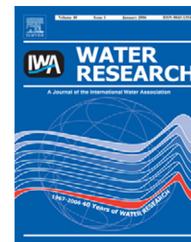
WATER RESEARCH 41 (2007) 3585–3594

Available at www.sciencedirect.com

journal homepage: www.elsevier.com/locate/watres



# Multiple lines of evidence to identify the sources of fecal pollution at a freshwater beach in Hamilton Harbour, Lake Ontario

*Thomas A. Edge*, Stephen Hill*

*National Water Research Institute, Water Science & Technology Directorate, Environment Canada, 867 Lakeshore Road, Burlington, Ont., Canada L7R 4A6*

ARTICLE INFO

*Article history:*
Received 18 January 2007
Received in revised form
1 May 2007
Accepted 7 May 2007
Available online 17 May 2007

*Keywords:*
*Escherichia*
Beach
Source tracking
Antimicrobial resistance

ABSTRACT

Multiple microbial source-tracking methods were investigated to determine the source of elevated *Escherichia coli* levels at Bayfront Park Beach in Hamilton Harbour, Lake Ontario. *E. coli* concentrations were highest in wet foreshore sand (114,000 CFU/g dry sand) and ankle-depth water (177,000 CFU/100 mL), declining rapidly in deeper waters. Many gull and geese droppings were enumerated each week on the foreshore sand within 2 m of the waterline. Both antimicrobial resistance analysis and rep-PCR DNA fingerprinting of *E. coli* collected at the beach and nearby fecal pollution sources indicated that *E. coli* in sand and water samples were predominantly from bird droppings rather than from pet droppings or municipal wastewater. Both methods indicated a trend of decreasing bird contamination, and increasing wastewater contamination, moving offshore from the beach. When foreshore sand was treated as a reservoir and secondary source of *E. coli*, waterborne *E. coli* were found to be more similar to sand isolates than bird or wastewater isolates out to 150 m offshore. Multiple lines of evidence indicated the importance of bird droppings and foreshore sand as primary and secondary sources of *E. coli* contamination in beach water at Bayfront Park.

Crown Copyright © 2007 Published by Elsevier Ltd. All rights reserved.

## 1. Introduction

Fecal contamination of beaches can present significant public health risks, loss of recreational opportunities, and costly impacts for local economies. Around the Great Lakes, almost one-third of the beaches in Canada and the United States had swimming advisories, postings, or closures in 2003 (Environment Canada and US Environmental Protection Agency, 2006). Diverse fecal contamination sources contribute to these beach advisories, including point sources such as municipal wastewater effluents, and non-point sources such as agricultural run-off and wildlife droppings. It is important to identify the source of fecal contamination at beaches in order to better understand public health risks and correctly target fecal pollution prevention actions.

Municipal wastewater is a familiar source of fecal contamination at beaches (Dorfman et al., 2004; Bower et al., 2006). While improvements continue to be made to control sources such as sewage treatment plant effluents and combined sewer overflows, beach closures persist in many communities around the Great Lakes. There is growing recognition that, in addition to point sources, a better understanding is needed of the significance of non-point sources of fecal contamination (Kinzelman et al., 2004). For example, fecal droppings from

*Corresponding author. Tel.: +905 319 6932; fax: +905 336 6430.
E-mail address: tom.edge@ec.gc.ca (T.A. Edge).

0043-1354/$ - see front matter Crown Copyright © 2007 Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.watres.2007.05.012

birds (Levesque et al., 1993), impervious surface runoff (Scopel et al., 2006), mats of *Cladophora* green alga (Whitman et al., 2003), and foreshore sand (Whitman and Nevers, 2003) can serve as non-point sources of fecal indicator bacteria adversely impacting recreational waters.

Beaches in Hamilton Harbour, Lake Ontario, have been frequently closed in recent years despite investments in municipal wastewater infrastructure and storage tanks to control combined sewer overflows (Hall et al., 2006). It had been assumed that beach closures were probably the result of municipal wastewater contamination. However, recent investigations have suggested that bird droppings might be a contributor to the elevated numbers of *Escherichia coli* in beach waters (Charlton and Milne, 2004; Edge and Hill, 2004, 2005). The following study applied multiple lines of evidence to determine the source of *E. coli* contaminating Bayfront Park Beach in Hamilton Harbour. The field of microbial source tracking has developed in recent years to provide a toolbox of methods that are available for identifying the source of fecal contamination in aquatic ecosystems (Simpson et al., 2002). However, the field is still evolving, and there is recognition that multiple lines of evidence are generally needed to resolve fecal contamination problems (USEPA, 2005a; Edge and Schaefer, 2006; Rochelle and De Leon, 2006). For this reason, antimicrobial resistance analysis (Whitlock et al., 2002) and rep-PCR DNA fingerprinting (Johnson et al., 2004) methods were applied in parallel, along with *E. coli* monitoring and beach observations, to determine the source of *E. coli* at Bayfront Park Beach.

## 2. Materials and methods

### 2.1. Study site and field observations

Hamilton Harbour is a 2150 ha embayment at the western end of Lake Ontario. It is situated in an urban setting surrounded by the cities of Hamilton and Burlington (population of 640,000 in 2001). Four municipal wastewater treatment plants discharge into the harbor area, and combined sewer overflow storage tanks occasionally overflow during storm events. There are large populations of ring-billed gulls (*Larus domesticus*) and Canada geese (*Branta canadensis*) around the harbor, and they are increasingly common in beach areas. Hamilton Harbour is listed as a Great Lakes Area of Concern, and beach closures are identified as one of the beneficial use impairments that are being addressed through a Remedial Action Plan (Hall et al., 2006). The harbor supports an active recreational environment for windsurfers and boaters, although beaches have often been closed in recent years as a result of high *E. coli* levels (O'Connor, 2003). Bayfront Park Beach is a 160 m crescent-shaped beach that is situated at the end of a promontory and set in a protective bay that reduces water circulation from the rest of the harbor. Over the 2004 bathing season, weekly observations were made of the number of animals and their fecal droppings around Bayfront Park Beach. Animals were enumerated on the beach and adjacent grassy areas, and fresh fecal droppings were counted along the beach within 2 m of the waterline.

### 2.2. Water, sand, and fecal sampling

Water and sand samples were collected at Bayfront Park Beach each Monday morning over the 2004 bathing season. Water samples were collected at the middle of the beach by wading out from the shoreline for ankle- and knee-depth samples. Additional surface water samples were collected by boat at about 150 m directly offshore of the beach at the mouth of the bay (6 m depth) and further offshore in the middle of the harbor (24 m depth). All water samples were collected in sterile bottles and returned on ice to the laboratory for analysis within several hours of collection. Two water samples were collected at each sampling location, and *E. coli* counts were expressed as the mean of the two replicates.

Sand samples were obtained from the wet foreshore sand within a meter of the waterline, and to a depth of about 15 cm, using a sterile plastic core (diameter = 2.5 cm). About 20 g of wet sand was recovered from the cores, placed in Whirlpak bags, and returned to the laboratory on ice for analysis within several hours of collection. Two adjacent sand cores were collected and *E. coli* counts were expressed as the mean of the two replicates.

