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Persistence of fecal indicator bacteria and associated genetic markers from wastewater treatment plant effluents in freshwater microcosms

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ABSTRACT

Limited information exists on the environmental persistence of genetic markers for fecal indicator bacteria (FIB) in treated wastewaters. Here, the decay rate constants of culturable cells and genetic markers for four diverse groups of FIBs, such as enterococci, *Clostridium*, *Escherichia coli*, and *Bacteroides*, were investigated in freshwater microcosms seeded with disinfected and non-disinfected secondary-treated wastewaters. Decay rate constants of genetic markers and culturable cells varied significantly among the different FIB groups. Water temperatures (winter vs. fall/spring/summer) significantly affected the decay of all genetic marker and cell types; however, genetic marker decay were not found to be significantly different in disinfected (chlorination/ultraviolet) and non-disinfected wastewater-seeded microcosms or, for example, lake- and river-receiving waters. No evidence was seen that decay rate constants of FIB genetic markers from treated wastewater were substantially different from those observed in similar, previously reported microcosm studies using raw sewage. Unexpected relationships between decay rate constants of genetic markers and culturable cells of *Bacteroides* were observed. Results suggest that decay rate constants of FIB genetic markers determined from other studies may be applicable to treated wastewaters. Results of this study should be informative for ongoing efforts to determine the persistence of FIB genetic markers relative to surviving pathogens after wastewater treatment.

Key words: fecal indicator bacteria, genetic marker, persistence, qPCR, wastewater treatment

HIGHLIGHTS

- The influence of wastewater treatment on the persistence of FIB genetic markers was examined.
- Diverse FIBs in different freshwaters and temperatures were investigated.
- Disinfection did not significantly affect genetic marker decay.
- Genetic marker decay rate constants were *Bacteroides* > *E. coli*, *Enterococcus* > *Clostridium*.
- Genetic markers may better predict the presence of pathogens than culturable FIB in disinfected wastewater.

INTRODUCTION

The U.S. Environmental Protection Agency (EPA) has accepted the use of a quantitative PCR (qPCR) method as a rapid alternative for monitoring recreational waters for fecal contamination (U.S. EPA 2012a). Fecal contaminations such as combined sewer overflows, leaky septic systems and runoff of land-deposited municipal, livestock and other fecal wastes are possible sources of microbial pathogens with human health risks in recreational waters (Gerba & Smith 2005; Nevers *et al.* 2014). However, treated wastewaters discharged by wastewater treatment plants (WWTPs) are considered to be a main source of pathogens in many of these waters (Payment *et al.* 2001; Koivunen *et al.* 2003; Ottoson *et al.* 2006a; Castro-Hermida *et al.* 2008; Carducci *et al.* 2009; Okoh *et al.* 2010; Hendricks & Pool 2012). The fate of microbial pathogens that may survive current wastewater treatment processes, therefore, remains an important question.

qPCR analysis methods have been used extensively to study the decay of fecal indicator bacterial (FIB) genetic markers as potential surrogates for microbial pathogens. A number of studies have investigated the persistence of such markers from

various FIB groups in marine and freshwater microcosms (Seurinck *et al.* 2005; Wery *et al.* 2006; Okabe & Shimazu 2007; Bae & Wuertz 2009; Bell *et al.* 2009; Walters & Field 2009; Walters *et al.* 2009; Jeanneau *et al.* 2012; Korajkic *et al.* 2014; Bae & Wuertz 2015; Cloutier & McLellan 2017). Most of these persistent studies have used raw sewage or fecal material as the seed material in microcosms. Findings from these studies may not accurately reflect the fate of genetic markers present in normal WWTP effluents. Under normal operating conditions, WWTP effluents have been treated by various primary and secondary processes and then typically disinfected by either chlorine or ultraviolet (UV) radiation. These treatment processes are known to reduce the concentrations and viability of the fecal microbial populations in the wastewater (George *et al.* 2002, Villanova *et al.* 2004; Ottoson *et al.* 2006b; Wéry *et al.* 2008; Varma *et al.* 2009; Wen *et al.* 2009). Less is known about the effects of these treatments on the persistence of the nucleic acid components of these organisms after they are released with the treated wastewater effluents into ambient receiving waters. Chern *et al.* (2014) reported no immediate significant reduction of FIB genetic markers caused by secondary wastewater disinfection processes. Other studies have reported differences between chlorination and UV irradiation with regard to reduction and viability of culturable bacteria (Blatchley *et al.* 2007; Crockett 2007), but similar studies have not examined the influence of these treatments on the persistence of FIB genetic markers.

