

## Influence of wastewater disinfection on densities of culturable fecal indicator bacteria and genetic markers

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### ABSTRACT

The US Environmental Protection Agency has proposed the use of quantitative polymerase chain reaction (qPCR) as a rapid alternative analytical method for monitoring recreational water quality at beaches. For qPCR to be considered for other Clean Water Act purposes, such as inclusion in discharge permits and use in Total Maximum Daily Load calculations, it is necessary to understand how qPCR detectable genetic markers are influenced by wastewater disinfection. This study investigated genetic markers for *Escherichia coli*, *Enterococcus*, *Clostridium* spp., *Bacteroides*, total Bacteroidales, as well as the human-associated *Bacteroides* markers, HF183 and HumM2, to determine which, if any, were influenced by disinfection (chlorination or ultraviolet light) of effluents from secondary wastewater treatment in different seasons. The effects of disinfection on culturable enterococci, *E. coli*, *Bacteroides*, and *C. perfringens* were also compared to their associated genetic markers. Disinfection of secondary treatment effluents significantly reduced culturable fecal indicator bacteria (FIB) but not genetic marker densities. No significant differences were observed in the responses of FIB culture and genetic marker densities to type of disinfection (chlorination vs UV) or season. Results of this study provide evidence that qPCR may not be suitable for monitoring efficacy of wastewater disinfection on the inactivation of bacterial pathogens.

**Key words** | disinfection, FIB, qPCR, wastewater

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### INTRODUCTION

To prevent pathogens from posing serious risks to public health, disinfection procedures are commonly used prior to discharging treated wastewater. Chlorination or ultraviolet light (Vanden Heuvel *et al.* 2010) are the two predominant disinfection methods used by wastewater treatment plants (WWTPs) (Chiu *et al.* 1999). Both methods have been shown to be effective in inactivating fecal indicator bacteria (FIB), which are routinely monitored as surrogates for fecal contamination in ambient waters (Tree *et al.* 2003; Nasser *et al.* 2006). Current culture methods for enumerating FIB produce results in 18–24 hours or more. Molecular-based methods such as quantitative polymerase chain reaction (qPCR) have been investigated because they can offer rapid enumeration of FIB in recreational waters (Rousselon *et al.* 2004; Siefring *et al.* 2008; Chern *et al.* 2009; Schriewer *et al.* 2010; Wade *et al.* 2008, 2010) and have been demonstrated

to allow timely water quality notifications at beaches to facilitate public health protection efforts (Griffith & Weisberg 2011). The US Environmental Protection Agency has recently provided the option for states to adopt a qPCR method for monitoring recreational water quality on a site-specific basis (U.S. EPA 2012).

To determine if qPCR can be applied to effectively monitor wastewater treatment efficacy, it is necessary to understand how qPCR-targeted genetic markers may be influenced by wastewater disinfection processes. Several studies have examined the influence of wastewater disinfection on genetic markers from individual or limited varieties of FIB organisms (He & Jiang 2005; Wery *et al.* 2008, 2010; Varma *et al.* 2009; Srinivasan *et al.* 2011). These studies, however, generally have not focused on directly comparing the effects of disinfection on a wide variety of different FIB

organisms, including organisms and/or genetic markers that may be applicable for human microbial source tracking (MST), or on the influence of different types of disinfection on these FIB organisms and their genetic markers. Additionally, Varma *et al.* (2009) examined the reduction of *Enterococcus* spp. and total Bacteroidales genetic markers through wastewater treatment processes using propidium monoazide incorporated with qPCR (PMA-qPCR) to distinguish between DNA from viable and non-viable cells. Greater reductions were observed in some cases with the use of PMA-qPCR compared with qPCR alone. However, limitations to the PMA-qPCR method, such as possible interference to light activation of the PMA dye by particulate matter in the samples and uncertainty about the dye's ability to penetrate cell membranes of viable but non-culturable cells, were encountered (Nocker *et al.* 2007a, b; Varma *et al.* 2009).

In this study we examined the effects of wastewater disinfection on the densities of genetic markers from a wide variety of different FIB organisms or groups of organisms by conventional qPCR analysis to determine which, if any, genetic marker may be applicable for monitoring wastewater disinfection efficacy. Markers for *Escherichia coli*, *Enterococcus* (total enterococci), *Clostridium* spp. (*C. perfringens* cluster; Rinttila *et al.* 2004), total Bacteroidales (Dick & Field 2004), *Bacteroides* (*B. fragilis* cluster; Okabe *et al.* 2007) as well as the human-associated *Bacteroides* markers, HF183 (Bernhard & Field 2000) and HumM2 (Shanks *et al.* 2009), were analyzed. Culturable enterococci, *E. coli*, *Bacteroides* (*B. fragilis* cluster), and *C. perfringens* reductions caused by disinfection of secondary treatment effluents were also compared to the disinfection effects on their associated genetic markers.

