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RESEARCH ARTICLE

The effect of *Paenibacillus* on IDEXX Enterolert results from freshwater stream environments

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Abstract

Enterolert, a fluorogenic substrate test, is used as a quantitative method for determining freshwater concentrations of *Enterococcus* for water quality indicators. However, there is some evidence from recent studies suggesting that Enterolert may not suppress false positives due to pollution sources in waterbodies. In this study, we evaluated this method by analyzing field water and sediment samples from four freshwater streams. We also performed a laboratory microcosm study from two of the stream sediments. The Enterolert method was investigated by phenotypic and genomic analyses for accuracy of isolating and quantifying *Enterococcus* and/or *Streptococcus*. Additionally, we tested isolates from Enterolert panels for antibiotic resistance. Results from the field and microcosm studies from initial to final time points indicated that false positives were predominantly *Paenibacillu*s spp. and other non-fecal indicator bacteria. Furthermore, the microcosm study indicated shifts from lactic acid to non-lactic acid bacteria between initial to final time points, but *Enterococcus* concentrations from Enterolert panels remained stable for the duration of the study for both stream sediments. Antibiotic resistance indicated no distinct pattern of resistance or susceptibility to a suite of antibiotics. However, all isolates tested were resistant to bacitracin and nalidixic acid. In conclusion, we found that Enterolert was not exclusively selective for *Enterococcus* from freshwater environments and that sediment and polluted waterbodies have the potential to skew the presumed concentrations. More research is needed to evaluate the effectiveness and selectivity of the medium used for the fluorogenic substrate test for *Enterococcus* enumeration.

Keywords Enterolert · *Enterococcus* · Freshwater · Stream · Sediment · *Paenibacillus*

Introduction

Fecal indicator bacteria (FIB) are important determinants of recreational water quality in freshwater streams and rivers. *Enterococcus* and *Escherichia coli* are the most used FIB as they are generally found in enteric environments and can indicate the presence of fecal contamination of waterbodies. While *E. coli* is a primary indicator species for freshwater, *Enterococcus* or enterococci have been used as a primary indicator for marine and freshwater environments to predict gastrointestinal diseases in humans from direct contact with contaminated water at certain density thresholds (USEPA 1986; USEPA 2012). Conventional methods used to determine *Enterococcus* concentrations in water were membrane filtration or multiple-tube fermentation prior to 1996 (Koide et al. 2007). As the importance of water quality testing for FIB increased, a new technique was developed to provide a standard, easy-to-use method for investigators to evaluate recreational

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waters (Budnick et al. 1996). A fluorogenic enzyme test, Enterolert, was developed to enumerate FIB in water samples using a most probable number method based on fluorescence of a matrix, or defined substrate, in a multi-well tray that inhibits non-enterococci species from fluorescing and promotes esculinase production by enterococci (Baird and Bridgewater 2017; Chen et al. 1996).

Results from initial trials by Chen et al. (1996) showed Enterolert effective for selecting *Enterococcus* spp., and Budnick et al. (1996) and Noble et al. (2010) indicated that Enterolert performed similarly to membrane filter and multiple tube fermentation techniques. Following initial studies and subsequent successful use by various entities, the method was formally adopted for use by the United States Environmental Protection Agency (USEPA) in 2003 (USEPA 2003). While regulatory entities have widely accepted this USEPA-approved method, early evidence from Kinzelman et al. (2003) found a low correlation between membrane filtration and the fluorogenic methods, and others expressed that more research was needed to determine its appropriateness for freshwater regulatory testing (Ferguson et al. 2013). Recent evidence from Peperzak and van Bleijswijk (2021) suggested that the fluorogenic method (Enterolert) has a potential for false positives in marine environments, caused predominantly by *Bacillus licheniformis*.

