The potential for beach sand to serve as a reservoir for *Escherichia coli* and the physical influences on cell die-off

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Introduction

Beach closings owing to faecal pollution are a global public health issue. In the Great Lakes, recreational water is monitored for *Escherichia coli* as an indicator organism for faecal contamination, and beaches are deemed unsafe when *E. coli* levels surpass 235 CFU (colony forming units) per 100 ml of water (USEPA 2003).

Abstract

Aims: The *Escherichia coli* burden at a Great Lakes urban beach was evaluated during the summer months to determine if sand served as a reservoir for *E. coli*, and if there was evidence of cell replication in situ. Field and laboratory studies investigated the effects of moisture, temperature and UV on *E. coli* densities in the sand.

Methods and Results: Sand samples (*n* = 481) were collected across three distinct transects of the beach, the top, a middle streamline, and the berm, over 15 sample days. The highest levels were found in the middle streamline, which was affected by stormwater discharge from nearby outfalls and roosting gulls; daily geometric mean levels of these seven sites ranged from 6700 to 40 900 CFU per 100 g of sand. *Escherichia coli* levels were greatest in samples with moisture levels between 15% and 19%, and were significantly higher than 0–4 and 20–24% ranges (*P* < 0.05). Pre- and post-rain samples at the beach demonstrated an increase in *E. coli* levels nearly 100-fold within 30 min, suggesting sand washout as a major mechanism for loading of *E. coli* into the beach waters. Rep PCR analysis of 160 isolates obtained from eight sites demonstrated that 21% of the isolates fell into one of the six clonal patterns, suggesting that bacteria may be able to replicate and possibly colonize beach sand. Sand field plots inoculated with *E. coli* cells containing pGFPuv that expresses GFP (green fluorescent protein) as a marker showed an initial two- to 100-fold increase at 24 h, depending on the temperature condition. The sand appeared to provide considerable protection from UV exposure as no significant difference was seen in cell densities within the first 2–4 cm of sand between exposed and unexposed plots (*P* < 0.05).

Conclusions: Beach sand may act as a reservoir for *E. coli*. Replication of cells appears to be one possible contributing factor to the persistently high levels, as indicated by both field studies and laboratory studies, and warrants further investigation. Moisture content of sand may also be a determinant of cell persistence in the sand environment.

Significance and Impact of the Study: *Escherichia coli* is used as an indicator organism for faecal pollution at most Great Lakes coastal beaches; therefore, a better understanding of how *E. coli* might survive, or possibly replicate, in the environment would improve interpretation of beach monitoring results.
Recreational water samples are rarely analysed for pathogens, as methods for the detection are complicated and expensive, and a thorough evaluation would require assaying for the hundreds of possible organisms linked to waterborne diseases (Toranzos and McFeters 1997). Therefore, often public health efforts to evaluate health risk rely solely on a single measurement of E. coli in a water sample.

Recent studies suggest that the beach sand environment of the coastal zone may act as a reservoir for E. coli (Wheeler-Alm et al. 2003; Whitman and Nevers 2003; Shibata et al. 2004). Previous studies show that faecal indicators can persist in sand throughout the year without much variation (Obiri-Danso and Jones 1999), and replication of cells has been suggested to occur (Roll and Fujioka 1997; Solo-Gabriele et al. 2000; Byappanahalli et al. 2003). These studies provide evidence that the ecology of faecal indicator organisms in the beach environment is complex, and does not follow first-order die-off kinetics, which diminishes the relationship between indicator bacteria levels and faecal pollution inputs.

Multiple factors influence survival times of E. coli in the environment, including exposure to UV light, nutrient availability, temperature, and predation (Lim and Flint 1989; Noble et al. 2004). Sediments have been shown to provide a favourable, non-nutrient limiting environment (Davies et al. 1995; Roll and Fujioka 1997), and numerous studies have documented reservoirs of faecal indicator bacteria in sediments (Hardina and Fujioka 1991; Desmarais et al. 2002; Byappanahalli et al. 2003). The sand environment may act similarly, providing nutrients and protection from UV light exposure (Wheeler-Alm et al. 2003; Whitman and Nevers 2003). The presence of indigenous micro-organisms have also been linked to diminished survival times for E. coli, as either a direct consequence of predation (Rhodes and Kator 1988; Davies and Bavor 2000), or secondary to competition for nutrients with native bacteria (Lim and Flint 1989).

