Environmental Microbiology (2006) **8**(3), 504–513 doi:10.1111/j.1462-2920.2005.00916.x

Population structure, persistence, and seasonality of autochthonous *Escherichia coli* in temperate, coastal forest soil from a Great Lakes watershed

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Summary

The common occurrence of *Escherichia coli* in temperate soils has previously been reported, however, there are few studies to date to characterize its source, distribution, persistent capability and genetic diversity. In this study, undisturbed, forest soils within six randomly selected 0.5 m^2 exclosure plots (covered by netting of 2.3 mm^2 mesh size) were monitored from March to October 2003 for *E. coli* in order to describe its numerical and population characteristics. Culturable *E. coli* occurred in 88% of the samples collected, with overall mean counts of 16 MPN g^{-1}, ranging from <1 to 1657 ($n = 66$). *Escherichia coli* counts did not correlate with substrate moisture content, air, or soil temperatures, suggesting that seasonality were not a strong factor in population density control. Mean *E. coli* counts in soil samples ($n = 60$) were significantly higher inside than immediately outside the exclosures; *E. coli* distribution within the exclosures was patchy. Repetitive extragenic palindromic polymerase chain reaction (Rep-PCR) demonstrated genetic heterogeneity of *E. coli* within and among exclosure sites, and the soil strains were genetically distinct from animal (*E. coli*) strains tested (i.e. gulls, terns, deer and most geese). These results suggest that *E. coli* can occur and persist for extended periods in undisturbed temperate forest soils independent of recent allochthonous input and season, and that the soil *E. coli* populations formed a cohesive phylogenetic group in comparison to the set of fecal strains with which they were compared. Thus, in assessing *E. coli* sources within a stream, it is important to differentiate background soil loadings from inputs derived from animal and human fecal contamination.

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Introduction

Non-point sources of fecal indicator bacteria (e.g. *Escherichia coli*) derived from a variety of habitats – beach sand, riparian sediments, soil and water – have previously been reported (Carrillo *et al*., 1985; Whitman *et al*., 1995; Fujioka *et al*., 1999; Byappanahalli, 2000; Solo-Gabriele *et al*., 2000; Byappanahalli *et al*., 2003; Whitman and Nevers, 2003). Over the years, there has been increasing evidence that soil is a major non-point source of indicator bacteria to environmental waters in tropical locations, such as Hawaii and Guam (Hardina and Fujioka, 1991; Fujioka *et al*., 1999; Byappanahalli, 2000). Although the reasons for their increased levels in water are not well understood, at least three important natural mechanisms – (i) surface/land run-off (Hardina and Fujioka, 1991; Fujioka *et al*., 1999); (ii) release and transportation of soil/sediment bacteria by tidal/wave action along banks (Solo-Gabriele *et al*., 2000; Boehm *et al*., 2002; Desmarais *et al*., 2002); and (iii) resuspension of sediment-borne bacteria into overlying water (Davies *et al*., 1995; An *et al*., 2002) – may, in part, explain increased levels of indicator bacteria in impacted waters.

The widespread environmental occurrence of *E. coli* is not limited to warm climates. Recent studies have shown that this indicator organism can similarly be found in temperate habitats, including creeks, submerged and wet sediments along creeks and rivers (Whitman *et al*., 1995; 1999; Byappanahalli *et al*., 2003), aquatic macrophytes (e.g. *Cladophora* and seaweed) (Anderson *et al*., 1997; Whitman *et al*., 2003a), nearshore beach sand (Alm *et al*., 2003; Kinzelman *et al*., 2003; Whitman and Nevers, 2003), and in backshore, deep subsurface sand (Whitman *et al*., 2003b), perhaps unrelated to point-source (e.g. sewage) contamination. Furthermore, preliminary evidence suggests that *E. coli* can be found in temperate forest soils (Byappanahalli *et al*., 2003), but its distribution, relative abundance and persistence over time in this environment has not been fully explored.

Table 1. Physical and chemical characteristics of soil at the study sites within Dunes Creek watershed.

Site	General description[a]	Moisture content (%)[b]	Particle size analysis (%)			Textural classification	Chemical analysis			Organic carbon (%)
			Sand	Silt	Clay		pH	*N* (%)	P (p.p.m)	
1	Partly sunny, low-lying area, ~7 m from creek margin	128	65	23	12	Sandy loam	7.1	0.773	6	21.3
2	Shaded, ~2 m from creek margin	31	84	10	6	Loamy sand	7.8	0.059	15	1.9
3	Shaded, ~5 m from creek margin	37	78	16	6	Loamy sand	5.9	0.333	8	5.2
4	Partly sunny, ~6 m from creek margin	49	77	17	6	Loamy sand	5.9	0.314	6	8.1
5	Mostly sunny, low-lying area with occasional flooding, ~4 m from creek margin	87	77	17	6	Loamy sand	6.2	0.575	5	13.0
6	Shaded, low-lying area with occasional flooding, ~6 m from wetland and ~20 m from creek margin	41	67	21	12	Sandy loam	6.4	0.233	5	5.3

a. All sites were forested areas covered with heavy leaf litter.
b. Average moisture content over the study period.

In this study, we selected soils of a protected natural area in order to avoid anthropogenic influences, such as presence of septic field, manure applications and urban run-off. This study (i) demonstrates the tenacious nature of *E. coli* in temperate forest soils; (ii) characterizes temporal-spatial distribution of soil-borne *E. coli*; and (iii) shows the genetic relatedness of the soil-borne *E. coli* to each other, and to other strains isolated from homeothermic animals.