Fecal samples were collected simultaneously with water and sand sampling. Municipal wastewater samples were obtained from combined sewer overflow storage tanks and three municipal wastewater treatment plant effluents (Hamilton Woodward, Dundas, and Waterdown Plants). Samples of feces from gulls, Canada geese, and mallard ducks (*Anas platyrhyncos*) were obtained from fresh fecal droppings on the beach in numbers approximating their representation on the beach. Additional fecal samples were collected from Canada geese droppings adjacent to the beach, and occasional dog droppings elsewhere in the Park. Fecal samples were also obtained from fresh droppings of stray dogs and cats at the City of Hamilton animal shelter. Fecal dropping samples were obtained using sterile culturette cotton swabs (BD Inc.). The swabs were stored on ice and returned to the laboratory for analysis within several hours of collection.

### 2.3. E. coli enumeration and isolation

Water and municipal wastewater effluent samples were analyzed by membrane filtration and *E. coli* enumeration was expressed as CFU/100 mL. Water samples were diluted and membrane filters were placed on chromogenic differential coliform (DC) agar media supplemented with cefsulodin (Oxoid Inc.) for 18 h incubation at 44.5 °C. Sterile water samples were filtered as negative controls. Sand samples were analyzed by a blender-based method and *E. coli* counts were expressed as CFU/gram of dry sand. Wet sand was weighed to 10 g and placed into 150 mL of phosphate buffer in a Waring blender. The sand was blended for 1 min and then left standing for another minute. The supernatant was then filtered following the membrane filtration procedure. Ten grams of wet sand was also dried overnight to get a dry weight conversion factor. Fecal swabs were streaked onto mFC agar (Difco Inc.) and incubated at 44.5 °C for 18 h. Isolates showing a typical blue color on mFC agar were selected for further *E. coli* identification confirmation tests. *E. coli* isolates

obtained from mFC agar or DC agar typically showed normal responses when grown on the other agar (data not shown).

*E. coli* were isolated from the weekly water, sand, and fecal samples to provide *E. coli* isolates representative of the beach area over the bathing season. Up to 12 *E. coli* isolates were randomly selected from DC agar plates for each water or sand sample. Between three and five *E. coli* isolates were randomly selected from mFC agar plates for each fecal swab. The isolates were picked with a sterile toothpick and streaked onto MacConkey agar (Difco Inc.) for overnight growth at 37 °C. Putative *E. coli* isolates on MacConkey plates were then tested for glucuronidase activity by growth and fluorescence in EC-MUG (Difco Inc.), and for indole production by growth in 1% (w/v) tryptone (Difco Inc.) and reaction with Kovac's reagent (Oxoid Inc.). Isolates positive for both tests were stored in 96-well Matrix plates (Matrix Technologies Corp., Hudson, NH) at −80 °C in tryptic soy broth and 15% (v/v) glycerol. *E. coli* ATCC 29194 and *Klebsiella* ATCC 33495 were used as positive and negative controls, respectively, during confirmation tests.

### 2.4. *Antimicrobial resistance analysis*

*E. coli* from 96-well Matrix plates were thawed and incubated overnight in a microplate containing 200 μL per well of EC-MUG broth at 44.5 °C. A 96-floating pin replicator (V&P Scientific, San Diego, CA) was used to transfer *E. coli* isolates to the surface of rectangular tryptic soy agar plates. The 12 antimicrobials (and three concentrations of each) used were as follows: ampicillin (5, 16, 32 μg/mL), cephalothin (5, 16, 32 μg/mL), chlorotetracycline (20, 40, 80 μg/mL), cloramphenicol (5, 16, 32 μg/mL), erythromycin (25, 50, 100 μg/mL), irgasan ( = triclosan) (0.01, 0.1, 0.5 μg/mL), kanamycin (1, 5, 16 μg/mL), oxytetracycline (1, 5, 16 μg/mL), penicillin G (25, 50, 100 U), streptomycin (1, 5, 16 μg/mL), sulfamethoxazole (50, 200, 512 μg/mL), and tetracycline (1, 5, 16 μg/mL). Agar plates were incubated for 18 h at 37 °C and growth of *E. coli* isolates on plates with antimicrobials was compared to their growth on control plates without antimicrobials. To quantify their relative growth, plates were scanned on a standard optical scanner as TIF files, and optical density readings of colonies were obtained with the BMNIA filter of Bionumerics ver. 4.0 (Applied Maths, Austin, TX) after rolling ball background subtraction. *E. coli* antimicrobial resistance was measured as a continuous variable (ratio of its optical density on the antimicrobial plate relative to the control plate) and as a binary variable (an isolate was considered resistant to an antimicrobial if its growth was $>0.73$ of its growth on a control plate without the antimicrobial). The value of 0.73 was derived as a practical threshold after examining several thousand *E. coli* isolates and determining the optimal optical density for discriminating between susceptible and resistant responses across different antimicrobials. When data were recorded as binary, *E. coli* isolates were occasionally found to be resistant at a high concentration of an antimicrobial, while also susceptible at a lower concentration. In these cases, the data were corrected and scored as resistant at the lower concentration. Negative control wells (blank wells) and positive control wells (wells with other *E. coli* strains with known profiles) were included on antimicrobial resistance plates. The reproducibility of the method for ratio data was assessed by repeatedly testing (six times) the profiles of 88 different *E. coli* isolates. The isolates were clustered, and it was found that the average similarity of an isolate to one of its replicates was 86%.

Prior to statistical analysis of antimicrobial resistance data, *E. coli* isolates with identical antimicrobial resistance binary profiles from the same fecal dropping or wastewater sample (or sand sample) were removed to reduce library bias. The resulting library of *E. coli* antimicrobial resistance profiles was analyzed by discriminant analysis (SAS, 1999—PROC DISCRIM procedure) using a non-parametric nearest-neighbor ($k = 5$) approach (Ritter et al., 2003). A two-way analysis of the library was performed to discriminate between bird and wastewater *E. coli* classes. Three-way analyses of the library were also performed to discriminate between bird, wastewater, and pet *E. coli* classes, and between bird, wastewater, and sand *E. coli* classes.

The performance of the library was evaluated by internal and external accuracy measures. The internal accuracy of the library was evaluated by calculating average rates of correct classification (ARCC) using resubstitution and the less-biased jack-knife method. A crossvalidation evaluation was also performed by selecting fecal samples from each source class, such that 30% of the *E. coli* isolates from each class were removed from the library. The removed isolates were then presented as “unknowns” for assignment to a source class. In addition, a mock database was constructed in which isolates were randomly assigned to each source group (bird or wastewater) to test whether, inadvertently, analysis of the randomized database would provide artifactual correct classifications. The external accuracy of the library was evaluated by its ability to predict the correct class for *E. coli* proficiency isolates collected independently from the library from duck droppings at LaSalle Park across the harbor ($n = 457$), water samples likely contaminated by wastewater from nearby Redhill and Stoney Creeks ($n = 55$), and sand samples from Beachway Park Beach on Lake Ontario outside the harbor ($n = 113$).

