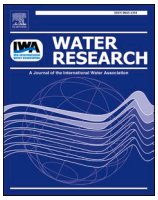
Water Research 242 (2023) 120137

Contents lists available at ScienceDirect

Water Research

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



Inclusion of hydrodynamic properties of bathing waters is critical in selecting faecal indicators to assess public health impacts of faecal contamination

Niamh A. Martin [a,1], Laura Sala-Comorera [a,1], Guanghai Gao [b], Aisling Corkery [b], Liam J. Reynolds [a], Tristan M. Nolan [a], Megan Whitty [a], John J. O'Sullivan [b], Wim G. Meijer [a,*]

[a] UCD School of Biomolecular and Biomedical Science, UCD Earth Institute and UCD Conway Institute, University College Dublin, Dublin 4, Ireland
[b] UCD School of Civil Engineering, UCD Dooge Centre for Water Resources Research and UCD Earth Institute, University College Dublin, Dublin 4, Ireland

ARTICLE INFO

Keywords:
Bathing water directive
Decay rate
Advection and dispersion
crAssphage
PMMoV
Faecal indicator bacteria

ABSTRACT

The EU Bathing Water Directive (BWD) requires member states to assess bathing water quality according to the levels of faecal indicator bacteria (FIB) in designated bathing areas. However, this criterion has two significant limitations given that the BWD does not; (i) account for differences in hydrodynamic properties of bathing waters and, (ii) assumes that all faecal pathogens decay equally in aquatic environments. This study simulated sewage discharge events in three hypothetical aquatic environments characterised by different advection and dispersion parameters in the solute transport equation. Temporal changes in the downstream concentration of six faecal indicators were determined in simulations that utilised measured decay rates of each faecal indicator from a programme of controlled microcosm experiments in fresh and seawater environments. The results showed that the decay rates of faecal indicators are not a critical parameter in advection dominant water bodies, such as in fast-flowing rivers. Therefore, faecal indicator selection is less important in such systems and for these, FIB remains the most cost-effective faecal indicator to monitor the public health impacts of faecal contamination. In contrast, consideration of faecal indicator decay is important when assessing dispersion and advection/dispersion dominant systems, which would pertain to transitional (estuarine) and coastal waterbodies. Results suggest that the inclusion of viral indicators, such as crAssphage and PMMoV, could improve the reliability of water quality modelling and minimise the risk of waterborne illnesses from faecal contamination.

1. Introduction

Bathing water quality in the European Union (EU) is governed by the EU Bathing Water Directive (2006) which classifies coastal and inland bathing waters as excellent, good, sufficient, and poor. These classifications are based on the 95th and 90th percentiles of the concentrations of two faecal indicator bacteria (FIB), *Escherichia coli* (*E. coli*) and intestinal enterococci, over a four-year period (EU, 2006). The levels of these two FIB are correlated to the risk of contracting a gastrointestinal and respiratory disease (Wade et al., 2003; Wade et al., 2006). In the context of recreational bathing waters, the presence of human-specific enteric pathogens, in particular human enteric viruses, is of particular concern regarding the risks of gastrointestinal illnesses (Kay et al., 1994; Fleisher et al., 1996). Although the implementation of this EU Directive has improved bathing water quality in the EU (World Health Organisation, 2007), significant shortcomings need to be addressed to update these regulatory standards in line with epidemiology. For example, the Bathing Water Directive does not consider the biological source of faecal contamination which impacts the risk of gastrointestinal and respiratory disease (Soller et al., 2010; Soller et al., 2014). This study addresses two major limitations which result from the use of just two types of faecal indicator bacteria as a proxy for faecally derived pathogens in the aquatic environment. These are the assumptions that; (i) all bathing waters have the same hydrodynamic properties and, (ii) that all faecally derived pathogens decay at the same rate in aquatic environments.

Although the EU Bathing Water Directive differentiates between inland and coastal bathing waters, the hydrodynamic characteristics of the waterbody are not considered–rivers, lakes and coastal waters being

* Corresponding author.
E-mail address: wim.meijer@ucd.ie (W.G. Meijer).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.watres.2023.120137
Received 22 March 2023; Received in revised form 26 May 2023; Accepted 27 May 2023
Available online 28 May 2023
0043-1354/© 2023 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

undifferentiated in this regard. This is a potentially important issue in that, the physical transport parameters of a waterbody which are governed by advection and dispersion, are important to assess the transport and fate of pathogenic organisms. Numerical models and frameworks have been developed to simulate the transport and fate of sewage associated microorganisms in both fluvial and coastal systems (Pascual-Benito et al., 2020; Jalliffier-Verne et al., 2016; Scroccaro et al., 2010; Bedri et al., 2015), and these models provide understanding of the environmental dynamics of infectious agents.

Several studies have shown that the decay rate of pathogens may be significantly different from those of *E. coli* and intestinal enterococci (Greaves et al., 2020). Furthermore, recent studies have shown conflicting results with regard to the quantifiable relationships between FIB and the presence of viral and protozoan waterborne pathogens such as cryptosporidium (Cabelli et al., 1982; Wu et al., 2011; Lalancette et al., 2014). Therefore, if FIB is not detected in a bathing water, it does not necessarily imply that faecally derived pathogens are not present. In recent years, several alternative faecal indicators or human associated microbial source tracking (MST) markers have been proposed that may be more suitable as a proxy for viral pathogens. These include somatic coliphages, F-RNA bacteriophages (Moce-Llivina et al., 2005; Havelaar, 1991), the *Bacteroidales*-associated human faecal marker HF183 (Seurinck et al., 2005; Green et al., 2014; Boehm and Soller, 2020), the Pepper Mild Mottle Virus (PMMoV) (Rosario et al., 2009) and crAssphage (Stachler et al., 2017; Dutilh et al., 2014).

This study aims to examine whether *E. coli* and intestinal enterococci are appropriate proxies for the presence of pathogens in the aquatic environment. We determined the decay rates of ten alternative faecal indicators and used these decay rates in hypothetical hydrodynamic models of waterbodies characterised by different advection and dispersion parameters. We show that decay rates of faecal indicators are not a critical parameter in advection dominant water bodies, and hence the use of *E. coli* and intestinal enterococci as FIB is appropriate. However, in dispersion dominant systems, consideration of the decay rate of potential faecal indicators is critical to assess the impact of faecal contamination on public health.

2. Materials and methods

2.1. Viral stocks

E. coli strain (ATCC 700,078) and *Salmonella typhimurium* strain WG49 (NCTC 12,484) were used for PhiX174 (ATCC13706-B1) and MS2 bacteriophages (ATCC 15,597-B1) propagation. Phage lysates were prepared by plating plaque-purified phage according to ISO 10,707–1:1995 and ISO 10,707–1:2000. The plaques were resuspended in Modified Scholten's broth (MSB) and tryptone-yeast extract-glucose (TYGB), respectively, and centrifuged at 3000 g for 10 min. The phage lysate was then filtered through a 0.22 μm filter (Dennehy and Turner, 2004).

2.2. Experimental design

The decay rates of culturable faecal markers and molecular markers in freshwater and seawater were determined using microcosm experiments. Freshwater and seawater were collected from the Grand Canal, Dublin, and Sandymount Strand in Dublin Bay. Wastewater influent (24-hour composite samples) was obtained from the Ringsend wastewater treatment plant in Dublin, Ireland.

Microcosms consisted of flasks (1 litre) containing fresh or seawater (500 ml) mixed with wastewater influent (25 ml). The microcosms were spiked with PhiX174 (final concentration 3.2×10^6 pfu/ml) and MS2 (final concentration 6.5×10^7 pfu/ml) and were maintained in the dark at 4 °C ± 2 °C and 20 °C ± 2 °C. Each microcosm was set up in duplicate.

Microcosms were sampled daily during the first week and subsequently every two days until day 21 to enumerate culturable markers, whereas molecular markers were quantified on days 2, 4, 6, 9, 11, 15, 21 and 30.

2.3. Enumeration of faecal indicator organisms

Water and wastewater influent samples (ten wastewater 24-h composite samples) were filtered through 0.45 μm nitrocellulose membranes (Thermo Fisher Scientific, USA) to enumerate faecal indicator bacteria. Membranes were placed on Tryptone Bile X-Glucuronide agar (Sigma-Aldrich, USA) at 37 °C for 4 h, followed by incubation at 44 °C for 18 h for the enumeration of *E. coli* (International Organization for Standardization, 2001). The levels of intestinal enterococci were quantified by incubating membranes on Slanetz and Bartley agar (Oxoid, USA) at 37 °C for 48 h. Membranes were subsequently transferred onto Bile Aesculin agar at 44 °C for 2 h to confirm positive intestinal enterococci colonies.