Results

Soil physical and chemical characteristics

The soil physical and chemical characteristics at the study sites are summarized in Table 1. The soil was generally sandy loam in origin with abundant litter detritus. Soils within the exclosures remained moist throughout the experimental period. Soil pH ranged from 5.9 to 7.1. Nitrogen and phosphorus levels were within the expected concentrations for the area soils. Mean (± SE) per cent organic carbon for the sites was 9.1 (± 2.9), with a range of 1.9–21.3.

Exclosure effects and distribution

Escherichia coli was commonly recovered from soils under all six exclosures throughout the study period (from March to October, 2003); the exclosures were randomly selected from a riparian stratum. While (exclosures) well separated from one another, they were located in an area of approximately 1.25 km^2 (Fig. 1). Mean *E. coli* counts in soil samples ($n = 60$) were significantly higher inside than immediately outside the exclosures ($F_{1,58} = 4.818$, $P = 0.03$). There was no significant difference, however, in mean *E. coli* densities within the exclosures ($F_{5,60} = 0.56$, $P = 0.73$); overall mean *E. coli* counts for these six sites was 1.2 log MPN g^{-1} ($n = 66$, SE = 0.1) (see also Fig. 2). Pearson correlation analysis suggested that *E. coli* counts at sites 1 and 2, and 4 and 6 were correlated ($r = 0.685$ and 0.634 respectively). Furthermore, there was no cor-

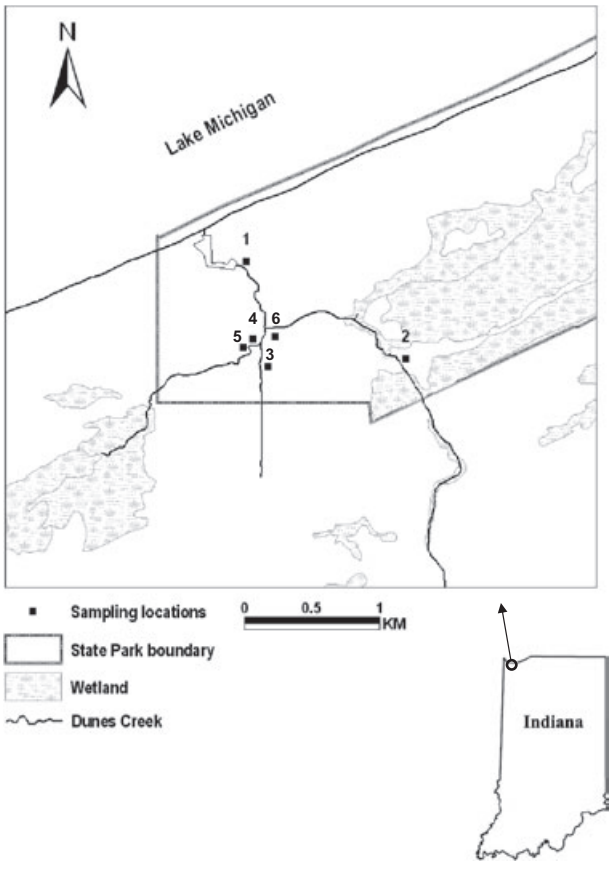

Fig. 1. Sampling locations and general characteristics of the Dunes Creek watershed within the Indiana Dunes State Park.