When the library was applied to assign water and sand *E. coli* isolates, an isolate was classified as “unknown source” when it could not be assigned to either bird or wastewater source classes with a probability of greater than 0.67. An *E. coli* isolate was classified as “unknown source” in three-way analyses when it could not be assigned to one of the three classes with a probability of greater than 0.5. These probability thresholds were chosen as a practical approach to minimizing incorrect classifications. A minimum detection percentage (Whitlock et al., 2002; Wiggins et al., 2003) was calculated based on misclassification rates to consider a conservative minimum limit for considering that a particular fecal source was present in water or sand samples.

### 2.5. *Rep-PCR DNA fingerprinting analysis*

Rep-PCR fingerprinting was performed using a BOX-PCR primer approach. A 96-pin replicator was used to transfer *E. coli* isolates to 96-well microplates containing 200 μL of tryptic soy broth in each well. Isolates were incubated at 37 °C for 16–18 h. In addition to the test isolates, four positive

controls with known BOX-PCR fingerprints and a negative control were added to each plate. Plates were centrifuged for 10 min at 3050 *g* to form a cell pellet. The cells were washed by removing the supernatant and resuspending the cells in 200 μL of sterile water. A PCR plate was filled with 5 μL of Lyse-N-Go reagent (Fisher Scientific, Nepean, Ont.) to which 5 μL of the cell suspension was added. Heating and cooling the suspension in a thermocycler as per the manufacturer's instructions lysed the cells, making the DNA available in a PCR stable solution. Fifteen microliters of master mix was created and added to achieve the following concentrations in the final 25 μL solution: 1 × Eppendorf HotMaster Taq buffer, 0.25 mM each dNTP, 5% (vol/vol) DMSO, 400 nM BOX primer (sequence 5′-CTACggCAAggCgACgCTgACg-3′), and 0.1 U/μL HotMaster Taq (Eppendorf, Mississauga, Ont.) and ultrapure water. The amplification cycling conditions were as follows: initial denaturation of 2 min at 94 °C, followed by 35 cycles of 20 s at 94 °C, 20 s at 60 °C, and 5 min at 65 °C, with a final extension of 5 min at 65 °C. Electrophoresis of the PCR products was done in a 1.25% agarose gel in TAE buffer with three rows of 50 wells. Three microliters of sample combined with loading dye was loaded into the wells. Three microliters of a $\frac{1}{2}$ dilution of Promega 1 kb ladder was used as a standard in four wells per row. A voltage of 170 V was applied until the bottom dye marker reached the end of the gel (approximately 3.5 h). The gel was stained in ethidium bromide for 30 min and destained in water for 20 min. Following staining, DNA bands were visualized by exposure to UV light and the image was captured at an exposure just below the saturation level of the brightest bands in the ladder.

Gel images were imported into Bionumerics ver. 4.00. Automatic lane and band calling were used; however, since most analyses were conducted using lane curves rather than band matchings, manual alterations were not made. DNA fingerprint comparisons were based on using a Pearson coefficient (0.28% optimization) and UPGMA clustering. Isolates that did not have at least one band with a volume of 2000 were removed to exclude failed amplifications. The reproducibility of the controls was found to be approximately 90%, which was the value used to remove *E. coli* isolates (clones) from the same fecal dropping or wastewater sample (or sand sample) to reduce library bias. Similar to antimicrobial resistance analysis, the *E. coli* rep-PCR DNA fingerprinting library was analyzed by two-way and three-way cluster analyses for birds, municipal wastewater, pets, and sand source classes. Performance of the DNA fingerprint library was evaluated in BioNumerics by simulating jack-knife-based ARCC using a maximum similarity measure and nearest-neighbor approach. Libraries were classified against themselves using $K = 7$, with nearest-neighbor source matches needing to be greater than 4 ($K = 7$ was used rather than $K = 6$ because one match would be the unknown isolate against itself, so there must be at least three other matches to a source before the isolate could be classified as such). ARCCs were expressed as a percentage of those isolates that could be identified after "unknown" source isolates were removed. When the DNA fingerprint library was applied to assign unknown water and sand isolates, they were compared to the fecal isolates using maximum similarity and a $K = 6$ nearest-neighbor approach. When a water or sand isolate had a tie with the number of nearest-neighbor matches for two fecal source classes, it was classified as "unknown source." Minimum detection percentages were calculated as they were for antimicrobial resistance analyses.

## 3. Results

Weekly monitoring results for cumulative numbers of bird droppings on foreshore sand and *E. coli* concentrations in ankle-depth water at Bayfront Park Beach are presented in Fig. 1. The highest concentrations of *E. coli* were found in ankle-depth water, dropping off rapidly at knee depth, and again at sites further offshore. The concentration of *E. coli* reached 177,000 CFU/100 mL in ankle-depth water on August 3. *E. coli* concentrations also peaked on this day at knee depth (8750 CFU/100 mL) and at the offshore bay (425 CFU/100 mL) and mid-harbor (162 CFU/100 mL) sites. *E. coli* numbers were otherwise less than 100 CFU/100 mL at the two offshore sites over the sampling period. High concentrations of *E. coli* were found in wet foreshore sand ranging from 248 to 114,000 CFU/g dry sand. The sand concentrations generally increased over the sampling period and exceeded 100,000 CFU/g dry sand on July 26 and August 3.

Birds were the only significant animal fecal source observed in the beach area over the sampling period. Ring-billed gulls were observed at every sampling time, with up to about 160 gulls observed on the beach on some days. Canada geese were also common, with numbers increasing noticeably in early June. Up to about 175 geese could be observed on the beach and surrounding grass areas on some days. Small numbers of mallard ducks were occasionally observed on the beach. While dogs were walked in Bayfront Park, they were very rarely seen on the beach and their fecal droppings were only occasionally observed elsewhere in the Park area. Large numbers of gull and geese droppings were deposited close to the waterline, and at times, droppings were observed directly in the water, and on the sand subject to waves washing up onto the beach. Up to 808 gull droppings were counted along the beach on sampling days in the early spring, while up to 707 Canada geese droppings were counted on the beach in late July. Weekly counts of gull or Canada geese droppings were not significantly correlated with ankle-depth *E. coli* concentrations at Bayfront Park Beach.

