Short Communication

Comparison of Fecal Indicator Bacterial Populations in Surface Waters of the Kalamazoo River, USA

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Surface waters along the Kalamazoo River, USA, were examined for occurrence and population trends of fecal indicator bacteria (FIB) with culture-based and culture-independent methods. The two methods recorded discrepancies in FIB counts, with the culture-independent method revealing more consistent numbers between the river sites. FIB cells that hybridized with the ECO1482 probe were highest in the downstream site, while the upstream site recorded higher ENF343 hybridized cells. Spatial and temporal differences in FIB populations were probably attributable to contrasting fecal pollution influences, vegetation type, varying environmental conditions as well as several in-stream factors between the two river sites.

Key words: fecal indicator bacteria, *in situ* hybridization, viable counts, river

Fecal indicator bacteria (FIB) are generally found and presumed to be released mainly from the gastrointestinal tracts of humans and animals into natural aquatic systems (e.g., 21, 31). Although FIB presence (particularly those of *Escherichia coli* and *Enterococcus faecalis*) is conventionally considered solely an indication of extraneous fecal contamination of natural waters, however they have been observed autochthonously in a variety of environments, including freshwater systems (9, 13), beach sand and sediments (33), and algal mats (23, 32), persisting and multiplying while in prolonged residence (8). In particular, cells of *E. faecalis* generally originate from human and birds, rarely from other animals' fecal materials (30) and known to survive longer in aquatic environments relative to *E. coli* cells (6), yet very little is known about *E. faecalis* ecology in natural systems. Therefore, given the ecological and increasing public health importance of FIB populations in the environment (17, 25, 29), we decided to conduct a temporal and spatial study along the reaches of the Kalamazoo River, located in an extensive watershed in the southwestern portions of the Lower Peninsula of Michigan, USA, to further enhance general knowledge regarding factors that may potentially influence the occurrence and persistence of these presumably allochthonous group of microorganisms in freshwater systems.

FIB detection and enumeration were determined with a combination of culture-dependent (viable plate counts) and culture-independent approaches (i.e., direct counts using 4',6-diamidino-2-phenylindole [DAPI] staining and fluorescent *in situ* hybridization [FISH]). Traditional counts methods are particularly advantageous for FIB detection, since they are relatively quick and inexpensive, with smaller optimal conditions than are typically required for most bacterial

assemblages in nature. However, they are comparatively inadequate for bacterial quantification, since natural bacterial populations are often difficult to grow with only a small fraction of natural assemblages actually cultivable (2, 12). In contrast, FISH since its advent as a powerful molecular tool, has been effectively utilized for the enumeration of individual bacterial cells in complex environments (2, 15, 34), and even though relatively fast and effective, some major problems have been often associated with its optimization and application (14, 28).

Triplicate water samples were collected weekly from the air-water interface (~0.2 m below the surface) along the river edges in sterile 250 mL polypropylene bottles, during spring run-off through midsummer (May 15th to July 24th 2007) from two sites along the north branch of the Kalamazoo River (42°12'55.152N, -84°48'24.908W, Fig. S1). The first site located upstream of the city's conventional sewage treatment plant had a characteristic open channel, dominated by short grasses that were covered by copious animal (gulls and geese) fecal droppings. The second site downstream (canopied by willow, cottonwood, silver maple, and ash trees along the river bank), represented a location mostly affected by direct effluent discharges from the sewage treatment plant. During sampling, water characteristics were measured using the YSI model 556 MPS multi-probe system (YSI, Concord, CA, USA) and presence of anions quantified by Ion chromatography using conductivity detectors (Dow Analytical Laboratory, Albion, MI, USA). Subsamples of collected water were preserved in 8% (w/v) paraformaldehyde and 1×phosphate-buffered saline solution for DAPI staining (24) and FISH analyses as previously described (15, 23), using oligonucleotide probes EUB338, ENF342, and ECO1482 to target the Domain *Bacteria* (1), *E. faecalis* (7) and *E. coli* (15), respectively. Membrane filtration technique was employed to determine total and fecal coliform abundance with m-Endo and mFC agar plates (3). Viable *E. coli*

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and *E. faecalis* numbers were also determined on modified mTEC and ME-EIA agar plates, respectively (USEPA, EPA-600/4-85/076 [1985], EPA 821-R-02-021 [2002]).