Limited research has been conducted to determine if the fluorogenic substrate method accurately quantifies *Enterococcus* indicators and suppresses non-*Enterococcus* species, in freshwater samples. Research for method development was from a series of urban runoff and beach samples. Ferguson et al. (2013) showed that species selectivity for the two fecal-associated *Enterococcus* species, *E. faecium* and *E. faecalis*, was lower than that of the marine environment water samples and had a higher percentage of plant associated species. Furthermore, recent studies indicate that *Enterococcus* sources may be non-enteric and in natural aquatic environments such as sediment and decayed organic matter, plants, soils, and animals (Boehm and Sassoubre 2014; Byappanahalli et al. 2012). Badgley et al. (2010) and Devane et al. (2020) revealed that reservoirs of *Enterococcus* and other pathogens of interest exist within the stream water column, sediment, and aquatic vegetation. Additionally, *Enterococcus* populations in the environment have been shown to vary in species distributions and antimicrobial resistance depending on the specific freshwater conditions and seasons (Alm et al. 2014; Cho et al. 2020; Lupo et al. 2012). For example, varying land use types and associated runoff or stream inputs (e.g., sediment, wastewater, and animal fecal material) can influence the abundance and species composition of *Enterococcus* species (Ran et al. 2013; Sidhu et al. 2014). Furthermore, others have indicated that environmental factors such as warmer temperatures and increased precipitation can impact the frequency of fecal origin *Enterococcus* species due to varying contamination sources and favorable temperature growth ranges (Cho et al. 2020).

More evidence is needed to understand how *Enterococcus* persists in freshwater environments, and how population shifts and method interferences could potentially alter our understanding of monitoring streams and rivers for recreational water quality. In this paper, we describe results from a study to investigate freshwater streams and lakes in Oklahoma for *Enterococcus* population trends in water and sediment, assess antimicrobial resistance of isolates from Enterolert panels, and identify isolates from positive Enterolert panel wells. The objectives of the study were to (1) evaluate *Enterococcus* species distributions in freshwater environments under stable (lab) and field (variable) conditions and (2) assess the fluorogenic substrate test method (IDEXX Enterolert) for selectivity of *Enterococcus* in freshwater samples.

Methods

Representative impaired waterbodies for *Enterococcus* on the 2020 USEPA 303(d) list that included varying stream size, geomorphology, and geographic locations were chosen for this study to limit bias of environmental conditions (ODEQ 2020). Field water and sediment samples were collected at four stream sites (Cat, Bird, Hogshooter, and Walnut Creek) at two time points: time 1 (week 1 [T_I]) in July to time final (week 10 [T_F]) in September. Samples were collected during a minimum of 5-day antecedent rainfall (< 0.254 mm) period. Water samples from field sites were collected in 120-mL sterile polystyrene bottles from the stream thalweg and waist-depth from lake shores using United States Geological Survey sampling procedures (USGS 2014). Positive control wastewater samples were collected from the City of Norman, OK, wastewater reclamation influent water. Water samples were cooled on ice to < 6 °C and processed within 24-h hold time using the fluorogenic substrate most probable number (MPN) method, as described in SM9230D (Baird and Bridgewater 2017). Sediment samples were collected from the stream thalweg in the upper 5 cm of the benthic substrate into sterile 290-mL polystyrene bottles as described in Graves et al. (2023). The volume of the resulting sediment sample bottles was comprised of roughly 100 mL of sediment and 150 mL of stream water. Immediately following collection, the samples were cooled and stored at < 6 °C. Sediment samples from Cat and Walnut Creek were used for the 31-day microcosm study. To establish a controlled and dark environment, the 290-mL polystyrene collection bottles had the lids loosely covered and the sides and bottoms tightly wrapped with aluminum foil. The sample containers were agitated at 100 revolutions per minute on a horizontal orbital shaker at constant room

temperature (22 °C) to promote aerobic interaction with the sediment as found in natural streambeds. Sample bottles were randomly selected in duplicate at day 1 (D_I) and day 31 (D_F) for analysis.

Sediment samples were processed using adapted soil microbial detachment and dispersal methods from Ogram et al. (2007). Tetrasodium pyrophosphate was added to sterile, reverse osmosis (RO) water and buffered to pH 7.0 to make a 2% sodium pyrophosphate solution. A sterile serological pipetter was used to decant water from the undisturbed sample bottle. The saturated sediment was dispersed by adding 200 mL of pyrophosphate solution, closing the bottle, and shaking manually for 2 min before 15 min of mixing at 200 revolutions per minute on a horizontal orbital shaker. The sediment and pyrophosphate sample mixtures were serially diluted (1:500, 1:1000, 1:10,000) with buffered (pH 7.0) sterile RO water and processed and analyzed as aqueous samples using SM9230D for *Enterococcus* (Baird and Bridgewater 2017). Similarly, we evaluated water samples using Enterolert from the same field locations to verify and compare them to the diluted sediment samples. Following completion of bacteria enumeration, IDEXX processed sample well trays were preserved at < 6 °C for short-term preservation (< 5 days) for Biolog processing and/or 16s sequencing. Representative streambed sediment was collected and analyzed to determine percent organic matter and particle size distribution using Loss on Ignition (LOI) methods and ASTM 6913 (Ball 1964).

