Environmental Science
Processes & Impacts

PAPER

View Article Online
View Journal



Cite this: DOI: 10.1039/c7em00316a

# Estimating the probability of illness due to swimming in recreational water with a mixture of human- and gull-associated microbial source tracking markers†

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Beaches often receive fecal contamination from more than one source. Human sources include untreated sewage as well as treated wastewater effluent, and animal sources include wildlife such as gulls. Different contamination sources are expected to pose different health risks to swimmers. Genetic microbial source tracking (MST) markers can be used to detect bacteria that are associated with different animal sources, but the health risks associated with a mixture of MST markers are unknown. This study presents a method for predicting these health risks, using human- and gull-associated markers as an example. Quantitative Microbial Risk Assessment (QMRA) is conducted with MST markers as indicators. We find that risks associated with exposure to a specific concentration of a human-associated MST marker (HF) are greater if the HF source is untreated sewage rather than treated wastewater effluent. We also provide a risk-based threshold of HF from untreated sewage at a beach, to stay below a predicted illness risk of 3 per 100 swimmers, that is a function of gull-associated MST marker (CAT) concentration.

Received 12th July 2017
Accepted 24th October 2017

DOI: 10.1039/c7em00316a

rsc.li/espi



**Environmental significance**

The contamination of natural waters with pathogens can cause gastrointestinal illness in swimmers. Microbial source tracking markers offer the possibility of identifying pathogen sources, but there is no framework for interpreting their concentrations. We use a risk-based framework to gain insight into the risk of illness associated with exposure to microbial source tracking markers from gull and human feces. At the same concentration, exposure to human markers from sewage is associated with a greater risk than human markers from treated effluent. When both sewage and gull contamination are present in water, they can both contribute to the risk. The framework presented here can be used for a wide range of MST markers either alone or in combination.

## 1 Introduction

Recreational beaches are routinely monitored for fecal indicator bacteria (FIB) to assess risks from swimming. The EPA recommends threshold FIB concentrations for states to adopt into their water quality standards[28] that are protective for primary contact recreation. EPA criteria were developed, in part, using results of epidemiology studies[12,82] that show an increase in the occurrence of swimmer gastrointestinal illness with increasing FIB concentration. The relationship between FIB and swimmer illness is generally robust when the source of FIB is treated effluent[81,82] and sometimes holds when the source is stormwater runoff.[3,15,16,34,38,70] FIB, however, can come from a variety of contamination sources, including many different types of animal feces[10] and environmental reservoirs including beach sand and wrack.[42,84]

To aid the remediation of fecal contamination, microbial source tracking (MST) protocols have been developed over the past two decades. Most MST protocols use animal host-associated fecal DNA markers (hereafter referred to as MST markers) to aid in FIB source identification. Common MST markers identify intestinal bacteria of various animal hosts and are measured using molecular methods such as polymerase chain reaction (PCR). There are MST markers for a number of hosts including human, pig, gull, cow, and dog feces.[8]

According to a large-scale method evaluation study conducted using fecal sources from California, USA, one of the best performing human-associated MST markers is HF183 [46] (hereafter referred to as HF). HF is located within the 16S rRNA gene of human-associated *Bacteroides* spp.[5,33,41] The same large-scale method evaluation study identified the LeeSeaGull marker,[47] which is located on the 16S rRNA gene of *Catellicoccus marimammalium* and is hereafter referred to as CAT, as one of the

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c7em00316a

best performing gull-associated MST markers.[68] These two markers are particularly important: HF because it targets human feces, which can contain a large number of infectious human enteric pathogens,[71] and CAT because it targets gull feces, which can contain zoonotic bacterial pathogens[64] and are a widespread source of FIB for coastal beaches.[1,17,31,32,49,62]

Although MST markers have demonstrated potential for source tracking, difficulty still remains in using them in practice. In particular, no thresholds exist for interpreting their concentrations. This study seeks to establish thresholds for MST markers at which the USEPA illness benchmark (~3 illnesses per 100 swimmers) is exceeded using quantitative microbial risk assessment (QMRA).[36] We perform QMRA with Monte-Carlo simulations to predict the probability of illness ($P_{ill}$) associated with swimming in water with varying concentrations of MST markers. This QMRA approach has been used previously to estimate the risks of swimming at beaches contaminated with FIB from different animal sources,[71] from mixtures of human sources,[63] and from mixtures of human and animal sources.[64,69] It has also been used to estimate risks associated with individual MST markers: HF from raw sewage,[7] and CAT from gull feces,[9] as well as directly from pathogens.[18]

The first objective is to determine whether the HF source affects the predicted risk. HF is abundant in human feces, raw sewage and septage,[40,46] as well as biologically treated and disinfected effluent from wastewater treatment plants.[4,14,51,73] Boehm *et al.*[7] proposed a risk-based threshold for HF in recreational water, assuming a fresh, raw sewage source. Because many pathogens are removed from sewage by biological wastewater treatment and disinfection processes,[71] it is expected that $P_{ill}$ associated with swimming in recreational water with HF from an effluent source is less than if the HF comes from a sewage source. Thus, the first objective evaluates this assumption. The second objective is to estimate $P_{ill}$ when recreational water contains a mixture of HF and CAT. Many beaches likely have more than one animal source of fecal pollution.[11,29,59,62,67]

This mixed source scenario will establish a framework for studying mixtures using a fecal source mixture relevant to beaches.[1,11,23,29,59,62,64,67] The framework we present here can be amended to consider different mixtures of MST markers.

# 2 Methods

## 2.1 Sample collection

Final effluent samples were collected from 32 wastewater treatment facilities along coastal California, the majority from around the San Francisco Bay Area ($n = 15$) and Los Angeles/Orange Counties ($n = 12$), and the rest along the central coast. The plants ranged in capacity from 0.09 million gallons per day (MGD) to 400 MGD. Ten plants discharge directly to the ocean, and 22 discharge to freshwater rivers which eventually make their way to the ocean. All of the treatment trains included secondary biological treatment and clarification, and a majority also included subsequent disinfection processes with chlorine ($n = 20$) or ultraviolet light ($n = 4$).

Sampling was allowed at the plants provided that results would be anonymous, and thus exact plant locations are not disclosed. Samples were collected in acid-washed or bleached bottles. The bottles were rinsed with the sample water before collection. At treatment plants with chlorine disinfection, the sample was taken after the final treatment step of dechlorination with sodium bisulfite. The samples were transported on ice to the laboratory and processed within 6 hours of collection.