Knowledge about the persistence of FIB genetic markers originating from treated wastewater could be useful in understanding how well these analytes perform as surrogates for similarly treated microbial pathogens and thus in their ability to predict potential health risks from these pathogens. In this study, we examined the relative persistence of genetic markers from a range of different FIB groups in freshwater microcosms seeded with non-disinfected or disinfected secondary-treated wastewater effluents based on determinations of their decay rate constants. The FIB groups included enterococci and *Escherichia coli*, both facultative anaerobic bacteria groups that are recommended by the U.S. EPA for water quality testing (U.S. EPA 1986, 2012a), and the strict anaerobe groups, *Clostridium* and *Bacteroides*, among which the former are known to be environmentally persistent due to their production of spores and the latter are commonly used as indicators in microbial source tracking. The decay rate constants of culturable organisms in each of these groups were also determined the influence of different disinfection methods (UV and chlorination). Experiments were performed in the winter, fall, spring and summer seasons to determine any temperature effects, and data from the fall and spring season experiments were pooled (fall/spring) due to their similarity in water temperatures. Two different sources of simulated receiving waters for the microcosms, such as Lake Michigan and the Ohio River, were selected for the study to represent different water body types that can be impacted by WWTP effluents. This study provides a direct comparison of the environmental persistence of a variety of different FIB groups and their associated genetic markers coming from treated wastewater including the effects of disinfection.

METHODS

Sample collection

Surface water samples (20 L) were collected in 2008 from Lake Michigan near Porter, IN and in 2009 from the Ohio River near California, OH. Samples were collected during the summer, spring/fall, and winter season in the mornings at approximately 9 AM. Both secondary non-disinfected and disinfected wastewater samples (1 L) were collected from four different WWTPs across southern Ohio. All plants used the activated sludge treatment process for secondary wastewater treatment. Two plants disinfected by UV radiation (daily average discharge of 5–16 million gallons daily (MGD)) and the other two by chlorination (daily average discharge of 27–144 MGD). All samples were held on ice during transport to the laboratory for immediate processing.

Microcosms

A total of nine microcosms were prepared for each of the six experiments (two receiving water sources, each collected in three different seasons). Each microcosm contained a total volume of 1.5 L in 2 L flasks with tops covered by aluminum foil. One of the nine microcosms was used as a control containing only Lake Michigan or Ohio River water. The remaining eight microcosms were a mixture of freshwater and 20% of the secondary-treated or disinfected, secondary-treated wastewater effluent collected from one of the four different treatment plants. Microcosms were stored in the dark at temperatures similar to the ambient Lake or River water temperature at the time of collection (4, 20 and 25 °C) with constant agitation on a New Brunswick shaker (Innova Model 2150, New Brunswick Scientific, Enfield, CT) at a speed of approximately 65 rpm throughout the study period. In this study, seasons correspond to the different water temperatures during

sample collection. Duplicate samples were collected for analysis on the initial day (D_0) followed by sampling on days 1, 2, 4 and 6.

Culture analysis

Volumes of 1, 5 and 10 mL water samples from each microcosm were filtered in duplicate through a 0.45 μm pore size (47 mm in diameter) cellulose nitrate membrane filter (Sartorius, Bohemia, NY). Filters were placed onto mEI (U.S. EPA 2002) agar and followed by incubation overnight at 41 °C for the enumeration of enterococci. For *E. coli* enumeration, modified mTEC (U.S. EPA 2014) agar was used and followed by incubation at 44.5 °C (with a 2-h initial incubation at 35 °C). Bacteroides Bile Esculin (BBE) (Livingston *et al.* 1978) agar was used and followed by incubation at 36 °C for 48 h for culturing of the *Bacteroides fragilis* group. Filters placed onto mCP (U.S. EPA 1996) agar were incubated overnight at 44.5 °C for the enumeration of *Clostridium perfringens*. Anaerobic conditions using the GasPak™ EZ container system (BD Diagnostics, Franklin Lakes, NJ) were used for incubation of the BBE and mCP plates. Presumptive *C. perfringens* colonies were confirmed as described in the Membrane Filter Method for *C. perfringens* in the Information Collection Rule Microbial Lab Manual (U.S. EPA 1996).

