ENTERIC BACTERIA AS ENVIRONMENTAL BIOINDICATORS IN STREAM WATER AND SEDIMENTS

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ABSTRACT

A combination of classical microbiological tests, API 20E, and amplified ribosomal DNA restriction analysis was used to examine effects of farmland and rural development on the distribution of populations of *Enterobacter* species along the stream. The enteric bacteria were examined in an agricultural stream, Ledbetter Creek in Murray, Kentucky, USA over a period of 2 years and were significantly higher compared to a pristine stream, Panther Creek, Tennessee, USA. Ledbetter Creek is an agricultural stream where interstitial waters were less aerobic, with high nitrate (0.268 mg/L), high turbidity (14.1 mg/L), and high total suspended solids (14.1 mg/L). Total area of the site was approximately 300 m^2. Five distinct patterns were differentiated among *Enterobacter* isolates recovered from stream water, streambed sediments and different hyporheic depths by ARDRA analysis. Different ARDRA types dominated at different depths. We concluded that *Enterobacter* species were heterogeneously distributed along the stream and distribution was influenced by site (streambed sediments, gravel bar, upwelling, downwelling), sampling time (winter, spring, summer, fall), occurrence of anaerobic zones, macronutrients, turbidity, and total suspended solids. The presence and movement of the enteric bacteria in the environment will significantly contribute to our understanding of bacterial populations in freshwaters and we suggest using enteric bacteria as environmental bacterial indicators.

INTRODUCTION

Indicator organisms are used globally as warning of possible contamination and as index of water quality deterioration. *Enterobacter* species are ubiquitous in the terrestrial and aquatic environments (e.g. DePaola et al. 1995; Pedersen and Jacobsen 1993), occur in animals and plants (e.g. Coleman et al. 1996; DePaola et al. 1995; Halda et al. 1991), and are often used as indicators of sewage contamination. These organisms are responsible for a wide array of human infections (Schaberg 1991), some of which can be life threatening but were rarely found as pathogens before widespread use of antibiotics. Prevalence of members of the family Enterobacteriaceae is a public health concern, since enteric bacteria are normal microflora of human (Coleman et al. 1996) and fish intestine (DePaola et al. 1995) that transfer antibiotic resistence under various conditions. Therefore, presence and movement of the enteric bacteria in the environment will significantly contribute to our understanding of bacterial populations in freshwaters. The effects of land-use practices that may alter the long-term health of stream ecosystems are only beginning to be understood (Ovreas et al. 1998). Nutrient inputs from agricultural fields and sewage inputs from septic systems adjacent to streams are transported via groundwater flow. Thus, groundwater discharge into streams will affect general water quality. Streambed sediments are often heterogenous as a result of depositional and erosional processes that create pool-riffle-pool sequences, which promote the variable surface flow patterns and establish hyporheic zones. Hyporheic zones are defined as saturated interstitial areas within the streambed and stream banks that contain advected surface waters (downwelling zones) or some proportion of surface water mixed with ground water (upwelling zones) (White et al. 1993). Alterations in spatial heterogeneity of sediments induce changes in sediment microbial communities and play a key role in shaping microbial distribution and diversity. The dynamics of streams make the sampling of microbial populations and communities during the year very difficult. However, completely reproducible and representative sampling is the prerequisite for studying the presence and distribution of indicator microorganisms. The objective of this study was to determine the distribution of enteric genotypes in stream sediments. The second objective was to interpret distribution patterns of *Enterobacter*

cloacae and *Enterobacter agglomerans* genotypes with respect to surface and subsurface water quality variables (inorganic nutrients, dissolved oxygen, turbidity, dissolved organic carbon, and total suspended solids).