## 2.2 HF quantification

Between 50 and 500 ml of effluent samples, depending on turbidity, were filtered through polycarbonate 0.4 μm pore size filters (EMD Millipore, Billerica, MA).[8] A filtration blank was run per 12 samples, and involved filtering approximately 250 ml of deionized (DI) water through a filter. The filters were stored at −80 °C (in a freezer or on dry ice) for a maximum of 60 days before DNA extraction. DNA was extracted from filters with a DNA-EZ ST1 kit (Generite, North Brunswick, NJ). This kit has been shown by others to perform well with acceptable efficiencies.[19,66] One extraction blank, without a filter, was run per 22 samples. Extracted DNA was stored for a maximum of 30 days at −20 °C before analysis.

HF concentrations were quantified with the HF183 quantitative polymerase chain reaction (qPCR) assay following the protocol of Haugland *et al.*,[41] as modified by Green *et al.*[33] for simplex qPCR. Standards consisted of a 167-base pair *Bacteroides dorei* reference sequence[33] inserted into a plasmid (Integrated DNA Technologies, Coralville, IA). Forward primers,[5] as well as reverse primers and probes,[33] are given in Table S1.† Standard curves from individual plates were pooled to create a master standard curve, with serial dilutions ranging from $10^0$ to $10^6$ copies per μl.

Standard curve performance descriptors are given in Table S2.† Analytical triplicates were performed on all standards, samples, and negative controls. qPCR was performed on an Applied Biosystems StepOne Plus Real Time qPCR instrument. One filtration blank, one extraction blank, and one no-template control of molecular-grade water were run on each 96-well plate. Copies per reaction measured by qPCR were converted to copies per 100 ml effluent using dimensional analysis taking into account the volumes filtered, and DNA extraction eluant volumes. The lower limit of quantification (LLOQ) was determined with the qPCR standard curve, as the lowest dilution (20 copies per reaction) at which all of the analytical triplicates amplified. There were 2 samples for which only 2 of the analytical triplicates amplified, and the amplification was less than 20 copies per reaction. Those samples were assigned a concentration of half the LLOQ for data analysis and plotting.

Inhibition was tested using the spike-and-dilute method.[13] Three wastewater effluent samples were chosen randomly to test for inhibition. Purified standard plasmid concentrate was added to sample DNA extracts to guarantee a minimum concentration of 750 copies per μl. The spiked sample extracts were then diluted fivefold in triplicate, for dilutions of 5×, 25×, and 125×. With the HF183 assay amplification efficiency of 94% (Table S2†), the expected $C_q$ difference between five-fold

 

dilutions is 2.55 ($1.88^{2.55} = 5$). If the difference in $C_q$ between dilutions is less than expected without dilution, then the sample is considered inhibited.

### 2.3 Fitting distributions

The effluent HF concentrations were $\log_{10}$ transformed. Probability density functions were created using the Distribution Fitting Application in MATLAB (Natick, MA). Distributions were fit separately to three data sets: (1) all effluent data; (2) the subset of data from plants that perform disinfection (either by chlorination or by UV treatment); and (3) the subset of data from plants that perform disinfection by chlorination only.

### 2.4 QMRA

QMRA was conducted to predict the $P_{ill}$ associated with swimming in recreational water with varying concentrations of MST markers from different fecal contamination sources. In addition to the concentration distribution for HF treated effluent reported in this study, MST marker data were obtained for HF in raw sewage from Shanks *et al.*[65] and for CAT in gull feces from Brown *et al.*[9]

Common enteric pathogens from sewage, effluent, and gull feces were taken from literature reviews conducted by Soller *et al.*[71] and Schoen and Ashbolt.[64] The use of reference pathogens is an accepted practice in QMRA studies.[52,71,80] Bacteria, protozoa, and a virus are included as reference pathogens to represent the fate and transport processes that vary among these categories.[71] The reference pathogens for sewage are *Campylobacter*, *Salmonella*, *E. coli* O157:H7, *Cryptosporidium*, *Giardia*, and norovirus, for effluent are *Cryptosporidium*, *Giardia*, and norovirus, and for gull feces are *Campylobacter* and *Salmonella*.

For a given concentration of an MST marker in recreational water, the QMRA calculates doses of each reference pathogen, and then predicts the probabilities of infection and illness associated with those doses. Matlab was used to run Monte Carlo simulations ($n = 10\,000$ trials for each MST marker concentration). In each trial, the model drew input variables from statistical distributions, described in the following section, to incorporate their inherent variability.

### 2.5 Estimating the reference pathogen dose

The pathogen dose, $\mu_{rp}$, of each reference pathogen from a certain source incidentally ingested during recreational water contact was estimated by using eqn (1):[71]

$$\mu_{rp}^{S} = \frac{C_{MST}}{F_{MST}^{S}} \times R_{rp}^{S} \times p_{rp}^{S} \times V \quad (1)$$

where S, rp, and MST denote the fecal source, reference pathogen, and MST marker, respectively, $C_{MST}$ is the concentration of the MST marker in ambient seawater [copies per volume], $F_{MST}^{S}$ is the concentration of the MST marker in the fecal source [copies per (volume or mass)], $R_{rp}^{S}$ is the concentration of a reference pathogen in the source [number of pathogens per (volume or mass)], $p_{rp}^{S}$ is the fraction of pathogenic species or serotypes that are infectious to humans [unitless], and $V$ is the volume of seawater ingested.

For a fixed value of $C_{MST}$, stepping by an order of magnitude at a time from $10^0$ to $10^5$ copies per 100 ml, a distribution of $\mu_{rp}^{S}$ was generated using a 10 000-trial Monte Carlo simulation. In the mixed-source model, $\mu_{rp}^{S}$ was calculated independently for each source, and then added together to find the total dose $\mu_{rp}$. Each of the remaining parameters on the right-hand side of eqn (1) was drawn randomly for a single trial from a distribution of values (Table 1).

Norovirus concentration data were obtained from meta-analyses of published studies.[24,56] Pouillot *et al.*[56] reported the $\log_{10}$-transformed reduction in concentration due to wastewater treatment, $L_{noro}$, rather than for wastewater effluent concentration directly. Therefore, the concentration of norovirus in effluent was calculated as $10^{R_{noro}^{sewage} - L_{noro}}$, where $R_{noro}^{sewage}$ is drawn randomly from a $\log_{10}$-normal distribution and $L_{noro}$ from a $\log_{10}$-uniform distribution with parameters given in Table 1.

### 2.6 Estimating the probability of illness

During each trial, the calculated dose was input to a dose–response relationship to predict the probability of infection due to a particular reference pathogen, $P_{inf,rp}$. The dose–response relationships were taken from feeding studies and outbreak data and vary in the mathematical form. They are summarized in Table 2.

