the source of fecal pollution. To track the source of fecal pollution, two broad categories of bacterial source tracking (BST) have been established including library-dependent - and library-independent methods. Library-dependent methods compare phenotypic or genotypic patterns in bacteria

collected from polluted sinks with those from potential sources. Library-dependent methods present considerable limitations, as they require the generation of isolate libraries containing potentially thousands of isolates collected from the pollution sink and each potential source (Wiggins et al., 2003). This large number is necessitated by the high diversity of environmental bacterial populations and broad geographic locations of potential sources. Because of the vast size of appropriate isolate libraries, library-dependent methods can result in excessive expenditure of time, labor and money (Johnson et al., 2004; Wiggins et al, 2003).

In contrast, library-independent methods do not require a reference isolate library. In fact, when compared with library-dependent methods, library-independent methods were shown to be superior for identifying the sources of five different fecal sources artificially added to a water sample (Griffith et al., 2003). Library independent methods are based on the presence or absence of genes associated with a target organism or function in an environmental sample. For example, specific human and animal DNA markers can be used to identify the host origin (animal vs. human) of fecal pollution (Bernhard and Field, 2000 a, b; Hamilton, et al., 2006). Library-independent analysis targeting the whole bacterial community has also become an important tool to investigate the ecology of environmental bacteria. For example, DGGE can provide fingerprints of microbial communities by separating PCR generated fragments that vary in nucleotide sequence by as little as one base pair (Muyzer

et al., 1993). While DGGE has been generally used to analyze the structure of microbial community in the soil and water environment (Sigler and Zeyer, 2004; Sekiguchi et al., 2002), samples collected at different times can be analyzed simultaneously, therefore allowing a temporal monitoring of bacterial community structure (Muyzer and Smalla, 1998). While library-dependent methods might require hundreds to thousands of isolates to construct a reference library, DGGE is a relatively rapid method without the necessity of a reference library.

DGGE can provide a relatively rapid diagnostic fingerprint useful for differentiating complex bacterial assemblages. Therefore, DGGE might represent a methodology to quickly identify geographic sources of fecal pollution. The effective use of DGGE requires the utilization of a gene target that can differentiate bacterial communities, thereby allowing a comparison of communities collected form pollution sinks and potential sources. Furthermore, a gene associated with an established fecal indicator would further enhance the use of DGGE. *uidA* is expressed in approximately 95% of environmental *E. coli* (Martins et al., 1993) and codes for the enzyme, *β*-glucuronidase, which has been shown to be an appropriate molecular target for *E. coli* detection (Bej et al.,1991). It has been reported that nucleotide sequence analysis of *β*-glucuronidase (*uidA*) from 182 human and animal fecal *E. coli* isolates resulted in 75% correct assignment to the primary host, suggesting the utility of *uidA* as a microbial source tracking target (Ram et al., 2004). PCR-DGGE of *uidA* has also shown as an effective tool to differentiate unique *E. coli* populations in the secondary

environment (Sigler and Pasutti, 2006; Farnleitner et al., 2000a). Therefore, PCR-DGGE of uidA as a library independent method can be used to rapidly identify the potential sources of bacterial pollution in the environment.

In 1996, a study commissioned by MBSP and the City of Toledo found that *E. coli* densities at LEB were highest in water samples collected from the east end of the beach and declined in samples collected from the west end (Anonymous, 2003). In addition, research conducted by our laboratory in the summer of 2004 reached the same conclusion (data not shown); that *E. coli* densities in water collected from the east end of the beach were consistently higher than densities in water at the west end. These studies led to the hypothesis that Berger Ditch, a drainage ditch located adjacent to the east end of the beach, might be a significant source of *E. coli* impacting the water quality of LEB. Prior to the development of this hypothesis, several studies have attempted to identify the pollution source. Lauber (2001) used FAME- (fatty acid methyl ester) and plasmid analyses of *E. coli* isolates from water collected from LEB, the Inland Lake Beach, and nearby ditches to ascertain the contribution of *E. coli* from Berger Ditch. The study did not conclusively define the source of fecal pollution at MBSP, as plasmid analysis was limited in tracing diffuse pollution sources. Further efforts by Francy et al. (2005) determined that the spatial distribution of *E. coli* could be explained by several environmental factors including rainfall, wind direction and wave height. However, no definitive identification of fecal contamination sources could be made. One variable that could have

complicated these studies is the presence of other nearby ditches. While Berger Ditch has been historically implicated as the primary source of bacterial pollution, additional sources such as McHenry- and Tobias Ditches, are located to the west of the beach and drain significant areas of the surrounding watershed into Lake Erie. Therefore, these sites could also have contributed to the bacterial pollution at the beach.

Most previous attempts to identify geographic sources of bacterial pollution have relied on point-in-time samplings. However, because seasonal patterns in climate, vegetation, and animal- and human activity are not consistent, it is likely that the sources, and therefore dynamics of bacterial pollution volume and composition, will change throughout the year. The collection of microbiological data throughout the year can provide several advantages over single season-, or point-in-time samplings. First, a long-term sampling effort can integrate many of the short-term, periodic issues that have been shown to affect *E. coli* densities in natural waters (Whitman and Nevers, 2004). These issues include environmental factors, such as UV radiation, wind speed and direction, wave height and relative lake level, temperature, rainfall and stream flow volume (Whitman et al., 2004; Lipp et al., 2001; Kinzelman et al., 2004). Additional factors include fecal inputs from wild and domestic animals, such as gulls (Lévesque et al., 1993), swan (Khatib et al., 2003) and cattle (Doran and Linn, 1979). If one or more of the aforementioned factors is strong, but short lived, and results in *E. coli* communities from one potential source that appear similar to those at a pollution sink, a one-time sampling might result in a conclusion that

inflates the true contribution of the source. In contrast, long-term (year-long) sampling might avoid this bias by allowing the integration of multiple point-in-time analyses. Furthermore, the estimation of correlation between *E. coli* densities in polluted sinks and potential sources requires values from multiple samplings. Finally, although summer is the primary season for water recreation, it is not the only season for water-enthusiasts. Sports such as windsurfing and fishing are performed at MBSP year-round (personal observation). Therefore, integrated, yearlong sampling can account for water quality conditions during secondary recreation seasons, as well as the primary recreation season.

The overarching goal of this study was to identify the geographic sources of bacterial pollution at LEB. This was addressed by performing DGGE community fingerprinting analysis on *E. coli* community from water collected from LEB, Berger-, McHenry- and Tobias Ditches, from October 2005 to September 2006.

The specific objectives of this study were to:

1. Use *E. coli* density correlations and *E. coli* community DGGE fingerprinting to identify the geographic sources of fecal pollution impacting water quality at LEB.
2. Monitor the year-long dynamics of *E. coli* densities and community structure in water collected from LEB and nearby ditches, including Berger-, McHenry- and Tobias Ditches to determine if the source ditch(es) is changing over time.

3. Confirm the results of DGGE by using a high-resolution library-dependent technique, BOX-PC on a library of *E. coli* isolates collected from the beach and three ditches during the swimming season (June to September, 2006).

Materials and Methods

Site description. Water samples were collected from LEB as well as three ditches used for storm water management, including Berger-, McHenry-, and Tobias Ditches (Fig. 1).

LEB. LEB is located in MBSP in northwest Ohio on the southern shore of Lake Erie. It is approximately 750 m in length and defined by six man-made embayments to limit wave energy, each approximately 100 m in length.

Berger Ditch. Berger Ditch is approximately 5,500 m long and flows from south to north. It starts far south before it connects with Wolf Creek (about 15,000 m long) approximately 2,400 m south of MBSP. Berger Ditch flows through varying land management systems, including residential areas, farmland, wetlands, forest, and ultimately through the MBSP's marina before emptying into Lake Erie directly adjacent to the east end of LEB. Berger Ditch is approximately one m wide at its headwaters, and it gradually becomes wider (~6 m) as it approaches the outfall to the lake. Berger Ditch continually flows throughout the year.

McHenry- and Tobias Ditches. McHenry Ditch is approximately 4,900 m long, flows south to north and eventually empties into Lake Erie approximately

900 m west of LEB. Tobias Ditch flows for 3,500 m through residential areas and farmland areas, and adjacent to a horse meadow (about 25 acres in area and containing 32 horses) before emptying into Lake Erie approximately 1,750 m from the west end of the beach (Fig. 1). Compared with Berger Ditch, McHenry- and Tobias Ditches are shorter, shallower and narrower, each less than one m wide. Neither ditch flows continually throughout the year, but both flow after rainfall. Therefore, it was not always possible to sample water from these two ditches. All three ditches are open to the air, except when they cross a road, at which point they are continued to an underground sewer pipe.

Water collection. Water samples were collected along LEB and the ditches as follows:

(i) Four sampling sites along Berger Ditch (the point where Wolf Creek and Berger Ditch merge, MBSP entrance, pre-marina and the mouth of Berger Ditch as it enters Lake Erie),

(ii) Three sites along LEB (west-most-, middle- and east-most embayments),

(iii) Two sites, representing the terminal (north-most) section of sewer pipe and mouth of McHenry Ditch and Tobias Ditch (Fig. 1).

Fig.1 Map of LEB and Berger-, McHenry- and Tobias Ditches.

Samples were collected monthly from October 2005 to September 2006 from all sites and twice per month from the beach sites from April 2006 to September 2006. Surface water (10-20 cm depth) from each site was collected in triplicate, sterile, 1 L plastic bottles and stored on ice until laboratory analysis was performed (within 6 hours after collection). Samples were usually collected between 11:00 am and 2:00 pm within 36 hours following a rainfall event (0.13-8 cm) that could make ensure that water in each ditch was positively flowing (south-to-north) into Lake Erie. The rainfall data were collected from NOAA, (http://www.weather.gov/climate/index.php?wfo=cle). Conversely, backflow would occur when northerly currents forced lake water backwards, up the ditch. Water samples from sampling sites under backflow conditions were not collected.

The mean *E. coli* density (CFU/100 mL) for each time point (month) was obtained by calculating the mean *E. coli* density at each site of the beach and in each ditch. Swimming activities usually take place during June to September, and this duration of time is the most important season for bacterial source tracking. Therefore, in this study, the samplings during June to September, as summing season were grouped to the swimming season, and the other eight months samplings from October 2005 to May 2006 were grouped into nonswimming season. To evenly compare the results between the swimming and nonswimming season, samplings from the nonswimming season were broke into halves. So, the total twelve months samplings were then expressed within the context of three arbitrary “seasons”: (i) Season One (nonswimming season, from

October, 2005 to January, 2006); (ii) Season Two (nonswimming season, from February, 2006 to May, 2006), and (iii) Season Three (swimming season, from June, 2006 to September, 2006). The temporal correlation of *E. coli* densities between ditch and beach samples was calculated using Statistical Analysis Software (v 9.1, SAS Institute, Inc).

***E. coli* enumeration.** *E. coli* densities in the water samples were determined following standard membrane filtration procedures (USEPA, 2000c). Specifically, three volumes (1 mL, 25 mL, 100 mL) of each water sample were passed through a nitrocellulose membrane (0.45 μm pore size). For the 1 ml volume, 1 ml of sample and 9 ml of 10 mM sodium phosphate buffer (pH 7.0) were filtered together to ensure adequate dispersal of the bacteria over the membrane. Membranes were placed onto Rapid *E. coli* 2 medium (BioRad Laboratories,Inc), and incubated upside down at 37^{o} C for 24 h. Following incubation, purple colonies were counted in the plates that contained between 10 and 100 colonies. Rapid *E. coli* 2 medium is a medium for the enumeration of *E. coli* and other coliforms that takes advantage of the activity of two enzymes: *β*-D-glucuronidase and *β*-D-galactosidase. To detect the activity of these two enzymes, the medium contains two chromogenic substrates: 4-methylumbelliferyl-β-D-glucuronide (MUG) and 5-bromo-4-chloro-3-indolyl-β-d-galactosidase (X-Gal). When *β*-D-glucuronidase cleaves MUG, colonies will turn blue, while *β*-D-galactosidase cleavage of X-Gal results in magenta colonies. *E. coli* strains express both enzymes simultaneously, resulting in purple colonies. Non *E. coli* coliforms,

which only express *β*-D-galactosidase, remain blue (Fig. 2). *E. coli* density was expressed as colony forming unit per 100 mL water (CUF/100 mL).