Our study investigated whether or not E. coli is capable of establishing a reservoir in beach sand, thereby, compromising its effectiveness as an indicator of faecal pollution. We also examined survival kinetics of E. coli under the specific conditions of temperature and desiccation stress, and exposure to UV irradiation, using sand plots containing natural biota. Understanding the processes that control prolonged survival, replication, or cell die-off would be important for determining the significance of elevated E. coli levels in water, and could provide the basis for identifying similarities and differences in how pathogenic organisms behave in the same ecological niche.

Materials and methods

Bradford Beach site

The study was conducted along Bradford Beach, an urban beach in Milwaukee on the shore of Lake Michigan (43°03′41″ N, 87°52′20″1″ W). This site is typical for an open water beach on the Great Lakes. The beach site is located 1.5 km from the Milwaukee Harbour, which receives discharge via three rivers from a large 850 square mile basin encompassing agricultural, suburban, and urban land uses. This beach site is impacted by two localized sources of contamination, including a large shore bird population (500 to >1000 birds on most days) and seven stormwater outfalls that discharge surface runoff from the adjacent street and bluff (approximately 3 km² of drainage area) directly to the beach.

Sand sampling

To determine the E. coli burden in the beach, 481 samples of sand were taken from the top 10 cm for 2–3 days per week during the time period from 28 June to 4 August 2004 for a total of 15 sample days. The three distinct regions chosen for sampling included: the top of the beach, the stream (where outfalls created an artificial stream as a result of the uneven slope of the beach), and the berm (defined as the portion of shoreline impacted by wave action) along the length of the beach. Daily sample surveys included 20–30 sites spaced 10–15 m that were marked for repeated sampling using handheld ground positioning system (GPS) device (Garmin, Olathe, KS, USA). Triplicate sampling in and around single sites was performed to determine CFU variation within a 10-m radius on a single day. The percent moisture was determined by wet and dry weight measurements of approximately 1 g of sand removed from the sample. Escherichia coli densities were determined by eluting cells from 50 g of sand with 300 ml of sterile water by vigorously stirring for 5 min, similar to other previously described methods for E. coli analysis in the sand (Kinzelman et al. 2004). The appropriate volume was vacuum filtered onto 0.45-µm nitrocellulose filters and transferred to mTEC (membrane filtration-total E. coli) agar (Becton, Dickinson, Sparks, MD, USA) plates for colony counts, according to the Environmental Protection Agency (EPA) original method for E. coli enumeration (USEPA 2000).

Beach water sampling

Water samples were collected at evenly spaced sites along the shore at the same time as the sand surveys. Additional
Strain typing

Clonal populations were evaluated as described previously (McLellan et al. 2001; Kinzelman et al. 2004). Approximately 160 E. coli from sand eight sites on Bradford Beach were selected from primary isolation on mTEC media, and inoculated into microtitre plates containing E. coli (EC) media supplemented with 4-methyl-ubelliferyl-β-D-glucuronide (MUG) (Remel, Lenexa, KS, USA) to test for beta-glucuronidase activity. Bacteria from individual microtitre plate wells were streaked to Luria–Bertani (LB) agar plates, incubated overnight, and isolated colonies were then confirmed for indole production using a colorimetric spot test of p-dimethylaminocinnamaldehyde (Remel, Lenexa, KS, USA). Previous work in our laboratory has demonstrated this procedure results in greater than 98% correct identification when verified with biochemical reactions using an API 20 E system (bio-Merieux, Lyon, France) (McLellan et al. 2001). The cells were inoculated into 96-well microtitre plates containing LB media and grown overnight at 30°C. Whole-cell preparations were made by centrifuging the microtitre plates, removing supernatant and resuspending in 5-mol l⁻¹ NaCl. This initial wash was followed by two washes with sterile Milli-Q water. Rep-PCR analysis was carried out using the whole cell preparations as DNA template and REP1R and REP2I primers, as described previously (Versalovic et al. 1991; Rademaker and de Bruijn 1997; McLellan et al. 2003). The PCR generated 12–24 distinct products ranging from 6 to 200 bp; however, bands <500 bp were not used for comparisons, as they were not distinctive among strains. Laboratory strain K12 was used as a control in every reaction to assure consistent amplification between PCR assay set-ups. Comparisons of DNA fingerprint patterns were made based on the Pearson coefficient with a 1% optimization and 1% tolerance setting using Bionumerics software v. 3.0 (Applied Maths, Kortrijk, Belgium).