MATERIALS AND METHODS

Site description

Ledbetter Creek drains an agriculturally impacted watershed located in Calloway County, western Kentucky, USA. The study site (Halda-Alija, Hendricks, and Johnston 2000) is located in a third-order reach approximately 100 meters upstream from where Ledbetter Creek discharges into Kentucky Lake reservoir. The average annual base-flow discharge is 0.065 m^3 s^{-1}. Streambed and gravel bar sediments at the study site are primarily sands, gravel and cobble with coarse gravel predominating. Other physical, chemical and hydrological characteristics of the stream are described in detail elsewhere (Hendricks, Halda-Alija, and White 1999).

Difficulties in hand-coring cherty, gravelly sediments at the study site led to the development of sediment chambers (Hendricks, Halda-Alija, and White 1999; Hendricks and Rice 1999) to facilitate spatial and temporal sediment sampling from subsurface gravel bar and hyporheic habitats. Duplicate chambers were buried within the gravel bar and within the streambed to just below the top of the water table or to just below the streambed sediment-water interface, respectively. The distance between upwelling and downwelling site was 25 m. Locations of burial within the gravel bar were dictated by subsurface water flowpath from upstream to downstream and within the hyporheic zone by locations of groundwater discharge and recharge (Hendricks, Halda-Alija, and White 1999). Sediment chambers were filled with sediments (particle size (3 mm) "native" to the stream. Sediments were collected, sieved (Standard Sieve Series), autoclaved, placed in the chambers and buried within the gravel bar or streambed to colonize with biofilm for a minimum of 2 months prior to retrieval for microbiological analyses.

Sediment sampling protocol

Surface streambed sediment samples were collected to a depth of 10 cm over a period of two years. Samples were taken in January, April, August, and November of 1997 close to upwelling and downwelling sites. Additionally, surface streambed sediment samples were collected near where sediment chamber samples were buried in January, June and September of 1998. Surface water flow was temporarily disrupted by placing an open-ended barrel through the water column onto the sediment. At each sampling point, three sediment samples were collected using 50-ml disposable sterile polypropylene centrifuge tubes with plug seal caps (Fisher Scientific, Pittsburgh, PA) and stored on ice. Microbiological analysis were initiated within 24 h of sample collection. Methods of characterization and storage of microorganisms are described elsewhere (Halda-Alija and Johnston 1999). However, prior to removing sediments water samples were withdrawn from tubing embedded within each depth interval for dissolved inorganic nutrient, dissolved oxygen and dissolved organic carbon analyses using a sterile 60-ml syringe. For nutrient analyses, water samples were placed into sterile Whirl-pak bags, placed on ice and transported to the lab. Chemistry sample processing was initiated within 4 h of sampling. Water samples collected for DOC analysis were acidified to pH 2 in the field. Dissolved oxygen was determined by a modified Winkler method and reagents were added in the field. Water samples were filtered (Whatman GFF, 0.7 µm pore size) in the lab prior analyzed with a Lachat autoanalyzer (Lachat Instruments, 1995).

Viable Bacteria Counts and Characterization of Enterobacter Isolates

Heterotrophic aerobic bacteria were estimated on R2A media (Difco Inc., Detroit, MI) as previously described (Halda-Alija and Johnston 1999). *Enterobacter* isolates were selected on MacConkey agar (Difco Inc., Detroit, MI) based on colony pigmentation. In addition, colony morphology and cell morphology were determined. Selected organisms were also characterized with an API 20E test (Bio- Merieux, SA, Marcy l'Etoile, France) and fatty acid analysis. American Type Culture Collection cultures were included in tests: *Enterobacter cloacae* ATCC 13047 and *Enterobacter agglomerans* ATCC 27155. Isolates were tentatively identified to either genus or species level by comparing their phenotypic characteristics with those of American Type Culture Collection type cultures. *Enterobacter* isolates were assigned to bacterial species (Table 1) by comparing their restriction patterns with those of reference strains (Halda-Alija and Johnston, 1999).

Statistical Analysis.

Stastistical analyses were conducted using SYSTAT v. 7.2.1. software (SPSS Inc., Chicago, IL).