Fig.2 *E. coli* and coliforms growing on Rapid *E. coli* 2 agar. *E. coli* in this agar shows purple color, and the other coliforms shows blue color.

For eventual DNA extraction (described below), large volumes of water (75 - 800 ml) were filtered and incubated (as described above) to generate lawns of *E. coli*. Incubation was performed at 44.5^{o} C for 24 h. This incubation temperature was higher than of the enumeration protocol to limit the growth of bacteria exhibiting lower optimal growth temperatures, thereby maximizing *E. coli* enrichment.

***E. coli* community DNA isolation.** DNA isolation from the enrichment membranes was performed according to the protocol of Sigler and Zeyer (2002). Specifically, each membrane containing an enriched *E. coli* community was cut into small pieces with sterile scissors and then transferred to a sterile, 2 ml microcentrifuge tube containing 0.4 - 0.5 ml of glass beads (0.10 mm diameter)

and 1.0 ml of DNA extraction buffer (5% SDS, 50 mM NaCl; 50 mM Tris-HCl, pH 7.6; 50 mM EDTA). Tubes were shaken with a Fast-Prep (Thermo Electron Corporation) instrument for 30 seconds at 5.5 ms^{-1}, followed by centrifugation for three minutes at 14,000 x g. The supernatant (~ 0.4 - 0.6 ml) was transferred to a new 1.5 ml microcentrifuge tube and purified by adding 0.5 volumes of both phenol (Tris-saturated, pH 8.0) and chloroform/isoamyl alcohol (24:1) (final volume ~ 1 ml).The solution was vortexed until milky (5 - 10 s) followed by centrifugation for three minutes at 14,000 x g. The aqueous phase containing the DNA was transferred to a new 1.5 ml tube and an equal volume of chloroform was added, vortexed (5 -10 s), followed by centrifugation for three minutes at 14,000 x g. The upper, aqueous phase was transferred to a new 1.5 ml tube. DNA was precipitated by adding 10% and 70% of the DNA extract volume of 3 M sodium acetate solution and isopropanol, respectively, Tubes were mixed by inverting several times by hand, and then centrifuged at 14,000 x g for 30 minutes at 10^{o}C. The supernatant was decanted by aspiration and the remaining DNA pellet was washed with 0.5 ml of ice-cold 70% ethanol by gently inverting the tube several times. The ethanol was aspirated and the DNA pellet was suspended in 50 µL of DNase/RNase-free water. Following quantification by spectrophotometry (A_{260}), each DNA sample was diluted to a concentration of approximately 100 µg mL^{-1} and stored at -20^{o} C.

***uidA* polymerase chain reaction.** A portion of the *uidA* gene of *E. coli* was amplified by PCR using primers UAL-1939 and UAR-2105, which target the

carboxyl coding region of the *uidA* gene (Bej et al., 1991). Each reaction contained the desired DNA at a concentration of 1 ng μl^{-1}, PCR buffer (1X), 2.5mM $MgCl_2$, BSA (bovine serum albumin) (1 mg/mL), 0.2 mM each dNTP, 0.3 µM each primer), 0.02 U μL^{-1} *Taq* DNA polymerase (Promega Corporation), and DNase/RNase-free water to a final volume of 50 µL. A positive control containing DNA isolated from *E. coli* strain DHα5, and a negative control containing no added DNA were also include in each PCR assay. The PCR program consisted of an initial denaturing cycle (94^{o}C for 5 min), 30 cycles of 94^{o}C for 45 s, 50^{o}C for 45 s, and 72^{o}C for 60 s, and a final extension cycle of 72^{o}C for 30 min. PCR products were screened by electrophoresis in 1x TAE buffer at 100 v for 15 min and archived by image analysis with a Gel Logic 200 system (Eastman Kodak Company).

DNA community analysis by DGGE. DGGE (Sigler et al., 2004) was performed in a Dcode DGGE system (BioRad, Laboratories, Inc) for 1000 V●h (200 V for 5 h) at 60^{o} C using a denaturing gradient of 40-55% (a 100% denaturing gel contains 7M urea and 40% formamide). Approximately 30 µL of PCR product was loaded into each lane, while a standard marker was loaded such that a maximum of five samples separated each marker well. The standard marker was generated by mixing the PCR products (16S rRNA primer (Muyzer et al., 1993)) of the DNA from six bacterial strains. These strains included *Pseudomonas aeruginosa, Ralstonia pickettii,* and four environmental *E. coli* strains. Following electrophoresis, the gel was stained for 15 min in 50 mL of a

1:10,000 dilution of GelStar nucleic acid stain (BioWhittacker, Inc), and band patterns were archived by an image analysis.

DGGE fingerprint patterns were analyzed with GelCompar II software (version 4.6, Applied Maths, Inc). DGGE fingerprints were normalized using abovementioned standard marker. The similarity of *E. coli* community fingerprints was calculated using the band-based, Dice coefficient (Dice, 1945) with 0% optimization and 3% band position tolerance settings in the GelCompar II software. Bands in each lane were picked by computer and then checked manually to avoid missassignment. Similarity coefficients were calculated for each fingerprint pair possibility, from which a similarity matrix was constructed. A dendrogram displaying the inferred relationship between all fingerprints was generated using the parameters automatically set by the computer.

***E. coli* isolate libraries and BOX-PCR analysis.** This study represented the first use of DGGE to identify geographic sources of fecal pollution. Therefore a confirmation of the method's validity was necessary. BOX-PCR is a library dependent method that has been shown to be an effective tool for differentiating human and animal sources of fecal pollution (Dombek et al., 2000). Therefore, in an effort to validate the results of the DGGE analyses, BOX-PCR analysis of *E. coli* libraries generated during a portion of the study was performed.

A library of *E. coli* colonies was generated during the summer swimming

season (June to September of 2006). Specifically, during each month, approximately 25 isolates were collected from the beach and each ditch, resulting in a total of 100 isolates from the four locations each month. The *E. coli* were collected from the same filter membranes that were generated during the enumeration analysis. Isolates were picked with streaking needles, transferred twice to fresh Rapid *E. coli* 2 agar and incubated (37^{o} C overnight). Pure, putative *E. coli* isolates were confirmed for identity by determining morphology on eosin-methylene blue agar (EMB) (OXOID, Inc). EMB agar is a differential medium used in the identification and isolation of Gram-negative enteric rods. *E. coli* colonies on EMB medium appear black, with a metallic green sheen (due to the fermentation-driven precipitation of methylene blue from the medium). The identify of the putative *E. coli* was further confirmed by PCR using 16S rRNA *E. coli* primers specific for *E. coli* (Sabat et al., 2000). DNA was isolated from each confirmed isolate using a Wizard SV 96 Genomic DNA Purification System (Promega Corporation). Following isolation, the DNA was quantified as described above and stored at -20^{o} C.

BOX PCR was performed according to the protocol of Rademaker et al. (1998). To facilitate the electrophoresis of the numerous BOX-PCR samples, three electrophoresis systems were used in this study; (i) Owl Model A1 Gator Large Gel Electrophoresis System (Owl Separation Systems), (ii) Fisher Biotech Horizontal Electrophoresis Midigel System (Fisher Scientific, Inc.), and (iii) a BioRad Sub-cell GT (BioRad, Laboratories, Inc.). Migration distance in these

three systems was approximately 13 cm, however to achieve comparable fingerprint separation, it was necessary to vary the running time used for each system (90 min, 120 min and 180 min for BioRad, Fisher and Owl systems, respectively). To assess the impact of different electrophoresis running times, a 100 bp DNA marker was loaded in several gel lanes and electrophoresed in each system for the duration of time mentioned above. The marker fingerprints were analyzed as described above, which revealed 99.99% similarity among the 68 marker test lanes (27 for Owl system, 23 for BioRad, and 18 for FisherBiotech), indicating that the selection of running times for each machine were appropriate and would not alter the analysis of fingerprint patterns.

BOX-PCR fingerprints were generated from 30 µl of each reaction mixture loaded onto a 1.5% agarose gel containing ethidium bromide (0.005 mg/L). A 100 bp DNA size ladder (Fisher Scientific, Inc.) was loaded into the two outside- and middle wells of the gel. Electrophoresis was performed as described above. Isolate fingerprints were archived by image analysis.

BOX-PCR fingerprints were analyzed by GelCompar II software as described above, except that normalization were performed using the 100 bp DNA ladder instead of the DGGE marker. In an effort to express the BOX-PCR fingerprint data in a manner that could validate the DGGE results, multidimensional scaling (MDS) was performed to provide a convenient visual interpretation of the similarity among the fingerprints of total *E. coli* isolates. MDS produces a 'map' in two or

more dimensions that the distance between isolates best approximates the relative similarity between isolates, allowing researcher to visualize proximity of isolates both within and among source groups (Ritter et al., 2003). MDS analysis was used in this study to classify BOX-PCR fingerprints in an effort to better understand relationships (e.g. separation or clustering) between the *E. coli* isolates based either on temporal or geographic patterns.

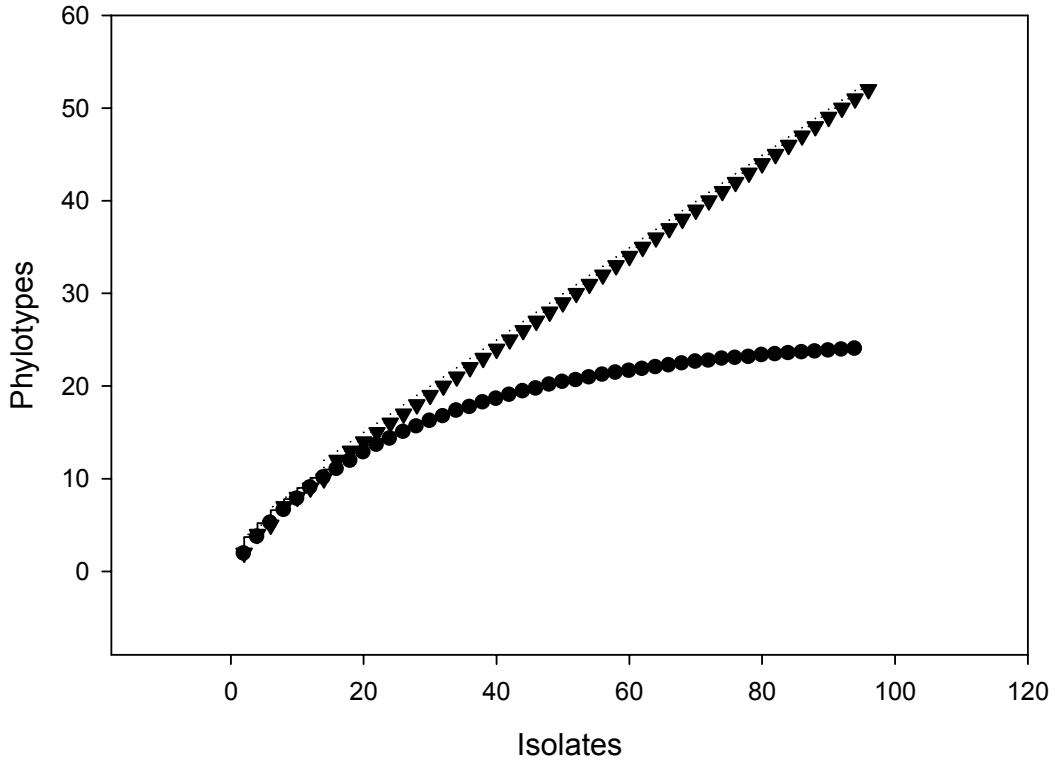
Jackknife analysis was performed (maximum similarity) to determine how accurately the BOX-PCR fingerprint similarity coefficients could predict the environment from which any given isolate was obtained. Briefly, the isolates were first manually assigned to the correct source groups (geographic areas), following which each isolate was individually removed from the database. Similarity between the removed isolate and each of the remaining isolates in each source group was calculated, based on which, a maximum group similarity coefficient was determined. The removed isolate was assigned to the source group that possessed the greatest maximum group similarity coefficient, and the percentage of accurately assigned isolates for each source group was then calculated, resulting in a rate of correct classification (maximum similarity) of the isolates collected from each location. If the rate of correct classification was high for each location, it meant that BOX-PCR fingerprints of the *E. coli* isolates based on the location of origin were discriminative, and vice-versa. The results of the DGGE analyses would be validated if they also showed poorly discriminative *E. coli* community fingerprints among various locations.