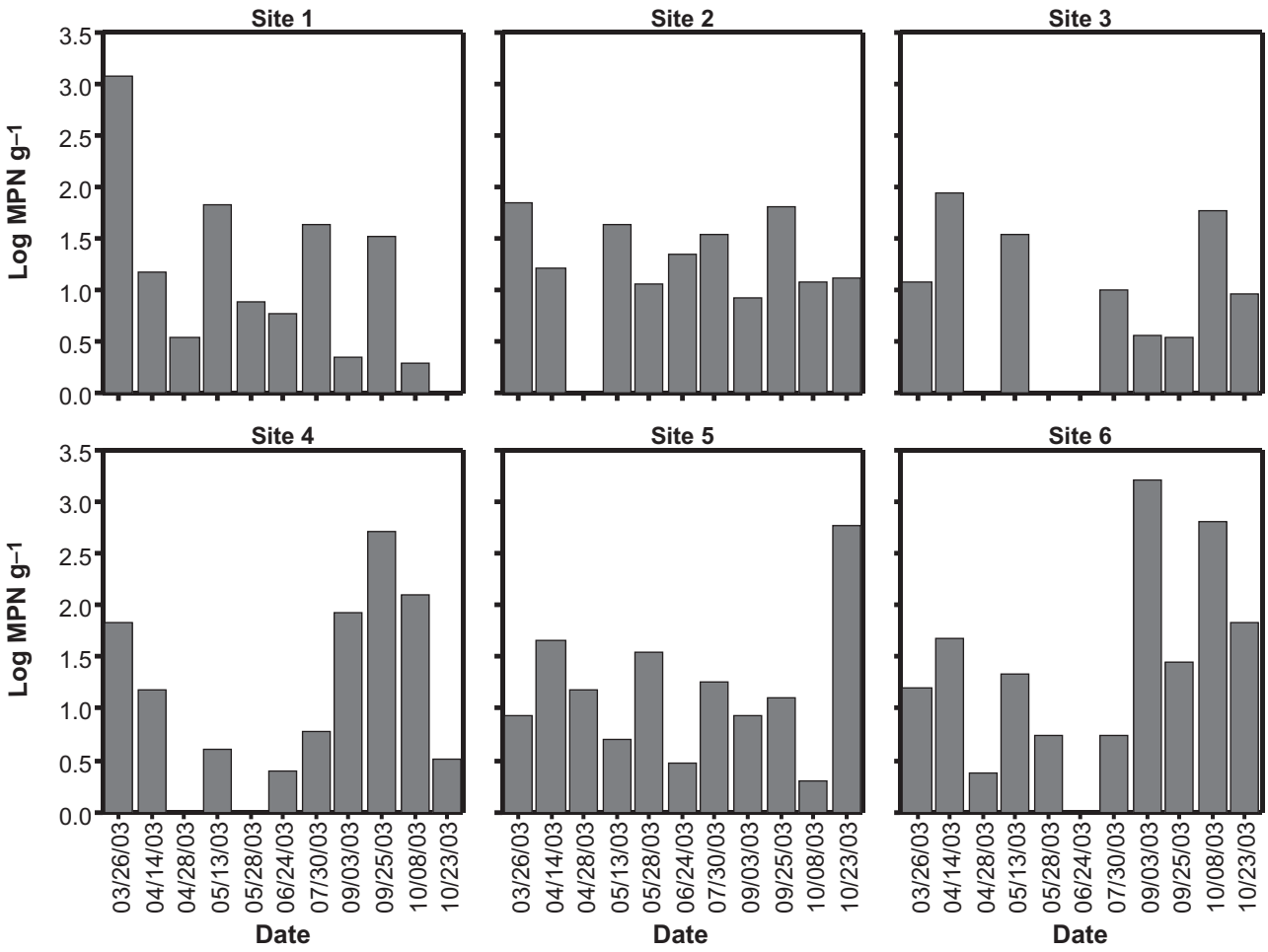

Fig. 2. *Escherichia coli* counts (log MPN g^{-1}) in soil over time for the six locations (sites 1–6) within Dunes Creek watershed. Samples ($n = 66$) were collected and analysed over a period of 8 months (March–October, 2003) to determine the ubiquity and persistence of *E. coli* in temperate forest soils.

relation between *E. coli* counts and moisture content, or air and soil temperatures ($P = 0.83$, 0.32 and 0.75, respectively), indicating that seasonality was not a strong factor in population density control. *Escherichia coli* counts in soil represented only a fraction (10^{-4}) of the culturable, heterotrophic bacteria (data not shown).

Intensive replicate sampling of soil inside and just outside of the exclosure at site 5 failed to show significant difference in mean *E. coli* density ($n = 48$, $P = 0.67$). Culturable *E. coli* counts ranged from 0 to 4.32 log MPN g^{-1} (mean 1.40, SE 0.13) (Fig. 3). Furthermore, *E. coli* within and around this plot (2.54 m^2) was not normally distributed as determined by the Kolmogorov-Smirnov (K-S) test ($P < 0.001$); K-S failed to reject log normal distribution ($P = 0.76$).

Escherichia coli *species confirmation*

The identity of coliform bacteria recovered from soil was confirmed by standard cultural, biochemical and genetic methods. All isolates had the ability to grow on mTEC agar, had β-D-glucuronidase activity, produced indole, and were urease-negative (APHA, 1998). Additional biochemical tests, as previously described by Dombek and colleagues (2000), further verified the isolates were *E. coli*. Six of the isolates, which were later used for DNA sequence analysis (see below), were speciated by API 20 E-test kits. These isolates were confirmed as *E. coli*, with good to excellent identification scores.

The taxonomic identity of isolates (DSP304, DSP307, DSP402, DSP405, DSP510 and DSP527) was further verified by nearly full-length sequence analysis of DNA encoding 16S rRNA. The polymerase chain reaction (PCR) primers used in these studies amplified an approximately 1.5-kb region of the 16S rDNA, encompassing nucleotides 27–1525 (*E. coli* MG1655 numbering). BLASTN analysis indicated that the sequenced regions from all six isolates had >98% nucleotide identity to the sequence of

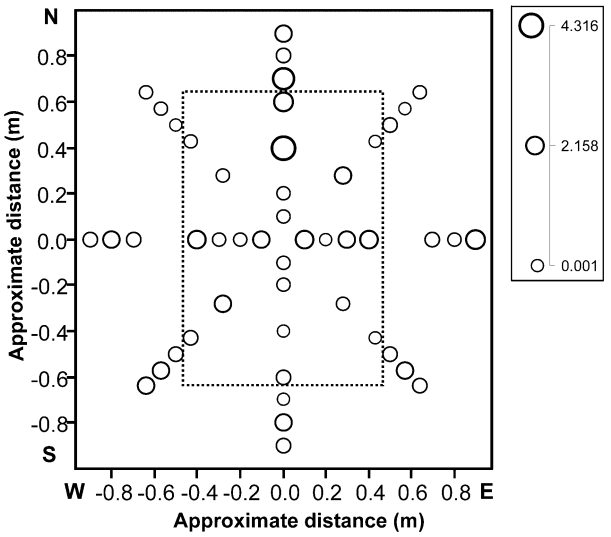

Fig. 3. Spatial variations in relative *E. coli* counts (log MPN g^{-1}) within an experimental plot measuring 2.54 m^2 ($n = 48$). Dotted rectangular box represents effective caged area. Relative *E. coli* concentrations in soil (log MPN g^{-1}) are shown by size-graded open circles.

16S rDNA from *E. coli* strain MG1655 (Accession number U00096). Taken together, these results indicate that the bacterial isolates recovered from the Dunes Creek forest soils are confirmed *E. coli* strains.