A total of 1966 *E. coli* isolates were collected from Bayfront Park area fecal sources (Table 1). Simultaneously, 1615 isolates were collected from water and sand samples at Bayfront Park Beach. *E. coli* isolates from municipal wastewater sources showed a higher frequency of antimicrobial resistance than *E. coli* from bird or pet droppings. The frequency of antimicrobial resistance was lowest in *E. coli* from beach sand and water samples. An evaluation of the two-way and three-way fecal source discriminatory analyses is provided in Table 2. Two-way antimicrobial resistance and rep-PCR analyses resulted in jack-knife ARCCs of 84% and 82%, respectively. Two-way analyses of antimicrobial resistance data found that using the ratio data provided a higher ARCC than binary data (72%), so ratio data were used in subsequent two-way analyses. Two-way analysis of the randomly assigned bird and wastewater *E. coli* isolates had a low jack-knife ARCC of

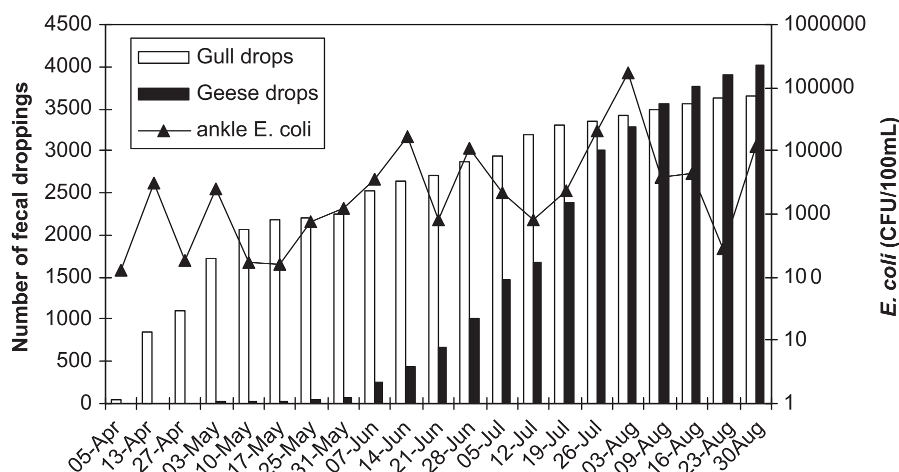



**Fig. 1 – Cumulative numbers of bird fecal droppings and concentration of *E. coli* in ankle depth water at Bayfront Park Beach in 2004.**

**Table 1 – Sources of *Escherichia coli* isolates for antimicrobial resistance and rep-PCR DNA fingerprinting analyses**

| Source | No. of *E. coli* isolates | | | | | |
|---|---|---|---|---|---|---|
| | Antimicrobial resistance analysis | | | Rep-PCR analysis | | |
| | No. of samples | Total | Decloned | No. of samples | Total | Decloned |
| Gulls | 166 | 390 | 348 | 69 | 165 | 119 |
| Canada geese | 183 | 454 | 409 | 81 | 200 | 152 |
| Ducks | 27 | 99 | 82 | 8 | 23 | 18 |
| Total birds | 376 | 943 | 839 | 158 | 388 | 289 |
| Dogs | 38 | 186 | 143 | 38 | 186 | 96 |
| Cats | 46 | 203 | 165 | 46 | 199 | 87 |
| Total pets | 84 | 389 | 308 | 84 | 385 | 183 |
| STP effluent | 58 | 373 | 317 | 53 | 194 | 173 |
| CSO tank | 22 | 261 | 211 | 19 | 196 | 143 |
| Total wastewater | 80 | 634 | 528 | 72 | 390 | 316 |
| Bayfront sand | 35 | 370 | 295 | 27 | 196 | 138 |
| Total | 575 | 2336 | 1970 | 341 | 1359 | 926 |

49.5%, similar to the result expected by chance in a two-way analysis (50%). The crossvalidation test of the two-way antimicrobial resistance analysis found that 80% of the removed isolates were correctly assigned to their source class. Evaluation of the external accuracy of the two-way antimicrobial resistance analysis found that 64% of duck isolates and 61% of suspected wastewater isolates were correctly assigned to their source class. Some three-way analyses (e.g. antimicrobial resistance) had lower ARCC values than two-way analyses, but were still much better than expected by chance for each class (33%). The cross-validation test of the sand three-way antimicrobial resistance analysis found that 62% of the removed isolates were correctly assigned to their source class. Evaluation of the external accuracy of this three-way antimicrobial resistance analysis found that 50% of duck isolates, 54% of suspected wastewater isolates, and interestingly, 88% of Beachway sand isolates were correctly assigned to their source class.

When *E. coli* from water and sand samples were classified in the two-way analysis, both antimicrobial resistance and rep-PCR methods clearly indicated that most *E. coli* in sand and shallow ankle- and knee-depth water were more similar to *E. coli* from birds rather than wastewater sources (Fig. 2). Birds were the only fecal source that consistently exceeded minimum detection percentages for both antimicrobial resistance and DNA fingerprinting analyses. The rep-PCR method suggested a trend toward increasing presence of *E. coli* from wastewater sources at offshore sites, although the DNA fingerprinting results were not above the minimum detection percentage.

In the pet three-way analysis of *E. coli* from water and sand, both methods still indicated the prominence of *E. coli* from

**Table 2 – Evaluation of the *Escherichia coli* library by antimicrobial resistance and rep-PCR DNA fingerprinting analyses**

| Discrimination analyses | N[a] | ARCC-1[b] | ARCC-2[c] | MDP[d] |
|---|---|---|---|---|
| *Bird-wastewater (2-way)* | | | | |
| Antimicrobial resistance analysis | 1367 | 90 | 84 | 19 |
| Rep-PCR DNA fingerprinting | 605 | ND[e] | 82 | 36 |
| *Bird-wastewater-pet (3-way)* | | | | |
| Antimicrobial resistance analysis | 1675 | 87 | 80 | 24 |
| Rep-PCR DNA fingerprinting | 788 | ND | 83 | 34 |
| *Bird-wastewater-sand (3-way)* | | | | |
| Antimicrobial resistance analysis | 1662 | 83 | 72 | 25 |
| Rep-PCR DNA fingerprinting | 743 | ND | 84 | 31 |

[a] Number of *E. coli* fecal isolates.
[b] Average rate of correct classification using resubstitution method.
[c] Average rate of correct classification using jack-knife method.
[d] Minimum detection percentage derived as described in Materials and methods.
[e] Not determined.

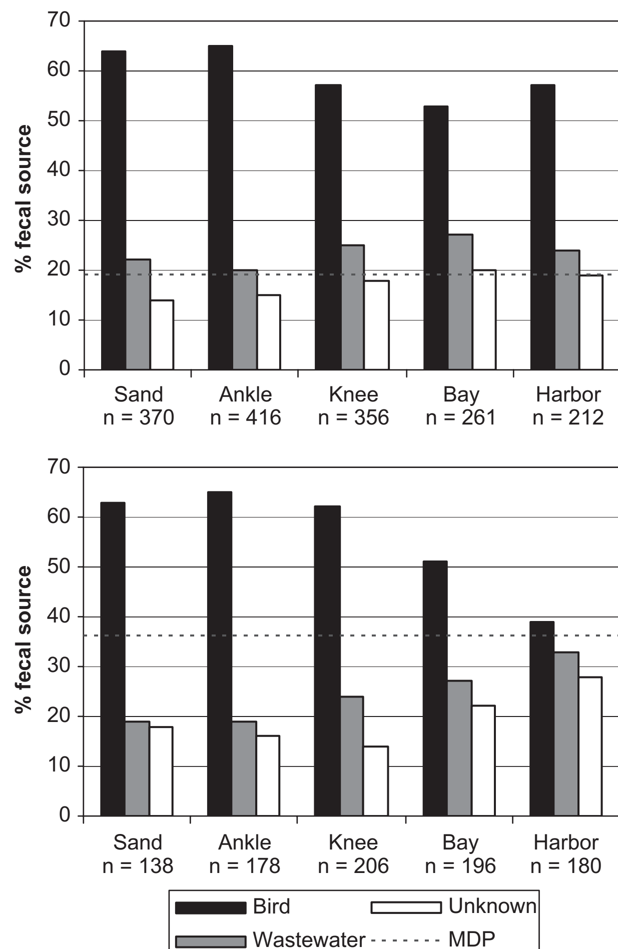



**Fig. 2 – Two-way assignment of *Escherichia coli* isolates in Bayfront Park Beach sand and water samples to bird or wastewater fecal sources by antimicrobial resistance (top) and rep-PCR DNA fingerprinting (bottom) analyses. MDP = minimum detection percentage.**