BOX- PCR reproducibility. To test whether the fingerprints of the same strain analyzed by BOX PCR in one gel were reproducible in other gels, DNA from five different *E. coli* isolates (e.g. strain A-E) was BOX-PCR amplified in five, separately mixed reactions followed by electrophoresis and image analysis as described above. The reproducibility of BOX-PCR was expressed as the fingerprint similarity (as calculated by GelCompar II) among the five repeated fingerprints generated from each of the five *E. coli* strains tested (e.g. A1-A5). The cut off value was calculated by averaging the similarity of five repeated fingerprints of each strain as shown in the calculation {[(similarity of A1-A5) + (similarity of B1-B5) + (similarity of C1-C5) + (similarity of D1-D5) + (similarity of E1-E5)] / 5}. This resulting average similarity was used as a threshold similarity, above which isolates can be considered identical. Using this threshold value, the number of *E. coli* phylotypes in the libraries could be determined and used for rarefaction analysis.

Since BOX-PCR analysis could require from hundreds to thousands of isolates to correctly discriminate the fecal sources from human to animals (Dombek et al., 2000; McLellan et al., 2003; Johnson et al., 2004). It was necessary to determine if our library size was appropriate. Rarefaction analysis is a technique to compare species richness among samples of different sizes and was performed by Analytic Rarefaction software (version1.3, University of Georgia) in an effort to determine whether the sample size is enough for

representing the richness of sample species. Each point stands for the expected species under certain sample size. Rarefaction curves are used to calculate the number of unique types associated with a given sampling effort. A high slope indicates a high likelihood that a novel member of the community will be sampled if sampling was to continue, and therefore a large proportion of the bacterial diversity has not been sampled. In contrast, a low slope (or zero slope in the most extreme case) indicates that repeated sampling will yield only a small number of additional species, therefore indicating appropriate sampling effort. For example, the straight line in Fig. 3 indicates a high likelihood that new phylotypes of *E. coli* will be sampled with continued sampling, which means the sampling size is inadequate, and further sampling is required. In contrast, the curved line shows that more intensive sampling will result in low likelihood of collecting new *E. coli* phylotypes.

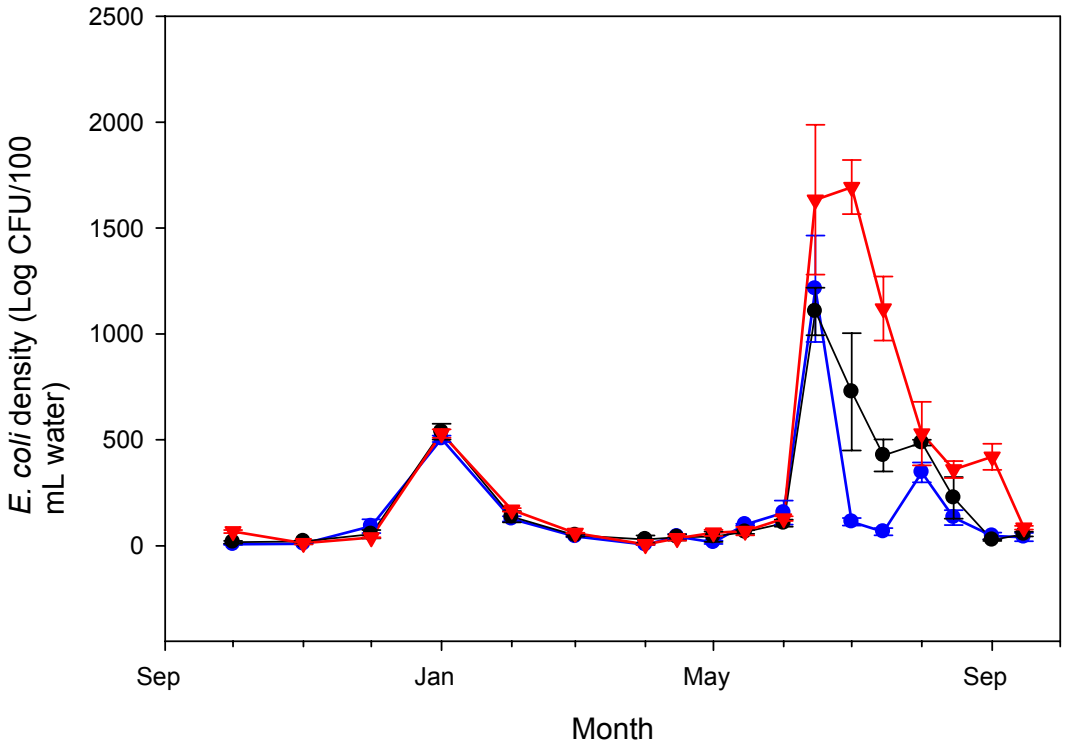
Fig. 3 Example of rarefaction analysis between appropriate- and inappropriate samplings. The circle (curved line) indicates the appropriate sampling, and the triangle (straight line) indicates the inappropriate sampling.



Results

***E. coli* enumeration.** *E. coli* densities in LEB (including east, middle and west) were dynamic, ranging from as low as 10 CFU/100 mL in November 2005 to as high as 1,700/100 mL in July 2006 (Fig. 4). In general, *E. coli* densities at three sites of the beach were more consistent in Seasons One and Two than in Season Three. Specifically, in Seasons One and -Two, *E. coli* densities in the three beach sites were low, less than 100 CFU/100 mL, except in January, when density was greater than 500 CFU/100 mL. In Season Three, *E. coli* densities in the three beach sites increased, beginning in mid-June, when *E. coli* density was approximately 1,600 CFU/100 mL in the east, and 1,200 CFU/100 mL in the west. While, *E. coli* density in the three beach sites decreased during the next three months, the density on the east side of the beach was always significantly higher than that of the west side ($p<0.05$). No significant difference in *E. coli* density between east and west sides was observed in Seasons One and Two ($p=0.44$).

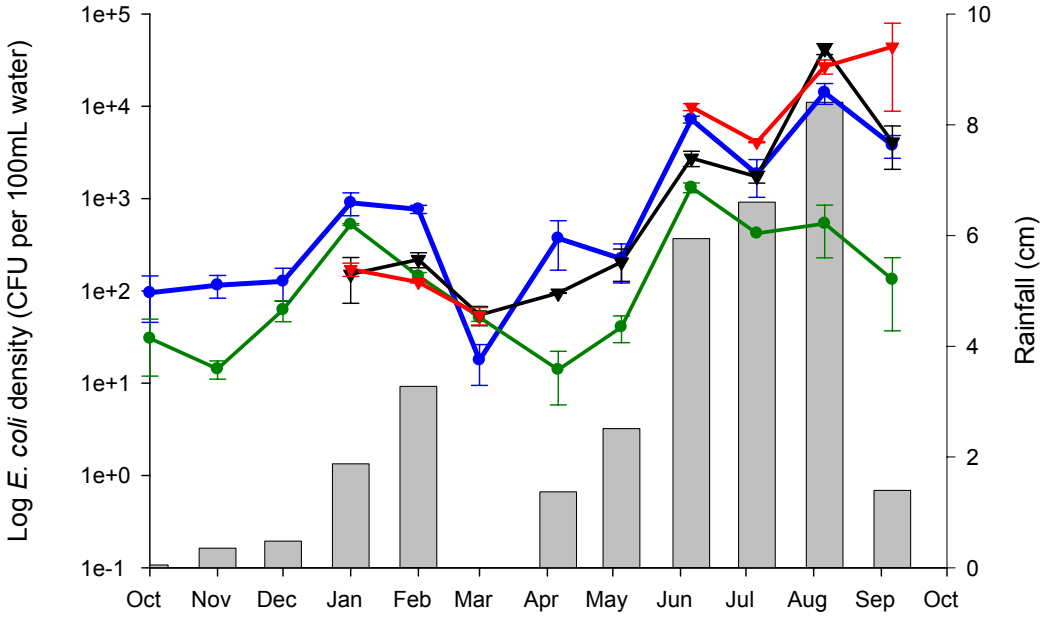
Fig. 4. *E. coli* densities at the west, middle and east sites of LEB from October, 2005 to September, 2006. Error bar indicates the standard error (n=3). Each line indicates *E. coli* density in each beach site. Red, black, and blue lines indicate *E. coli* densities in east, middle and west sites of the beach, respectively.



Average *E. coli* densities in each ditch- and beach water sample were variable throughout the year, ranging from 10^1 to 10^5 CFU/100 mL (Fig. 5). *E. coli* densities in the ditches and the beach were consistent during Seasons One and -Three. During Season One, *E. coli* densities in Berger Ditch and the beach increased over time. In Season Three, densities in all ditches and the beach increased from June to August, and decreased in September. However, in Season Two, the trend of *E. coli* densities in ditches and the beach was inconsistent, as densities in Berger Ditch decreased in March, and then increased in April, while densities in the beach decreased from February to April. As compared with *E. coli* densities in ditches, the densities at the beach were consistently lower, ranging from 10^1 to 10^3 CFU/100 mL.

The average rainfall depth during the four days prior to sampling and the sampling day of each sampling event was highest during Season Three (5.6 cm) and lowest during Season One (0.7 cm), with intermediate rainfall occurring during Season Two (1.8 cm). Rainfall depth was significantly correlated with *E. coli* densities in three of the four sites: LEB (r=0.68), Berger Ditch (r=0.80) and McHenry Ditch (r=0.67). *E. coli* density in Tobias Ditch was not correlated with rainfall volume (r=0.12).

Fig. 5. Combination of rainfall data and monthly changes of *E. coli* composite densities in water collected from each ditch and the beach. There were some missing points of McHenry and Tobias Ditches resulting from unavailable sampling due to no water flowing. Each bar indicates the rainfall depth during the four days prior to sampling and the sampling day. Each line indicates the average *E. coli* densities from all the sites of each ditch and the beach. Specifically, the blue (Berger Ditch), black (McHenry Ditch), red (Tobias Ditch) and green (LEB) lines indicate mean *E. coli* densities. Error bars indicates the standard error (n=3). Missing points of the lines are due to unavailable sampling because of little or no water flow in certain ditch(es).



Correlations between ditch and beach *E. coli* densities. The correlation of *E. coli* densities between each ditch and the beach were variable (Table 1). For example, during Season One, the *E. coli* density in the beach water was significantly correlated with densities in Berger Ditch ($r=0.98$, $p<0.05$). In contrast, *E. coli* densities in McHenry and Tobias Ditches were negatively correlated with those at the beach.

During Season Two, the *E. coli* densities in all ditches correlated with densities at the beach. Specifically, correlation coefficients between Berger-, McHenry-, and Tobias Ditches and LEB were 0.63, 0.59 and 0.94, respectively ($p < 0.05$) (Table 1).

During Season Three, the *E. coli* densities in each ditch were uncorrelated ($r< 0.2$) with those at the beach water.

Table 1. Results of seasonal correlation tests based on *E. coli* densities of water sample from each ditch and the beach. An asterisk indicates $p<0.05$; n indicates the number of comparisons.