Population genetics

Forty-nine *E. coli* isolates collected from three different exclosures during the study were examined for their genetic relatedness by horizontal fluorophore-enhanced repetitive extragenic palindromic polymerase chain reaction [rep-PCR (HFERP)]. The soil isolates could be divided into two major clusters (I and II), which were separated from each other at a similarity value of approximately 62% (Fig. 4). Cluster I isolates comprised a single group, while those in cluster II consisted of two subgroups, A and B. Although all the isolates were related to one another, the strains were not identical. The two subgroups in Cluster II were differentiated at a similarity value of 74%, and there were no outliers. Moreover, there was no apparent relationship between *E. coli* genotype (as determined by HFERP) and site of isolation, suggesting that there is some degree of genetic diversity within and between isolates from the different exclosures.

Comparison to library strains

The soil-borne *E. coli* isolates represented a relatively unique group, distinct from other *E. coli* isolates in the library tested, except for a few goose isolates (Fig. 5). The first and second discriminants accounted for 82% of the variation, and together the first three discriminants accounted for 97% of the variation. Because the *E. coli* isolates in the library represent some of those thought to be found in a northern-wooded, lake-associated ecosystem, our analyses suggest that the soil-borne *E. coli* isolates were relatively distinct from reference fecal isolates (to which they were compared).

In vitro *moisture effects*

Escherichia coli density in the fresh soil (87% moisture) at the beginning of the experiment was 3.0 logs g $soil^{-1}$,

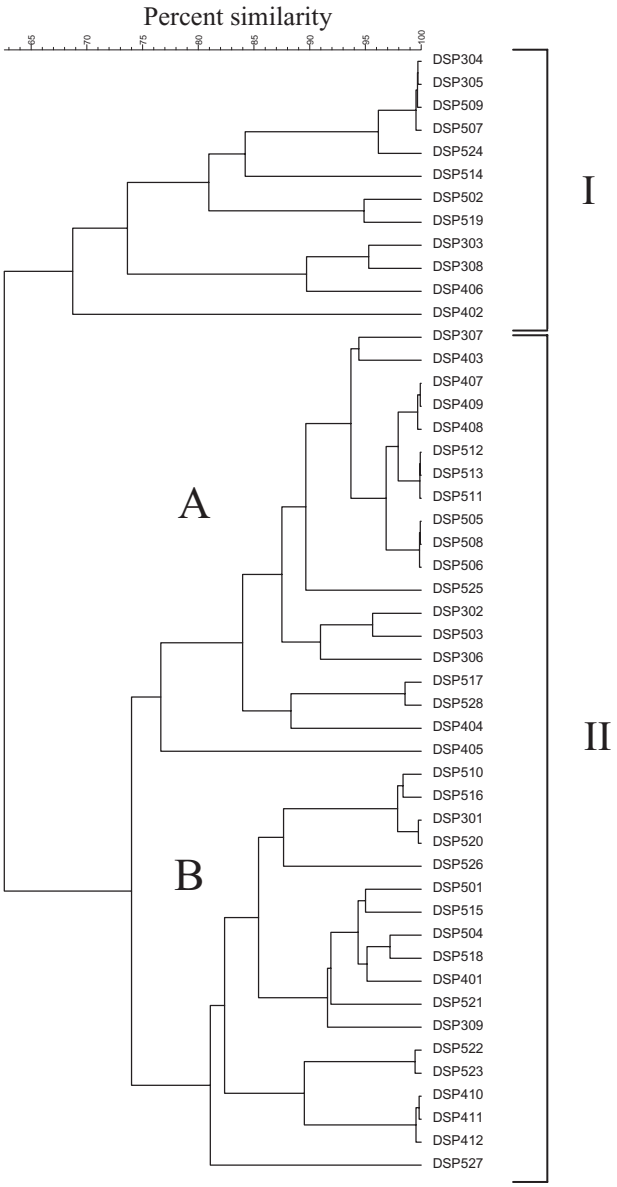

Fig. 4. Dendrogram showing the relatedness of *E. coli* strains isolated from Dunes Creek soils as determined by HFERP DNA fingerprint analysis. Fingerprints were generated using the Box A1R primer and relationships were determined using the cosine, curve-based, correlation coefficient and the UPGMA clustering method.

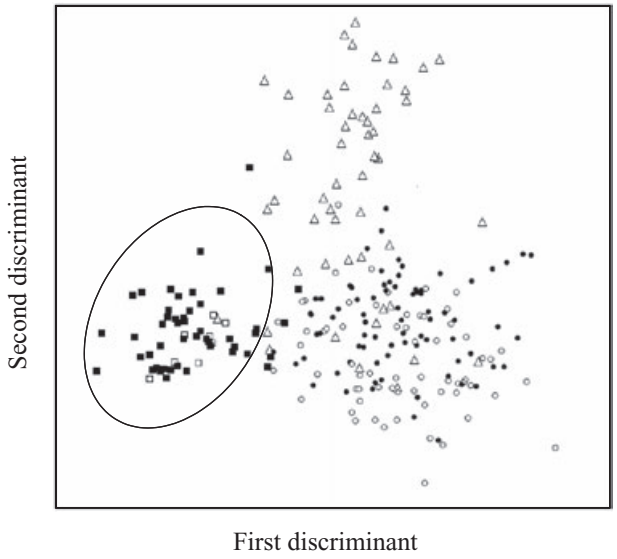

Fig. 5. Multivariate analysis of variance (MANOVA) of HFERP DNA fingerprints from *E. coli* strains obtained from soil and animal sources. A binary band-matching character tables were analysed by MANOVA, accounting for variance. The *E. coli* isolates were obtained from soils (■), geese (□), terns (●), deer (△) and gulls (○). Only the first two discriminants are presented, the first is shown by the distance along the *x*-axis, and the second discriminant is represented by the distance along the *y*-axis.

however, its counts decreased exponentially during the 70-h desiccation at 24 ± 1°C (Fig. 6). By the end of 70 h, there were only four (culturable) cells g soil^{-1}. In contrast, *E. coli* counts in the non-desiccated (control) soil remained more or less stable, with total counts decreasing by only about 0.5 log in 70 h.

