

## Seasonal persistence and population characteristics of *Escherichia coli* and enterococci in deep backshore sand of two freshwater beaches

Muruleedhara N. Byappanahalli, Richard L. Whitman, Dawn A. Shively, W. T. Evert Ting, Charles C. Tseng and Meredith B. Nevers

### ABSTRACT

We studied the shoreward and seasonal distribution of *E. coli* and enterococci in sand (at the water table) at two southern Lake Michigan beaches—Dunbar and West Beach (in Indiana). Deep, backshore sand (~20 m inland) was regularly sampled for 15 months during 2002–2003. *E. coli* counts were not significantly different in samples taken at 5-m intervals from 0–40 m inland ( $P = 0.25$ ). Neither *E. coli* nor enterococci mean counts showed any correlation or differences between the two beaches studied. In laboratory experiments, *E. coli* readily grew in sand supplemented with lake plankton, suggesting that *in situ* *E. coli* growth may occur when temperature and natural organic sources are adequate. Of the 114 sand enterococci isolates tested, positive species identification was obtained for only 52 (46%), with *E. faecium* representing the most dominant species (92%). Genetic characterization by ribotyping revealed no distinct genotypic pattern (s) for *E. coli*, suggesting that the sand population was rather a mixture of numerous strains (genotypes). These findings indicate that *E. coli* and enterococci can occur and persist for extended periods in backshore sand at the groundwater table. Although this study was limited to two beaches of southern Lake Michigan, similar findings can be expected at other temperate freshwater beaches. The long-term persistence of these bacteria, perhaps independent of pollution events, complicates their use as indicator organisms. Further, backshore sand at the water table may act as a reservoir for these bacteria and potentially for human pathogens.

**Key words** | beach sand, enterococci, *Escherichia coli*, groundwater, indicator bacteria persistence

**Muruleedhara N. Byappanahalli**  
**Richard L. Whitman** (corresponding author)  
**Dawn A. Shively**  
**Meredith B. Nevers**  
United States Geological Survey,  
Great Lakes Science Center, Lake Michigan  
Ecological Research Station,  
1100 North Mineral Springs Road, Porter,  
Indiana 46304,  
USA  
Tel: +1 (219) 926-8336  
Fax: +1 (219) 929-5792  
E-mail: rwhitman@usgs.gov

**W. T. Evert Ting**  
**Charles C. Tseng**  
Purdue University Calumet,  
Department of Biological Sciences,  
2200 169<sup>th</sup> Street, Hammond,  
Indiana 46323,  
USA

### INTRODUCTION

Several studies have shown that fecal indicator bacteria, such as *E. coli* and enterococci, can routinely be recovered in beach sand (Twining, *et al.* 1993; Ghinsberg, *et al.* 1994; Whitman, *et al.* 1994; Oshiro & Fujioka 1995), with concentrations often exceeding those in the water column (Obiri-Danso & Jones 1999; Alm, *et al.* 2003; Whitman & Nevers 2003). The reasons for their occurrence in beach sand are not well-understood; however, there are two

plausible hypotheses for this phenomenon—(1) direct fecal contamination or concentration by sand and (2) long-term persistence and perhaps growth under permissible conditions.

Recent data collected at a Chicago beach demonstrated that *E. coli* persists in foreshore subsurface sand, and the sand may become a source of beach water contamination (Whitman & Nevers 2003). Evidence suggests that *E. coli*

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may grow in the sand under certain conditions (Whitman & Nevers 2003; Whitman *et al.* 2003). Over the years, studies on indicator bacterial occurrence in beach sand have generally focused on areas adjacent to the shoreline—foreshore (Ghinsberg *et al.* 1994; Whitman *et al.* 1994; Alm *et al.* 2003; Whitman & Nevers 2003). One potential difficulty with this approach is determining whether sand-borne indicator bacteria are indigenous to the system or represent transient, residual populations from external sources, such as run-off, aquatic birds, or lake water. Backshore beach areas are relatively less subject to these influences, and it is unknown whether indicator bacteria can occur and persist for an extended time in subsurface sand far from the shoreline and common contamination sources. Moreover, the occurrence, persistence, and possible growth of indicator bacteria in backshore areas may have serious implications on important issues, such as beach water quality and microbial source tracking. The overall purpose of this study was to describe the seasonal occurrence and distribution of *E. coli* and enterococci in subsurface backshore beach sand at the water table.