RESULTS

Samples from streambed sediments, gravel bars, and hyporheic zone gave rise to colonies showing different morphologies on MacConkey agar. A total of 145 colonies were obtained from streambed and at upwelling and downwelling zones in June 1998 (Table 1). The bacterial strains isolated on MacConkey agar were mostly assigned to *E. cloacae* and *E. agglomerans* by API 20E and an analysis of the restriction patterns produced by amplified DNA coding for 16S rRNA (ARDRA) (Halda-Alija and Johnston, 1999). Restriction analysis of the amplified DNA of each sample with the enzyme *Hpa* II enabled five ARDRA patterns to be recognized (Table 1). Among them, ARDRA patterns 1 and 4 were the same as those obtained with the 16S DNAs from reference strains *E. cloacae* ATCC 13047 and *E. agglomerans* ATCC 27155, respectively. ARDRA pattern 2 had one additional fragment (396 bp) when compared to *E. cloacae* ATCC 13047. Moreover, the results of API 20E showed that strains with ARDRA patterns 1, 2, and 3 had the characteristic metabolic activities of *E. cloacae* and ARDRA patterns 4 and 5 have the characteristic metabolic activities of *E. agglomerans*.

Spatial variation of *E. cloacae* and *E. agglomerans* ARDRA patterns was determined in June 1998 among streambed, upwelling and downwelling sites (Table 1). The total area analyzed was approximately 300 m^2. Occurrence of *Enterobacter* isolates was more frequent in upwelling zones compared to downwelling zones. No significant difference was found in the distribution of ARDRA patterns among streambed, upwelling, and downwelling zones, however, accept for ARDRA pattern 4. Significant difference for ARDRA pattern 4 was found between upwelling and downwelling zones.

Abundance of culturable enteric bacteria was higher at the agricultural stream (12.9% in upwelling zone), compared to the pristine stream (Table 2) where occurrence of enteric bacteria (as a percentage of total number of heterotrophic bacteria) did not exceed 4.6%. Highly similar *E. cloacae* and *E. agglomerans* strains were obtained from the pristine stream, Panther Creek, Tennessee located 40 miles apart.

Ledbetter Creek is an agricultural stream with relatively low nitrate input (data not presented here). This agricultural stream has high turbidity (14.1 mg/L) and high total suspended solids (14.1 mg/L). Anoxic zones occurred frequently within the agricultural gravel bar subsurface sediments and upwelling hyporheic zone (data not presented here). *Enterobacter* species are facultative anaerobes and relative predominance in subsurface gravel bar sediments and upwelling hyporheic zone may be attributed to occurrence of anoxic zones. Enteric bacteria obtained from Ledbetter Creek and enumerated on MacConkey agar were correlated with other subsurface physicochemical parameters (temperature and nutrient concentrations) (Table 3) along hydrological flowpaths

DISCUSSION

The aim of this study was to investigate different *Enterobacter* genotypes in order to assess the effects of farmland and rural development on the distribution along an agricultural stream. The differences between pristine and agricultural site may be attributed to higher ammonia loads, higher temperatures, and more frequent occurrence of anoxic zones at the Ledbetter Creek. However, enteric bacteria are of little numerical significance compared to *Pseudomonas fluorescens*, *Pseudomonas putida*, *Micrococcus*, *Alcaligenes*, *Bacillus*, and *Acinetobacter* in pristine (Halda-Alija and Johnston 1999) and agricultural stream (data not presented here). Additionally, population sizes of *Pseudomonas fluorescens*, *Pseudomonas putida*, *Micrococcus*, *Alcaligenes*, *Bacillus*, and *Acinetobacter* did not reflect environmental differences among sites. Enteric bacteria showed significant differences between pristine and agricultural streams, which indicates that enteric bacteria may be used as environmental indicators.