The soils that had been dried for various periods (29–70 h) were re-wetted to simulate recurring cycles of substrate desiccation and re-hydration, as might be expected under natural conditions. Following re-wetting, culturable *E. coli* counts increased by 1.3- to 11.4-fold depending upon duration of prior desiccation; maximum recovery occurred in re-wetted soil that had been desiccated for 70 h, followed by 27 and 48 h desiccated soils. These results suggest that *E. coli,* like most soil bacteria, are sensitive to soil desiccation and its relative density may fluctuate with moisture content and antecedent soil conditions.

Discussion

Escherichia coli *occurrence in natural environments*

The primary objective of this study was to investigate the persistence, spatial-temporal distribution and genetic characteristics of *E. coli* in temperate forest soils. The idea that *E. coli* can occur and may grow in soil is contrary to the generally held paradigm that fecal indicator bacteria are resident populations only in the intestinal tracts of warm-blooded animals, and remain transient in external environments. However, studies conducted in several geographically and climatically distinct regions (e.g. Hawaii, Guam, Puerto Rico, Indiana, south Florida and Australia) have suggested that *E. coli* can occur, and may even grow, in natural environments – water, soils, sediments, beach sand and plants (Carrillo *et al*., 1985; Bermudez and Hazen, 1988; Hardina and Fujioka, 1991; Ashbolt *et al*., 1997; Byappanahalli and Fujioka, 1998; 2004; Fujioka *et al*., 1999; Solo-Gabriele *et al*., 2000; Whitman and Nevers, 2003; Power *et al*., 2005).

In this study, we found soil as a potential habitat for the persistent, perhaps resident, *E. coli* populations in temperate conditions. While our studies showed that *E. coli* can occur in temperate forest soils, albeit at low densities, it also had the ability to persist for extended periods in these habitats, suggesting that it is not a transient organism in soil but perhaps part of the natural microflora. Even if this is not the case, its population resiliency suggests that soil-borne *E. coli* should be treated as background concentration in source and impact evaluation investigations.

Long-term persistence of E. coli *in temperate forest soils*

For *E. coli* to be considered as an integral part of the natural soil biota, it must consistently be found in soils over time with lack of input from fecal sources and under conditions in which culturable cells can be recovered. In this study, we recovered *E. coli* from the soils throughout

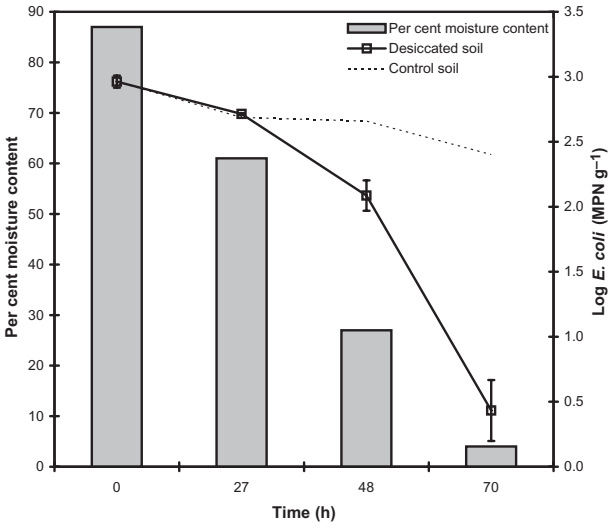

Fig. 6. Effect of soil desiccation on culturable *E. coli* counts under laboratory conditions (24 ± 1°C); vertical lines represent ±1 SE.

the 8-month study period, indicating that its occurrence in area soils was rather common and uninterrupted. The patchiness of *E. coli* distribution was not surprising because microbial occurrence, distribution and abundance in natural environments are not homogenous (Wollum and Cassel, 1984; Atlas and Bartha, 1998; Nunan *et al*., 2001). As we used exclosures to cover the sampling sites, *E. coli* presence in soils was most likely not due to immediate or direct fecal contamination from potential sources, such as wildlife or human waste. The higher *E. coli* densities within the exclosures were perhaps due, in part, to the microenvironmental conditions, such as shading and increased humidity, provided within; however, these conditions are likely also present in shaded and protected areas of dense forest canopies. Besides, the exclosures were necessary to protect the sites from intrusion and possible contamination from resident wildlife. While this study could not be extended to cover the entire winter season (December 2003 to March 2004) due to frozen soil conditions and snow cover, samples collected in January and February 2004 contained *E. coli,* albeit in low numbers (0.69 log MPN g^{-1}, $n = 12$). This indicates that *E. coli* can persist and survive even in frozen soils.

In the current study, *E. coli* recovered from forest soils represented mostly culturable (viable) cells; however, we are aware that a fraction of the cells cultured may represent recovery of stressed or viable but non-culturable (VBNC) organisms, upon reversion, under favourable environmental conditions, such as available moisture, nutrients and optimal temperature; the re-wetting experiment supports this observation. Over the years, the VBNC state has been recognized as an important adaptive strategy employed by bacteria for long-term survival in the external environment (Roszak and Colwell, 1987). Thus, based on our findings, we conclude that *E. coli* is a common component of the natural soil microflora in the Dunes Creek forest soils. Our findings not only confirm previous reports of the natural occurrence of *E. coli* in tropical/subtropical soils (Hardina and Fujioka, 1991; Fujioka *et al*., 1999; Solo-Gabriele *et al*., 2000) but also temperate soils (Byappanahalli *et al*., 2003) experiencing repeated freezing and thawing.