All statistical analyses including student *t*-tests, ANOVA and multiple range test (Student Newman Keuls test) were performed with SPSS for windows (version 15, SPSS, Chicago, IL, USA). Relationships between FIB populations and environmental variables were examined using linear regression and Pearson correlation analysis. Bacterial numbers were log-transformed before performing statistical analyses. Statistical significance was set at $p \leq 0.05$.

Overall, differences were observed in the mean values of most of the environmental variables between the two river sites. ANOVA analysis showed differences in the mean values of water pH ($F=66.28$, $df=1$, $p<0.0001$), dissolved oxygen ($F=20.24$, $df=1$, $p<0.0001$) and conductivity ($F=205.28$, $df=1$, $p<0.0001$) between the two sites, however, no significant differences were found in the mean water temperature values between sampling dates ($F=0.26$, $df=1$, $p=0.610$). Significant differences were also detected in the concentrations of chloride ($F=49.1$, $df=1$, $p<0.0001$), fluoride ($F=44.0$, $df=1$, $p<0.0001$), sulfate ($F=8.47$, $df=1$, $p=0.006$), and nitrate between the sites ($F=175.37$, $df=1$, $p<0.0001$). The date by site interactions for fluoride, nitrate and sulfate were however not significantly different, an indication that the concentrations of these anions were stable between the sites over the sampling period. Significant differences were recorded in the oxidation-reduction potential (ORP) between the two sites ($F=19.64$, $df=1$, $p<0.0001$), and among sampling dates ($F=38.39$, $df=10$, $p<0.0001$).

Viable counts revealed total bacterial numbers to be similar between the sites ($F=0.15$, $df=1$, $p=0.700$), with mean values of 8.8×10^5 CFU mL⁻¹ and 9.5×10^5 CFU mL⁻¹ in the upstream and downstream sites, respectively. Also similar were counts of total coliform between the sites ($F=0.008$, $df=1$, $p=0.93$), with numbers in the upstream site showing strong correlations with water conductivity ($R=0.7$). In contrast, we observed fecal coliform to be significantly different between the sites ($F=16.62$, $df=1$, $p<0.0001$), with more cells detected on average in the downstream site (7.1×10^3 CFU 100 mL⁻¹) than in the upstream site (2.9×10^3 CFU 100 mL⁻¹), also correlating negatively with dissolved oxygen ($R=-0.5$) at this particular site, but not at the downstream site of the river. When *E. coli* CFU numbers were examined, counts were similar between the two sites ($F=2.05$, $df=1$, $p=0.157$), with an average counts of 0.9×10^4 CFU 100 mL⁻¹ and 1.2×10^4 CFU 100 mL⁻¹ in the upstream and downstream sites, respectively. In the upstream site, *E. coli* CFU counts correlated with both water conductivity and ORP ($R=0.5$, respectively). *E. coli* CFU counts accounted for between 0.1% and 69.3% of total CFU counts in the upstream site and between 0.04% and 101.3% in the downstream site. In contrast, viable *E. faecalis* counts (when detected) were significantly higher in the downstream site than in the upstream site ($F=34.66$, $df=1$, $p<0.0001$) and also varied significantly among sampling dates ($F=94.46$, $p<0.0001$). For the most part, *E. faecalis* ranged from below detection levels to about 1.0% and 0.8% of total viable counts in the upstream and downstream sites, respectively.