DNA extraction

Duplicate aliquots of 50 mL from each microcosm were filtered through a 0.4 μm pore size (47 mm in diameter) polycarbonate membrane filter (GE Osmonics, Minnetonka, MA). Filters were placed into 2 mL screw cap extraction tubes prefilled with 0.3 g of glass beads. Cells were lysed after the addition of 600 μL of AE Buffer (Qiagen, Valencia, CA) containing 0.2 μg mL^{-1} of salmon DNA (Sigma, St. Louis, MO) into the extraction tubes followed by bead milling and then centrifugation as described previously by Haugland *et al.* (2005). DNA extracts were stored at 4 °C no longer than 24 h prior to qPCR analysis.

Amplification of target sequences

qPCR was used to estimate the concentrations of genetic markers of enterococci, *E. coli*, *Clostridium* spp. and the *B. fragilis* cluster in the microcosms. Primers and TaqMan® hydrolysis probe sequences used to amplify the genetic markers are shown in Supplementary Material, Table S1. For the purpose of consistency, this paper will subsequently refer to the group of *Bacteroides* species detected by the qPCR (Okabe *et al.* 2007) and the species detected by BBE culture medium as *B. fragilis* group. Similarly, the *Clostridium* species detected by the qPCR (Rinttilä *et al.* 2004; Supplementary Material, Table S1) and by mCP culture medium will both be referred to as *Clostridium* unless otherwise specified. Total reaction volumes of 25 μL containing 12.5 μL of TaqMan® Universal PCR Master Mix or Gene Expression Master Mix for the *E. coli* assay (Applied Biosystems, Foster City, CA), 2.5 μL of 2 mg L^{-1} bovine serum albumin, 1 μmol L^{-1} of each primer, 80 nmol L^{-1} of probe and 5 μL of 5-fold diluted extracts were used. Amplification conditions consisted of an initial incubation at 50 °C for 2 min then 95 °C for 10 min followed by 40 PCR cycles of 95 °C for 15 s and 60 °C for 1 min. All reactions were carried out in an ABI Model 7900 DNA thermal cycler (Applied Biosystems, Foster City, CA).

Controls

Calibrator cells of *E. coli* (ATCC #25922), *Enterococcus faecalis* (ATCC# 29212), *C. perfringens* (ATCC# 13124) and *Bacteroides thetaiotaomicron* (ATCC # 29741), a member of the *B. fragilis* group (Okabe *et al.* 2007), were prepared as previously described by Chern *et al.* (2009). Approximately 1×10^6 *E. coli*, 4.6×10^4 *C. perfringens*, 3×10^5 *E. faecalis* and 3.0×10^5 *B. thetaiotaomicron* cells were present in each calibrator sample. Serial dilutions of calibrator sample extracts were prepared to determine amplification efficiency and detection limits for each of the assays. qPCR analyses for salmon DNA that was added to all samples at identical concentrations prior to DNA extraction were used as a control to determine total DNA recovery in the extracts and to signal potential PCR interference (Haugland *et al.* 2005; U.S. EPA 2012b). All samples were analyzed in duplicate. Filter blanks and no template controls were also analyzed as negative controls along with the calibrator samples and unknown test samples.

Estimation of genetic and culturable marker decay rate constants

Concentrations of each genetic marker were estimated as calibrator cell equivalents (CCE) by the delta–delta C_t calculation method as previously described (Chern *et al.* 2011, 2014) using the mean amplification efficiency value generated from multiple analyses of serially diluted calibrator sample extracts. CCE estimates below the mean detection limits were adjusted to

one-half of the mean detection limit values. Geometric mean concentration estimates from duplicate filters of each sample were calculated and extrapolated to 100 mL sample volumes. Concentrations by culture were expressed as $\log_{10}$ colony-forming units (CFUs). Rates of decay for each FIB group, both genetic markers and culture-based, in the microcosm were then estimated using the first-order Chick–Watson kinetic model (Chick 1908; Watson 1908): $\ln (C/C_0) = -kt$, where C_0 is the initial concentration (CFU or CCE per 100 mL), k is the rate constant (day^{-1}) and C is the concentration (CFU or CCE per 100 mL) at time t, in days. Average decay rate constants over the four different FIBs are sometimes referred to the convenience of discussion, particularly when their trends are similar. This is not meant to imply that these decay rate constants are independent of FIB. Intra-FIB differences are discerned later in the discussion.

Statistical analysis

Analyses of variance (ANOVAs) were performed separately for criterion variables (CCE, CFU) as well as their differences (CCE – CFU). The model utilized the following as independent, explanatory, variables:

- type of organism (four indicator classes),
- season (winter vs. spring/summer/fall),
- source of spiked freshwater (river, lake) and
- effluent disinfection status (non-disinfected, chlorine-disinfected, UV-disinfected).