In addition, differences in the effectiveness of using chlorination or UV light to inactivate various culturable fecal organisms have been reported, but robust data comparing how chlorination and/or UV impacts the densities of FIB genetic markers are limited (Blatchley *et al.* 2007; Crockett 2007). Our study included analyses of disinfected and non-disinfected secondary treatment effluents from WWTPs using different disinfection methods. Samples from each WWTP were also collected seasonally to examine potential influences of this factor on disinfection efficacy.

## METHODS

### Sample collection

One liter of secondary treatment effluent was collected both before and after disinfection from four treatment plants across southern Ohio during each sampling event. All plants used the activated sludge process for secondary treatment. Two of these plants used ultraviolet radiation while the other two used chlorination to disinfect. Sodium thiosulfate (1 mL of a sterile 10% solution per liter of wastewater sample) was immediately added to each sample that was disinfected by chlorination. Samples were collected once during the winter, summer, and spring or fall (similar temperatures were observed in the spring and fall) seasons for two consecutive years. All samples were held on ice during transport to the laboratory for immediate processing.

### Culture analysis

Duplicate volumes of serially diluted wastewater samples were filtered through a 0.45 µm pore size (47 mm in diameter) cellulose nitrate membrane filter (Sartorius, Bohemia, NY). Filters were placed onto mEI (enterococci), modified mTEC (*E. coli*), *Bacteroides* Bile Esculin (BBE) (*B. fragilis* cluster, Livingston *et al.* 1978) and mCP (*C. perfringens*) agar plates and incubated overnight at 41 °C, 44.5 °C (with 2 hour initial incubation at 35 °C), 36 °C (for 2 days), or 44.5 °C, respectively. Both BBE and mCP plates were incubated under anaerobic conditions using the GasPak™ EZ container system (BD Diagnostics, Franklin Lakes, NJ). Confirmation of presumptive colonies was performed as described in the Information Collection Rule Microbial Lab Manual (EPA 600-R-95-178) ([www.epa.gov/nerlcwww/documents/icrmicro.pdf](http://www.epa.gov/nerlcwww/documents/icrmicro.pdf)).

### DNA extraction

Ten milliliters of each wastewater sample was 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 tubes filled with 0.3 g of glass beads and 600 µL of AE Buffer (Qiagen,

Valencia, CA) containing  $0.2 \mu\text{g mL}^{-1}$  of salmon DNA. Cells were lysed by bead beating as described by Haugland *et al.* (2005). DNA extracts were stored at  $4^\circ\text{C}$  and analyzed within 24 h.

### Quantitative PCR

Amplification of target DNA sequences occurred in a total reaction volume of  $25 \mu\text{L}$  containing  $12.5 \mu\text{L}$  of TaqMan<sup>®</sup> Universal or Gene Expression (polymerase with reduced bacterial DNA for detection of *E. coli*) PCR Master Mix (Applied Biosystems, Foster City, CA),  $2.5 \mu\text{L}$  of  $2 \text{ mg/mL}$  bovine serum albumin,  $1 \mu\text{M}$  of each primer,  $80 \text{ nM}$  of probe and  $5 \mu\text{L}$  of five-fold diluted extracts. Real-time simplex qPCR assays used in this study and corresponding target organisms are shown in Table 1. Reaction conditions consisted of an initial incubation at  $50^\circ\text{C}$  for 2 min then  $95^\circ\text{C}$  for 10 min followed by 40 PCR cycles of  $95^\circ\text{C}$  for 15 s and  $60^\circ\text{C}$  for 1 min. All reactions were carried out in an ABI Model 7900 DNA thermal cycler (Applied Biosystems, Foster City, CA).