## METHODS

### Site description

In this report we refer to the backshore beach as the relatively flat area behind the berm and isolated from all but the highest lake surges (Thompson & Baedke 1997); sometimes, it is referred to as the upper or far beach. Two recreational beaches under the management of the United States National Park Service (Indiana Dunes National Lakeshore, Porter, Indiana) and located near the southern tip of Lake Michigan were selected: 1) Dunbar Beach, a residential area but lightly used lakefront, and 2) West Beach, a natural area heavily used as a recreational beach. Whilst there has been an indication of salt intrusion from water softeners at a beach near Dunbar, neither West nor Dunbar Beach has any evidence of domestic waste (sewage) input (Twinning *et al.* 1993). Beach closures due to high *E. coli* levels ( $> 235$  colony-forming units (CFU)/100 ml) occur a few times a year at Dunbar but only rarely at West Beach (Whitman *et al.* 1999). A schematic representation of

the backshore beach area with sampling locations is illustrated in Figure 1.

The backshore sands are derived primarily from up drift lakebed and bluff erosion (Cressey 1928). Texturally, these sands are medium to coarse (in composition), clean and well sorted. These beach sands are interspersed with layers of gravel that overlie glacial till lakebed deposits. The water table is generally at a depth of 1.0–1.5 m from the surface. Wave surges only reach backshore sands during the more significant storms of the seasons and are most common in late-fall and winter, when most onshore erosion occurs. Shelf ice often develops during the coldest months (January and February), helping to protect the shores during high waves. In our study, the sampling year 2002 was uncharacteristically warm, and prolonged shelf ice development did not occur.

### Sampling strategy

#### Shoreward *E. coli* distribution in subsurface saturated sand

At Dunbar Beach, sand samples were collected at the groundwater table at 5-m intervals from shoreline to 40 m inland (sampling depth ranged from 0.4–1.85 m, with a mean of 1.03 m). Sand thickness at Dunbar Beach was about 2.0–2.4 m (Shabica & Pranschke 1994). Initially, the surface and subsurface sand was excavated using a clean shovel. Then, a posthole digger was used to collect the moist sand samples; adequate care was taken to prevent cross-contamination or mixing of the top sand with moist subsurface sand.

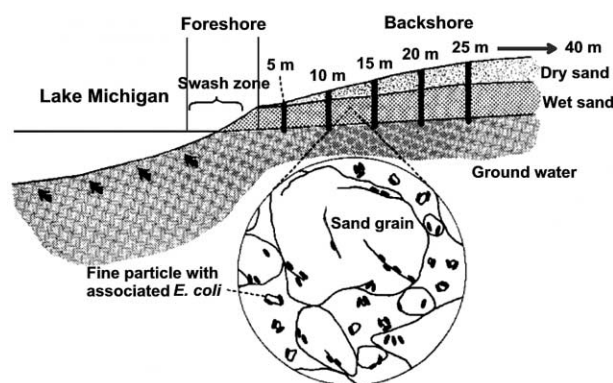


Figure 1 | Schematic representation of the backshore beach area and sampling locations.

In addition, between samplings, the posthole digger was rinsed thoroughly with 70% alcohol, and then washed several times with sterile, distilled water. At each location, quadruplicate samples were collected, placed on ice in a cooler and transported to the laboratory. Samples were analyzed within four to six hours of collection.