Identification and genetic diversity of E. coli *in temperate soils*

While previous studies examining the occurrence of *E. coli* in soils have used cultural methods (e.g. growth and reaction on selective and differential media) for isolating and identifying *E. coli* (Hardina and Fujioka, 1991; Solo-Gabriele *et al*., 2000), we used a polyphasic approach, consisting of cultural, biochemical and genetic analyses, to isolate and identify the soil-borne *E. coli.* The cultural and biochemical methods, including the API 20 E system, clearly identified that the isolates tested were *E. coli.* In addition, the nearly full-length analysis of DNA encoding 16S rRNA showed that all the tested strains had >98% nucleotide sequence identity to the same gene in the sequenced *E. coli* strain MG1655.

Genetic diversity studies showed that the soil-borne *E. coli* strains generally clustered together yet appeared genetically diverse both within and among exclosures. These data suggest that the soil-borne *E. coli* strains may represent a separate group relative to the majority of isolates obtained from animals inhabiting temperate, coastal forests. Moreover, genetically diverse *E. coli* strains may have colonized these soils over time.

Although our DNA fingerprint library was relatively small, Bionumerics bootstrap subroutine analyses nevertheless indicated that 14 soil isolates showed some degree of genetic relatedness ($P > 0.9$) to *E. coli* isolated from birds (terns and geese); and two isolates were related to *E. coli* originating from deer. Thus, it is possible that some of the soil *E. coli* strains may have originated from fecal deposits of animals inhabiting the forest ecosystem and over time became established as part of the normal soil microbiota. However, at this point, the initial origin of the soil-borne *E. coli* remains uncertain, and a more detailed study is required that uses a larger and more extensive DNA fingerprint database, which encompasses strains isolated from other forest-dwelling animals, such as rodents, raccoon and opossum.

Escherichia coli *growth potential in temperate soils*

There is circumstantial evidence that *E. coli* may grow in temperate pasture/agricultural soils and in stream sediments that are impacted by grazing animals (Crane *et al*., 1980; Faust, 1982; Gary and Adams, 1985; Howell *et al*., 1996). Whether *E. coli* can grow in natural (temperate) soils not influenced by such sources has not been investigated before, although there is growing evidence for this possibility in natural tropical soils (Byappanahalli and Fujioka, 1998; 2004; Solo-Gabriele *et al*., 2000; Fujioka and Byappanahalli, 2001; 2003; Desmarais *et al*., 2002). Two potential factors support the hypothesis that *E. coli* can actively grow in temperate soils: (i) warm soil conditions during summer months (June to September); and (ii) nutrient availability. As *E. coli* growth requirements are relatively simple (Byappanahalli and Fujioka, 1998; Byappanahalli, 2000), and it has the ability to synthesize its cellular components from just glucose and minerals (Andrews, 1991), it is possible that *E. coli* can grow in the organically rich, moist Dunes Creek forest soils (Table 1), at least during summer months. The empirical evidence from this study, especially the occurrence and long-term

persistence in soil (Fig. 2), isolation of relatively distinct population within exclosures (Fig. 5), recovery and growth following (soil) re-hydration, and growth in natural soil under reduced competition (e.g. in the presence of bile salts, an inhibitor of non-fecal bacteria; data not shown), supports this observation.

Implications and future directions

Previously, there was some indication that Dunes Creek forest soils harboured *E. coli* (Byappanahalli *et al.*, 2003), however, the extent of its distribution, survival capability and population characteristics in these soils were mostly descriptive. The current findings show that *E. coli* occurrence in the study area soils is rather widespread, persistent and independent of short-term input. The primary source of *E. coli* remains ill-defined, and could possibly be quite varied because once introduced it remains viable for an extended period. Environmentally, *E. coli* occurrence in Dunes Creek forest soils may mean that it (*E. coli*) can readily be transported to streams and adjacent coastal waters by run-off, soil/sediment erosion and aeolian processes. The year-round presence of *E. coli* in relatively high numbers in Dunes Creek watershed strongly supports this phenomenon. While we concede there are human and animal input that have yet to be defined, their delivery is less likely to account for the occurrence, distribution and predictable levels found both in riparian soils and waterways. Thus, even in the absence of any known contamination sources, *E. coli* levels in impacted waters may remain high as a result of frequent input from soil.

The fading absolute paradigm that *E. coli* can be reliably used to detect and tract human waste is being qualified and now extended. As our understanding of microbial ecology advances, the acceptance that *E. coli* does indeed live within natural habitat and under suitable conditions may thrive. More studies on its autecology and limiting factors beg for investigations. The dynamics of bacterial transport is another fruitful area of inquiry. Regardless, in the pursuit of microbial source identification, the bacteria held within the soils and sand of indeterminate origin should be taken into serious consideration because they may indeed influence contamination studies.

While this study was limited to relatively less disturbed, moist forest soils rich in organic matter, additional studies are necessary to determine the occurrence of *E. coli* and its growth potential in other temperate soils, with different chemical and physical characteristics. Also, future studies should focus on ecological characteristics (e.g. survival strategies, growth requirements, microbial interactions and population genetics) to better explain if *E. coli* and other fecal indicator bacteria (e.g. enterococci) have the ability to accumulate, persist, and perhaps even grow in soil environments in temperate locations.