The double-agar layer technique was used to enumerate somatic coliphages and F-RNA specific phages using *E. coli* strain (ATCC 700,078) and *S. typhimurium* strain WG49 (NCTC 12,484) as a host, as described in ISO 10,705–2:2000 and 10,705–1:1995.

2.4. Nucleic acid extraction

DNA was extracted from water samples (10 ml) after filtering through 0.22 μm mixed cellulose ester membranes (Thermo Fisher Scientific, USA). Membranes were divided into 2 ml screw cap tubes for nucleic acid extraction using DNeasy PowerSoil Pro-Kit (Qiagen, Germany). RNA was directly extracted from water samples (250 μl) with the RNeasy PowerMicrobiome Kit (Qiagen, Germany). DNA from wastewater influent samples was extracted using DNeasy Blood and Tissue kit (Qiagen, Germany) as described by Sala-Comorera et al. (2021), and RNA from wastewater influent samples was concentrated using 100 kDa Centricon Plus-70 filters and extracted using RNeasy PowerMicrobiome Kit (Qiagen, Germany) (Reynolds et al., 2022).

2.5. Molecular marker quantification

Gene targets were quantified using previously described qPCR and RT-qPCR assays with TaqMan probes and SYBR green assays (Table 1) on the Roche Lightcycler 96 platform (Roche Diagnostics, Switzerland). Amplification reactions were performed in a 20 μl reaction mixture using LightCycler, FastStart Essential DNA Probes Master, FastStart Essential DNA Green Master or Multiplex RNA Virus Master as described by the manufacturer (Roche Diagnostics, Switzerland). Primer sequences and concentrations, and thermal cycling conditions for each gene, are given in Table 1.

Standard curves were generated using 10-fold dilutions of gBlock Gene Fragments (Integrated DNA Technologies, USA) or cloned target genes (Reynolds et al., 2020; Balleste et al., 2020). The limit of detection (LOD) of each assay was determined as the lowest number of nucleic acid target in each template volume, detected in at least 95% of replicates (Rutledge and Stewart, 2008). The limit of quantification (LOQ) was calculated as the lowest concentration of the target quantified within 0.5 standard deviations of the $\log_{10}$ concentration. The amplification efficiency of every reaction was calculated by using the slope of the linear regression lines employing the following equation (Rutledge and Cote, 2003):

$$E = 10^{-\left(\frac{1}{\text{slope}}\right)} - 1 \quad (1)$$

For every set of samples, a negative control, positive control, and four dilutions of purified plasmid or gBlock Gene Fragments for construction of a standard curve were added. The efficiency for all runs was between 93 and 102% (Table S1).

Table 1
Inactivation parameters of each indicator in freshwater and seawater, 95% confidence intervals (CI) have been included. s: stable for the duration (30 days) of the experiment.

	Microcosm	Temp (°C)	Model	k_{mean} (day $^{-1}$)		95% CI	
Culturable *E. coli*	Freshwater	4 °C	Biphasic	[a]s	[b]0.45		0.52 to 0.38
		18 °C	Biphasic	[a]0.44	[b]0.25	[a]0.59 to 0.36	[b]0.29 to 0.19
	Seawater	4 °C	Biphasic	[a]s	[b]0.49		0.62 to 0.33
		18 °C	Linear		1.10		1.29 to 0.92
E. coli DNA	Freshwater	4 °C	Linear		0.23		0.29 to 0.17
		18 °C	Linear		0.25		0.29 to 0.21
	Seawater	4 °C	Linear		0.21		0.27 to 0.16
		18 °C	Biphasic	[a]0.65	[b]s	[a]0.77 to 0.53	
Culturable Intestinal enterococci	Freshwater	4 °C	Biphasic	[a]0.1	[b]0.33	[a]0.15 to 0.04	[b]0.4 to 0.28
		18 °C	Biphasic	[a]0.59	[b]0.55	[a]0.62 to 0.56	[b]0.58 to 0.52
	Seawater	4 °C	Linear		0.09		0.11 to 0.07
		18 °C	Biphasic	[a]0.51	[b]0.54	[a]0.54 to 0.48	[b]0.56 to 0.52
Enterococcus faecalis DNA	Freshwater	4 °C	Linear		s		
		18 °C	Linear		0.21		0.25 to 0.17
	Seawater	4 °C	Linear		s		
		18 °C	Linear		0.10		0.16 to 0.05
Somatic Coliphages	Freshwater	4 °C	Linear		s		
		18 °C	Biphasic	[a]s	[b]0.28		0.32 to 0.24
	Seawater	4 °C	Linear		s		
		18 °C	Biphasic	[a]s	[b]0.25		0.31 to 0.21
F-RNA bacteriophages	Freshwater	4 °C	Linear		0.12		0.16 to 0.09
		18 °C	Linear		0.97		1.07 to 0.87
	Seawater	4 °C	Linear		0.25		0.29 to 0.21
		18 °C	Linear		0.55		0.61 to 0.5
MS2 RNA	Freshwater	4 °C	Linear		s		
		18 °C	Linear		0.19		0.23 to 0.15
	Seawater	4 °C	Linear		0.09		0.16 to 0.02
		18 °C	Linear		0.19		0.23 to 0.14
HF183	Freshwater	4 °C	Linear		0.24		0.29 to 0.19
		18 °C	Linear		0.71		0.77 to 0.65
	Seawater	4 °C	Biphasic	[a]s	[b]0.31		0.45 to 0.17
		18 °C	Linear		0.50		0.57 to 0.43
PMMoV RNA	Freshwater	4 °C	Linear		s		
		18 °C	Linear		s		
	Seawater	4 °C	Linear		s		
		18 °C	Linear		s		
crAssphage_2	Freshwater	4 °C	Linear		s		
		18 °C	Linear		0.22		0.25 to 0.19
	Seawater	4 °C	Linear		0.19		0.24 to 0.14
		18 °C	Linear		0.14		0.16 to 0.11

[a] Decay rate 1 (k_1),.
[b] Decay rate 2 (k_2).

2.6. Mathematical model for sewage overflow event simulation

Under steady and uniform flow fields, the solute transport equation can be simplified to (Runkel, 1996):

$$\frac{\partial C}{\partial t} = -U\frac{\partial C}{\partial x} + D\frac{\partial^2 y}{\partial x^2} - kC \tag{2}$$

where C = concentration; t = time; U = velocity; x = distance; D = dispersion coefficient; k = decay rate.

The analytical solution of the above equation was adopted in this study to model the concentration of various faecal indicators downstream of a human sewage spill, at $x = 0$, for a finite time period, τ (O'Loughlin and Bowmer, 1975; Runkel, 1996).

For $t \leq \tau$, the solution is:

$$C(x,t) = \frac{C_0}{2}\left\{\exp\left(\frac{-kx}{U}\right)\mathrm{erfc}\left(\frac{x - Ut[1+2\eta]}{2\sqrt{Dt}}\right)\right\} \tag{3}$$

and for $t > \tau$, the solution is:

$$C(x,t) = \frac{C_0}{2}\exp\left(\frac{-kx}{U}\right)\left\{\mathrm{erfc}\left[\frac{x - Ut(1+2\eta)}{2\sqrt{Dt}}\right] - \mathrm{erfc}\left[\frac{x - U(t-\tau)(1+2\eta)}{2\sqrt{D(t-\tau)}}\right]\right\} \tag{4}$$

where C_0 = initial concentration at the point of spill ($x = 0$) and $\eta = \frac{kD}{U^2}$ is the estuary number.

Three hypothetical water systems were considered for the simulation of sewage discharges into different water bodies. The first was an advection dominant water system with $U = 0.2\ m/s$ and $D = 5\ m^2/s$ to reflect a typical river system, the second was an advection/dispersion dominant system with $U = 0.05\ m/s$ and $D = 50\ m^2/s$, characteristic of an estuarine or coastal system, and the third was a dispersion dominant water system with $U = 0.001\ m/s$ and $D = 1\ m^2/s$, typical of a freshwater lake. To determine the impact of the hydrodynamic properties on the spatial concentration of faecal indicators in the different water systems, six faecal indicators reflecting a variety of decay rates were selected for analytical modelling. Indicators included *E. coli*, intestinal enterococci, crAssphage, PMMoV, HF183 and somatic coliphages, and all were simulated in a hypothetical sewage spill of 2 h duration over a 10 km distance for 200 h. The initial concentrations (Table S4) of the simulated indicators and molecular markers at the point of spill were the calculated means determined from untreated influent sewage samples ($n = 10$) taken from the Ringsend WWTP. The decay rates for each indicator, determined from the microcosm experiments (Section 2.2) were used in these simulations.