### Seasonal *E. coli* and enterococci distribution

From January-May 2002 beaches were sampled weekly, and from June 2002-May 2003, samples were collected monthly. During sampling, five random replicate sand samples were taken 20 m inland at each beach. Care was taken to avoid sampling locations from previous visits by keeping track of the sampling points; this was accomplished using a GPS and measuring from a fixed point on the beach to identify the starting location/point; no July or August (2002) samples were taken. Mean sampling depth at Dunbar was 0.67 m, with a range of 0.24–1.1 m; mean sampling depth at West Beach was 0.62 m, with a range of 0.22–1.15 m.

### Laboratory growth experiment

Fresh sand samples collected from 2 m inshore were augmented with plankton, collected by Wisconsin Net (80- $\mu$ m mesh) near the Washington Beach breakwater in Michigan City, Indiana, on December 4, 2001. In the laboratory, 700 g of sand was mixed with 138 ml of net-plankton, consisting mainly of microcrustaceans, rotifers and filamentous algae (Stoermer & Tuchman 1979; Makarewicz & Lewis 1989). The same amounts of sand and phosphate buffered water were used as a control (unamended sand). Sub-samples (25 g) of the plankton-sand mixture and the control sand were incubated separately in 200 ml plastic bottles at 23.5°C. Triplicate samples were analyzed on days 0, 1, 2, 3, 4, 5, and 7 for *E. coli* counts by membrane filtration method using mTEC agar (*Standard Methods for the Examination of Water and Wastewater* 1998).

### Sample analysis

All sand and water samples were analyzed for *E. coli* using the standard membrane filtration (MF) method or

Colilert-18 system (IDEXX, Inc., Westbrook, Maine, USA). Enterococci were enumerated similarly using Enterolert (IDEXX, Inc.). Colilert-18 and Enterolert are based on defined substrate technology (Edberg *et al.* 1990; Fricker & Fricker 1996), and these methods have been used extensively before to analyze soil/sediment samples for *E. coli* and enterococci (Solo-Gabriele *et al.* 2000; Desmarais *et al.* 2002; Byappanahalli *et al.* 2003a). Bacterial counts were expressed as most probable number (MPN) or colony-forming units (CFU)/100 g dry weight of the sample. All sets of analyses for *E. coli* and enterococci included suitable blanks and standard cultures of *E. coli* (ATCC 25922) and *E. faecalis* (ATCC 19433).

### Enterococci identification

Presumptive cultures of enterococci from Enterolert were isolated on mEnterococcus agar. Upon additional confirmatory tests for these bacteria (esculin hydrolysis, negative catalase reaction, positive growth in brain heart infusion broth at 45°C and in 6.5% NaCl) the pure cultures were then stored at –80°C for later use. A total of 114 confirmed enterococci isolates (collected from Dunbar Beach) were speciated using the API 20 Strep system (bioMerieux, Inc., Hazelwood, Missouri, USA).

### Genetic characteristics of sand-borne *E. coli* as determined by ribotyping

A total of 235 *E. coli* isolates collected between January 2002 and May 2003 from Dunbar and West Beach were used for ribotyping, a DNA fingerprinting technique that has extensive applications in epidemiological and bacterial source tracking approaches (Scott *et al.* 2002). Bacterial samples were prepared per the standard protocol–RiboPrinter® Sample Preparation Ready Reference (DuPont Qualicon, Wilmington, Delaware, USA). In brief, pure cultures of *E. coli* were streaked on tryptic soy agar plate and incubated at 35°C overnight. Using a sterile colony-pick, bacterial growth from the plate was transferred to a microcentrifuge tube containing 100  $\mu$ l of sampling buffer and the mixture was then briefly agitated to release the cells; 30  $\mu$ l of the bacterial suspension was transferred to a sampling well on a carrier. After loading

the carrier with eight bacterial samples, the carrier was then placed in a treatment station and heated to 80°C for 10 minutes to kill off the cells. To each sampling well, 5 µl each of lysing agent A and lysing agent B was added. The subsequent steps—(1) DNA preparation and rDNA digestion by *Hind*III, (2) separation and transfer, (3) membrane processing and probe hybridization, and (4) detection and processing of image—were all automated and occurred within the RiboPrinter® system.