Experimental procedures

Site description

Field studies and soil collections were conducted within the Dunes Creek watershed, a small coastal stream located along the shore of southern Lake Michigan in north-west Indiana, USA (N41°37′, W87°05′); the study site is located within the boundaries of Indiana Dunes State Park and Indiana Dunes National Lakeshore. The forested areas studied are well protected and have low recent human influence. Over 90% of the watershed is natural area, with approximately one-third of this classified as aquatic or wetland habitat. Sampling sites were dominated by white oak and were well above the normal flood zone of Dunes Creek. The watershed is a natural habitat for a variety of birds and rodents (squirrels, mice, chipmunk, voles, shrews), deer, raccoon and opossum, but human habitation is confined to campgrounds, park housing for managers, and residential communities well outside the park.

For soil physical and chemical analyses, samples (approximately 40.0 g) were collected from each of the six exclosures at least three times over the course of the study; the subsamples were pooled and thoroughly mixed, and a representative sample for each exclosure was analysed for physical and chemical characteristics. The soil and vegetation characteristics of the study sites are summarized in Table 1.

Microbiological analysis of soil samples

Soil and sediment samples were analysed for *E. coli* by using the Colilert-18 system (IDEXX, West Brook, ME) as previously described (Byappanahalli *et al.*, 2003). Aliquots (100 ml) of 10-fold dilutions of soil samples containing Colilert-18 reagent were aseptically transferred into distribution trays (IDEXX Quanti-Tray, 2000), sealed, and incubated at 35°C for 18 h. Wells fluorescing upon exposure to long-wavelength UV light were counted to determine the most probable number (MPN) of *E. coli* 100 ml^{-1}. Unless otherwise stated, all bacterial counts (MPN) are expressed g dry weight of $soil^{-1}$. *Escherichia coli* strain ATCC 25922 served as positive control for all analyses.

Culturable, heterotrophic bacteria in soil samples were enumerated on one occasion using R2A agar (Difco Laboratories, Becton-Dickinson, Sparks, MD). Triplicate aliquots (200 μl) from several dilutions were spread-plated on R2A agar, and plates were incubated at 25°C for 72 h. Plates from suitable dilutions were used to determine the densities (colony-forming units, cfu) of heterotrophic bacteria; counts were expressed g dry weight of $soil^{-1}$.

Isolation and identification of E. coli

Escherichia coli was isolated from soils and sediments as follows: bacterial growth in wells from Quanti-Trays that fluoresced was streaked onto the surface of cellulose ester membrane (Advantec MFS, Pleasanton, CA) placed on mTEC agar (Difco, Detroit, MI). The plates were incubated at 44.5°C

for 20–22 h, and yellow or yellow-brown colonies that appeared on the membrane were then initially confirmed as *E. coli* by substrate analyses (APHA, 1998). Well-isolated colonies from mTEC agar plates were subcultured onto MacConkey agar (Difco), and pure colonies were later confirmed for β-glucuronidase activity on nutrient agar (Difco) containing 4-methylumbelliferyl-β-D-glucuronide, MUG. Confirmed *E. coli* isolates were stored at –80°C in tryptic soy broth containing 10% glycerol, until used.

Additional confirmatory tests for *E. coli* were carried out using procedures previously described (Dombek *et al*., 2000). The six isolates, which were later used for DNA sequence analysis, were speciated using API 20 E-test kits (bioMerieux, St Louis, MO). Forty-nine *E. coli* isolates collected from five exclosures (5–15 isolates exclosure^{-1}) over several samplings were later used in genetic studies (see below).

Horizontal fluorophore enhanced rep-PCR (HFERP)

The genetic relatedness of 49 soil *E. coli* isolates to each other and to those from other sources in a DNA fingerprint library was determined by horizontal, fluorophore-enhanced repetitive extragenic palindromic-PCR (HFERP) analysis and 6-FAM fluorescently labelled Box A1R primers. Polymerase chain reaction and electrophoresis conditions were performed as previously described (Johnson *et al*., 2004). The library contained HFERP-generated DNA fingerprints of *E. coli* isolated from fecal materials of deer, geese, beaver, terns and gulls that are commonly found in near-shore forested areas (Hieb *et al*., 2004). The library, which was constructed from animal isolates obtained around Lake Superior and adjacent watersheds, near Duluth, Minnesota, contained 242 unique DNA fingerprints and was constructed as previously described (Johnson *et al*., 2004).

HFERP images were captured using a Typhoon 8600 Variable Mode Imager (Molecular Dynamics/Amersham Biosciences, Sunnyvale, CA) as previously described (Johnson *et al*., 2004), and processed using ImageQuant image analysis software (Molecular Dynamics/Amersham Biosciences). Gel images were analysed by BioNumerics v.2.5 software (Applied-Maths, Sint-Martens-Latem, Belgium) and normalized using the Genescan-2500 ROX internal lane standard. DNA fingerprint similarities were calculated by the curve-based cosine coefficient, and dendrograms were generated based on the unweighted pair-group method using arithmetic averages (UPGMA). Relatedness of isolates to each other and to those in the DNA fingerprint library was determined as previously described by Johnson and colleagues (2004). The identification of isolates was carried out by Bionumerics ID bootstrap analysis; only isolates that have a *P*-value >0.9 were recorded. Clustering of isolates was accomplished by multivariate analysis of variance (MANOVA), a form of discriminant analysis, accounting for variance (Dombek *et al*., 2000). Five groups were specified: deer, geese, gulls, terns and soil bacteria. Only the first two discriminants were used for graphical display.

