Science of the Total Environment 573 (2016) 826–830

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Short Communication

# Correlations between pathogen concentration and fecal indicator marker genes in beach environments

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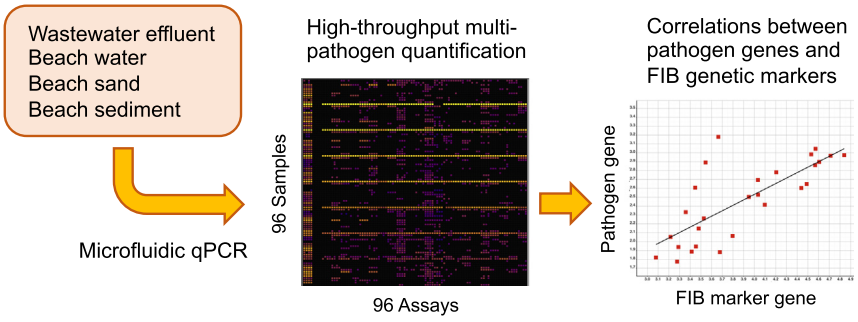
## HIGHLIGHTS

- Concentrations of various pathogens and FIB marker genes were directly quantified.
- Multiple pathogen genes were detected in beach water, sand, and sediment samples.
- Wastewater effluent and waterfowl may contribute to pathogen loading of Duluth harbor.
- Sample type influenced correlations between pathogens and FIB marker genes.
- Appropriate FIB markers need to be chosen for water/sand quality monitoring.

## GRAPHICAL ABSTRACT




## ARTICLE INFO

*Article history:*
Received 7 June 2016
Received in revised form 17 August 2016
Accepted 18 August 2016
Available online xxxx

Editor: D. Barcelo

*Keywords:*
Water quality
Fecal indicator
Pathogens
Genetic markers
Quantitative PCR

## ABSTRACT

The concentrations of several potential pathogens were measured using a microfluidic quantitative PCR (MFQPCR) platform in beach water, sand, and sediment samples collected in Duluth, MN. Among the 19 pathogen marker genes examined, *eaeA* from *Escherichia coli* and *plc* from *Clostridium perfringens* were most frequently detected in all samples. In beach water and wastewater samples, positive correlations were observed between quantities of potential pathogens and most of the fecal indicator genetic markers. Such correlations, however, were not frequently observed in sand and sediment samples. Our results suggest that the behavior of potential pathogens and FIB may vary by sample type and source of contamination. Consequently, appropriate FIB marker genes need to be chosen for reliable water/sand quality monitoring.



## 1. Introduction

Currently, levels of fecal indicator bacteria (FIB), such as *Escherichia coli* and enterococci, are monitored to evaluate the quality of water (e.g., recreational, drinking, and irrigation water) and to estimate the risks of human exposure to enteric pathogens (Boehm and Sassoubre, 2014; Edberg et al., 2000). For this purpose, culture-based approaches, including the membrane filter and most probable number methods are widely used (USEPA, 2002, 2009). Relationships between these FIB and pathogen concentrations, however, have not been well correlated in past studies (Ishii et al., 2014b; Oster et al., 2014). This is partly due to the occurrences of *E. coli* and enterococci

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http://dx.doi.org/10.1016/j.scitotenv.2016.08.122
0048-9697/

of non-fecal origin (Byappanahalli et al., 2012; Ishii and Sadowsky, 2008).

Culture-independent approaches, such as quantitative PCR (qPCR) are increasingly being used to measure concentrations of FIB genetic markers for more rapid and accurate water quality monitoring. While the *uidA*, Entero1, AllBac and HF183 marker genes/assays have been used to quantify *E. coli*, enterococci, total *Bacteroides*, and human *Bacteroides*, respectively (Eichmiller et al., 2013), relationships between concentrations of FIB and pathogen marker genes are still not well known. Without knowledge of these associations, it is difficult to assess health risks associated with contact with water and beach sands.

Recent technological developments now allow for the direct detection and quantification of multiple pathogens present in water samples. Several methods capable of achieving this goal have been reported, including microarrays, multiplex qPCR, and microfluidic qPCR (MFQPCR) platforms (Ishii et al., 2014a; Lemarchand et al., 2004). Among these, MFQPCR is promising for future water quality assessment because it can provide quantitative information on a large number of pathogens, which is essential for quantitative microbial risk assessment (QMRA) (Ishii et al., 2014b). The MFQPCR technique has been used to monitor and assess the quality of river, lake, and irrigation water systems (Byappanahalli et al., 2015; Ishii et al., 2014a, 2014b). However, application of MFQPCR has not been extensively tested to evaluate the microbiological quality of a beach system (e.g., beach water, sand, and sediment) and a potential source of pathogens (e.g., wastewater effluent). Because sand and sediment are important parts of a beach system impacting human health, their microbiological quality needs to be evaluated (Phillips et al., 2011; Whitman et al., 2014; Zhang et al., 2015a, 2015b).

Therefore, the objectives of this study were to: (1) analyze the occurrences of 19 pathogen maker genes in a beach system and wastewater effluent by using the MFQPCR technique, and (2) examine the relationship between FIB marker genes and pathogen concentrations in these matrices.