RiboPrint® patterns of all isolates were analyzed with the BioNumerics™ software (Applied Maths, Kortrijk, Belgium). Band positions of each RiboPrint® were optimized at 1.0%, and the position tolerance was also set at 1.0%. To minimize the influence of faint and inconsistent bands, both the minimum height and surface area was set at 0. Similarity between any two patterns was calculated using Pearson correlation, which is based on densitometric curves comparison.

To determine the genetic diversity of the sand isolates, a dendrogram of the 235 RiboPrint® patterns was constructed (data not shown) using the unweighted pair group method with arithmetic average (UPGMA) to reflect similarities in the matrix. For clarity, the results are presented graphically in multi-dimensional scaling (MDS) along two axes, with ribotypes partitioned by season (winter 2002 vs. spring 2003), year (March 2002 vs. March 2003), and within season (January-February 2002).

### Statistical analyses

Statistical analyses were performed using SPSS software, version 12.0. The data were log-transformed to meet parametric assumptions of equality of variances and normal distribution. The statistical significance level was set at  $P = 0.05$ , unless otherwise stated.

Genotypic similarity of isolates was determined using a Pearson correlation. Non-metric multidimensional scaling was used to configure the sample similarities. Multi-dimensional scaling examines the absolute similarity values and attempts to satisfy conditions of the rank similarity matrix, resulting in a visual configuration of sample results.

## RESULTS

### Shoreward *E. coli* distribution

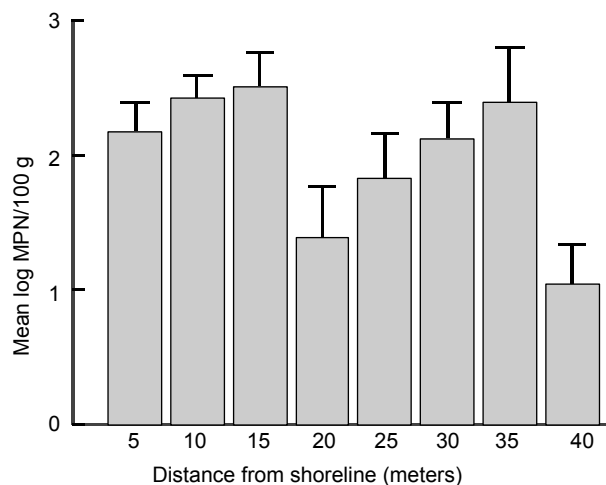
There was no significant difference in *E. coli* counts in sand at 5-m intervals from the shoreline to 40 m inland ( $F_{7,47} = 1.34$ ,  $P = 0.25$ ). *E. coli* distribution in the sand was patchy; mean *E. coli* density was  $426 \pm 167$  (S.E.) MPN/100 g, with a range of 43 MPN/100 g at 40 m to 1,775 MPN/100 g at 35 m (Figure 2). The widespread occurrence of *E. coli* in subsurface backshore sand—from shoreline to 40 m inland—is an indication that indicator bacteria, such as *E. coli*, are rather common in this habitat. Subsequent studies were focused only at 20 m inland since this distance seemed to provide best representation of average conditions (e.g., minimal contamination from external sources, minimal influence from wave surges, depth of water table) of the backshore environment.

### Seasonal *E. coli* and enterococci distribution

There was no significant difference in mean densities of *E. coli* and enterococci between the two beaches over the 12-month study ( $P = 0.75$  and  $P = 0.88$ ). Further, neither *E. coli* nor enterococci numbers were significantly correlated between the two beaches ( $P = 0.53$  and  $P = 0.26$ ). The overall log mean (S.E.) *E. coli* and enterococci counts were  $1.1 (\pm 0.09)$  and  $1.1 (\pm 0.15)$  MPN/100 g at Dunbar and  $1.2 (\pm 0.14)$  and  $1.1 (\pm 0.08)$  MPN/100 g at West Beach (see also Figures 3(a) and (b)). Since sand samples were collected in a habitat that is presumably unfavorable for fecal indicator bacteria and in a different substrate, comparison with the lake water is difficult.