In the feeding and outbreak studies, the exact dose that individuals ingest is not known; rather, the mean dose is estimated. In QMRA, we are considering the effect of an exact dose, and therefore it is necessary to use the conditional dose–response relationships that correspond to the averaged dose–response relationships that are reported by feeding and outbreak studies.[35]

The dose–response data for *Cryptosporidium* and *Giardia* have been fit with an exponential model (eqn (2)).[26,60] The exponential model assumes that there is a constant probability, $r$, of a single pathogen causing infection in a human host.[52]

$$P_{inf,rp} = 1 - (1 - r)^{\mu_{rp}} \quad (2)$$

For *Salmonella*,[76] *E. coli* O157:H7,[77] *Campylobacter*[75] and norovirus,[77] $r$ is not constant among human hosts, but rather is described by a beta distribution. In this case, the conditional dose–response curve takes the beta-binomial form given in eqn (3).[52]

$$P_{inf,rp} = 1 - \frac{B(\alpha, \beta + \mu_{rp})}{B(\alpha, \beta)} \quad (3)$$

where $B$ is the standard beta function, and $\alpha$ and $\beta$ are parameters for beta-distributed mean host sensitivities.[35]

For norovirus, a fractional Poisson dose–response model, also given in Table 2, has been proposed more recently.[54] The fractional Poisson model includes a parameter for the fraction of perfectly susceptible hosts, as well as a parameter for aggregate size.[54] The beta-binomial model assumes disaggregated virus particles.[78]

Table 1 Distribution parameters for variables used to estimate the dose $\mu_{rp}^{S}$ (eqn (1)). *n* refers to colony forming units (CFUs) or the most probable number (MPN) for *E. coli* O157:H7, *Campylobacter* and *Salmonella*; oocysts for *Cryptosporidium*, cysts for *Giardia*, and genomes for norovirus. ND: not detected; NA: not applicable (not present in this source). $R_{rp}$ is the concentration of a reference pathogen [number of pathogens per (L or g)], $F_{MST}$ is the concentration of the MST marker [copies per (ml or g)], $p_{rp}$ is the fraction of pathogenic species or serotypes that are infectious to humans [—], and *V* is the volume of seawater ingested [ml].

| | | Source | | |
|---|---|---|---|---|
| Variable | | Sewage | Disinfected effluent | Gull feces |
| $R_{rp}$ [*n*/L or g] | *E. coli* O157:H7 | −1, 3.2[a,f] | ND[f] | ND[f] |
| | *Campylobacter* | 3.0, 4.6[a,g] | ND[g] | 3.3, 6[a,g] |
| | *Salmonella* | 0.5, 5.0[a,i] | ND[i] | 2.3, 9.0[a,h] |
| | *Cryptosporidium* | −0.5, 3.0[a,j] | −1.3, 1.6[a,j] | ND[h] |
| | *Giardia* | −0.3, 4.2[a,j] | −1.3, 2.8[a,j] | ND[h] |
| | Norovirus | 4.7, 1.5[b,k] | −4.6, −1.1[d,l] | NA[h] |
| $F_{MST}$ [copies per ml or g] | | 5.2, 0.57[b,m] | 3.6, 2.4[c,n] | 8.7, 8.3[c,h] |
| $p_{rp}$ [—] | | 1[f] | 1[f] | 0.01, 0.4[e,f] |
| *V* [ml] | | 1.15, 0.045[b,o] | 1.15, 0.045[b,o] | 1.15, 0.045[b,o] |

[a] Upper and lower bounds of a $\log_{10}$-uniform distribution. [b] Mean and standard deviation of a $\log_{10}$-normal distribution. [c] Scale and shape parameters of a $\log_{10}$-Weibull distribution. [d] Upper and lower bounds of $\log_{10}$-uniform removal during treatment. [e] Upper and lower bounds of a uniform distribution. [f] Garcia-Aljaro *et al.*[30] [g] Arimi *et al.*,[2] Koenraad,[44] and Stampi *et al.*[72] [h] Brown *et al.*[9] [i] Koivunen *et al.*[45] and Lemarchand and Lebaron.[48] [j] Dungeni and Momba,[22] Harwood *et al.*,[39] and Kitajima *et al.*[43] [k] Eftim *et al.*[24] [l] Pouillot *et al.*[56] [m] Boehm *et al.*[7] [n] This study. [o] Dufour *et al.*[21]

In the present study, the two norovirus dose–response relationships were used in conjunction to define a dose–response "envelope", following the method of Soller *et al.*[70] The fractional Poisson relationship predicts a lower illness rate than the beta-binomial relationship. Thus, these relationships define the lower and upper bounds of the envelope, respectively. Each trial sampled the $P_{inf,noro}$ envelope by randomly weighting the lower and upper bounds and summing them.

In general for all reference pathogens, it is assumed that illness is contingent upon infection, such that the probability of illness for a particular reference pathogen, $P_{ill,rp}$, is found by multiplying $P_{inf,rp}$ by the probability of illness given infection, $P_{ill|inf,rp}$. $P_{ill|inf,rp}$ has been described as a constant value drawn from a uniform distribution for all reference pathogens,[27] but has also been described as the dose-dependent function given in eqn (4) for *Salmonella*[79] and *Campylobacter*.[75] The dose-dependent $P_{ill|inf,rp}$ relationships were developed based on data from both feeding and outbreak studies. They include response data for not only adults, but also children, as well as responses to lower pathogen doses. The effect of using these relationships instead of constant values was examined with a sensitivity analysis, as described below.

Eqn (4) represents a case in which a greater dose results in greater pathogen growth inside the host, and thus a longer period of infection during which illness may occur.[76] At low doses, infection is less likely to result in illness, but as dose increases, the rate of infection approaches the rate of illness. $\eta$ and $\rho$ are parameters describing the distribution of infection

Table 2 Reference pathogen dose–response curves for the probability of infection for a dose of a reference pathogen, $P_{inf,rp}$, and relationship or distribution for the probability of illness given infection, $P_{ill|inf}$. The "constant" entries for *Campylobacter* and *Salmonella* (below the double horizontal line) replaced the "dose-dependent" entries for these pathogens in the sensitivity analysis

| Reference pathogen | $P_{inf,rp}$ model | $P_{inf,rp}$ parameters | $P_{ill\|inf,rp}$ | Reference |
|---|---|---|---|---|
| *E. coli* O157:H7 | $1-\frac{B(\alpha,\beta+\mu_{rp})}{B(\alpha,\beta)}$ | $\alpha=0.248$, $\beta=48.8$ | 0.2–0.6[a] | Teunis *et al.*[77] |
| *Campylobacter* (dose-dependent) | | $\alpha=0.024$, $\beta=0.011$ | $\eta=3.6\times10^{-9}$, $\rho=2.4\times10^{8}$[b] | Teunis *et al.*[75] |
| *Salmonella* (dose-dependent) | | $\alpha=0.0085$, $\beta=3.14$ | $\eta=69.0$, $\rho=8.23$[b] | Teunis *et al.*[79] |
| Norovirus, upper bound | | $\alpha=0.04$, $\beta=0.055$ | 0.3–0.8[a] | Teunis *et al.*[78] |
| Norovirus, lower bound | $P(1-e^{\mu_{rp}/\lambda})$ | $P=0.72$, $\lambda=1106$ | 0.3–0.8[a] | Messner *et al.*[54] |
| *Cryptosporidium* | $1-(1-r)^{\mu_{rp}}$ | $r=0.09$ | 0.2–0.7[a] | EPA[26] |
| *Giardia* | | $r=0.0199$ | 0.2–0.7[a] | Rose and Gerba[60] |
| *Campylobacter* (constant) | $1-\left(1+\frac{\mu_{rp}}{\beta}\right)^{-\alpha}$ | $\alpha=0.145$, $\beta=7.59$ | 0.1–0.6[a] | Medema *et al.*[53] |
| *Salmonella* (constant) | | $\alpha=0.3126$, $\beta=2884$ | 0.2 | Haas *et al.*[37] |