Season One

Correlation between	Pearson correlation coefficient	n
Berger/Beach	0.98*	12
McHenry/Beach	-0.73	3
Tobias/Beach	-0.70	3

Season Two

Correlation between	Pearson correlation coefficient	n
Berger/Beach	0.63*	12
McHenry/Beach	0.59*	12
Tobias/Beach	0.94*	6

Season Three

Correlation between	Pearson correlation coefficient	n
Berger/Beach	0.13	12
McHenry/Beach	-0.14	12
Tobias/Beach	-0.56	12

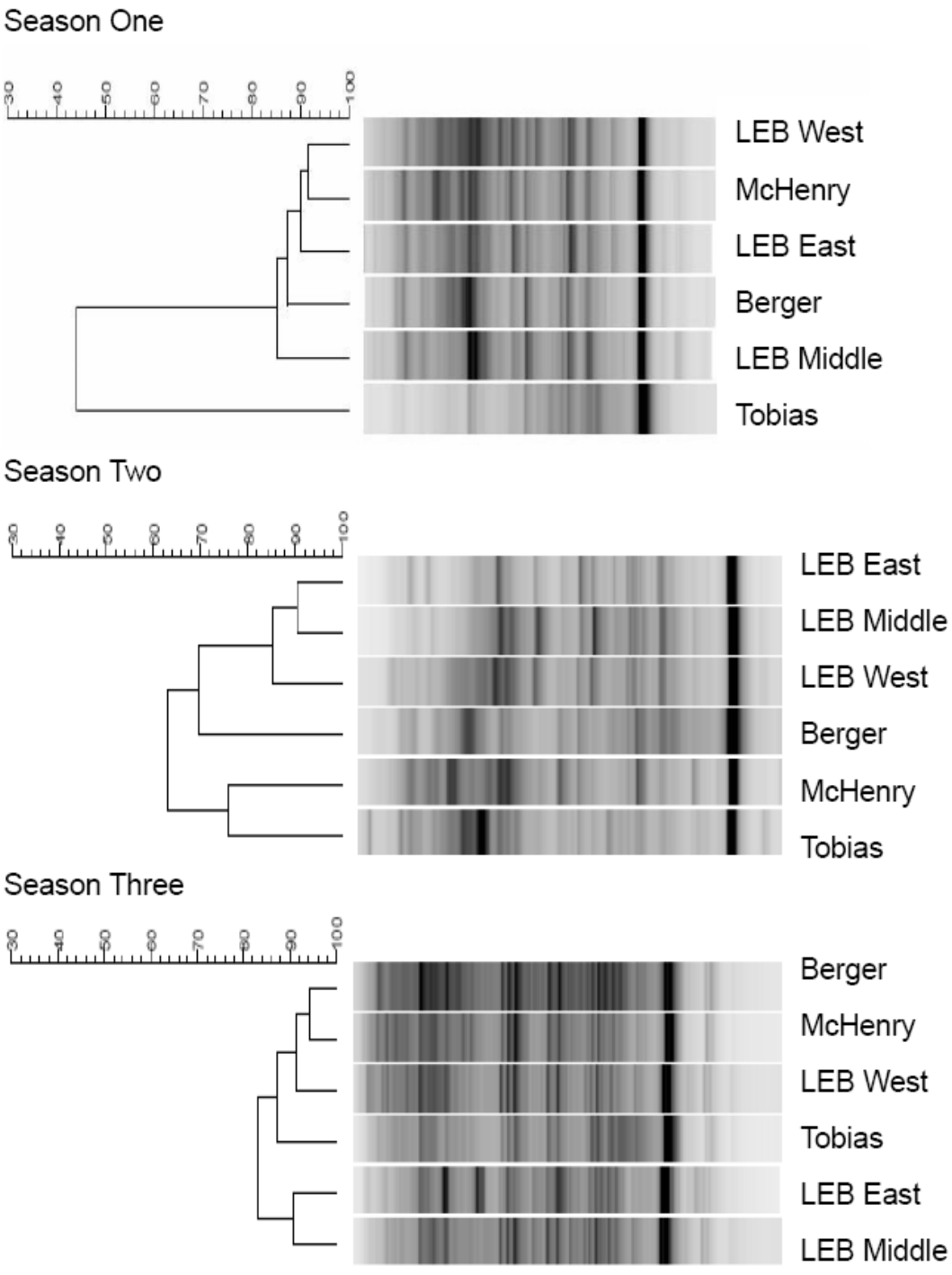
***E. coli* community structure.** In an effort to characterize the dynamics of the bacterial pollution at LEB, fingerprints were generated that represented the *E. coli* community in each ditch and the three beach sites. These fingerprints were assessed to determine the temporal dynamics of *E. coli* and were compared to determine community similarity among the sites, under the assumption that high similarity of *E. coli* communities of the ditch and beach samples would imply a source-sink relationship between the sites. Dendrogram analysis of community fingerprints showed that in Season One, *E. coli* communities in Berger Ditch, Tobias Ditch and the beach (west, middle and east sites) were 85% similar, forming a distinct cluster, to which the Tobias Ditch communities were 44% similar.

Analysis of fingerprints generated from *E. coli* communities during Season Two showed that all communities were more similar to each other (62% overall similarity) than those from Season One (44%). Distinct clustering (85% similarity) was observed among *E. coli* community fingerprints generated from the beach sites. Of the three ditches, Berger Ditch harbored *E. coli* communities most similar to those of the beach sites (69%), followed by McHenry- and Tobias Ditches (62%).

E. coli community fingerprints from Season Three were more similar to each other than those from Seasons One and Two, exhibiting 88% overall similarity. Within this tight clustering, *E. coli* communities from the different sites were highly

similar indicating that the communities from the three ditches were all similar to those at the beach.

Fig. 6 Dendrogram analysis of DGGE gel of *uidA* amplified from *E. coli* community obtained from water in each ditch and the beach three sites (LEB west, LEB middle and LEB east) among three seasons. Denaturing gradient is from 40 to 55%. Scale indicates the percentage of similarity of *E. coli* community fingerprints among different locations.

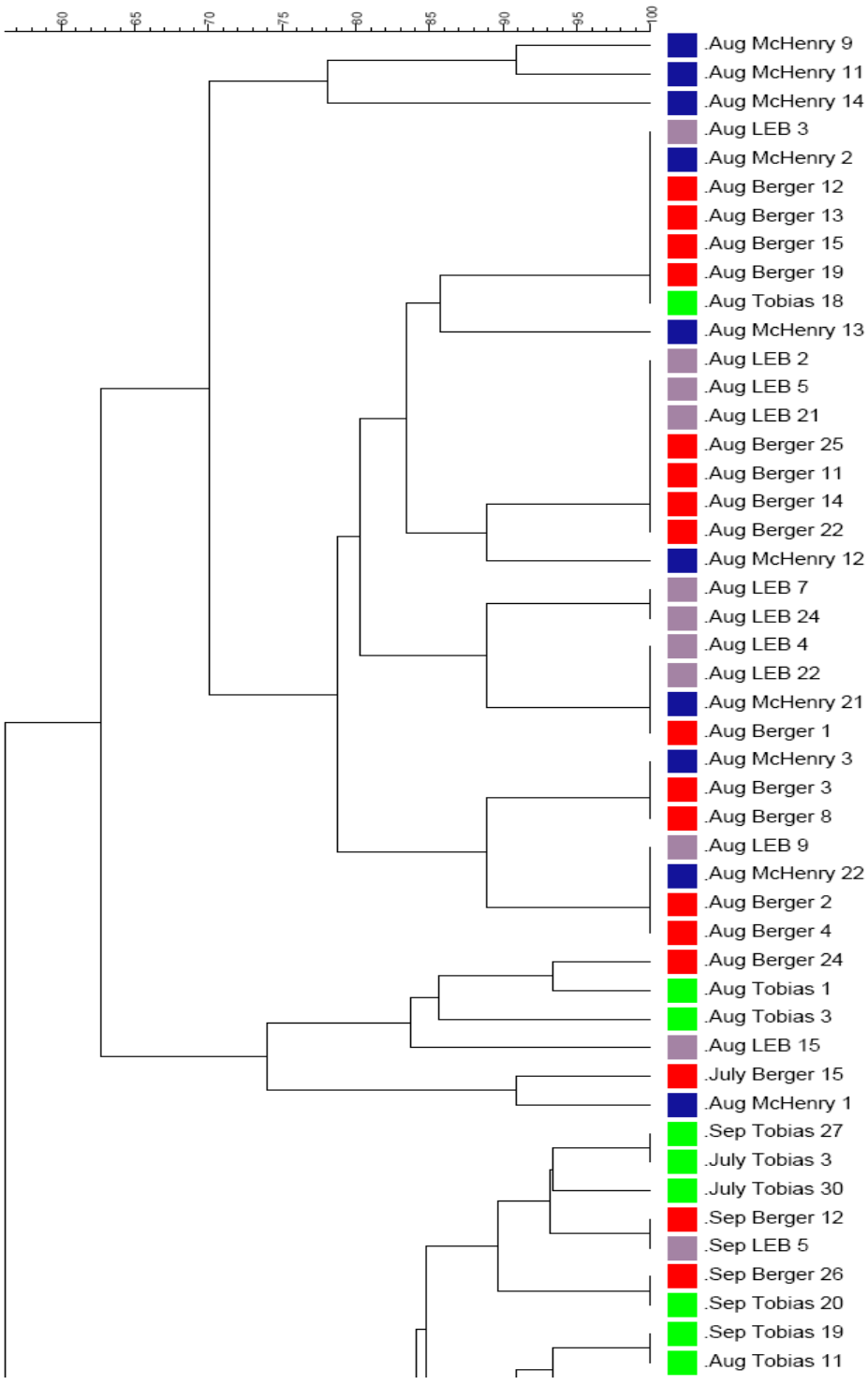


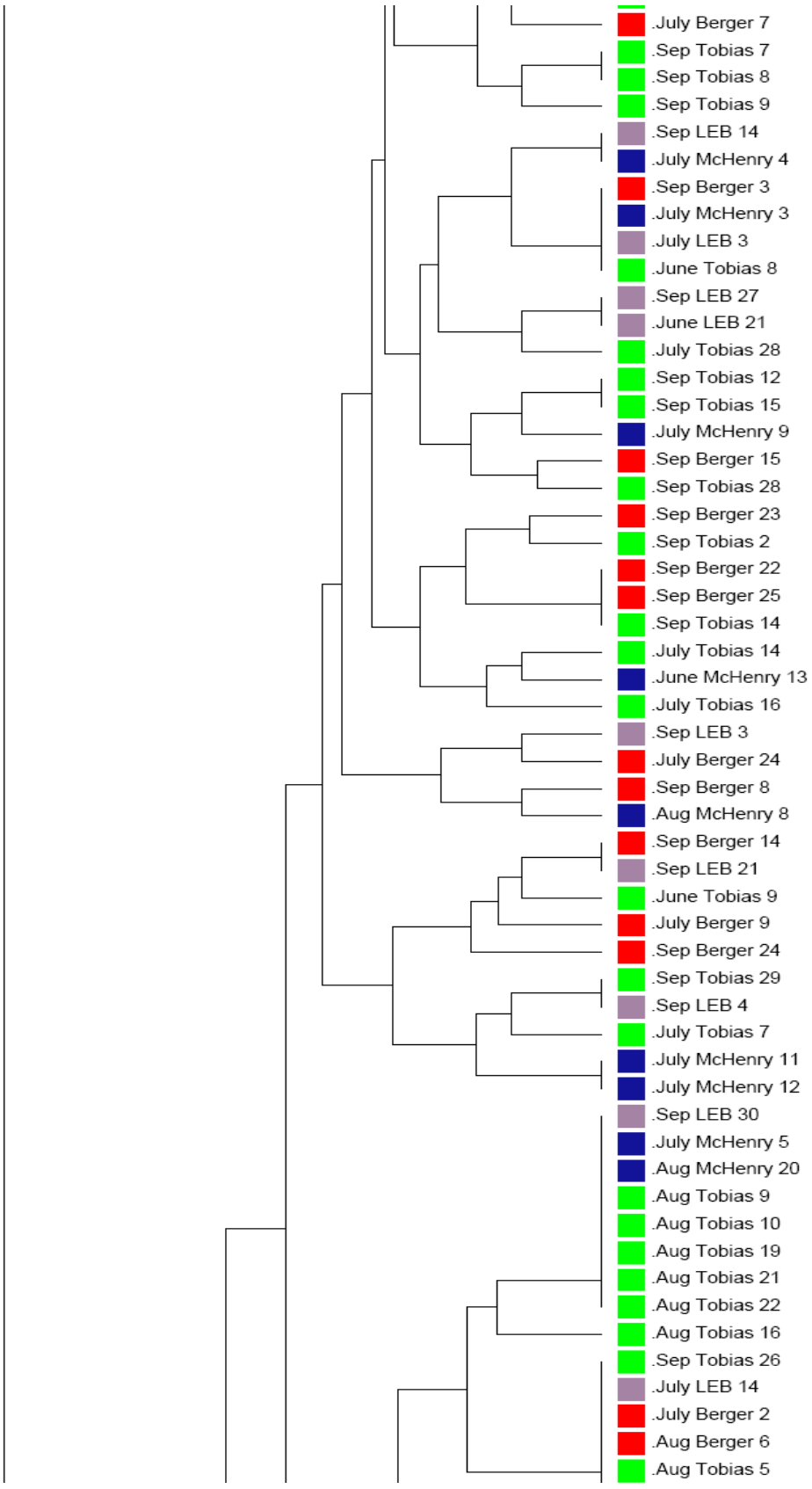
Confirming the results of the *E. coli* community structure analyses. Since this study represented the initial use of DGGE fingerprinting to identify geographic sources of bacterial pollution, BOX PCR, an established, library dependent method of source identification, was used to validate the DGGE results. To limit the resource expenditure associated with library dependent methodology, BOX-PCR validation of DGGE was limited to samples collected during Season Three, since this duration of time represented the swimming season and the most sensitive season for source tracking. Cluster analysis of the 358 BOX-PCR fingerprints revealed that 83 distinct *E. coli* phylotypes (using the 85% similarity threshold as described in Materials and Methods) were collected during the Season Three sampling (Fig. 7). Similar *E. coli* phylotypes were present in all three ditches and the beach sites, as no geographic-specific clustering of the *E. coli* phylotypes were observed. For example, multidimensional scaling (MDS) (Fig. 8) failed to separate the geographic origin of the phylotypes. Similar results were obtained following Jackknife analysis, as the rates of correct geographic classification of the *E. coli* isolates were low to moderate, ranging from 53% to 71% (Table 2).