All potential interactions among the above were included, yielding a total of 10 potential explanatory variables. The four WWTP sources of wastewater used for spiking were modeled as a random factor, representative of WWTPs in general. Based on the estimated mean decay rate constants as modeled via SAS version 9, p-values (significance level = 0.05) were used in the evaluation of the difference between seasons, disinfected and non-disinfected secondary wastewater effluent and disinfection methods with respect to stepwise $\log_{10}$ reductions for the individual CCE, CFU and CCE–CFU. ANOVA results for significant model variables are tabulated separately to facilitate discussion in the results section.

RESULTS

Performance of analytical methods

Amplification efficiency estimates of each qPCR assay were generated from the slope of the serial dilution analyses and ranged from 0.92 to 1.04. Results indicated that the mean calibrator cell detection limits, which were estimated as described in Chern *et al.* (2011), were approximately 86, 315, 75 and 134 CCE per 50 mL filtered water sample volume based on analyses of 5-fold extract dilutions for the enterococci, *E. coli*, *B. fragilis* group and *Clostridium* assays, respectively. No inhibition or contamination was detected from analysis of the salmon DNA sample processing controls and from analysis of the filter blanks and no template controls. Analyses of positive and negative control test organisms along with sterility controls were performed with each culture method and exhibited acceptable results.

Decay rate constants in freshwater microcosms seeded with secondary-treated wastewater

After the addition of wastewater into the lake- or river-receiving water microcosms, genetic marker concentrations increased, on average, by approximately 0.6 logs for *Clostridium* up to 1.3 logs for *enterococci* (Table 1), and the concentrations of the different markers increased substantially in at least 84% (37/44) of the microcosms, indicating that most of the markers initially present in the majority of these microcosms originated from the added wastewater. A similar or greater increase in concentration was seen for culturable organisms in the seeded microcosms; however, levels of culturable organisms in the microcosms seeded with disinfected wastewater were not sufficiently high to accurately determine decay rate constants. Consequently, decay rate constants for culturable organisms were based on the non-disinfected wastewater-seeded microcosms only in this study. Overall, decay rate constants of the cultured FIB or genetic FIB marker did not significantly differ in the different receiving water microcosms, i.e. lake vs. river (Table 2), except for the genetic marker decay rates in winter where slower decay occurred in river than lake water ($p < 0.0001$) (Table 2). No significant differences were observed in the averaged decay rate constants of the genetic markers from the four FIB groups in freshwater microcosms seeded with the disinfected and non-disinfected secondary-treated wastewater effluent (Table 3). In addition, the type of disinfection (chlorination vs. UV) had no significant effect on the rates of genetic marker decay (Table 3). Decay rate constants of the averaged FIB culture data and of the averaged genetic marker data for the four FIB groups were not significantly different

Table 1 | Initial mean $\log_{10}$ concentrations and standard deviations of target organisms in WWTP effluent-seeded and -receiving water only freshwater microcosms determined by molecular (CCE per 100 mL) and culture (CFU per 100 mL) measurements[a]

Target organisms	Microcosm category	*Clostridium*	*E. coli*	Enterococci	*B. fragilis* group
CCE	Non-disinfected effluent	3.67 (0.68)	3.81 (0.65)	3.72 (0.65)	4.20 (1.26)
	Disinfected effluent	3.89 (0.52)	3.84 (0.69)	3.72 (0.56)	4.54 (1.18)
	Receiving water only[b]	3.14 (3.26)	2.79 (2.82)	2.43 (2.35)	3.52 (3.64)
CFU	Non-disinfected effluent	2.43 (0.45)	3.35 (0.48)	2.29 (0.76)	2.18 (0.74)
	Disinfected effluent	1.92 (0.54)	2.14 (0.66)	1.01 (0.88)	1.16 (0.65)
	Receiving water only[b]	0.60 (3.76)	0.53 (3.21)	0.09 (2.86)	0.50 (4.26)

[a]Mean values from all microcosms in the study within each category that contained initially quantifiable target organism concentrations. Standard deviations of $\log_{10}$ concentrations are given in parentheses.
[b]A single receiving water was collected for each experiment ($n = 6$). The means and standard deviations are pooled over lake and river water and therefore based on only 2 degrees of freedom. Note that the three lake samples differed over a 1 log range, while the river, over about one-half log.