### Genetic characteristics of *E. coli* isolates

Genetic characteristics of *E. coli* were based on ribotyping results (i.e., the number of restriction sites in the rRNA genes that were cleaved by *Hind*III). Multi-dimensional scaling (MDS) along two axes for *E. coli* ribotypes—partitioned by season (winter 2002 vs. spring 2003), year (March 2002 vs. March 2003), and within season (January-February 2002)—are shown in Figure 4A–C.

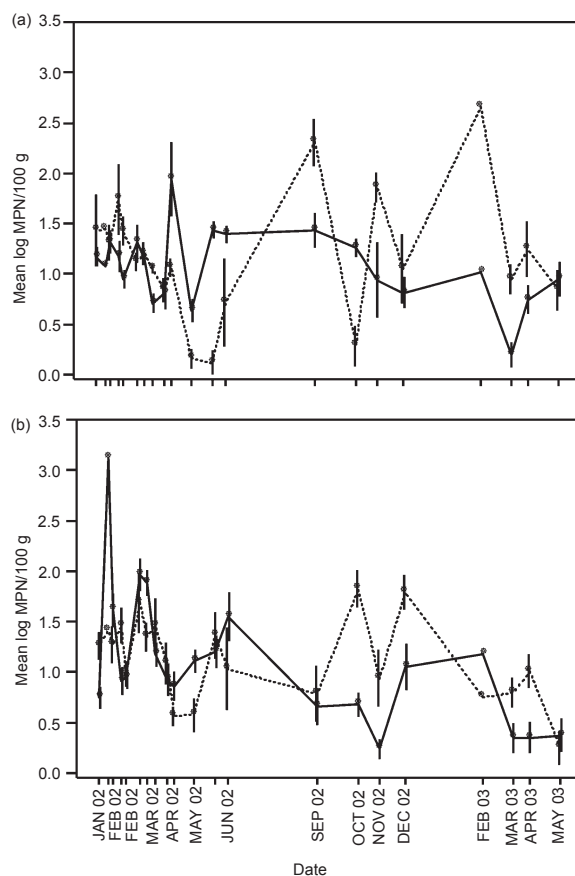


**Figure 2** | *E. coli* density in deep, saturated sand (at Dunbar Beach) as a function of distance from the shoreline. Error bars represent (+1 S.E.).

During the course of this study, *E. coli* isolates were collected under different climatic conditions (e.g., spring, summer, winter). There appeared to be some ribotype similarities among *E. coli* isolates in winter 2002, with several clustering together, and also similarity with ribotypes found in spring 2003 (Figure 4A). There was some overlap between seasons, but generally isolates were dissimilar (did not share the same ribotype). There were similar ribotypes identified in March 2002 and in March 2003 and also some overlap between years (Figure 4B). The similarity among ribotypes was more pronounced over the short-term, with several similar ribotypes emerging in January and February of 2002 (Figure 4C). Again, there were far more individual ribotypes among the isolates, indicating that heterogeneous ribotypes may be found over a short time (Jenkins *et al.* 2003).