Isolate analysis

Isolates were selected from a subset of the field and laboratory samples at initial and final times for identifying isolates in water and sediment through phenotypic and genomic analyses. These included Enterolert panels from field samples of the four stream sites and the two microcosm sets as identified above. Furthermore, isolates from all of the Enterolert panels were screened to determine phenotypic traits to characterize the enumerated bacteria densities. Isolates from Enterolert panels at T_I and T_F for the field study and at D_I and D_F for the microcosm study were used to determine the predominant species for each time point and location. To validate methodologies between sediment and water samples, additional isolates from water samples from the field study week 10 samples were analyzed. Additionally, isolates of *Enterococcus faecalis* were obtained from the raw wastewater samples, and *Paenibacillus licheniformis* was isolated from selective screening methods for positive controls using the lactic minimal medium (LMM) agar method as described below.

Biolog phenotypic methods were used to identify isolates from Enterolert panels. Enterolert panels were sprayed with sterile 70% isopropyl alcohol (CiDehol 70, Decon Labs) and allowed to air dry at room temperature. Ten positive wells from each panel were sampled using BD allergy syringes (# 305541), and 5 µL was streaked onto plates of LMM agar. Plates were incubated at 37 °C. Dominant colony types were plated further until pure cultures were obtained. Most Enterolert panels sampled and cultured had a single colony type. Cultures were screened for Gram reaction (Hardy Diagnostics kit GK400A), cell morphology (phase microscopy), motility, and catalase reaction. If Enterolert wells contained more than one colony type, they were scored positive as a fecal indicator bacteria (FIB) if at least one was identified as an FIB.

LMM agar was adapted from LMM medium described by Ralph S. Wolfe, which was used in microbial diversity laboratories at the universities of Illinois, Massachusetts, Oklahoma, and elsewhere since the 1960s (Tanner 2007). The recipe for the LMM agar is (/L)10 mL mineral solution; 10 mL vitamin solution; 1 mL trace metal solution; 0.5 g K_2HPO_4; 8 g yeast extract (BD 212750 = Gibco 212750); 10 g glucose; 15 g purified agar (Oxoid LP0028). Precipitated chalk (2 g/L; $CaCO_3$) was added for the detection of acid-producing colonies. LMM agar could support the growth of isolates that may not grow on TSA or BUG-blood agar (data not shown).

Isolates at initial and final times of the studies were identified using Biolog's GEN III system (Sandle et al. 2013), and microplates were scored by eye. The identity of approximately 20% of isolates was confirmed by 16S rDNA sequence analysis. Genomic DNA were extracted with the Promega Wizard® Genomic DNA purification kit according to the manufacturer's instructors for Gram-positive bacteria (Promega Corp. Madison, WI) and quantified with the Qubit dsDNA broad range assay (ThermoFisher Scientific, Waltham, MA, USA). The 16S rRNA gene was amplified by PCR with primers FD1 and 1492R and with Taq DNA polymerase and ThermoPol® buffer (New England BioLabs, Inc., Ipswich, MA) (Elnahas et al. 2017; Turner et al. 1999; Weisburg et al. 1991).

Antibiotic resistance

Water samples were collected from two reservoirs (Mountain Lake and Lake Thunderbird) and four stream locations (Bluff, Crooked Oak, Washington, and West Elm Creek) for antibiotic resistance and susceptibility screening. Samples were collected from these additional locations to further evaluate and understand potential differences between water types and various expected levels of pollution. Isolates from field water sample Enterolert panel wells (as described earlier in the "Methods" section) were evaluated for antibiotic susceptibility using the method as in Bauer et al. (1966), often referred as the Kirby-Bauer antibiotic disc assay. A total of 35 isolates were screened for antibiotic resistance

or sensitivity. The antibiotics tested were ampicillin (AM), 10 µg; bacitracin (B), 0.04 units; carbenicillin (CB), 100 µg; cefoxitin (FOX), 30 µg; doxycycline (D), 30 µg; erythromycin (E), 15 µg; gentamicin (GM), 10 µg; nalidixic acid (NA), 30 µg; sulfathiazole (ST), 250 µg; tetracycline (TE), 30 µg; trimethoprim (TMP), 5 µg; and vancomycin (A), 30 µg.