Table 2 | FIB culture and genetic marker decay rate constants observed in seeded fresh lake and river water microcosms in spring/summer/fall seasons vs. winter

	Matrix	Lake	River	*p*-value
CCE	Spring/summer/fall	0.262	0.213	0.0766
	Winter	0.193	−0.101	<0.0001
CFU[a]	Spring/summer/fall	0.225	0.221	0.9205
	Winter	0.089	0.073	0.6979

[a]Data from only non-disinfected wastewater-seeded microcosms were used to calculate CFU decay rate constants. Standard errors of estimated decay rate constant: 0.025.

Table 3 | Genetic marker decay rate constants observed in non-disinfected vs. disinfected effluent-seeded freshwater microcosms by the disinfection method and seasons

	Disinfection	Cl	UV	None	*p*-value[a]
CCE	Summer/spring/fall	0.280	0.202	0.232	0.3062
	Winter	0.108	0.003	0.027	0.2861

[a]Probability under hypothesis is that all three means are equal. Standard error of estimated decay rate constants: 0.028 (summer/spring/fall) and 0.0.040 (winter).

between the summer and spring/fall seasons, so decay rate constants from these seasons were combined in further analyses (Tables 4 and 5).

However, significant differences in decay rate constants of both culturable organisms and genetic markers were detected between winter and summer/spring/fall seasons and among the different FIB groups in the seeded microcosms ($p < 0.05$) (Tables 4 and 5). Differences between the decay rate constants of culturable organisms and their genetic markers also differed significantly among the different FIB groups in the summer/spring/fall season but not in the winter season (Table 5).

Table 4 | Decay rate constants observed in seeded freshwater microcosms by the season

Seasonality	Summer/spring/fall	Winter	Seasonal difference	*p*-value[b]
CCE	0.238	0.046	0.192	0.0084
CFU[a]	0.223	0.081	0.142	0.0017

[a]Data from only non-disinfected wastewater-seeded microcosms were used to calculate CFU decay rate constants.
[b]*p*-value under the null hypothesis that zero seasonal difference is nil. Standard errors of estimated decay rate constants: 0.018 (CCE, summer/spring/fall), 0.025 (CCE, winter), 0.004 (CFU, summer/spring/fall), 0.005 (CFU, winter).

Table 5 | FIB culture and genetic marker decay rate constants observed in freshwater microcosms by seasons

Target organism	Season	*Clostridium*	*E. coli*	Enterococci	*B. fragilis* group	*p*-value[a]
CCE[b]	Summer/spring/fall	0.079^{L}	0.239^{M}	0.204^{M}	0.428^{H}	<0.0001
	• Spring/fall	0.084	0.025	0.213	0.432	<0.0001
	• Summer	0.074	0.023	0.195	0.425	<0.0001
	Winter	0.080^{H}	0.089^{H}	0.049^{H}	-0.034^{L}	0.0011
CFU[c]	Summer/spring/fall	-0.022^{L}	0.257^{H}	0.352^{H}	0.305^{H}	<0.0001
	• Spring/fall	0.020	0.305	0.406	0.338	<0.0001
	• Summer	0.016	0.209	0.252	0.280	<0.0001
	Winter	0.012	0.157	0.117	0.037	0.0124
Difference (CFU–CCE)[d]	Summer/spring/fall	-0.104^{H} (0.01)	0.007^{L} (0.80)	0.114^{L} (0.01)	-0.112^{H} (<0.01)	0.0020
	Winter	−0.065 (0.15)	0.052 (0.24)	0.061 (0.18)	0.075 (0.11)	0.0583

[a]With respect to decay rate constants within the season. ^{L,M,H} Indicate low/middle/high values, respectively, among decay rate constants grouped by significant differences. Within the same row, any two target organisms with same superscript are *not* significantly different from one another. Two target organisms with different superscripts are significantly different. Note that none of the CFU-based constants were significantly different among themselves in winter and thus the absence of superscript notations. Standard errors of estimated decay rate constants: 0.013 (CCE, summer/spring/fall), 0.018 (CCE, winter), 0.016 (CFU, summer/spring/fall), and 0.23 (CFU, winter).

[b]Combined data from non-disinfected and disinfected wastewater-seeded microcosms were used to calculate CCE decay rate constants.

[c]Data from only non-disinfected wastewater-seeded microcosms were used to calculate CFU decay rate constants.

[d]Differences between CFU and CCE decay rate constants (based on comparable data) with *p*-values are indicated in parentheses.