[a] Lower and upper bounds of a uniform distribution.[27] [b] Eqn (4) parameters.

 

duration.[79] $P_{ill|inf,rp}$ values/parameters for all reference pathogens are given in Table 2.

$$P_{ill|inf,rp} = 1 - (1 + \eta\mu_{rp})^{-\rho} \quad (4)$$

Finally, the probability of illness due to exposure to the combination of reference pathogens, $P_{ill,sum}$, is found with eqn (5), which combines separate and statistically independent exposures:[58,71]

$$P_{ill,sum} = 1 - \prod_{rp}(1 - P_{ill,rp}) \quad (5)$$

### 2.7 Human sources

The first QMRA was conducted to estimate the risk of swimming in water with different levels of HF from a treated effluent source. Based on the available published data, there are no significant differences in the removal/inactivation of *Giardia*, *Cryptosporidium*, or norovirus between chlorination and UV disinfection processes.[56,61] The $R_{rp}^{effluent}$ distributions (Table 1) represent both process types. For consistency, $F_{HF}$ values were drawn from the HF concentration distribution containing values from disinfected effluent, both chlorinated and UV-disinfected (Fig. 1b).

In the QMRA examining risk predicted from exposure to HF from treated effluent, the ratio $f = C_{HF}/F_{HF}$ represents the volume of treated effluent present per volume of ambient water [ml per 100 ml]. The upper physical limit of $f$ is 100 ml treated effluent per 100 ml ambient water. Some of the simulated effluent trials run at high values of $C_{HF}$, in which a low value of $F_{HF}^{effluent}$ was drawn, violated the limit on $f$. In those cases, the model drew a new value from the $F_{HF}^{effluent}$ distribution until $f$ was less than 100 ml effluent per 100 ml ambient water. Drawing a new value of $F_{HF}^{effluent}$ is justified, as the effluent QMRA scenario considers bathing water contaminated with HF from effluent only.

### 2.8 Mixed sources

The second QMRA was conducted to estimate the risk of swimming in water with a mixture of MST markers. Most beaches have more than one source of fecal contamination, and wildlife is a potential, but poorly understood, source. In this study, we use gulls as an example, for two reasons. First, they are often present at beaches.[1,17,23,47,49] Second, they have a lower "potency" than human feces, that is, a lower per-mass likelihood of human infection.[64,71]

The QMRA was run by performing Monte Carlo simulations inside nested loops. The outer loop stepped $C_{HF}$ incrementally from $10^0$ to $10^5$ copies per 100 ml, and the inner loop stepped $C_{CAT}$ incrementally from $10^0$ to $10^5$ copies per 100 ml. Distributions of $P_{ill,sum}$ were found for each combination of HF and CAT concentrations.

The reference pathogens for gull feces, *Campylobacter* and *Salmonella*,[64] are also found in raw sewage.[71] The literature values of concentrations in raw sewage and gull feces, as well as the fraction of infectious species/strains in gull feces, are given in Table 1. The doses of infectious pathogens were calculated independently for each source, and then added together. The summed dose was then entered into the corresponding dose–response relationships to find $P_{inf,rp}$ and $P_{ill,rp}$.

### 2.9 Sensitivity analysis

Two sensitivity analyses were conducted to examine how individual model components affected risk predictions. The first sensitivity analysis was conducted for the effluent scenario. It tested the effects of changes in the input variables following the method of Xue *et al.*[83] Briefly, the 25th, 50th, and 75th percentiles (p25, p50, and p75, respectively) of each model input variable were calculated from the variable's distribution. The model was run with the p25 and p75 values of a given variable to find a single value of $P_{ill,sum}$, while holding all other model variables constant at their median values. The ratio of $P_{ill,sum}$ at the p75 value to $P_{ill,sum}$ at the p25 value was calculated. p75 : p25 = 1 indicates no change in $P_{ill,sum}$ due to a changing input value; p75 : p25 > 1 indicates an increase in $P_{ill,sum}$ with an increasing input value, and p75 : p25 < 1 indicates a decrease in $P_{ill,sum}$ with an increasing input value. The model is most sensitive to variables where $|p75 : p25| \gg 1$.

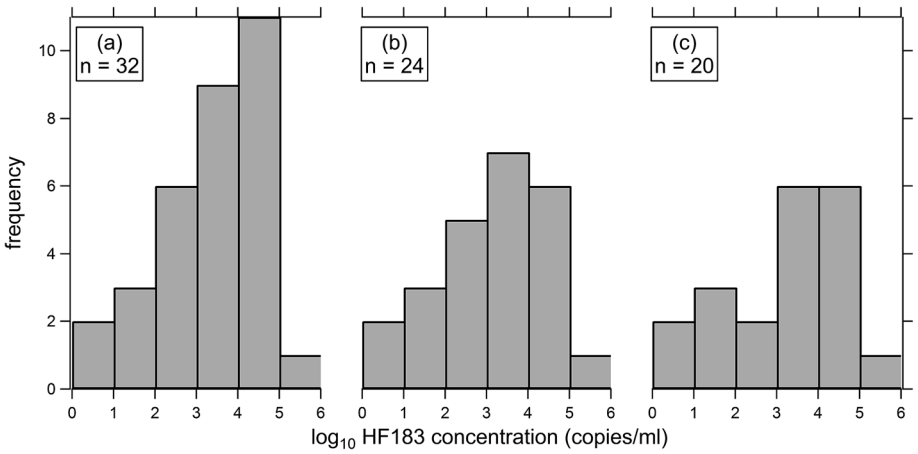


Fig. 1 Histograms of the effluent data from (a) all treatment plants, (b) treatment plants with chlorine or UV disinfection, and (c) treatment plants with chlorine disinfection.