Total bacterial numbers (enumerated by DAPI staining

direct count method) were similar between the two river sites ($F=1.84$, $df=1$, $p=0.18$) and sampling dates ($F=1.20$, $df=10$, $p=0.320$). Whereas number of cells that hybridized with the Domain *Bacteria* probe (enumerated by FISH) differed significantly between the sites ($F=16.54$, $df=1$, $p=0.034$) but not among sampling dates ($F=1.69$, $df=10$, $p=0.113$) as well as the date by site interaction ($F=1.12$, $p=0.368$). Overall, the abundance of Domain *Bacteria* represented from between 18.7% to 64.7% in the upstream and 8.7% to 35.1% in the downstream sites of total counts (*i.e.* DAPI-stained cells). The numbers of *E. coli* cells enumerated with the ECO1482 probe were significantly higher in the downstream than the upstream site (Fig. 1A; $F=46.22$, $df=1$, $p<0.00001$) and among sampling dates ($F=2.82$, $df=10$, $p=0.009$). Generally, bacterial cells that hybridized with the ECO1482 probe ranged from 1.5% to 12.8% in the upstream site and between 4.1% and 12.2% and downstream sites of total cells (DAPI-stained cells). In contrast to results observed with ECO1482 hybridized cells between the sites, the abundance of cells that hybridized with the ENF343 probe was higher in the upstream site than the downstream site (Fig. 1B; $F=7.89$, $df=1$, $p=0.007$), but the differences between sampling dates were found not to be significant ($F=1.73$, $df=10$, $p=0.103$). Cells that hybridized with the ENF343 probe ranged from 0.6% to 3.9% and 1.1% to 2.3% of total direct counts in both the upstream and downstream sites, respectively.

Generally, the FIB counts (with the exception of *E. faecalis* cells) were persistently high and comparable to those typically observed in similar aquatic environments (9, 23, 30). However, the two enumeration methods revealed contrasting results, especially in the detection of *E. faecalis* cells at the two river sites. An indication that cells of *E. faecalis* probably entered the viable but non-culturable (VBNC) state as often observed while under starvation in oligotrophic environments (20). Also, the low fecal coliform numbers relative to counts of *E. coli* might also be attributable to the inefficiency of the traditional methods in detecting and accurately quantifying the cells in environmental samples. Our results revealed clear differences in FIB spatial distribution between the two river sites. In particular, ECO1482 hybridized cells were relatively higher at the downstream than the upstream site, contrary to the distribution of the cells that hybridized with the *E. faecalis* probe. This appeared to be a strong indication that while *E. coli* influxes are probably associated with inputs of waste water effluents at this downstream site, the high *E. faecalis* recorded at the upstream site might be linked to animal fecal droppings washed with direct runoff from the adjacent watershed as observed previously (19). More importantly, contributions by several site-specific factors including stream sediment type (4, 9–11, 22, 27) as well as riparian vegetation (23, 32, 33) could have also influenced the persistence observed in the FIB populations between the sites. Specifically, it has been shown that stream sediments and riparian vegetation can serve as sources of nutrients to and significant repositories and reservoirs of FIB cells (4, 9, 10, 12, 22), from where they potentially multiply while in prolonged residence (8) and gradually released with erosional events into river reaches (27).

Most of the FIB populations were observed to be persistently high throughout the period of study at the two sites,

Fig. 1. Temporal changes in (A) *Escherichia coli* numbers enumerated with ECO1482 FISH probe and (B) *Enterococcus faecalis* numbers enumerated with ENF342 FISH probe at the (◆) upstream and (■) downstream sites. Values are means ($\pm$ SE) of triplicate samples.

with both *E. coli* and *E. faecalis* cells hybridized by FISH accounting for approximately 12% and 4% of total direct cell counts, respectively. These trends somewhat contradicts earlier reports regarding FIB survival and persistence rates, which have typically shown the opposite, often associating higher persistence of *E. faecalis* than *E. coli* cells to faster protozoa predation of the latter in aquatic systems (7, 10, 17, 18). We consider other important factors that co-vary with river hydrodynamics such as river discharge rate and changes in several environmental variables to have also accounted for FIB abundance in these sites (5, 16, 26). Although, water temperature appeared not to be a strong influencing factor in our study; however, it is generally considered one of the environmental factors that most negatively affect FIB survival in aquatic systems (6). The direct impact of precipitation during sampling on influxes of extraneous FIB cells with animal fecal materials (most especially of *E. faecalis* cells at the upstream site) as well as release of sequestered bacterial cells and organic matter from sediment matrixes and plant biofilms cannot be over-emphasized along the river channels (10, 32, 33).

In conclusion, our results corroborate similar studies that have previously observed temporal and spatial differences in FIB distribution in freshwater systems (e.g., 8, 16), but most importantly further reveal the preference for culture-independent enumeration approaches in accurately delineating FIB

ecology and population dynamics in their response to constant anthropogenic disturbances in aquatic environments.

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