### Quantification of FIB densities

Calibrator cells of *E. coli* (ATCC #25922), *Enterococcus faecalis* (ATCC# 29212), *Clostridium perfringens* (ATCC# 13124), and *Bacteroides thetaiotaomicron* (ATCC # 29741) were prepared for the EC23S857, Entero1, Cperf and GenBac3 and Human-Bac1 qPCR assays (Table 1), respectively, as previously described by Chern *et al.* (2009). Approximately  $1.0 \times 10^6$  *E. coli*,  $4.6 \times 10^4$  *C. perfringens*,

$3.0 \times 10^5$  *E. faecalis* and  $3.0 \times 10^5$  *B. thetaiotaomicron* cells were present in each calibrator sample. Amplification efficiency estimates were generated from analyses of serially diluted genomic DNA extracts of each of these organisms with the corresponding assays (Table 1). Estimated target sequences per reaction were  $3.3 \times 10^1$ ,  $1.0 \times 10^2$ ,  $4.0 \times 10^2$ ,  $4.0 \times 10^3$  and  $4.0 \times 10^4$  copies for *E. faecalis*, *C. perfringens*, and *B. thetaiotaomicron*, and  $2.3 \times 10^2$ ,  $7.0 \times 10^2$ ,  $2.8 \times 10^3$ ,  $2.8 \times 10^4$  and  $2.8 \times 10^5$  copies for *E. coli*. Serially ten-fold diluted plasmid DNA standards with estimated target sequences ranging from  $10^1$  to  $10^5$  copies per reaction were analyzed to generate master standard curves for the HumM2 assay and for the HF183 assay,  $4.0 \times 10^1$ ,  $1.0 \times 10^2$ ,  $4.0 \times 10^2$ ,  $4.0 \times 10^3$  and  $4.0 \times 10^4$  copies per reaction were analyzed (Table 1). Densities of the HF183 and HumM2 genetic markers were estimated directly from the master standard curves (Shanks *et al.* 2009; Haugland *et al.* 2010) and marker densities of all other FIB were estimated as calibrator cell equivalents (CCE) by the  $\Delta\Delta\text{Ct}$  comparative Ct calibration model (Haugland *et al.* 2005, 2009). Analyses using the Sketa2 qPCR assay for salmon DNA in each sample were used as a control to detect sample matrix interference (Haugland *et al.* 2005). Previously proposed guidelines for this control assay state that sample analysis results that are three or more cycle quantification units (Cq) greater than the mean of the corresponding calibrator Cq are unacceptable owing to matrix interference and these samples should be reanalyzed after an additional five-fold dilution of the extract. No sample analysis results were encountered in this study, however, that indicated matrix interference based on this criterion. All samples were

**Table 1** | Real-time PCR assays

Assay name	Target organisms	Reference	Calibration model parameter values for this study
Entero1	<i>Enterococcus</i> (total enterococci)	Ludwig & Schleifer (2000)	$E = 0.94$
GenBac3	Bacteroidales (total Bacteroidales)	Siefring <i>et al.</i> (2008)	$E = 0.96$
Human-Bac1	<i>Bacteroides</i> ( <i>B. fragilis</i> cluster)	Okabe <i>et al.</i> (2007)	$E = 1.01$
Cperf	<i>Clostridium</i> spp. ( <i>C. perfringens</i> cluster)	Chern <i>et al.</i> (2009)	$E = 0.92$
EC23S857	<i>Escherichia coli</i>	Chern <i>et al.</i> (2011)	$E = 1.04$
Sketa2	Salmon DNA	Haugland <i>et al.</i> (2005)	Not determined
HF183	Human-associated <i>Bacteroides</i>	Haugland <i>et al.</i> (2010)	$Y = -3.44x + 37.04$
HumM2	Human-associated <i>Bacteroides</i>	Shanks <i>et al.</i> (2009)	$Y = -3.13x + 37.84$

$E$  = Amplification efficiency.

analyzed in duplicate. Filter blanks and no template controls were also analyzed with each batch of unknown samples.

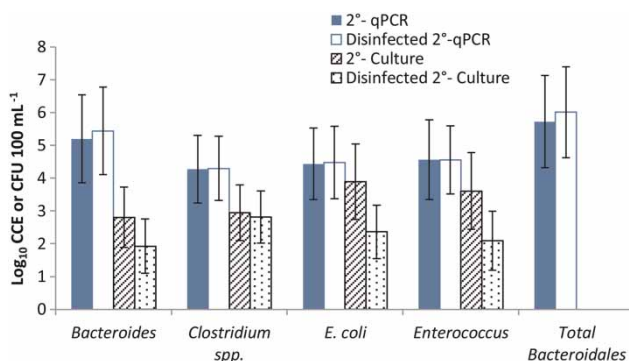
### Statistical analysis

Log<sub>10</sub> means and standard errors of means were determined for each FIB before and after disinfection. Differences in density for each FIB genetic marker were analyzed via a linear model with factors of publicly owned treatment works (POTW), season, treatment stage, and method of disinfection. Log<sub>10</sub> means tended to exhibit large variances, hence large standard errors, within pre- and post-disinfection stages, due to considerable variability in the FIB density from sample to sample in secondary treatment stage effluents and subsequent carryover of this variability after disinfection. However, the variance of the differences in log<sub>10</sub> means from pre- to post-disinfection, relevant to the linear model analysis, tended to be much smaller owing to relatively similar log reductions regardless of the initial level of FIB. Analyses were completed using SAS version 9 (SAS Institute Inc., Cary, NC).