The second sensitivity analysis was conducted for the sewage and gull scenarios. It investigated the effect of using a different pair of $P_{inf,rp}$ and $P_{ill|inf,rp}$ for both *Campylobacter* and *Salmonella*. It was not conducted for effluent because these two pathogens are generally not detected in effluent. Our sensitivity analysis compared a pair of equations we will refer to as "constant", consisting of a beta-Poisson $P_{inf,rp}$ and a constant value of $P_{ill|inf,rp}$, to the pair we will refer to as "dose-dependent" given by eqn (3) and (4). We compare pairs, rather than individual equations, because the studies which developed eqn (3) and (4)[75,79] fit $P_{inf,rp}$ and $P_{ill|inf,rp}$ relationships simultaneously to outbreak data, because the rates of infection and illness were not known separately. The equations used herein as the constant pair were developed independently in separate studies. All equations are summarized in Table 2. Linear regressions of median $P_{ill,sum}$ for the two cases were compared.

# 3 Results

## 3.1 Effluent HF concentration distributions

HF was detected in all but two of the 32 treated effluent samples. The two samples with HF concentrations below the LLOQ came from facilities using chlorine disinfection. HF was not detected in any of the process blanks. No sample inhibition was detected using the spike-and-dilute tests. The data set is provided in Table 4.

The data sets of $\log_{10}$ transformed concentrations from (1) all effluent, (2) disinfected effluent, and (3) chlorine-disinfected effluent were described by Weibull distributions with scale parameters $a$ and shape parameters $b$. The parameter values $\pm$95% confidence intervals for all effluent data were $a = 3.7 \pm 0.48$ and $b = 2.8 \pm 0.85$, for disinfected effluent data were $a = 3.6 \pm 0.64$ and $b = 2.4 \pm 0.83$, and for the chlorine-disinfected effluent data were $a = 3.6 \pm 0.77$ and $b = 2.2 \pm 0.85$. The histograms for all data and disinfected effluent data are shown in Fig. 1. Most of the effluent HF concentrations, regardless of the occurrence or type of disinfection, fall between $10^3$ and $10^5$ copies per ml.

## 3.2 QMRA for human fecal sources

Box and whisker plots in Fig. 2 show predicted $P_{ill,sum}$ distributions as a function of $\log_{10} C_{HF}$. $P_{ill,sum}$ increases with increasing $C_{HF}$. The medians of the $P_{ill,sum}^{effluent}$ and $P_{ill,sum}^{sewage}$ distributions exceed the 0.03 threshold (3 cases of illness per 100 swimmers) when $C_{HF} > 10^5$ and $10^3$ copies per 100 ml, respectively. The median $P_{ill,sum}^{effluent}$ is less than the median $P_{ill,sum}^{sewage}$ at all $C_{HF}$.

Linear regressions were performed to compare the relationships between the $\log_{10} P_{ill,sum}$ and $\log_{10} C_{HF}$ for effluent and sewage (Fig. 3). The regression for the median $P_{ill,sum}$ had a slope, $y$-intercept, and $R^2$ of 0.71, $-4.6$, and 0.94, respectively, for effluent, and 0.76, $-4.0$, and 0.98, respectively, for sewage. Based on the regression, the median of the $P_{ill,sum}$ distribution exceeds the threshold of 0.03 when $C_{HF} = 10^{4.3}$ copies per 100 ml for effluent, and when $C_{HF} = 10^{3.2}$ copies per 100 ml for sewage. Linear regressions for the 25th and 75th percentiles were used to bound a range of $C_{HF}$ at which risk equals 0.03. This range is $10^{3.0} < C_{HF} < 10^{5.7}$ copies per 100 ml for effluent

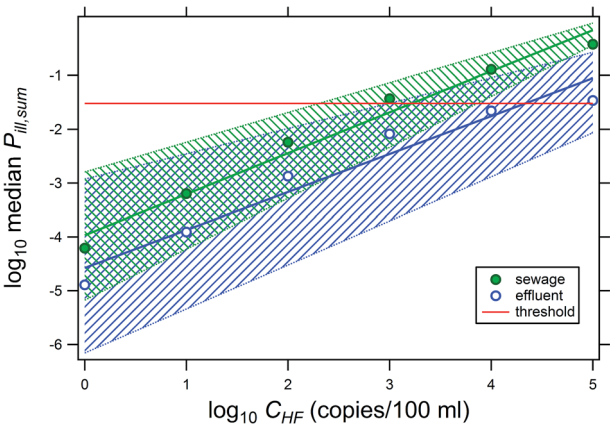


Fig. 3 Median values of the $P_{ill,sum}$ distributions for sewage and effluent, plotted with linear regression lines. Shading indicates the area between the 25th and 75th percentiles: green for sewage and blue for effluent. The red line indicates the threshold of 3 cases of illness per 100 swimmers.

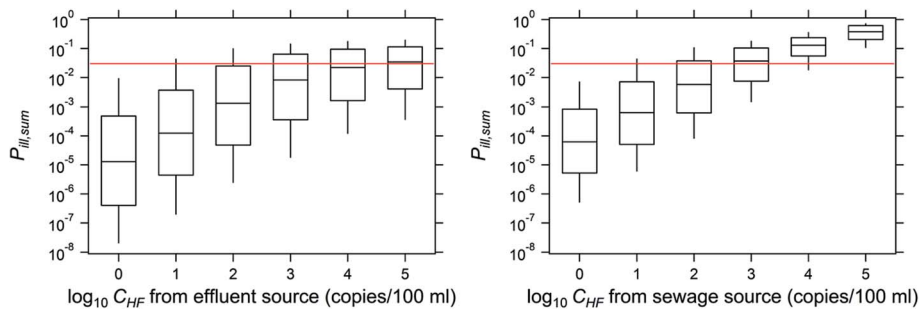


Fig. 2 Box and whisker plots of the $P_{ill,sum}$ distributions *versus* $\log_{10} C_{HF}$ from disinfected effluent (left) and untreated sewage (right). Midlines of boxes represent the medians (50th percentile) of the distributions, tops and bottoms of boxes represent 75th and 25th percentiles, respectively, and upper and lower whiskers represent the 90th and 10th percentiles, respectively. The red line indicates the threshold of 3 cases of illness per 100 swimmers.

 

and $10^{2.3} < C_{HF} < 10^{3.9}$ copies per 100 ml for sewage. The results for sewage are consistent with those reported by Boehm *et al.*[7]

$P_{ill,rp}^{effluent}$ data for individual pathogens are shown in Fig. S1–S3.† Comparison of these figures shows that norovirus contributes the most to $P_{ill,sum}^{effluent}$, with relatively low contributions from *Cryptosporidium* and *Giardia*. Norovirus also contributes the most to $P_{ill,sum}^{sewage}$ (data not shown), a result also observed by Boehm *et al.*[7]

At high values of $C_{HF}$, a random draw of $F_{HF}^{effluent}$ was frequently too low to result in that level of ambient contamination, resulting in an *f* violation (Table 3). In other words, based on the levels of $F_{HF}$ measured in effluent, it is unlikely that effluent would contaminate surface water at a concentration greater than $10^4$ copies per 100 ml.