birds rather than from wastewater or pets in sand and shallow water (Fig. 3). However, unlike rep-PCR results, antimicrobial resistance analysis indicated *E. coli* from pets in ankle-depth water, and a greater prominence of *E. coli* from wastewater at offshore sites. When sand was treated as a reservoir and secondary source of *E. coli* in the three-way analysis, both methods indicated *E. coli* from ankle- and knee-depth water were mostly similar to *E. coli* from sand samples, rather than bird droppings or wastewater sources (Fig. 4). The prominence of *E. coli* from sand seemed to extend out to the mouth of the bay sampling site about 150 m offshore. Both methods also indicated that a transition occurred between knee depth and the mouth of the bay where *E. coli* from wastewater became more prominent than *E. coli* from birds.

## 4. Discussion

The highest concentrations of *E. coli* in water at Bayfront Park Beach were found in ankle-depth water, dropping rapidly as one moved offshore. Water samples from ankle-depth water exceeded Ontario provincial recreational water quality guidelines (geometric mean of 100 *E. coli* CFU/100 mL) at every sampling time. The *E. coli* concentrations in ankle-depth water reached as high as 177,000 CFU/100 mL, and were probably related to the protected nature of Bayfront Park Beach providing less water circulation and increased residence time of nearshore waters. The finding of such *E. coli* concentration gradients in beach waters has also been reported at other Great Lakes beaches (Whitman and Nevers, 2003; Sampson et al., 2005; US EPA, 2005b; Kleinheinz et al., 2006). At present, it is uncertain if high *E. coli* levels in shallow water present an increased public health risk for children who commonly play there. Epidemiology studies conducted to date at beaches have typically measured indicator bacteria densities in waters of swimming depth, and have addressed risks to adult swimmers rather than to infants and toddlers (US EPA, 2005b).

High concentrations of *E. coli* were found in the wet foreshore sand at Bayfront Park Beach, reaching over 100,000 CFU/g dry sand on two sampling occasions. *E. coli* concentrations in foreshore sand have been reported at other Great Lakes beaches, ranging from around 10 CFU/g dry sand (Alm et al., 2003) to $1.1 \times 10^4$ CFU/100 mL (Whitman and Nevers, 2003), and 20,000 CFU/g dry sand (Kinzelman et al., 2004). Whitman and Nevers noted that proper expression of *E. coli* counts in wet sand is unresolved. As there are no standard methods to measure *E. coli* in sand, it is difficult to compare results from Bayfront Park Beach with other studies.

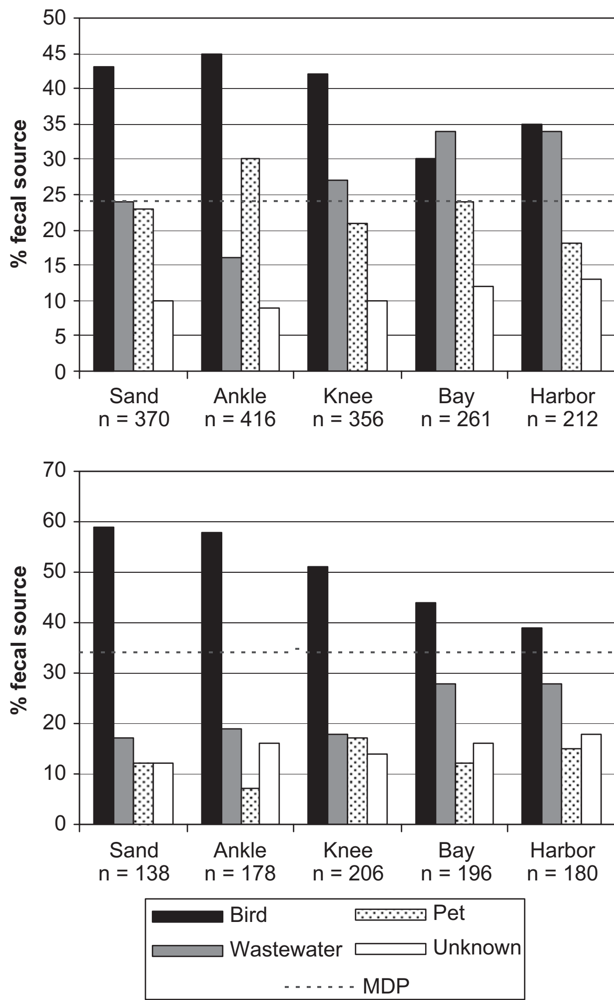



**Fig. 3 – Three-way assignment of *Escherichia coli* isolates in Bayfront Park Beach sand and water samples to bird, wastewater, or pet fecal sources by antimicrobial resistance (top) and rep-PCR DNA fingerprinting (bottom) analyses. MDP = minimum detection percentage.**

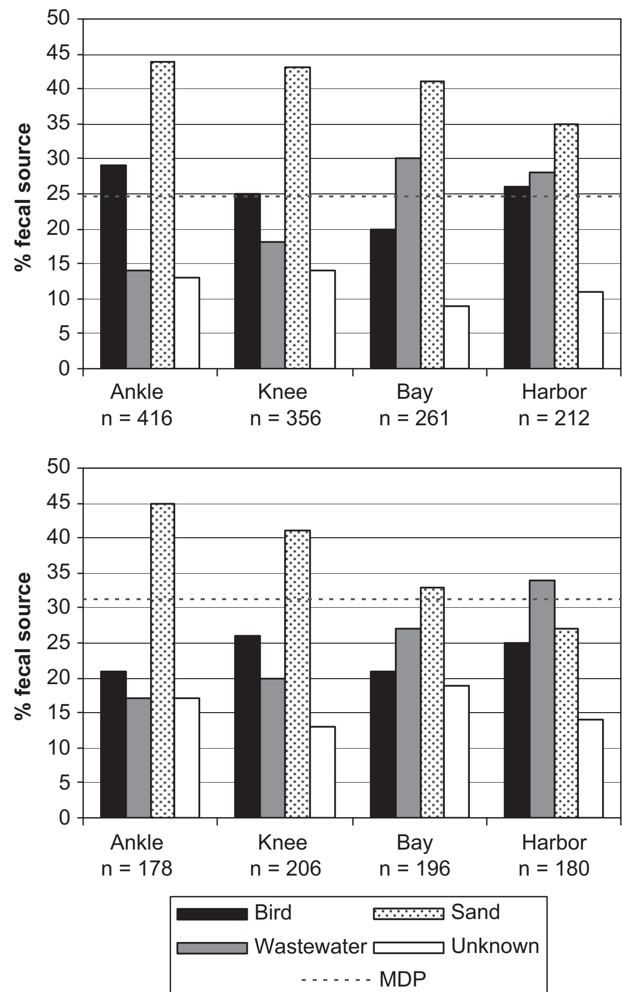



**Fig. 4 – Three-way assignment of *Escherichia coli* isolates in Bayfront Park Beach water samples to bird, wastewater, or sand sources by antimicrobial resistance (top) and rep-PCR DNA fingerprinting (bottom) analyses. MDP = minimum detection percentage.**

In our source-tracking study, we chose to use a blender-based extraction method in a rigorous attempt to recover a representative sample of *E. coli* cells including those that might be in biofilms or more closely adhering to sand particles. Irrespective of measurement method, high numbers of *E. coli* in sand relative to adjacent beach water suggests that foreshore sand can serve as a potential reservoir and non-point source of *E. coli* (Whitman and Nevers, 2003; Alm et al., 2003; Kinzelman et al., 2004).