Sand field plot design

Multiple 61 x 122 x 20-cm deep modules were filled with sand from the Bradford Beach site. Sand was collected from areas of the beach that are not impacted by outfall water and background levels of E. coli were negligible (≤5 CFU per 100 g of sand). The sand was not sterilized, so that background level of indigenous organisms would be present. Plots were hydrated to near 30% water w/v at the start of experiments with stored rainwater, unless otherwise specified. The sand was homogeneously saturated by mixing in large plastic tubs and plot replicates were placed on the roof top in full sun exposure, and were subjected to fluctuating temperatures of daytime and night time cycles. For incubation under laboratory conditions, sand plot experiments were performed in duplicate in 25-cm diameter x 30-cm deep plastic pots filled with sand collected from the Bradford Beach sites. The large and small plots were loaded with either 500 or 50 ml of E. coli JM109 cells containing plasmid pGFPuv (Clontech, Palo Alto, CA, USA) grown overnight to saturation in Luria broth, which corresponded to approximately 3 x 10⁸ cells ml⁻¹.

Prior to loading, cells were centrifuged at 5000 rev min⁻¹ for 15 min, and washed 5x with Milli-Q water to remove any residual media. Cells were resuspended in Milli-Q water, and the load was applied by adding cells during the water saturation process for experimental plots. Unloaded plots served as controls. For initial experiments, saturated culture was used as the inoculum to test the survival of cells in stationary phase, which is known to produce resistance to various stressors, including temperature stress. Similar experiments were performed with inocula grown to mid-log (c. 5 x 10⁶ cells ml⁻¹) rather than stationary phase to investigate whether or not the survival characteristics were differentially modulated with respect to growth phase.

Temperature experiments

Stationary phase E. coli JM109 were inoculated onto sand plots as earlier and incubated at 4°C, room temperature (RT), 37, 44-5°C, and outside in the sand plots on the rooftop under fluctuating temperature conditions for 19 days. Experiments comparing stationary and mid-log phase cells were conducted for 72 h. For each temperature, duplicate 10-g portion (70 g for rooftop experiments) was collected and cells harvested by eluting cells from sand in 10x v/w of phosphate-buffered saline (PBS). Aliquots were taken at 1, 4, 6, 11, and 19 days postinoculation for the stationary phase experiment, and at 24, 48, and 72 h for the stationary vs mid-log phase experiment. Cell densities were determined by plating eluent dilutions.
on LB agar plates containing 40-μg ml⁻¹ ampicillin. Colonies were confirmed as JM109 by visualization under UV light.

UV experiment

Five sand plots were seeded with 50 ml of E. coli JM109 culture, grown overnight, containing vector pGFPuv. Each plot was exposed to UV light under a Nuaire Safety Cabinet (model # nu-425-600; Plymouth, MN, USA) for 20, 40, 80, 160, and 320 s. Sand samples from each plot, approximately 50 g, were eluted with 300-ml sterile water by stirring for 5 min. Escherichia coli levels were determined in 50-ml aliquots, as described earlier, using LB agar plates supplemented with 40-μg ml⁻¹ ampicillin. The biological safety cabinet used for these experiments is reported to kill E. coli in 7040 μW s cm⁻² at 100 μW s cm⁻² output.

Statistical analyses

Differences in E. coli levels of the various regions, weather conditions, and moisture content were evaluated using a Student’s t-test (two tailed) with equal variance.