2.7. Data analysis

Data were analysed and plotted using GraphPad Prism9 (GraphPad Software, USA). Decay rates for culturable microorganisms and molec-

ular markers in freshwater and seawater were calculated assuming the first order exponential model (Chick, 1908), defined by the equations:

$$N_t = N_0 e^{-kt} \quad (5)$$

$$\mathrm{Ln}\left(\frac{N_t}{N_0}\right) = -kt \quad (6)$$

where N_t and N_0 are the concentrations of the viable microorganism or the molecular marker in the microcosm at time t and time 0, respectively, and k is the decay rate. A linear and a biphasic decay model was determined for each indicator using segmented pairwise regression. A break point between segments was identified, and decay rate constants (k_1 and k_2) were calculated for each of the two segments. The best fit model was determined by comparing the linear and biphasic decay models using the Akaike information criteria (AIC), r^2, RMSE and Wald-Wolfowitz runs test (Supplemental material).

The time required to reduce the concentration by 1 $\log_{10}$ unit (T_{90}) and 2 $\log_{10}$ units (T_{99}) was determined as follows:

$$T_{90} = \frac{-\ln(0.1)}{k} \quad (7)$$

$$T_{99} = \frac{-\ln(0.01)}{k} \quad (8)$$

3. Results

3.1. Decay of culturable faecal indicator bacteria and their associated marker genes in fresh and seawater

To compare the decay rates of different faecal indicators in freshwater and seawater, we first determined the culturable and molecular bacterial indicator decay rates of FIB included in the Bathing Water Directive (2006) in a microcosm system (Fig. 1). The decay profile of culturable *E. coli* was biphasic in all microcosm experiments with the exception of the seawater microcosm at 18 °C (Table 1, 2). For both freshwater and seawater at the lower temperature (4 °C), *E. coli* remained stable for five days and seven days, respectively, after which decay rates for the two waters became similar ($k = 0.45$ days^{-1} T_{90}= 5.1, k = 0.49 days^{-1} T_{90}=4.7 days, respectively). *E. coli* in freshwater at 20+/−2 °C exhibited a rapid decay in the first phase ($k = 0.43$ days^{-1}, T_{90}=5.2 days), followed by a slower decay in the second phase (k=0.24 days^{-1}, T_{90}=9.3 days). The highest decay rate was observed in seawater at 18 °C (k=1.10 days^{-1}, T_{90}= 2.1 days).

In contrast to culturable *E. coli*, the decay profile of the *E. coli* 16S rRNA gene was linear for all experimental conditions, except for seawater at 18 °C (Table 1, 2). Under these conditions, the gene marker decayed rapidly in the first phase (k=0.65 days^{-1}, T_{90}=3.5 days) and remained stable for the remaining 13 days of the experiment. In contrast, the *E. coli* gene marker was more stable in freshwater at 18 °C (T_{90}=9.3 days). At 4 °C, the decay rates of the *E. coli* gene marker were comparable in freshwater and seawater (k=0.21–0.23, T_{90}= 9.9–10.7 days).

Similar to culturable *E. coli,* intestinal enterococci were characterised by a biphasic decay profile in three out of four conditions. However, the two faecal indicators showed different decay profiles (Fig. 1a, c & Fig. S1a, c). The intestinal enterococci decay was higher (T_{90}=3.9 days) than that for *E. coli* (T_{90}=5.2 days) in freshwater at 18 °C, whereas intestinal enterococci were more stable in seawater at 18 °C (T_{90}=4.5 days) than *E. coli* (T_{90}=2.1 days). At 4 °C, intestinal enterococci remained reasonably stable in freshwater and seawater for the first 10 days, but from this time, levels decayed more rapidly in freshwater than in seawater (T_{90}=22.9 and 26.3 days, respectively). Similarly, the *Enterococcus faecalis* gene marker remained constant for the duration of the experiment at 4 °C. However, and somewhat surprisingly, the decay rate of this gene in freshwater at 18 °C was 2-fold higher than in seawater (k=0.21 days^{-1}, T_{90}=11 days and k=0.10 days^{-1}, T_{90}= 22.2 days, respectively).

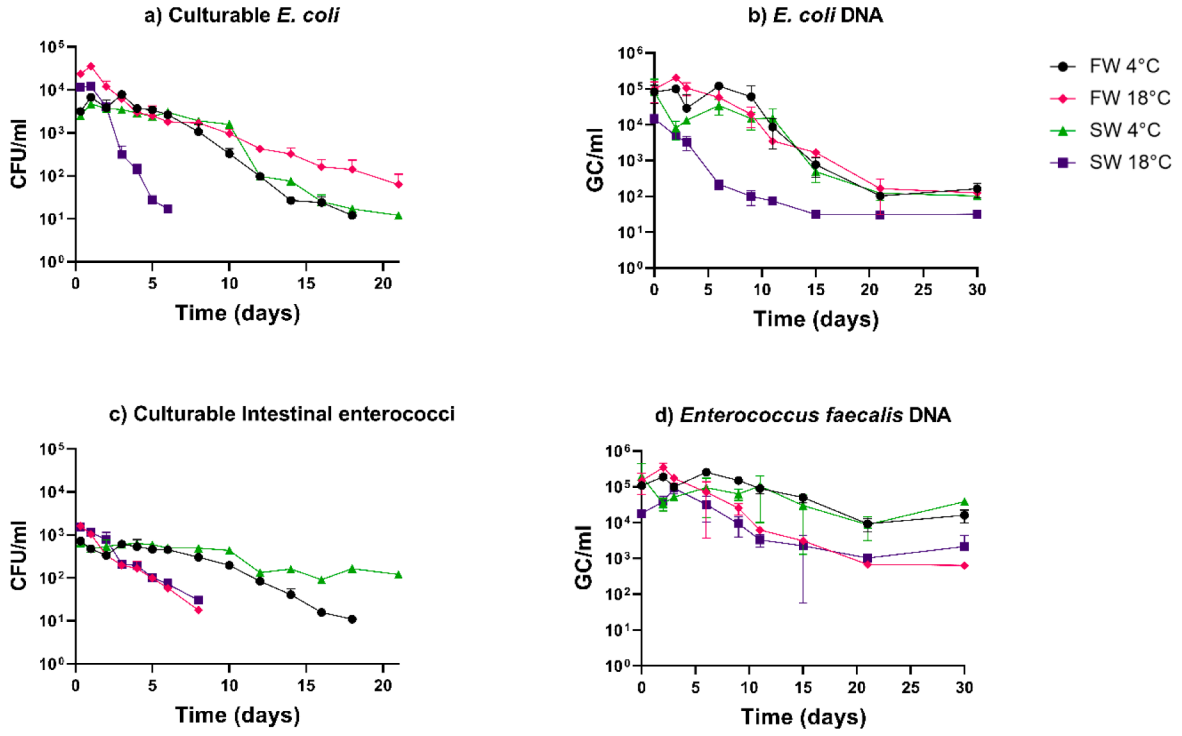


Fig. 1. Decay of a) culturable *E. coli,* b) *E. coli* DNA, c) culturable intestinal enterococci, and d) *Enterococcus faecalis* DNA in freshwater (FW) and seawater (SW) at 4 °C and 18 °C. Data points represent mean values obtained from duplicate experiments. Error bars represent standard deviation. CFU/ml: Colony forming units per millilitre, GC/ml: gene copies per millilitre.

Table 2
T_{90} and T_{99} (days) with 95% confidence intervals (CI) for all indicators. The turning point (TP): the day at which decay rate 1 (k_1) changes to decay rate 2 (k_2), s: stable for the duration (30 days) of the experiment.