Data analysis

Results from the Biolog analysis were analyzed by descriptive statistics to determine metrics such as percent false positives and percent FIB. Percent-positive fecal indicator bacteria (FIB_p) was determined by the total number of isolates from Enterolert wells identified as belonging to the 19 spp. of *Enterococcus* and *Streptococcus* listed in Table 9230:1 of *9230 Fecal Enterococcus/Streptococcus Groups* in Baird and Bridgewater (2017), divided by the total number of isolates for each location/time point. Total isolates were all isolates recovered from ten positive (fluorescing) Enterolert panel windows from each individual sample. A Fisher's exact test conducted in R was used to evaluate significance of FIB percentages, and any population shifts between initial and final time points of the study (Fisher 1934; R Core Team 2013). Species identified were compared and evaluated as a fecal or non-fecal *Enterococcus* source and screened by morphological characteristics for Enterolert accuracy where false positives are indicated. All data analyses were performed in R and Excel.

Results and discussion

Enterolert MPN summary

Enterococcus water concentrations from the 10-week field study from Cat, Bird, Hogshooter, and Walnut Creek water samples ranged from 171 to 1040 MPN (Most Probable Number) 100 mL^{-1} with an average of 457 MPN 100 mL^{-1} and 101 to 1300 MPN 100 mL^{-1} with an average of 499 MPN 100 mL^{-1} for July (T_I) and September (T_F), respectively. The sediment *Enterococcus* concentrations from the field ranged from 3.9 to 490 MPN per gram and an average of 224 MPN per gram for T_I and ranged from 4.6 to 36 MPN per gram with an average of 19 MPN per gram for T_F samples.

The sediment *Enterococcus* concentrations from the laboratory microcosm experiment at day 1 (D_1) were 1300 MPN per gram and 5.4 MPN per gram, and at day 31 (D_F), concentrations were 500 MPN per gram and 5.6 MPN per gram for Cat and Walnut, respectively.

The pH and water temperature were recorded for all field samples between time points with an average pH of 8.0 and 7.9 and average water temperature of 24.0 and 24.9 °C at T_I and T_F, respectively. Furthermore, pH was measured in the microcosm samples and was 7.7 and 7.4 for Cat and 8.3 and 8.5 for Walnut at T_I and T_F, respectively.

Enterolert panel isolates

Sediment samples were analyzed by phenotypic methods to identify isolates from Enterolert panels on D_1 and D_F of the microcosm sampling period. The Walnut and Cat Creek microcosm results showed that while sediment *Enterococcus* counts remained stable for the 31-day period, the FIB_P percentage decreased from 82 to 0% for Walnut. Cat Creek decreased from 9 to 0% FIB_P for Cat Creek between D_1 and D_F with Enterolert sediment concentrations of 1320 and 500 MPN per gram, respectively. A Fisher's test was performed on both microcosms, and significant differences between time points were shown for Walnut ($p = 0.01$) and no significant difference ($p > 0.05$) for Cat. Results show that while the panels indicated positive for a FIB, false positives were indicated for 100% of the samples after 31 days with the majority of the isolates (65%) identified by Biolog as *Paenibacillus* spp. Furthermore, D_1 isolates from the Cat samples resulted in 63% of isolates identified as *Paenibacillus* spp.

Even though the *Enterococcus* numbers, as determined by Enterolert panels, in Walnut and Cat Creek were stable over the 31-day microcosm period, the Enterolert-positive population quickly shifted away from lactic acid bacteria (LAB) to the non-lactic rods, false positives. Specifically for Walnut Creek, the FIB_P isolates as indicated by Gram-positive, catalase-negative morphologies went from 82% of total isolates on D_I to 63% on day 2, then averaged 14% (SD ± 5%) for days 5 through D_F (Table 1). Most species identifications were found using Biolog, and some were confirmed by 16S partial sequence analysis on D_I and D_F. All other LAB isolates from this study were catalase-negative, Gram-positive, nonmotile cocci in pairs and short chains, and the unidentified non-LAB were catalase-positive, Gram-positive, motile rods. These were similar to *Paenibacillus* spp. isolates, especially as they all gave unusual reactions on Biolog plates: all false positives, including the negative control well, using protocol A; a yellow color instead of the usual purple color, due to further reduction of the indicator, in the positive control well using protocol B.