Summer/spring/fall conditions (warmer water) had the greatest effect on accelerating *B. fragilis* genetic marker decay, while *Clostridium* genetic marker decay showed a relatively little seasonal variation (Table 5). Cultured enterococci, *E. coli* and *B. fragilis* group shared similar decay rate constants that were faster than cultured *Clostridium* in the summer/spring/fall season, while in the winter cultured enterococci and *E. coli* both were observed to have slightly faster decay rate constants than the cultured *B. fragilis* group and *Clostridium* markers. This contrasted with the similar decay rate constants of *Clostridium*, *E. coli* and enterococci genetic markers that decayed faster than *B. fragilis* group genetic markers in the winter (Table 5). Except for *Clostridium*, summer/spring/fall conditions accelerated both culturable organism and genetic marker decay rate constants than winter conditions. These results are also illustrated in Figure 1 where the persistence of both cultured FIB and their associated genetic markers was generally greater in the winter season than in the summer/spring/fall seasons.

DISCUSSION

Results of this study could provide important new insights into the persistence of genetic markers from FIB target organisms occurring in secondary-treated wastewater. Season or temperature had the strongest influence on the decay of these markers among the environmental parameters tested but only in terms of winter vs. summer, spring and fall conditions, even though the magnitude of this effect differed among the various FIBs. These results are consistent with previous results, indicating that water temperature was one of the single most important environmental factors in determining FIB genetic marker decay (Balleste & Blanch 2010; Bae & Wurtz 2015). However, wastewater disinfection and type of wastewater disinfection did not have a significant effect on the persistence of these markers in this study.

The persistence of culturable FIB has been reported to be influenced by numerous biotic and abiotic factors in the environment (Scheuerman *et al.* 1988; Okabe & Shimazu 2007; Jackson *et al.* 2008; Bell *et al.* 2009; Balleste & Blanch 2010; Korajkic *et al.* 2013). FIB genetic markers have been described as more environmentally persistent than corresponding culturable organisms (Walters *et al.* 2009; Korajkic *et al.* 2014). This conclusion was supported by results from this study for the traditional FIB groups, *E. coli* and enterococci. However, the larger decay rate constants seen for the *B. fragilis* group and *Clostridium* genetic markers compared to the culturable organisms in summer/spring/fall in this study appeared to differ from this more widely seen trend and may warrant further investigation. It should also be noted that there was no functional culture medium available to enumerate the same group of *Clostridium* species that was detected by the *Clostridium* genetic marker assay (see Supplementary Material, Table S1) or vice versa. Therefore, comparisons of decay rate constants between *C. perfringens* culture and the genetic markers detected by the qPCR assay in this study may not be appropriate. In contrast, it is believed that a highly similar group of species is detected by the selective culture medium and the genetic marker assay used