	Microcosm	Temp (°C)	TP	T_{90} (CI)		T_{99} (CI)	
Culturable *E. coli*	Freshwater	4 °C	5.0	[a]s	[b]5.1 (4.4–6)	[a]s	[b]10.3 (8.8–12.1)
		18 °C	5.0	[a]5.2 (3.9 - 6.4)	[b]9.3 (7.9–12.1)	[a]10.5 (7.8–12.7)	[b]18.7 (15.8–24.2)
	Seawater	4 °C	7.8	[a]s	[b]4.7 (3.7–6.9)	[a]s	[b]9.3 (7.4–13.9)
		18 °C			2.1 (1.7–2.5)		4.2 (3.5 - 5)
E. coli DNA	Freshwater	4 °C			9.9 (7.9–13.5)		19.8 (15.8 - 27)
		18 °C			9.3 (7.9–10.9)		18.6 (15.8–21.9)
	Seawater	4 °C			10.7 (8.5–14.4)		21.5 (17–28.7)
		18 °C	8.0	[a]3.5 (2.9 - 4.3)	[b]s	[a]7.04 (5.9–8.6)	[b]s
Culturable Intestinal enterococci	Freshwater	4 °C	7.7	[a]22.9 (15.4 - 57.6)	[b]6.9 (5.7–8.2)	[a]s	[b]13.9 (11.6–16.4)
		18 °C	11.5	[a]3.9 (3.7 - 4.1)	[b]4.2 (3.9 - 4.4)	[a]7.8 (7.4–8.2)	[b]8.3 (7.9–8.8)
	Seawater	4 °C			26.3 (20.9–32.9)		s
		18 °C	11.3	[a]4.5 (4.2–4.8)	[b]4.2 (4.1–4.4)	[a]9.0 (8.5–9.5)	[b]8.5 (8.2–8.8)
Enterococcus faecalis DNA	Freshwater	4 °C			S		s
		18 °C			10.9 (9.2–13.5)		21.9 (18.4–27)
	Seawater	4 °C			S		s
		18 °C			22.2 (14.3 - 46)		s
Somatic Coliphages	Freshwater	4 °C			S		s
		18 °C	3.9	[a]s	[b]8.3 (7.2–9.5)	[a]s	[b]16.7 (14.3 - 19)
	Seawater	4 °C			S		s
		18 °C	4.0	[a]s	[b]9.2 (7.4–10.9)	[a]s	[b]18.4 (14.8–21.9)
F-RNA bacteriophages	Freshwater	4 °C			18.9 (14.3–25.5)		s
		18 °C			2.4 (2.1–2.6)		4.8 (4.3–5.8)
	Seawater	4 °C			9.1 (7.9–10.9)		18.2 (15.8–21.9)
		18 °C			4.2 (3.7–4.6)		8.3 (7.5–9.2)
MS2 RNA	Freshwater	4 °C			S		s
		18 °C			12.2 (10–15.3)		24.3 (20–30.7)
	Seawater	4 °C			26.1 (14.3- s)		s
		18 °C			12.4 (10–16.4)		24.8 (28.7 - s)
HF183	Freshwater	4 °C			9.6 (7.9–12.1)		19.2 (15.8–24.2)
		18 °C			3.3 (2.9–3.5)		6.5 (5.9 - 7)
	Seawater	4 °C	11.0	[a]s	[b]7.3 (5.1–13.5)	[a]s	[b]14.6 (10.2 - 27)
		18 °C			4.6 (4–5.3)		9.3 (8–10.7)
PMMoV RNA	Freshwater	4 °C			S		s
		18 °C			S		s
	Seawater	4 °C			S		s
		18 °C			S		s
crAssphage_2	Freshwater	4 °C			S		s
		18 °C			10.4 (9.2–12.1)		20.8 (18.4–24.2)
	Seawater	4 °C			12 (9.6–16.4)		24 (19.1–32.9)
		18 °C			17.2 (14.4–0.9)		s

[a] Decay rate 1 (k_1),.
[b] Decay rate 2 (k_2).

3.2. Decay of infectious bacteriophages and their associated marker gene in fresh and seawater

Somatic coliphages and F-specific RNA bacteriophages are abundant in human faeces and have been suggested as indicators of human viral pathogens of faecal origin (Balleste et al., 2021; Jebri et al., 2017). Somatic coliphages followed a biphasic decay profile in freshwater and seawater at 18 °C, which remained stable for four days. However, from this time, decay was similar in both microcosms (k_2= 0.28 and 0.25 $days^{-1}$ for freshwater and seawater). Unlike the decay of bacterial indicators at 4 °C, somatic coliphages remained stable in all treatments for the duration of the experiment at this temperature (Fig. 2a).

Both F-RNA bacteriophages and the MS2 RNA markers followed a linear decay model (Table 1, 2). Interestingly, the two infectious bacteriophages decayed at very different rates, F-RNA bacteriophages decaying more rapidly than somatic coliphages under all conditions, most notably in freshwater at 18 °C. At 4 °C, F-RNA decay rates were similar in both fresh and seawaters (k = 0.12 and 0.25 $days^{-1}$). A similar decay profile was observed for the MS2 marker, which was stable for the duration of the experiment (30 days). Furthermore, at 18 °C, the F-RNA decay rate was 1.8-fold higher in freshwater (k = 0.97 $days^{-1}$, T_{90}= 2.4 days) than in seawater (k = 0.55 $days^{-1}$, T_{90}= 4.2 days). In contrast, the decay rates for MS2 RNA were similar at 4 °C, with T_{90} values of 12.2 and 12.4 days, respectively, being determined in freshwater and seawater.

3.3. Decay of human sewage associated molecular marker genes in fresh and seawater

We evaluated the decay rate of the crAssphage, PMMoV and HF183, which are used as human source tracking markers (Fig. 2d, e, f & Table 1,2). The HF183 marker decayed more rapidly at 18 °C in freshwater (k=0.71 $days^{-1}$, T_{90}= 3.3 days) than in seawater (k=0.50 $days^{-1}$, T_{90}= 4.6 days). At 4 °C, the HF183 marker remained stable for 11 days in seawater before it began to decay. The crAssphage marker was more persistent than the HF183 marker under all conditions. For example, at 18 °C under both conditions, the T_{90} values for crAssphage were around three times higher than that of HF183 (Table 2). However, similar to the HF183 marker, crAssphage levels decreased 1.7-fold faster in freshwater (k=0.22 $days^{-1}$, T_{90}= 10.4 days) than in seawater at 18 °C (k=0.14 $days^{-1}$, T_{90}= 17.2 days). Moreover, the crAssphage marker remained stable in freshwater at 4 °C for the duration of the experiment. In comparison to all other indicators, the viral PMMoV RNA marker was the only indicator to remain stable under all tested conditions for the entire experiment (30 days), and therefore, no decay parameters were determined for this marker.

3.4. Sewage overflow event simulation in different water systems

As described in Section 2.6, six (of 10 in Table 2) faecal indicators were simulated in a hypothetical sewage spill of 2 h duration over a 10

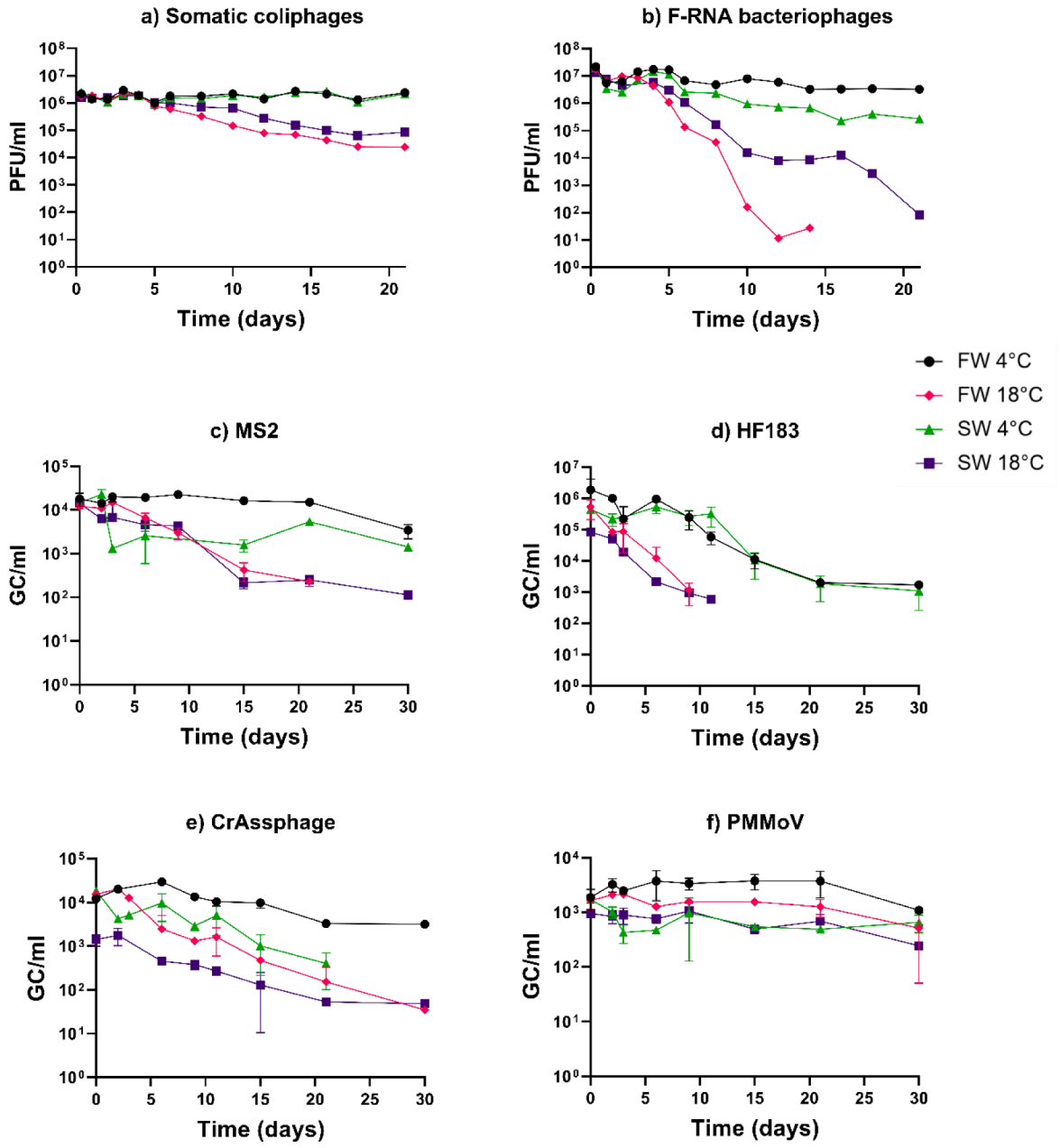


Fig. 2. Decay of a) culturable somatic coliphages, b) F-RNA bacteriophage, c) MS2 gene marker, d) HF183, e) crAssphage, f) PMMoV in freshwater (FW) and seawater (SW) at 4 °C and 18 °C. Data points represent mean values obtained from duplicate experiments. Error bars represent standard deviation. PFU/ml: Plaque forming units per millilitre. GC/ml: gene copies per millilitre.