The LAB from the D_I samples from Walnut microcosm sediment were presumptive enterococcal FIB. Predominant isolates from Walnut were identified as *Enterococcus mundtii*, usually associated with plants but also isolated from a wide variety of other sources (Švec and Franz 2014). Conversely, Cat isolates on D_I were primarily *Paenibacillus* species that are not commonly associated with a human health risk (Grady et al. 2016). D_F isolates for both Cat and Walnut resulted in a 100% decrease in FIB_P isolates with the predominate isolates being *Paenibacillus* and/or unidentified

Table 1 Summary of isolates from Enterolert panels analyzed from the Walnut Creek microcosm stream sediment for a 31-day study duration. *FIB*, fecal indicator bacteria. FIB percent (%) total is calculated as the number of FIB isolates divided by the total isolates

Day	Total isolates	FIB isolates	FIB % total	*Enterococcus* sediment (MPN g^{-1})
1	11	9	82%[1]	5
2	8	5	63%	17
5	9	2	22%	9
10	10	2	20%	6
17	12	1	8%	5
24	11	1	9%	4
31	11	0	0%[1]	6

[1]FIB isolates were confirmed by Biolog and/or 16s rDNA sequencing from day 1 and day 31

non-LAB, which were Gram-positive, catalase-positive, motile rods (Table 2).

Furthermore, field sediment samples from four streams (creeks) were analyzed by phenotypic methods using an identical approach from the microcosm study to identify isolates from Enterolert panels during a 10-week period. No discernable trend was observed between the July (T_I) and September (T_F) time periods for Enterolert panels from sediment concentrations in field samples (Table 3). A higher average percentage of FIB_P isolates were found in the sediment samples at T_F (50%) compared to T_I (27%). Water samples analyzed from T_F sampling time points resulted in an average of 71% FIB_P isolates. Results from the Fisher's exact test indicated that no significant difference ($p = 0.13$) was shown between T_I and T_F for all locations and isolates. Individual sites were evaluated, similarly, and no significant differences ($p > 0.05$) were shown indicating that no change in population was found by chance.

However, when evaluating percent differences between T_I and T_F, the average FIB_P was 27% and 50%, respectively, indicating a quantitative shift increase of fecal indicators between time points. FIB_P isolates for all locations averaged 32% (SD ± 39%) for T_I and 27% (SD ± 8%) T_F and an average of 69% (SD ± 29%) for all time points. The results indicate that species in the streams are dynamic and that shifts in FIB occurred frequently during the 10-week sampling period for all locations. For example, at Bird Creek, isolates from Enterolert panels processed from stream sediment samples were identified as 10%, 30%, 90%, 90%, 50%, 60%, 100%, 80%, 10%, and 30% FIB_P for consecutive weeks 1 through 10, respectively, with no apparent predictable pattern. *Paenibacillus* spp. were the predominant false-positive species identified in 25% of all isolates identified from field sediment and water sample Enterolert panels.

A high percentage (> 90%) of the non-FIB from sediment windows from the microcosm and field samples (113 of 531) were isolates of *Paenibacillus*, including *P. apiarus*, *P. borealis*, *P. dendritiformis*, *P. graminis*, *P. thiaminolyticus*, and *P. woosongensis*. Other species recovered from sediment windows were *Aeromonas veronii*, *Burkholderia multivorans*, *Carnobacterium divergens* (a LAB), *Gemella palaticanis*, and *Streptococcus acidominimus* (a LAB). Thirteen of 119 windows from aqueous stream sample Enterolert panel windows were non-FIB, but these were a diverse group of bacteria including *Carnobacterium gallinarium* (a LAB), *Cellulomonas hominis*, *Chryseobacterium humi*, *Enterococcus canintestini* (a LAB), *Lactococcus garviae* (a LAB), *Jonesia denitrificans*, *Ochrobactrum intermedium*, *Paenibacillis sanguinis*, *P. thinaminolyticus*, and *Proteus mirabilis* (Tables 2 and 4). The resulting identifications

Table 2 Isolate species identifications from Enterolert panels from two microcosms at two time periods, D_I and D_F, from two stream sediment sources. Ten windows from each Enterolert panel for each site and time period were sampled for identification tests. The number of multiple identifications (if any) from each sample location and time are denoted by parentheses next to the binomial nomenclature

Location	Sample	Day 1 (D_I) Identification	Day 31 (D_F) Identification
Cat Microcosm	Sediment	*Enterococcus faecium* (1)* *Paenibacillus sp.* (2) *P. dendritiformis* (3) *P. thiaminolyticus* (2) *Gemella* sp. (1) *G. palanticanis* (1) Unidentified non-LAB (1)	*Paenibacillus sp.* (2) *P. dendritiformis* (5) Unidentified non-LAB (5)
Walnut Microcosm	Sediment	*E. faecalis* (1)* *E. faecium* (1)* *E. mundtii* (6)* *E. gallinarium* (1)* *Paenibacillus borealis* (1) Unidentified non-LAB (1)	*Carnobacterium divergens* (1) *Paenibacillus* sp. (2) *P. dendritiformis* (3) *P. thiaminolyticus* (1) *Staphylococcus epidermis* (1) Unidentified non-LAB (4)