Since Bayfront Park Beach was posted for most of the bathing season, people were rarely seen on the beach, and ring-billed gulls and Canada geese were the only animals regularly observed. Gulls were regularly observed standing at the water's edge, and their fecal droppings were observed directly in the water or on the wet sand subject to wave action. Canada geese, and their droppings, became more numerous on the beach at the beginning of June. These gull and geese droppings would have been a significant source of *E. coli*. Gould and Fletcher (1978) studied caged gulls and found that individual gulls could produce between 34 and 62 fecal droppings in 24 h. Alderisio and DeLuca (1999) found that gull feces had $3.68 \times 10^8$ fecal coliforms per gram of feces, while the geese had $1.53 \times 10^4$ fecal coliforms per gram of feces. Fogarty et al. (2003) reported *E. coli* numbers in gull feces from a Chicago beach as high as $1.9 \times 10^9$ CFU/g of feces. The gull and Canada geese droppings on Bayfront Park Beach would have provided a continuous loading of *E. coli* into foreshore sand over the bathing season.

Both antimicrobial resistance and rep-PCR DNA fingerprinting methods indicated the importance of *E. coli* contamination from bird droppings rather than from pet droppings or municipal wastewater sources at Bayfront Park Beach. We chose to interpret the microbial source-tracking results in a more qualitative sense, since the basis for drawing accurate quantitative conclusions has not been well established (Griffith et al., 2003; Stoeckel et al., 2004; Moore et al., 2005; US EPA, 2005a; Stoeckel and Harwood, 2007). The results from antimicrobial resistance analyses were similar to those from

other studies in finding higher frequencies of resistance in *E. coli* from municipal wastewater than from wildlife fecal sources (Guan et al., 2002; Edge and Hill, 2005; Salmore et al., 2006). They were also consistent with DNA microarray analyses, which found antimicrobial resistance genes more common in *E. coli* from the middle of Hamilton Harbour than in ankle-depth water at Bayfront Park Beach (Hamelin et al., 2006). The results from both microbial source-tracking methods, as well as enumeration of *E. coli* in sand and water samples, and observations of numerous bird fecal droppings provide multiple lines of evidence to indicate that birds were a more prominent source of *E. coli* contaminating Bayfront Park Beach than wastewater or pet sources over the 2004 bathing season.

Bird droppings have been reported to contribute to impairment of water quality at other beaches around the Great Lakes area (Standridge et al., 1979; Levesque et al., 1993; Whitman and Nevers, 2003; McLellan and Salmore, 2003). It was notable that the bird droppings could contribute to concentrations of *E. coli* as high as 177,000 CFU/100 mL in ankle-depth water at Bayfront Park Beach. Abulreesh et al. (2004) reported levels of *E. coli* up to 300,000 CFU/100 mL in British amenity ponds impacted by ducks and geese. Kirschner et al. (2004) reported levels of *E. coli* reaching 13,000 CFU/100 mL in shallow saline pools, whose fecal inputs were exclusively from birds such as gulls, geese and ducks. Such high *E. coli* concentrations are more typical of those measured at sources like stormwater or combined sewer overflow outfalls (Salmore et al., 2006; Bower et al., 2006; Scopel et al., 2006). While these high levels of *E. coli* are suggestive of human health risks, the risks associated with shallow beach water contaminated by high levels of *E. coli* from bird sources remain uncertain. While health risks might be lower than if the *E. coli* were from municipal wastewater sources, bird droppings can also be a source of pathogens (Levesque et al., 2000; Jones, 2005).

Both microbial source-tracking methods suggested that the frequency of *E. coli* from municipal wastewater sources seemed to be higher at sites further offshore in Hamilton Harbour. Four municipal wastewater treatment plants discharge into the harbor, and combined sewer overflow storage tanks occasionally overflow during storm events. It is likely that these sources of municipal wastewater contributed to *E. coli* contamination in the offshore waters. Hamelin et al. (2006) found that *E. coli* from the middle of Hamilton Harbour more commonly possessed virulence and antimicrobial resistance genes than *E. coli* isolates collected from ankle-depth water at Bayfront Park Beach. The possibility of sporadic municipal wastewater contamination from storm events, and continuous bird dropping contamination from beach sand, presents water-sampling challenges for microbial source-tracking studies. Our weekly water-sampling regime did not specifically capture wet weather events, and thus represents an integration of weekly *E. coli* contamination at Bayfront Park Beach waters over a whole bathing season. Microbial source-tracking water-sampling designs will need to be applied at the appropriate scale to the problem they are addressing (e.g. determining the predominant source of fecal contamination for a specific event, or for a whole bathing season).

The persistence of *E. coli* in foreshore sand is a poorly understood complication for applying microbial-source tracking methods at beaches. Gordon et al. (2002), Topp et al. (2003), and McLellan (2004) identified the differential survival of *E. coli* strains in secondary habitats outside the gut as a problem for microbial-source tracking studies. If there is significant differential survival of *E. coli* strains in beach sand, then the *E. coli* strain composition in the sand may no longer closely reflect the *E. coli* strain composition in the original fecal source (e.g. goose dropping). In addition, foreshore sand may serve as a reservoir for fecal indicator bacteria allowing them to persist for long periods of time and be resuspended in beach water through wave actions (LeFevre and Lewis, 2003; Whitman and Nevers, 2003; Kinzelman et al., 2004). In this case, resuspended *E. coli* may not be a reliable reflection of recent sources of fecal contamination. McLellan (2004) suggested that this might have accounted for the surprisingly low diversity of *E. coli* rep-PCR DNA fingerprints in beach water, and their unexpectedly low frequency of resemblance to *E. coli* from nearby gulls at Lake Michigan beaches.

When foreshore sand was treated as a reservoir and secondary source of *E. coli* at Bayfront Park Beach, both microbial source-tracking methods found that *E. coli* in the adjacent beach water were more similar to *E. coli* from the sand than from bird droppings or wastewater sources. It is possible the sand *E. coli* isolates may have originated largely from birds, but represent a unique subset of bird isolates with different survival characteristics, better enabling them to persist in sand and be mobilized into adjacent beach water. The similarity between *E. coli* in sand and water samples seemed to extend to the mouth of the bay about 150 m offshore, suggesting that beach sand was a continuous active source of *E. coli* loading into adjacent water over the beach season rather than a passive sink. These results are consistent with Whitman and Nevers (2003), who argued that while there is a continuous bidirectional flux of *E. coli* between sand and water, there was a net movement of *E. coli* from the sand lakeward at a Lake Michigan beach. The complexity of *E. coli* fluxes at the sand–water interface raises questions for microbial source-tracking studies, the appropriate grooming and management practices for reducing *E. coli* concentrations in sand, and for understanding the reliability of *E. coli* as an indicator of health risks in wet foreshore sand and shallow beach water where children play.