km distance for 200 h. Data for three indicators (a faecal bacterial species - *E.coli*, a phage - crAssphage and a virus–PMMoV) are shown in Fig. 3. Data for intestinal enterococci, somatic coliphages and the human marker HF183 exhibited similar trends and are shown in Fig. S3. In the simulation of the advection dominant (river) system (Fig. 3(a), (d) and (g)), the sewage spill was shown to have minimal impact over time on the downstream water quality, but the peak concentrations further downstream were shown to be significantly higher than for both the advection/dispersion dominant estuarine/ coastal (Fig. 3(b), (e) and (h)) and dispersion dominant (lake) systems (Fig. 3(c), (f) and (i)). All indicators displayed a sharp rise and fall over a relatively short period of time (Fig. 4(a)). The rate of decay had only a limited impact on the downstream indicator concentrations, as the T_{90} and T_{99} values were shown to be greater than the time it took for the indicators to be distributed over the 10 km model length. In contrast, the downstream concentration was impacted for a much longer period in the estuarine/ coastal system, but with a lower peak concentration compared to the river system. The decrease in concentration was observed over a considerably longer time period in the estuarine/ coastal and lake systems than the river system (Fig. 4). Furthermore, the concentration of indicators with higher decay rates, such as *E. coli*, intestinal enterococci and HF183, were shown to decrease more rapidly than the stable indicators, such as somatic coliphages and PMMoV (Fig. 4(b), (c)) in the estuarine/ coastal and lake systems. In the lake system, none of the faecal indicators travelled further than 7 km from the point of discharge over the 200-hour simulation period (Fig. 3(c), (f) and (i)). The stable indicators, such as somatic coliphages (Fig. S3(i)) and PMMoV (Fig. 3(i)) were shown to continue spreading to further downstream cross-sections after the 200-hour period, and until the concentration stabilised, while indicators with higher decay rates, such as *E. coli*, (Fig. 3(c)) intestinal enterococci (Fig. S3(c)), and HF183 (Fig. S3(f)), were shown to not impact the further downstream cross-sections, since at the end of the

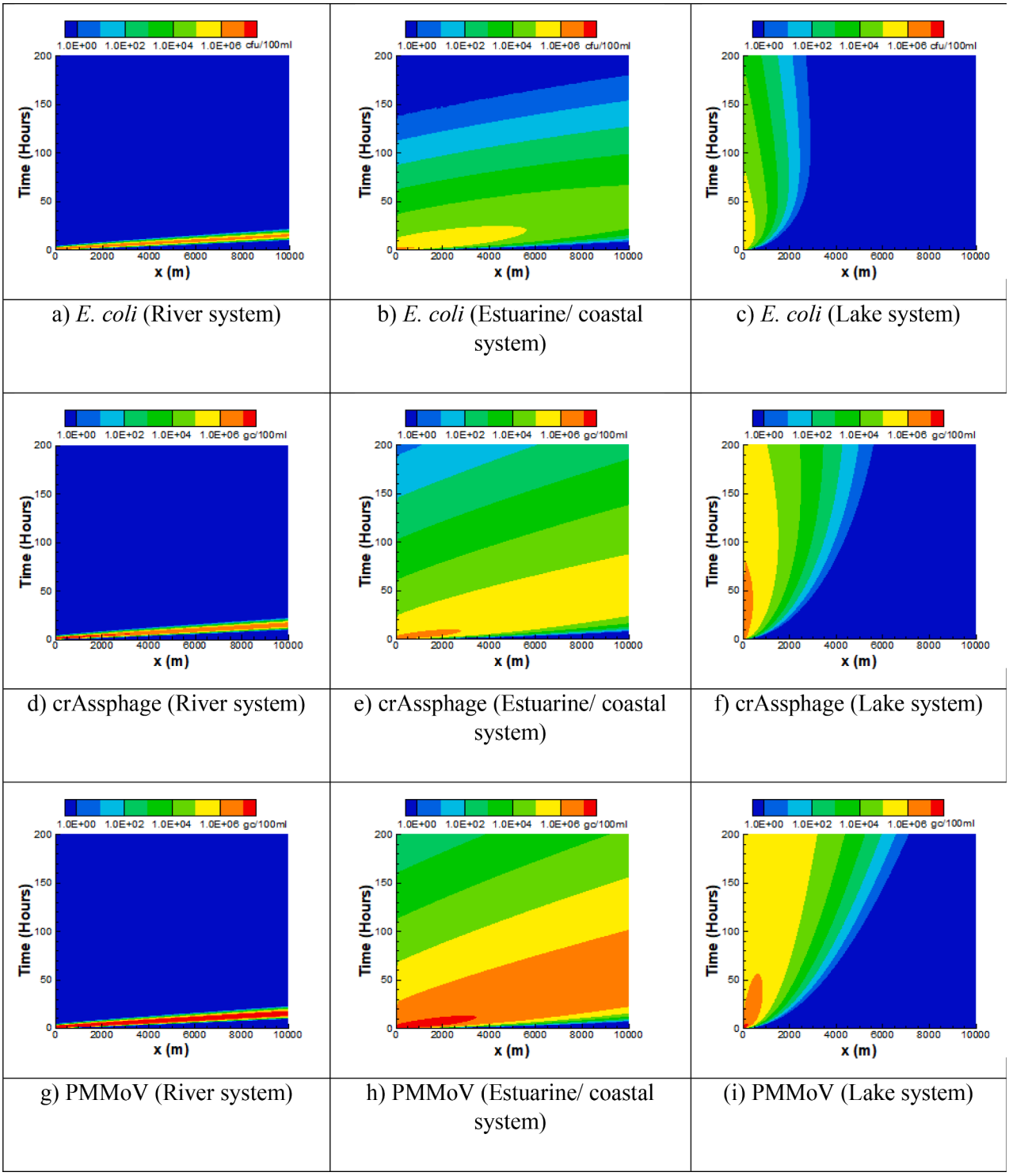


Fig. 3. Simulation of the faecal indicator concentration distribution over a 10 km area for 200 h for; culturable *E. coli* in a) advection dominant river system, b) advection/dispersion dominant estuarine/ coastal system, and c) dispersion dominant lake system; crAssphage in d) advection dominant river system, e) advection/ dispersion dominant estuarine/ coastal system, and f) dispersion dominant lake system; PMMoV in g) advection dominant river system, h) advection/dispersion dominant estuarine/ coastal system, and i) dispersion dominant lake system. X (m): 10,000 m area.

200-hour simulation, the areal extent of the simulated domain being impacted was already diminishing.

4. Discussion

The use of FIB to assess bathing water quality and the accompanying risk of contracting gastrointestinal and/ or respiratory disease, as required by the EU Bathing Water Directive (2006), has led to a significant increase in bathing water quality throughout the European Union (World Health Organisation, 2007). However, the EU Bathing Water Directive does not consider the hydrodynamic properties of a given waterbody, nor does it consider the decay rates of faecal indicators in the aquatic environment. A systematic review of decay rates of mammalian viruses of public health concern in aquatic environments, including