*Fecal indicator bacteria species that are listed as enterococci or streptococci fecal indicators in Baird and Bridgewater (2017)

Table 3 Summary of isolates from Enterolert panels at four stream sampling locations at two time periods, T_I and T_F. *FIB*, fecal indicator bacteria. FIB percent (%) total is calculated as the number of FIB isolates divided by the total isolates

Site	Total isolates	FIB isolates	FIB % total	*Enterococcus* concentration[1,2]
July (T_I)—sediment				
Bird	14	0	0%	490
Cat	10	2	20%	410
Hogshooter	9	1	11%	40
Walnut	9	7	78%	3.9
September (T_F)—sediment				
Bird	10	2	20%	4.6
Cat	11	5	45%	36
Hogshooter	9	6	67%	23
Walnut	9	6	67%	13
September (T_F)—water				
Bird	10	9	90%	100
Cat	11	9	82%	450
Hogshooter	10	6	60%	440
Walnut	10	5	50%	140

[1] *Enterococcus* sediment units: MPN g^{-1}

[2] *Enterococcus* water units: MPN 100 mL^{-1}

indicate that Enterolert panels are not exclusive for selection of the targeted FIB for freshwater samples, and these isolates are potentially not considered a human health concern as many are of environmental origin (Devane et al. 2020). Furthermore, these isolates could create interferences and variability in most probable number calculations due to the high percentage of false positives and non-target selection characteristics of the medium.

Isolate identification showed that many isolates were not FIB and potentially were from other non-enteric sources. Many of the more recently added FIB from Baird and Bridgewater (2017) would be considered of animal origin (*E. hirae*, *E. columbae*, *E. cecorum*, *E. saccharolyticus*, and *E. asini*) or of environmental origin (*E. casseliflavus* and *E. mundtii*) rather than of human origin (Švec and Franz 2014). Additionally, the list of fecal enterococci/streptococci increased from four species in 1985 (Greenberg et al. 1985) to 18 species in 2017 (Baird and Bridgewater 2017); n.b., *Streptococcus bovis* is a synonym of *Streptococcus equinus* as shown in the List of Prokaryotic names with Standing in Nomenclature (Parte et al. 2020). However, limited information is available for the increase of fecal indicator species on this list. Many of these species that are not of animal origin are often associated in the same environmental conditions as fecal-origin bacteria except these LAB spp. identified may have been isolated from contaminated water samples (Korajkic et al. 2018). More research is needed to assess whether the fecal indicators added between 1985 to 2017 are of concern to human health in primary body contact recreation freshwaters and how to apply methodology for accurately identifying targeted species.

Enterolert false positives

The most widely used application of Enterolert is for routine bacterial enumeration of *Enterococcus* in non-potable and potable water sources. However, we also evaluated this method to evaluate the efficacy of Enterolert with introduction of sediment by using sediment dispersion methods, diluting the sediment sample, and processing them as water samples as described in "Methods" section. The fluorogenic substrate in Enterolert is 4-methyl-umbelliferyl-β-D-glucoside, an analog of esculin (James et al. 1997). The same β-D-glucosidase that cleaves esculin also cleaves this fluorogenic substrate, releasing the chromophore. The medium used for Enterolert may have an inducer of this esculin hydrolase, and any esculin hydrolysis-positive species could give a positive fluorescent result in an Enterolert window. The published false-positive species for Enterolert, *Bacillus licheniformis* (Peperzak and van Bleijswijk 2021), is esculin positive (Logan and De Vos 2009). Most spp. of *Paenibacillus* are esculin positive (Priest 2009).