### Enterococci species

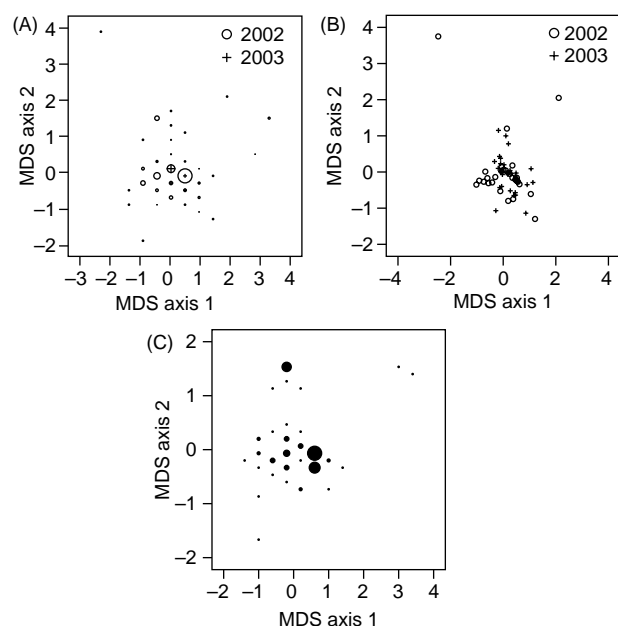
Of the 114 enterococci isolates tested, a positive species ID was obtained for only 52 (46%), with *E. faecium* representing the most dominant (92%) of the identified species; other species included *E. casseliflavus* and *E. durans*. While 33 (29%) isolates could only be identified to the genus level, the rest (29 confirmed enterococci isolates) could not be identified due to low discrimination at species level or ambiguous test reactions.



**Figure 3** | Temporal variations in *E. coli* (dashed line) and enterococci (solid line) counts over time at (a) West Beach and (b) Dunbar Beach; subsurface sand samples (near groundwater table) were collected 20 m from shoreline. Error bars represent ( $\pm 1$  S.E.).

### Growth patterns of *E. coli* in plankton-amended and unamended beach sand

Net-plankton ( $\sim 80 \mu\text{m}$  mesh size) was used to simulate the composition and density of the organisms that might be incorporated into beach sand by natural processes such as wave surge and spray. In the unamended (control) sand, *E. coli* counts remained relatively stable during the first 5 days but declined exponentially during the next 48 hours to almost undetectable levels (Figure 5). However, the addition of net-plankton to the sand resulted in a significant increase (about 2 logs) in *E. coli* numbers over 24 hours, with numbers gradually decreasing over the next six days. At the end of the experiment (day 7), *E. coli* counts were approximately 1 log higher than initial counts. These results

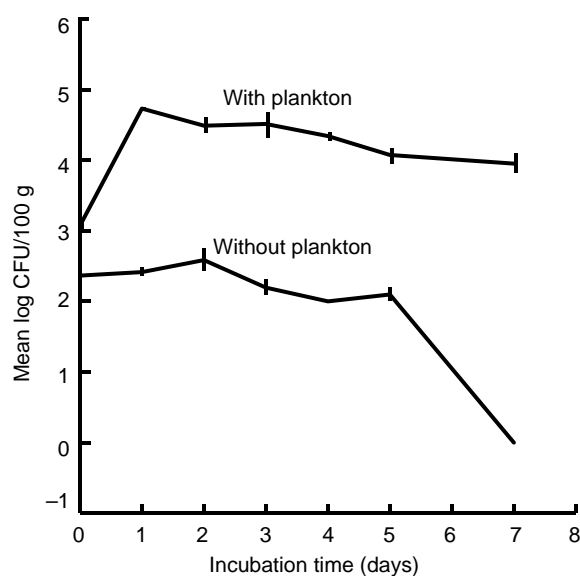


**Figure 4** | Multi-dimensional scaling (MDS) along two axes for *E. coli* genotypes—partitioned by (A) season (winter 2002 vs. spring 2003), (B) year (March 2002 vs. March 2003), and (C) within season (January-February 2002). MDS is a two-dimensional visualization of the Pearson correlation matrix. MDS plot has been converted to point bins (15 by 15) to indicate groupings of similar genotypes. Size-graded symbols (Figure 4A and 4C) denote similar genotypes.

provide evidence for potential growth of indicator bacteria in moist surface (0–10 cm) beach sand, particularly under permissible conditions—warm summer months and when nutrients (e.g., derived from plankton, algae, detritus) are available. These findings are consistent with reports of indicator bacterial growth in other natural environments, such as soil and water (Ashbolt *et al.* 1997; Byappanahalli & Fujioka 1998; Solo-Gabriele *et al.* 2000; Gauthier & Archibald 2001; Desmarais *et al.* 2002).