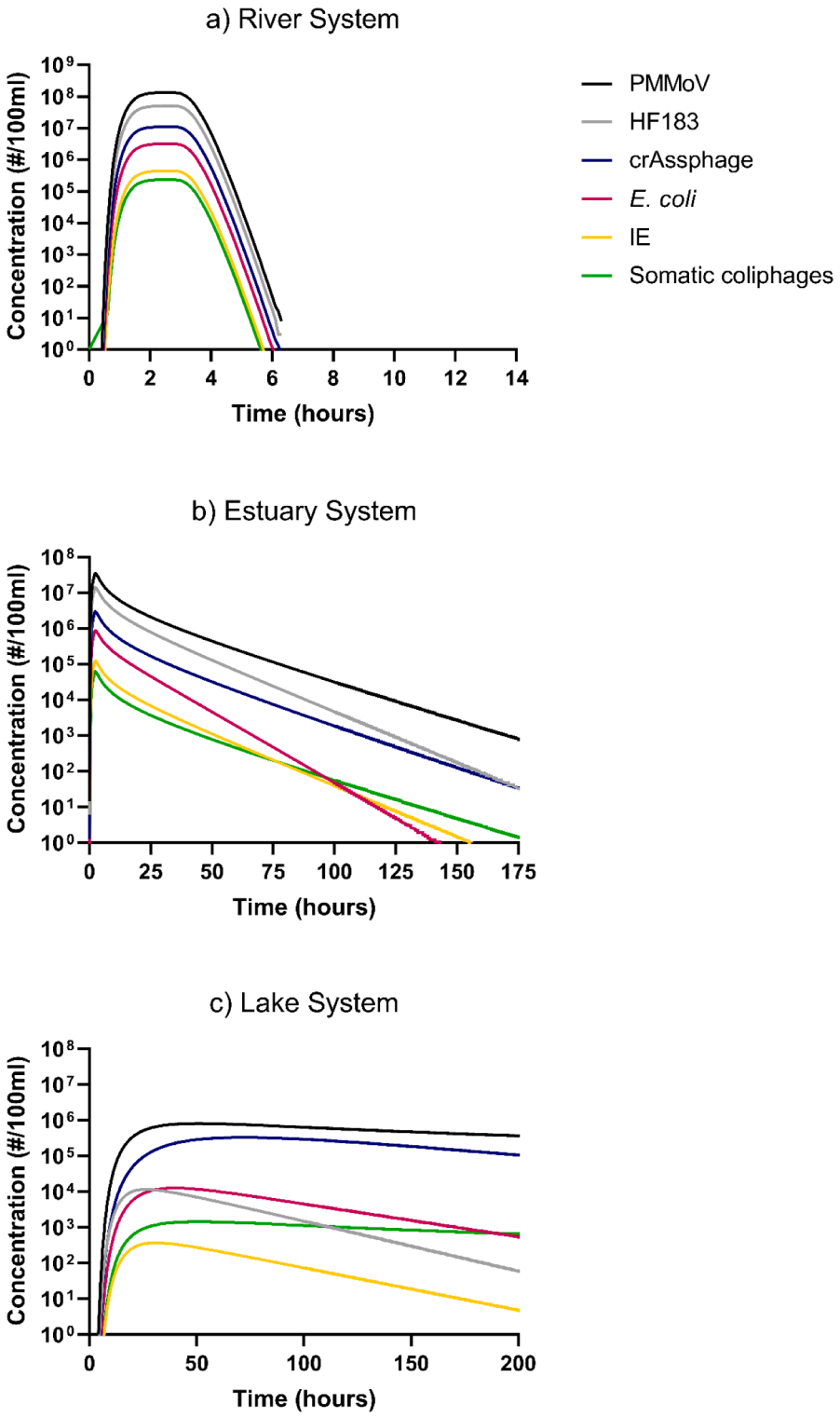


Fig. 4. Concentration of microbial indicators at 1000 m downstream of a simulated sewage spill for a) advection dominant river system, b) advection/dispersion dominant estuarine/ coastal system, and c) dispersion dominant lake system.

Norovirus, Rotavirus and Enterovirus, reported k-values that are considerably smaller than those of *E. coli* and intestinal enterococci. The authors of this review conclude that FIB may therefore have limited use in assessing the risks to public health by mammalian enteric viruses introduced by faecal contamination (Boehm et al., 2019). Our study considered whether the two FIB in the Bathing Water Directive (*E. coli* and intestinal enterococci) are sufficient in different waterbodies with differing hydrodynamic properties to fully assess public health risks associated with faecal contamination. With *E. coli* and intestinal enterococci, we determined decay rates (from experimental microcosms) of four additional faecal indicators in hypothetical simulations of river, estuarine/ coastal and lake systems to identify the appropriate faecal indicators, with some not currently included in the Bathing Water Directive, to adequately reflect the public health risks associated with waterborne pathogens.

The model simulation results show that the decay rate of each

indicator had a minimal impact on the concentration levels of faecal indicators in advection dominant (river) systems. All faecal indicators showed a similarly sharp rise and fall in concentration and were below the detection limit within six hours. The rate at which the marker concentration increased and decreased in the simulated domain was substantially higher than the corresponding measured decay rates in this study. This means that the transport profiles of mammalian viruses of public health concern with low k-values, e.g., Norovirus, Rotavirus or Enterovirus under these conditions is highly similar to those of FIB which have a greater k-value (Boehm et al., 2019). Therefore, the choice of a particular faecal indicator is not critical in advection dominant (river) systems systems, and in these, FIB remain the most cost-effective indicators to assess public health risks associated with faecal contamination. However, of the *approximately* 22,000 designated bathing waters across the EU, 6% are river systems. The vast majority (over 85%) of EU designated bathing areas are coastal with the remainder comprising the non-river 'inland' waterbodies (European Environment Agency, 2021).

In less dynamic waterbodies that include estuarine/ coastal water bodies and lakes, faecal indicator selection is critical in assessing faecal pollution. Dispersion rather than advection is the primary driver of contaminant transport in these settings. In advection/dispersion (estuarine/ coastal settings) and dispersion (lake settings) simulations, the faecal indicators were observed in the model domain for longer time periods, as the water movement is slower than in the advection driven model. The lake simulation showed that the faecal indicators remained detectable within seven km of the discharge point over a 200-hr simulation period, suggesting that water quality remains compromised for a longer period of time than that for the estuarine/coastal and river systems that were simulated. Furthermore, after 4–5 days in an estuarine/ coastal system, the levels of intestinal enterococci are shown to decrease below the no-observed-adverse-effect levels (NOAELs) of less than 40 CFU/100 ml (WHO, 2003). The NOAEL applies to healthy adult bathers exposed to marine water and does not consider other activities nor individuals with a lower immunity, such as children, the elderly or immunocompromised persons. The simulation shows that when intestinal enterococci levels fall below the NOAEL, other indicators with k-values similar to some human viral pathogens are still present at substantial levels, indicating that a risk to public health may remain. Overall, the indicator specific decay rates were shown to have greater impacts on reductions in faecal indicator concentrations in scenarios where the T_{90} values were lower than the rate of dispersal. It should also be noted that the observed T_{90} values for FIB are comparable to other studies, ranging from 1.52 to 6.19 days and 1.15 to 5.46 days for culturable *E. coli* and intestinal enterococci, respectively (Balleste et al., 2018). Ahmed et al. (2021) have reported a biphasic decay profile for culturable *E. coli* under similar conditions as reported here.

In both the freshwater and seawater simulations, the bacteriophage markers were shown to persist for longer periods than the culturable and molecular bacterial indicators. Therefore, these markers are arguably more suitable as a proxy for viral pathogens because, as previously indicated, those viral pathogens have k-values that are considerably smaller than those of bacterial indicators. Somatic coliphage and F-RNA phage plaque assays are valuable as they measure the level of infectious particles in the water. However, as is the case for *E. coli* and intestinal enterococci, culturable bacteriophages and the MS2 marker are not exclusively associated with human contamination. The crAssphage and PMMoV markers are more representative of human viral pathogens in water quality analysis, as they have been shown to be correlated to the presence of human-specific gastrointestinal viruses in the aquatic environment (Jennings et al., 2020; Gonzalez-Fernandez et al., 2021). Moreover, Crank et al. (2019) demonstrated a relationship between observed PMMoV and crAssphage concentrations and the probability of bather illness in sewage-polluted waters. PMMoV and crAssphage markers are also highly abundant in sewage (Kato et al., 2018; Wu et al., 2020; Crank et al., 2020), with no seasonal fluctuations reported. In addition, studies have shown a good correlation between the crAssphage marker and other faecal indicators, such as FIB, HF183 and somatic coliphages (Ahmed et al., 2020; Balleste et al., 2019; Sala-Comorera et al., 2021). Furthermore, the k-values observed for crAssphage are within the range observed in other studies, for example Ahmed et al. (2021). Other studies have reported a higher persistence for PMMoV under similar conditions (Greaves et al., 2020). In comparison to other stable indicators, i.e., somatic coliphages, the PMMoV was detectable for the longest time period because of its high sewage content and low decay rate. It is, therefore, a suitable indicator in situations where a stable marker is required to monitor long-term spatial trends of a sewage spill because the decrease in PMMoV levels is due to the dispersal rate rather than its decay rate.

Monitoring human pathogens in the environment remains challenging. Adopting a more conservative approach by selecting a viral marker with a higher decay rate and higher concentration could improve the reliability of water quality monitoring in estuarine/ coastal waterbodies and lakes. Our results confirm that choosing the most appropriate faecal indicator markers is dependent on the type of water body and the nature of the spill. The study results point toward the increased level of public health protection that could be realised by using site-specific risk-control measures for different recreational water bodies depending on the resources available to responsible authorities, regulatory agencies and policymakers.