The stream substrates of the two sample locations were analyzed for sediment properties with D_{50} particle sizes of 5.08 and 0.38 mm and percent organic matter of 1.2% and 0.4% for Cat and Walnut, respectively. With the difference in stream substrate types and organic matter, potential interferences in the Cat samples may be due to the organic matter percentage. Zimmer-Faust et al. (2017) and others have indicated that organic material may have a positive effect on the viability and diversity of bacterial communities within stream environments. Additionally, sediments are known to increase the persistence of *Enterococcus* in the water column

Table 4 Isolate species identifications from Enterolert panels from four field stream sample locations at two time periods, T_I and T_F. Ten windows from each Enterolert panel for each site and time period were sampled for identification tests. The number of multiple identifications (if any) from each sample location and time are denoted by parentheses next to the binomial nomenclature

Location	Sample	July (T_I) Identification	September (T_F) Identification
Bird	Sediment	*Paenibacillus apiarius* (10) *Aeromonas veronii* (1) *Aeromonas* sp. (1) *Bacillus* sp. (1) *Paenibacillus* sp. (1)	*Enterococcus mundtii* (3)* *Ochrobactrum* sp. (5) *Micrococcus* sp. (1) *Paenibacillus* sp. (1)
Cat	Sediment	*E. casseliflavus* (2)* *Ochrobactrum* sp. (4) *Paenibacillus* sp. (1) *P. thiaminolyticus* (2) *P. apiarius* (1)	*E. faecalis* (2)* *E. mundtii* (3)* *Carnobacterium divergens* (1) *Micrococcus* sp. (1) *P. thiaminolyticus* (2) Unidentified LAB (2)
Hogshooter	Sediment	*E. faecalis* (1)* *Burkholderia multivorans* (1) *Paenibacillus* sp. (1) *P. apiarius* (1) *P. thiaminolyticus* (5)	*E. faecalis* (6)* Unidentified LAB (3)
Walnut	Sediment	*E. casseliflavus* (4)* *E. mundtii* (3)* *Enterococcus* sp. (1) *P. thiaminolyticus* (1)	*E.faecalis* (3)* *E. mundtii* (3)* *Enterococcus* sp. (1) *P. sanguinis* (1) *P. thiaminolyticus* (1)
Bird	Water	-[1]	*Enterococcus mundtii* (9)* *Paenibacillus* sp. (1)
Cat	Water	-	*E. canintestini* (1)* *E. faecalis* (4)* *E. gallinarum* (3)* *E. mundtii* (3)*
Hogshooter	Water	-	*E. faecalis* (6)* Unidentified LAB (4)
Walnut	Water	-	*E. casseliflavus* (2)* *E. dispar* (1)* *E. faecium* (1)* *E. mundtii* (1)* *Cellulomonas hominis* (2) *Chryseobacterium humi* (1) *Kocuria* sp. (1) *Proteus mirabilis* (1)

[1]One time period was used (T_F) to compare water versus sediment samples

*Fecal indicator bacteria species that are listed as enterococci or streptococci fecal indicators in Baird and Bridgewater (2017)

and could potentially create interferences with the medium and isolation of targeted species in Enterolert panels (Graves et al. 2023; Haller et al. 2009). Typical freshwater stream environments often carry a sediment load such as the sediment samples in this study that we analyzed as water samples (Stocker et al. 2019). The water samples collected and analyzed were typically less turbid than the diluted sediment samples, which had a known sediment load introduced into each water sample and may have been a contributing factor that resulted in fewer false positives as shown by similar enumeration methods (USEPA 2000). The variability of FIB_P with no distinct pattern identified between sediment and water samples indicates that the addition of sediment seems to increase irregularities in positively selecting targeted indicator species.

Results from the microcosm study correspond with the field species identification where high percentages of false-positive indicators were present in positive Enterolert wells. We found that regardless of the FIB_P counts in all samples, false positives were consistent throughout all panels and had an abundance of non-FIB_P bacterium (both LAB and non-LAB) between each individual panel. Furthermore, we

Table 5 Antibiotic resistances of isolates recovered from Enterolert panels from six waterbodies. [a]The antibiotics tested were ampicillin (AM), bacitracin (B)[d], carbenicillin (CB), cefoxitin (FOX), doxycycline (D), erythromycin (E), gentamicin (GM), nalidixic acid (NA)[d], sulfathiazole (ST)[d], tetracycline (TE), trimethoprim (TMP), and vancomycin (VA)[d]. The site locations were Bird (B), Crooked Oak (CO), Lake Thunderbird (LT), Mountain Lake (ML), Washington Creek (WA), West Elm (WE), and Norman Wastewater Reclamation Influent (WW)

Site	n[b]	AM_c	CB	FOX	E	GM	D	TE	TMP
B	6	1	3	5	6	1			
CO	1			1					
LT	1			1			1		
ML	1								1
WA	7		3	4	4	1	5	4	
WE	1			1	1				
WW	6		1	6	5	5	1	1	1

[1]Number of resistant isolates

[b]Number of isolates from the site

[c]Antibiotics and concentrations in the "Methods" section

[d]Bacitracin (B) and nalidixic acid (NA) are not shown in the table as all 35 isolates tested were resistant to both antibiotics

used *P. licheniformis* and *E. faecalis* as presumed positive controls, and both indicators resulted in positive fluorescence in all 96 Enterolert panel wells for both species with an approximate cell count of 500 cells per mL.