## RESULTS AND DISCUSSION

### Influence of disinfection on culturable FIB and genetic markers

The densities of all culturable FIB groups were reduced by disinfection of the secondary treatment effluents (Figure 1;



**Figure 1** | Mean Log<sub>10</sub> densities of culturable FIB (CFU) ( $n = 14$ ) and qPCR-estimated FIB (CCE) ( $n = 22$ ) in secondary-treatment effluents before (2') and after (disinfected 2') disinfection, based on results from all WWTP samples. Vertical bars indicate the standard errors of the means among WWTP samples within each respective treatment phase.

Table 2). In all cases, the reductions were significant ( $p < 0.001$ ) based on paired comparisons of pre- and post-disinfection densities within WWTPs. This finding was consistent with results reported by Srinivasan *et al.* (2011). Differences in the levels of reductions of each culturable FIB group due to disinfection were also statistically significant ( $p < 0.001$ ) except between culturable enterococci and culturable *E. coli* (data not shown). In contrast, none of the FIB genetic markers was significantly reduced by disinfection. This result may be attributable to the inability of qPCR to distinguish between nucleic acids from live and dead cells (Nocker *et al.* 2007a, b; Varma *et al.* 2009). Our observations were consistent with previous studies, where levels of genetic markers quantified by qPCR were not substantially reduced by disinfection of secondary treatment stage effluents (He & Jiang 2005; Varma *et al.* 2009; Srinivasan *et al.* 2011). Results from other researchers have suggested possible reductions in *Enterococcus* spp. and *E. coli* genetic markers in response to disinfection in a WWTP using a different treatment process (Lavender & Kinzelman 2009).

### Effect of type of disinfection and season on culturable FIB and genetic markers

No significant differences were observed between the effects of type of disinfection used (chlorination or UV) and reductions in culturable FIB or genetic marker densities. Season also did not significantly influence the effect of disinfection on densities of culturable FIB or genetic markers for *Enterococcus*, *E. coli*, *Bacteroides*, *Clostridium*, and total Bacteroidales (data not shown).

### Human MST markers in secondary wastewater treatment stage effluents

In contrast to the markers from the more general FIB groups, either non-detectable or non-quantifiable densities of the HF183 and HumM2 genetic markers, that are currently being used or considered for MST applications, were observed in the majority of the secondary treatment stage effluent samples from the WWTPs in this study. Similar to a previous report (Shanks *et al.* 2009) which showed lower densities of the HumM2 marker in effluents from

**Table 2** | Geometric mean densities of culturable FIB (CFU) and qPCR-estimated FIB (CCE) in secondary-treated wastewater before (2°) and after (disinfected 2°) disinfection

Method	Wastewater	Log <sub>10</sub> CCE or CFU per 100 mL				
		<i>Bacteroides</i>	<i>Clostridium</i> <sup>a</sup>	<i>E. coli</i>	<i>Enterococcus</i>	Total Bacteroidales
qPCR	2°	5.20 (1.34)	4.27 (1.03)	4.43 (1.09)	4.56 (1.21)	5.72 (1.41)
	Disinfected 2° (Cl and UV)	5.44 (1.33)	4.30 (0.98)	4.48 (1.11)	4.56 (1.04)	6.01 (1.39)
	Cl Disinfected	5.54 (1.76)	4.40 (1.35)	4.75 (1.52)	4.78 (1.42)	6.38 (1.89)
	UV Disinfected	5.32 (2.01)	4.18 (1.38)	4.15 (1.57)	4.28 (1.52)	5.56 (2.02)
Culture	2°	2.80 (0.92)	2.95 (0.85)	3.90 (1.15)	3.61 (1.17)	–
	Disinfected 2° (Cl and UV)	1.93 <sup>b</sup> (0.83)	2.82 <sup>b</sup> (0.80)	2.36 <sup>b</sup> (0.81)	2.09 <sup>b</sup> (0.90)	–
	Cl Disinfected	2.61 (1.21)	3.10 (1.14)	2.80 (1.16)	2.41 (1.32)	–
	UV Disinfected	1.24 (0.87)	2.53 (1.05)	1.92 (1.06)	1.77 (1.00)	–

Standard error in parentheses.