5. Conclusions

This study investigated whether the two FIB that underpin the current implementation of the EU Bathing Water Directive (namely, *E. coli* and intestinal enterococci) are sufficient for assessing the public health risks associated with faecal contamination in bathing waters. The model simulation results demonstrated that decay rates of faecal indicators are not a critical parameter in advection dominant water bodies, as the faecal indicators will be below the limit of detection before the viability or integrity of their genetic material is lost. Therefore, the choice of *E. coli* and intestinal enterococci as FIB are appropriate. However, in dispersion and advection/dispersion dominant systems, which includes the vast majority of EU bathing waters, the consideration of the decay rates of potential faecal indicators is critical to assess the impact of faecal contamination on public health. Therefore, more consideration should be given to marker selection when modelling waterbodies such as coastal beaches and estuaries. Inclusion of stable viral markers, such as crAssphage and PMMoV, will improve the reliability of water quality modelling and minimise the risk of waterborne illnesses from faecal contamination.

Funding sources

This research (Acclimatize) was part funded by the European Regional Development Fund through the Ireland Wales Cooperation Programme.

CRediT authorship contribution statement

Niamh A. Martin: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Writing – original draft. **Laura Sala-Comorera:** Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Writing – original draft. **Guanghai Gao:** Methodology, Formal analysis, Data curation, Writing – original draft. **Aisling Corkery:** Methodology, Formal analysis, Data curation, Writing – original draft. **Liam J. Reynolds:** Investigation. **Tristan M. Nolan:** Investigation, Formal analysis, Data curation. **Megan Whitty:** Investigation. **John J. O'Sullivan:** Methodology, Writing – original draft. **Wim G. Meijer:** Conceptualization, Methodology, Formal analysis, Data curation, Writing – original draft, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2023.120137.

References

Ahmed, W., Payyappat, S., Cassidy, M., Harrison, N., Besley, C., 2020. Sewage-associated marker genes illustrate the impact of wet weather overflows and dry weather leakage in urban estuarine waters of Sydney, Australia. Sci. Total Environ. 705, 135390 https://doi.org/10.1016/j.scitotenv.2019.135390.

Ahmed, W., Toze, S., Veal, C., Fisher, P., Zhang, Q., Zhu, Z., Staley, C., Sadowsky, M.J., 2021. Comparative decay of culturable faecal indicator bacteria, microbial source tracking marker genes, and enteric pathogens in laboratory microcosms that mimic a sub-tropical environment. Sci. Total Environ. 751, 141475 https://doi.org/10.1016/j.scitotenv.2020.141475.

Balleste, E., Blanch, A.R., Mendez, J., Sala-Comorera, L., Maunula, L., Monteiro, S., Farnleitner, A.H., Tiehm, A., Jofre, J., Garcia-Aljaro, C., 2021. Bacteriophages are good estimators of human viruses present in water. Front. Microbiol. 12, 619495 https://doi.org/10.3389/fmicb.2021.619495.

Balleste, E., Demeter, K., Masterson, B., Timoneda, N., Sala-Comorera, L., Meijer, W.G., 2020. Implementation and integration of microbial source tracking in a river watershed monitoring plan. Sci. Total Environ. 736, 139573 https://doi.org/10.1016/j.scitotenv.2020.139573.

Balleste, E., Garcia-Aljaro, C., Blanch, A.R., 2018. Assessment of the decay rates of microbial source tracking molecular markers and faecal indicator bacteria from different sources. J. Appl. Microbiol. https://doi.org/10.1111/jam.14058.

Balleste, E., Pascual-Benito, M., Martin-Diaz, J., Blanch, A.R., Lucena, F., Muniesa, M., Jofre, J., Garcia-Aljaro, C., 2019. Dynamics of crAssphage as a human source tracking marker in potentially faecally polluted environments. Water Res. 155, 233–244. https://doi.org/10.1016/j.watres.2019.02.042.

Bedri, Z., O'Sullivan, J.J., Deering, L.A., Demeter, K., Masterson, B., Meijer, W.G., O'Hare, G., 2015. Assessing the water quality response to an alternative sewage disposal strategy at bathing sites on the east coast of Ireland. Mar. Pollut. Bull. 91, 330–346. https://doi.org/10.1016/j.marpolbul.2014.11.008.

Boehm, A., Soller, J., 2020. Refined ambient water quality thresholds for human-associated fecal indicator HF183 for recreational waters with and without co-occurring gull fecal contamination. Microb. Risk Anal. 16, 100139 https://doi.org/10.1016/j.mran.2020.100139.

Boehm, A.B., Silverman, A.I., Schriewer, A., Goodwin, K., 2019. Systematic review and meta-analysis of decay rates of waterborne mammalian viruses and coliphages in surface waters. Water Res. 164, 114898 https://doi.org/10.1016/j.watres.2019.114898.

Cabelli, V., Dufour, A., McCabe, L., Levin, M., 1982. Swimming-associated gastroenteritis and water quality. Am. J. Epidemiol. 115, 606–616. https://doi.org/10.1093/oxfordjournals.aje.a113342.

Chick, H., 1908. An investigation of the laws of disinfection. J. Hyg. (Lond) 8, 92–158. https://doi.org/10.1017/S0022172400006987.

Crank, K., Li, X., North, D., Ferraro, G.B., Iaconelli, M., Mancini, P., La Rosa, G., Bibby, K., 2020. CrAssphage abundance and correlation with molecular viral markers in Italian wastewater. Water Res. 184, 116161 https://doi.org/10.1016/j.watres.2020.116161.

Crank, K., Petersen, S., Bibby, K., 2019. Quantitative microbial risk assessment of swimming in sewage impacted waters using crassphage and pepper mild mottle virus in a customizable model. Environ Sci Technol Lett 6, 571–577. https://doi.org/10.1021/acs.estlett.9b00468.

Dennehy, J.J., Turner, P.E., 2004. Reduced fecundity is the cost of cheating in RNA virus φ 6. Proc. R. Soc. Lond. B Biol. Sci. 271, 2275–2282. https://doi.org/10.1098/rspb.2004.2833.

Dutilh, B.E., Cassman, N., McNair, K., Sanchez, S.E., Silva, G.G., Boling, L., Barr, J.J., Speth, D.R., Seguritan, V., Aziz, R.K., Felts, B., Dinsdale, E.A., Mokili, J.L., Edwards, R.A., 2014. A highly abundant bacteriophage discovered in the unknown sequences of human faecal metagenomes. Nat. Commun. 5, 4498. https://doi.org/10.1038/ncomms5498.

EU, 2006. Directive 2006/7/EC of the European Parliament and of the Council of 15 February 2006 concerning the management of bathing water quality and repealing Directive 76/160/EEC. Off. J. Eur. Union 64, 14.

European Environment Agency (2021). European bathing water quality in 2021. Available at: https://www.eea.europa.eu/publications/bathing-water-quality-in-2021/european-bathing-water-quality-in-2021 [Accessed 03 June 2022].

Fleisher, J.M., Kay, D., Salmon, R.L., Jones, F., Wyer, M.D., Godfree, A.F., 1996. Marine waters contaminated with domestic sewage: nonenteric illnesses associated with bather exposure in the United Kingdom. Am. J. Public Health 86 (9), 1228–1234. https://doi.org/10.2105/AJPH.86.9.1228.

Gonzalez-Fernandez, A., Symonds, E.M., Gallard-Gongora, J.F., Mull, B., Lukasik, J.O., Rivera Navarro, P., Badilla Aguilar, A., Peraud, J., Brown, M.L., Mora Alvarado, D., Breitbart, M., Cairns, M.R., Harwood, V.J., 2021. Relationships among microbial indicators of fecal pollution, microbial source tracking markers, and pathogens in Costa Rican coastal waters. Water Res. 188, 116507 https://doi.org/10.1016/j.watres.2020.116507.

Greaves, J., Stone, D., Wu, Z., Bibby, K., 2020. Persistence of emerging viral fecal indicators in large-scale freshwater mesocosms. Water Res. X 9, 100067. https://doi.org/10.1016/j.wroa.2020.100067.

Green, H.C., Haugland, R.A., Varma, M., Millen, H.T., Borchardt, M.A., Field, K.G., Walters, W.A., Knight, R., Sivaganesan, M., Kelty, C.A., Shanks, O.C., 2014. Improved HF183 quantitative real-time PCR assay for characterization of human fecal pollution in ambient surface water samples. Appl. Environ. Microbiol. 80, 3086–3094. https://doi.org/10.1128/AEM.04137-13.

Havelaar, A., 1991. Bacteriophages as model viruses in water quality control. Water Res. 25, 529–541. https://doi.org/10.1016/0043-1354(91)90126-B (Oxford).