### 3.3 QMRA for mixed sources

As discussed in the previous section, the median of the $P_{ill,sum}^{sewage}$ distribution is always greater than that of $P_{ill,sum}^{effluent}$ for a given $C_{HF}$. There are many cases of beaches with an unknown HF source. In those cases, assuming that the source is sewage is conservative with respect to protecting public health. The QMRA herein for a mixture of HF and CAT was therefore conducted assuming a sewage source of HF.

The contour plot of $P_{ill,sum}$ as a function of $C_{HF}$ and $C_{CAT}$ in ambient water is shown in Fig. 4. The contours were created using IGOR Pro (Wavemetrics) which uses Delaunay triangulation to interpolate between QMRA-generated data points. Curvature in the contour lines of constant $P_{ill,sum}$ indicates that both sewage and gull feces contribute to $P_{ill,sum}$ at the corresponding levels of HF and CAT, whereas no curvature indicates that only one source contributes to $P_{ill,sum}$. For example, when $C_{HF} = 10^4$ copies per 100 ml, the $P_{ill,sum}$ is constant (no curvature) at 0.15 as $C_{CAT}$ increases from $10^0$ to $10^3$ copies per 100 ml, indicating that the presence of gull feces does not contribute to the risk at that level of sewage contamination. On the other hand, when $C_{HF} = 10^2$ copies per 100 ml, $P_{ill,sum}$ depends on $C_{CAT}$, as lines of constant $P_{ill,sum}$ are curved at all $C_{CAT}$ concentrations. At the lowest levels of $C_{CAT}$ or $C_{HF}$ tested, the output of the mixed source model agrees with the output of the individual gull feces and sewage models, respectively (Brown *et al.*[9] and Fig. 2). As $\log_{10} C_{CAT}$ goes to zero, the threshold $C_{HF} = 10^{2.8}$ copies per 100 ml. This value is consistent with Fig. 2, in which the median at $C_{HF} = 10^3$ copies per 100 ml

Table 3 Number of times that the constraint of 100 ml effluent per 100 ml ambient seawater was violated and $F_{HF}$ was redrawn. The total number of draws was 10 000 + the number of *f* violations

| $\log_{10} C_{HF}$ (copies per 100 ml) | Number of *f* violations | Percentage of draws resulting in violation |
|---|---|---|
| 0 | 0 | 0 |
| 1 | 0 | 0 |
| 2 | 0 | 0 |
| 3 | 480 | 4.6% |
| 4 | 2223 | 18% |
| 5 | 4879 | 33% |

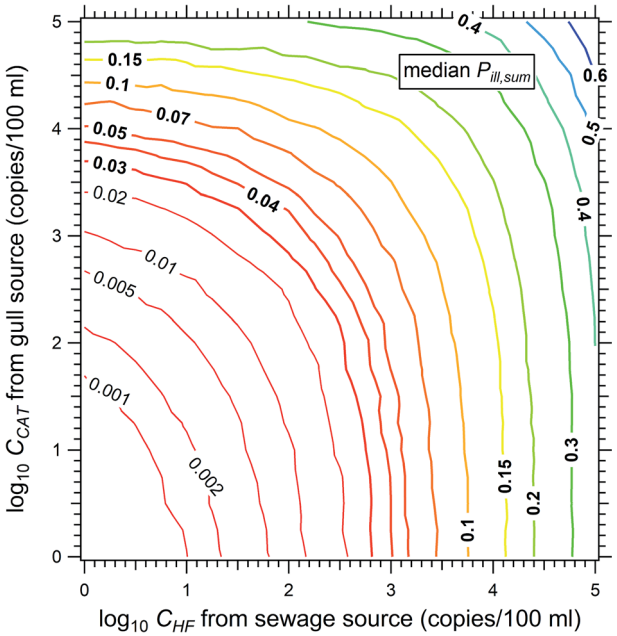


Fig. 4 Median $P_{ill,sum}$ at different mixed concentrations of the HF183 human marker ($C_{HF}^{sewage}$) and the gull marker ($C_{CAT}$) in recreational water.

exceeds the threshold illness risk of 0.03. This value is slightly different from the threshold value for sewage obtained by linear regression of median $\log_{10} P_{ill,sum}$ *versus* $\log_{10} C_{HF}$ ($10^{3.2}$ copies per 100 ml). The difference can be seen in Fig. 3; at $C_{HF} = 10^3$ copies per 100 ml the risk predicted by the regression is less than the median risk from the QMRA run.

The threshold level of HF at a beach with both sewage-sourced HF and gull-sourced CAT to maintain a risk level of 0.03 depends on CAT concentrations *via* the following empirical formula:

$$\log_{10} C_{HF} = 2.95 + \frac{-2.85}{(\log_{10} C_{CAT} - 4.55)^2 + 0.26} \quad (6)$$

where $C_{HF}$ and $C_{CAT}$ are in the units of copies per 100 ml. This formula was obtained using the curve fitting function in IGOR PRO to fit values from the $P_{ill,sum} = 0.03$ contour in Fig. 4 to the falling limb of a Lorentzian peak; this provided the best empirical fit to the data. According to this formula, for example, when $C_{CAT} = 10^2$ copies per 100 ml, then $C_{HF}$ should not exceed $10^{2.5}$ copies per 100 ml.

### 3.4 Sensitivity analysis

The sensitivity analysis of the effluent scenario (Table S3†) showed that at low levels of $C_{HF}$ the model is most sensitive to the concentration of norovirus in effluent, $R_{noro}$, while at high levels of $C_{HF}$ the model is approximately equally sensitive to $R_{noro}$ and the concentration of HF in effluent, $F_{HF}$. For all $C_{HF}$, the third-ranked sensitivity parameter was the removal of norovirus in wastewater treatment, $L_{noro}$.