BOX-PCR analysis was in higher ability to classify *E. coli* isolates on a temporal (month of collection) scale. For example, a temporal pattern of *E. coli* phylotypes were detected by MDS analysis of the BOX-PCR fingerprints (Fig. 9). Furthermore, Jackknife analysis showed that the *E. coli* isolates were correctly classified to the month during which they were collected at rates ranging from

69% to 86% (Table 3).

Fig. 7. Cluster analysis of total 358 *E. coli* isolate fingerprints generated by BOX-PCR. Scale indicates the similarity of fingerprints among isolates. Different color indicates various locations from where *E. coli* isolates were collected. Blue, red, green and purple indicate isolates collected from McHenry-, Berger, Tobias Ditches, and LEB, respectively.

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.Sep LEB 27
.June LEB 21
.July Tobias 28
.Sep Tobias 12
.Sep Tobias 15
.July McHenry 9
.Sep Berger 15
.Sep Tobias 28
.Sep Berger 23
.Sep Tobias 2
.Sep Berger 22
.Sep Berger 25
.Sep Tobias 14
.July Tobias 14
.June McHenry 13
.July Tobias 16
.Sep LEB 3
.July Berger 24
.Sep Berger 8
.Aug McHenry 8
.Sep Berger 14
.Sep LEB 21
.June Tobias 9
.July Berger 9
.Sep Berger 24
.Sep Tobias 29
.Sep LEB 4
.July Tobias 7
.July McHenry 11
.July McHenry 12
.Sep LEB 30
.July McHenry 5
.Aug McHenry 20
.Aug Tobias 9
.Aug Tobias 10
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.Aug Tobias 22
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.Sep Tobias 26
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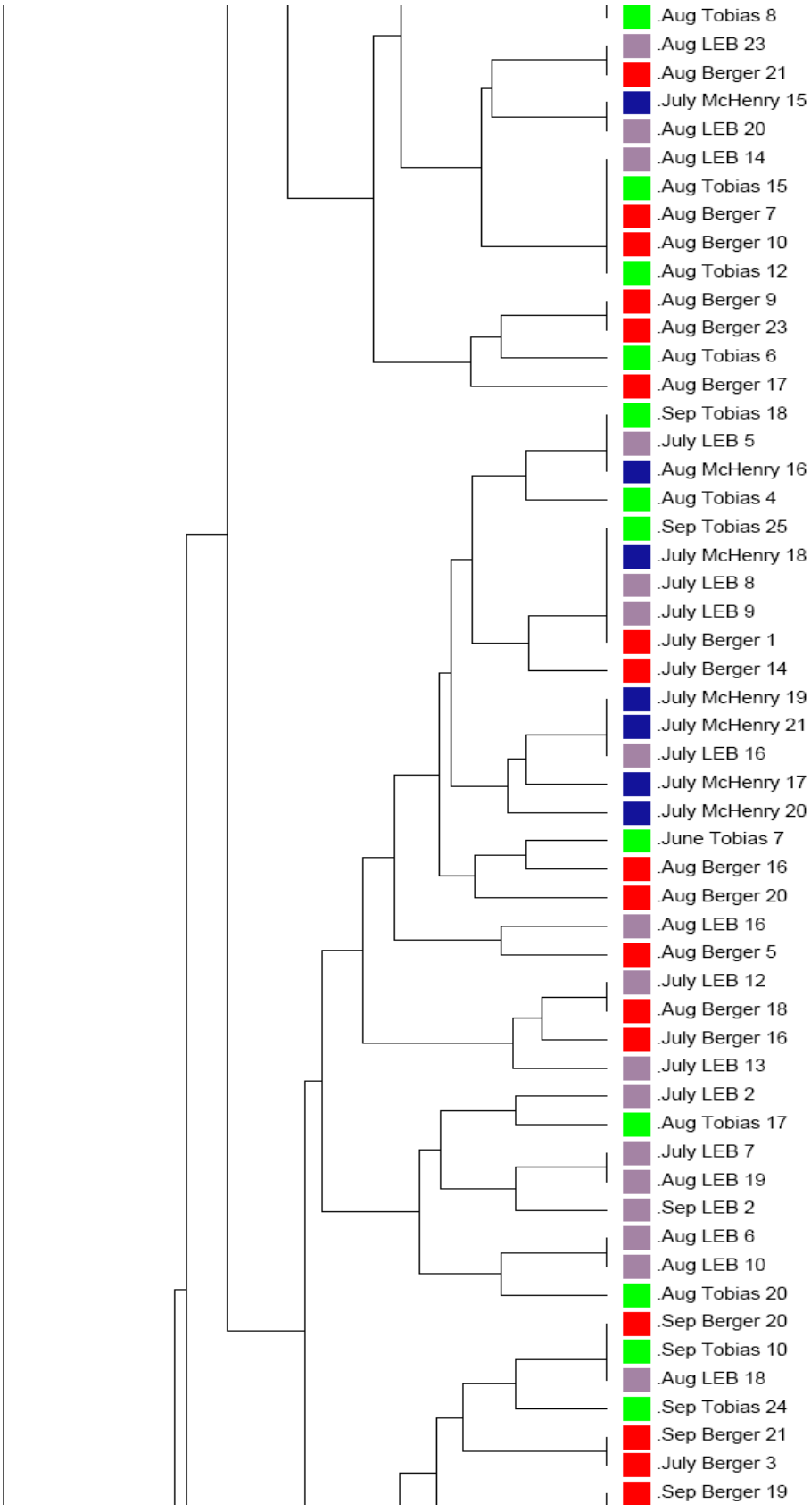
.Aug Berger 6
.Aug Tobias 5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.Aug Berger 9
.Aug Berger 23
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.Aug Berger 20
.Aug LEB 16
.Aug Berger 5
.July LEB 12
.Aug Berger 18
.July Berger 16
.July LEB 13
.July LEB 2
.Aug Tobias 17
.July LEB 7
.Aug LEB 19
.Sep LEB 2
.Aug LEB 6
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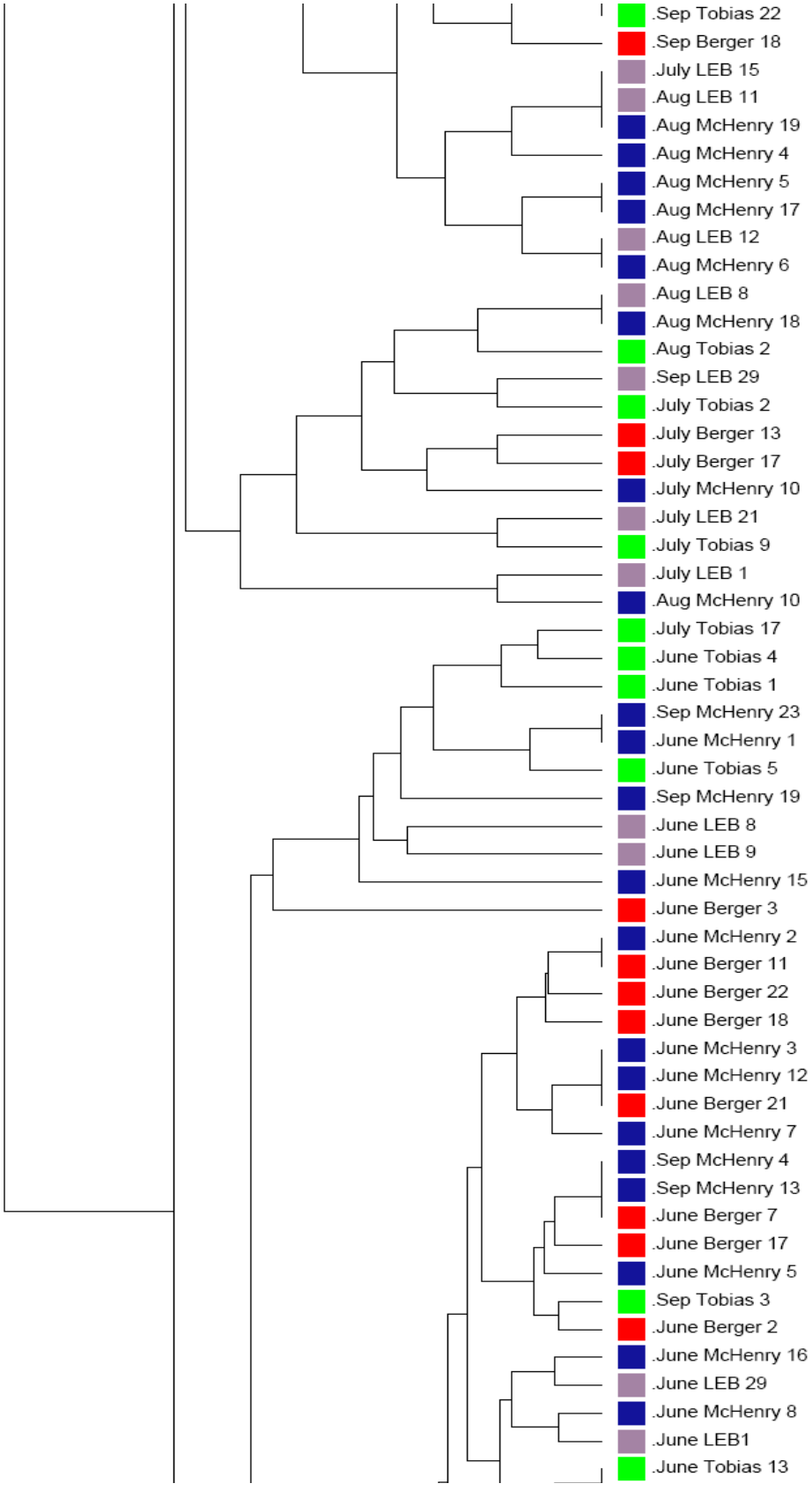
.Aug LEB 18
.Sep Tobias 24
.Sep Berger 21
.July Berger 3
.Sep Berger 19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.Aug LEB 8
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.Sep LEB 29
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.June Berger 3
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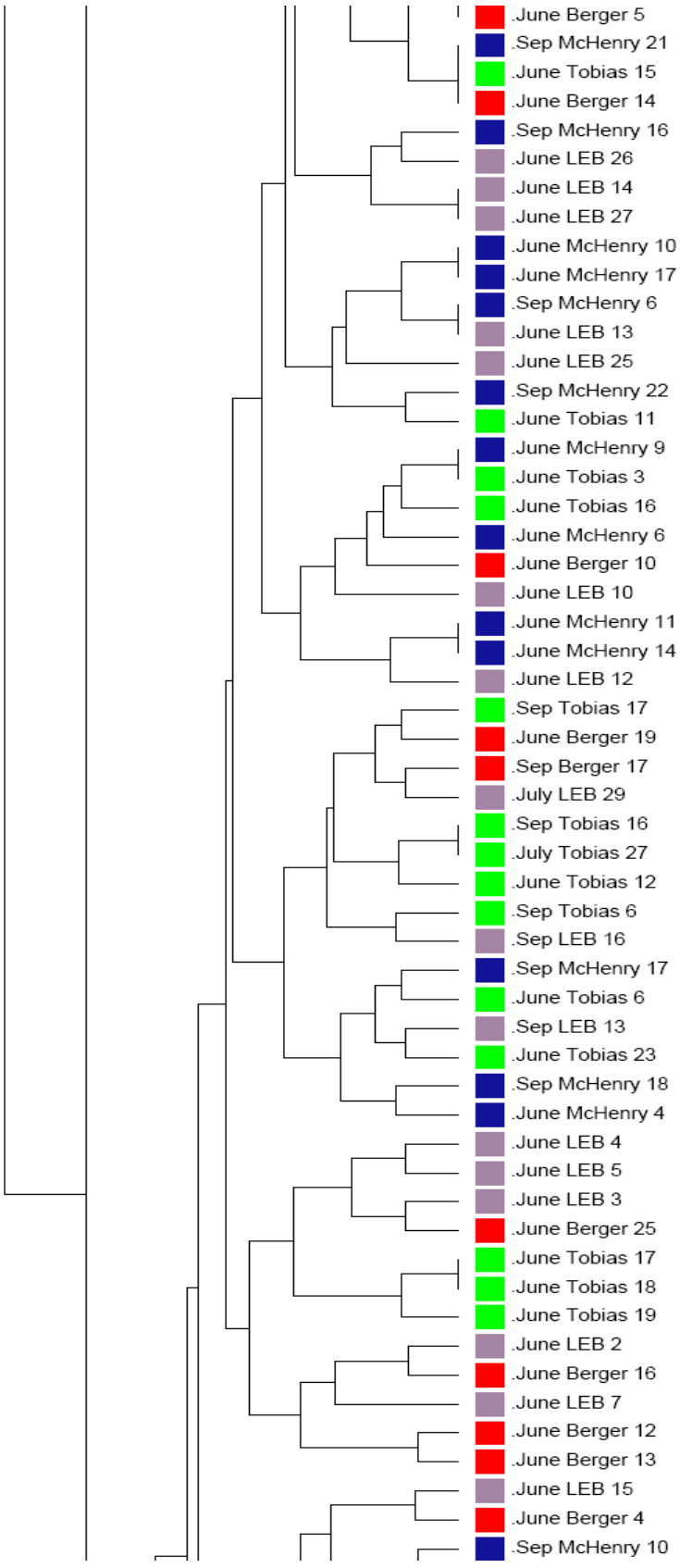
.June McHenry 8
.June LEB1
.June Tobias 13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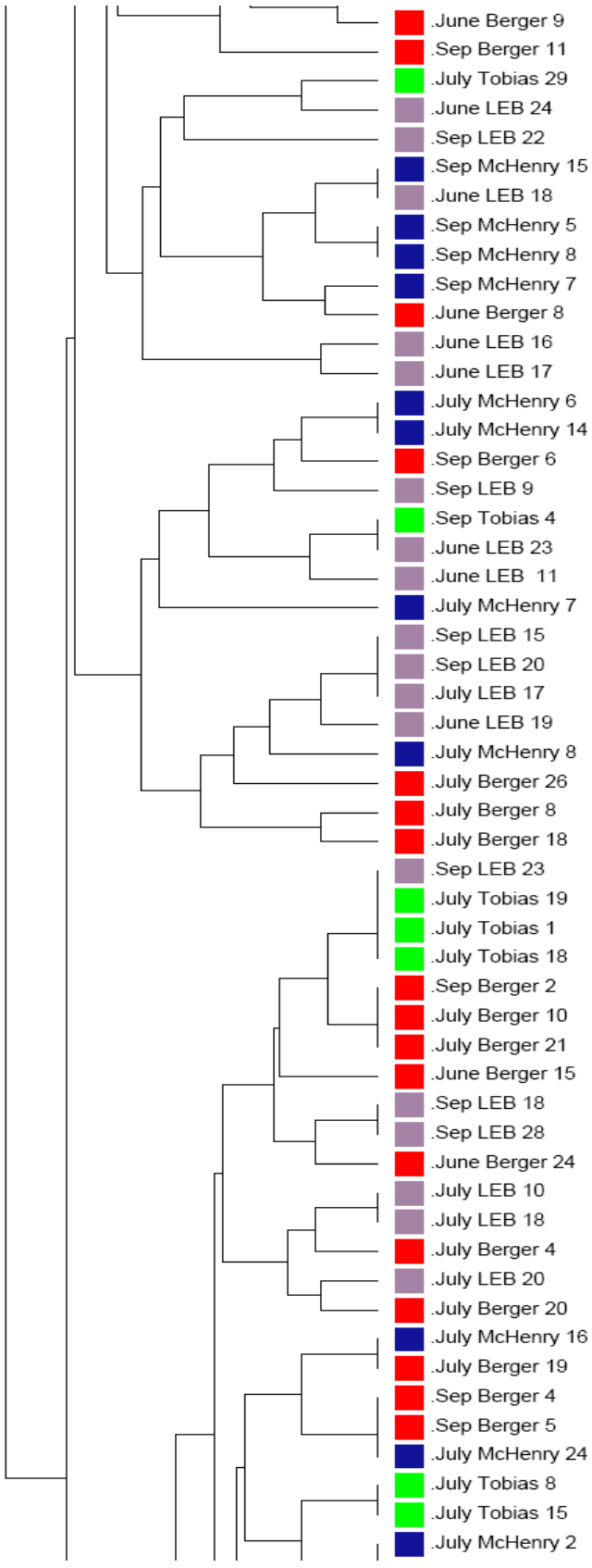
.June Berger 4
.Sep McHenry 10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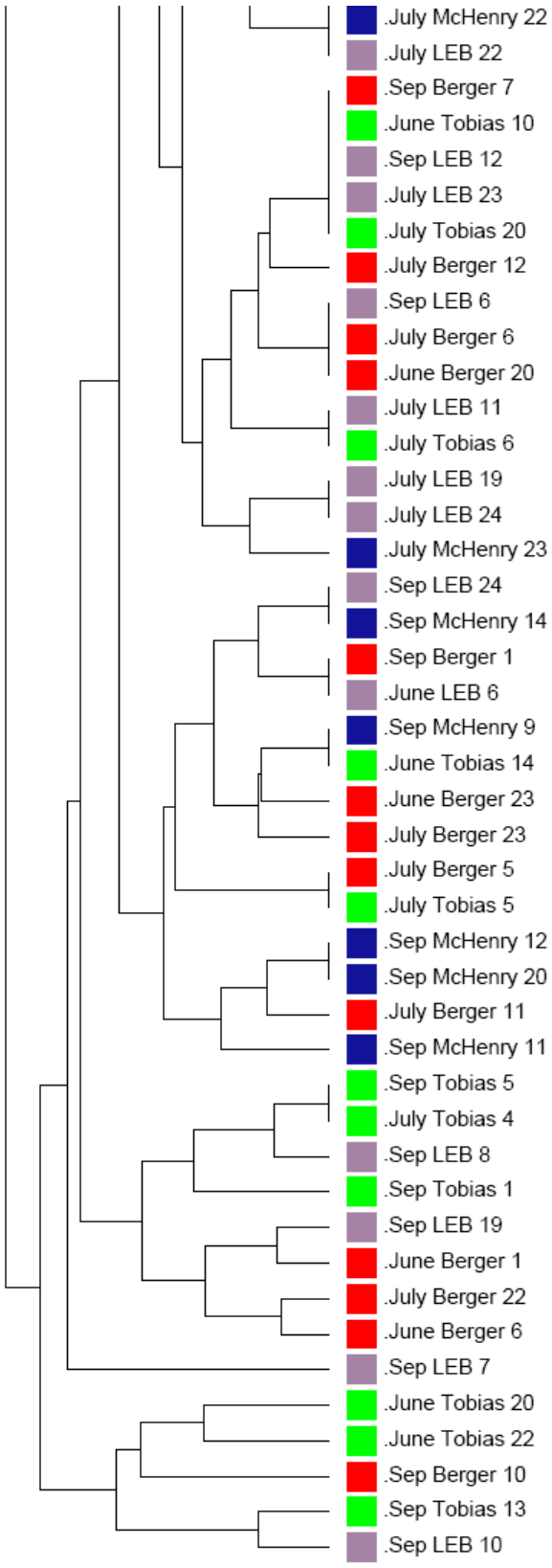
.July McHenry 22
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.Sep Berger 1
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.Sep McHenry 9
.June Tobias 14
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.July Tobias 5
.Sep McHenry 12
.Sep McHenry 20
.July Berger 11
.Sep McHenry 11
.Sep Tobias 5
.July Tobias 4
.Sep LEB 8
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.Sep LEB 19
.June Berger 1
.July Berger 22
.June Berger 6
.Sep LEB 7
.June Tobias 20
.June Tobias 22
.Sep Berger 10
.Sep Tobias 13
.Sep LEB 10