The library-dependent microbial source-tracking methods applied in this study provided results consistent with other lines of evidence to indicate that bird fecal droppings and foreshore sand were more prominent sources of *E. coli* contamination at Bayfront Park Beach than pet droppings or municipal wastewater. Similar results have been reported elsewhere in the Great Lakes, where more localized non-point sources of fecal contamination have unexpectedly been prominent causes of elevated *E. coli* levels at beaches rather than familiar point sources like municipal wastewater outfalls (McLellan and Salmore, 2003; Scopel et al., 2006). While *E. coli* library-dependent methods have disadvantages in terms of the costs and complexities of library building, they have advantages when validated library-independent methods for key fecal sources (e.g. birds) do not yet exist, and when results need to be communicated to end users who make decisions

using *E. coli* as a water quality indicator. Though more research is required to evaluate *E. coli* as a fecal source identifier, antimicrobial resistance and rep-PCR DNA fingerprinting analyses in this study provided useful results for identifying the most prominent source of fecal contamination over the temporal and spatial boundaries of a bathing season at Bayfront Park Beach on Lake Ontario.

## 5. Conclusions

1. *E. coli* library-based microbial source-tracking methods using antimicrobial resistance analysis and rep-PCR DNA fingerprinting identified the relative prominence of sources of fecal pollution over a bathing season at a freshwater beach on Lake Ontario, Canada.
2. Bird fecal droppings can be an important source of *E. coli* contamination in foreshore sand of temperate freshwater beaches.
3. Foreshore sand can serve as a significant reservoir of *E. coli*, and an important secondary source of *E. coli* contamination into adjacent beach waters.
4. A better understanding is needed of the survival and ecology of *E. coli* at the sand–water interface of beaches to inform sand-grooming practices and beach-management decisions to protect public health.

## Acknowledgments

Many thanks to Diana Benetteraj, Murray Charlton, Charlotte Curtis, Bailey Davis, Jason Demelo, Fargol Firouzchian, Jeannine Guindon, Tammy Kim, Leichelle Little, Alyssa Loughborough, Lauren McShane, Gabriela Midence, Jacqui Milne, Keira Pereira, Kinga Smolen, Flora Suen, and Laura Swystun from the National Water Research Institute, Environment Canada, and to Carol Dunn and Greg Gowing from the City of Hamilton.

REFERENCES

Abulreesh, H.H., Paget, T.A., Goulder, R., 2004. Waterfowl and the bacteriological quality of amenity ponds. J. Water Health 2, 183–189.

Alderisio, K.A., DeLuca, N., 1999. Seasonal enumeration of fecal coliform bacteria from the feces of ring-billed gulls (*Larus delawarensis*) and Canada geese (*Branta canadensis*). Appl. Environ. Microbiol. 65, 5628–5630.

Alm, E.W., Burke, J., Spain, A., 2003. Fecal indicator bacteria are abundant in wet sand at freshwater beaches. Water Res. 37, 3978–3982.

Bower, P.A., Scopel, C.O., Jensen, E.T., Depas, M.M., McLellan, S.L., 2006. Detection of genetic markers of fecal indicator bacteria in Lake Michigan and determination of their relationship to *Escherichia coli* densities using standard microbiological methods. Appl. Environ. Microbiol. 71, 8305–8313.

Charlton, M., Milne, J., 2004. Hamilton Harbour water quality update. RAP Technical Team, Research and Monitoring Report: 2003 Season. May 2004, Burlington, Ont., Canada, pp. 60–65.

Dorfman, M., Stoner, N., Merkel, M., 2004. Swimming in sewage. Natural Resources Defense Council and the Environmental Integrity Project. February, 2004, 68pp. 〈www.nrdc.org〉.

Edge, T.A., Hill, S., 2004. Microbial source tracking with *Escherichia coli* in Hamilton Harbour. RAP Technical Team, Research and Monitoring Report: 2003 Season. May 2004, Burlington, Ont., Canada, pp. 90–91.

Edge, T.A., Hill, S., 2005. Occurrence of antimicrobial resistance in *Escherichia coli* from surface waters and fecal pollution sources near Hamilton, Ontario. Can. J. Microbiol. 51, 501–505.

Edge, T.A., Schaefer, K.A. (Eds.), 2006. Microbial source tracking in aquatic ecosystems: the state of the science and an assessment of needs. National Water Research Institute, Burlington, Ont. NWRI Scientific Assessment Report Series No. 7 and Linking Water Science to Policy Workshop Series, 26pp.

Environment Canada and US Environmental Protection Agency, 2006. Beach advisories, postings and closures—Indicator #4200. State of the Great Lakes 2005. Cat. No. En161-3/0-2005E-PDF, pp. 161–164.

Fogarty, L.R., Haack, S.K., Wolcott, M.J., Whitman, R.L., 2003. Abundance and characteristics of the recreational water quality indicator bacteria *Escherichia coli* and enterococci in gull faeces. J. Appl. Microbiol. 94, 865–878.

Gordon, D.M., Bauer, S., Johnson, J.R., 2002. The genetic structure of *Escherichia coli* populations in primary and secondary habitats. Microbiology 148, 1513–1522.

Gould, D.J., Fletcher, M.R., 1978. Gull droppings and their effects on water quality. Water Res. 12, 665–672.

Griffith, J.F., Weisberg, S.B., McGee, C.D., 2003. Evaluation of microbial source tracking methods using mixed fecal sources in aqueous test samples. J. Water Health 1, 141–151.

Guan, S., Xu, R., Chen, S., Odumeru, J., Gyles, C., 2002. Development of a procedure for discriminating among *Escherichia coli* isolates from animal and human sources. Appl. Environ. Microbiol. 68, 2690–2698.

Hall, J.D., O'Connor, K., Ranieri, J., 2006. Progress toward delisting a Great Lakes area of concern: the role of integrated research and monitoring in the Hamilton Harbour remedial action plan. Environ. Monit. Assess. 113, 227–243.

Hamelin, K., Bruant, G., El-Shaarawi, A., Hill, S., Edge, T.A., Bekal, S., Fairbrother, J.M., Harel, J., Maynard, C., Masson, L., Brousseau, R., 2006. A virulence and antimicrobial resistance DNA microarray detects a high frequency of virulence genes in *Escherichia coli* from Great Lakes recreational waters. Appl. Environ. Microbiol. 71, 4200–4206.

Johnson, L.K., Brown, M.B., Carruthers, E.A., Ferguson, J.A., Dombek, P.E., Sadowsky, M.J., 2004. Sample size, library composition, and genotypic diversity among natural populations of *Escherichia coli* from different animals influence accuracy of determining sources of fecal pollution. Appl. Environ. Microbiol. 70, 4478–4485.