Results

Field studies to assess sand reservoir of Escherichia coli

High levels of E. coli were found throughout the sampling period. A wide range of values were found for the stream and berm samples; therefore, the geometric mean was calculated for sites in each region. The geometric mean of E. coli levels at each site for the entire sampling period is shown in Fig. 1. Overall there was a significantly higher E. coli burden in the sand at the water line (designated the berm) and in the centre on the beach (designated the stream) where a series of stormwater outfalls discharge, than at the top of the beach which receives no stormwater inputs (P < 0.05). In addition, the E. coli levels in the stream region were significantly higher than the berm region (P < 0.05). Escherichia coli levels at the berm and stream were approximately two orders of magnitude higher than the top of the beach (Fig. 2). Triplicate sampling around single sites showed that there was variation within as little as 10-m distance between sites, but each transect as a whole appeared to stay consistent throughout as demonstrated by the observation that 75% of the samples taken from the stream and berm fell within 1 log of the median and 90% of samples were less than 2 logs from the median (Fig. 2). No samples from the berm or stream were below 100 CFU per 100 g of sand, suggesting that E. coli was evenly distributed across the majority of the beach area.

Large differences were seen in the E. coli levels observed in samples from sand when there had been precipitation in the preceding 24 h (Fig. 3). This effect
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Clonal populations of *Escherichia coli* in the sand

In order to investigate whether the extremely high levels of *E. coli* in some of the sand samples could be in part attributed to replication, *E. coli* strains were evaluated by DNA fingerprinting to determine if genetically identical or clonal populations were present. On the whole, a high degree of genetic diversity was observed among those isolated from the beach sand (Fig. 4a). However, at individual sites, multiple isolates were found to have identical Rep PCR fingerprint patterns (Fig. 4b). Less frequently, identical patterns were found in isolates obtained from different sites. In all, 34 of the 160 isolates (21%) analysed could be placed into six clonal groups. Clonal groups were defined as three or more isolates showing >90% similarity based on Pearson coefficient. All clonal banding patterns were identical with the exception of band intensity in some isolates. A total of 17 pairs of isolates with identical patterns were also noted.

Temperature effects on *Escherichia coli* survival in sand plots

Laboratory experiments demonstrated that at all temperatures, there was an increase in *E. coli* cell density in the initial 24 h, followed by the loss of culturable cells over time. The sand plots that simulated actual beach conditions, e.g. rooftop plots with fluctuating temperatures, demonstrated the largest initial cell increased 100-fold (2 log), and followed by a gradual decrease in cell densities. At 44°C, an initial sevenfold increase was noted, and cell densities did not fall below 75% after 5 days. No cells were recovered from 50 and 55°C conditions.

Sand plot experiments comparing survival times of stationary and mid-log cells demonstrated similar results with an initial increase followed by gradual decreases in cell numbers (Fig. 6). The mid-log cultures at 4°C, RT, 37, 44°C, produced a 1–3 log increase at 24 h. Stationary phase cells only increased twofold at RT and 44°C, but interestingly, reached approximately the same density as the mid-log cells.

Effect of UV irradiation on *Escherichia coli*

There was no observed die-off in *E. coli* levels in sand exposed to high doses of UV irradiation. No decrease in cell numbers was observed at all of the time points of 0–6 min in sand plots exposed to UV; *E. coli* levels were similar to...
unexposed control plots. Controls consisting of *E. coli* in liquid culture demonstrated a 94–99% reduction in cell numbers at all time points following UV exposure. This suggests that the major portion of cells in the sand plots were protected from UV exposure, and UV could not account for cell loss observed on rooftop sand plots.

**Discussion**

Field sampling of an urban beach located on Lake Michigan demonstrated a substantial *E. coli* burden within the sand across the entire 0.5-km stretch of beach. Neither
the stream nor the berm regions showed levels below 500 CFU per 100 g. For the stream samples, more than 75% of the values for *E. coli* fell within one order of magnitude of each other, which indicates the presence of a uniformly high load (Fig. 2). The levels found at the berm were also uniformly high, but not to the extent as that of the stream area, suggesting that wave action may wash out *E. coli* at sites near the water line. Our results were consistent with what was reported in another study at a Lake Michigan beach, where a fluctuation between water and nearshore sand (rise and fall pattern) was noted. These authors concluded that the sand is a source of *E. coli* that is found in water, rather than bacteria in water washing up on the beach (Whitman and Nevers 2003). The current study’s findings are also consistent with studies that reported fluctuations in indicator bacteria levels corresponding to tidal cycles, suggesting bacteria are washed into the water, thereby elevating indicator organism numbers (Solo-Gabriele et al. 2000).