International Organization for Standardization, 2001. ISO 16649-1:2001 - Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of beta-glucuronidase-positive Escherichia coli — Part 1: Colony-count technique at 44 °C using membranes and 5-bromo-4-chloro-3-indolyl. Available at: https://www.iso.org/standard/29823.html [Accessed June 23, 2021].

Jalliffier-Verne, I., Heniche, M., Madoux-Humery, A.S., Galarneau, M., Servais, P., Prévost, M., Dorner, S., 2016. Cumulative effects of fecal contamination from combined sewer overflows: management for source water protection. J. Environ. Manag. 174, 62–70. https://doi.org/10.1016/j.jenvman.2016.03.002.

Jebri, S., Muniesa, M., Jofre, J., Farnleitner, A., Blanch, A., 2017. General and host-associated bacteriophage indicators of faecal pollution. Global Water Pathogens Project, Part 2 Indicators and Microbial Source Tracking Markers. Michigan State University, E. Lansing, MI, UNESCO. http://www.waterpathogens.org/book/coliphage.

Jennings, W.C., Galvez-Arango, E., Prieto, A.L., Boehm, A.B., 2020. CrAssphage for fecal source tracking in Chile: covariation with norovirus, HF183, and bacterial indicators. Water Res. X 9, 100071. https://doi.org/10.1016/j.wroa.2020.100071.

Kato, R., Asami, T., Utagawa, E., Furumai, H., Katayama, H., 2018. Pepper mild mottle virus as a process indicator at drinking water treatment plants employing coagulation-sedimentation, rapid sand filtration, ozonation, and biological activated carbon treatments in Japan. Water Res. 132, 61–70. https://doi.org/10.1016/j.watres.2017.12.068.

Kay, D., Jones, F., Wyer, M.D., Fleisher, J.M., Salmon, R.L., Godfree, A.F., Zelenauch-Jacquotte, A., Shore, R., 1994. Predicting likelihood of gastroenteritis from sea bathing: results from randomised exposure. Lancet North Am. Ed. 344, 905–909. https://doi.org/10.1016/S0140-6736(94)92267-5.

Lalancette, C., Papineau, I., Payment, P., Dorner, S., Servais, P., Barbeau, B., Di Giovanni, G.D., Prevost, M., 2014. Changes in Escherichia coli to Cryptosporidium ratios for various fecal pollution sources and drinking water intakes. Water Res. 55, 150–161. https://doi.org/10.1016/j.watres.2014.01.050.

Moce-Llivina, L., Lucena, F., Jofre, J., 2005. Enteroviruses and bacteriophages in bathing waters. Appl. Environ. Microbiol. 71, 6838–6844. https://doi.org/10.1128/AEM.71.11.6838-6844.2005.

O'Loughlin, E.M., Bowmer, K.H., 1975. Dilution and decay of aquatic herbicides in flowing channels. J. Hydrol. Amst 26, 217–235. https://doi.org/10.1016/0022-1694(75)90004-9.

Pascual-Benito, M., Nadal-Sala, D., Tobella, M., Balleste, E., Garcia-Aljaro, C., Sabate, S., Sabater, F., Marti, E., Gracia, C.A., Blanch, A.R., Lucena, F., 2020. Modelling the seasonal impacts of a wastewater treatment plant on water quality in a Mediterranean stream using microbial indicators. J. Environ. Manag. 261, 110220 https://doi.org/10.1016/j.jenvman.2020.110220.

Reynolds, L.J., Sala-Comorera, L., Khan, M.F., Martin, N.A., Whitty, M., Stephens, J.H., Nolan, T.M., Joyce, E., Fletcher, N.F., Murphy, C.D., 2022. Coprostanol as a Population Biomarker for SARS-CoV-2 Wastewater Surveillance Studies. Water 14, 225. https://doi.org/10.3390/w14020225 (Basel).

Reynolds, L.J., Sala-Comorera, L., Martin, N.A., Nolan, T.M., Stephens, J.H., Gitto, A., O'Hare, G.M.P., O'Sullivan, J.J., Meijer, W.G., 2020. Correlation between antimicrobial resistance and faecal contamination in small urban streams and bathing waters. Sci. Total Environ. 739, 140242 https://doi.org/10.1016/j.scitotenv.2020.140242.

Rosario, K., Symonds, E.M., Sinigalliano, C., Stewart, J., Breitbart, M., 2009. Pepper mild mottle virus as an indicator of fecal pollution. Appl. Environ. Microbiol. 75, 7261–7267. https://doi.org/10.1128/AEM.00410-09.

Runkel, R.L., 1996. Solution of the advection-dispersion equation: continuous load of finite duration. J. Environ. Eng. 122, 830–832. https://doi.org/10.1061/(ASCE)0733-9372(1996)122:9(830.

Rutledge, R.G., Cote, C., 2003. Mathematics of quantitative kinetic PCR and the application of standard curves. Nucleic. Acids. Res. 31, e93. https://doi.org/10.1093/nar/gng093.

Rutledge, R.G., Stewart, D., 2008. Critical evaluation of methods used to determine amplification efficiency refutes the exponential character of real-time PCR. BMC Mol. Biol. 9, 96. https://doi.org/10.1186/1471-2199-9-96.

Sala-Comorera, L., Reynolds, L.J., Martin, N.A., Pascual-Benito, M., Stephens, J.H., Nolan, T.M., Gitto, A., O'Hare, G.M.P., O'Sullivan, J.J., Garcia-Aljaro, C., Meijer, W.G., 2021. crAssphage as a human molecular marker to evaluate temporal and spatial

variability in faecal contamination of urban marine bathing waters. Sci. Total Environ. 789, 147828 https://doi.org/10.1016/j.scitotenv.2021.147828.
Scroccaro, I., Ostoich, M., Umgiesser, G., De Pascalis, F., Colugnati, L., Mattassi, G., Vazzoler, M., Cuomo, M., 2010. Submarine wastewater discharges: dispersion modelling in the Northern Adriatic Sea. Environ. Sci. Pollut. Res. 17, 844–855. https://doi.org/10.1007/s11356-009-0273-7.
Seurinck, S., Defoirdt, T., Verstraete, W., Siciliano, S.D., 2005. Detection and quantification of the human-specific HF183 Bacteroides 16S rRNA genetic marker with real-time PCR for assessment of human faecal pollution in freshwater. Environ. Microbiol. 7, 249–259. https://doi.org/10.1111/j.1462-2920.2004.00702.x.
Soller, J.A., Schoen, M.E., Bartrand, T., Ravenscroft, J.E., Ashbolt, N.J., 2010. Estimated human health risks from exposure to recreational waters impacted by human and non-human sources of faecal contamination. Water Res. 44, 4674–4691. https://doi.org/10.1016/j.watres.2010.06.049.
Soller, J.A., Schoen, M.E., Varghese, A., Ichida, A.M., Boehm, A.B., Eftim, S., Ashbolt, N. J., Ravenscroft, J.E., 2014. Human health risk implications of multiple sources of faecal indicator bacteria in a recreational waterbody. Water Res. 66, 254–264.
Stachler, E., Kelty, C., Sivaganesan, M., Li, X., Bibby, K., Shanks, O.C., 2017. Quantitative CrAssphage PCR assays for human fecal pollution measurement. Environ. Sci. Technol. 51, 9146–9154. https://doi.org/10.1021/acs.est.7b02703.
Wade, T.J., Calderon, R.L., Sams, E., Beach, M., Brenner, K.P., Williams, A.H., Dufour, A. P., 2006. Rapidly measured indicators of recreational water quality are predictive of swimming-associated gastrointestinal illness. Environ. Health Perspect. 114, 24–28. https://doi.org/10.1289/ehp.8273.
Wade, T.J., Pai, N., Eisenberg, J.N., Colford Jr, J.M., 2003. Do U.S. Environmental Protection Agency water quality guidelines for recreational waters prevent gastrointestinal illness? A systematic review and meta-analysis. Environ. Health Perspect. 111, 1102–1109. https://doi.org/10.1289/ehp.6241.
WHO, 2003. Guidelines for safe recreational water environments. Coast. Fresh Waters 1, 1–219.
World Health Organisation, 2007. Bathing water quality: fact sheet No. 1.4. 4. Available at: https://apps.who.int/iris/handle/10665/366454 [Accessed].
Wu, J., Long, S., Das, D., Dorner, S., 2011. Are microbial indicators and pathogens correlated? A statistical analysis of 40 years of research. J. Water Health 9, 265–278. https://doi.org/10.2166/wh.2011.117.
Wu, Z., Greaves, J., Arp, L., Stone, D., Bibby, K., 2020. Comparative fate of CrAssphage with culturable and molecular fecal pollution indicators during activated sludge wastewater treatment. Environ. Int. 136, 105452 https://doi.org/10.1016/j.envint.2019.105452.