ARDRA has been used to delineate population structure of enteric bacteria; two different genotypes were recognized within *E. cloacae* and three different genotypes were recognized within E. *agglomerans*. ARDRA is suitable in dividing single species into different genotypes (Ingianni et al. 1997). The information on the occurrence and distribution of *E. cloacae* and *E. agglomerans* genotypes would be virtually impossible to obtain without the application of molecular biological

techniques. This is the first study, which correlates genotypes of each studied species with the specific, distinct spatial location in stream sediments. Our study demonstrated the persistence of *E. cloacae* and *E. agglomerans* genotypes in the stream ecosystem for the period of 4 months (June-September 1998). Enteric bacteria harbor genetic diversity, but this diversity is organized into a limited number of genetically distinct clones (Whittam et al. 1983).

The effect of environmental disturbances on the distribution and movement of *Enterobacter* genotypes was studied in aquatic environment. Studies that have avoided the species problem by determining ecological function have been moderately successful. In the present study, we correlated the occurrence and relative distribution of E. *cloacae* and *E. agglomerans* genotypes with physico-chemical characteristics of the stream ecosystem. Spatial heterogeneity of streambed sediments affected the distribution of *Enterobacter* species in this agricultural stream. The sediment microbial colonization chambers allowed a consistent and representative sampling of hyporheic and gravel bar sediments and appear to be reasonable devices for examining water chemistry and distribution and occurrence of bacterial isolates (Halda-Alija, Hendricks, and Johnston 2000), microbial activities, and biogeochemical transformations within the hyporheic zone beneath streambeds or at the groundwater-surface water interface adjacent to streams (Hendricks and Rice 2000). *E. cloacae* and *E. agglomerans* were heterogeneously distributed along the stream. Heterogeneous distribution of microbial biomass and its activities has been observed previously in soil. Environmental factors, such as temperature, predation, occurrence of anaerobic zones, and organic loading, can affect the composition of a microbial community and the structure of bacterial populations (Lee and Fuhrman 1991). Occurrence of anoxic zones, temperature and nutrients affected the distribution of enteric bacteria at Ledbetter Creek. In conclusion, other factors (e.g. temperature, occurrence of anaerobic zones), besides pollution and agricultural run-off may influence the distribution and occurrence of environmental bioindicators.

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Table 1. Distribution of *E. cloacae* and *E. agglomerans* genotypes in streambed sediments and hyporheic zone at Ledbetter Creek

		No. of isolates		
Strain	ARDRA patterns[a]	**Stream bed**	**Upwelling**	**Downwelling**
E. cloacae *LC H1*	1	12	8	6
E. cloacae LC 10	2	9	12	10
E. cloacae LC 33	3	6	10	12
E. agglomerans LC H2	4	11	11	3
E. agglomerans LC 13	5	6	15	14
Total		44	56	45

a Restriction patterns of amplified 16S rDNA genes digested with *Hpa* II.

Table 2. Occurrence of enteric bacteria, *Pseudomonas* sp., and *Bacillus* sp. in different environments as the percentage of the total number of isolates

Environment	Genus		
	Enterobacter	Pseudomonas	Bacillus
Winter			
Pristine stream	-	32.7	9.6
Agricultural stream	3.7	29.8	10.2
Rhizosphere (*Zea mays*)	-	-	-
Rhizosphere (*Juncus effusus*)	5.2	10.5	19.7
Summer			
Pristine stream	4.6	23.8	14.9
Agricultural stream	11.35	25.1	14.3
Rhizosphere (*Zea mays*)	15.3	24.7	15.6
Rhizosphere (*Juncus effusus*)	-	-	-

Table 3. Influence of physicochemical parameters on the occurrence of enteric bacteria. Significance levels at which the null hypothesis was rejected are given as * = $p < 0.05$, n.s.= not significant

Parameter	Occurrence of enteric bacteria in winter 1998	Occurrence of enteric bacteria in summer 1998	Combined summer and winter 1998
NH_4	n.s.	*	*
NO_3	*	*	*
SRP	n.s.	*	*
DOC	n.s.	*	*
DO	n.s.	*	*
AFDM	n.s.	*	*
T	n.s.	*	*