<sup>a</sup>*C. perfringens* for culture.<sup>b</sup>Significant reduction (*p*-value < 0.001) in mean log<sub>10</sub> density after disinfection.

the primary wastewater treatment stage at 20 different WWTPs across the USA, this marker was detected less frequently than the HF183 marker in the secondary treatment stage effluents (Table 3). However, owing to their low densities, neither of these more specific genetic markers could be evaluated for effects of disinfection or seasonal effects in this study.

Results indicated that approximately 10 copies of the HumM2 marker could be quantified per reaction; however, 40 copies per reaction of the HF183 marker were needed (data not shown). These per reaction lower limits of quantitation translated to method limits of 1,200 copies per 10 mL of water sample for HumM2 and approximately 4,800 copies per 10 mL of sample for the HF183 assay with the total extract volumes used in this study. The filtration and extraction of larger water sample volumes, analyses of larger portions of the DNA extracts from the samples and/

or use of extract concentration methods yielding higher densities of target DNA (Shanks *et al.* 2010) may be needed to improve analytical sensitivity for quantifying these MST markers in secondary treatment stage effluents. Substantial removal of suspended organisms during secondary wastewater treatment also may contribute to the low densities of these more specific markers that were observed in the effluents. In effluents from primary treatment, HF183 genetic marker densities were reported to be at least 1–2 logs greater than the highest possible densities inferred from our results for secondary treatment stage effluents (Haugland *et al.* 2010). This is also supported by Srinivasan *et al.* (2011) where between 1 and 3.11 logs of *E. coli*, enterococci, and *B. thetaiotaomicron* densities were removed prior to chlorination in comparison to only 0.12–0.40 log removal after chlorine treatment. Overall, these results suggest that further studies using more sensitive methods are needed to determine the densities of human specific genetic markers in secondary treatment stage effluents and consequently the feasibility of using these markers as indicators of WWTP impacts on ambient waters.

**Table 3** | Detection frequency of HF183 and HumM2 Genetic Markers in non-disinfected and chlorine or UV disinfected secondary-treated wastewater (2°)

Marker	Wastewater	Samples analyzed	Samples detected	Percent detection
HF183	Nondisinfected 2°	22	13	59
	Cl disinfected 2°	12	7	58
	UV disinfected 2°	10	7	70
HumM2	Nondisinfected 2°	22	5	23
	Cl disinfected 2°	12	0	0
	UV disinfected 2°	10	5	50

### Implications for determining overall wastewater treatment efficacy

While qPCR increasingly has been investigated for its ability to provide same-day water quality results in recreational waters (Converse *et al.* 2009; Griffith *et al.* 2009; Lavender & Kinzelman 2009; Griffith & Weisberg 2011), results from

this study suggest that the densities of genetic markers from a variety of different FIB, as determined by the qPCR method, are not substantially reduced by current WWTP disinfection practices. Consequently, qPCR analyses for FIB genetic markers do not appear to be suitable to evaluate the efficacy of wastewater disinfection on the inactivation of bacterial pathogens. However, given that two of the three groups of pathogens of public health concern in wastewater, that is, enteric viruses and protozoa, are less effectively attenuated by the disinfection regimes commonly used at WWTPs (Chang *et al.* 1985; Tree *et al.* 1997), reliance on only a culturable bacterial indicator of fecal contamination that is quite susceptible to disinfection in current discharge permits may not provide sufficient protection of the recreational or drinking water source designated uses. Indeed, EPA's recently concluded NEEAR study, conducted at beaches primarily impacted by secondary treated and disinfected WWTP effluents, demonstrated a significant association between reported gastrointestinal illness and the enterococcus qPCR method used in the current study (Wade *et al.* 2006, 2008, 2010). Further analysis of this epidemiological data provided evidence that enteric viruses, such as norovirus, could account for the vast majority of those gastrointestinal illnesses in NEEAR (Soller *et al.* 2010). These results point to a need for discharge permits that include metrics to cover all three main groups of pathogens so as to protect the designated use and provide effective public health protection. Additional studies that further define the relationships between removal or decay rates of FIB genetic markers and viral and protozoan pathogens both during wastewater treatment and in the environment are needed to fully assess the potential usefulness of these markers as surrogates for these two non-bacterial pathogen groups.

## CONCLUSIONS

Current WWTP disinfection practices did not substantially reduce FIB genetic marker densities. Therefore, qPCR may not be suitable for monitoring the efficacy of wastewater disinfection on the inactivation of bacterial pathogens. More studies are needed to understand the relationship between the fates of genetic markers and viral and protozoan

pathogens through the wastewater treatment process and in the environment.

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