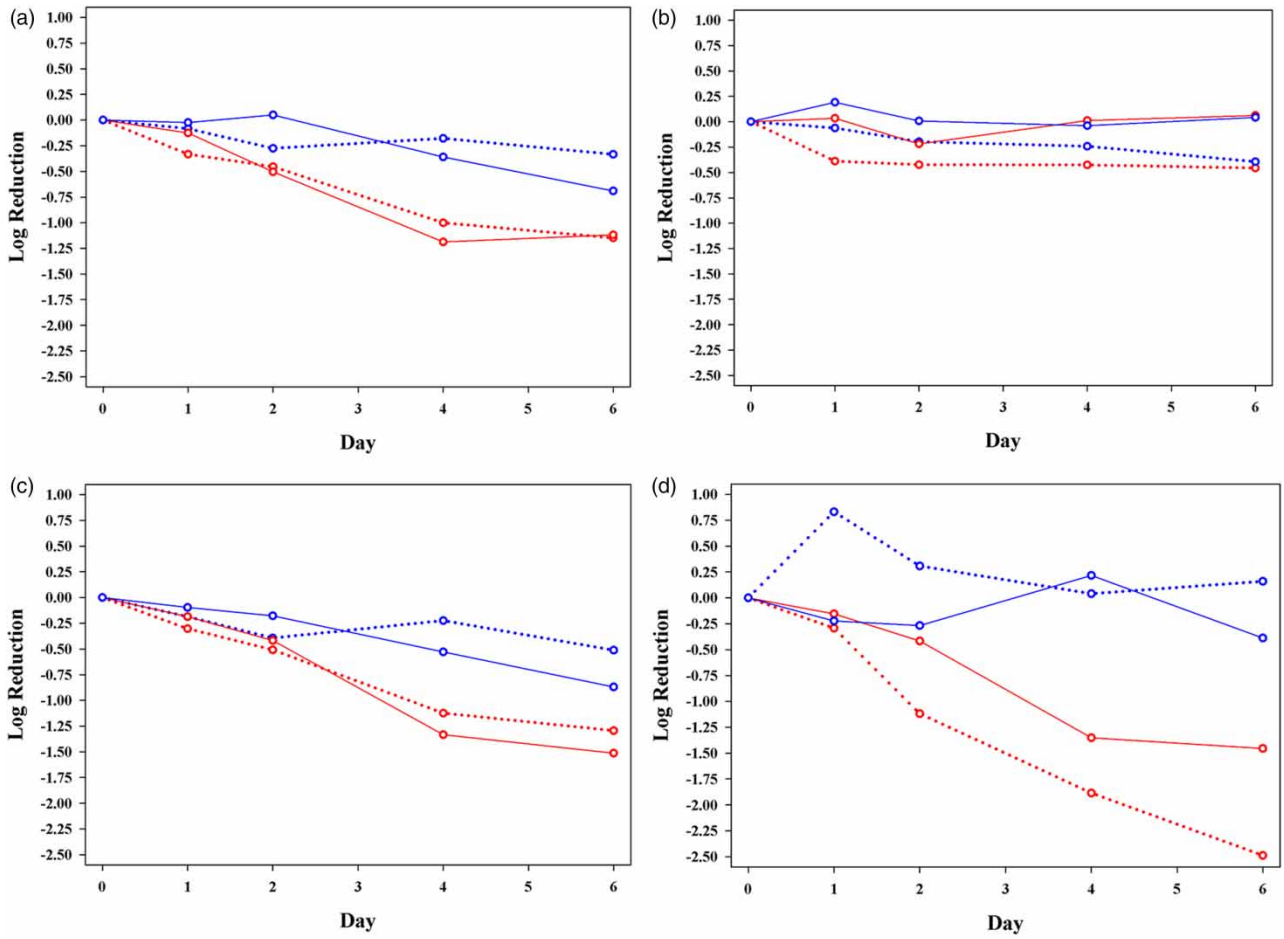

Figure 1 | Decay curves of (a) enterococci, (b) *Clostridium*, (c) *E. coli*, and (d) *B. fragilis* group culturable markers (solid lines) and their associated genetic marker (dotted lines) in secondary-treated wastewater-seeded freshwater microcosms in the summer/fall/spring (red) and winter (blue) seasons. Genetic marker data from both disinfected and non-disinfected wastewater-seeded microcosms. Culturable data from only non-disinfected wastewater-seeded microcosms. Standard deviations are reported in Table 1. Please refer to the online version of this paper to see this figure in colour: http://dx.doi.10.2166/wh.2021.152.

in this study for the *B. fragilis* group. Similar trends in differences between culture and qPCR decay rate constants have been reported in another study using two specific *Bacteroides* strains from this group as seeding material (Balleste & Blanch 2010). At least in this study, using treated secondary effluents as the seed material for the microcosms, a reasonable hypothesis for the faster decay rate of *B. fragilis* group genetic markers than culturable cells could be that most of the qPCR target sequences are being contributed by cells that have already died and are deteriorating even before disinfection. This hypothesis is supported by the relatively high concentrations of *B. fragilis* CCE compared with CFU estimates in the D_0 microcosms containing non-disinfected as well as disinfected wastewater as shown in Table 1, although care should be taken when comparing these estimates (Haugland *et al.* 2014).

One common observation reported in several studies investigating culturable FIB persistence is that *Clostridium* is more persistent while *Bacteroides* is less persistent than other FIB indicators even when using different sources of these microbes to seed microcosms (Fiksdal *et al.* 1985; Lisle *et al.* 2004; Hijnen *et al.* 2009). These relationships could be extended to the genetic markers examined in this study where the *Clostridium* marker was also found to be the most persistent while the *B. fragilis* group marker was the least persistent, at least in summer, spring and fall conditions. The differences seen between the decay rate constants of genetic markers from the different FIB groups hold open the possibility that the fate of one of these groups may more closely mirror the fate of viral or protozoan pathogen groups in at least some environmental conditions.