Fig.8. MDS analysis of *E. coli* isolate fingerprints based on locations from which they were collected

● : LEB
● : Berger Ditch
● : McHenry Ditch
● : Tobias Ditch

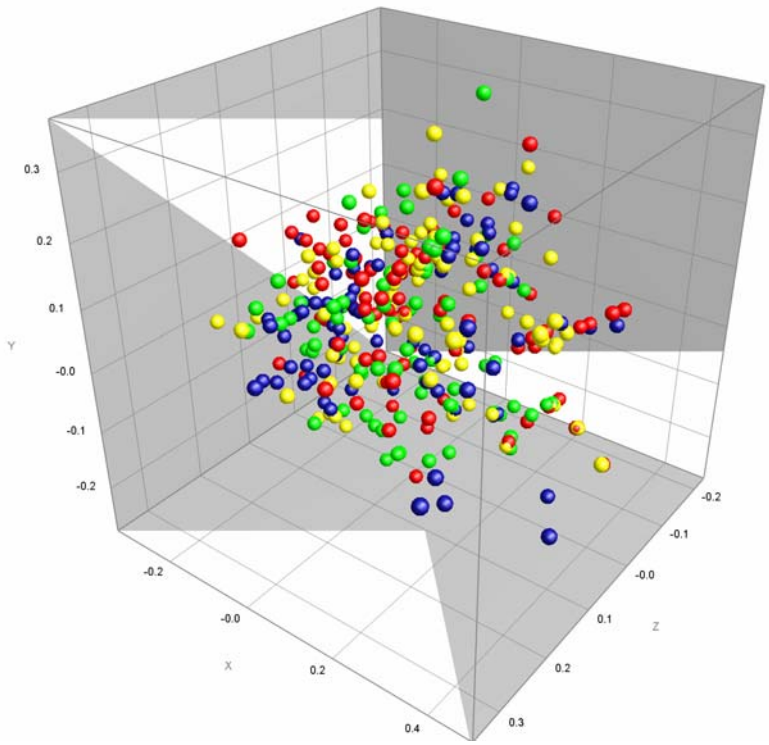


Fig. 9. MDS analysis of *E. coli* isolate fingerprints based on time when they were collected

● : June
● : July
● : August
● : September

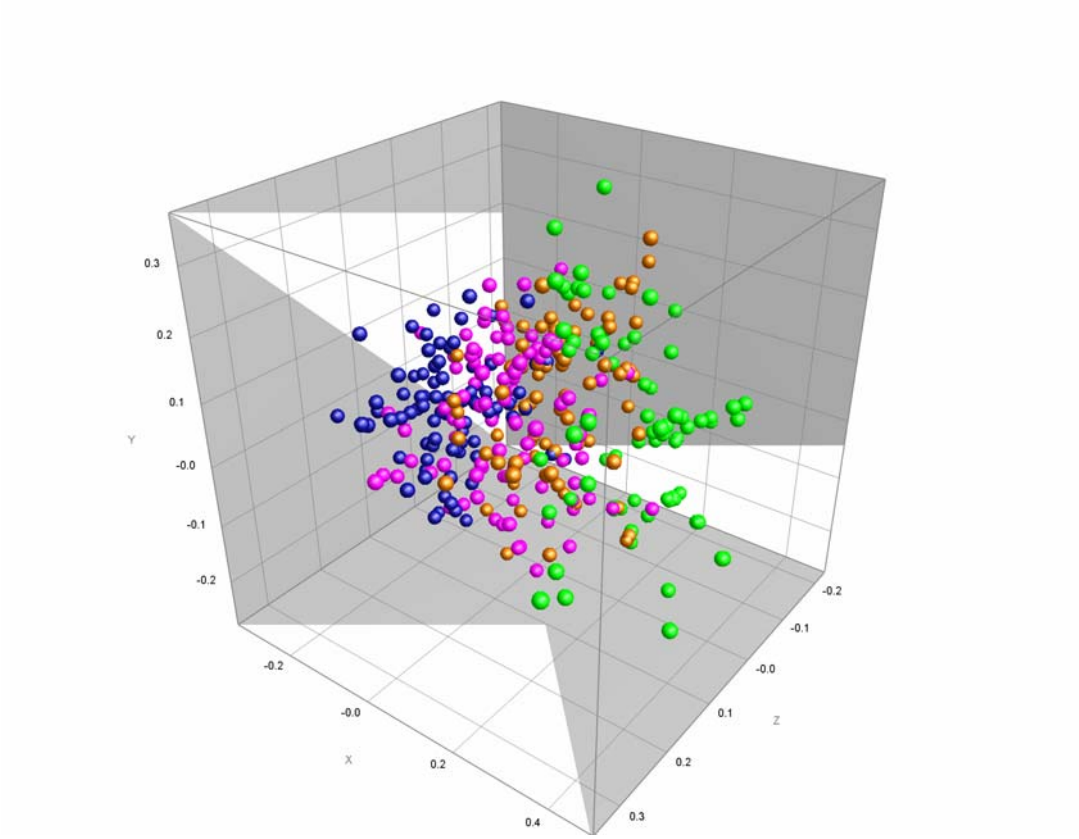


Table 2. Jackknife analysis showed the percentage of correct classification of *E. coli* isolates based on the location (ditches and the beach) they were collected