The comparison of the linear regressions of $P_{ill,sum}^{sewage}$ for the constant and dose-dependent cases is shown in Fig. S4.† Recall

Table 4 Concentrations of the human fecal marker in the final effluent from coastal California wastewater treatment plants, measured by using the HF183 assay. Average and standard deviations are taken from qPCR analytical triplicates

| Sample ID | Disinfection type | Average concentration (copies per ml) | Standard deviation (copies per ml) |
|---|---|---|---|
| 1 | None | 10 069 | 362 |
| 2 | None | 1888 | 33 |
| 3 | None | 3443 | 90 |
| 4 | None | 122 | 3 |
| 5 | None | 11 556 | 162 |
| 6 | None | 17 178 | 277 |
| 7 | None | 14 744 | 48 |
| 8 | None | 14 524 | 384 |
| 9 | Chlorine | 590 808 | 23 943 |
| 10 | Chlorine | 115 | 2 |
| 11 | Chlorine | 41 | 11 |
| 12 | Chlorine | 3[a] | 0 |
| 13 | Chlorine | 12 269 | 696 |
| 14 | Chlorine | 91 | 3 |
| 15 | Chlorine | 1744 | 84 |
| 16 | Chlorine | 2[a] | 0 |
| 17 | Chlorine | 196 | 9 |
| 18 | Chlorine | 5297 | 147 |
| 19 | Chlorine | 19 | 2 |
| 20 | Chlorine | 6218 | 52 |
| 21 | Chlorine | 6733 | 126 |
| 22 | Chlorine | 26 284 | 902 |
| 23 | Chlorine | 17 025 | 1191 |
| 24 | Chlorine | 47 665 | 410 |
| 25 | Chlorine | 39 637 | 3595 |
| 26 | Chlorine | 78 122 | 6099 |
| 27 | Chlorine | 6127 | 246 |
| 28 | Chlorine | 6667 | 172 |
| 29 | UV | 682 | 12 |
| 30 | UV | 176 | 31 |
| 31 | UV | 7353 | 58 |
| 32 | UV | 755 | 41 |

[a] Indicates a concentration less than the detection limit that was replaced by a value of half the LLOQ.

that each $P_{ill|inf,rp}$ is paired with a specific $P_{inf,rp}$; constant $P_{ill|inf,rp}$ with a beta-Poisson $P_{inf,rp}$, and dose-dependent $P_{ill|inf,rp}$ with a beta-binomial $P_{inf,rp}$ (Table 2). At $C_{HF} < 10$ copies per 100 ml, the constant case predicts slightly greater risk, and at $C_{HF} >$ 10 copies per 100 ml, the dose-dependent case predicts greater risk that increases with $C_{HF}$. For the constant case, the linear regression of median $P_{ill,sum}^{sewage}$ crosses the illness threshold of 0.03 at $C_{HF} = 10^{3.5}$ copies per 100 ml. For gull feces (Fig. S5†), the predicted risk is much less using the constant case; the linear regression of median $P_{ill,sum}^{gull}$ crosses the illness threshold of 0.03 at $C_{CAT} = 10^{5.6}$ copies per 100 ml, compared to $C_{CAT} = 10^{3.8}$ copies per 100 ml for the dose-dependent case (Brown *et al.*[9] and Fig. S5†). Because *Salmonella* and *Campylobacter* are the only reference pathogens in gull feces, this choice has a greater effect on $P_{ill,sum}^{gull}$ than on $P_{ill,sum}^{sewage}$. The contour plot of $P_{ill,sum}$ as a function of $C_{HF}$ and $C_{CAT}$ generated for the constant case is shown in Fig. S6.† It is clear from this plot that the choice of one pair of equations *versus* the other has a large effect on the extent to which gulls contribute to risk at beaches with gull and sewage contamination.

# 4 Discussion

## 4.1 HF in effluent

HF was detected in 94% of the effluent samples, indicating that the MST DNA markers remain intact through biological treatment and disinfection. HF has been measured previously in treated wastewater effluent at concentrations ranging from $10^{2.4}$ to $10^5$ copies per ml,[4,25,51,73] similar to the range of $10^3$ to $10^5$ copies per ml found in this study. It should be noted that MST markers may be detected in not only living, but also dead bacterial cells.

## 4.2 QMRA for sewage and effluent exposure

For a given $C_{HF}$, median risk is predicted to be greater if the source of the human marker is sewage than if the source is disinfected effluent. The $P_{ill,sum}$ distributions for sewage and effluent do, however, overlap at all values of $C_{HF}$. This overlap indicates that exposure to sewage does not always carry greater risk than exposure to treated effluent, due to the considered range of values of various QMRA parameters.

The regression between $C_{HF}$ and $P_{ill,sum}$ for effluent suggests that the median $P_{ill,sum}^{effluent}$ exceeds the illness benchmark of 0.03 when $C_{HF} = 10^{4.3}$ copies per 100 ml. However, at $C_{HF} > 10^4$ copies per 100 ml, median $P_{ill,sum}^{effluent}$ no longer appears to increase linearly, but rather begins to asymptote. $P_{ill,sum}^{sewage}$ also appears to asymptote at $C_{HF}$ at greater concentrations, above the risk threshold. This non-linear behavior is due to the increasing number of $f$ violations with increasing $C_{HF}$. Each time $f$ is violated, $F_{HF}$ is redrawn, so a restricted range of $F_{HF}$ is input to the QMRA. More than 10% of the runs for the effluent scenario resulted in $f$ violations when $C_{HF} > 10^4$ copies per 100 ml.

$f$ violations occur when the randomly drawn $F_{HF}$ value is low relative to $C_{HF}$, resulting in a calculation that describes a physically impossible situation in which the volume of effluent in the ambient water sample is greater than the total volume of the water sample. The $f$ violations thus indicate that $C_{HF} > 10^4$ copies per 100 ml may require the concentration of HF in the source material to be greater than the values observed in treated effluent, as occurs in raw sewage or feces. The concentrations of HF in raw sewage have been measured previously at $10^{7.2}$ to $10^{8.6}$ copies per 100 ml [7,51] and in feces at $10^{6.9}$ copies per mg.[46]

Our finding that seawater contaminated with treated effluent is unlikely to elevate risk beyond the threshold contrasts with several important epidemiology studies, which established illness-indicator relationships at effluent-impacted beaches.[12,81,82] One possibility to explain this disparity is that, at the beaches where those epidemiology studies took place, an unknown source of untreated sewage was present. This possibility was also suggested by Soller *et al.*[71] and Schoen *et al.*,[63] who concluded that the observed ENT concentrations and illness incidence were unlikely to occur simultaneously without a sewage source.

Another possible explanation is that this study underestimates the risk from exposure to treated effluent. Such underestimation could occur due to differences in treatment efficacy among chlorination processes. At the plants in this study that

 

use chlorine disinfection, the chlorine is generally present as chloramines rather than free chlorine, either because chloramines are added directly or because nitrogen species are present in the effluent that react with chlorine to form chloramines (multiple treatment plant operators, personal communication, Nov. 2016). Chloramines are much less effective than free chlorine in inactivating viruses,[6] including murine norovirus,[20] a culturable surrogate for norovirus. Because norovirus is the dominant contributor to $P_{ill,sum}$, the extent of its inactivation is key to predicting risk. Current water quality standards are based on fecal indicator bacteria, not viruses, so treated effluent may meet regulatory standards but still contain norovirus.