Faecal pollution in stormwater and other types of non-point source runoff generally originates from multiple, unrelated host sources and has been shown to contain a diverse array of *E. coli* strains (Parveen et al. 1999; McLellan et al. 2003; McLellan 2004). However, cells that have undergone division and are derived from a common parent cell would appear genetically identical. Rep PCR DNA fingerprint analysis of individual *E. coli* isolated from the sand demonstrated 21% of the isolates fell into one of the six clonal groups (Fig. 4b). This finding suggests that the replication of organisms in the sand environment may, in part, account for the observed *E. coli* levels, rather than simple accumulation of cells (McLellan et al. 2001; Kinzelman et al. 2004). Similar findings were reported for *E. coli* isolates obtained from temperate soils in Lake Superior watersheds, where highly genetically similar *E. coli* (using Rep PCR analysis) were isolated from specific sites (Ishii et al. 2006). Further studies are needed to determine if the sand *E. coli* population persists overtime, suggesting it is a naturalized population, which is what was found for the *E. coli* in Lake Superior soils (Ishii et al. 2006). However, our sand plot experiments further support the suggestion that *E. coli* is able to replicate in the sand environment, as an increase in cell numbers was noted during the initial 24 h of the experiment. Actual growth, rather than accumulation of faecal indicator bacteria has been documented in numerous studies in tropical and subtropical climate (Roll and Fujioka 1997). However, these studies add to the growing body of knowledge that such complications apply to temperate climate beaches (Whitman and Nevers 2003). Given the high impact *E. coli* sand reservoirs may have on beach monitoring results, and the reliance on this organism as an indicator of faecal pollution, cellular processes, such as replication outside of the host, warrant further investigation.

Environmental factors, such as organic nutrients, contribute to a suitable environment for bacteria (Lim and Flint 1989; Whitman and Nevers 2003). Bacterial survival has been found to increase with the amount of organic matter in sand or soil (Desmarais et al. 2002). In the field studies, the stream area of the beach corresponded to the path of the stormwater outfall discharge, offering a constant nutrient supply. Throughout the summer season, an increasing amount of vegetation was noted in the stream area, demonstrating that the stream area contained enough nutrients to support plant growth. In addition, *Cladophora* strandings occurred on shore throughout the summer, most likely adding nutrients to the berm region, and perhaps contributing to the prolonged survival (Whitman et al. 2003). These nutrient sources most likely contributed to the persistent and increasing levels found in these two sampling regions. In further support of the conclusion that sand is a favourable environment in terms of nutrients, the sand plot experiments showed an increase in cell numbers during the first 24–96 h of the experiment (Fig. 5).

*Escherichia coli* JM109 that expressed GFP (green fluorescent protein) provided a means of quantifying and tracking cells in sand replicates. This strain is a genomically characterized, well-used lab strain; however, may not completely represent how a natural *E. coli* strain may behave under similar stressors. Other studies employing *E. coli* laboratory strain XL-1 blue labelled with GFP demonstrated the ability of these cells to enter the viable but nonculturable (VBNC) state under various environmental stressors. In addition, studies have also noted differences in the ability to attach to glass slides for a laboratory strain compared with four of eight wild-type strains that were tested (Castonguay et al. 2006).

Stationary-phase cells are noted for their resistance to various stressors, including heat stress (Gross 1996). We expected that if temperature stress was a major factor for initial cell die-off, stationary phase cells would show higher survival rates than mid-log phase cells at higher temperatures. Interestingly, there were no differences in survival times between stationary and mid-log culture. Rather, cell numbers were not reduced, but actually increased at all temperatures, including 44.5°C and the rooftop, which can exceed this temperature. Overall, ambient temperature conditions corresponded to the longest survival times, both in the laboratory (23 to 25°C) and in sand plots placed on the rooftop, which were subjected to a larger temperature fluctuation. Our findings may be explained by the following: The 20-h stationary phase culture used to load sand replicates had not entered death phase. Cells were, therefore, able to resume replica-
tion using limited, but available nutrients in the native sand and behaved similarly to the mid-log culture. In addition, any protective factors accumulated under stationary phase conditions did not modulate survival in the sand plot environment. Alternatively, these temperature conditions were not the major modulator of cell survival in the sand plots. Overall, these findings suggest that sand is a favourable rather than stressful environment within the temperature range used in these experiments. These findings in the sand environment vary from other studies of *E. coli* inactivation in natural waters and microcosm experiments, which have been found to be highly influenced by temperature (Mancini 1978; Rhodes and Kator 1988; Noble et al. 2004).