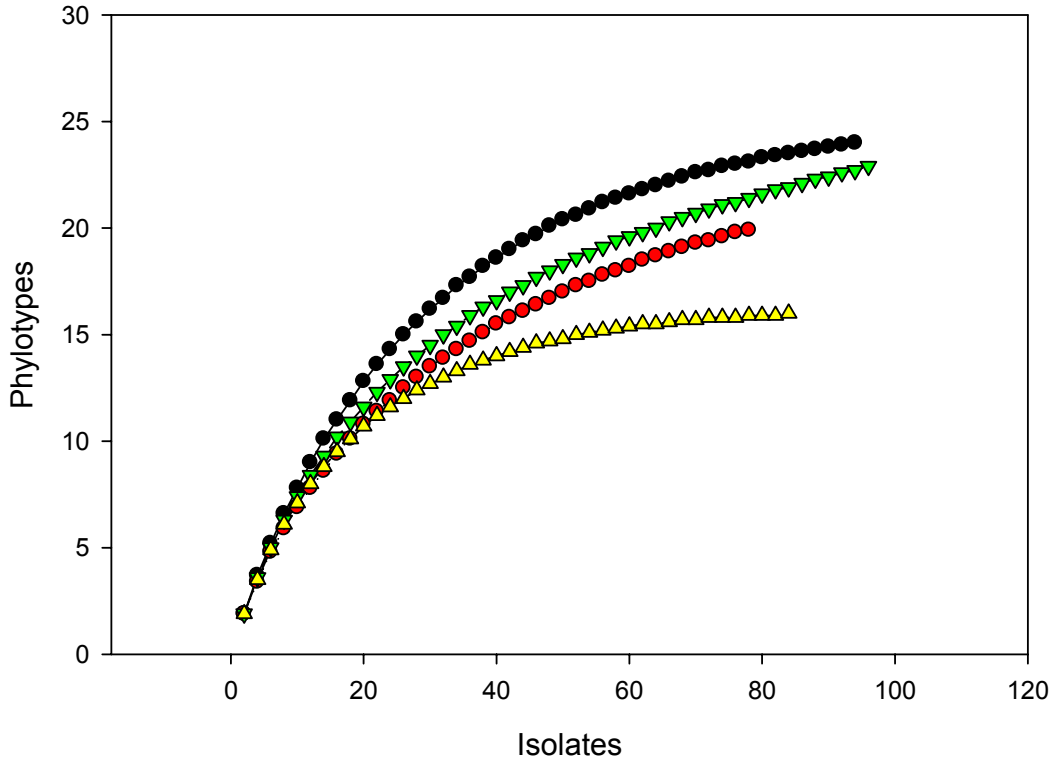
	Tobias	Berger	LEB	McHenry
Tobias	**70.93**	26.53	26.32	16.46
Berger	17.44	**57.14**	13.68	15.19
LEB	6.98	12.24	**53.68**	7.59
McHenry	4.65	4.08	6.32	**60.76**

Table 3. Jackknife analysis showed the percentage of correct classification of *E. coli* isolates based on the time (month) they were collected

	August	July	June	September
August	**85.88**	10.00	0.00	5.32
July	12.94	**73.33**	12.36	12.77
June	0.00	7.78	**78.65**	12.77
September	1.18	8.89	8.99	**69.15**

As with all library dependent analyses, it was necessary to determine if our *E. coli* library was of appropriate size. To test if the number of *E. coli* isolates selected from each site was large enough to draw meaningful conclusions about the BOX-PCR analysis, and therefore the validity of the DGGE analysis, rarefaction analysis was implemented. As shown in Fig. 10, rarefaction curves for all *E. coli* collections showed a strong trend towards saturation (slope of zero) with increased sampling effort. This suggested that the number of *E. coli* in the four isolate libraries was reasonable and more sampling would not have appreciably increased the number of *E. coli* phylotypes.

Fig. 10. Rarefaction analyses of *E. coli* isolates collected from each ditch and the beach during Season Three. Every point of each line indicates the number of expected *E. coli* phylotypes based on the number of isolates collected. Black, green, red and yellow lines indicate the rarefaction curves of *E. coli* isolates from LEB, Berger-, McHenry- and Tobias Ditches, respectively.



Discussion and Conclusion

The purpose of this study was to determine the geographic source of bacterial pollution impacting LEB in MBSP over the course of one year. This was performed by characterizing *E. coli* density and community structure in Lake Erie and several drainage ditches thought to contribute bacterial pollution. While all sites exhibited varying densities throughout the year, the *E. coli* density in LEB water (10^1 to 10^3 CFU/100 mL) was consistently one to two orders of magnitude lower than densities in the three ditches (10^1 to 10^5 CFU/100 mL)(Fig. 5). This observation was not surprising, as it is likely that *E. coli* exiting the ditch onto the foreshore beach was either diluted by lake water or was associated with sediments that dropped out of the water column close to the east side of the beach. Those populations that remained in deep water were likely less detectable, as less sediment resuspension will occur in deeper water. For example, Whitman and Nevers (2003) found *E. coli* density of water sample collected at 90 cm depth of water in foreshore beach was about half of that at 45 cm depth. Furthermore, the variation observed in both *E. coli* density and community structure was expected, given the position of LEB on Lake Erie. LEB is located in the Western Basin of Lake Erie, which is more shallow than the central and eastern portions of the lake. For example, the average depth of Lake Erie is 19 meters, however, the average depth in the Western Basin is only 7.4 meters (USEPA, 2006). This discrepancy in water level will result in greater

impact of currents with regard to water movement and sediment resuspension and movement. This dynamic will have a profound impact on water quality since sediment can harbor fecal bacteria and be resuspended in turbulent water (Obiri-Danso and Jones, 1999; Jamieson et al., 2005).

Previous work showed that *E. coli* density was consistently higher at the east end of LEB than at the west end (Sigler, unpublished data), which suggested that Berger Ditch, located at the east end of the beach, was a principal contributor to fecal pollution in LEB. During Season Three, we observed a similar phenomenon (Fig. 4), *E. coli* densities were significantly higher at the east end of the beach than at the west end ($p=0.047$). While this result again suggested a role of Berger Ditch in the elevated *E. coli* densities in LEB during Season Three, no significant difference in *E. coli* densities was observed across the beach during Seasons One and -Two ($p=0.48$, 0.36, respectively). Several possible reasons exist for this seasonal contribution. Berger Ditch is the longest ditch surveyed in this study, and originates in Wolf Creek, which has been shown to harbor high densities of *E. coli* (~46,000 CFU/100 mL) (Anonymous, 2003). Therefore, it is a logical assumption that Berger Ditch might experience a greater degree of potential fecal inputs than the other ditches. For example, agricultural inputs, in particular those associated with biosolids application, can dramatically increase the *E. coli* density in ditches that receive drain water from amended fields. Land application of biosolids is a popular practice in northwest Ohio, and is currently performed on several fields adjacent to Berger Ditch. Scarbro (2006) found that

densities of *E. coli* in water draining into Berger Ditch from a biosolids-amended field increased from 100 to approximately 8,500 CFU/100 mL. This increase was detected as long as three months after the biosolids application. Since Berger Ditch is not only the longest of the ditches, but also carries the highest volume of water to Lake Erie compared with the other two ditches, increased animal activity (waste inputs from muskrats, possum, groundhogs, deer, wild ducks, birds and geese) (Struffolino, personal communication) during Season Three (with increasing temperature) would further separate its contribution from the other ditches. For example, animals are more active in the summer than in winter, as there are more available resources (e.g. food). Agricultural activity during early and late summer will also increase the potential for farmland leaching after rainfall. Therefore, Berger Ditch, as the longest ditch surveyed in the study, might have more potential sources such as wild animals and farmland leaching that separate its contribution from the other ditches. Further separation of Berger Ditch during Season Three likely resulted from increased rainfall, which combined with the large size of Berger Ditch, led to greater overall discharge into the lake when compared of McHenry- and Tobias Ditches. Under these conditions, the output from Berger Ditch during Season Three could have resulted in higher *E. coli* densities at the east end of the beach, while simple dilution of the bacteria as they were transported to the west end resulted in lower densities at the other beach sites.

E. coli densities in both beach and ditch samples during Season Three was

one to two orders of magnitude greater than densities observed in Seasons One and -Two (Fig. 5). This can be explained primarily by the frequency and amount of rainfall that occurred during Season Three. NOAA rainfall data indicated that 49 rainfall events occurred during Season Three, resulting in 45 cm of precipitation, which was higher than the rain frequency and volume during Season One (42 events, 26.4 cm) and Two (45 events, 31.2 cm). Our data revealed a significant correlation between rainfall amount and *E. coli* densities at the beach and in Berger- and McHenry Ditches (r=0.68, 0.8, 0.67, respectively). It has been shown that storm water runoff can increase water turbidity by disturbing the underlying sediment/sand in ditches and beach water (Atherholt et al., 1998), an activity that has been shown to release and resuspend previously trapped *E. coli* and other potential pathogenic bacteria into the water column (Grimes, 1975). Furthermore, stormwater/rainfall can also transport contaminants, such as agriculture manure, and wildlife fecal material into ditches and streams that eventually empty into receiving waters (Mallin, et al., 2000).

It has been previously assumed that a significant correlation between *E. coli* densities at the beach and a specific ditch might indicate a source-sink relationship. During Season One, only the *E. coli* density in Berger Ditch was correlated ($r=0.981$, $p<0.05$) with that of the beach, while densities in all three ditches correlated (for Berger Ditch and the beach, $r=0.628$, $p=0.0256$; McHenry Ditch and the beach, $r=0.589$, $p=0.0435$; Tobias Ditch and the beach, $r=0.943$, $p=0.0048$) with those from the beach during Season Two. However, during

Season Three, no correlation was observed between any of the three ditches and the beach. The inconsistency of correlation among the three seasons suggested that inputs other than the three ditches play a role in the dynamic *E. coli* densities observed in this study. For example, Lévesque et al (1993, 2000) studied the influence of gull droppings on the composition and number of potentially pathogenic bacteria in beach water. *Staphylococcus aureus, Aeromonas spp., Campylobater spp., Salmonella spp.,* and fecal coliforms were found to be major microflora in gull droppings, while total bacteria densities were variable, ranging from 10^4 to 10^8 CFU per g (Lévesque et al., 2000). LEB is a habitat for both resident- and migratory birds, including gulls and roosting waterfowl, as evidenced by the presence of their droppings on the beach. More importantly, however, gulls are rarely found visiting or inhabiting the ditches. This suggests that the presence of gulls might reduce the correlation between ditch- and beach *E. coli* densities by augmenting the beach *E. coli* numbers independent of those in the ditches.

Another potential factor driving differences between ditch and beach *E. coli* densities is the role of beach sand as pathogen reservoirs. Microorganisms are a significant component of beach sand. It was shown that *E. coli* could sustain population densities over 2,000 CFU/100 mL in beach sand during summer months without continual lake, human or animal input (Whitman and Nevers, 2003). The study also found that *E. coli* densities in sand were, on average, 5 to 10 times higher than densities in adjacent swimming waters. In addition, the

study found a significant correlation between *E. coli* in beach sand and the next day's water *E. coli* concentration, suggesting that *E. coli* in beach sands influence water densities. Beyond simply inhabiting beach sands, *E. coli* can grow in sands (Alm et al., 2006). In the current study, *E. coli* density in the beach water was similar to or higher than densities in the ditches in March when there was no rain, indicating other sources such as sand may play a role in the elevated *E. coli* in the beach water. Therefore, the transport of *E. coli* from sand to the beach water might explain, in part, the weak correlation between beach- and ditch *E. coli* densities.