The meta-analysis of norovirus removal during wastewater treatment used herein[56] does not differentiate chlorination treatments by the particular chlorine species that are present. Assuming that the norovirus removal distribution, $L_{noro}$, contains data from both free chlorine and chloramine disinfection processes, the QMRA will calculate lower norovirus concentrations in effluent than it would from chloramine disinfection data alone. On the other hand, the distribution of HF concentration, $F_{HF}$, in this study represents only effluent samples that were disinfected with chloramines. Combining relatively high HF concentrations (measured in chloramine-disinfected effluent) with relatively low norovirus concentrations (measured in free chlorine-disinfected effluent) could underpredict risk from exposure to effluent.

The sensitivity analysis indicates that reducing the uncertainty in $R_{noro}$, $F_{HF}$, and $L_{noro}$ will most reduce the uncertainty of $P_{ill}$ estimates. This analysis reflects the fact that norovirus is the dominant reference pathogen in creating risk. It also suggests that future measurements of HF in wastewater effluent, to better characterize its distribution, are warranted.

### 4.3 QMRA for mixed sources

Most beaches have more than one source of fecal contamination. Here we considered a case in which both HF, with an assumed sewage source, and CAT are simultaneously measured in ambient seawater. Both sources contribute to $P_{ill,sum}$, and the risk-based threshold for HF, defined as the concentration of HF at which the risk exceeds 0.03, depends on $C_{CAT}$. $C_{CAT}$ was reported to be between $10^{1.9}$ and $10^{3.4}$ for beaches with low to moderate gull contamination.[49] At these $C_{CAT}$, the threshold $C_{HF}$ would be $10^{2.8}$ and $10^{1.1}$, respectively, according to eqn (6).

Several studies have measured human and gull markers within the same sampling period at a beach.[11,59,67] In particular, at a marine beach in California, Riedel *et al.*[59] found chronically high levels of CAT, $10^4$ to $10^5$ copies per 100 ml, and sporadically detected HF at concentrations of $10^3$ to $10^4$ copies per 100 ml. Based on our analysis, in that case, the gull contamination would result in risk above the threshold even without human contamination. Human contamination would greatly increase the risk, to as high as 30–40 cases of illness per 100 swimmers. However, our sensitivity analysis shows that using beta-Poisson $P_{inf,rp}$ and constant $P_{ill|inf,rp}$ for *Salmonella* and *Campylobacter* predicts much lower risk from exposure to gull feces. For the marine beach in California, risk would remain below the illness threshold when only gull contamination was present. The highest levels of measured human contamination would increase risk to approximately 8 cases of illness per 100 swimmers. Future studies that clarify which $P_{inf,rp}$ and $P_{ill|inf,rp}$ relationships are appropriate for recreational water exposure are clearly necessary.

Fig. 4 and S6† may be used as a tool for beach managers to assess whether a particular beach exceeds the $P_{ill,sum}$ threshold based on measurement of two MST markers. Interactions between MST markers from additional animal hosts warrant investigation by this method. Exposure to cattle feces during recreational swimming, for example, is more likely to cause illness in humans than exposure to an equivalent amount of gull feces.[71] Future work could test interactions between cattle-associated MST markers and HF.

### 4.4 Study limitations

One limitation of the current study is that we only assess the risk of exposure to fresh fecal contamination, but do not consider the effects of aging of contamination in the environment. After fecal contaminants are released into the environment, the constituent pathogens and MST markers are expected to decay over time. The decay rates are likely to vary among different pathogens and MST markers, potentially affecting the risks associated with exposure to MST markers. For example, if the HF in sewage decays more quickly than the pathogens, then $P_{ill,sum}$ will be greater than that predicted by the current model. Thus, although this study offers a starting point for beach managers to interpret measurements of MST markers in seawater, a better understanding of how these species decay in the environment is critical for the successful application of QMRA to beaches.

Another limitation is that the literature values for pathogen concentration distributions and dose–response relationships used in this QMRA were compiled from various sites and circumstances that may not apply to every specific site. For example, pathogen concentration distributions from sources were compiled from the literature and could deviate from actual values at specific beaches. However, at the present time, they represent the best available information and our Monte Carlo approach embraced the expected variation in those parameters.

The dose–response relationships used in this QMRA were developed from feeding and outbreak studies, which contain sparse data at the low doses we expect from environmental exposure. Although they are widely used in QMRA applications, there is considerable uncertainty in applying these relationships to recreational water contact. For norovirus, we dealt with this uncertainty following the approach of Soller *et al.*,[70] who found that the weighted model used herein predicted illness risk that agreed with the results of a concurrent epidemiology study of surfers in Southern California by Arnold *et al.*[3] Future studies that similarly reconcile dose–response curves with environmental epidemiology studies would be extremely valuable. Our sensitivity analysis shows that risk predictions for exposure to gull feces, as well as mixtures of gull feces and

sewage, are very different depending on the $P_{inf,rp}/P_{ill|inf,rp}$ pair used for *Salmonella* and *Campylobacter*. It is not clear whether the constant or dose-dependent pair of equations is more appropriate. In particular, studies that focus on *Campylobacter* would improve our understanding of risk at gull-contaminated beaches, because *Campylobacter* is the reference pathogen that contributes the most to risk from gull feces.[9]

Another limitation of the method presented here is that MST markers are not 100% specific to the animal hosts they are designed to track. In particular, the gull fecal marker is not fully specific to gull feces, but is also found in pigeon feces.[68] Pigeon feces may contain *Campylobacter*[57] and *Salmonella*,[74] as well as other zoonotic pathogens,[50,55] but the concentrations and fractions of infective species are likely different from those in gull feces. For beaches with large pigeon populations, therefore, it may be necessary to expand the ranges of these parameters in the simulations.

# 5 Conclusions

• HF was almost always detected in treated effluent samples, albeit at lower concentrations than in raw sewage of human feces.

• Given the concentrations of HF in treated effluents, treated effluent is unlikely to be responsible for HF in ambient waters above $10^4$ copies per 100 ml.

• At the same concentration in ambient water, HF from a sewage source presents a greater level of risk than HF from a treated effluent source.

• To remain below a risk threshold of 0.03, the allowable level of HF will depend on the level of CAT at a beach.

# Conflicts of interest

There are no conflicts to declare.

# Acknowledgements

This publication was prepared by the authors under NOAA Grant #NA14OAR4170075, California Sea Grant College Program Project #R/RCC-06, through NOAA's National Sea Grant College Program, U.S. Dept. of Commerce. The statements, findings, conclusions and recommendations are those of the author(s) and do not necessarily reflect the views of California Sea Grant, NOAA or the U.S. Dept. of Commerce. Additional funding was provided by NSF Grant #CBET-1334359. The authors express special thanks to Pat Leahy at the Caltech Kerckhoff Marine Laboratory for providing bench space, and to the many wastewater treatment plant personnel who assisted with effluent sample collection.

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