For the microcosms to achieve winter temperature conditions and to minimize the number of different variables, all microcosms in this study were kept in controlled temperature rooms in the dark. The reported decay rate constants should be conservative since sunlight is known to accelerate the decay of culturable cells (Davies-Colley *et al.* 1999; Sinton *et al.*

2002). Results by Dong *et al.* (2014), using artificially constructed microcosms with pure cultures of *Bacteroides ovatus*, also indicated that DNA decayed faster in daylight conditions under full spectrum irradiation than in the dark; however, the effect of sunlight on accelerating the decay of *E. coli*, enterococci and *Bacteroides* genetic markers from sewage under more natural conditions was less evident (Wanjugi *et al.* 2016). The relative and/or absolute decay rate constants of *E. coli*, enterococci and *Bacteroides* genetic markers in this study appeared to be consistent with those reported in other studies for these organisms from raw sewage while under dark conditions (Wanjugi *et al.* 2016; Liang *et al.* 2017).

As with most small-scale controlled microcosm studies, there are limitations in the experimental approach of this study and the conclusions that can be drawn. For example, the volumes used for each analysis substantially decreased the total volumes of the microcosms over the period of the experiments which may have affected other parameters. If any such effects occurred, they would presumably have affected the decay rate constants equally for all potential factors that were investigated (organism, disinfection type, temperature, etc.). The primary focus of this study was not to exhaustively investigate the many environmental factors that could contribute to the decay of FIB and their genetic markers which, as discussed above, have been investigated in numerous other studies, but rather to focus on the differences in decay rate constants between different groups of potentially relevant FIB and investigate just a few parameters that could specifically affect the decay of these organisms and their genetic markers in treated wastewater effluents. Another potential limitation of this study was suggested by the relatively low concentrations of some of the different FIB groups and their genetic markers in some of the wastewater-seeded microcosms compared to corresponding receiving water microcosms. This observation would suggest that decay rate constant estimates for the FIB groups and their genetic markers in these microcosms could be strongly influenced by the decay of the indigenous organisms in the receiving waters. These microcosms, however, provided only a small percentage of the data used to estimate the overall FIB decay rate constants in this study and are thus believed not to strongly influence these estimates.

This study presents results from four WWTPs. We treated these as a random selection of WWTPs, adding the variance among plants to the more stable within plant variability. This allowed projection of the studied WWTP results to WWTPs in general. Adding WWTPs to this base would enable increased power in evaluating effects and in representativeness of the results. The same holds true for incorporating additional sources of receiving waters, which, in our case, were regarded as fixed effects out of necessity, given that only two sources were used. Differing receiving water characteristics could potentially yield valuable information on microbial decay relative to source water properties.

CONCLUSIONS

This study presents genetic marker decay rate constants from a range of FIB target groups originating from treated wastewaters. Knowledge of such decay rate constants should aid in determining if genetic markers may be suitable to serve as surrogates for pathogens in these effluents. The relationship between the persistence of FIB markers and enteric viruses and protozoa is of particular interest because both have been shown to be less effectively attenuated than culturable bacteria by the disinfection regimes commonly used at WWTPs (Chang *et al.* 1985; Tree *et al.* 1997). In addition, monitoring for genetic markers may be a better option for predicting the fate of pathogens in disinfected wastewater since, as reflected by this study, the lower concentrations of culturable bacterial indicators from these sources can make them more difficult to accurately quantify. Despite this, a limitation to the accurate determination of decay rate constants of genetic markers originating from treated wastewaters in receiving waters can be the relative concentration of these markers in the wastewaters compared to the receiving waters in microcosm studies of this nature. Studies on other WWTPs and source waters are needed to confirm the extent that results from the present study apply to a diverse range of conditions. In addition, further studies are needed to compare the persistence of FIB and their genetic markers with pathogens. Such studies could support the development of generalized models for predicting the health risks posed by WWTP effluents in ground water (Shende & Chau 2019) or at nearby recreational beaches (Boehm *et al.* 2018). Genetic markers from enterococci have already been shown to be predictive of associated health risks at WWTP-impacted beaches (U.S. EPA 2012a). Monitoring genetic markers could further aid in the routine assessment of water quality and in the protection of public health at other recreational beaches.

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NOTICE

Information has been subjected to U.S. EPA peer and administrative review and has been approved for external publication. Any opinions expressed in this paper are those of the authors and do not necessarily reflect the official positions and policies of the U.S. EPA. Any mention of trade names or commercial products does not constitute endorsement or recommendation for use.

CONFLICT OF INTEREST

None declared.

DATA AVAILABILITY STATEMENT

All relevant data are available from an online repository or repositories (https://doi.org/10.23719/1519085).

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