In addition to providing a suitable habitat, sand may also act as a physical barrier protecting cells from solar and UV irradiation that has been shown to influence cell inactivation (Noble et al. 2004). Whitman et al. (2004) reported that insolation, rather than UV radiation alone primarily inactivates *E. coli* in the water column, but these factors only marginally affected concentrations in turbid waters. Our experiments with sand plots used high doses of UV radiation, which should have resulted in 99% mortality for unprotected cells. Instead, we observed no decrease in cell numbers in comparison with unexposed controls. Cell loading was carried out by adding cells to the top of the plot, and allowing the liquid to filter through the sand; therefore, these results show that the passive distribution of cells result in the majority of the load residing below the surface and protected from direct exposure. Such distribution dynamics may be applicable on a large scale at beach sites, where stormwater loads are discharged across beaches and allowed to infiltrate into the sand, e.g. a protected environment.

In field experiments, moisture content corresponded to *E. coli* sand burden, and the *E. coli* levels in sand on wet days, when there was rainfall within 24 h, was significantly higher than dry days (Fig. 3). Low moisture or drying conditions have been associated with decreased cell survival or growth limiting conditions (Avery and Bunic 2003; Byappanahalli and Fujioka 2004). In addition, wet sand and high moisture content have been found to correlate to high *E. coli* levels in contaminated sediments and sands (Byappanahalli et al. 2003; Wheeler-Alm et al. 2003). Cycles of wetting and drying have been suggested to either cause resuscitation of cells or propagation of a subpopulation, with the end result of cell number increases posthydration (Ravel et al. 1995; Solo-Gabriele et al. 2000). The extent of cell resuscitation and recovery may be dependent on the magnitude and dynamics of the rehydration process (Mille et al. 2003).

A number of environmental stressors can induce a VBNC state in bacteria, such as *E. coli*, in which cells may be resuscitated under favourable conditions (Boaretti et al. 2003). Most studies have focussed on oligotrophic waters, and the ability of *E. coli* to survive through this mechanism has not been explored for the sand environment. In oligotrophic microcosms, induction and resuscitation of *E. coli* to and from the VBNC state was linked to the presence of organic molecules and inversely proportional to temperature (Na et al. 2006). Attachment to biotic and abiotic material has been shown to accelerate the entry of enterococci – another type of faecal indicator bacteria, into a VBNC state (Signoretto et al. 2005). Under environmental conditions in beach sand, organic materials and microbiota may influence *E. coli* survival via this mechanism. Some of the environmental conditions that have been shown to influence persistence of *E. coli* include temperature (Craig et al. 2004), the presence of particulate and organic matter (Craig et al. 2004), water current force and velocity (Jamieson et al. 2005), and associations with biofilm (Banning et al. 2003). Interestingly, in this study, *E. coli* levels were found to greatly increase in the beach sand following rain events (Fig. 3), possibly through resuscitation of VBNC cells. Identification of the factors that promote survival through induction of a VBNC state might lead to better control measures for prolonged persistence of faecal indicator in beach sand.

Our studies suggest that high *E. coli* densities in the sand may be a result of a combination of factors that include *in situ* replication after initial deposition of *E. coli*. This brings into question the reliability of *E. coli* as an indicator of faecal pollution, and challenges our current methods for analysing indicator levels as a surrogate to pathogen levels. In addition, the increasing evidence that sand is a favourable, rather than a hostile environment for *E. coli*, raises the question as to the potential for pathogenic bacteria to persist or multiply in a sand environment (Rhodes and Kator 1988; Wheeler-Alm et al. 2003; Elmanama et al. 2005). On the whole, these findings warrant further investigation into the survival mechanisms of indicator organisms and pathogens that may inhabit beach and coastal zones.

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