As the Enterolert method is a USEPA-approved method for recreational freshwater quality criteria, true numbers of FIB as indicated in Baird and Bridgewater (2017) could be misrepresented for concentration requirements for impaired waterbodies. Furthermore, selectivity for FIB_P in freshwater may be skewed by false-positive indicators such as *Paenibacillus* spp. and not representative of the waters evaluated, especially if the waters have contributing sediment or pollution from sources such as wastewater or urban runoff (Ferguson et al. 2013; Peperzak and van Bleijswijk 2021; Suzuki et al. 2012). Overall, the results indicate that the number of species identified from the microcosm and field studies are of varying origin in the environment and suggest that more emphasis in selectivity of target fecal indicators should be considered when using Enterolert for analyzing freshwater samples.

Antibiotic resistance

Isolates from field water and sediment samples were evaluated for antibiotic resistance and susceptibility to understand potential predictors and characterization of *Enterococcus* spp. from freshwater samples processed using Enterolert methods. Isolates were cultured and recovered from direct plating of water samples and isolation from positive Enterolert panel windows. Results indicated that all 35 isolates were resistant to bacitracin and nalidixic acid. Bacitracin, in general, targets Gram-positive bacteria (Dubos 1939), but our set of isolates contradicted this conclusion. However, nalidixic acid is generally more effective against Gram-negative bacteria, so the resistance observed may not be unusual (Cook et al. 1966). All but one isolate was resistant to sulfathiazole, which was a *Lactococcus garvieae* recovered from an Enterolert window inoculated with water from Crooked Oak. Additionally, sulfathiazole is a broad-spectrum antibiotic; therefore, the resistance observed here was not expected but has been documented from fecal samples (Middleton and Ambrose 2005). We found limited differences between the overall antibiotic resistance of isolates between direct isolation (plating) or from an Enterolert window. The only isolates ($n = 4$) that were resistant to vancomycin were all isolated directly from the Norman Wastewater Reclamation Influent: *Enterococcus faecium*, *Leuconostoc lactis*, *Paenibacillus anaericanus*, and *Weissella halotolerans*. *Enterococcus* or Gram-positive species displayed an overall higher susceptibility to most of the other antibiotics selected (Table 5). Given the potential for antibiotic-resistant bacterium in the environment, more information is needed to understand which antibiotics have the potential to suppress non-indicator bacterium for inclusion into selective medium for bacteria quantification testing such as the fluorogenic substrate tests.

Conclusions

Freshwater fecal indicators are important for designating impairment status and related potential for human health impacts. However, considerations for applicable methods used in freshwater waterbodies should be evaluated further for regulatory decisions to properly target and quantify fecal indicators. We investigated the Enterolert method using a combination of field and laboratory freshwater stream samples and determined potential interferences in *Enterococcus*

enumeration. This work provides evidence that false positives can be dominant in freshwater stream samples using the fluorogenic substrate method for *Enterococcus* and that population shifts away from FIB can occur in both field and laboratory environments over short time periods. Also, it is not apparently predictable when and where a high percentage of false-positive results will occur (e.g., as shown above in the time point results for the Bird Creek field study). In addition, many of our identified LAB isolates from Enterolert panels could be considered of environmental or animal origin. *Paenibacillus* spp. (non-LAB) were the predominant false-positive indicators among all isolates identified in sediment and water samples regardless of sample location, type, or time period. Antibiotic resistance from various waterbodies provided initial evidence of resistance to nalidixic acid and bacitracin that are not typical of Gram-positive species.

Our work coincides with and adds to the evidence of Peperzak and van Bleijswijk (2021) where they found that false positives were in seawater samples with the genus *Bacillus* as the primary cause of interference. However, more studies are required to understand the impacts of sediment, organics, and other potential interactions and influences on the isolation of targeted Gram-positive FIB. Furthermore, research is needed to improve the fluorogenic substrate technology, such as Enterolert, to suppress non-FIB in freshwater samples.

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Declarations

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