Algae have been shown to provide protection to pathogens, thereby serving as a reservoir that can enhance *E. coli* densities in beach water (McLellan and Jensen, 2005). In the current study, during the August and September samplings, algal mats were observed along the shore of Lake Erie Beach (personal observation). These algae were in dark blue in color, filamentous, and connected to form an extensive mat that covered the foreshore beach. Algal mats maintain high moisture content, protect bacteria from sunlight and are rich in nutrients, thus providing an appropriate habitat for bacterial survival and growth (Whitman et al., 2003; Byappanahalli et al., 2003). The contribution of pathogens harbored in algal mats is likely limited to the beach environment, as mats were never observed in or around any of the ditches, thereby providing another potential bacteria source exclusive to the beach.

Finally, humans and their related activities have been shown to impact the bacteriological quality of recreational water. Since human activity is concentrated on the beach at MBSP and not in the ditches, they represent an exclusive source of *E. coli* for the beach. Human inputs result from poor hygiene (improperly disposed of baby diapers or poorly toilet trained children) (Keene, et al., 1994) and motorboats that disturb and resuspend the sediment near the beach (Hilton and Phillips, 1982), resulting in the liberation of trapped bacteria in the sediments into the beach water (Grimes, 1975). Bathing activities can also elevate *E. coli* in beach water via shedding of indicator bacteria or resuspension of contaminated sediments (Obiri-Danso and Jones, 1999). Furthermore, people who spend time both on the beach and in the water can also play a role in transporting bacteria from the sand to the water (Sherry, 1986).

The results of *E. coli* community fingerprinting analyses by DGGE showed that the *E. coli* communities in three ditches and the beach were similar (with the exception of Tobias Ditch during Season One) but seasonally dynamic (Fig. 6). Because these results were based on *E. coli* community composition and not simply on densities, the relationships between the *E. coli* communities observed in beach- and ditch water were likely more reliable, as densities cannot provide reliable assessment of from where the bacteria originated.

In Seasons One and -Three, the fingerprints of *E. coli* communities from all ditches were at least 85% similar to those from Lake Erie Beach, with the

exception of Tobias Ditch in Season One (discussed below). The similarity of *E. coli* community fingerprints among the ditches and the beach is expected, as the three ditches were all located in close approximate to Maumee Bay State Park. Berger Ditch (the easternmost of the ditches) and Tobias Ditch (the westernmost) are separated by approximately 2200 m (McHenry Ditch lies between Berger- and Tobias Ditches). Along this ca. 2 km of shoreline is found a mixture of impervious surfaces, residential areas and farmland, and no single land use type defines the drainage area of any one of the ditches. Therefore, it is possible that common sources of *E. coli* could be represented by each of the ditches. Common sources in this region might include residential septic tank leaking, wild animal input, paved surface runoff, and agricultural runoff and leaching. For example, a survey by the Lucas County Public Health Department suggested 40% of the residential septic systems in the drainage area of MBSP might not function properly (Sinsabaugh and Glatzer, 1999). It has also been reported that 79% of all agricultural lands in the Maumee River basin were cropland (Hess, 1995) and a considerable proportion of these are subject to biosolids application. Thus, significant rainfall, particularly in Season Three (Fig. 6), appears to augment the bacterial load into Lake Erie, contributing to the elevated *E. coli* at Lake Erie Beach.

In Season Two, the similarity of the beach *E. coli* community fingerprints to those from the ditches decreased, likely due to any of a number of environmental factors. The average air temperature in February and March was -1^{O}C and 3^{O}C,

respectively, with at least on heavy snow (18 cm) in one precipitation event. The low temperature might have lowered the average *E. coli* densities in the three ditches by lowering the inputs into the ditches (e.g. lowered activities of animals), and ultimately resulting in little contribution to the beach. Because snowfall does not infiltrate soil or run offsite like rainfall does, a lag in the ditch discharge of snow precipitation will occur until snowmelt (Yang et al., 2003). When temperatures increase, with a concomitant increase in rainfall (March and April, Fig. 6), accumulated sediments with attached *E. coli* are likely released from the ditch bottoms and discharged into the lake. This build-up and eventual release might drive differences in the *E. coli* communities among the ditches and the beach, especially if the built up sediments harbor *E. coli* from numerous, temporally driven sources.

Compared to community fingerprints from McHenry- and Tobias Ditches, those from Berger Ditch were usually higher in similarity to fingerprints from the beach, suggesting the consistent contribution of Berger ditch to the pollution at the beach. The similarities of the *E. coli* community fingerprints between Berger Ditch and the beach were 87%, 70% and 93% in Seasons One, -Two and -Three respectively (Fig. 6), while McHenry- (88%, 65%, and 83%) and Tobias Ditches (46%, 62%, and 83%) were less similar. The high similarity of *E. coli* communities from Berger Ditch to those from the beach was expected, primarily as a result of factors characterizing Berger Ditch that was mentioned above. Overall, the data suggested a yearlong contribution from Berger Ditch; however,

the contribution of each potential point source within the ditch has not been studied and requires further investigation. Since McHenry Ditch is located 900 m from the west end of the beach, and primary interest concerning bacterial pollution has surrounded Berger Ditch, the role of McHenry Ditch in polluting the beach has not been investigated. It is likely however, that McHenry Ditch is involved in the pollution, as fingerprints from Season One were over 88% similar to those from the beach.

The similarity of *E. coli* fingerprints in Tobias Ditch to those from the beach increased from 46% to 83% throughout the three sampling seasons. The primary driver of this difference is likely the small size of Tobias Ditch, when compared with the size of McHenry- and Berger Ditches. Because of the comparatively low volume of water flowing through Tobias Ditch, it is subject to occasional periods of little or no flow. This was especially evident during Season One, as little to no water was flowing during three out of the four sampling events. It is clear that this low level of water activity limited the contribution of Tobias Ditch to the pollution at Lake Erie Beach during Season One. In contrast, more frequent and heavier rainfall (Fig. 6), probably led to more consistent water flow in the ditch during Seasons Two and -Three. As was described above, it is likely that periods of little or no water flow allowed for the accumulation of *E. coli* in ditch sediments that were released en-masse during periods of heavy rainfall (e.g. Season Three), resulting in contribution to the bacterial pollution at Lake Erie Beach.

Since this study represents the first application of DGGE to identify potential geographic sources of bacterial pollution, it was prudent to validate the results by analyzing a subset of the samples with an established, library-dependant method. Therefore, BOX PCR (Dombek et al., 2000) was used to confirm the results of the DGGE analysis of *E. coli* communities. The 358 isolates collected during Season Three represented a sampling of the isolates present throughout the entire swim season. Three analyses associated with BOX-PCR, including cluster analysis, multidimensional scaling analysis and jackknife analysis, were used to analyze the BOX-PCR results and validate the results of DGGE.

Overall, the analysis of BOX-PCR fingerprints confirmed the results of the Season Three DGGE analysis, revealing that the *E. coli* communities from each site were similar, and geographically non-discernable (Fig. 7). The results of MDS and jackknife analyses showed that the *E. coli* communities could not be classified based on location of origin (Fig. 8) but were easily classified based on time (Fig. 9). While multidimensional scaling analysis can provide a convenient visual interpretation of the similarity among the fingerprints of *E. coli* isolates by classifying these isolates according to the similar fingerprint characteristics (Ritter et al., 2003), jackknife analysis tests the discriminating capability of mixed *E. coli* isolates into their environment of origin (Dombek et al., 2000). Both analyses revealed the similarity of the *E. coli* communities sampled during Season Three, regardless of geographic origin, which confirmed the results of the DGGE

analysis.

To accurately validate the DGGE results, MDS and jackknife analyses were dependent on an isolate library of appropriate size. The number of phylotypes of *E. coli* represented in our library was determined by calculating the number of fingerprint clusters that formed above an empirically derived similarity threshold of 85% (described in Materials and Methods). In other words, if the similarity of *E. coli* isolate fingerprints was above 85%, they were considered as identical phylotypes. Using a similar method of threshold determination, Kinzelman et al. (2004) found 87% similarity to be an appropriate threshold value for computer recognition of identical fingerprint patterns. Rarefaction analysis was used to ultimately test the validity of the library size. For each library (based on the location of origin), the rarefaction curves became asymptotic, indicating that more intensive sampling would not add additional phylotypes to the collection (Fig. 10). While the sample size of *E. coli* isolates necessary for geographic source tracking is not standardized, reports suggest that host origin library sizes can vary from one hundred to more than two thousand *E. coli* isolates from human and various animals (Dombek et al.,2000; Hartel et al., 2002; McLellan et al., 2003; Johnson et al., 2004). The number of isolates necessary is dependant on the variation in genetic structure (Gordon, 1997; Souza et al., 1999), host specificity (Gordon and Lee, 1999), and temporal changes in alleles (DNA sequences) (Pupo and Richardson, 1995; Gordon, 1997). Despite reports of large library sizes, increasing the size of libraries does not always improve classification and can

even result in decreased discriminating capability (Johnson et al., 2004), possibly as a result of the presence of clonal strains (multiple strains collected from a single host) (McLellan, 2003), or increased errors accumulated from gel-to-gel variation (Johnson et al., 2004).

Although numerous studies have attempted to identify sources of bacterial pollution, the majority have drawn conclusions based on a limited sampling period, varying from a one time sampling to sampling collected over several months (Dombek et al., 2000; Kinzelman et al., 2004; Ram et al., 2004). However, no evaluation has been performed to check the reliability of short-term sampling. This study has shown that environmental and climatic variability including variability in temperature, rainfall, light, water level, available nutrients, salinity, and environmental pollutants could change the strain composition of fecal contamination over time (Lipp et al., 2001; Kinzelman et al., 2004; Whitman et al., 2004; Tassoula, 1997; Korhonen and Martikainen, 1991; Pathak and Bhattacherjee, 1994) and affect the results of source identification. For example, in the current study it was found that annual variability in the abovementioned factors could impact the geographic sources of bacterial pollution in Lake Erie Beach. For example, during Season Two, DGGE analysis revealed low similarity between communities from the beach and each ditch, indicating that the ditches might not represent the principal contributors to the bacterial pollution. In contrast, analysis of Season Three samples showed that all ditch communities were similar to those from the beach, and likely contributing to the pollution.

Therefore, understanding the true dynamics and ecology of bacterial pollution requires long-term sampling of fecal indicator bacteria.

The results of this study indicated that all the three ditches contributed to the bacterial pollution in Lake Erie Beach at certain times, particularly in Season Three. It also suggested that common sources might be contributing to all the ditches and therefore to the beach. The common sources might be animal fecal material, farmland leaching and (or) septic tank leaking. Therefore, it is necessary to study all three ditches to identify which common source attributed to the elevated *E. coli* in both the ditches and the beach. Equally important, this study also showed that the fingerprints of *E. coli* isolates from the beach and ditches were changing from month to month during Season Three, which indicated the sources of pollution in LEB varied during Season Three. This is an interesting finding, as the common sources such as septic tank leaking and farmland leaching might not change from month to month. Therefore, further investigation is required to determine the common source (either host or geographic) that leads to variability in the content of *E. coli* in the surveyed locations of this study.

Based on the data in this study, we found both the *E. coli* density and community structure in Lake Erie Beach and the three ditches were dynamic and seasonally variable during the year-long sampling period. The weak correlation between *E. coli* density in the ditch and beach water demonstrated that monitoring

bacterial densities is limited in its utility for bacterial source tracking and is best combined with complimentary methods. One such method highlighted in this study is DGGE, which effectively described the relationship of *E. coli* communities in the ditches and the beach. While results suggested that all three ditches sampled were involved in the bacterial pollution at some time, the contribution was likely seasonally based. The contribution from Tobias Ditch, in particular, was highly rainfall dependent, while Berger Ditch was the most consistent contributor of the three ditches, regardless of rainfall. BOX-PCR of *E. coli* libraries collected during Season Three validated the results of DGGE analysis, indicating that DGGE was a reliable method for rapidly screening the putative geographic sources of bacterial pollution in Lake Erie Beach.

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