Jones, K., 2005. Flying hazards: birds and the spread of disease. Microbiol. Today 32, 174–178.

Kinzelman, J., McLellan, S.L., Daniels, A.D., Cashin, S., Singh, A., Gradus, S., Bagley, R., 2004. Non-point source pollution: determination of replication versus persistence of *Escherichia coli* in surface water and sediments with correlation of levels to readily measurable environmental parameters. J. Water Health 2, 103–114.

Kirschner, A.K.T., Zechmeister, T.C., Kavka, G.G., Beiwl, C., Herzig, A., Mach, R.L., Farnleitner, A.H., 2004. Integral strategy for evaluation of fecal indicator performance in bird-influenced saline inland waters. Appl. Environ. Microbiol. 70, 7396–7403.

Kleinheinz, G.T., McDermott, C.M., Leewis, M.C., Englebert, E., 2006. Influence of sampling depth on *Escherichia coli* concentrations in beach monitoring. Water Res. 40, 3831–3837.

LeFevre, N.M., Lewis, G.D., 2003. The role of resuspension in enterococci distribution in water at an urban beach. Water Sci. Technol. 47, 205–210.

Levesque, B., Brousseau, P., Simard, P., Dewailly, E., Meisels, M., Ramsay, D., Joly, J., 1993. Impact of the ring-billed gull (*Larus delawarensis*) on the microbiological quality of recreational water. Appl. Environ. Microbiol. 59, 1228–1230.

Levesque, B., Brousseau, P., Bernier, F., Dewailly, E., Joly, J., 2000. Study of the bacterial content of ring-billed gull droppings in relation to recreational water quality. Water Res. 34, 1089–1096.

McLellan, S.L., 2004. Genetic diversity of *Escherichia coli* isolated from urban rivers and beach water. Appl. Environ. Microbiol. 70, 4658–4665.

McLellan, S.L., Salmore, A.K., 2003. Evidence for localized bacterial loading as the cause of chronic beach closings in a freshwater marina. Water Res. 37, 2700–2708.

Moore, D.F., Harwood, V.J., Ferguson, D.M., Lukasik, J., Hannah, P., Getrich, M., Brownell, M., 2005. Evaluation of antimicrobial resistance analysis and ribotyping for identification of faecal pollution sources in an urban watershed. J. Appl. Microbiol. 99, 618–628.

O'Connor, K.M., 2003. Remedial action plan for Hamilton Harbour: Stage 2 Update 2002. Prepared by Hamilton Harbour RAP Stakeholder Forum. Hamilton Harbour Remedial Action Plan Office, Canada Centre for Inland Waters, Burlington, Ont., Canada.

Ritter, K.J., Carruthers, E., Carson, C.A., Ellender, R.D., Harwood, V.J., Kingsley, K., Nakatsu, C., Sadowsky, M.J., Shear, B., West, B., Whitlock, J.E., Wiggins, B.A., Wilbur, J.D., 2003. Assessment of statistical methods used in library-based approaches to microbial source tracking. J. Water Health 1, 209–223.

Rochelle, P., De Leon, R., 2006. Workshop on microbial source tracking. Final Report 03-HHE-3. Water Environment Research Foundation, Alexandria, VA, 106pp.

Salmore, A.K., Hollis, E.J., McLellan, S.L., 2006. Delineation of a chemical and biological signature for stormwater pollution in an urban river. J. Water Health 4, 247–262.

Sampson, R.W., Swiatnicki, S.A., McDermott, C.M., Kleinheinz, G.T., 2005. *Escherichia coli* at Lake Superior recreational beaches. J. Great Lakes Res. 31, 116–121.

SAS Institute Inc., 1999. SAS/STAT User's Guide, Version 8. SAS Institute Inc., Cary, NC, 3884pp.

Scopel, C.O., Harris, J., McLellan, S.L., 2006. Influence of nearshore water dynamics and pollution sources on beach monitoring outcomes at two adjacent Lake Michigan beaches. J. Great Lakes Res. 32, 543–552.

Simpson, J.M., Santo Domingo, J.W., Reasoner, D.J., 2002. Microbial source tracking: state of the science. Environ. Sci. Technol. 36, 5279–5288.

Standridge, J.H., Delfino, J.J., Kleppe, L.B., Butler, R., 1979. Effect of waterfowl (*Anas platyrhynchos*) on indicator bacteria populations in a recreational lake in Madison, Wisconsin. Appl. Environ. Microbiol. 38, 547–550.

Stoeckel, D.M., Harwood, V.J., 2007. Performance, design, and analysis in microbial source tracking studies. Appl. Environ. Microbiol. 73, 2405–2415.

Stoeckel, D.M., Mathes, M.V., Hyer, K.E., Hagedorn, C., Kator, H., Lukasik, J., O'Brien, T.L., Fenger, T.W., Samadpour, M., Strickler, K.M., Wiggins, B.A., 2004. Comparison of seven protocols to identify fecal contamination sources using *Escherichia coli*. Environ. Sci. Technol. 38, 6109–6117.

Topp, E., Welsh, M., Tien, Y.-C., Dang, A., Lazarovits, G., Conn, K., Hong, Z., 2003. Strain-dependent variability in growth and survival of *Escherichia coli* in agricultural soil. FEMS Microbiol. Ecol. 44, 303–308.

US Environmental Protection Agency, 2005a. Microbial Source Tracking Guide, EPA/600-R-05-064. Office of Research and Development. United States Environmental Protection Agency, Cincinnati, OH, 150pp.

US Environmental Protection Agency, 2005b. The EMPACT Beaches Project: Results from a Study on Microbiological Monitoring in Recreational Waters, EPA 600/R-04/023, August, 2005. Office of Research and Development, United States Environmental Protection Agency, Cincinnati, OH, 74pp.

Whitlock, J.E., Jones, D.T., Harwood, V.J., 2002. Identification of the sources of fecal coliforms in an urban watershed using antimicrobial resistance analysis. Water Res. 36, 4273–4282.

Whitman, R.L., Nevers, M.B., 2003. Foreshore sand as a source of *Escherichia coli* in nearshore water of a Lake Michigan beach. Appl. Environ. Microbiol. 69, 5555–5562.

Whitman, R.L., Shively, D.A., Pawlik, H., Nevers, M.B., Byappanahalli, M.N., 2003. Occurrence of *Escherichia coli* and enterococci in *Cladophora* (Chlorophyta) in nearshore water and beach sand of Lake Michigan. Appl. Environ. Microbiol. 69, 4714–4719.

Wiggins, B.A., Cash, P.W., Creamer, W.S., Dart, S.E., Garcia, P.P., Gerecke, T.M., Han, J., Henry, B.L., Hoover, K.B., Johnson, E.L., Jones, K.C., McCarthy, J.G., McDonough, J.A., Mercer, S.A., Noto, M.J., Park, H., Phillips, M.S., Purner, S.M., Smith, B.M., Stevens, E.N., Varner, A.K., 2003. Use of antimicrobial resistance analysis for representativeness testing of multiwatershed libraries. Appl. Environ. Microbiol. 69, 3399–3405.