16S rRNA sequence analysis

Six *E. coli* isolates were chosen for nearly full-length 16S rDNA sequence analysis based on their clustering in dendrograms constructed from DNA fingerprints (Fig. 5). Two isolates each from the three clusters, I, IIA and IIB, were selected for 16S rDNA analysis: DSP304, DSP307, DSP402, DSP405, DSP510 and DSP527. *Escherichia coli* isolates were streaked onto Plate Count Agar (Difco, Detroit, MI) and grown overnight at 37°C. Single colonies were picked with a 1-μl sterile inoculating loop and suspended in 100 μl of 0.05 M NaOH, heated at 95°C for 15 min, and centrifuged at 10 000 r.p.m. for 5 min. Supernatants were diluted 10-fold, and 1-μl aliquots were used as template for PCR using 16S rRNA gene primers 27F, 5′-AGAGTTTGATCMTGGCTCAG-3′, and 1525R, 5′-AAGGAGGTGWTCCARCC-3′ (Lane, 1991). Reaction mixtures (100 μl) contained 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.5 μM each primer, 0.16 μg μl^{-1} bovine serum albumin, 0.1% Triton-X 100 and 2.5 unit of *Taq* DNA polymerase (Promega, Madison, WI). Polymerase chain reaction was performed using MJ Research PTC 100 Thermal Cycler (MJ Research, Waltham, MA) with the following condition: 95°C for 5 min, followed by 30 cycles consisting of 95°C for 30 s, 55°C for 30 s and 72°C for 90 s. After final extension at 72°C for 5 min, PCR reaction mixtures were stored at 4°C.

Electrophoresis was performed with 1.2% SeaKem LE agarose gel (FMC, Rockland, ME) at 90 V for 2.5 h. DNA fragments (approximately 1.5 kb) were recovered from gels using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) and directly used as templates for sequencing using the following primers: 27F, 519R, 530F, 907R, 926F, 1100R, 1392R, 1406F and 1525R (Lane, 1991). Sequences were aligned and identified by using the BLASTN algorithm (http://www.ncbi.nlm.nih.gov/BLAST/).

Widespread occurrence and persistence of E. coli *in riparian forest soils*

The occurrence and persistence of *E. coli* in forest soils of the Dunes Creek watershed was investigated during March to October 2003, covering three distinct seasons – spring, summer and early autumn. Six randomly selected quadrants (each measuring 0.5 m^2) were selected for intensive sampling. The quadrants selected were well separated from one another and were located in an area of approximately 1.25 km^2. The quadrants were selected based on the following considerations: (i) representativeness of the location, particularly in terms of soil and vegetation characteristics, and topography; (ii) ready access; and (iii) minimum environmental disturbance from human activities. Sampling sites were protected from external contamination by using 1.0 m by 0.5 m exclosures covered with nylon mesh (2.30 mm^2 mesh size). Exclosures were routinely inspected for animal disturbance, intrusion, or nearby defecation. Soils under exclosures were sampled once in March, every 2 weeks during April and May, once a week in June and July, and then every 2 weeks in September and October; no samples were collected in August. Three sub-samples (each approximately 15 g) were randomly and aseptically collected from each site, pooled and mixed thoroughly; homogenized sub-samples (usually 10 g) were used for analyses. No locations were sampled more than once. Samples were kept on ice and analysed within 4 h of collection. Air and soil temperatures were recorded at each site using a digital thermometer. Soil

samples were also collected outside the exclosures to account for exclosure effects.

Effect of soil desiccation on E. coli *counts*

Laboratory mesocosm experiments were carried out to determine the effects of desiccation on *E. coli* levels in soil. Surface soil (0–6 cm depth, 88% initial moisture content) was collected from site 5, homogenized, and four 190-g subsamples were separately transferred to ~740-ml plastic containers. Soil in the containers was allowed to dry at 24 ± 1°C, which corresponds to the average maximum air temperature during summertime (June to September). Soil in each container was mixed thoroughly at least twice each day. A control sample was prevented from desiccation by covering the container with a lid. Containers were randomly removed after 27, 48 and 70 h of desiccation, which corresponded to a moisture loss of 29%, 69% and 95% respectively. For determining moisture content, 10 g of fresh soil was placed in a 100°C oven for 24 h and the weight differential was recorded; per cent moisture was calculated by the following formula:

$$(\text{weight of fresh sample} - \text{weight of dry sample})/\text{weight of dry sample} \times 100$$

While viable counts of *E. coli* in control soils were monitored during all time intervals, soils in the other containers were analysed only at the end of the corresponding desiccation period. All desiccated soils were stored in airtight containers at 4°C for later use.

The desiccated soils were re-wetted (to their original moisture level) with sterile distilled water to simulate the cyclical processes of desiccation and re-wetting that occur under natural conditions. Re-wetted soils were incubated at 25°C and analysed for changes in *E. coli* densities over the next 48 h. There were two replicates per desiccated soil treatment.

Statistical analyses

Statistical analyses and graphical presentations of all soil and bacterial count data were performed using SPSS, Version 12.0 (SPSS, Chicago, IL). Statistical procedures were performed on $\log_{10}$-transformed data to meet parametric assumptions of equality of variances and normal distribution. Non-parametric tests (e.g. K-S test) were used to test normality. ANOVA and correlation analysis was used to compare means. Unless otherwise stated, statistical significance was set at $\alpha = 0.05$.

Acknowledgements

This study was funded, in part, by grants from Sea Grant and the University of Minnesota Agricultural Experiment Station (to M.J.S.). We thank Meredith Nevers and Douglas Wilcox, USGS, for their critical review of the manuscript. Special thanks to Stacey Byers, USGS, who helped in sample collection and analysis. This article is Contribution 1338 of the USGS Great Lakes Science Center.

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