## 2. Materials and methods

### 2.1. Sample collection

Water, sand, and sediment samples were collected from a beach in the Duluth-Superior Harbor, Duluth, MN, monthly from June to October in 2010 and from May to September in 2011 (Eichmiller et al., 2013). A total of 89 samples (29 water, 30 sand, and 30 sediment samples) were obtained from this location. The beach is located approximately 100 m from the wastewater effluent discharge pipe, from which samples ($n = 30$) were also obtained. Turbidity, flow volume, and chlorine concentration data for the wastewater effluent were collected from a local water quality monitoring program (http://www.lakesuperiorstreams.org/streams/stream_data.html). Wind speed and rainfall data were collected from a local weather station (http://www.wunderground.com/q/zmw:55814.1.99999?sp=KMNDULUT34).

### 2.2. DNA extraction

DNA was extracted from the samples as described previously (Eichmiller et al., 2013). In brief, wastewater effluent and water samples were filtered through 0.45-μm-pore nitrocellulose filters (Millipore, Billerica, MA). The filter was cut into four pieces by using a sterile razor blade. DNA was extracted directly from the sliced filters by using PowerSoil DNA Isolation Kits (MoBio Laboratories, Carlsbad, CA). The same kit was used to extract DNA from homogenized sand and sediment samples (1 g each). The DNA was stored at −20 °C until used for conventional qPCR and MFQPCR.

### 2.3. MFQPCR and conventional qPCR

The MFQPCR technique was done using a 96.96 Dynamic Array chip (Fluidigm, South San Francisco, CA) and a BioMark HD Reader (Fluidigm) as described previously (Ishii et al., 2013). In order to increase the target molecules, specific target amplification (STA) reactions, which do not influence quantitative information (Ishii et al., 2013), were performed before MFQPCR, as described in Ishii et al. (2013). In this study the following potential pathogens were quantified using the identified assessed genes: total *E. coli* (*ftsZ* and *uidA*), potentially pathogenic *E. coli* (*eaeA*, $stx_1$, and $stx_2$), *Shigella* spp. (*ipaH* 7.8, *ipaH* all, and *virA*), *Salmonella* spp. (*invA* and *ttrC*), *Campylobacter jejuni* (*cadF* and *ciaB*), *Clostridium perfringens* (*cpe* and *plc*), *Legionella pneumophila* (*mip*), *Listeria monocytogenes* (*iap* and *hlyA*), *Vibrio cholera* (*ctxA*), and *Vibrio parahaemolyticus* (*tdh*) (Ishii et al., 2013). We considered the detection of these genes as the occurrences of potential pathogens, although some genes are occasionally present in non-infectious bacteria (e.g., non-pathogenic *eaeA*-positive *E. coli*) (Leroy et al., 1994). The assays were performed in quadruplicate. The amplification efficiencies ranged from 90 to 110% with the linear dynamic ranges from 2 to $2 \times 10^6$ copies per μL DNA. The goodness-of-fit ($r^2$) values for the standard curves were >0.99.

The DNA was also used to measure concentrations of FIB marker genes by conventional qPCR methods (Eichmiller et al., 2013). The target genes were Entero1 (for total enterococci; Ludwig and Schleifer, 2000), AllBac (for total *Bacteroides* spp.; Layton et al., 2006), and HF183 (for human-associated *Bacteroides*; Bernhard and Field, 2000). The amplification efficiencies ranged from 90 to 114% with the linear dynamic ranges from 3 to $3 \times 10^5$ copies per 5 μL DNA template. The $r^2$ values for the standard curves were >0.99.

### 2.4. Statistical analysis

Quantitative values of the 19 genes obtained by MFQPCR were processed as described by Ishii et al. (2014b). Quantitative values from MFQPCR and conventional qPCR were log-transformed to satisfy assumptions of normal distribution and equality of variances. Replicate values ($n = 4$) were averaged. When concentrations of target genes were below detection limit, we predicted the values by using the robust regression on order statistic (robust ROS) (Helsel, 2012; Palarea-Albaladejo and Martín-Fernández, 2015). This was repeated for 1000 times by the Monte Carlo method and the mean predicted values were assigned to each sample. The robust ROS and Monte Carlo method were used when frequency of below-detection-limit samples was smaller than 80%. We did not predict values when below-detection-limit samples were >80% because currently-available models cannot accurately predict values with such data (Helsel, 2012).

Pearson's product-moment correlation coefficient was used to evaluate correlations between the different genes (Helsel, 2012) and to evaluate the correlations between targeted genes with environmental variables (e.g., rainfall, wind speed, effluent turbidity, and chlorine concentration). Student's *t*-test was used to evaluate the detection frequency of genes in different samples. Correlations were considered significant at $\alpha = 0.05$. All statistical analyses were done using R version 3.2.0.

## 3. Results and discussion

### 3.1. Occurrence of potential pathogens in beach and wastewater environments

Several potential pathogens were detected in the wastewater and beach sands, sediments, and water samples collected in Duluth, MN (Fig. 1). Overall, total *E. coli* was most frequently detected by MFQPCR (Table 1). The *ftsZ* and *uidA* were present in 63–100% and 93–100% of samples respectively. Potentially pathogenic *C. perfringens* (*plc*)