## DISCUSSION

Throughout the study period, *E. coli* and enterococci counts in the subsurface sand at the water table remained relatively stable except for occasional elevated counts that were mostly attributed to patchiness of microbial distribution and relative abundance in natural habitats (Wollum & Cassel 1984; Nunan *et al.* 2001; Byappanahalli *et al.* 2003a), or perhaps sporadic events of growth under certain conditions (Fujioka & Byappanahalli 2001; Whitman & Nevers 2003;



**Figure 5** | *In vitro* growth of *E. coli* in beach sand amended or unamended with net-plankton from Lake Michigan. Error bars represent ( $\pm 1$  S.E.).

Byappanahalli *et al.* 2003b). Two significant consequences of these findings are that (1) both *E. coli* and enterococci occur in beach sand at the water table, 20 m from the shoreline throughout the year, and (2) these bacteria are not transient but a component (albeit, less dense) of the population of beach microflora described by Whitman and Nevers (2003). Our observation is that *E. coli* and enterococci are ubiquitous throughout the wetted beach apart from the swash zone where sunlight and turbulence may limit their occurrence. The findings confirm the widespread seasonal distribution of these bacteria throughout the beach.

Only 52 (46%) of the enterococci isolates could be speciated, with *E. faecium* representing the most dominant species (48/52, 92%); other identified species included *E. casseliflavus* and *E. durans*. It is possible that the sand harbored other *Enterococcus* spp.; however, the phenotypic tests of the API 20 Strep identification system were inadequate to provide IDs for all the isolates tested. These results support previous observations that phenotypic approach alone is insufficient to identify (speciate) environmental enterococci (Muller *et al.* 2001).

Genetic analysis of the sand-borne *E. coli* isolates by ribotyping did not indicate the selection of any specific ribotype (genotype) over time; instead, numerous ribotypes

were found in the sand. Therefore, it is possible that the sources of fecal indicator bacteria (*E. coli*, enterococci) in the deep, subsurface sand environment are likely numerous and passively depositional—perhaps arising from a mixture of animal seeding (e.g., shore birds), and introduction from occasional storms. This, coupled with the quantitative decline in spring *E. coli* densities that were sustained through much of the summer, suggest that fall and winter *E. coli* populations are reintroduced to the subsurface sand through fecal infiltration with rain or snow melt and wave surges.

The public health significance of these findings remains unexplored; however, an immediate area of concern may be whether human pathogens (bacteria, viruses, protozoa) can similarly persist for extended periods in deep, subsurface sand. It is possible that the surface-bound pathogens from various sources (e.g., birds, run-off) may be transported to subsurface sand near the water table by natural processes. Once deposited in this environment, they are protected from external stresses (desiccation, radiation, freezing). In this situation, the pathogens can survive for extended periods and may even grow (particularly bacteria) under suitable conditions. Additional studies are nonetheless needed to demonstrate if indicator bacteria are commonly found in backshore subsurface sand under different climatic (temperate, tropical/subtropical) and water (fresh, estuarine, marine) conditions.

Previously, studies have shown that contaminated groundwater is responsible for outbreaks of numerous water-borne diseases in the US (Macler & Merkle 2000). Based on our findings, we identify two important areas of immediate concern—(1) recreational beaches, where visitors can come into contact with potential pathogens in the sand (e.g., children digging deep below the sand), and (2) shallow well water used for showers or drinking water fountains at the beach. Further research should address these identified problems.

## CONCLUSIONS

In conclusion, *E. coli* and enterococci can persist for extended periods in backshore sand at the groundwater table. Although this study was limited to two beaches of southern Lake Michigan, similar findings can be expected at

other temperate freshwater beaches. The origin of these bacteria, whether indigenous or derived from outside sources, is speculative but we have presented evidence of a population that exists in equilibrium and is responsive to increased nutrients and permissible growth conditions and, presumably, seasons. Public health implications of this poorly studied habitat deserve scientific attention in a variety of climates and biomes.

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M. N. Byappanahalli and R. L. Whitman contributed equally to this work.

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